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**BIRTH OUTCOMES ASSOCIATED WITH HEAVY METAL  
EXPOSURES IN THE SURROUNDINGS OF A FORMER  
CHEMICAL PLANT IN TÂRNĂVENI, ROMANIA**

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Thesis submitted for partial fulfillment of the requirement for the degree of  
Masters of Public Health (MPH) in Environmental Health Science at  
Yale School of Public Health

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Thesis Advisor: Dr. Caroline Johnson

Second Reader (also the Project Investigator): Dr. Eugen Gurzau

## **Abstract**

Heavy metal exposures have long been a public health concern, research on this field may promote policies and regulations to restrict or limit certain industrial practices. This study aims to evaluate potential associations between toxic heavy metal exposures via contaminated environmental media and the frequency of the birth defects in the designated area: Tarnaveni, Romania; historically contaminated by a former chemical plant. In 2018, a pilot investigation conducted on the site demonstrated that chromium (Cr), lead (Pb), and manganese (Mn) are common heavy metal contaminants found. Soil concentrations for all three metals exceeded normal background levels according to Romanian Environmental Law. This study highlights a mixed approach in terms of using both soil (environmental) and blood (biofluid) samples to identify exposures, stratifying the results into individual effects of different metals, and integrating the effects from all exposures. Data from 30 pregnant women in the area were collected and heavy metal concentrations were measured (Cr, Pb, Mn in soil and blood; in addition to Arsenic and Cadmium in blood) and correlated these exposures levels to birth outcomes. The estimated exposure intakes were calculated for the population using a probabilistic method. The end-point specific hazard quotients (HQs) and hazard index (HI) were calculated to evaluate the non-carcinogenic health risk. A spatial analysis was performed to investigate the relationship of metal concentration and cases. By assessing the population needs and communities' health, I hope to raise awareness and potentially promote further studies in this subject.

**Keywords:** Heavy metals, environmental health science, industrial contamination, community exposure, birth defects, soil, Romania

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## Table of Contents

Abstract.....	1
Acknowledgement.....	2
List of Tables and Figures.....	4
Introduction.....	5
Review of Studies Relevant to the Problem.....	7
<i>Chromium</i>	
<i>Lead</i>	
<i>Manganese</i>	
Research Design.....	13
<i>Study design and population sample</i>	
<i>Soil sampling and analysis</i>	
<i>Blood samples collection and analysis</i>	
<i>Statistical analysis</i>	
<i>Hazard Index and Hazard Quotient</i>	
<i>Spatial analysis</i>	
Presentation and Analysis of Findings.....	22
Limitations and Confounders.....	32
Conclusion.....	34
References.....	36
Appendix	

## List of Tables and Figures

*Table 1. Heavy Metals Target-Organ Toxicity Dose (TTD)/Minimal risk levels (MRLs) of targeted metals (Arsenic, Cadmium, Chromium, Manganese, and Lead)*

*Table 2. Potential Health Effects of Concern for Intermediate and Chronic Oral Exposure to the Mixture Lead, Arsenic, Cadmium, Chromium, and Manganese*

*Table 3. Demographic characteristics of the population sample in Tarnaveni, Romania*

*Table 4. Heavy metal concentration in blood of the population sample in Tarnaveni, Romania*

*Table 5. Heavy metal concentration in blood of cases in Tarnaveni, Romania*

*Table 6. Heavy metal concentration in blood of controls in Tarnaveni, Romania*

*Table 7. Heavy metal concentration in soil from the study area in Tarnaveni, Romania*

*Table 8. Distribution of Selected Characteristics of the Study Population in Tarnaveni, Romania*

*Fig. 1. Map of Tarnaveni, Romania*

*Fig. 2. Spatial distribution of soil and blood samples of patients in Tarnaveni, Romania*

*Fig. 3. Correlation Plot of Continuous Variables (Age, Metal Concentrations in Blood, Metal Concentration in Soil, Distance from Chemical Plant, Gestation Age, Weight, and Height)*

*Fig. 4. Linear regression and p values among gestation age and Mn concentrations in the blood samples*

*Fig. 5. Linear regression of the metal concentrations in the residential soil samples and distance from a former chemical plant in Tarnaveni, Romania*

### **Appendix**

*Fig. 6. Histogram showing the distribution and log transformation of the metal concentrations in soil*

*Fig. 7. Histogram showing the distribution and log transformation of the metal concentrations in blood*

*Fig. 8. Boxplot showing the distribution of the metal concentrations in soil*

*Fig. 9. Boxplot showing the distribution of the metal concentrations in blood*

*Table 9. Heavy metal concentration difference between cases and controls*

*Table 10. Hazard Quotient and Hazard Index of end-point specific outcomes*

## Introduction

Human exposure and environmental pollution from heavy metals have been rising rapidly in recent decades due to an exponential increase in use of various industrial and technological applications (Tchounwou *et al.*, 2012). Industrial sites may be contaminated for years even after the manufacturing activities have been terminated. Living in close proximity to contaminated sites may lead to higher exposure to heavy metals, which is associated with increased risk of cancer, neurological diseases, and endocrine-related disorders (Jaishankar *et al.*, 2014). Pregnant women, in particular, are more vulnerable from exposures to toxicants as these chemicals may affect fetal development, causing adverse birth outcomes (Bank-Nielsen *et al.*, 2019).

A pilot study conducted in 2018 demonstrated that chromium (Cr), lead (Pb), and manganese (Mn) are common heavy metal contaminants generated by found in a former chemical plant in Romania. Soil concentrations for all three metals exceeded normal background levels according to Romanian Environmental Law (Mihaileanu *et al.*, 2019). These heavy metals are naturally occurring constituents in the earth's crust and mantle and can be found in air, soil, water, and food (Singh *et al.*, 2011; Huang *et al.*, 2017). Trace measures of heavy metals are naturally present in the human body, since some (such as Cr (III), Mn) and are essential nutrients for biochemical and physiological functioning (Jaishankar *et al.*, 2014; Tutic *et al.*, 2015). However, heavy metals are also considered to be toxic in certain concentrations. The World Health Organization (WHO) identified that Cr, Mn, Pb, arsenic (As), and cadmium (Cd) are of major public health concern as they are systemic toxicants that may influence or damage multiple organ systems even at lower exposure levels (above the normal levels found physiologically in the body) (Bank-Nielsen *et al.*, 2019).

The objective of this study is to assess the birth outcomes associated to environmental exposures from heavy metals (Cr, Mn, Pb) contamination generated by a former chemical plant in Tarnaveni, Romania. Heavy metals may bind to proteins and accumulate in different tissues in our body such as heart, brain, kidneys, bone, liver and thereby disrupting functions in these vital organs (Singh *et al.*, 2011; Engwa *et al.*, 2019). Common health risks from these toxic metals include neurological disorders, endocrine disruption, cardiovascular dysfunction, kidney and skeletal damages, and even carcinogenic effects (Maurya *et al.*, 2019; Singh *et al.*, 2011). The most common exposure pathways for heavy metals are through inhalation and ingestion of water and food from contaminated sources. Therefore, I am interested in investigating the exposure route and potential prenatal health effects in this specific community. Initially, the source and levels of heavy metals in soil in the contaminated area is studied. Then, biomonitoring data from metal concentrations measured in blood in the exposed communities is analyzed, to obtain an overall dose in the human body from all exposure pathways, and then correlate potential birth outcomes related to the levels of heavy metals in maternal blood and soil collected. The study highlights a mixed approach in terms of using both environmental and biofluid samples, integrating the effects from all exposures, and stratifying the results into individual effects of different metals. To the best of my knowledge, this is the first study that evaluates the extent of heavy metals exposure from both soil and blood samples in communities of a former industrial site in Romania to correlate with potential adverse pregnancy outcomes.



## Review of Studies Relevant to the Problem

Epidemiological evidence has demonstrated that prenatal exposure to heavy metals can adversely influence fetal development and growth in a dose-response relationship, or even induce deaths in the embryo and fetus (Wai *et al.*, 2017; Bank-Nielsen *et al.*, 2019). For instance, Cr can cross the placenta, and there may be excessive exposure which would influence the development of the fetus if blood Cr levels of mothers are raised by environmental factors. Similar to general health effects from heavy metals exposures, some of the birth defects studied include endocrine disruption, musculoskeletal defects, cardiovascular defects, genital malformations, and premature rupture of membranes leading to preterm birth (Li *et al.*, 2008; Ni *et al.*, 20; Huang *et al.*, 2017). There is a higher risk of preterm births as well as an inverse relation to birth weight and birth size for prenatal Pb exposure (Zheng *et al.*, 2016). Potential neurodevelopmental consequences of excess Mn have only recently been investigated, with a few studies reporting a neurodevelopmental impact from co-exposures to Mn and other environmental contaminants (Bellinger *et al.*, 2013). Results for exposure to Mn are inconsistent in different study populations, with some studies showing association to exposure and low birth weight while some did not find significant results (Zheng *et al.*, 2016). Few data are available on the effect of prenatal exposure from Cr and Mn in contaminated industrial sites and risks of birth. Most studies focus on the effect of single chemical exposure or an overall health outcome, but pregnant women are exposed to complex mixtures in the real-world (Bellinger *et al.* 2013). Strategies for tackling the issue of interactions between mixtures needed to be developed. The individual effects of each heavy metals are evaluated as followed.

## *Chromium*

Chromium is the seventh most abundant element on earth, naturally found in rocks, animals, plants, soil, and in volcanic dust and gases. It is estimated that 33 tons of total Cr are released annually into the environment. Cr's presence has increase greatly with various anthropogenic activities. The most common oxidation states are Trivalent Chromium ( $\text{Cr}^{3+}$ ), and hexavalent Chromium ( $\text{Cr}^{6+}$ ). According to their different oxidation states, their toxicity is different and this is mainly due to their difference in cell membrane permeability. Hexavalent  $\text{Cr}^{6+}$  has better penetrating power and thus relatively toxic. Trivalent  $\text{Cr}^{3+}$  occurs naturally in the environment and is an essential nutrient.  $\text{Cr}^{6+}$  and Cr metal are generally produced by industrial processes (ATSDR; Jaishankar *et al.*, 2009). Cr does not usually remain in the atmosphere, but is deposited into the soil and water and can change from one form to another. Cr compounds are greatly persistent in water sediments (ATSDR; Jaishankar *et al.*, 2009).

Cr can be found in air, soil, and water after release from the manufacture, use, and disposal of Cr-based products, and during the manufacturing process. The route of exposure is complex, including inhalation, ingestion, skin/dermal and eye contact. Exposure to Cr occurs from ingesting contaminated food or drinking contaminated water, breathing contaminated workplace air, or skin contact during use in the workplace, and even in cigarette smoke as Cr is a component of tobacco smoke. (ATSDR) Living near uncontrolled hazardous waste sites containing Cr or industries that use Cr also increase the risk of or level of exposure. A large number of workers are potentially exposed to Cr. Occupational exposure has been a major concern because of the high risk of Cr-induced diseases in industrial workers occupationally. (Tchounwou *et al.*, 2012). Drinking or bathing in water containing Cr are both ways of exposure. It may also enter the body through

dermal contact. The general population is most likely to be exposed to trace levels of Cr in the food that is eaten. The uptake of Cr<sup>6+</sup> compounds through the airways and digestive tract is faster than that of Cr<sup>3+</sup> compounds. Most of the Cr leaves the body in the urine within a week, although some may remain in cells for several years or longer.

In general, affected organ systems included Renal (Urinary System or Kidneys), Respiratory (From the Nose to the Lungs) and Immune system. Exposures to high levels of Cr<sup>6+</sup> may also produce effects on the liver, gastrointestinal, cardiovascular and neurological hematological systems, and possibly the blood (ATSDR; Jaishankar *et al.*, 2009). In terms of birth outcomes, Cr can cross the placenta, and may lead to excessive exposure and influence the development of the fetus if blood Cr levels of mothers are raised by environmental pollution. Animal studies have shown adverse birth outcome such as low birth weight (LBW, <2500 g), preterm births (PTBs, <37 weeks of completed gestation), and birth defects like endocrine disruption, musculoskeletal defects, neural-tube defects, cardiovascular defects, genital malformations, and others (McDermott *et al.*, 2015). In the CDC website (updated 2015), it explicitly stated that: “There are no studies that have looked at the effects of Cr exposure on children. It is likely that children would have the same health effects as adults. We do not know whether children would be more sensitive than adults to the effects of Cr. No studies showing that it causes birth defects in humans.” Therefore, unlike metals like Pb, Cd, mercury (Hg), and As, which is well documented that their exposures produce a wide variety of adverse birth outcomes in humans, the effects on birth outcomes of Cr exposure are still unclear.

## *Lead*

Lead is a naturally occurring heavy metal that are widely distributed across the world. It has been used prominently in various anthropogenic activities, such as mining, manufacturing, burning of fossil fuels, waste incineration, etc. (Tchounwou *et al.*, 2012). There are also a lot of Pb-containing products (e.g., Pb-based paints, pigments, and glazes; pesticides, electrical shielding; plumbing; storage batteries; solder; and welding fluxes). Common sources in household are Pb-based paints and Pb-pipes, which had been a major problem on childhood Pb poisoning in the United States. Pb does not naturally degrade in the environment and it can be can be transported and transferred between air, water, and soil. The route of exposure of Pb is mainly through in inhalation of contaminated dust in ambient air, and ingestion of contaminated foods and drinking water (ADSTR, 2020).

Pb is one of the most studied heavy metal, the toxicity and health effects of Pb are well investigated. There is an extensive database in effects on humans, including children and infants, established from the large amount of epidemiological studies. Pb is transferred and transported throughout our body and is retained for decades. Therefore, adverse health effects of Pb have been observed in every organ system, including neurological, renal, cardiovascular, hematological, immunological, reproductive, and developmental effects (ADSTR; Tchounwou *et al.*, 2012; Tutic *et al.*, 2015). The association of blood lead levels (PbB) and IQ (intelligence quotient) in children is renowned. International Agency for Research on Cancer (IARC) has classified inorganic Pb compounds as probably carcinogenic to humans (Group 2A). Adverse health effects have been observed even with low doses of Pb.

Exposure to Pb during pregnancy is of special concern, since Pb absorbed by the pregnant mother is readily transferred to the developing fetus (Tchounwou *et al.*, 2012). Developing fetuses and children are most vulnerable to neurotoxic effects due to Pb exposure. Human evidence corroborates animal findings, studies have evaluated developmental effects including adverse birth outcomes including reduced birth weight and preterm delivery, birth defects, neural tube defects, neurodevelopmental abnormalities, decreased anthropometric measures in children, and delayed puberty, associated with Pb exposure (ADSTR; Tchounwou *et al.*, 2012; Tutic *et al.*, 2015).

Pb exposures are generally measured in blood in term of PbB. While some studies also investigate the associations between maternal bone Pb and birth outcome, maternal PbB and/or cord PbB are used as the biomarker for exposure in most studies evaluating developmental effects in infant or child. The database for related studies is dominated by environmental exposure studies with PbB  $\leq 10$   $\mu\text{g/dL}$ , showing inconsistent results and mixed evidence for the effects of birth outcomes (ADSTR).

### *Manganese*

Manganese is also one of the most abundant metals in Earth's crust, found in over 100 minerals. It does not occur in the environment in its pure metal form, but combined with other substances such as oxygen, sulfur, and chlorine (ATSDR; Jaishankar *et al.*, 2009; WHO). It is an essential element for both humans and animals for proper functioning as it is required for the functioning of many cellular enzymes. It occurs naturally in most foods and may be added to food products as well as for nutritional supplements. It is mainly used in the manufacture of iron and steel alloys and products such as batteries, glass, fertilizers and fireworks (ATSDR; WHO).

The primary source of exposure is ingestion of Mn containing food. Most Mn ingested will leave human body in feces within a few days. Occupational exposure and inhalation of Mn near industrial sources is also common. Surface water and groundwater may contain Mn in addition to soils that may erode into these waters. Human activities are responsible to Mn contamination in some areas.

Although Mn is an essential nutrient, excess Mn exposure may cause neurotoxicity. Health effects from high levels of Mn include effects on nervous system, respiratory system and behavioral changes (ATSDR; Jaishankar *et al.*, 2009; WHO). Mn is present in all tissues of the body, with highest levels in the liver, kidney, pancreas and adrenals (WHO). It may accumulate preferentially in certain regions of the brain in infants. Mn in brain may cause DNA damage and chromosomal aberrations and was toxic to the embryo and fetus. Very few studies are conducted on the developmental effect of Mn exposure. Most of the studies did not find increases in birth defects or low birth weight in the fetus. There are experimental studies on fetal development which investigate the Mn toxic effect, that showed exposure to Mn results in a decrease in fetal weight and retardation of the development of the skeleton and internal organs (Grazuleviciene *et al.* 2009; Rahman *et al.*, 2013). Results for exposure to Mn are inconsistent in different study populations and limited data is available on the effect of prenatal exposure from Mn.

## Research Design

This thesis is a research on heavy metal exposure and birth outcomes. There are quantitative data (heavy metal concentration in blood samples and soil samples) and qualitative data (questionnaire). The samples collected investigated exposure routes including ingestion (soil and vegetable samples) and overall internal exposure (blood sample concentration). There are three components regarding the method of analysis: statistical analysis, a scoring system from the Hazard Index in evaluating the critical effects of the heavy metals on specific outcomes, and spatial analysis. A mixture approach is used and results are interpreted in a way that will be significant to the local community or the study field.

### *Study design and population sample*



Figure 1. Vector Map of Târnăveni, Romania (satellites.pro)

Târnăveni is a town in Mureș County, Transylvania, located on the Târnava Mică River in central Romania as shown in *Figure 1*. There are 22,075 inhabitants with a density of 420/km<sup>2</sup> (Romania National Institute of Statistics, 2011). There was a chemical plant that produced chemical products containing bichromate located close to the town that operated until 2007 (Mihaileanu *et al.*, 2019). Millions of tons of chemical wastes were discharged. Heavy metals such

as Cr<sup>6+</sup> and Mn were residues from the former industrial platform, in close proximity to the town and river.

A cross-sectional study was conducted at the Gynecology and Obstetrics Department of the Municipal Hospital “Dr. Gheorghe Marinescu” in Tarnaveni between July 2016 and January 2019. The study recruited 30 hospital patients, who were pregnant women carrying a baby with a congenital malformation. Congenital malformations were defined as structure and/or function anomalies of organs/systems and were diagnosed by the gynecologist during pregnancy (before delivery) by ultrasound examination. The study included only pregnant women carrying a baby with a congenital malformation, who had lived in the Tarnaveni area for more than five years. Before hospital discharge, information is also abstracted on health status, gynecological history, other disease history, smoking status from clinical observation sheets, and collected more information on the participants’ lifestyle and the place where they live (their residence) based on a questionnaire survey.

All study participants provided written informed consent, prior to study participation. The research protocol was approved by the “Iuliu Hatieganu” University of Medicine Ethics Committee (Institutional Review Board (IRB) approval number 356/02.06.2015).

#### *Soil sampling and analysis*

Thirteen soil samples were collected from the residential areas where our study participants lived. The soil samples were analyzed for metals concentrations (total Cr, Pb, and Mn) by atomic absorption spectrometry at the Environmental Health Center (EHC) laboratory in Cluj-Napoca.



The sampling locations were selected based on the location of the patient and recorded distance from the former chemical plant. An eTrex global positioning system (GPS) handheld device (Garmin International, Inc., Olathe, USA) was used to document latitude and longitude coordinates at each soil sampling location. A clean stainless-steel shovel was used to collect approximately 500 g of soil from the topsoil layer (0–10 cm depth) into metal-free polyethylene containers, at each sample point, and stored in the laboratory under standard protocol until analysis.

Soil samples were dried at room temperature on paper trays. The samples were then grounded and sieved to a size smaller than 0.25 mm. Total Cr, Pb, and Mn concentration in soil were measured by atomic absorption spectrometry according to the ISO 11047/1999 standard. A reference sample of quality control (QC) soil material (NIST 2709) was also processed following the same procedure.

#### *Blood samples collection and analysis*

The blood samples from study participants were collected in the hospital by trained medical personnel (nurse), in 2 mL plastic containers containing anticoagulant. The samples were labeled with a unique subject identification number and stored 4°C at Tarnaveni hospital until analysis. Isothermal containers filled with ice bags were used for sample transportation to the EHC laboratory, where the samples were analyzed for metals by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS).

The blood samples were prepared for analysis by diluting 10-fold of 2% (v/v) NH<sub>4</sub>OH, 1g/L EDTA, 1% (v/v) Propanol, and 0.05% (v/v) Triton X-100 in an alkali mixture. The same

alkali mixture was used to prepare the calibration standards, blanks, and the internal standard. The calibration standards concentrations for all the metals were 0 µg/L, 0.1 µg/L, 0.5 µg/L, 1 µg/L, 3 µg/L and 5 µg/L. After calibration, the prepared blood samples were analyzed by ICP-MS using a PlasmaQUANT MS ELITE device (Analytik Jena, Jena, Germany). A detailed description of the chemical method is provided by Analytik Jena (Analytik Jena AG, 2016). Blood concentrations of As, Cd, Cr, Mn, and Pb are exported in the study database.

### *Statistical analysis*

Statistical analyses of the data were performed with R (Version 3.5.1) (under the terms of the Free Software Foundation's GNU General Public License). Statistical significance was defined as  $p < 0.05$  for a two-tailed test. Descriptive statistics were first generated in R studio. Then, a correlation plot was created to demonstrate all of the continuous variables to observe potential correlations between the variables. Visual inspections are made on the normality of each variable's distribution and tried natural log transformation to check if there are log-normal distributions (Appendix). Skewness, kurtosis, outliers and extreme values are also checked, but there were some limitations due to the small sample size and missing information. Each category between cases and controls were compared. Multiple linear regression was used to evaluate the relationship between metal concentrations in soil and the distance from the chemical factory, as well as effects of metal concentrations in blood on gestational age, and the association between metal concentrations in blood and/or soil and death in fetus and embryo.

## *Hazard Index*

Contaminated hazardous waste sites generally contain a mixture of pollutants. U.S. Department of Health and Human Services Public Health Service, Agency for Toxic Substances and Disease Registry (ATSDR) and the National Toxicology Program together developed a mixtures program to investigate the potential health hazards and to fulfill the legislative mandate from the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). The mixtures program determines the health effects of mixed substances and established a guidance manual that outlines methods for assessments. They proposed Interactive Profiles that evaluate data on the toxicology of individual chemicals as well as their joint actions or interactions from both invitro and animal studies. Exposure-based assessment approaches are then recommended.

The Interactive Profile on Pb, As, Cd, and Cr were created in ATSDR since they are common constituents in soil found in industrial and manufacturing waste sites. Information for Mn was obtained in another profile. As it is a mixture in soil, the primary route of exposure is likely to be oral, with possible dermal contact. To study the health outcomes (birth defects) in this study, the critical effects of each metal and their Minimal risk levels (MRLs) and the end-points specific effects with the Target-Organ Toxicity Dose (TTD) was examined to check whether there were any additive interactions. Heavy Metals TTD and MRLs of corresponding targeted metals are then listed in *table 1*. The limits or no observed adverse effects level (NOAEL) were derived from different pathways: ingestion, dermal and some by inhalation, according to data availability. It was very difficult to derive a strict standard due to limitations in various study designs and reporting, as well as result inconsistency across studies for the same target organ.

<b>Metal Mg/kg/day</b>	<b>EPA RfD</b>	<b>NOAEL</b>	<b>LOAEL</b>	<b>ATSDR</b>	<b>TTDNEURO</b>	<b>TTDRENAL</b>	<b>TTDCARDIO</b>	<b>TTD HEMATO</b>
Arsenic (As)	0.0003 Chronic	0.0008 Dermal	0.014 Dermal	0.0003 Chronic Oral	0.0003	0.09	0.0003	0.0006
Cadmium (Cd)	0.0005 Water 0.001 Food	0.005 Water 0.01 Food	N/A	0.0002 Chronic Oral	0.0002	0.0002 (MRL)	0.005	0.0008
Chromium (Cr) (VI)	0.003 chronic oral^	2.5 mg Cr (VI) /kg/day] (EPA)	0.002 mg Cr (VI)/m <sup>3</sup>	0.000005 mg Cr/m <sup>3</sup> inhalation MRL#	0.01	0.01	N/A	0.003
Manganese (Mn)	0.14	0.047	0.05	chronic inhalation MRL#	0.07	N/A	N/A	N/A
Lead (Pb)	N/A	N/A	N/A	No MRLs	10*	34	10	10

**Table 1.** Heavy Metals Target-Organ Toxicity Dose (TTD)/Minimal risk levels (MRLs) of targeted metals (Arsenic, Cadmium, Chromium, Manganese, and Lead)

^chronic inhalation RfC of 0.0001 mg Cr (VI)/m<sup>3</sup> for Cr (VI) particulates, based on a benchmark concentration of 0.016 mg Cr(VI)/m<sup>3</sup>

\*CDC level of concern

#the upper end of the range of the estimated safe and adequate daily dietary intake of 200g Cr/kg/day

##provisional guidance value for total dietary intake of 0.07 mg Mn/kg/day

The critical effects are different for the targeted metals are different and they each affect a wide range of target organs and endpoints. Some of the target organs are common between two or more metals. A review of the potential health hazards is summarized in *table 2* (ATSDR, 2020). According to the hazard listed, relevant endpoints exposure risks were then calculated.

<b>Arsenic</b>	<b>Cadmium</b>	<b>Chromium</b>	<b>Manganese</b>	<b>Lead</b>
Dermal lesions Cardiovascular Hematological Renal Neurological Cancer	Renal Cardiovascular Hematological Hepatic Neurological Testicular	Hematological Hepatic Renal Neurological Testicular	Neurological	Neurological Hematological Cardiovascular Renal Testicular

**Table 2.** Potential Health Effects of Concern for Intermediate and Chronic Oral Exposure to the Mixture Lead, Arsenic, Cadmium, Chromium, and Manganese

*Hazard Quotient (HQ)* and *hazard Index (HI)* are often used in evaluating non-carcinogen risks, especially for chronic effects. HI of the total end-point risk is the sum of HQ of each chemical, which is calculated by Exposure of the metal divided by the target organ toxicity dose or RfC. The same applied to renal, cardiovascular and hematological effects, with HQ of the chemical that has corresponding effects on the specific effect in the organ. For example, the Endpoint-specific HI for neurological toxicity of the mixture was derived as followed:

$$HI_{NEURO} = \frac{E_{Pb}}{CDC\ PbB_{Pb\ NEURO}} + \frac{E_{As}}{TTD_{As\ NEURO}} + \frac{E_{Cd}}{TTD_{Cd\ NEURO}} + \frac{E_{Cr(VI)}}{TTD_{Cr(VI)\ NEURO}} + \frac{E_{Mn}}{TTD_{Mn\ NEURO}}$$

As shown in *Table 2*, all five metals have shown potential neurological health effects. Thus, each of the HQ from the 5 different metals are summed up. The exposure (E) is originated from the metal intake, which was the measured metal concentration in soil with a consideration of other factors, which was shown below. If the End-point specific HI > 1, it implied a risk or health hazard on targeted health outcomes (ATSDR IR Profile, 2020).

The exposure for each metal is calculated with the following equation:

$$E \text{ or Intake (mg/kg*day)} = \frac{CS*IR*CF*EF*ED}{BW*AT}$$

Where:

CS = Chemical Concentration in Soil (mg/kg)

IR = Ingestion Rate (mg soil/day)

CF = Conversion Factor (10<sup>-6</sup> kg/mg)

EF= Exposure Frequency (days/months) – 30 days full exposure window for pregnancy

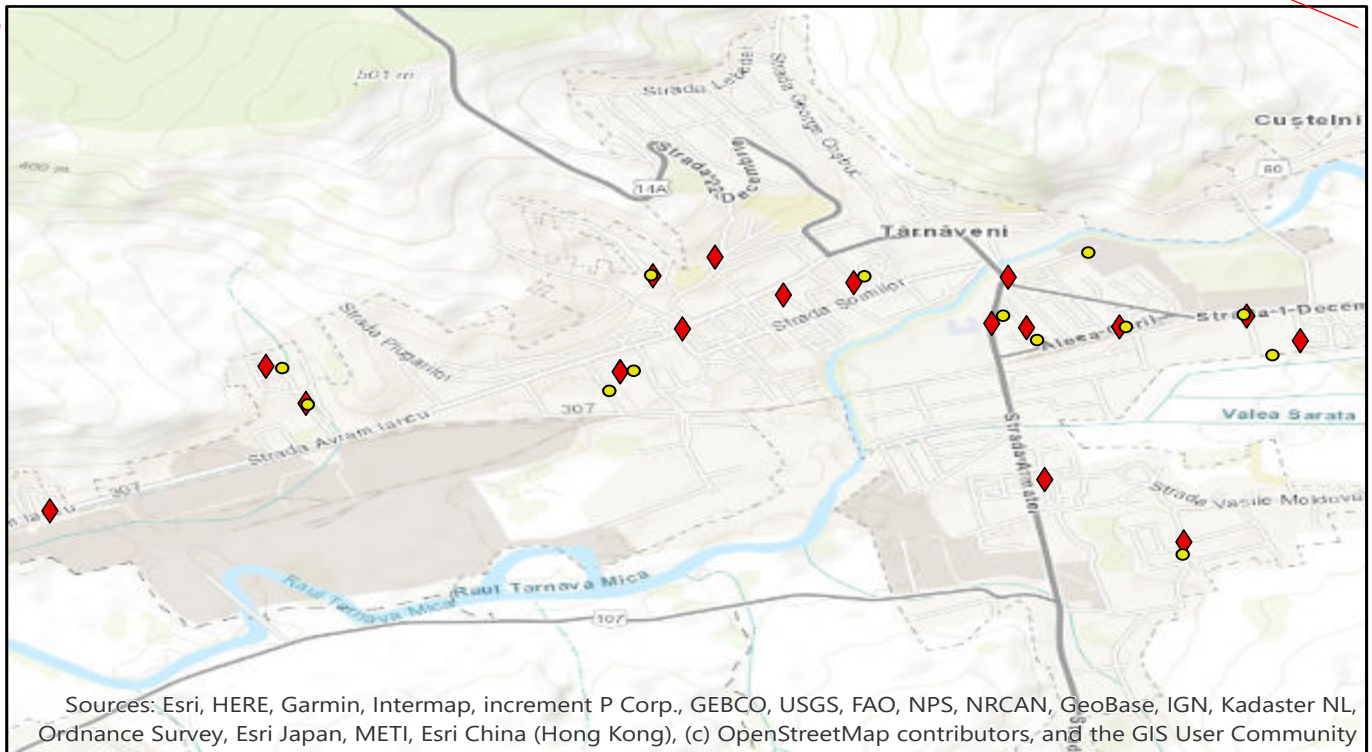
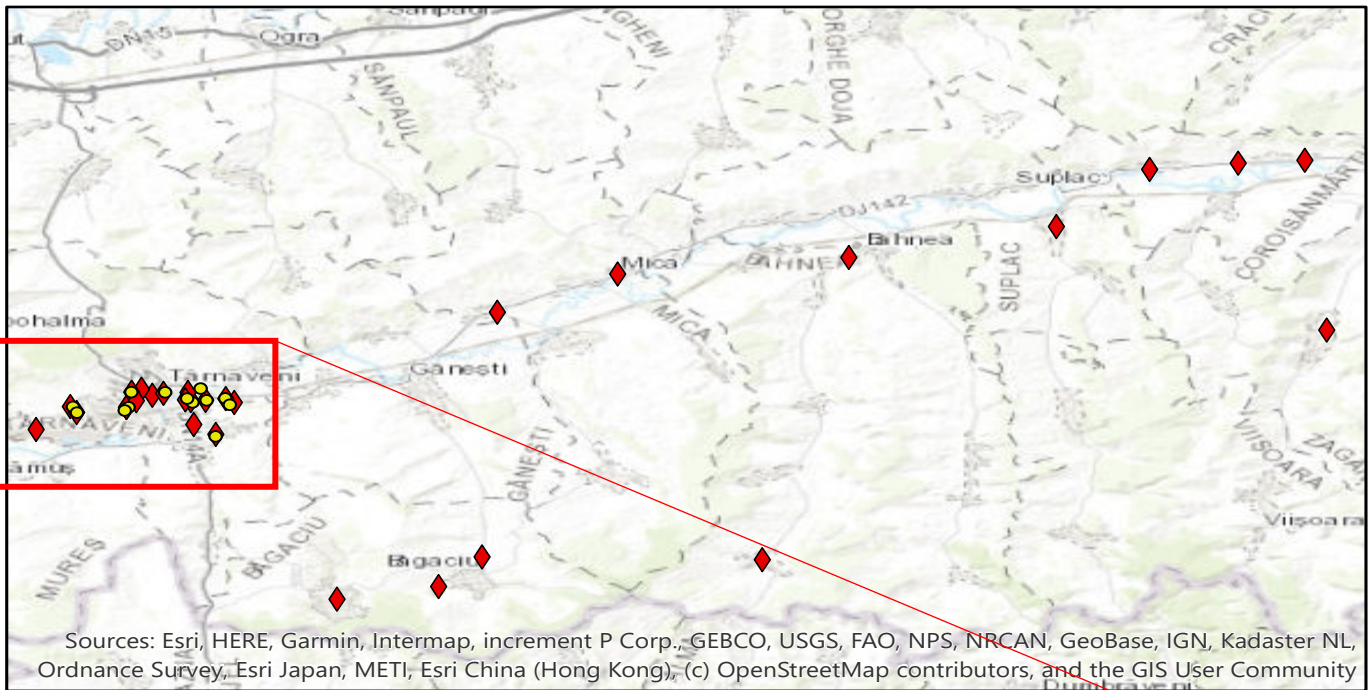
ED= Exposure Duration (months) – 9 months full exposure window for pregnancy

BW = Body Weight (kg)

The exposure dose was obtained by multiplying the metal concentration in soil by the ingestion rate and exposure window divided by averaged exposure time and body weight. For ingestion rate, 100g daily ingestion was assumed for adult women from EPA's exposure factor handbook and need to be converted through a conversion factor ( $10^{-6}$  kg/mg). The exposure frequency and exposure duration can be cancelled out with the averaging time as we were examining the daily dose to compare to the reference dose. Exposure dose in units of mg/kg bw(body weight)/day were resulted and compared to the reference dose.

### *Spatial Analysis*

The locations for the samples were mapped as followed using ArcGIS PRO. An eTrex global positioning system (GPS) handheld device (Garmin International, Inc., Olathe, USA) is used to record the latitude and longitude coordinates for soil samples location, whereas street addresses of the patients were recorded from the questionnaire. *Fig. 2* displays the soil and patient distribution in the study area, Târnăveni, Romania. Soil samples for patients from regions outside of the town (Abus, Bagaciu, Bahnea, Coroisanmartin, Delenii, Idiciu, Mica, Odrihei, Zagar) were excluded from the final analysis. The red legends denote locations of patients while the yellow circles represent where the soil sample was collected. The grey area above the Târnava Mică River was the location of the former chemical plant that this study is concerned. As observed in the generated map, residents are generally located by the river, which also passes through the chemical plant. Historic contaminations of river water by the plant is highly possible, affecting the heavy metal concentrations in soil in different regions.



*Fig. 2 Spatial distribution of soil and blood samples of patients in Tarnaveni, Romania*

## Presentation and Analysis of Findings

The population sample consists of 30 hospital patients from the Tarnaveni area. Eighteen patients (62%) live less than 5km from the former chemical plant. There are 23 cases and 7 controls that had a normal pregnancy. There were three twin cases. The demographic characteristics of the patients (mothers) are shown in *Table 3*. The age of pregnant women ranged from 14-40 years, with a median age of 24. The gestation age ranged from 6 to 40 weeks, with a median of 20.5 weeks. 62% of the sample population lived less than 5km from the former chemical plant. The ratio of smokers is 1:2, and 25% of non-smokers reported ETS exposures. None of the patients in the study reported drug or alcohol consumption during their pregnancy. In this study, eight (26.66%) of the mothers experienced death in embryo, the remaining 15 cases (50%) had diagnosed birth defects in their fetus, three of the cases with birth defects gave birth. Four mothers had a history of miscarriage or premature birth during a prior pregnancy. 37% are Roma population with a generally lower education background and employment. Among the birth defects, 20% of the cases have problems in the cardiovascular system, 13% in the nervous system, and 10% in the urogenital system.



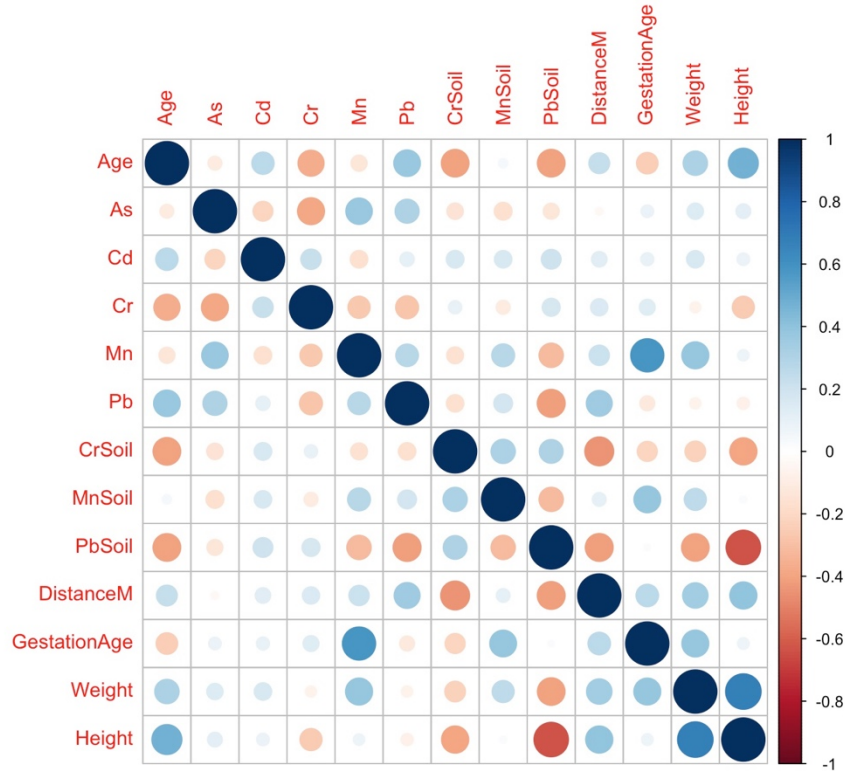
*Descriptive Statistics*

**Table 3**

Demographic characteristics of the population sample in Tarnaveni, Romania

<b>Demographic variables</b>	<b>Characteristics</b>	<b>N (%)</b>	<b>Mean</b>	<b>Min.</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>Max.</b>
Maternal age (years)			25.27	14	19	24	30.75	40
	<20	11(37%)						
	20-30	11(37%)						
Weight (kg)	>30	8 (26%)						
			53.72	45	50	52	55	75
	<50	7 (24%)						
	50-55	15 (52%)						
Height	>55	7 (24%)						
	*Missing n=1							
			166.2	150	162	168	170	180
	<160	5 (17%)						
	160-170	19 (66%)						
Gestation age (weeks)	>170	5 (17%)						
	*Missing n=1							
			21.20	6	9	20.5	32.75	40
	<10	9 (30%)						
Distance from the former chemical plant (km)	10-30	11 (37%)						
	>30	10 (33%)						
			8.983	0.2	2.05	3.6	14.8	27.9
	<5	18 (62%)						
Employment	5-10	1 (3%)						
	>10	10 (35%)						
	*Missing n=1							
	Unemployed	14 (48%)						
Education levels	Employed	13 (45%)						
	Homemaker	2 (7%)						
	*Missing n=1							
	No Schooling	5 (17%)						
	Primary School	4 (13%)						
Secondary School	4 (13%)							
High school/ Vocational School	12 (40%)							
College/university	5 (17%)							

Ethnicity	Romanian	12 (40%)
	Roma	11 (37%)
	Hungarians	6 (20%)
	Others	1 (3%)
Smoking during pregnancy	No	20 (67%)
	Yes	10 (33%)
Birth Defects	Pregnancy (Control)	7 (23%)
	Absence of fetal cardiac activity	8 (27%)
	Cardiovascular system	6 (20%)
	Nervous system	4 (13%)
	Urogenital system	3 (10%)
	Oral cavity	2 (7%)
Pregnancy monitoring	<Once a trimester	8 (29%)
	Once a trimester	14 (50%)
	>Once a trimester	6 (21%)
	*Missing n=2	
Prenatal Care	<1 Month	10 (33%)
	>1 Month	20 (67%)
Prenatal Vitamin Use	No	18 (78%)
	Yes	5 (22%)
	*Missing n=7	
Time at current residence	<5 years	3 (10%)
	5 - 9 years	2 (7%)
	10- 14 years	9 (30%)
	>15 years	16 (53%)
Source of exposure near home	No	23 (77%)
	Yes	7 (23%)
Total		
		30 (100%)



*Fig. 3 Correlation Plot of Continuous Variables (Age, Metal Concentrations in Blood, Metal Concentration in Soil, Distance from Chemical Plant, Gestation Age, Weight, and Height)*

*Fig. 3* demonstrates the correlation plot of all continuous variables of my data, including Age, Metal Concentrations in Blood, Metal Concentration in Soil, Distance from Chemical Plant, Gestation Age, Weight, and Height. A preliminary analysis of the relationship of various factors is derived from visual inspection of this plot. For instance, Cr and Pb concentrations in soil increases as the proximity is closer to the former chemical plant. There is also an interesting negative correlation between gestation age and Mn concentration in blood. Further investigations will then be made. The normality of each variable’s distribution and natural log transformation is checked to see if there are log-normal distributions for regressions (See Appendix).

**Table 4**

Heavy metal concentration in blood of the population sample in Tarnaveni, Romania

<b>Metal</b>	<b>Mean (µg/dl)</b>	<b>Range</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>90<sup>th</sup> percentile</b>
Arsenic (As)	0.1945	0.0044- 0.8237	0.0328	0.0874	0.3202	0.6058
Cadmium (Cd)	0.0983	0.0413- 0.3574	0.0644	0.0710	0.1197	0.1562
Chromium (Cr)	0.5445	0.3617- 1.3666	0.4412	0.4824	0.5668	0.6744
Manganese (Mn)	1.963	0.85- 3.506	1.613	1.852	2.265	2.775
Lead (Pb)	2.005	0.644- 8.082	1.155	1.657	1.987	3.1611

**Table 5**

Heavy metal concentration in blood of cases in Tarnaveni, Romania

<b>Metal</b>	<b>Mean (µg/dl)</b>	<b>Range</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>90<sup>th</sup> percentile</b>
Arsenic (As)	0.1801	0.0044- 0.6903	0.0333	0.0820	0.2681	0.5605
Cadmium (Cd)	0.0912	0.0413- 0.2123	0.0623	0.0690	0.1155	0.1510
Chromium (Cr)	0.5548	0.3878- 1.3666	0.4412	0.4905	0.5668	0.6625
Manganese (Mn)	1.908	1.024- 3.506	1.565	1.848	2.082	2.573
Lead (Pb)	2.162	0.93- 8.082	1.211	1.678	2.260	3.470

**Table 6**

Heavy metal concentration in blood of controls in Tarnaveni, Romania

<b>Metal</b>	<b>Mean (µg/dl)</b>	<b>Range</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>90<sup>th</sup> percentile</b>
Arsenic (As)	0.2416	0.0124- 0.8237	0.0531	0.0890	0.3299	0.6626
Cadmium (Cd)	0.1217	0.0642- 0.3574	0.0687	0.0791	0.1069	0.2178
Chromium (Cr)	0.5110	0.3617- 0.73	0.4253	0.4741	0.5804	0.6692
Manganese (Mn)	2.145	0.85- 3.306	1.762	1.965	2.683	2.960
Lead (Pb)	1.489	0.644- 2.183	1.147	1.635	1.834	2.067

**Table 7**

Heavy metal concentration in soil from the study area in Tarnaveni, Romania

<b>Metal</b>	<b>Mean (mg/kg)</b>	<b>Min.</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>90<sup>th</sup> percentile</b>	<b>Max.</b>
Chromium (Cr)	34.76	18.54	22.81	26.96	45.20	56.05	71.36
Manganese (Mn)	780.1	482.6	646.5	760.1	905.5	1006.5	1281.5
Lead (Pb)	51.81	25.43	30.50	44.48	62.52	91.02	136.72

Table 4,5,6,7 provided a detailed summary of the geometric mean and distribution of the heavy metal concentrations in blood and in soil. The range of heavy metals in soil is 18.54-71.36 mg/kg for Cr, 482-1281.5 mg/kg for Mn and 25.43-136.72 mg/kg for Pb, while the Romanian regulatory exposure alert threshold level for residential soils is 100 mg/kg, 1500 mg/kg, 50 mg/kg respectively (Mihaileanu *et al.*, 2019). It is worth noted that more than a quartile of the sample's Pb concentration has exceeded the alert threshold level. An interesting observation is that the blood level concentration of Mn and Pb are relatively similar but very different in soil concentration, suggesting that exposure to Pb may potentially came from different sources. There is no apparent difference in concentrations between cases and controls by visual inspection. More evaluations can be found in the appendix with other visual presentations.

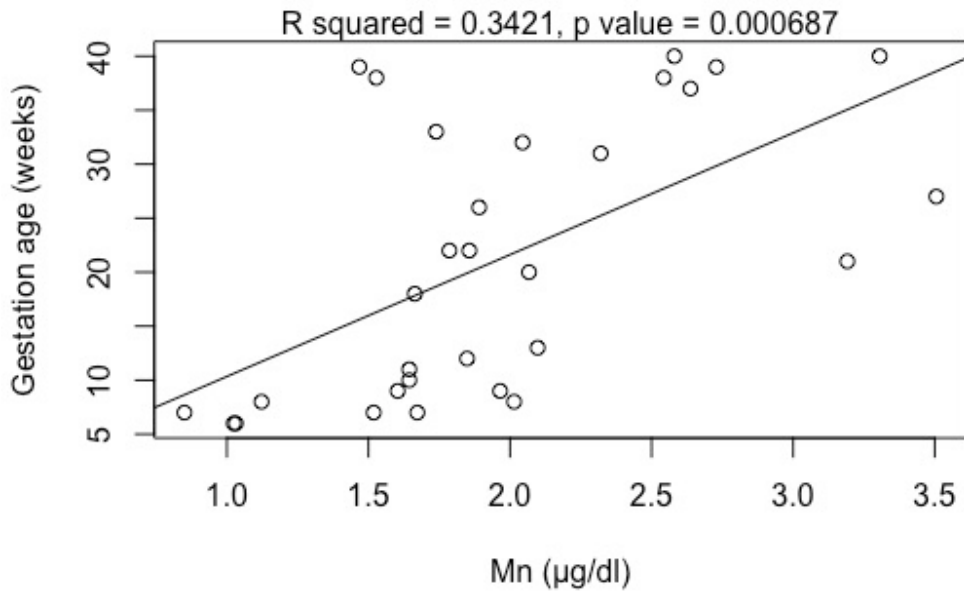
The distribution of selected characteristics between cases and controls is shown in table 8. Pearson's Chi-squared test was used to determine the p-value. Categorical variables for age, education level, employment, gestation age, distance from the chemical plant, pregnancy monitoring, malformation and time of residency are created for comparison. However, all factors displayed no statistical significance despite birth defects (malformation). This may be due to very small sample size (23 cases and 7 controls).

**Table 8.** Distribution of Selected Characteristics of the Study Population in Tarnaveni, Romania (N=30).

	Case (N=23)		Control (N=7)		p-values
	Number	%	Number	%	
<b>Age (years)</b>					
<20	9	39.2	2	28.6	<b>0.4206</b>
20-30	7	30.4	4	57.1	
>30	7	30.4	1	14.3	
<b>Education (years)</b>					
≤9	11	47.8	2	28.65	<b>0.5363</b>
10-15	9	39.2	3	42.9	
≥16	3	13.0	2	28.65	
<b>Employment</b>					
No	13	56.5	3	42.9	<b>0.7445</b>
Yes	9	39.2	4	57.1	
Missing	1	4.3			
<b>Gestation age (weeks)</b>					
<10	7	30.4	2	28.6	<b>0.2458</b>
10-30	10	43.5	1	14.3	
>30	6	26.1	4	57.1	
<b>Smoking during pregnancy</b>					
No	15	65.2	5	71.4	<b>0.7602</b>
Yes	8	34.8	2	28.6	
<b>Distance from the former chemical plant (km)</b>					
<5	14	60.9	4	57.1	<b>0.1123</b>
5-10	0	0	1	14.3	
>10	9	39.1	1	14.3	
Missing			1	14.3	
<b>Malformation</b>					
Absence of fetal cardiac activity	8	34.8			<b>&lt;0.0001</b>
Cardiovascular system	6	26.1			
Nervous system	4	17.4			
Urogenital system	3	13.0			
Oral cavity	2	8.7			
Pregnancy			7	100	
<b>Pregnancy Monitoring</b>					
<once a trimester	7	30.4	1	14.3	<b>0.4177</b>
=once a trimester	9	39.2	5	71.4	
<once a trimester	5	21.7	1	14.3	
missing	2	8.7			

Prenatal Care							
<1 Month	6	26.1	4	57.1	<b>0.127</b>		
>1 Month	17	73.9	3	42.9			
Time at current residence							
<5 years	2	8.7	1	14.3	<b>0.4634</b>		
5 - 9 years	0	0	2	28.6			
10- 14 years	8	34.8	1	14.3			
>15 years	13	56.5	3	42.9			
Vitamin Use							
No	13	56.5	5	71.4	<b>0.7595</b>		
Yes	4	17.4	1	14.3			
missing	6	26.1	1	14.3			
Source of exposure near home							
No	17	73.9	6	85.7	<b>0.518</b>		
Yes	6	26.1	1	14.3			

Linear regression was used to evaluate the relationship between metal concentrations in soil, and the distance from the chemical factory, metal concentrations in blood on gestation age, and the association between metal concentrations in blood and/or soil and fetus' death in the embryo. In terms of the metal concentrations in blood and embryo death, none of the metals show statistical significance individually, but Mn showed a slightly negative linear relationship with embryo death when multiple regression was performed. As shown in *fig. 4*, Mn was also significant (p-value: 0.000687) when predicting gestation age, while other variables were statistically insignificant.

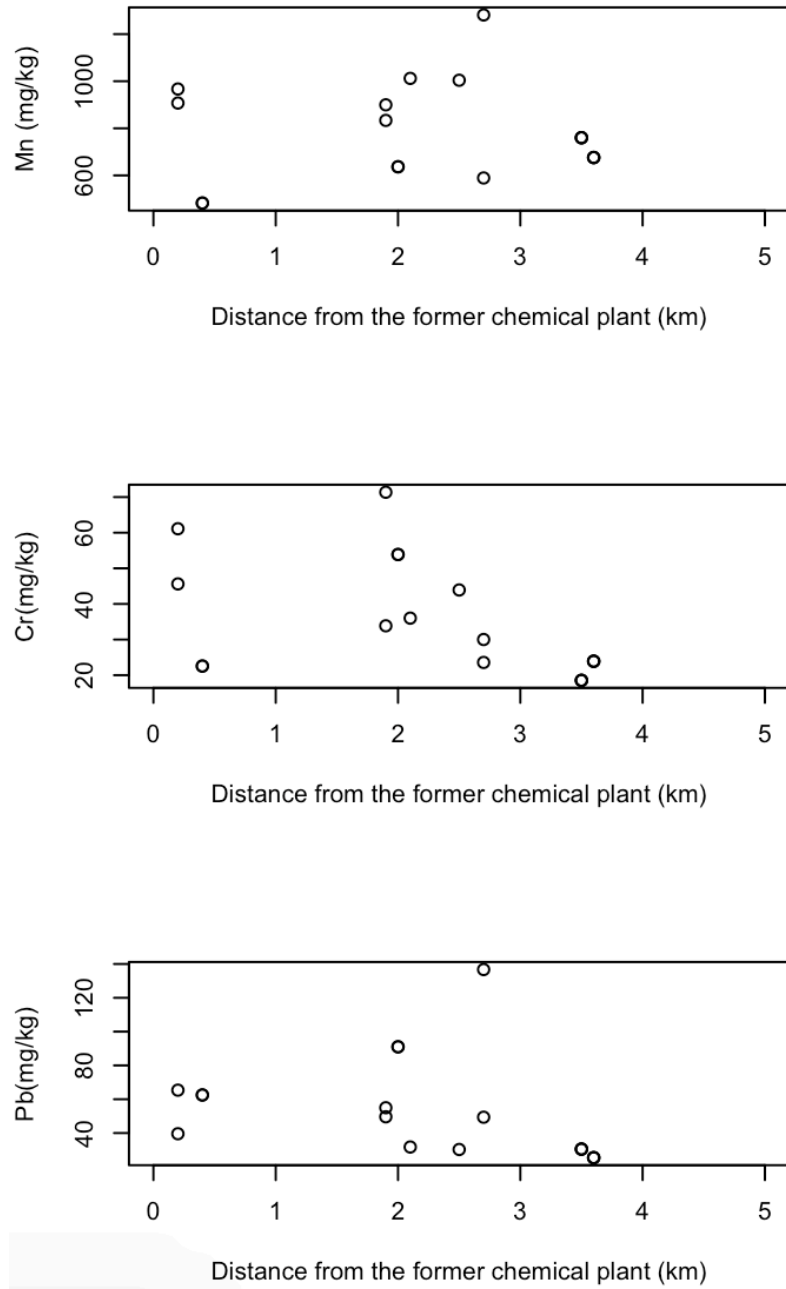


*Fig. 4 Linear regression and p values among gestation age and Mn concentrations in the blood samples*

Linear regression was performed on soil metal concentrations and the distance and proximity from the former chemical plant. The Cr concentration in soil was significantly correlated with a p-value of 0.0484 and 0.2221 multiple R-squared. The results were plotted in *Fig. 5*. The p-values for Mn and Pb concentrations were 0.904 and 0.16824 respectively.

With regard to the Hazard quotient and Hazard Index, all of the estimated HI of different end-points were below 1.00 (Appendix), indicating that exposure to the total concentrations of Cr, Mn, and Pb in soil did not pose a serious health hazard to the patients. The HI was slightly higher for cardiovascular effects which might reflect in the greater proportion of cardiovascular events seen in the babies (26.1%; *Table 8*). Since Cr, Mn, and Pb are all capable in crossing the placenta, the risks on fetus or embryo was hypothesized to be relatively proportional to the risks of mother.





*Fig. 5 Linear regression of the metal concentrations in the residential soil samples and distance from a former chemical plant in Tarnaveni, Romania*

## Limitations and Confounders

The biggest limitation in the study is the sample size, which may have limited the ability to observe statistically significant correlations and differences, whether it is a strong association or just random incidence. A small sample size has a higher variability and therefore lower reliability, which is decreasingly representative of the entire population. It also led to biases as well as reduces the power of a study and increases the margin of error. There is a generally a strong interaction between age, lifestyle factors, like smoking status and alcohol intake, social-economic status, nutritional factors during pregnancy, which complicates the proper interpretation of associations. Failure to account for these factors may attenuate or strengthen the apparent associations between heavy metal exposure and the outcome, depending on the direction of the effect of the variable on the outcome.

There are great difficulties as it is assessing a mixture of heavy metals, because their real-world interaction effects are still in explorations. Metal-metal interactions in body or soil were not accounted. Other exposed chemicals that may affect the toxicity of the target metal should be considered. For example, Pb is released from the mother's bones along with calcium during pregnancy. Various assumptions were made during the calculation of HI, such as uniform intake of metals from the soil across the entire pregnancy period, which are important limitations that may have influenced results in the calculation. The end-point effects were calculated with respect to the mother's exposure and intake. Assumptions in calculating the intake may also not uphold in the actual situations. The risks for fetuses or embryo might not be necessarily identical to the risks in mother, as metal transformation from mother to fetuses can be affected by dose penetration or other unknown variables. When considering the reference dose for each end-point, people should

also be alerted that there is an inhalation to ingestion extrapolation for RfC to RfD since human studies are limited.

Other sources of metals were unclear in this study. To further assess the overall impact of the historical chemical plant site to the surrounding environment, other exposure pathways should be assessed to obtain a greater overview of all routes of exposures and sources of contaminations. Adults absorb 35–50% of Pb through drinking water, and the absorption rate for children may be greater than 50% (Tchounwou *et al.*, 2012). Water sampling may provide more insight in account to the high BPb level in this study. It is important to note that many heavy metals may accumulate in the food chain via contaminated vegetables, especially Cr in root vegetables. By carrying out proper vegetable sampling and analysis, concentrations of metals are measured in vegetable samples collected from the area, and the transfer of heavy metals in soil to crops ingested can be examined. The exposure via contaminated environmental media would also be better evaluated.

Occupational exposure of patients should be recorded. Information such as occupation of other family member (father) in the household or average daily time that patient spent at residency can be collected. Although the effect of smoking was not statistically significant in this study, it is also essential to know that heavy metals such as As, Cd, Cr, and Pb are used in cigarette (Caruso *et al.*, 2013; Janaydeh *et al.*, 2019), and may be vital when considering the heavy metal exposures and the health effects.

## Conclusion

This study provided preliminary evidence of the effects of heavy metal exposure (Cr, Mn, Pb) from a former industrial region on birth outcomes. In this study, a positive relationship between Cr concentration in soil and the distance of the former chemical plant is reported, which is a logical reflection as the plant has a history of storing chemical waste from Cr compounds. The relatively similar blood level concentration of Mn and Pb but very different concentration in soil suggested for other sources of Pb exposure. There is a correlation between blood Mn concentration and gestational age and death of embryo and fetus. Although each of the end-point specific hazard indexes did not exceed 1, the potential health risks of the mixture of heavy metals exposure and their targeted organs matched with the malfunctions or birth defects found in the cases.

In terms of future studies, a larger cohort with a higher sample size is recommended. Occupational exposure should be considered and we could also set and/or compare controls with other populations for comparisons. Other exposure pathways can be assessed to obtain a greater overview of all routes of exposures and sources of contaminations, and further assess the overall impact of the historical chemical plant site to the surrounding environment. Air and water sampling may be considered to examine exposures through inhalation and water ingestion. The heavy metal concentration in water also provide information in how metals are transported or transferred in various media. Vegetables sampling in the area is recommended to observe contamination of the local food chain. Investigations can be made on the transfer of heavy metals in soil to crops ingested. Child development is important when considering heavy metals exposure, therefore, a cohort that focuses on longer-run can be proposed. In-depth analyses on all interactions between heavy metals-heavy metals (e.g. Cr on Mn) in human body will be very helpful in this field. It is

also recommended to develop better strategies for tackling the issues that would reflect real-world exposure effects. With the difficulties in characterizing the toxicities, we should develop good strategies for tackling the issues that would reflect real-world exposure effects.

Few studies have addressed non-occupational exposure to environmental toxins and heavy metals contamination, and that little is known about the long-term effect for children and women exposed during childhood or the reproductive and developmental period. Adverse pregnancy outcomes were associated with heavy metals exposure is also overlooked, with conflicting results of different studies on the threats to the fetus at low levels of exposure to heavy metals and trace elements (Zheng *et al.*, 2014). Therefore, it is important to further evaluate the association between historical environmental heavy metal contamination and its health effects, especially in vulnerable population.

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## Appendix

Fig. 6

Histogram showing the distribution and log transformation of the metal concentrations in soil

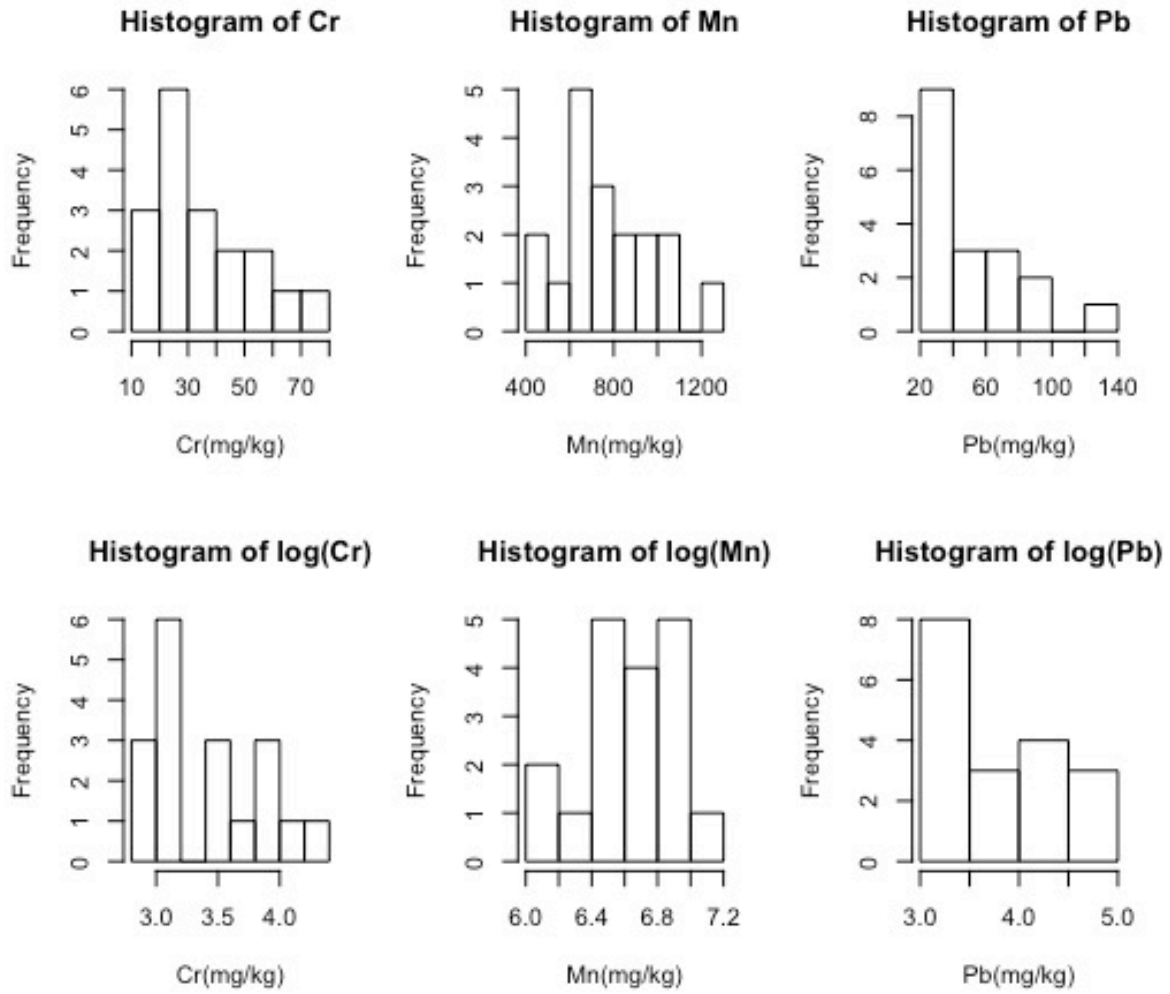




Fig. 7

Histogram showing the distribution and log transformation of the metal concentrations in blood

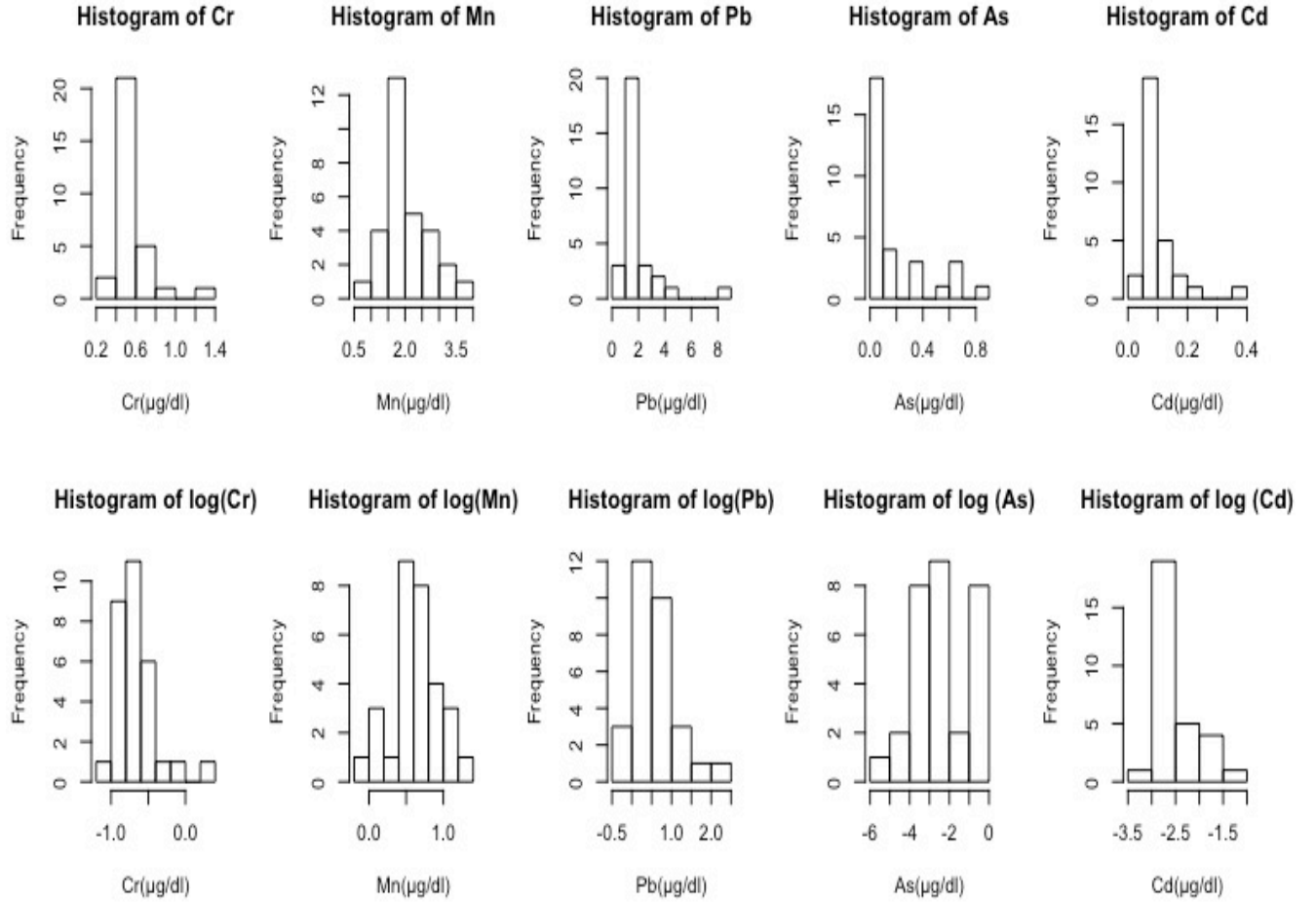


Fig. 8

Boxplot showing the distribution of the metal concentrations in soil

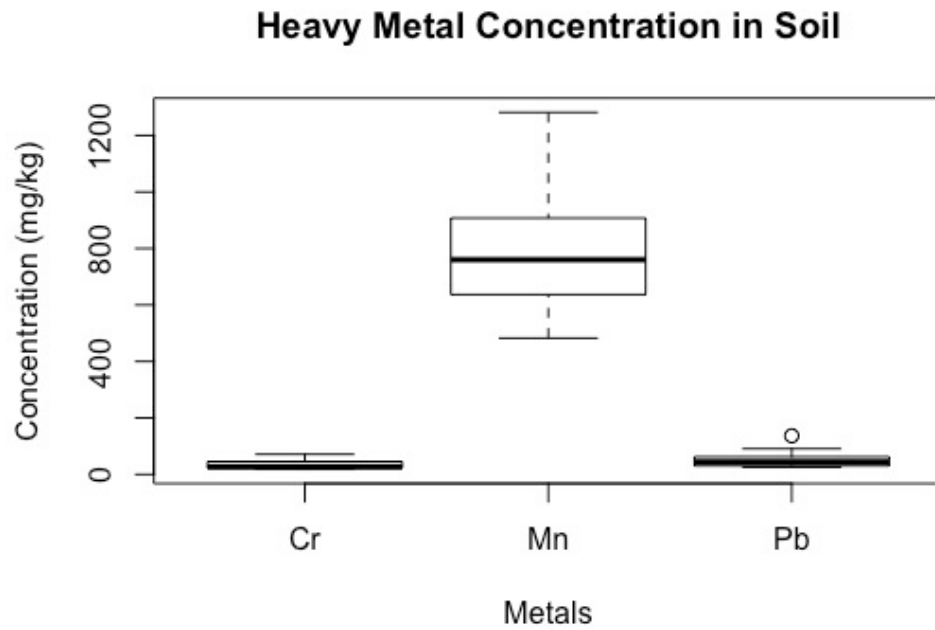
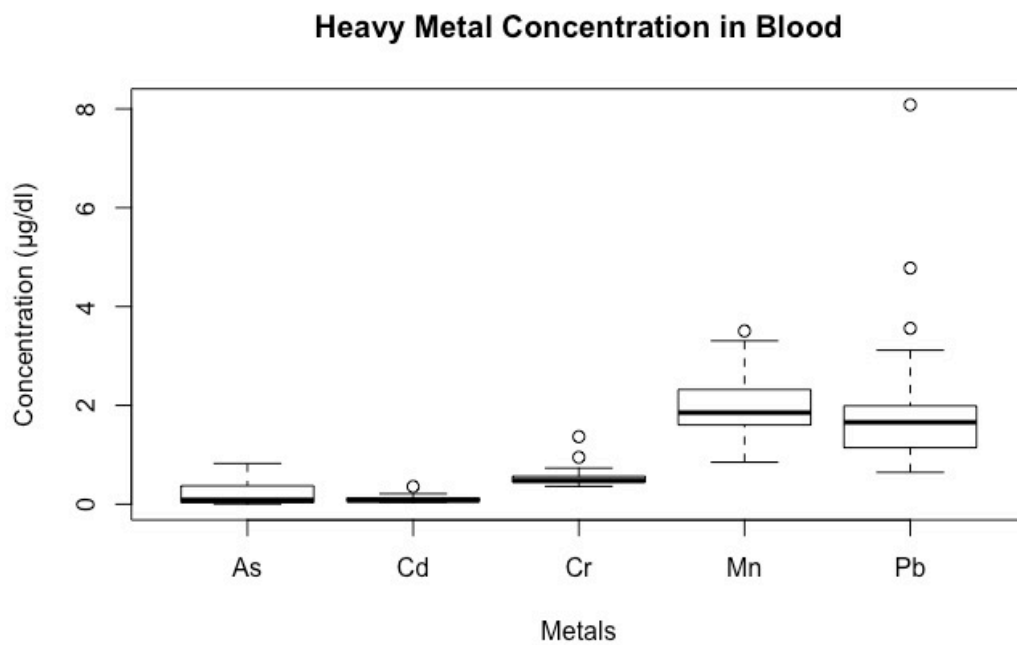


Fig. 9

Boxplot showing the distribution of the metal concentrations in blood



**Table 9**  
Heavy metal concentration difference between cases and controls

<b>Metal</b>	<b>Mean (µg/dl)</b>	<b>Min.</b>	<b>25<sup>th</sup> percentile</b>	<b>Median</b>	<b>75<sup>th</sup> percentile</b>	<b>90<sup>th</sup> percentile</b>	<b>Max.</b>
Arsenic (As)	-0.0615	-0.008	-0.0198	-0.007	-0.0618	-0.1021	-0.1334
Cadmium (Cd)	-0.0305	-0.0229	-0.0064	-0.0101	0.0086	-0.0668	-0.1451
Chromium (Cr)	0.0438	0.0261	0.0159	0.0164	-0.0136	-0.0067	0.6366
Manganese (Mn)	-0.237	0.174	-0.197	-0.117	-0.601	-0.387	0.2
Lead (Pb)	0.673	0.286	0.064	0.043	0.426	1.403	5.899

**Table 10**  
Hazard Quotient and Hazard Index of end-point specific outcomes

ID	HQ neuro Bpb	HI Nuero	HI Neuro*Bp	HQ CrRenal	HQ PbRenal	HQ PbRenal	HI Renal	HI Renal*bp	HQ PbCardic	HI Cardio B	HI Hemato Bpb
1	0.1979					0.0582059		0.0582059		0.1979	0.1979
2	0.1678	0.0241005	0.1918954	0.0047813	1.496E-07	0.0493529	0.004781445	0.0541342	0.2543242	0.1678	0.183737652
3	0.1498	0	0.1498	0		0.0440588		0.0440588		0.1498	0.1498
4	0.8082	0	0.8082	0		0.2377059		0.2377059		0.8082	0.8082
5	0.093	0.0301931	0.1231741	0.0112247	0.26769201	0.0273529	0.27891672	0.0385777	0.9101528	0.093	0.130415703
6	0.1279	0.0219095	0.1498049	0.0043466	0.07480125	0.0376176	0.07914788	0.0419643	0.2543242	0.1279	0.142388775
7	0.093	0.0301931	0.1231741	0.0112247	0.26769201	0.0273529	0.27891672	0.0385777	0.9101528	0.093	0.130415703
8	0.1934	0.0333264	0.2267172	0.0118935	0.16168551	0.0568824	0.173579056	0.0687759	0.5497307	0.1934	0.233045152
9	0.1192	0	0.1192	0		0.0350588		0.0350588		0.1192	0.1192
10	0.199	0	0.199	0		0.0585294		0.0585294		0.199	0.199
11	0.1143	0.0224743	0.1367458	0.00491	0.40210435	0.0336176	0.407014377	0.0385277	1.3671548	0.1143	0.130666747
12	0.1132	0	0.1132	0		0.0332941		0.0332941		0.1132	0.1132
13	0.1142	0.0442908	0.1584763	0.0135804	0.19225615	0.0335882	0.205836558	0.0471686	0.6536709	0.1142	0.159468017
14	0.1745	0.0355224	0.2100141	0.0050024	0.14522032	0.0513235	0.150222684	0.0563259	0.4937491	0.1745	0.19117456
15	0.0644	0.0183128	0.0827003	0.0045122	0.18389202	0.0189412	0.188404223	0.0234534	0.6252329	0.0644	0.079440662
16	0.1722	0	0.1722	0		0.0506471		0.0506471		0.1722	0.1722
17	0.1518	0	0.1518	0		0.0446471		0.0446471		0.1518	0.1518
18	0.2183	0	0.2183	0	0.11645453	0.0642059	0.116454533	0.0642059	0.3959454	0.2183	0.2183
19	0.1967	0.0254324	0.2221263	0.0037086	0.08969626	0.0578529	0.093404904	0.0615616	0.3049673	0.1967	0.209062157
20	0.4777	0.0241005	0.5017954	0.0047813	0.07480125	0.1405	0.079582543	0.1452813	0.2543242	0.4777	0.493637652
21	0.1678	0	0.1678	0		0.0493529		0.0493529		0.1678	0.1678
22	0.1635	0.02051	0.1840051	0.0029908	0.08969626	0.0480882	0.092687102	0.0510791	0.3049673	0.1635	0.173469482
23	0.3558	0.0339978	0.3897868	0.0075234	0.14590204	0.1046471	0.153425433	0.1121705	0.4960669	0.3558	0.380877992
24	0.1098	0.016648	0.1264366	0.004102	0.18389202	0.0322941	0.187994023	0.0363961	0.6252329	0.1098	0.123473329
25	0.254	0.033448	0.2874421	0.0066694	0.09336542	0.0747059	0.100034781	0.0813752	0.3174424	0.254	0.276231197
26	0.1613	0.0195634	0.1808587	0.0028528	0.08969626	0.0474412	0.092549063	0.050294	0.3049673	0.1613	0.170809352
27	0.1517	0	0.1517	0		0.0446176		0.0446176		0.1517	0.1517
28	0.1102	0.0360388	0.146233	0.0084465	0.08903888	0.0324118	0.097485381	0.0408583	0.3027322	0.1102	0.138355014
29	0.282		0.282			0.0829412		0.0829412		0.282	0.282
30	0.3117		0.3117			0.0916765		0.0916765		0.3117	0.3117