CARDIOVASCULAR EFFECTS OF LEAD AND MERCURY AND THEIR MIXTURES IN RATS

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By

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ABSTRACT

Cardiovascular diseases are the major cause of death worldwide. It is a group of diseases, which affect the heart, the vasculature and the brain. Lifestyle and metabolic risk factors are major contributors to cardiovascular ill-health. In addition to these risk factors, a growing number of scientific studies show that some environmental pollutants, e.g. lead and mercury, can adversely affect cardiovascular health. Despite the increasing amount of knowledge from human and animal studies, cardiovascular effects of lead, mercury species or their mixtures are not well understood. It is also unknown if safe exposure thresholds for these metals exist or the underlying mechanisms of action for the elicitation of cardiovascular toxicity.

The first set of studies had the objectives to elucidate the range of effects of single exposure to lead, inorganic mercury or methylmercury on the cardiovascular system. Therefore, male Wistar rats were exposed to a broad range of doses of lead, inorganic mercury or methylmercury for four weeks through the drinking water. Cardiovascular health of the rats was assessed by measuring the blood pressure and the cardiac electrical activity after four weeks of exposure, while the heart function and blood flow in the carotid artery was measured at baseline and at the end of the exposure duration. The study showed that all three metals differ in their effects on the cardiovascular system. Lead showed bi-phasic dose-response curves for several cardiovascular end-points. No cardiovascular effects were observed for inorganic mercury, while methylmercury showed linear dose-response curves. Based on these results, safe levels of exposure for lead and methylmercury were derived.

The second study applied the same experimental design as the previous study in order to investigate the cardiovascular effects of combined exposures to lead, inorganic mercury and methylmercury. The mixture ratios were based on reference and exposure values published in the scientific literature. The adverse cardiovascular effects, which were observed for single exposures were reversed for the mixtures indicating antagonism. In contrast to single exposures, mixtures negatively affected the electrical activity of the heart (synergism), which could lead to arrhythmias and heart failure.

The third set of studies focused on the exploration of oxidative stress, kidney function and damage, and global DNA methylation as potential mechanisms of action for the development of elevated blood pressure. Results for lead showed an increase in oxidative stress but not mercury. While only lead was associated with kidney damage, only inorganic mercury was related to altered global DNA methylation. Methylmercury appears to elevate blood pressure through a not investigated

mechanism. Therefore, oxidative stress and kidney damage seem to be associated with elevated blood pressure but not global DNA methylation.

Overall, the research presented in this thesis shows that lead, inorganic mercury and methylmercury and their mixtures have the ability to adversely affect the cardiovascular system. However, each metal affected the cardiovascular system differently and surprisingly, mixtures showed antagonism or synergism depending on the examined end-point, which was reflected in the results of the mechanistic study. As health problems of the cardiovascular system, e.g. hypertension, occur mainly in the adult population and in particular the elderly, cardiovascular effects should be considered as an important end-point for this age group in addition to neurodevelopmental effects in children.

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LIST OF ABBREVIATIONS

AL	Acceptable level
AOP	Adverse Outcome Pathway
BLL	Blood Lead Level
BMD	Benchmark dose
BMDL	Benchmark dose lower bound
BP	Blood Pressure
CCAC	Canadian Council on Animal Care
CVDs	Cardiovascular diseases
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDV	End-diastolic volume
EFSA	European Food Safety Authority
ESV	End-systolic volume
GSH	Glutathione
GSSG	Glutathione disulfide, oxidized glutathione
Hg(II)	Inorganic mercury ($HgCl_2$)
HR	Heart rate
KIM-1	Kidney Injury Molecule 1
LOAEL	Lowest observed adverse effect level
MeHg(I)	Organic mercury (mono-methylmercury-chloride, CH_3-Hg-Cl)
MOA	Mechanism of Action
NHANES	National Health and Nutrition Survey

NO	Nitric oxide
NOAEL	No observed adverse effect level
Pb(II)	Lead acetate
pTDI	Provisional Tolerable Daily Intake
RfD	Reference Dose
ROS	Reactive Oxygen Species
SV	Stroke volume
TWI	Tolerable Weekly Intake
US EPA	United States Environmental Protection Agency

1 GENERAL INTRODUCTION

1.1 Introduction

Non-communicable diseases, such as cardiovascular diseases (CVDs) or cancer, have exceeded the number of deaths from communicable diseases (Laslett et al., 2012). Presently, CVDs are responsible for more than 17 million per year, which renders this disease group the major cause of death worldwide. Taking into account that the number of deaths is expected to pass 23 million by 2030, heart attacks and stroke, as the most important representatives for CVDs pose an important public health problem (WHO, 2014). Cardiovascular diseases are chronic and characterized by long disease duration, slow progression and their potential prevention through a reduction of risk factors (WHO, 2013).

During previous decades, more and more risk factors were discovered and were categorized as behavioral, physiological/metabolic and socio-economic risk factors. Unhealthy behaviors include cigarette smoking, lack of exercise, high salt diets or excess consumption of alcohol (Mendis et al., 2011). These risk factors can lead to metabolic disturbances (Danaei et al., 2009) by increasing blood pressure (hypertension), blood sugar (diabetes), blood lipids (hyperlipidemia) or body weight (overweight/obesity) (Laslett et al., 2012), which constitute risk factors of their own. In contrast to the behavioral risk factors, CVDs can also be the result of the patient's genetic make-up, gender or age. Finally, the socio-economic environment, e.g. low income and education status, and psychological factors contribute to the development of atherosclerosis, which is a risk factor of its own for CVDs (Mendis et al., 2011). Atherosclerosis is characterized by a complex pathological process, which leads to the narrowing of blood vessels. The risk for a heart attack or stroke increases with the number of existing risk factors (The Conference Board of Canada, 2010).

While the prevalence of some risk factors, such as tobacco use, declined in many parts of the world, obesity and hypertension increased (Laslett et al., 2012). Since 1980, the number of obese men and women doubled worldwide, reaching 10% for men and 14% for women (Malik et al., 2013). However, obesity rates passed 30% in North America, in parts of Latin America, North Africa and the Middle East (Malik et al., 2013). A similar trend can be observed with blood pressure. While in 1980 about 600 million adults over the age of 25 years were diagnosed with hypertension, in 2008, this number had risen to almost one billion corresponding to about 40% (Mendis et al., 2011).

Over the next 15 years, it is expected that the costs associated with CVDs will rise from US$863 billion to US$20 trillion (Mensah et al., 2014). These costs can be allocated to direct health costs, such as hospitalization, medication and physician care, and indirect costs, which result in economic

losses (Tarride et al., 2009). The impaired health of patients suffering from CVDs leads to a reduced quality of life through disabilities and premature death (Tarride et al., 2009).

1.1.1 Rationale

There is increasing evidence that exposure to lead or mercury species is a risk factor for the development of cardiovascular diseases. In particular, the association between lead exposure and hypertension is considered to be causal (Nawrot et al., 2002; Navas-Acien et al., 2007; Scinicariello et al., 2011). Although the picture for mercury species is less clear, studies exist which indicate an association between mercury exposure and cardiovascular effects (Vupputuri et al., 2005; Bautista et al., 2009; Lim et al., 2010; Roman et al., 2011). As CVDs can be prevented by a reduction of risk factors, it is worth investigating the cardiovascular effects of exposures to lead and mercury species in more detail.

The overall purpose of this research was to elucidate the cardiovascular effects of exposure to lead or mercury species alone or their mixtures and to investigate underlying mechanisms of action for cardiovascular toxicity. Due to the limited availability of studies on a broad variety of cardiovascular end-points and often inconclusive results, the first step was to investigate the effects of single metal exposure on the cardiovascular system. In a second step, effects of combined exposures, which better reflect the real-life situation of humans, on the cardiovascular system were assessed. The last step was to explore different mechanisms of actions on how single and combined metal exposures induce cardiovascular toxicity.

1.1.2 Hypotheses

As part of this research project the following hypotheses were examined:

1. Exposure to lead (Pb(II)), inorganic mercury (Hg(II)) or methylmercury (MeHg(I)) will adversely affect blood pressure, heart function, blood flow and electrical activity of the heart in rats.

2. Concomitant exposure to Pb(II), Hg(II) and MeHg(I) will aggravate the cardiovascular effects of single metal exposures.

3. The three metals Pb(II), Hg(II) and MeHg(I) will induce cardiovascular toxicity through the same mechanism of action.

1.1.3 Objectives

The objectives of this research project were investigated by the following three experiments:

Experiment 1: Which are the effects on the cardiovascular system due to single exposure to Pb(II), Hg(II) or MeHg(I) in adult male rats?

1. To investigate cardiovascular health, blood pressure, heart function, blood flow and cardiac electrical conductivity were measured in adult male rats.
2. To derive safe exposure levels for Pb(II), Hg(II) and MeHg(I), which are protective against hypertension, from the animal studies for the human adult population.

Experiment 2: What are the effects on the cardiovascular system due to combined exposure to Pb(II), Hg(II) and MeHg(I) in adult male rats?

1. To determine mixture effects, such as synergism or antagonism, from mixtures containing Pb(II), Hg(II) and MeHg(I).
2. To investigate the influence of the ratio between Pb(II), Hg(II) and MeHg(I) on the cardiovascular end-point.
3. To evaluate if mixture effects of Pb(II), Hg(II) and MeHg(I) on the cardiovascular system can correctly be predicted from single metal exposures.

Experiment 3: Through which mechanism(s) of action do Pb(II), Hg(II) and MeHg(I) and their mixtures elicit cardiovascular toxicity?

1. To investigate oxidative stress levels, kidney function and global DNA methylation status as potential mechanisms of action.
2. To identify differences between single metal exposure and combined exposure on cardiovascular end-points.

1.2 Organization of the dissertation

The research in this dissertation is presented in manuscript format following the style requirements of peer-reviewed journals. The introduction in Chapter 1 and the literature review in Chapter 2 provide an overview of the overall research topic. Each manuscript-based chapter (Chapters 3 – 5) starts with a preface, which links the specific content of the manuscript to the thesis as a whole. The

remainder of the chapters follows the structure of a scientific article: abstract, introduction, materials and methods, results, discussion and conclusions.
The three manuscript-based research chapters address the following objectives:

Chapter 3: Investigate the range of adverse cardiovascular effects resulting from single exposure to Pb(II), Hg(II) or MeHg(I) in rats to broaden the understanding of relevant cardiovascular end-points,

Chapter 4: Explore the cardiovascular effects of combined exposures to Pb(II), Hg(II) and MeHg(I) and evaluate the usefulness of single metal exposure effects for predicting mixture effects,

Chapter 5: Analyze effects of single and combined exposures of Pb(II), Hg(II) and MeHg(I) on reactive oxygen species/antioxidant balance, kidney function and global DNA methylation status to understand how these mechanisms of action interact in the development of cardiovascular toxicity.

The final chapter (Chapter 6) interprets the major research results as a whole and provides recommendations based on open questions for future research. This chapter is followed by a reference section (Chapter 7). Chapter 8 contains appendices A-C. Appendix A includes supplementary data to Chapter 3. Appendix B presents results on cardiovascular effects from single and combined exposures to Pb(II), Hg(II) and MeHg(I), which were not published in the scientific literature. Appendix C includes permissions for the use of accepted manuscripts in the thesis.

2 LITERATURE REVIEW

2.1 Introduction

Cardiovascular diseases are a group of diseases, which affect the brain, heart and blood vessels (Mendis et al., 2011). With growing age and depending on the number of existing risk factors, the risk of CVDs increases. Therefore, adults and in particular elderly are the main population group affected by this group of diseases.

2.2 Parameters of cardiovascular health

2.2.1 Blood and pulse pressure

Blood pressure is the pressure of the blood on the arterial walls. The systolic blood pressure value (higher value) represents the pressure at the time when the blood is ejected from the heart and the diastolic blood pressure (lower value) when the ventricles are filling with blood (Levy and Pappano, 2007a).

A standard method to assess cardiovascular health is the measurement of the blood pressure because it can be measured fast and non-invasively in humans. The two organs, which are responsible for blood pressure regulation, are the brain and the kidneys (Osborn, 2005; Herrera and Coffman, 2012). However, the detailed mechanism is not entirely known. The blood pressure can be regulated through the heart rate, stroke volume and the total peripheral resistance (Raven and Chapleau, 2014). The brain has the ability to influence the heart, kidneys, blood vessels and adrenal medulla through the autonomic nervous system, consisting of the sympathetic and parasympathetic arm (Osborn, 2005). Through the release of chemicals and hormones into the blood, the heart contractility can be altered and thus, changing the cardiac output, which is the product of the heart rate and stroke volume. Furthermore, the brain can adjust the blood volume through the kidneys and alter the resistance and capacitance of the blood vessels. The kidneys have the ability to control long-term blood pressure regulation through sodium excretion (Herrera and Coffman, 2012).

Although blood pressure varies throughout the day to adapt to changing environmental conditions, a constantly increased blood pressure is considered to be an important risk factor for the development of CVDs. A formalized classification scheme for blood pressure allows the identification and categorization of humans as normotensive or hypertensive (Table 2.1).

Table 2.1. Blood pressure classification (WHO/ISH, 2003)

Category	Systolic (mmHg)	Diastolic (mmHg)
Optimal	< 120	< 80
Normal	< 130	< 85
High-normal	130-139	85-89
Grade 1 hypertension (mild)	140-159	90-99
Grade 2 hypertension (moderate)	160-179	100-109
Grade 3 hypertension (severe)	≥ 180	≥ 110

With regard to the development of CVDs, it is also important to identify additional risk factors in the patients, because of their accumulative nature (Table 2.2).

Table 2.2: Risk stratification table (WHO/ISH, 2003)

	Blood pressure (mmHg)		
Other risk factors and disease history	***Grade 1***	***Grade 2***	***Grade 3***
No other risk factors	Low risk	Medium risk	High risk
1-2 risk factors	Medium risk	Medium risk	High risk
3 or more risk factors, target-organ damage, associated clinical conditions	High risk	High risk	High risk

Probability of developing a major cardiovascular event within the next 10 years: Low risk: < 15%; medium risk: 15-20%; high risk: > 20%

Although the prevalence of hypertension in the adult population is high, in the majority of patients the cause remains often unknown (essential hypertension) and only symptoms are treated (Chen, 2012). In half of the cases, the reason for the presence of increased blood pressure can be related to genetics. However, most of the time more than one gene is involved. The other half can be attributed to epigenetic mechanisms, i.e. interaction between genes and the environment. Environmental factors or risk factors, e.g. smoking, stress, overweight, can positively contribute to the development of hypertension. Secondary hypertension exists if an underlying disease, e.g. diabetes mellitus or chronic renal disease, is the cause (Bateman et al., 2012).

Based on the blood pressure measurements, the pulse pressure can be calculated by subtracting the diastolic blood pressure value from the systolic value. Changes in pulse pressure can indicate arterial stiffness and an increased risk for atherosclerosis.

2.2.2 Heart function

The heart consists of four chambers, two atria and two ventricles. The blood coming from the systemic cycle enters the heart through the right atrium. From here the blood flows to the right ventricle and enters the pulmonary cycle. The blood re-enters the heart through the left atrium and flows then to the left ventricle (Levy and Pappano, 2007b). With every beat of the heart, blood is ejected from the left ventricle into the aortic arc. The heart function can be assessed by determining the end-systolic and end-diastolic volumes, whose difference provides the stroke volume. Another parameter for heart function is the cardiac output, which is the product of the heart rate and the stroke volume.

2.2.3 Blood vessels and blood flow

The structure of blood vessels depends on their main function. Big arteries, e.g. the aorta, consist of a high amount of elastic tissue allowing the change from pulsatile to laminar flow (Pappano, 2008). The small capillaries, which facilitate the exchange of nutrients and oxygen, basically consist of only the endothelium. Measuring the wall thickness or diameter of major arteries, such as the carotid, or different blood flow parameters, it is possible to evaluate the health of the artery.

2.2.4 Cardiac electrical activity

The heart is innervated by the autonomic nervous system. However, the different chambers of the heart are not all depolarized or repolarized at the same time but in a specific order. The electrical activity of the heart can be recorded with an electrocardiograph and made visible on an electrocardiogram (ECG) (Pappano, 2008). The first peak in an ECG (P wave) corresponds to the activation of the atria. While the atria relax, the ventricles are activated resulting in a second wave (QRS complex). The following T wave reflects the repolarization of the ventricles. Based on the ECG, it is possible to identify irregularities in the heart beat, activation and relaxation of the chambers (arrhythmia), which can indicate disturbances in the electrical activity of the heart.

2.3 Lead

2.3.1 Occurrence and exposure

The Earth's crust naturally contains ores including lead, mainly as galena (PbS), anglesite ($PbSO_4$) or cerussite ($PbCO_3$). About 30% of the worldwide lead reserves are located in North America (ATSDR, 2007) with Canada being among the top 10 producers and suppliers of refined lead (Panagapko, 2009). Lead is mainly used in the production of lead-acid storage batteries. These

types of batteries play an important role in the car and communications industries. Due to its resistance to corrosion, it is used in paints for iron and steel and in roofs. It is also used in a variety of screens because of its ability to prevent the release of potentially damaging radiation. Additional uses are in plastic piping and decorative glass (Panagapko, 2009). Overall, lead possesses many useful properties, which makes it a desirable substance in various industries. Workers employed in these industries can be exposed to lead fumes and dust at their work place. Therefore, regulations are in place to restrict the amount and exposure duration in occupational settings. Additionally, the implementation of safety measures, e.g. lead-free areas, allows to minimizing exposure to lead. In the case of an accident, high exposures for a short term can occur.

Because of the extensive use of lead in manufacturing, processing and production facilities, it is released into the environment, where it is ubiquitously present. Through the deposition of lead from the air on agricultural soils or water surfaces, it will reach the food chain rendering food and water the main exposure sources for the general population. Tobacco plants have the ability to accumulate lead from the soil, which renders cigarettes and cigarette smoke another potential source for lead. In older homes, lead-containing paint might constitute an additional exposure source (ATSDR, 2007).

To protect the general population from ill-health due to lead exposure, governmental agencies, e.g. Health Canada, have established an intervention level for lead of 10 µg/dL blood for adults and 5 µg/dL blood for children based on neurological effects. At this level, it is recommended to take action to reduce exposure. Generally, the blood lead levels (BLL) in the Canadian population are below 2 µg/dL blood (Statistics Canada, 2008). However, as adverse effects were observed at blood levels < 5 µg/dL, no reference values are defined.

2.3.2 Toxicokinetics

The main route of exposure for lead is by ingesting food and water. Once the lead particles have reached the gastro-intestinal tract, they can be absorbed into the blood circulation. The absorption rate depends on a number of different factors, such as nutritional status, age, meal status (ATSDR, 2007). In the blood, lead is mainly found in the erythrocytes with a half-life of about 30 days (Rabinowitz et al., 1976). With the blood, lead is distributed in the whole organism but will eventually be deposited in bones where it can remain for decades. Excretion occurs through urine and feces.

2.3.3 Toxic effects

2.3.3.1 Neurotoxicity

Traditionally, lead is considered to act as a neurotoxicant with neurodevelopmental toxicity as the most sensitive end-point. Hence, the majority of studies investigate the neurobehavioral effects of lead exposure on children as the most vulnerable population group. Children show decreased

cognitive and academic skills at a BLL ≤ 5 µg/dL (Lanphear et al., 2000). In an adult population recruited from the third National Health and Nutrition Examination Survey (NHANES III) a BLL of ≤ 25 µg/dL did not result in any adverse neurobehavioral effects (Krieg et al., 2005). Due to life-long accumulation of lead in the bones, the elderly might be another vulnerable population group. Although the association between bone lead and cognitive tests is weak (Weisskopf et al., 2007), the coordination between vision and motor skills might be adversely affected.

2.3.3.2 Cardiovascular toxicity

The scientific literature provides sufficient evidence to consider the relation between lead exposure and the development of hypertension as causal (Nawrot et al., 2002; Navas-Acien et al., 2007; Hara et al., 2014). As the prevalence of hypertension increases with age, adults and elderly might be the most vulnerable population group for impaired cardiovascular health due to lead exposure. Few studies explored other cardiovascular end-points than blood pressure. Peripheral arterial disease (PAD) is a disease affecting the blood vessels, whose blood flow is limited due to the presence of atherosclerosis. Navas-Acien et al (2004) found a positive association between mean BLLs of 2 µg/dL and PAD. This association could not be confirmed for urinary lead (Navas-Acien et al., 2005). Similar BLLs also showed an increased risk of mortality from heart attacks and strokes (Menke et al., 2006; Schober et al., 2006). A study by Cheng et al (1998) showed a relation between a BLL of 5.8 µg/dL and disturbances of the cardiac electrical conductivity. The lengths of the QT and QRS intervals increased with higher lead body burden.

2.3.3.3 Nephrotoxicity

Lead has also the ability to impair kidney function resulting in secondary hypertension. Data from large human studies, such as NHANES and the Normative Aging Study, indicate a direct relation between lead body burden and chronic kidney disease and hypertension (Muntner et al., 2003; Tsaih et al., 2004). Similarly, BLLs ≤10 µg/dL were associated with a reduced glomerular filtration rate (Kim and Lee, 2012) or kidney damage (Sommar et al., 2013).

2.4 Mercury

2.4.1 Occurrence and exposure

Similar to lead, mercury is naturally occurring in the Earth's crust. Mercury vapor can be released into the environment naturally through volcanic eruptions or from natural sinks like the ocean but also through human activities, such as burning of coal or waste (Clarkson, 2002). This process indicates the start of global mercury cycling. Once the mercury vapor has reached the atmosphere, it can be oxidized and deposited to the ground by rainfall. The Hg(II) is biomethylated to MeHg(I) followed by accumulation in predatory fish and seafood, which constitutes one of the major exposure sources for humans. Dental amalgam, which contains 50% mercury, provides a second source for

human exposure (Bellinger et al., 2006). In an occupational setting, dentist and other dental staff might be at risk during the preparation and placing of amalgam fillings (Richardson, 2003), unless suitable protection is applied (Goodrich et al., 2013b). The general population will be exposed to mercury vapor starting with the dental treatment. As the amalgam fillings will continue to release mercury vapor, the low level exposure becomes long-term (Mutter, 2011; Richardson et al., 2011).

2.4.2 Toxicokinetics

MeHg(I) and mercury vapor are both easily absorbed. The lipophilicity of MeHg(I) facilitates the absorption from the gastro-intestinal tract into the blood (Clarkson et al., 2007). This chemical property also allows MeHg(I) to cross membranes, such as the blood-brain or blood-placenta barriers (Ballatori, 2002). It also has a high affinity to thiol-groups and can easily bind to biomolecules, e.g. glutathione or cysteine (Bridges and Zalups, 2005). If MeHg(I) binds to cysteine, the resulting molecule resembles the amino acid methionine. By mimicking this neutral amino acid, MeHg(I) can use transporters to cross membranes and to cause toxic effects (Bridges and Zalups, 2005). Instead of normal amino acids, the MeHg(I)-cysteine compound is incorporated into hair, which makes it a good biomarker for MeHg(I) exposure. Finally, MeHg(I) is mainly excreted through the feces.

As a gas, mercury vapor has the ability to diffuse through membranes and thus cross the blood-brain and blood-placenta barriers leading to a high absorption rate (Clarkson et al., 2007). Once it has reached the blood, it is quickly oxidized to Hg(II), which is assumed to be the toxic form (Clarkson et al., 2007). With the blood, the Hg(II) will be transported to other organs, e.g. spleen, liver or kidneys, where it might accumulate. Although a small amount of the mercury vapor is exhaled, most of it will be excreted as Hg(II) through the urine and feces (Clarkson et al., 2007).

2.4.3 Toxic effects

2.4.3.1 Neurotoxicity

As Pb(II), mercury is classically considered to be a neurotoxicant with the developing brain as the main target organ. Due to two major accidents poisoning people in Japan and Iraq, the neurotoxic effects of MeHg(I) are well known.

From 1950 to 1970, a factory released MeHg(I) into the Minamata River, Japan, which accumulated in fish in this area. Because of the consumption of this contaminated fish, people living around Minamata Bay were chronically exposed to MeHg(I) (Minamata disease) (Ekino et al., 2007). Although children are more sensitive to neurotoxicity because of their still developing brains, the exposure was high enough to also cause neurotoxicity in the adult population. In the early 1970s,

another MeHg(I) poisoning occurred in Iraq because of contaminated seed grain (Bakir et al., 1980). As the exposure was sufficiently high, children as well as adults were affected.

To study neurodevelopmental effects from MeHg(I) contained in the diet, two large cohort studies were started in the 1980s. One study location is the Faroe Islands between Iceland and the United Kingdom. The other study was carried out in the Seychelles, which are east of the African coast and north of Madagascar. Due to their different locations and climates, the diets differed substantially. In the Faroe Islands the main exposure source for MeHg(I) is whale meat, while in the Seychelles, it is ocean fish and seafood. Both studies focused on neurobehavioral end-points in children to assess developmental neurotoxicity. In the Faroe Islands, cognitive deficits, e.g. in language skills, were found. However, the overall picture is not entirely clear (Grandjean et al., 2012). Analyzing the potential contribution of polychlorinated biphenyls to the neurobehavioral deficits, it was concluded that their effect was minor in comparison to MeHg(I) (Grandjean et al., 2012). In the Seychelles, no association between prenatal exposure to methylmercury and neurophysiological skills were observed (Davidson et al., 2011). The researchers could also not find an association between prenatal exposure to mercury vapor through amalgam fillings of the mother and neurodevelopmental effects in the children (Watson et al., 2011). Although MeHg(I) causes neurotoxic effects at higher doses as observed in Japan and Iraq, the neurodevelopmental effects of prenatal MeHg(I) exposure in children are less clear.

Dentists and dental staff are occupationally exposed to mercury vapor when handling amalgam fillings. Several research groups have studied the relation between occupational exposure to mercury vapor in dentists and dental staff and neurobehavioral effects. Although behavioral changes were only subtle, Echeverria et al (1995) could show a positive relation in dentists between urine mercury as a biomarker for elemental mercury exposure and reduced mental concentration, emotional lability and mood scores. Similarly, dental assistants (Moen et al., 2008; Hilt et al., 2009) experienced more often psychosomatic symptoms, memory and concentration problems, and fatigue.

2.4.3.2 Cardiovascular toxicity

Although no causative relation as with Pb(II) and hypertension exists, there is growing scientific evidence for an association between mercury exposure and adverse effects on the cardiovascular system (Roman et al., 2011; Houston, 2014). The general population is mainly exposed to MeHg(I) through the consumption of fish and seafood. At the same time, fish also contains heart-protective compounds, such as long-chain n-3 fatty acids. Therefore, study results might differ for fish and non-fish eaters. Fillion et al (2006) studied a fish-eating Amazonian population in Brazil. Hair mercury was positively associated with blood pressure, although overall blood pressure was rather low.

Vupputuri (2005) could not show an overall relation between mercury exposure and blood pressure. However for non-fish eating women aged from 16-49 years an increase in systolic blood pressure was observed. Bautista et al (2009) found a positive association between blood and hair mercury and increased blood pressure. Valera et al studied the relation between environmental mercury exposure and cardiovascular end-points in a number of different populations and countries. Inuit adults from Nunavik, Canada, are exposed to MeHg(I) through the consumption of marine mammals and predatory fish as part of their traditional diet (Valera et al., 2008). Valera et al (2008; 2009) found that blood mercury was positively associated with systolic blood pressure and pulse pressure. Dietary mercury exposure reduces heart rate variability (Lim et al., 2010; Valera et al., 2008), which is a sign for impaired cardiac autonomic activity through parasympathetic dysfunction. Mercury level was associated with increased risk for heart attacks (Guallar et al., 2002). Goodrich et al (2013b) investigated the relation between MeHg(I) exposure (hair as biomarker) and mercury vapor (biomarker urine) and blood pressure. Hair mercury was positively associated with blood pressure, while urine mercury was negatively associated with blood pressure.

The association between MeHg(I) exposure and the development of hypertension is corroborated by animal studies by Grotto et al. Wistar rats received MeHg(I)-chloride for the duration of 100 days at a dose of 100 µg/kg-bw/d by gavage (Grotto et al., 2009). After only four weeks of exposure, the systolic blood pressure (30 mmHg) was increased in exposed rats compared to the control group. In a follow-up study, rats were exposed to MeHg(I) simulating the diet of an Amazon riverside population (Grotto et al., 2011) by adding fish from a contaminated area in Northern Brazil and uncontaminated fish from an area in Southern Brazil. The final rat diet contained 20% fish and 80% normal rat food, which corresponds to the contribution of fish to the overall food consumption in the Amazonian population. After 11 weeks of exposure, the rats exposed to the contaminated fish showed an increase in systolic blood pressure (20 mmHg).

The second source for mercury exposure is dental amalgam fillings with dental staff and people with dental amalgam fillings as the target groups. Siblerud (1990) observed a positive association between people with amalgam fillings and increased blood pressure and decreased heart rate. After the removal of the amalgam fillings, the people reported an improvement of cardiovascular health and overall better well-being. As dental staff is exposed to mercury vapor by preparing and handling amalgam fillings, this professional group is of particular interest. The health status of dentists was evaluated by analyzing pharmacy utilization data (Duplinsky and Cicchetti, 2012). The overall conclusion of this study was that the dentists had more health problems than the control group due to a higher consumption of e.g. cardiovascular medications (Duplinsky and Cicchetti, 2012).

2.4.3.3 Nephrotoxicity

Mercury vapor is quickly oxidized to Hg(II), which accumulates in the kidneys. Therefore, mercury in urine provides a good biomarker for exposure to elemental mercury (Clarkson et al., 2007). While high exposure to Hg(II) can cause renal failure, low-level exposures have only subtle effects on biomarkers for kidney function (Li et al., 2012). Glutathione-S-transferases, which indicate damage of the proximal tubules, were elevated in children bearing dental amalgam (Geier et al., 2014). People living close to a mercury mine in China (Li et al., 2012) showed increased values for serum creatinine and serum urea nitrogen, which are signs for impaired kidney function, for increasing blood mercury levels.

2.5 Mechanisms of action

Lead and mercury are systemic toxicants and can elicit toxicity through a number of different mechanisms of actions (MOAs), which can occur in parallel or interact with each other. The best-known MOA for metals is the production of oxidative stress by impairing the balance between reactive oxygen species (ROS) and antioxidants. These ROS can disturb biochemical pathways, damage cells or react with other biomolecules. If this happens in the kidneys, they might not be able anymore to carry out their control function to regulate the blood pressure. Epigenetic mechanisms are gaining more and more importance in the development of non-communicable diseases, e.g. CVDs. As metals have the potential to affect gene expression, a consequence could be the impairment of cardiovascular health.

2.5.1 Oxidative stress

Oxidative stress occurs when the balance between reactive oxygen species, e.g. superoxide, hydrogen peroxide, or nitrogen species (RNS) and antioxidants is disturbed. Both parts of this system play important roles in the regulation of physiological functions. Nitric oxide (NO) as a representative of an RNS acts as a vasodilator and hence, is an important for the blood pressure regulation. However, if due to an imbalance the amount of ROS increases, then these molecules can harm DNA or biomolecules in general (Valko et al., 2007).

Metals are well known to have the ability to increase the production of ROS through Fenton-like reactions:

$$M^{(n)} + O_2^{-}\cdot \rightarrow M^{(n-1)} + O_2$$
$$M^{(n-1)} + H_2O_2 \rightarrow M^{(n)} + HO\cdot + OH^{-}$$

Fig. 2.1. Fenton-like reactions (Ercal et al., 2001)

In case of an imbalance of the ROS/antioxidant system, various defense mechanisms are employed (Valko et al., 2007): 1) preventative mechanisms, 2) repair mechanisms, 3) physical defenses and 4) antioxidant defenses. Glutathione (GSH) is an important antioxidant in the prevention of oxidative damage. However, both lead and mercury have a high affinity for thiol-groups and by binding to these groups of glutathione result in glutathione inactivation (Bridges and Zalups, 2005).

Studies have shown that Hg(II) causes an imbalance between ROS and antioxidants in several ways. It was shown that Hg(II) increases the production of O_2^- in the vasculature (Wiggers et al., 2008) and thus oxidative stress. The radical O_2^- can then damage the endothelium, which is an important producer of the vasodilator NO. As a consequence, the bioavailability of NO is reduced (Wiggers et al., 2008). Hg(II) also contributes to oxidative stress by oxidizing GSH and has the ability to cause cell damage (Wolf and Baynes, 2007). MeHg(I) is also cytotoxic and increases oxidative stress but through raised NO levels (Huang et al., 2008). Due to its high affinity to thiol-groups, MeHg(I) also reacts with GSH resulting in its depletion (Hagele et al., 2007). Mercury vapor does not seem to increase oxidative stress (Goering et al., 2002). In biological systems, Pb(II) induces oxidative stress by reducing NO availability (Gonick et al., 1997) and the depletion of GSH (Vaziri et al., 2000). Instead of measuring the amount of molecules, increased enzyme activities of e.g. superoxide dismutase, catalase or glutathione peroxidase are also an indicator of oxidative stress (Farmand et al., 2005). In summary, Pb(II) and Hg species have the ability to reduce the availability of the vasodilator NO by e.g. cytotoxicity, and to increase the number of ROS. At the same time, the antioxidant GSH is inactivated, which aggravates the effect of oxidative stress on the organism. Finally, by disturbing the balance between ROS and antioxidants, Pb(II) and Hg species will increase the blood pressure and the risk for CVDs (Vaziri, 2008; Houston, 2014).

To evaluate the amount of oxidative stress caused by the exposure to Pb(II) or Hg species and their mixtures, the 3-nitrotyrosine levels in blood were measured. Under normal circumstances, 3-nitrotyrosine is very low. In the case of increased oxidative stress production, NO is decreased, while O_2^- is increased. Both molecules react with each other and from peroxynitrite, which then reacts with tyrosine residues in proteins.

$$NO + O_2^- \rightarrow ONOO^- + \text{tyrosine} \rightarrow \text{3-nitrotyrosine}$$

Therefore, an increase of 3-nitrotyrosine is a sign of increased oxidative stress.

In addition to assess oxidative stress, glutathione levels were measured as a representative of an antioxidant. In the event of oxidative stress, the reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG) (Wolf and Baynes, 2007). The ratio of GSSG to GSH is generally small

because a healthy cell contains more GSH than GSSG. Hence, an increased ratio is deemed to be a sign for oxidative stress.

2.5.2 Kidney damage

Lead and Hg species are known nephrotoxicants, which will impair kidney function with increasing body burden. As the kidneys play an important role in the regulation of blood pressure due to their ability to regulate sodium excretion, kidney damage can be considered as a metabolic risk factor for CVDs. Therefore, kidney function was assessed as a MOA for lead and Hg species toxicity for cardiovascular health.

Creatinine originates from muscle metabolism and as muscle mass is quite stable on a day-to-day basis, creatinine is as well. Furthermore, it is freely filtrated by the nephrons in the kidney and can be used as a marker for overall kidney function. Creatinine clearance is a clinical standard for assessing kidney function by providing an estimate of the glomerular filtration rate, which equals the volume of blood being cleared of creatinine through urine excretion per minute. It is defined as the product of the creatinine concentration in urine and urine flow divided by the creatinine concentration in plasma. However, creatinine clearance is not a very sensitive marker (Vlasakova et al., 2014) because healthy nephrons have the ability to compensate for damaged ones. Therefore, a decrease in creatinine clearance is a sign for substantial nephron damage (Schnellmann, 2008). Despite this weakness, blood and urine creatinine concentrations were measured, urine flow obtained from the literature and the creatinine clearance was calculated, which is regularly obtained in patients.

To complement the creatinine clearance test with a more sensitive marker, levels of the kidney injury molecule 1 (KIM-1) levels in urine were measured. KIM-1 is a protein, which is expressed by the tubular epithelial cells in the kidney due to injury. In comparison to other markers for kidney function (Vlasakova et al., 2014), it is a sensitive marker for evaluating damage of the kidney tubules.

2.5.3 Epigenetic mechanism

Epigenetics describes the interactions between genes and the environment. In contrast to mutations in genetics, epigenetic effects do not alter the DNA sequence. Three epigenetic mechanisms regulate the expression of proteins by switching genes on and off. The best studied mechanism is DNA methylation. DNA methyltransferases (DNMTs) transfer methyl-groups from S-adenosylmethionine (SAM) to cytosine-guanine (CpG) dinucleotides at the carbon-5 position in cytosine. The second mechanism is the modification of histones, which organize DNA into nucleosomes. Modification, e.g. deacetylation or methylations, takes place at the N-terminal tails resulting in a silencing of the gene (Fuks, 2005). Furthermore, histone modifications can interact with DNA methylation in order to regulate gene expression (Hashimoto et al., 2010). The last mechanism

refers to the non-coding RNA (ncRNA), which consist of long and small ncRNA (Cheng et al., 2012). While small ncRNA participate in the regulation of gene expression through the silencing of genes, long ncRNA play a role in genomic imprinting (Choudhuri, 2011).

An increasing body of studies indicates the involvement of epigenetic mechanisms in a broad variety of diseases. Therefore, it is likely that these mechanisms are a potential mode of action for the development of cardiovascular diseases because of exposure to lead or mercury species. It can be assumed that these metals either affect directly or indirectly genes, which are involved in the maintenance of cardiovascular health. However, little scientific evidence exists to substantiate this relation.

For recent exposure, blood lead levels are the preferred biomarker, while bone lead is an indicator for long-term exposure. Wright et al (2010) found a positive association between bone lead and DNA methylation but not with blood lead levels. This might indicate that the total body burden due to chronic lead exposure is necessary for changes in DNA methylation. As cardiovascular diseases are more pronounced after a certain age, this might support the link between lead bone levels and the development of CVDs. In a study by Kovatsi et al (2010), blood lead was related to altered methylation of the *p16* promoter gene, which is involved in the death of neurons. Although this rather indicates a mechanism for neurotoxicity, cardiovascular health could indirectly be impaired through disturbances in the autonomic nervous system. Histone modifications are not attributed to lead exposure (Fragou et al., 2011).

As MeHg(I) is considered to be very toxic to the developing fetus, a couple of studies exist, which investigated prenatal exposure to methylmercury. Pregnant rats were exposed to high doses of methylmercury (Desaulniers et al., 2009). The off-spring had decreased mRNA levels for DNMT and DNA methylation in the liver. In a study by Onishchenko (2008) prenatal exposure to MeHg(I) lead to signs of depression in the off-spring through hypomethylation of the brain-derived neurotrophic factor. Mercury decreased the methylation of growth factors required for normal neurodevelopment (Waly et al., 2004). It was also observed that mercury blood levels are directly associated with increased methylation of the glutathione-S-transferase M1 gene, indicating a risk for oxidative stress (Hanna et al., 2012). In contrast, an indirect relation exists between DNA methylation and brain mercury levels (Pilsner et al., 2010). Overall, no study was identified, which investigated the effect of lead and/or mercury exposure on genes relevant for cardiovascular health.

A small number of studies investigated the effect of altered DNA methylation of enzymes relevant for cardiovascular homeostasis exploring critical steps in DNA methylation as a potential mechanism of action. Hypomethylation of the enzyme 11 beta-hydroxysteroid dehydrogenase type 2 (11beta-

HSD2) reduces its activity. At the same time, mineralocorticoid receptors are activated, which result in increased sodium retention in the kidneys and as a consequence in an increase in blood pressure (Fragou et al., 2011; Friso et al., 2008). Another epigenetic mechanism of action for the development of hypertension could be the DNA methylation of the *ace-1* genes as shown by Riviere (2011). These genes code for the angiotensin-converting enzyme (ACE), which plays an important role in blood pressure regulation. The enzyme ACE facilitates the conversion of angiotensin I to angiotensin II, a vasopressor, and deactivates the vasodilator bradykinin. Another important vasodilator NO could be affected in a similar way. Chan et al (2004) observed in a number of different cell types that hypomethylation of the gene for the endothelial NO synthase (eNOS) decreases the expression of the eNOS mRNA leading to a reduced availability of NO. While these molecular studies provide information on the mechanism of action, studies analyzing the global DNA methylation status are able to link an epigenetic mechanism to a physiological outcome and thus, complement the picture. Smolarek et al (2010) analyzed the global DNA methylation status in the blood of patients with essential hypertension. Hypomethylation was associated with severity of hypertension. Similarly, in a study by Baccarelli et al (2010) hypomethylation of blood LINE-1 was associated with increased risks for ischemic heart disease, stroke and total mortality. In the Chinese population in Singapore, Kim et al (2010) explored if DNA methylation could be used as a biomarker for cardiovascular diseases, such as heart attacks or strokes, or metabolic risk factors, e.g. hypertension, diabetes. Although the study indicates an association between DNA methylation and increased blood pressure or cardiovascular diseases, this can only be considered as a very first step towards the development of an epigenetic biomarker for cardiovascular diseases.

Based on the scientific literature, it seems very likely that DNA methylation constitutes a potential mechanism of action for the development of CVDs, including CVDs due to exposure to lead or mercury exposure. The actual mechanism of action will have a high grade of complexity because of the high number of genes involved in the maintenance of cardiovascular health. As the amount of studies investigating this topic is very limited, it is not possible to conclusively say there is a role for DNA methylation in the development of CVDs.

As part of this research, the amount of 5-methyl-2'deoxycytidine in urine was analyzed as a marker for global DNA methylation. The purpose was to explore the effect of single and combined exposures to lead and mercury species on the overall DNA methylation status and to potentially link this epigenetic mechanism to cardiovascular effects.

2.6 Human health risk assessment

Risk assessments estimate the potential risk on human health by taking into account the toxicity of the substance and exposure. Depending on the amount of available data, this process can be time-consuming and challenging.

2.6.1 Risk assessment of single metal exposure

A risk assessment is a defined process, which consists of four steps. The first step is the hazard identification, which evaluates inherent properties of the substance, which might cause adverse effects. The dose-response assessment (step 2) and the exposure assessment (step 3) can be carried out in parallel and complement each other providing the necessary information for the risk characterization (step 4). While step 2, investigates potential health problems at defined exposures, step 3 explores exposures in the population group in question. Findings from these steps are required to finalize the risk assessment (U.S. EPA, 2012). Conclusions based on the outcome of a risk assessment will identify the type and size of a potential risk in the general population as well as vulnerable groups, e.g. children.

In case of a sufficient database for the substance and the most sensitive end-points, governmental agencies establish a safe dose of exposure. The U.S. EPA calls this dose reference dose (RfD) and defines it as “an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. No-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) can be derived from the dose-response curves (U.S. EPA, 2014b). The RfD can be derived from a NOAEL, LOAEL, or benchmark dose, with uncertainty factors generally applied to reflect limitations of the data used” (U.S. EPA, 2014c). If the NOAEL or LOAEL were derived from animal data, uncertainty factors for inter- and intra- species variability can be applied to determine safe exposure levels for the protection of human health. Additional uncertainty factors can be applied to protect vulnerable groups, such as children, elderly, immunocompromised people. The U.S. EPA (2014b) defines a BMD as “a dose that produces a predetermined change in response rate of an adverse effect (called the benchmark response or BMR) compared to background”. Depending on the agency and country, these safe doses are called RfD by U.S. EPA or provisional tolerable daily intake (pTDI) by Health Canada and in Europe.

No RfD or pTDI exist for lead exposure and developmental neurotoxicity or adult nephrotoxicity because the available database indicates that even low exposures have an adverse effect so that no safe dose exists. Using the BMD approach, EFSA (2012b) has derived $BMDL_{s_{01}}$ identifying a 95^{th} percentile lower confidence limit of the benchmark dose of 1% extra risk for developmental

neurotoxicity in children, cardiovascular toxicity and nephrotoxicity in adults with 0.5, 1.5 and 0.63 µg/kg-bw/d, respectively.

Mercury toxicity depends on its chemical form. Hence, safe exposure levels are established for organic mercury, namely MeHg(I), and Hg(II). Based on the U.S. EPA Integrated Risk Information System (IRIS) (U.S. EPA, 2014d) the RfD for MeHg(I) is 0.1 µg/kg-bw/d for neurodevelopmental effects, while the RfD for Hg(II) is 0.3 µg/kg-bw/d with autoimmune effects as end-point. In contrast, to a daily exposure dose, EFSA (2012a) established tolerable weekly intakes (TWI). The TWI of Hg(II) was determined as 4 µg/kg-bw for nephrotoxicity, which corresponds to a daily tolerable intake of 0.6 µg/kg-bw/d. MeHg(I) has a TWI of 1.3 µg/kg-bw for neurodevelopmental effects, which equals a daily tolerable intake of 0.2 µg/kg-bw/d. Discrepancies in the doses considered as safe can be explained with the use of different datasets and end-points. However, it can be assumed that the human population is protected by these values due to the application of uncertainty factors in their calculations.

2.6.2 Risk assessment of metal mixture exposure

The human population is generally exposed to a mixture of substances because of the ubiquitous presence of chemicals in the environment. However, experiments in animals or cells often use only one substance to evaluate its toxicity. Although studies in humans would allow studying mixture effects, e.g. by measuring total mercury and BLLs, it is rarely done. Hence, risk assessments of mixtures often present a big challenge because of the lack of suitable and sufficient data.

The US EPA guidelines for the health risk assessment of chemical mixtures (U.S. EPA, 1986) provide guiding principles on how to assess the risk of chemical mixtures. A critical step in the risk assessment is to obtain sufficient data on exposure and toxicity of the mixture of concern. In case, this is not possible, it is suggested to consider data on similar mixtures, e.g. same components but different concentrations, or on single components of the mixture. Depending on the quality and quantity of the available data, the conclusions of the risk assessment will contain varying degrees of uncertainty.

Government agencies have established safe levels of exposure for many chemicals, e.g. Pb and Hg, which allows the risk assessor to estimate the risk of a mixture for human health by calculating the hazard index (HI) (U.S. EPA, 1986). In the case of HI, the safe level of exposure is called acceptable level (AL). As with full risk assessments, the HI assumes additivity of substances and is defined as

$$HI = E_1/AL_1 + E_2/AL_2 + \ldots + E_i/Al_i$$

with E_i = exposure level to the i^{th} toxicant and AL_i = maximum acceptable level for the i^{th} toxicant.

The assumption of additivity of mixture components indicates that the single mixture components do not interact with each other. This may lead to an under- or overestimation of risk due to synergistic or antagonistic effects. As interaction data are often missing, further uncertainty is introduced.

3 CARDIOVASCULAR RESPONSES TO LEAD ARE BIPHASIC, WHILE METHYLMERCURY, BUT NOT INORGANIC MERCURY, MONOTONICALLY INCREASES BLOOD PRESSURE IN RATS

3.1 Author contributions

Tanja M. Wildemann carried out the animal experiment, including the daily care of the animals, measurements of the cardiovascular effects, termination and tissue collection and processing. Furthermore, she conducted the statistical analysis and prepared the manuscript.
Naghmeh Mirhosseini assisted in the termination of the animals, sample collection and processing and provided comments on the manuscript.

Steven D. Siciliano provided scientific input, provided comments on the manuscript and supported the research financially through his research grant.

Lynn P. Weber provided scientific input and guidance, edited the manuscript and supported the research financially through her research grant.

3.2 Preface

Most studies on cardiovascular effects of Pb(II) and mercury species originate from epidemiological studies, which have the inherent disadvantage of limited control over confounding factors, such as smoking or comorbidities. For ethical reasons, it is also not possible to carry out invasive interventions. To be able to investigate the association between Pb(II), Hg(II) and MeHg(I) exposure and a broad range of cardiovascular effects, we opted for an animal model. Using rats as our model, we were able to explore cardiovascular effects, such as blood pressure, heart function, blood flow and electrical activity of the heart, at different doses of these three metals in a controlled environment. This allowed us to derive dose-response curves for each metal and the selected cardiovascular end-points and to determine safe thresholds of exposure.
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3.3 Abstract

Cardiovascular diseases, such as heart attack and stroke, are the major cause of death worldwide. It is well known that a high number of environmental and physiological risk factors contribute to the development of cardiovascular diseases. Although risk factors are additive, increased blood

pressure (hypertension) is the greatest risk factor. Over the last two decades, a growing number of epidemiological studies associate environmental exposure to lead or mercury species with hypertension. However, cardiovascular effects beyond blood pressure are rarely studied and thresholds for effect are not yet clear. To explore effects of lead or mercury species on the cardiovascular system, normal male Wistar rats were exposed to a range of doses of lead, inorganic mercury or methylmercury through the drinking water for four weeks. High-resolution ultrasound was used to measure heart and vascular function (carotid artery blood flow) at baseline and at the end of the exposure, while blood pressure was measured directly in the femoral artery at the end of the 4-week exposure. After 4 weeks, blood pressure responses to lead were biphasic. Low lead levels decreased blood pressure, dilated the carotid artery and increased cardiac output. At higher lead doses, rats had increased blood pressure. In contrast, methylmercury-exposed rats had increased blood pressure at all doses despite dilated carotid arteries. Inorganic mercury did not show any significant cardiovascular effects. Based on the current study, the benchmark dose level 10% ($BMDL_{10}$s) for systolic blood pressure for lead, inorganic mercury (based on systemic toxicity) and methylmercury are 1.1, 1.3 and 1.0 µg/kg-bw/d, respectively. However, similar total mercury blood levels attributed to inorganic mercury or methylmercury produced strikingly different results with inorganic mercury having no observable effect on the cardiovascular system but methylmercury increasing systolic and pulse pressures. Therefore, adverse cardiovascular effects cannot be predicted by total blood mercury level alone and the mercury species of exposure must be taken into account.

3.4 Introduction

Heavy metals, such as lead (Pb) and mercury (Hg), are mobilized in the environment mainly through anthropogenic sources, such as mining and industrial activities, rendering them ubiquitously present in the environment (ATSDR, 2007; Health Canada, 2013; EFSA, 2012a). The principal exposure route for lead is oral through food and drinking water (Health Canada, 2013; ATSDR, 1999; EFSA, 2012a). The absorption of lead depends on a variety of factors, such as age or the nutritional status of the person. In the body, lead is bound to the erythrocytes and more than 90% of the total body burden in adults can be found in the bones (ATSDR, 2007). Two mercury species are relevant for human exposure, namely elemental mercury (Hg^0) and methylmercury (MeHg). While exposure to elemental mercury primarily arises through inhalation from dental amalgam fillings (Richardson, 2014), methylmercury accumulates in predatory fish and seafood, which leads to dietary exposure in humans (Clarkson, 2002). Elemental mercury is vaporous and hence very mobile. It can easily cross the blood brain barrier, but is also quickly oxidized to inorganic mercury in the blood and other tissues (Clarkson et al., 2007), leading to deposition of inorganic mercury in target organs such as

liver or kidneys. Methylmercury is readily absorbed in the gut and distributes in all tissues, including the brain. It can be converted in the body to inorganic mercury or methylmercury-cysteine which can mimic the amino acid methionine (Clarkson et al., 2007; Ballatori, 2002). Thus, human environmental mercury exposure can be modeled with inorganic mercury and methylmercury.

Although both lead and mercury are considered to have neurological effects as their primary toxicity, a growing body of epidemiological research associates lead exposure (mostly blood lead levels) with adverse cardiovascular health. Data from population-based studies, including the National Health and Nutrition Examination Survey (NHANES) or the Normative Aging Study, show clear associations between low blood lead levels (≤ 10 µg/dl) and hypertension (Scinicariello et al., 2011; Cheng et al., 2001; Glenn et al., 2003; Nash et al., 2003). Only a few studies using the NHANES database examined additional cardiovascular end-points beyond hypertension. Specifically, positive associations were shown between blood lead levels (≥ 2 µg/dL) and increased mortality due to myocardial infarction and stroke (Menke et al., 2006; Schober et al., 2006), increased risk of peripheral arterial disease (Navas-Acien et al., 2004) or abnormalities in electrocardiogram (ECG) data consistent with left ventricular hypertrophy (Schwartz, 1991). A few rat studies support a cause-effect relationship between chronic lead exposure and hypertension (Nowack et al., 1993; Ding et al., 1998; Fiorim et al., 2011). Furthermore, Skoczynska et al (2014) exposed rats to lead through the drinking water and then measured heart function with cardiac magnetic resonance imaging (MRI). This study showed that the cardiac ejection fraction and fractional area change in the lead-exposed rats were significantly reduced in comparison to the control rats indicating impaired cardiac function. There is evidence for a causal relationship between blood lead levels and hypertension as well as additional cardiac effects but thresholds for adverse effects remain unclear.

The connection between mercury exposure and cardiovascular health is much less clear. In human studies, the most common biomarker of exposure is total mercury levels in blood which is generally thought to indicate recent exposure to inorganic and organic mercury (Health Canada, 2010). Another biomarker of exposure is mercury levels in hair samples which are generally thought to relate to methylmercury exposure (Li et al., 2008). Some epidemiological studies (Bautista et al., 2009; Valera et al., 2009; Pedersen et al., 2005) found a positive association between blood or hair mercury levels and an increased risk for hypertension and pulse pressure. However, other studies in humans (Vupputuri et al., 2005; Johansson et al., 2002; Mozaffarian et al., 2011; 2012; Valera et al., 2011a; Park et al., 2013) did not find a link between blood mercury levels and blood pressure or cardiovascular diseases (Mozaffarian et al., 2011). Higher mercury levels in hair or blood samples were associated with reduced heart rate variability (a known risk factor for cardiovascular death) in Koreans (Lim et al., 2010), Nunavik Inuits (Valera et al., 2008) and Canadian Cree adults (Valera et al., 2011a). Furthermore, Salonen et al (2000) found a positive association between hair mercury

levels and carotid atherosclerosis in a Finnish cohort study. Finally, dentists and dental staff are occupationally exposed to mercury vapor, which is quickly metabolized to inorganic mercury, through the handling of dental amalgam. Using pharmacy utilization data, Duplinsky and Cicchetti (2012) showed that dental professionals at the age of 45 and above have a higher prescription rate for cardiovascular medication than the general population, further supporting the link between higher mercury exposure and increased risk for cardiovascular disease. In summary, while human studies show an association between mercury exposure and cardiovascular health, the specific mercury species responsible and the thresholds for cardiovascular effect remain unclear.

Animal studies have also suggested a link between mercury and adverse cardiovascular effects. In rat studies, although inorganic mercury (50 µg $HgCl_2$/ml in the drinking water for 320 days or 4.6 µg/kg injected loading dose plus daily 0.07 µg/kg/day i.m.) failed to significantly affect blood pressure (Carmignani et al., 1983; Blanco-Rivero et al., 2011; Furieri et al., 2011a) other studies using the same doses have reported increased vasoconstriction in isolated arteries (Blanco-Rivero et al., 2011; Golpon et al., 2003; Furieri et al., 2011b) or reduced cardiac contractility in isolated rat hearts (Furieri et al., 2011a; Souza de Assis et al., 2003). In contrast, methylmercury exposure in rats (0.5 mg MeHg/kg-bw oral gavage for 23 – 28 days or a 10-fold higher dose) led to latent increases in systolic blood pressure either at 42 days or immediately after exposure, respectively (Wakita, 1987) . In another study, rats gavaged with 100 µg/kg-bw/d methylmercury for 100 days also had significantly increased systolic blood pressure after four weeks (Grotto et al., 2009). Therefore, animal studies are highly suggestive of a causative association between methylmercury exposure and adverse cardiovascular effects, while the relationship between inorganic mercury and cardiovascular effects are unclear. However, thresholds for adverse cardiovascular effects for both mercury species require clarification.

We hypothesized that exposures to lead, inorganic mercury or methylmercury will adversely affect cardiovascular function in rats. In order to investigate adverse effects of lead versus differing mercury species on the cardiovascular system, as well as the thresholds for these effects, we examined effects of a broad range of metal doses in normal male Wistar rats. In order to generate data that could be used to assess risk to humans through oral exposure, rats were exposed to lead acetate, mercury chloride or mono-methylmercury chloride for 28 days via the drinking water. Cardiovascular end-points evaluated included cardiac and vascular function, assessed by high-resolution ultrasound in B-mode and power Doppler mode, respectively; direct (intravascular) blood pressure was measured in the femoral artery, and cardiac electrical activity through electrocardiography (ECG) analyses at the end of the 4-week exposure.

3.5 Materials and methods

3.5.1 Animals

Male Wistar rats (250 – 300 g) were purchased from Charles River Laboratories, Canada and acclimatized for one week before the start of the experiment. The animals were housed singly at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). They were housed at 22°C under a 12:12h-light dark cycle with free access to standard rat chow. All experiments were approved by the University of Saskatchewan's Animal Research Ethics Board and carried out according to the guidelines of the Canadian Council on Animal Care (CCAC).

For the duration of four weeks, rats were exposed (n = 5-6) to either lead acetate (Pb(II)), mercury chloride (Hg(II)) or mono-methylmercury chloride (MeHg(I)) through *ad libitum* drinking water (tap water with 0.2% nitric acid). Rats were exposed to broad ranges of lead acetate (4, 7, 14, 29, 57, 357, 1607, 45000 µg/kg-bw/d) or mercury chloride (7, 14, 29, 57, 357, 2000, 4000, 8000 µg/kg-bw/d) or mono-methylmercury chloride (4, 7, 14, 29, 57, 357, 1607 µg/kg-bw/d) based on published studies and LD_{50} (Grotto et al., 2009; Carmignani et al., 1983; 1992; Malvezzi et al., 2001; Jin et al., 2012). Average water consumption was assumed to be 10-12 ml/kg-bw/d, which would result in an expected water consumption of 40-50 ml per day and rat in this study. Based on these values for water consumption, the metal concentrations in the drinking water were calculated to achieve the above mentioned dose ranges. Control rats (n = 9) received tap water with 0.2% nitric acid. All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

3.5.2 High-resolution cardiovascular ultrasound, arterial blood pressure and electrocardiography

At baseline and after four weeks of exposure, heart function and blood flow in the carotid artery of the anesthetized rats (isoflurane 5% and oxygen 1mL/min to induce, isoflurane 1-3% and oxygen 0.5mL/min for maintenance) were examined using a high-resolution B-mode and power Doppler ultrasound, respectively (Visualsonics Vevo 770, Toronto, ON, Canada). Prior to anesthesia, rats were injected with 3 ml saline (s.c.) and placed on a heated platform controlled by a rectal thermometer to maintain body temperature at 37°C. All measurements were carried out as described and performed previously in our laboratory (Boon, 2011; Jadhav et al., 2013). Briefly, for cardiac function the length of the left ventricle in systole and diastole were each measured in triplicate views of the parasternal long axis in B-mode followed by three cross-sectional areas (A1, A2, A3) taken at the parasternal short axis using Visualsonics software or exporting TIFF images for off-line analysis of left ventricular free wall thickness (Image-Pro Express 6.0, MediaCybernetics Inc, USA, 2006). Ventricular length and cross-sectional area values were used in Simpson's rule (Boon, 2011) to calculate left ventricular volumes at end-systole (ESV) and end-diastole (EDV). An image of the carotid artery just prior to the bifurcation was taken first in B-mode to determine arterial luminal

diameter and arterial wall thickness, followed by power Doppler to measure pulse-wave velocity. Stroke volume (SV) was calculated as the difference between EDV and ESV, while cardiac output was calculated as the product of heart rate (HR) and SV, which are presented after 4 weeks of exposure. Due to large inter-individual variation evident at baseline (see Supplemental Table S8.1 for baseline data), results for free wall thickness of the heart (Figure 3.3), carotid diameter and free wall thickness as well as peak velocity (Figure 3.4) are presented as the difference between week 4 exposure and baseline values for each individual rat.

After the final ultrasound examination, rats were allowed to recover for 24 h before measurement of blood pressure, heart rate and electrical activity of the heart using Lead II on a Powerlab system and analysed using LabChart 7.0 (software (ADInstruments, Colorado Springs, CO, USA), followed immediately by euthanasia and tissue collection. Prior to blood pressure and ECG measurements, rats were injected with 3 ml saline (s.c.) and placed on a 37°C heating blanket to maintain body temperature. A saline-filled pressure catheter connected to a PowerLab was inserted into the femoral artery for intravascular blood pressure measurement after calibration with a mercury column (ADInstruments, Colorado Springs, CO, USA). Averaged values from blood pressure, heart rate and ECG (PR, QRS and QT intervals) over 10 minutes were used in statistics. Blood and pulse pressure as well as heart rate are presented after 4 weeks of exposure.

3.5.3 Blood analysis

Lead and mercury levels in whole blood samples were analyzed by the Prairie Diagnostic Services Inc. located at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The blood samples were digested with concentrated nitric acid (70%) in a Mars Microwave Accelerated Reaction System (CEM, Matthews, NC, USA) and digested samples analyzed with Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Scientific X Series 2, Waltham, MA, USA) against standard reference materials.

3.5.4 Statistical analysis

All results expressed as mean ± standard deviation (SD). Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnov and Bartlett's tests. All data satisfied these requirements for parametric statistics after outliers were removed. Differences among groups were detected by one-way Analysis of Variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post-hoc test (GraphPadPrism, GraphPad Software, Inc, San Diego, CA, USA). A $p < 0.05$ was considered significant. NOAELs (no observed adverse effects level) and LOAELs (lowest observed adverse effects level) were directly derived from the study results based on increases in systolic blood pressure since this is the endpoint with the clearest link to increased cardiovascular risk. The highest dose of a given metal producing no statistically significant increase in pressure was

determined to be the NOAEL, while the lowest dose producing a significant increase in pressure was considered the LOAEL. An alternative to the NOAEL-LOAEL approach is the benchmark dose (BMD) lower-limit approach using a 10% confidence (BMDL10). We used US EPA BMD software 2.5 (U.S. EPA, 2014a) to calculate BMDL10s for systolic blood pressure for the three metals. For lead, we did not include doses which produced a decrease in blood pressure since this effect is not clearly associating with adverse cardiovascular risk. Instead we used an exponential model and doses of 29 µg/kg-bw/d and higher, which showed either no effect or an increase on blood pressure. For both Hg species a polynomial model fit and the full dose range was used.

3.6 Results

3.6.1 Growth performance and blood metal analysis

All doses of lead were sub-lethal since no mortalities were observed over the 4-week period of exposure. Lead-exposed rats showed a significantly greater weight gain at low to moderate doses (ranging from 4 – 57 µg/kg-bw/d) compared to rats from the control group (Table 3.1). In contrast, rats from the three highest lead dose groups gained a similar amount of weight as control rats (Table 3.1). No significant differences among lead-exposed rats compared to control rats were observed in the relative weights of brain, heart, kidney and liver (expressed as percent body weight; Table 3.1). However, spleen weight tended to be higher in all lead-exposed groups compared to control, achieving significance in 7, 29, 357 and 45,000 µg/kg-bw/d lead groups (Table 3.1). In contrast to lead, inorganic mercury exposure at the highest dose of 8000 µg/kg-bw/d showed severe signs of toxicity (significant weight loss, reduced food and water intake, reduced grooming; Table 3.1). This toxicity was evident within a week and was severe enough that all rats of this treatment group had to be terminated after only two of the four-week exposure (values for rats after 2-weeks exposure shown in Table 3.1). The rats from this highest dose group would have likely died before the end of the 4-week exposure and thus this highest dose was considered a 100% lethal dose. All rats from lower inorganic mercury doses examined in this study did not exhibit obvious toxicity and thus were considered sub-lethal. After 2-weeks exposure to the highest inorganic mercury, relative brain and heart weight were significantly higher in this 8000 µg/kg-bw/d group compared to control. In addition, inorganic mercury exposure for 4 weeks at the next two highest doses plus the highest dose after 2-weeks exposure led to higher kidney weights compared to control (i.e. kidney weight increased for Hg(II) ≥ 357 µg/kg-bw/d; Table 3.1). No mortalities were observed due to methylmercury exposure at any of the doses examined in the current study. In contrast to inorganic mercury, methylmercury-exposed rats showed only mild signs of toxicity (reductions in grooming, food intake and weight gain) and only at the highest dose of 1607 µg/kg-bw/d (Table 3.1). Furthermore, this highest methylmercury group showed increased brain, increased kidney and decreased liver weights

compared to control rats (Table 3.1). Therefore, all methylmercury doses in this study could be considered sub-lethal.

Table 3.1. Morpholometric values and mortality of rats exposed to lead acetate, mercury chloride or monomethyl-mercury chloride versus control (0.9% nitric acid) in the drinking water for four weeks.

Experimental dose (µg/kg-bw/d)	Change in bodyweight (g)	Brain/body weight (%)	Heart/body weight (%)	Kidneys/body weight (%)	Liver/body weight (%)	Spleen/body weight (%)	Mortality (%)
Control							
0	113 ± 19	0.38 ± 0.05	0.26 ± 0.02	0.66 ± 0.05	3.58 ± 0.36	0.20 ± 0.02	0
Pb(II)							
4	188 ± 23****	0.41 ± 0.04	0.26 ± 0.04	0.70 ± 0.03	3.86 ± 0.37	0.21 ± 0.04	0
7	224 ± 27****	0.39 ± 0.03	0.26 ± 0.03	0.75 ± 0.03	4.03 ± 0.33	0.25 ± 0.02**	0
14	165 ± 17**	0.42 ± 0.05	0.29 ± 0.01	0.77 ± 0.07	4.33 ± 0.32	0.25 ± 0.02	0
29	209 ± 34****	0.41 ± 0.06	0.25 ± 0.01	0.71 ± 0.08	3.54 ± 0.31	0.24 ± 0.04*	0
57	217 ± 32****	0.41 ± 0.04	0.26 ± 0.02	0.70 ± 0.06	3.85 ± 0.30	0.22 ± 0.03	0
357	106 ± 22	0.40 ± 0.03	0.26 ± 0.02	0.70 ± 0.04	3.71 ± 0.31	0.24 ± 0.04*	0
1607	121 ± 29	0.39 ± 0.03	0.24 ± 0.01	0.70 ± 0.08	3.78 ± 0.34	0.21 ± 0.02	0
45000	113 ± 33	0.38 ± 0.03	0.24 ± 0.01	0.70 ± 0.04	3.81 ± 0.37	0.26 ± 0.03***	0
Hg(II)							
7	133 ± 14	0.41 ± 0.03	0.25 ± 0.02	0.71 ± 0.08	3.59 ± 0.30	0.22 ± 0.01	0
14	97 ± 19	0.40 ± 0.03	0.26 ± 0.02	0.67 ± 0.06	3.50 ± 0.22	0.20 ± 0.01	0
29	130 ± 31	0.41 ± 0.01	0.25 ± 0.03	0.68 ± 0.07	3.55 ± 0.31	0.19 ± 0.04	0
57	112 ± 10	0.40 ± 0.02	0.26 ± 0.02	0.70 ± 0.04	3.28 ± 0.36	0.20 ± 0.02	0
357	137 ± 18	0.41 ± 0.02	0.25 ± 0.02	0.76 ± 0.05*	3.42 ± 0.45	0.22 ± 0.02	0
2000	88 ± 26	0.37 ± 0.07	0.25 ± 0.02	0.83 ± 0.10***	3.41 ± 0.29	0.24 ± 0.03	0
4000	90 ± 24	0.40 ± 0.02	0.26 ± 0.02	0.87 ± 0.07****	3.58 ± 0.40	0.21 ± 0.03	0
8000	-72 ± 92****	0.59 ± 0.11****	0.30 ± 0.03**	1.17 ± 0.16****	3.70 ± 0.46	0.21 ± 0.09	100

Values shown are mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 in Fisher's LSD after 1-way ANOVA. n=5-6 rats/group

Table 3.1. Morpholometric values and mortality of rats exposed to lead acetate, mercury chloride or monomethyl-mercury chloride versus control (0.9% nitric acid) in the drinking water for four weeks (continued).

Experimental dose (µg/kg-bw/d)	Change in body weight (g)	Brain/body weight (%)	Heart/body weight (%)	Kidneys/body weight (%)	Liver/body weight (%)	Spleen/body weight (%)	Mortality (%)
MeHg(l)							
4	120 ± 24	0.44 ± 0.04**	0.25 ± 0.02	0.64 ± 0.07	3.53 ± 0.09	0.20 ± 0.02	0
7	103 ±10	0.41 ± 0.02	0.25 ± 0.02	0.64 ± 0.05	3.12 ± 0.49*	0.18 ± 0.03	0
14	124 ± 35	0.40 ± 0.03	0.25 ± 0.02	0.64 ± 0.04	3.53 ± 0.36	0.19 ± 0.02	0
29	109 ± 7	0.39 ± 0.04	0.25 ± 0.02	0.66 ± 0.04	3.37 ± 0.31	0.19 ± 0.02	0
57	97 ± 22	0.42 ± 0.02	0.27 ± 0.02	0.68 ± 0.06	3.49 ± 0.15	0.19 ± 0.02	0
357	121 ± 35	0.39 ± 0.01	0.25 ± 0.02	0.69 ± 0.03	3.40 ± 0.13	0.18 ± 0.01	0
1607	38 ± 22****	0.47 ± 0.04****	0.26 ± 0.03	0.77 ± 0.05***	2.87 ± 0.39***	0.19 ± 0.02	0

Values shown are mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 in Fisher's LSD after 1-way ANOVA. n=5-6 rats/group

Actual water consumption measured in the current study (30 ± 4 ml/day) was slightly lower for all groups compared to the 50 ml/day reported in the literature (Table 3.2). While lead and inorganic mercury exposures both did not have an effect on water consumption (except at the highest and lethal inorganic mercury dose of 8000 µg/kg-bw/d – water consumption not shown for this group) compared to control, methylmercury exposure significantly and dose-dependently decreased water consumption (Table 3.2). Despite the lower overall water consumption and decreasing trend with higher methylmercury, estimated metal consumption was lower, but still close to the targeted daily doses for all three metal species examined in this study (Table 3.2). Limited resources prevented us from measuring actual lead and mercury blood levels in all treatment groups. Therefore, whole blood samples were analyzed for lead and methylmercury treatment groups where an adverse cardiovascular effect was observed plus the next lower dose without an adverse effect in order to determine both lowest observed adverse effects levels (LOAEL) and no-observed adverse effects levels (NOAEL). However, since no adverse cardiovascular effects were observed for any sub-lethal inorganic mercury group, the four highest doses were analyzed instead. In general, rats exposed to lead showed a dose-dependent increase in blood lead levels (Table 3.2). Mercury analysis revealed that rats exposed to 57 µg/kg-bw/d inorganic mercury had blood mercury levels that were not significantly different from control rats, while the three highest inorganic mercury doses resulted in statistically significant increases in total blood mercury (Table 3.2). In contrast, methylmercury exposure ≤ 357 µg/kg-bw/d showed significant (and immensely higher than inorganic mercury), dose-dependent increases in total mercury blood levels (Table 3.2). The exception to this dose-dependent behavior of methylmercury was the highest dose of 1607 µg/kg-bw/d which showed a sharp decrease in mercury blood levels compared to the next lowest dose, but still higher than control rat levels (Table 3.2).

Table 3.2. Estimated lead and mercury exposure, target drinking water concentrations and resulting blood levels in rats after four weeks exposure via drinking water.

Target dose (µg/kg-bw/d)	Actual water consumption (ml/d)	Nominal drinking water concentration (µg(ml)	Estimated dose consumed (µg/kg-bw/d)	Actual Pb level in blood (µg/L)	Actual total Hg level in blood (µg/L)
Control					
0	33 ± 5	0	0	1.4 ± 1.2	21 ± 14
Pb(II)					
357	33 ± 3	5	330 ± 30	17 ± 7	-
1607	36 ± 5	21	1270 ± 680	86 ± 29***	-
45000	38 ± 2	600	46000 ± 2600	350 ± 75****	-
Hg(II)					
57	32 ± 4	0.8	50 ± 7	-	14 ± 1
357	33 ± 4	5	330 ± 40	-	28 ± 10*
2000	30 ± 3	27	1600 ± 140	-	186 ± 44****
4000	26 ± 3	53	2800 ± 330	-	178 ± 26****
MeHg(I)					
4	27 ± 4**	0.05	3 ± 0.4	-	65 ± 37
7	29 ± 4	0.1	6 ± 1	-	193 ± 165
14	29 ± 3*	0.2	11 ± 1	-	318 ± 146
29	27 ± 1**	0.4	22 ± 1	-	346 ± 193
57	28 ± 3*	0.8	45 ± 5	-	1930 ± 1316
357	27 ± 2**	5	270 ± 15	-	14,507 ± 4631
1607	22 ± 2****	21	1100 ± 94	-	226 ± 69*

Values shown are mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 in Fisher's LSD after 1-way ANOVA. n=5-6 rats/group.

3.6.2 Blood and pulse pressure

Blood pressure at 4 weeks of exposure showed differences in response compared to control rats, depending on both the type and dose of metal (Figure 3.1). First, lead showed a biphasic dose response curve with significant decreases in systolic, diastolic and pulse pressures at lower doses (7 µg - 29 µg Pb(II)/kg-bw/d respectively; Figure 3.1). However, at higher lead exposures (1607 µg Pb(II)/kg-bw/d and above), rats instead had significantly higher systolic and diastolic blood pressure without a difference in pulse pressure compared to control rats (Figure 3.1). When we next consider responses to inorganic mercury, rats exposed to this metal, regardless of dose, did not show any significant effect on the systolic, diastolic or pulse pressures (Figure 3.1). Finally, methylmercury significantly increased systolic, diastolic and pulse pressures at all doses starting at 7 µg MeHg(I)/kg-bw/d and above for systolic and pulse pressures, but from 14 µg MeHg(I)/kg-bw/d and above for diastolic blood pressure (Figure 3.1). No changes were observed for heart rate after 4 weeks of exposure (see Figure 3.2).

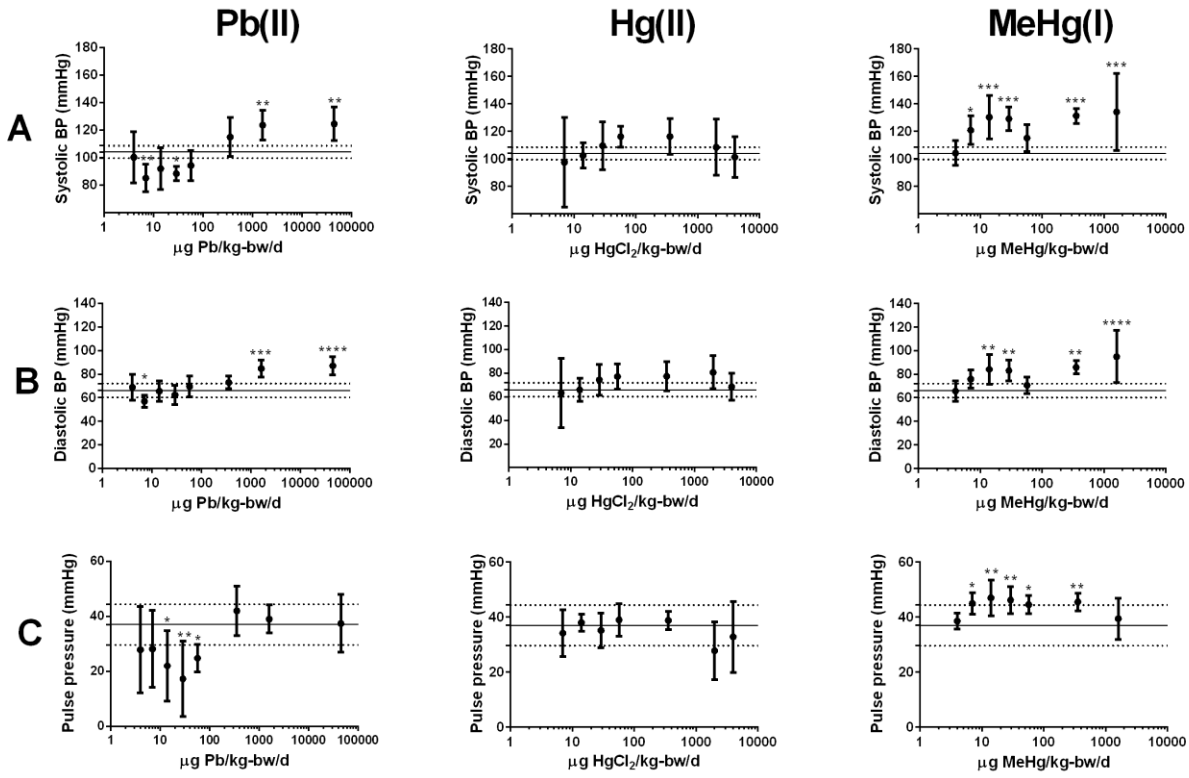

Fig. 3.1. Blood and pulse pressure in rats exposed to lead (left column), inorganic mercury (middle column) and methylmercury (right column) for four weeks via the drinking water. Results for systolic blood pressure (A), diastolic blood pressure (B) and pulse pressure (C) are shown as mean± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p < 0.05, **p <0.01, ***p <0.001, ****p < 0.0001 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.

3.6.3 Cardiac function and hemodynamics

After 4 weeks of exposure, the heart rate, end-diastolic (EDV), end-systolic (ESV) and stroke volumes (SV) were measured in control and metal-exposed rats. Lead exposure did not significantly affect heart rate, but did increase stroke volume and subsequently cardiac output at intermediate doses (14, 29 and 57 µg/kg-bw/d lead; Figure 3.2) where lower blood pressure was also observed (compare to Figure 3.1). However, at higher lead doses (above 57 µg/kg-bw/d lead), stroke volume and cardiac output were no longer significantly different from control values (Figure 3.2). In comparison, inorganic and organic mercury did not significantly affect any of the cardiac parameters examined (heart rate, stroke volume or cardiac output) compared to control (Figure 3.2). Finally, methylmercury exposure significantly decreased stroke volume at 14 µg/kg-bw/d, but not at any other dose and had no significant effect on heart rate or cardiac output at any dose (Figure 3.2). Increased SV was evident with lower, but not higher doses of lead (Figure 3.2). Both EDV and ESV increased at these same lower doses of lead (Supplemental Figure S8.1), with the increased SV due to a larger increase in EDV. Despite the changes in SV, EDV and ESV at lower lead doses, lead at all doses had no significant effect on ejection fraction compared to control (Supplemental Figure S8.1). In contrast to lead, neither inorganic mercury nor methylmercury had any significant effect on EDV, ESV or ejection fraction compared to control (Supplemental Figure S8.1).

Cardiac excitation was also measured by electrocardiogram (ECG) at the end of the 4-week exposures to these metals. No significant changes in the PR, QRS or QT intervals after 4 weeks of exposure were observed for any of the metals at any dose compared to control (data not shown) which is consistent with the observed lack of effect on heart rate for any of these metals.

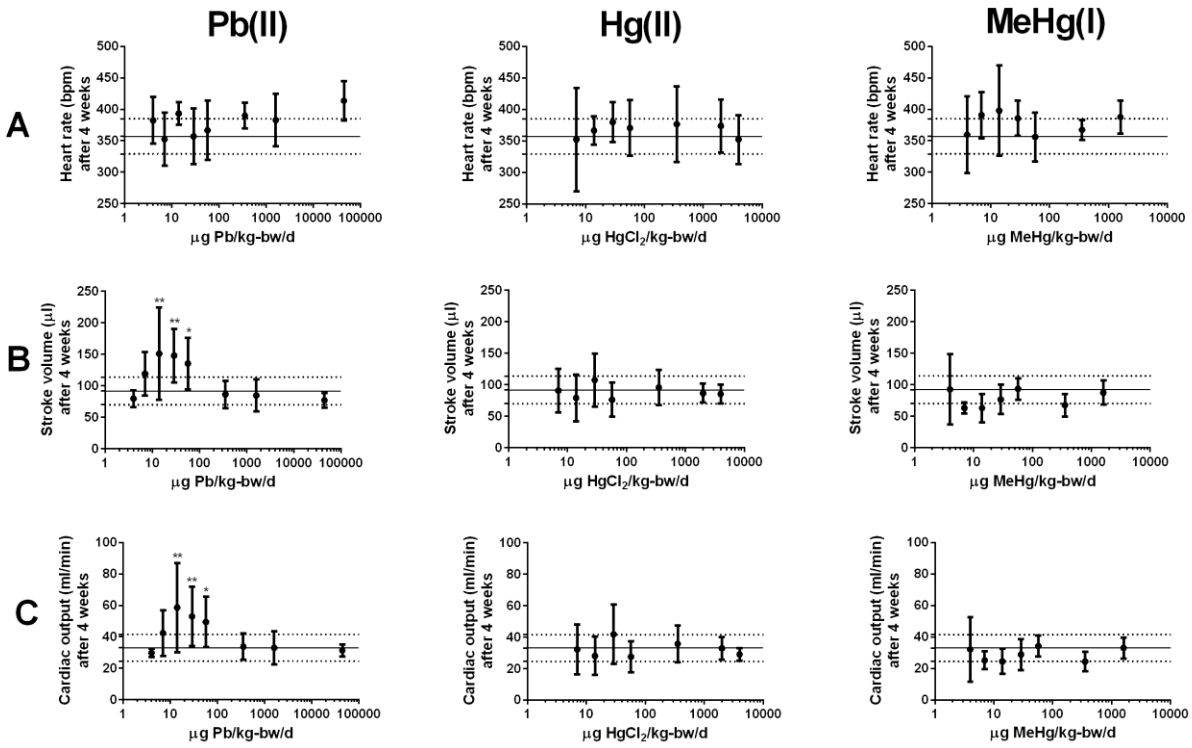

Fig. 3.2. Heart rate, stroke volume and cardiac output in rats exposed to lead (left column), inorganic mercury (middle column) and methylmercury (right column) for four weeks via the drinking water. Results for heart rate (A), stroke volume (B), cardiac output (C) are shown as mean ± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05, **p < 0.01 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.

Some minor changes in left ventricular free wall thickness displayed as difference between week 4 and baseline values were noted at a few doses of some metals in this study. After 4 weeks of exposure to lead, left ventricular free wall thickness was not significantly different from control at systole, but did show a significant increase during diastole at one intermediate dose where lower blood pressure was also observed (14 µg Pb(II)/kg-bw/d; Figure 3.3). In contrast, inorganic mercury exposure at all doses failed to significantly alter free wall thickness compared to control (Figure 3.3). However, methylmercury exposure resulted in significantly decreased left ventricular free wall thickness during systole without a change during diastole at two intermediate doses (29 and 57 µg MeHg(I)/kg-bw/d; Figure 3.3).

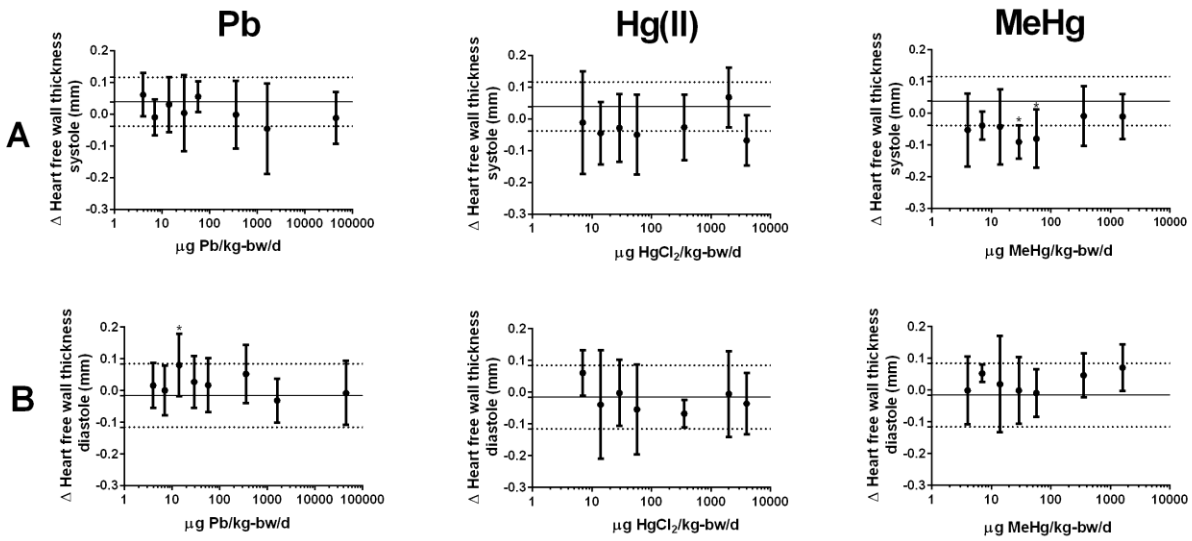

Fig. 3.3. Change in left ventricular wall thickness from baseline to week-4 of oral exposure to lead (left column), inorganic mercury (middle column) and methylmercury (right column). Results are shown as mean ± SD during systole (A) or diastole (B): For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.

The carotid artery diameter, wall thickness and peak blood velocity showed different patterns of response after exposure to different metals (Figure 3.4) represented as the difference between week 4 and baseline values. Lead again showed a biphasic dose-response curve for carotid artery diameter, carotid wall thickness and carotid blood velocity (Figure 3.4). Lead significantly increased the carotid artery diameter, while significantly decreasing carotid artery peak blood velocity at the lowest to moderate doses (4 - 357 µg/kg-bw/d lead) with a return to control values at higher doses (Figure 3.4). In contrast, carotid artery wall thickness was also significantly greater than control at the lowest lead dose to 57 µg/kg-bw/d lead, with a return to control values at higher levels (Figure 3.4). Similar to all other blood pressure and cardiac end-points, inorganic mercury failed to show any significant effect on carotid artery morphology or blood flow compared to control (Figure 3.4). Finally, methylmercury-exposed rats had significantly greater carotid diameters at a few doses, but there was no clear dose-response pattern (higher at 4, 57 and 1607 µg/kg-bw/d methylmercury), but no significant effects on the carotid artery wall thickness or peak blood velocity were observed in methylmercury-exposed rats at any dose compared to control (Figure 3.4).

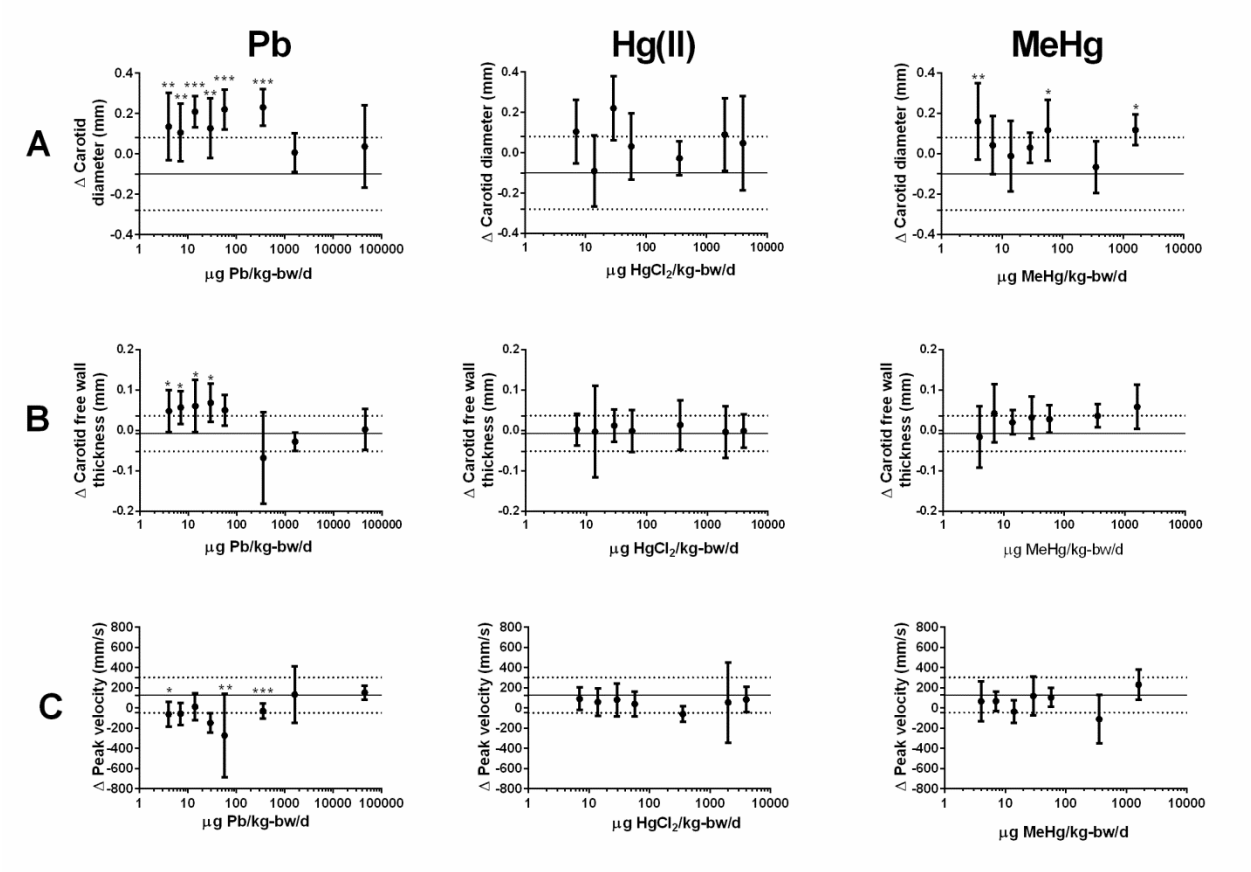

Fig. 3.4. Change in carotid artery luminal diameter, wall thickness and peak pulse-wave velocity from baseline to week-4 of oral exposure to lead (left column), inorganic mercury (middle column) and methylmercury (right column) for four weeks via the drinking water. Results for carotid luminal diameter (A), carotid wall thickness (B) and carotid artery pulse-wave velocity (C) are shown as mean ± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05, **p <0.01, ***p <0.001 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.

3.7 Discussion

This study confirmed that both lead and methylmercury have adverse cardiovascular effects using oral exposure in a normal rat model and then determined thresholds for these adverse effects. Lead at doses ≥ 1607 µg/kg-bw/d and methylmercury at doses ≥ 7 µg/kg-bw/d increased blood pressure (15-20 mmHg) above controls, whereas inorganic mercury had no effect on any of the evaluated cardiovascular end-points. The observed increase in blood pressure induced by lead and methylmercury exposure was sufficient to turn a normotensive individual into a pre-hypertensive or overtly hypertensive individual. Therefore, lead and methylmercury, but not inorganic mercury, potentially contribute to increased cardiovascular risk but more work is needed to link our sub-chronic exposure of 4 weeks in rats to the continuous, environmental exposures experienced by the human adult population.

3.7.1 Cardiovascular effects of lead

In addition to the well-known neurotoxicological effects, lead also interferes with the biosynthesis of hemoglobin causing lead-induced anemia (Jang et al., 2011). Although red blood cells were not examined in this study, spleen weight was increased, which could be a consequence of removing damaged blood cells. Aside from anemia, blood pressure is a well-studied cardiovascular endpoint for lead because it is easy to examine and facilitates comparison between animals and humans (Navas-Acien et al., 2007; Cheng et al., 2001; Glenn et al., 2003; Nash et al., 2003). Dose-response curves for lead were biphasic for blood and pulse pressures. Compared to controls, low levels of lead decreased systolic, diastolic blood pressure and pulse pressure and dilated the carotid artery, which is consistent with the observed decrease in blood pressure. These physiological effects would also reduce the peak blood velocity in the carotid artery (Pappano, 2008). However, when blood pressure and arterial resistance decrease due to arterial dilation, the baroreceptor response can provide compensation to maintain blood pressure primarily via increased sympathetic stimulation of cardiac function (Pappano, 2008). Thus, it is possible, although not directly measured in the current study, that the observed increased stroke volume and resulting increase in cardiac output at low lead doses are compensatory mechanisms elicited by the baroreceptor response. An argument against a baroreceptor response is the fact that greater end-diastolic filling was the mechanism responsible for the increased stroke volume, an effect generally mediated by increased preload and blood volume rather than rapid compensatory mechanisms like the baroreceptor. Another possible explanation for higher cardiac output with low-dose lead, which is not confirmed by existing literature, is a direct cardio-stimulatory effect of lead at low doses. A couple of studies are available showing a direct acute inhibitory effects of both lead and mercury on the cardiac contractile machinery leading to suppressed cardiac contractility (Souza de Assis et al., 2003;Vassallo et al., 2008). Finally, the prolonged dilation of the carotid artery in this experiment resulted in increased wall thickness possibly through changes in the blood flow quality or via a direct proliferative effect of lead on the vascular wall. Higher lead exposure significantly increased diastolic and systolic, but not pulse pressures. This observation is in agreement with a large number of previous studies indicating a causal relationship between lead exposure and hypertension (Cheng et al., 2001; Glenn et al., 2003; Nash et al., 2003; Navas-Acien et al., 2007). In the current study, higher systolic and diastolic pressure occurred without changes in cardiac function (heart rate, stroke volume and cardiac output) or vascular function (carotid artery diameter, carotid wall thickness or carotid pulse wave velocity), compared to control. Our study did not show any changes in the electrocardiograms (ECG), while data from the Normative Aging Study indicated a positive association between lead body burden and prolonged duration of the QT interval (Cheng et al., 1998; Eum et al., 2011). The resulting discrepancy may be due to species differences or to exposure length/dose differences. Lead-induced hypertension may be due to disturbances in systems involved in long-term blood pressure

control such as the renin-angiotensin system or kidney function (Vargas Robles et al., 2007). While this latter possibility would be consistent with the observed increase in diastolic filling, direct investigation of possible mechanisms of effects was beyond the scope of the current study.

3.7.2 Cardiovascular effects of mercury

The current study found that inorganic mercury did not alter any of the measured cardiovascular parameters, including blood pressure despite having similar total mercury blood concentrations as the methylmercury dosed group. The lack of blood pressure effect of inorganic mercury agrees well with a study by Blanco-Rivero (2011), in which rats were injected with 4.6 µg $HgCl_2$/kg-bw (i.m.) and received 0.07 µg/kg-bw/d maintenance dose for 30 days. In the human population, the main exposure source for inorganic mercury is dental amalgam which releases elemental mercury and is quickly oxidized in the body to Hg(II) after inhalation (Clarkson et al., 2007). In contrast to our results, Goodrich et al (2013b) identified an association between decreased systolic blood pressure and mercury levels in the urine, which can be an indicator for inorganic mercury exposure primarily from dental amalgam fillings. These discrepancies between the current study, Blanco-Rivero (2011) and Goodrich et al (2013b) are likely due to differences in the toxicokinetics of inorganic and elemental mercury and the different exposure routes, namely oral, intramuscular injection and inhalation. In our study, which did not detect any change in the wall thickness of the carotid artery, we used using high-resolution ultrasound. In contrast, Aguado et al (2013) used a pressure myograph and found a reduced wall thickness in the thoracic aorta and mesenteric arteries of inorganic mercury-exposed rats. This may be due to use of different arteries or methodological differences. Taken together, we can conclude that inorganic mercury by itself does not exert cardiovascular effects, but the possibility that elemental mercury does exert cardiovascular effects. Similar total mercury blood levels attributed to inorganic mercury or methylmercury differed in their cardiovascular effects. While inorganic mercury showed no effects on the cardiovascular system, methylmercury caused significant effects on systolic and pulse pressures and appears to exert much greater cardiovascular toxicity compared to inorganic mercury at similar or lower total mercury blood levels. Therefore, adverse cardiovascular effects cannot be predicted by total blood mercury level alone. Methylmercury doses closer to the higher end of our dosing range 500 µg/kg-bw/d methylmercury by gavage for 28 days (Wakita, 1987), 100 µg MeHg/kg-bw for 100 days (Grotto et al., 2009) or a diet high in MeHg-contaminated fish to simulate human consumption fed to rats for 12 weeks (Grotto et al., 2011) increased blood pressure either latently (significant only weeks after the exposure period) or after a longer exposure duration compared to the current study. We were able to observe significant increases in systolic blood pressure at much lower doses (starting at 7 µg/kg-bw/d methylmercury), which is most likely due to the more sensitive method of blood pressure measurement used in the current study (intravascular, direct blood pressure method) compared to

the previous studies where a much less sensitive and less reliable method was used (tail cuff plethysmography). Although no significant cardiac effect was observed at any methylmercury dose in the current study, a significant vascular effect (increased carotid artery diameter) was observed at an even lower dose of 4 µg/kg-bw/d methylmercury than the blood pressure effect. However, the direct effect of increased carotid diameter would be to reduce peripheral resistance and decrease blood pressure while the opposite effect was observed in this study. The effect of methylmercury on the carotid artery diameter was inconsistent for all doses. Therefore the blood pressure values were taken as more reliable and used for derivation of threshold of effect below. In contrast to inorganic mercury exposure, methylmercury exposure increases blood pressure but the mechanism is unclear.

3.7.3 Blood metal levels and thresholds for adverse cardiovascular effect of lead and mercury

In the current study, control rat blood lead levels were 1 µg/L (or 0.1 µg/dL), which is lower than the level of 13.4 µg/L (or 1.34 µg/dL) in the general population (Health Canada, 2013). Exposures to 357 and 1607 µg Pb(II)/kg-bw/d produced blood lead levels of 2 ± 0.7 and 9 ± 3 µg/dL respectively, which are below the lead guidance value of 10 µg/dL (Healey et al., 2010), while the highest exposure group (at 45,000 µg Pb(II)/kg-bw/d) produced levels comparable to occupational exposures (≥ 25 µg/dL) (CDC, 2011) to lead. Thus, our range of lead doses is suitable for determining if current thresholds for effects are sufficiently protective. The mercury reference values of 20 – 100 µg/L (Statistics Canada, 2008) were not exceeded by the control rats (21 ± 14 µg/L) and lowest dose groups of both mercury species, i.e. 57 µg Hg(II)/kg-bw/d (14 ± 1 µg/L) and 4 µg MeHg(I)/kg-bw/d (65 ± 37 µg/L). Inorganic mercury showed a slow dose-dependent increase in blood levels, while the highest dose of methylmercury showed a sharp decrease in total mercury levels probably due to reduced water consumption, weight loss and signs of toxicity.

As increased blood pressure is the most important risk factor for cardiovascular diseases, systolic blood pressure was selected as critical end-point. NOAELs and LOAELs were directly derived from the study results. NOAEL and LOAEL for lead were 57 µg/kg-bw/d and 357 µg/kg-bw/d, respectively. As inorganic mercury did not result in any cardiovascular effect, it was not possible to derive a LOAEL. The NOAEL was determined at 4000 µg/kg-bw/d. For methylmercury, a NOAEL of 4 µg/kg-bw/d and a LOAEL of 7 µg/kg-bw/d were derived. An alternative to the NOAEL-LOAEL approach is the benchmark dose (BMD) approach. The $BMDL_{10}$s calculated for lead, inorganic mercury and methylmercury were 1.1, 1.3 and 1.0 µg/kg-bw/d, respectively. The European Food Safety Authority (EFSA, 2012b) published a $BMDL_{01}$ for lead and cardiovascular effects of 1.5 µg/kg-bw/d. Although these estimates are remarkably close, discrepancies can be explained by the use of different datasets and species differences.

A large number of studies in humans exists investigating human exposure to low levels of methylmercury on a chronic basis, largely through the consumption of fish and seafood. Several epidemiological studies show a positive association between the intake of sea mammals, fish and seafood and blood pressure increase. Comparing blood pressure values of Danes (blood mercury of 2.2 µg/L) and Greenlanders (blood mercury of 10.8 µg/L) showed that Greenlanders following a traditional diet had higher blood and pulse pressures with increasing mercury blood levels (Pedersen et al., 2005) which is strikingly similar to our observations with methylmercury in rats. A study in the Brazilian Amazon (Fillion et al., 2006) showed a positive relation between mercury hair levels of 17.8 µg/g (we calculated: blood Hg 71.2 µg/L based on conversion ratios in Clarkson (2007)) and higher blood pressure, although the overall prevalence of hypertension was low. Valera et al carried out a number of studies evaluating different population groups, such as Inuit, French Polynesians, whose traditional diet is rich in fish and seafood. In Nunavik Inuits a positive relation between blood mercury (50 – 133 µg/L) and systolic blood and pulse pressure was observed (Valera et al., 2008; 2009). These findings are in contrast to the results from studies in French Polynesians (teenager: blood Hg 8 µg/L; adults: blood Hg 15 µg/L) (Valera et al., 2011b) or in Cree adults (blood Hg 15 µg/L) (Valera et al., 2011a) which did not show any significant relationship to blood pressure. These blood levels are similar to the mercury blood level of 65 µg/L from the lowest methylmercury dose in our rat study, which did not detect a significant change in blood pressure compared to control. However, the next dose of 7 µg/kg-bw/d methylmercury resulted in higher mercury blood level at 193 µg/L and an increase in blood pressure, which is similar to the highest end of the range reported in the above human studies. In human studies, confounding factors, including consumption of environmental pollutants, beneficial nutrients as well as co-existence of diseases like obesity, diabetes, cigarette smoking or dental health, can potentially modulate cardiovascular function. Therefore, considering these confounding factors, the fact that the methylmercury levels producing significant increases in blood pressure in rats in the current study are close to the observed human range reporting associations provides high confidence in these results.

3.7.4 Strengths and limitations

The strength of our study is the use of highly sensitive imaging and blood pressure measurement techniques for the examination of a broad range of cardiovascular end-points, including blood pressure as an important biomarker in human and animal studies with clear clinical benchmarks for health consequences in humans (WHO/ISH, 2003), and better characterization of vascular or cardiac effects. Additionally, the high number of metal doses, allowed for better identification of thresholds for adverse cardiovascular effects. Study limitations were the short exposure duration (4 weeks) in comparison to long-term human exposure, the use of a male only rat cohort and the small

sample size per group. However, despite the small group size, the higher sensitivity of our measurement methods allowed us to detect changes at lower thresholds than reported previously.

3.8 Conclusions

To our knowledge, this was the first study to investigate cardiovascular effects of lead and mercury exposure using high resolution imaging techniques (high-resolution ultrasound) along with direct, intravascular blood pressure measurements in rats. We confirmed the positive association between oral lead exposure and higher blood pressure. However, of note was the clear biphasic dose-response to lead, with lower doses decreasing blood pressure and higher doses causing sufficient increase in blood pressure to make an individual hypertensive (15-20 mmHg increase). Low lead doses seemed to have a greater effect on the vasculature where vasodilation lowered blood pressure. Lead is known to substitute for calcium ions (Ballatori, 2002), thereby blocking smooth muscle calcium channels and offering a potential explanation for the observed vasodilation with low-dose lead. In contrast, direct effects of low-dose lead on cardiac calcium channels which would impair cardiac contractility were not detected since instead an increase in stroke volume was observed in the current study. The observed increase in stroke volume with low-dose lead would be consistent with either the decreased afterload or a baroreceptor response to vasodilation. Then, at higher doses of lead in this study, blood pressure increased despite a return to control levels of cardiac function. While we do not know the mechanism(s) explaining how high-dose lead increased blood pressure, it likely relates to effects on other blood pressure regulating systems that function independent of cardiac or neural effects, such as increased renin-angiotensin-aldosterone system activation, vasoconstriction from angiotensin II and/or increased blood volume. Elucidating mechanisms of metal effects in the cardiovascular system is the focus of future investigations.

In this study, we also shed light on the relationship between mercury species and cardiovascular health. While inorganic mercury did not affect cardiovascular health in our study and therefore cannot be used as a surrogate for environmental mercury exposure, methylmercury clearly poses a risk factor for hypertension and should be considered in assessment of methylmercury risk to human health. In the future, more studies are needed to elucidate mechanisms by which adverse cardiovascular effects of lead and mercury occur as well as examining the influence of nutritional factors or co-existence of disease states that may further increase the cardiovascular risk of these metals.

4 COMBINED EXPOSURE TO LEAD, INORGANIC MERCURY AND METHYLMERCURY SHOWS DEVIATION FROM ADDITIVITY FOR CARDIOVASCULAR TOXICITY IN RATS

4.1 Author contributions

Tanja M. Wildemann was responsible for the daily care of the animals, measurement of cardiovascular effects, termination and tissue collection/processing. She carried out the statistical analysis of the data and prepared the manuscript.

Lynn P. Weber provided scientific input and guidance, provided comments on the manuscript and supported the research financially through her research grant.

Steven D. Siciliano provided scientific input, edited the manuscript and supported the research financially through his research grant.

4.2 Preface

The human population is generally exposed to mixtures of Pb(II), Hg(II) and MeHg(I). However, due to a lack of data on the relevant mixtures, risk assessments are often a challenge. For this case, we have data available on cardiovascular effects from single exposures to Pb(II), Hg(II) and MeHg(I) and their mixtures. This allowed us to directly observe how combined exposures affected the cardiovascular system but also to compare them to the single exposures.

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4.3 Abstract

Environmental exposure to metal mixtures in the human population is common. Mixture risk assessments are often challenging due to a lack of suitable data on the relevant mixture. A growing number of studies show an association between lead or mercury exposure and cardiovascular effects. We investigated the cardiovascular effects of single metal exposure or co-exposure to methylmercury, inorganic mercury and lead. Male Wistar rats received four different metal mixtures for 28 days through the drinking water. The ratios of the metals were based on reference and environmental exposure values. Blood and pulse pressure, cardiac output and electrical activity of the heart were selected as end-points. While exposure to only MeHg(I) increased systolic blood pressure and decreased cardiac output, the effects were reversed with combined exposures (antagonism). In contrast to these effects, combined exposures negatively affected the electrical activity of the heart (synergism). Thus, it appears that estimates of blood total Hg levels need to be

paired with estimates of what species of mercury dominate exposure as well as whether lead co-exposure is present to link total blood Hg levels to cardiovascular effects. Based on current human exposure data and our results, there may be increased risk of cardiac events as a result of combined exposures to Hg(II), MeHg(I) and Pb(II). This increased risk needs to be clarified by analyzing lead and Hg exposure data in relation to cardiac electrical activity in epidemiological studies.

4.4 Introduction

Metals occur ubiquitously in the environment due to natural and anthropogenic sources although environmental concentrations differ depending on the geographical areas. In contrast to organic chemicals, metals have unique properties, such as not being degradable over time or destroyable (U.S. EPA, 2007). Metals are generally found as mixtures with other metals. Therefore, the United States Environmental Protection Agency (U.S. EPA, 1986) recommends assessing the risks originating from combined metal exposures by calculating a hazard index (HI; Eq. 1):

$$HI = E_1/AL_1 + E_2/AL_2 + \ldots + E_i/AL_i$$

The hazard index is defined by the sum of ratios of the exposure (E) to the maximum acceptable level (AL) for i^{th} toxicants. Depending on the number of end-points, in which the risk assessor is interested, more than one HI can be determined.

The human population is generally exposed to a varying number of metals, at the same time leading to chronic, low level exposures. Among the metals of concern, lead and mercury species are important environmental pollutants. Lead (Pb(II)) exposure mainly occurs through the drinking water and food (ATSDR, 2007; EFSA, 2012b; Health Canada, 2013) with increased absorption likely occurring in children and elderly due to their age and nutrition status. For mercury, exposure occurs primarily through dental amalgam or food. Dental amalgam contains about 50% mercury (Richardson and Allan, 1996), and once the fillings are placed in the patient's teeth, mercury vapor will be continuously released and inhaled by the person. The inhaled mercury vapor is able to easily penetrate the blood-brain barrier and oxidation to inorganic mercury rapidly occurs in a number of different tissues and organs, such as brain or kidneys (Clarkson et al., 2007). In contrast, to dental amalgams, where final exposure is to Hg(II), food exposure to mercury is primarily via methylmercury (MeHg(I)). Due to its lipophilic properties, MeHg(I) has a high absorption rate from the gut and is distributed throughout the entire body. It can be incorporated into proteins mimicking the amino acid methionine (Clarkson et al., 2007; Ballatori, 2002) or can be converted into inorganic mercury.

Traditionally, lead and mercury are considered to be neurotoxicants with the developing central nervous system as the most sensitive end-point (Ekino et al., 2007; Bakir et al., 1980; Finkelstein et al., 1998; Sanders et al., 2009). However, Pb(II) or Hg exposure is also associated with

cardiovascular effects. Large epidemiological studies, e.g. the National Health and Nutrition Examination Survey (NHANES), have provided enough evidence to consider the association between Pb(II) exposure and increased blood pressure as causal (Navas-Acien et al., 2007; Glenn et al., 2003) in every major population group included in NHANES. Hypertension is considered to be the most important risk factor for cardiovascular diseases. These cardiovascular diseases are responsible for the majority of deaths worldwide (WHO/ISH, 2003). Laboratory based experiments found that rats exposed to Pb(II) through the drinking water (Skoczynska et al., 2014) had impaired heart function with a decreased cardiac ejection fraction. Epidemiological studies investigating Hg exposure and cardiovascular effects have produced contradicting results. On one hand, results from human studies (Bautista et al., 2009; Valera et al., 2009; Pedersen et al., 2005) demonstrated a relation between Hg exposure and hypertension and pulse pressure. On the other hand, a number of epidemiological studies (Vupputuri et al., 2005; Johansson et al., 2002; Mozaffarian et al., 2012; Valera et al., 2011a) could not show an association between Hg exposure and hypertension or cardiovascular diseases in general (Mozaffarian et al., 2011).

Governmental agencies have been considering hypertension as an important end-point for adults for Pb(II) or Hg exposure. The European Food Safety Authority (EFSA, 2012b) deemed the available body of literature for Pb(II) as sufficient to suggest a 95^{th} percentile lower confidence limit of the benchmark dose of 1% extra risk ($BMDL_{01}$) for systolic blood pressure in adults. A $BMDL_{01}$ of 1.5 µg/kg-bw/d was determined as critical concentration. Due to the limited amount of studies on cardiovascular effects, the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2004) based the tolerable weekly intakes (TWIs) for Hg(II) and MeHg(I) on developmental neurotoxicity with 4 µg/kg-bw (0.57 µg/kg-bw/d) and 1.6 µg/kg-bw (0.23 µg/kg-bw/d) respectively.

Generally, human exposure to Pb(II) and Hg through the environment is below the established reference values for a single exposure. In the European Union, environmental exposures were estimated as 0.039 µg/kg-bw/d for MeHg(I), 0.08 µg/kg-bw/d for Hg(II) (EFSA, 2012a) and 0.68 µg/kg-bw/d for Pb(II) (EFSA, 2012b). Calculating the HI based on environmental exposure and reference values (Eq. 2), a HI of 0.7 is obtained.

$$HI = E_{MeHg(I)}/AL_{MeHg(I)} + E_{Hg(II)}/AL_{Hg(II)} + E_{Pb(II)}/AL_{Pb(II)} = 0.039/0.23 + 0.08/0.57 + 0.68/1.5 = 0.7$$

Hence, no adverse health effect is expected and the general population is considered to be protected. However, these estimations are based on the assumption that there is no deviation from additivity between toxicant effects and thus, there is considerable uncertainty associated with this HI.

Although humans are generally exposed to more than one chemical at the same time, data for combined exposures are scarce, which renders risk assessment of chemical mixtures challenging.

Additional uncertainty is inserted by using a toxicity end-point, e.g. developmental effects, which might be of minor importance for the susceptible group, such as elderly. To investigate cardiovascular effects of combined exposure to Pb(II), Hg(II) and MeHg(I), rats were exposed orally to four different mixtures containing these metals for four weeks. The concentration ratios were based on the ratios among published reference values or environmental exposure values. Cardiovascular end-points, such as blood pressure, cardiac output and electrical conductivity of the heart were selected considering that adults and in particular the elderly population might have an increased risk of cardiovascular diseases due to chronic Pb(II) and Hg exposure. We hypothesized that combined exposure to lead acetate, mercury chloride and mono-methylmercury aggravates adverse effects on the cardiovascular system due to synergistic effects.

4.5 Materials and methods

4.5.1 Animals

Male rats (Wistar strain, 250 - 300 g) were obtained from Charles River Laboratories, Senneville, QC, Canada. The animals were housed in single cages, at 22°C room temperature and a 12:12h-light dark cycle with access to standard rat chow *ad libitum* at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The experiment was approved by the University of Saskatchewan's Animal Research Ethics Board and carried out according to the guidelines of the Canadian Council on Animal Care (CCAC).

After one week of acclimatization, the experiment was initiated. Rats were exposed (n = 5 per group) for four weeks to either lead acetate (Pb(II); 357 or 1607 µg/kg-bw/d), mercury chloride (Hg(II); 57 or 357 µg/kg-bw/d) or mono-methylmercury chloride (MeHg(I); 29 or 357 µg/kg-bw/d) or a mixture of all three metals via the drinking water (tap water with 0.2% nitric acid). The dose of single metal exposure was comparable to the dose in the mixtures. The control group (n = 6) received tap water with 0.2% nitric acid. All chemicals were ordered from Sigma-Aldrich (Oakville, ON, Canada).

4.5.2 Calculation for combined exposures

The classical method to carry out experiments with combined exposures is to use a factorial design. However, these types of experiments require a large number of treatment groups and hence animals (Casey et al., 2006). Adhering to the principles of the 3Rs (Reducing, Refining, Replacing), the fixed-ratio ray design (Brunden and Vidmar, 1989) was instead applied for this study (Table 4.1). One fixed-ratio ray (R) was based on the ratio between reference levels for methylmercury, inorganic mercury and Pb(II). A second fixed-ratio ray (10R) was also based on the reference levels ratio but doses were multiplied by 10. A third fixed-ratio ray (E) was based on environmental exposure values for methylmercury, inorganic mercury and Pb(II). The fourth fixed-ratio (10E) was again based on environmental exposure values multiplied by 10.

The European Food Safety Authority (EFSA, 2012b) published a $BMDL_{01}$ for Pb(II) and systolic blood pressure as end-point of 1.5 µg/kg-bw/d. The Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2004) set a tolerable weekly intake (TWI) of 1.6 µg/kg-bw for methylmercury which would equal 0.23 µg/kg-bw per day. JECFA (FAO/WHO, 2011; 2004) also established a TWI of 4 µg/kg-bw for inorganic mercury corresponding to 0.57 µg/kg-bw per day. Both TWIs were confirmed by EFSA. Based on these reference values the ratio is MeHg(I) : Hg(II) : Pb(II) = 1 : 3 : 7 (Table 4.1). As MeHg(I) is the most toxic of these metals, the MeHg(I) dose was considered to be the starting point. In a previous study (Wildemann et al., 2015) a dose of 29 µg/kg-bw/d increased systolic blood pressure. Therefore, this dose was used for MeHg(I). The first fixed-ratio ray (R) for reference values applied 29 µg MeHg(I)/kg-bw/d, 87 µg Hg(II)/kg-bw/d and 203 µg Pb(II)/kg-bw/d. For the second fixed-ratio ray (10R) based on reference values, the doses were multiplied by 10. Hence, the doses were 290 µg MeHg(I)/kg-bw/d, 870 µg Hg(II)/kg-bw/d and 2030 µg Pb(II)/kg-bw/d (Table 4.1). The same approach was used for fixed-ratio rays based on environmental exposures. EFSA estimated European environmental exposure at 0.039 µg/kg-bw/d for MeHg(I), 0.08 µg/kg-bw/d for Hg(II) (EFSA, 2012a) and 0.68 µg/kg-bw/d for Pb(II) (EFSA, 2012b). This provides a ratio of MeHg(I) : Hg(II) : Pb(II) = 1 : 2 : 17. As starting dose 29 µg MeHg(I)/kg-bw/d was used again. The third fixed-ratio ray (E) based on environmental exposure applied the following ratio MeHg(I) : Hg(II) : Pb(II) = 29: 58 : 493 µg/kg-bw/d. To calculate the doses for the fourth fixed-ratio ray (10E), the doses from ray 3 (E) were multiplied by 10 resulting in 290 µg MeHg(I)/kg-bw/d, 580 µg Hg(II)/kg-bw/d and 4930 µg Pb(II)/kg-bw/d (Table 4.1).

Table 4.1. Ratios for combined exposures

	Group label	MeHg(I) (µg/kg-bw/d)	Hg(II) (µg/kg-bw/d)	Pb(II) (µg/kg-bw/d)
Reference values	R	0.23	0.57	1.5
Ratio		1	3	7
Environmental exposure values	E	0.039	0.08	0.68
Ratio		1	2	17
Experimental ratios				
Reference values (I)	R	29	87	203
Reference values (II)	10R	290	870	2030
Environmental exposure values (I)	E	29	58	493
Environmental exposure values (II)	10E	290	580	4930

4.5.3 High-resolution cardiovascular ultrasound, arterial blood pressure and electrocardiography

In the anesthetized rats, heart function, blood pressure and cardiac conductivity was measured at baseline and after four weeks of exposure. A high-resolution B-mode ultrasound (Visualsonics Vevo 770®, Toronto, ON, Canada) was used to measure the stroke volume according to methods previously used in this laboratory (Wildemann et al., 2015). For stroke volume, in systole and diastole the length of the left ventricle was measured in triplicate in parasternal long axis view in B-Mode and three cross-sectional areas (A1, A2, A3) in parasternal short axis view. Applying Simpson's rule (Boon, 2011), these measurements were used to calculate left ventricular volumes at end-systole (ESV) and end-diastole (EDV). The stroke volume (SV) was calculated as the difference between EDV and ESV, while cardiac output was calculated as the product of heart rate (HR) and SV.

After four weeks of exposure and a 24 h recovery after the last ultrasound examination, intravascular blood pressure in the femoral artery, heart rate and a Lead-(II)-electrocardiogram (ECG) were measured using PowerLab with LabChart software (ADInstruments, Colorado Springs, CO, USA) as the data acquisition system according to previously established methods in this laboratory (Wildemann et al., 2015). All measurements were recorded for 10 minutes and averaged.

4.5.4 Blood analysis

After four weeks of exposure, whole blood (5-9 samples/dose) were collected and sent for analysis to the Prairie Diagnostic Services Inc., Western College of Veterinary Medicine, University of Saskatchewan (Saskatoon, SK, Canada). In addition to the blood samples, deionized ultra-filtered water, feed, tap water and prepared drinking water of controls were analyzed for quality control. The samples were prepared through a nitric acid (69-70%) digestion in a Mars Microwave Accelerated Reaction System (CEM, Matthews, NC, USA). The prepared samples were analyzed with Inductively-Coupled Plasma Mass Spectrometry *(*ICP-MS*)* (Thermo Scientific X Series 2, Waltham, MA, USA).

4.5.5 Statistical analysis

Results are presented as mean ± standard deviation (SD). Normality and homogeneity of variance of the data were evaluated using Kolmogorov-Smirnov and Bartlett's tests. Unpaired student's t-tests were carried out between control and exposure groups, and among exposure groups (GraphPadPrism, GraphPad Software, Inc., San Diego, CA, USA). Exposure groups consisted of one-metal exposure (MeHg(I), Hg(II), Pb(II)) or three-metal exposures (MeHg(I), Hg(II) and Pb(II)). A $p<0.05$ was considered significant.

4.6 Results

4.6.1 Growth performance and blood analysis

There were no obvious signs of gross toxicity or mortalities after exposure to single or metal mixtures after four weeks of oral exposure. Exposures to one metal (29 MeHg(I), 57 Hg(II), 357 Pb(II) µg/kg-bw/d) were chosen in such a way that they are comparable to the corresponding doses in the metal mixtures (Table 4.1). The ratios for the metal mixtures were based on ratios for human reference values (1:3:7 MeHg(I):Hg(II):Pb(II)) and environmental exposures (1:2:17 MeHg(I):Hg(II):Pb(II)). Methylmercury was selected as the starting point for the calculation of the ratios because it is more toxic than Hg(II) and Pb(II). The MeHg(I) dose of 29 µg/kg-bw/d as a single toxicant significantly increased systolic (129 ± 9 mmHg) and diastolic blood pressure (83 ± 9 mmHg) in comparison to control (104 ± 5/66 ± 6 mmHg) (Wildemann et al., 2015). Doses for Hg(II) and Pb(II) were then calculated based on the ratios of human reference values and environmental exposures. No significant changes were identified in bodyweight gain, or organ weight in relation to total bodyweight in any treatment group after four weeks compared to control (Table 4.2).

Table 4.2. Organ weights in rats after four weeks oral exposure to mixtures of metals in drinking water

Experimental dose ratio (µg/kg-bw/d)	Δ Bodyweight (g)	Brain/body weight (%)	Heart/body weight (%)	Kidneys/body weight (%)	Liver/body weight (%)	Spleen/body weight (%)
Control						
0	134 ± 26	0.35 ± 0.07	0.27 ± 0.03	0.68 ± 0.05	3.74 ± 0.34	0.21 ± 0.02
Mixture experiment						
R (29 : 87 : 203)	114 ± 23	0.40 ± 0.06	0.26 ± 0.02	0.68 ± 0.03	3.56 ± 0.12	0.21 ± 0.03
10R (290 : 870 : 2030)	108 ± 11	0.41 ± 0.03	0.27 ± 0.03	0.64 ± 0.27	3.05 ± 1.27	0.22 ± 0.06
E (29 : 58 : 493)	115 ± 18	0.34 ± 0.08	0.27 ± 0.02	0.67 ± 0.10	3.48 ± 0.33	0.20 ± 0.02
10E (290 : 580 : 4930)	139 ± 19	0.33 ± 0.07	0.26 ± 0.02	0.71 ± 0.06	3.71 ± 0.21	0.21 ± 0.04

Single exposure to MeHg(I) decreased water consumption ($p<0.05$), while exposures to Hg(II), Pb(II) or mixtures had no effect on water consumption (Table 4.3). Furthermore, exposure to only MeHg(I) resulted in lower total blood Hg levels than when MeHg(I) was part of a mixture of metals. For example, exposure to 29 µg/kg-bw/d MeHg(I) resulted in total Hg blood levels of 346 ± 193 µg/L (Table 4.3). Whereas when MeHg(I) was part of a mixture at the same dose level, i.e. 29 µg/kg-bw/d, total Hg blood levels increased ($p<0.05$). As the contribution of Hg(II) to blood levels can be considered negligible as the low Hg(II) dose levels by itself resulted in 15 ± 1 µg/L mercury in blood, it can be assumed that MeHg(I) availability is increased in combination with Hg(II) exposure but not because of Pb(II) exposure. This mixture dependence was also dependent on the ratios, with the increase induced by the regulatory ratio 290% greater than that for the environmental ratio which increased total Hg blood levels by only 200%. Hg(II) was higher and Pb was lower in the regulatory ratio mixture compared to the environmental ratio and the increase in total blood Hg levels was associated with the regulatory ratio. The total Hg blood levels were well above the human mercury reference values of 20-100 µg/L (Statistics Canada, 2008) for a Hg(II) dose of 357 µg/kg-bw/d, MeHg(I) doses of 29 and 357 µg/kg-bw/d and all mixtures (Table 4.3).

In contrast to MeHg(I), mixtures decreased ($p<0.05$) Pb(II) blood levels from 86 ± 29 µg/L at a dose of 1607 µg/kg-bw/d to 50 ±18 µg/L at 2030 µg/kg-bw/d in the higher regulatory ratio (290:870:2030 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) (Table 4.3). Furthermore, the blood Pb(II) levels also depended on the ratios ($p<0.05$), with the lower environmental ratio (29:58:493 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) (15 ± 5 µg/L) decreasing the blood levels by ca. 12 % and the lower regulatory ratio (29:87:203 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) by 47%. The blood Pb(II) levels from the lower Pb(II) doses, i.e. 357 µg/kg-bw/d for Pb(II) only and 203 and 493 µg/kg-bw/d as part of the mixture, produced blood levels of Pb(II) comparable to the Pb guidance value of 10 µg/dl (Healey et al., 2010). The higher Pb(II) doses of 1607 µg/kg-bw/d for Pb(II) exposure and 2030 and 4930 µg/kg-bw/d within mixtures resulted in blood Pb(II) levels (50 – 86 µg/L) comparable to values found in workers exposed to lead in an occupational setting (Table 4.3).

Table 4.3. Exposure and blood analysis for lead and total mercury

Anticipated ratio (µg/kg-bw/d)	Water consumption ml/d	Nominal drinking water concentration (µg/ml)	Estimated dose consumed (µg/kg-bw/d)	Blood Pb(II) levels (µg/L)	Total blood Hg levels (µg/L)
Control					
0	37 ± 6	0	0	1.4 ± 1.2	21 ± 14
MeHg(I)					
29	27 ± 1**	0.4	22 ± 1	-	346 ± 193
357	27 ± 2**	5	270 ± 15	-	14,507 ± 4631
Hg(II)					
57	32 ± 4	0.8	51 ± 7	-	14 ± 1
357	33 ± 4	5	330 ± 40	-	28 ± 10
Pb(II)					
357	33 ± 3	5	330 ± 30	17 ± 7	-
1607	36 ± 5	21	1270 ± 680	86 ± 29	-
Mixture study					
R (29 : 87 : 203)	31 ± 4	0.4 : 1 : 3	25 : 62 : 186	9 ± 3	1000 ± 660*
10R (290 : 870 : 2030)	32 ± 3	4 : 11 : 27	256 : 704 : 1728	50 ± 18	15,500 ± 4800
E (29 : 58 : 493)	33 ± 3	0.4 : 0.7 : 7	25 : 46 : 462	15 ± 5	680 ± 190*
10E (290 : 580 : 4930)	30 ± 2	4 : 8 : 66	240 : 480 : 3960	80 ± 24	11,200 ± 4100

Ratios are presented as MeHg(I):Hg(II):Pb(II); results are presented as the mean ± SD. *p=0.01-0.05, **p=0.001-0.01, ***p=0.001-0.0001, ****p<0.0001

4.6.2 Blood and pulse pressure

Higher doses of MeHg(I) increased blood and pulse pressure, while mixtures reduced the values to control level. At lower exposure levels (Figure 4.1A), MeHg(I) at a dose of 29 µg/kg-bw/d increased ($p<0.05$) systolic blood pressure compared to Hg(II) alone. In contrast, combinations of metals at higher environmental exposure ratios of (290:580:4930 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d), lowered ($p<0.05$) systolic and diastolic blood pressure as well as pulse pressure (Figure 4.1B) to control levels. This decrease in blood and pulse pressure appeared to be linked to differential responses of the blood and pulse pressure to mixtures in comparison to single exposures to MeHg(I), Hg(II) and Pb(II). For example, at both low (29 µg/kg-bw/d) and high (357 µg/kg-bw/d) doses, exposure to only MeHg(I) increased ($p<0.05$) systolic blood pressure (129 ± 9 and 131 ± 5 mmHg respectively) compared to Hg(II) alone doses (116 ± 8mmHg) and the control group (117 ± 11 mmHg). Pulse pressure was lower ($p<0.05$) in Hg(II) (39 ± 3 mmHg) and Pb(II) (39 ± 5 mmHg) alone exposed animals compared to MeHg(I) (46 ± 3 mmHg) at the higher doses.

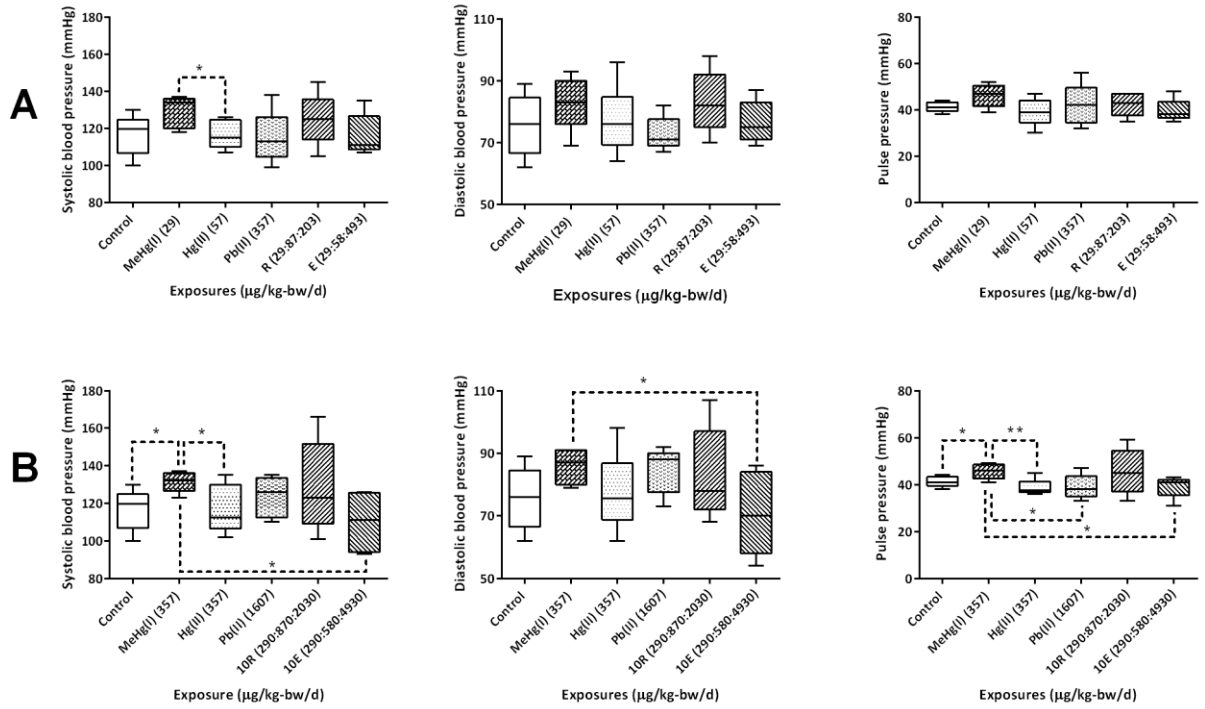

Fig. 4.1. Blood pressure and pulse pressure. Results are shown after four weeks of exposure. Row A: systolic and diastolic blood pressure and pulse pressure for one-metal exposure of methylmercury (29 µg/kg-bw/d), Hg(II) (57 µg/kg-bw/d), Pb(II) (357 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 29:87:203) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 29:58:493); row B: systolic and diastolic blood pressure and pulse pressure for one-metal exposure of methylmercury (357 µg/kg-bw/d), Hg(II) (357 µg/kg-bw/d), Pb(II) (1607 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 290:870:2030) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 290:580:4930); unpaired student's t-test *p = 0.01-0.05, **p = 0.001 – 0.01

4.6.3 Heart function

Similar to the effects observed for blood and pulse pressure, metal mixtures reversed the adverse effects on heart function associated with single metal exposure to control level (Figure 4.2). Cardiac output responded differently to single metal exposure compared to mixtures. Exposure at the high MeHg(I) (357 µg/kg-bw/d) alone showed a 40% lower cardiac output than the higher reference value ratios (290:870:2030 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) (39 ± 12 ml/min) reversing this effect to control levels.

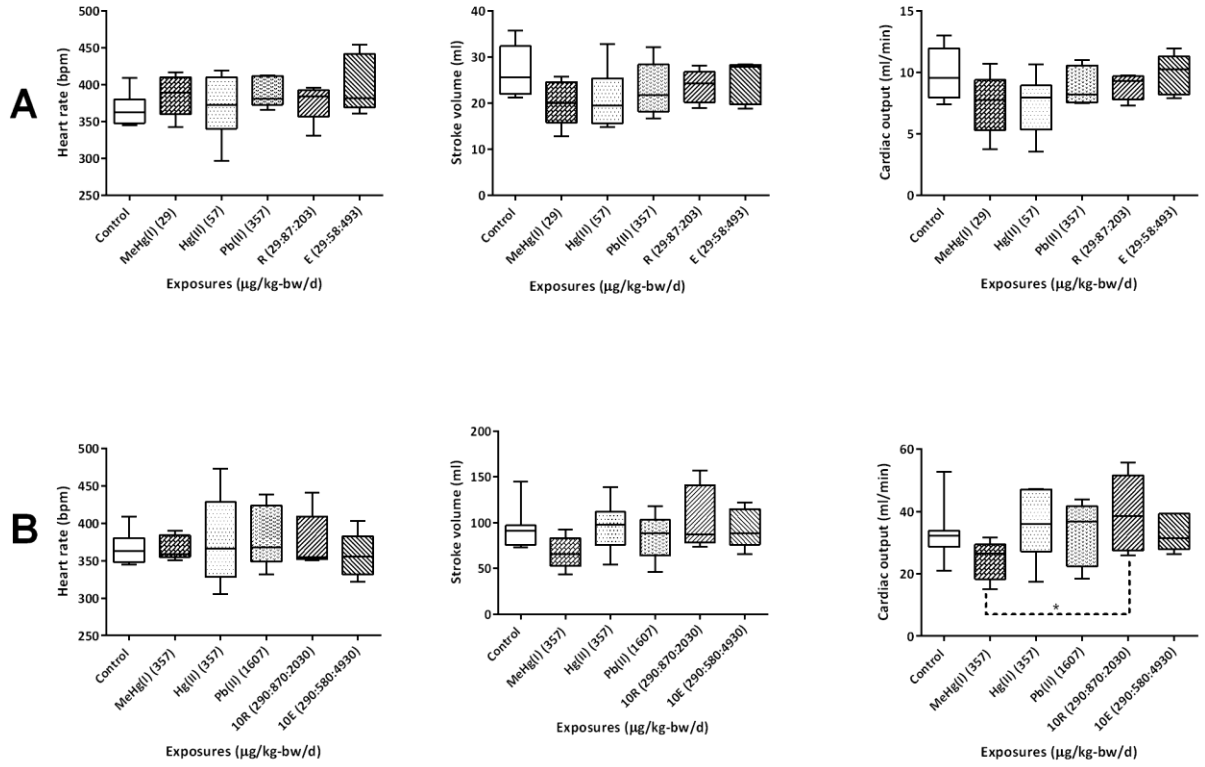

Fig. 4.2. Heart function as indicated by heart rate, stroke volume and cardiac output. Results are shown after four weeks of exposure. Row A: heart rate, stroke volume and cardiac output for one-metal exposure of methylmercury (29 µg/kg-bw/d), Hg(II) (57 µg/kg-bw/d), Pb(II) (357 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 29:87:203) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 29:58:493); row B heart rate, stroke volume and cardiac output for one-metal exposure of methylmercury (357 µg/kg-bw/d), Hg(II) (357 µg/kg-bw/d), Pb(II) (1607 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 290:870:2030) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 290:580:4930); unpaired student's t-test *p = 0.01-0.05, **p = 0.001 – 0.01

4.6.4 Cardiac electrical conductivity

In contrast to blood and pulse pressure (Figure 4.1) and heart function (Figure 4.2), mixtures produced an adverse effect on cardiac electrical conductivity while single metal exposures were comparable to control levels (Figure 4.3). Ratios based on human reference values (29:87:203 and 290:870:2030 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) increased ($p<0.05$) the duration of the QRS interval in comparison to single metal exposures and the environmental ratios. As an example, the highest increase of 40% was observed between exposure to Hg(II) and the reference value ratios. At the higher reference value ratio (290:870:2030 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d), the duration of the QT interval was increased by about 20% compared to Pb(II) alone and controls ($p<0.05$). In contrast, the PR interval followed the previous trend where single metal exposures produced a prolonged PR interval, while mixtures normalized this adverse effect. For example, exposure to lower levels of

Hg(II) (57 µg/kg-bw/d) alone prolonged the PR interval in comparison to MeHg(I) (29 µg/kg-bw/d) alone and lower dose mixtures (29:87:203 and 29:58:493 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d).

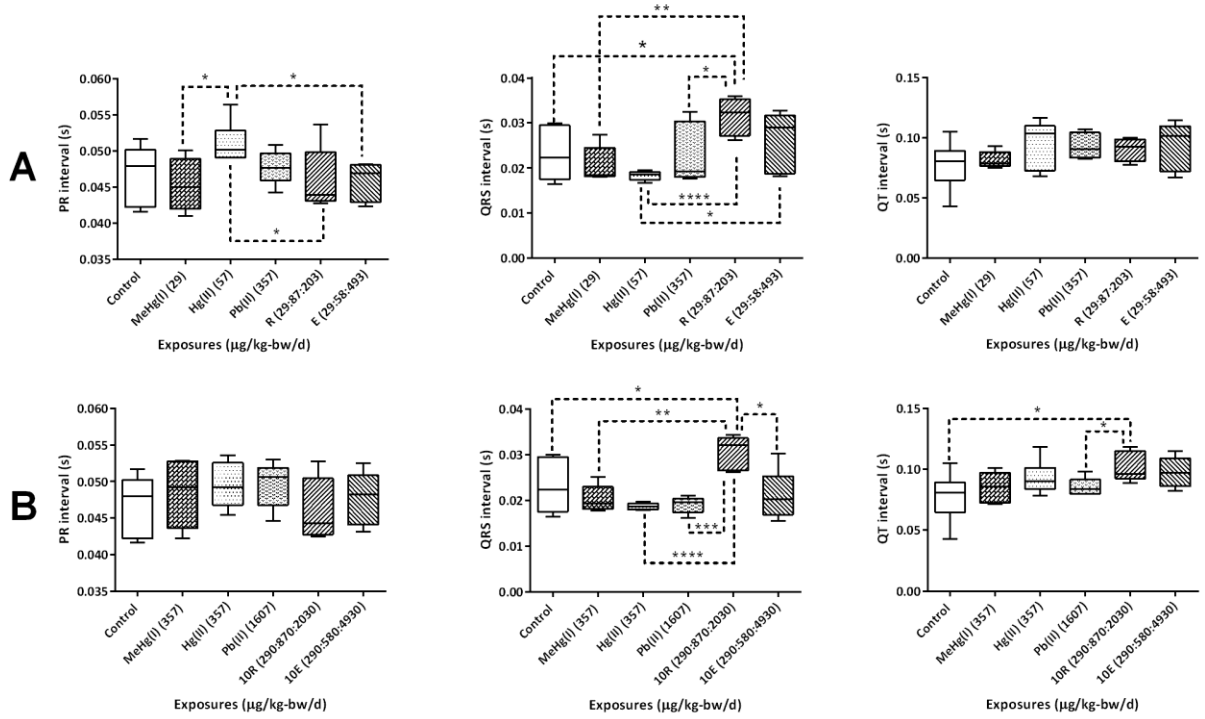

Fig. 4.3. Electrical conductivity of the heart by electrocardiogram (ECG). Results are shown after four weeks of exposure. Row A: QT, QRS and QT intervals for one-metal exposure of methylmercury (29 µg/kg-bw/d), Hg(II) (57 µg/kg-bw/d), Pb(II) (357 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 29:87:203) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 29:58:493); row B: QT, QRS and QT intervals for one-metal exposure of methylmercury (357 µg/kg-bw/d), Hg(II) (357 µg/kg-bw/d), Pb(II) (1607 µg/kg-bw/d) and reference value ratio (MeHg(I):Hg(II): Pb(II) = 290:870:2030) and environmental exposure ratio (MeHg(I):Hg(II): Pb(II) = 290:580:4930); unpaired student's t-test *p = 0.01-0.05, **p = 0.001 – 0.01

4.7 Discussion

In the current study exposure to mixtures of lead, organic mercury and inorganic mercury ameliorated the adverse effects of single metal exposures on some cardiovascular parameters, e.g. blood pressure. In contrast, mixtures deteriorated cardiac electrical behaviour while single metal exposures had no effect. Although there is increasing evidence in the literature that exposure to single metals like Pb(II) or Hg adversely affect the cardiovascular system, no other work so far has investigated how metal mixtures influence cardiovascular toxicity. However, some studies with non-cardiovascular end-points indicate a potential interaction between Pb(II) and Hg species. An *in vitro* study (Fortier et al., 2008) demonstrated that the reduced viability of lymphocytes due to MeHg(I)

exposure was mitigated by mixtures containing MeHg(I), $CdCl_2$ and $PbCl_2$. In contrast, in an epidemiological study with Inuit children exposed to lead, polychlorinated biphenyls and methylmercury (Boucher et al., 2012), developmental effects of Pb exposure were aggravated by Hg exposures. Therefore, our study and other mixture studies clearly demonstrate that extrapolating adverse effects of metal mixtures cannot be predicted by effects of single metal exposures.

4.7.1 Blood levels

Blood levels for total Hg and Pb(II) are important biomarkers for recent metal exposure with the absorption rate of Hg species depending on their chemical properties (Yokel et al., 2006). While absorbed Hg(II) is quickly removed from the blood and accumulates in the kidneys (Bridges and Zalups, 2005), MeHg(I) binds to the erythrocytes due to a higher affinity for SH-groups than Hg(II). The total Hg blood level is mainly driven by MeHg(I), whereas in our study the blood levels for Pb(II) are lower in mixtures than in comparable exposures to only Pb(II). The chemical similarity of Hg(II) and Pb(II) might enable them to compete for access to the same metal transporter, e.g. divalent metal transporters (Ballatori, 2002), resulting in a reduced absorption of Pb(II). Similarly to Hg, Pb(II) has a high affinity to SH-groups (Al-Modhefer et al., 1991), which seems to limit the binding of Pb(II) to SH-groups in comparison to MeHg(I).

4.7.2 Blood and pulse pressure

The result that mixtures reverse the effects on blood and pulse pressure of single metal exposures is particularly surprising because total Hg blood levels in the mixture groups are higher than single MeHg(I) exposures. While single exposure to MeHg(I) increased blood and pulse pressure, Hg(II) and Pb(II) had no effect. However, in a mixture, the Hg(II) and Pb(II) interact with MeHg(I) in an antagonistic way so that the overall effect is zero. Our results may explain why epidemiological data on the link between mercury and blood pressure are mixed. Epidemiological studies have reported positive relationships between Hg hair levels and increased blood pressure in Amazonians (Fillion et al., 2006) or between blood Hg levels and systolic blood pressure in Nunavik Inuits (Valera et al., 2008; 2009), but no relationship between blood or hair Hg levels and hypertension in Cree adults (Valera et al., 2011a) or Polynesians (Valera et al., 2011b). Using NHANES data from 2003 – 2006, Park et al (2013) found a negative association between urine, but not blood, levels of Hg and blood pressure. Similarly, hair Hg levels were positively related to blood pressure, while urine Hg levels were inversely related to blood pressure in a study of dental staff (Goodrich et al., 2013b).

Our data suggests that a simple analysis of total body Pb or Hg levels is not sufficient to unravel the link between environmental exposures and adverse cardiovascular effects and underlines the importance of the metal species regardless of bioavailability. For example, despite similar blood Hg

levels, exposures to MeHg(I) and the mixture with the higher reference value ratio (290:870:2030 MeHg(I):Hg(II):Pb(II); µg/kg-bw/d) in the current study had dramatically different blood pressure outcomes. Because of similar blood Hg levels in our study, the possibility of bioavailability differences as explanation is being ruled out. An alternative explanation is that speciation differs between the single metal and mixture groups, leading to differences in the adverse outcome pathway between MeHg(I) and Hg(II). However, the relationship between different Hg species and the exact pathway by which they exert adverse effects is not known. Both Hg species increase oxidative stress in the body (Wiggers et al., 2008; Farina et al., 2011) but the target organs might be different. Inorganic mercury mainly accumulates in the kidneys (Bridges and Zalups, 2005), which play an important role in the long-term regulation of blood pressure via the renin-angiotensin system. On the other hand, methylmercury can easily cross the blood-brain barrier and possibly influence the blood pressure through the autonomic nervous system (Pappano, 2008).

4.7.3 Heart function

While heart rate and stroke volume were not changed, cardiac output was affected by metal exposure. As with blood pressure, MeHg(I) and Hg(II) had differing effects on the cardiac output, which was decreased by MeHg(I) while Hg(II) and Pb(II) did not have an effect. This is in contrast with Skoczynska et al (2014), who observed that the cardiac ejection fraction in Pb(II) exposed rats was reduced using magnetic resonance imaging. The discrepancy might be due to the use of two different imaging techniques, magnetic resonance imaging versus high-resolution ultrasound. Although cardiac output was reduced when the rats were exposed to MeHg(I), combined exposures to all three metals did not affect any of these end-points. This indicates an antagonistic interaction between MeHg(I), Hg(II) and Pb(II).

4.7.4 Electrical conductivity

In contrast to other end-points, mixtures caused increased adverse effects on cardiac electrical activity. The different intervals represent different phases of the cardiac cycle. The PR interval reflects the activity of the atrioventricular node (Leffler et al., 1994) and was, in our study, mainly affected by inorganic mercury exposure. A Hg(II) dose of 57 µg/kg-bw/d produced a prolonged PR interval in this study, which completely disappeared in combined exposures with MeHg(I) and Pb(II). The QRS interval, which represents the depolarization of the ventricles, was most sensitive to combined metal exposure in our study. A prolonged QRS or QT interval is associated with increased risk of arrhythmias and sudden cardiac death (Okin et al., 2000). Ratios based on reference values show a significant prolongation of the QRS and QT intervals. The main difference between regulatory and environmental ratios is the higher amount of Hg(II) in the regulatory ratios. This suggests that the electrical activity of the heart might be more susceptible to Hg(II) than to MeHg(I)

or Pb(II) exposure. As exposures to only one metal did not show an effect in our study, the combined exposure effect indicates synergism. In contrast, to our study, Eum et al (2011) and Cheng et al (1998) found prolonged QT and QRS intervals with increasing Pb(II) levels in the tibia and patella by analyzing data from the Normative Aging Study. The average age of men in the Normative Aging Study was more than 60 years and low level exposure to Pb(II) for decades would have resulted in a substantial body burden. On the other hand, the rats in our study were only exposed to Pb(II) for four weeks so that the overall body burden is expected to be lower and we did not measure bone Pb levels, so we cannot directly compare studies. The discrepancy in the effect of Pb(II) exposure on QT and QRS intervals is probably the result of the different exposure durations and as a consequence body burdens.

4.7.5 Population level effects

HI calculations based on literature values indicate no increased risk for the general population due to combined exposures to MeHg(I), Hg(II) and Pb(II). However, the calculation of the HI is based on the assumption that the doses are additive and that the metals have a similar modes of action (MOA) (U.S. EPA, 2007). Based on our results, it appears that these metals are not additive and that the mechanism of action of MeHg(I) may differ from Hg(II). A potential source for increased uncertainty of the HI is the fact that the only reference value for a cardiovascular end-point exists for Pb(II), while reference values for mercury are based on developmental effects, which mainly affect children.

Comparing the calculated risk from combined exposures to MeHg(I), Hg(II), and Pb(II) for the general population with our study results depends on the selection of cardiovascular end-points. On one hand, mixtures showed antagonism for blood and pulse pressure and heart function, which would confirm that the public does not have an increased risk for ill-health based on the outcome of the risk assessment. On the other hand, combined exposures to MeHg(I), Hg(II) and Pb(II) indicated synergistic effects resulting in impaired electrical conductivity in the heart. Hence, for this cardiovascular end-point, the outcome of the risk assessment is not protective for human health.

4.8 Conclusions

According to our knowledge, this study is the first to investigate combined exposures of MeHg(I), Hg(II) and Pb(II) based on the fixed-ratio ray design and examine adverse cardiovascular end-points. We observed that MeHg(I) and Hg(II) affect the cardiovascular system differently showing the importance of the chemical form of the metal. Furthermore, Pb(II) does not affect the cardiovascular system in the same way as MeHg(I) or Hg(II). We also found deviation from additivity (synergism and antagonism) for the cardiovascular end-points measured. As we observed synergism for electrical conductivity, cardiac electrical activity might be the most critical end-point to monitor in future studies. An important first step could be to re-analyze data from the major epidemiological

studies, such as NHANES and the Normative Aging Study, to investigate the relation between hair/urine Hg plus blood Pb(II) levels and the electrical activity of the heart. Results from these studies could provide further information on the cardiovascular effects of combined exposures to MeHg(I), Hg(II) and Pb(II) specific to humans.

5 THE MECHANISMS ASSOCIATED WITH THE DEVELOPMENT OF HYPERTENSION AFTER EXPOSURE TO LEAD, MERCURY SPECIES OR THEIR MIXTURES DIFFERS WITH THE METAL AND THE MIXTURE RATIO

5.1 Author contributions

Tanja Wildemann carried out the animal experiment and biochemical analysis of the selected tissues. She analyzed the data statistically and prepared the manuscript.

Steven Siciliano provided scientific input, commented on the manuscript and supported the research financially through his research grant.

Lynn Weber provided scientific input and guidance, edited the manuscript and supported the research financially through her research grant.

5.2 Preface

To our surprise, the combined exposures reversed most of the adverse cardiovascular effects of single metal exposures with the exception of the cardiac electrical activity. By exploring the underlying mechanisms of action, namely oxidative stress, kidney damage and DNA methylation, we could show that each metal on its own differs in its mechanism of action as well as their mixtures supporting the physiological observations.

Submitted to *Toxicology* as Wildemann, T.M., Siciliano, S.D., Weber, L.P. (2015) The mechanisms associated with the development of hypertension after exposure to lead, mercury species or their mixtures differs with the metal and the mixture ratio.

5.3 Abstract

Hypertension is considered to be the most important risk factor for the development of cardiovascular diseases. Beside life-style risk factors, exposure to lead and mercury species are increasingly discussed as potential risk factors. Despite an increasing number of mechanistic studies, the underlying mechanism by which exposure to lead and mercury disturb blood pressure regulation is not understood. Potential mechanisms are oxidative stress production, kidney damage and epigenetic mechanisms, such as DNA methylation, all of which can interact to cause dysregulation of blood pressure. Male rats (Wistar) were exposed to lead, inorganic mercury or methylmercury or two mixtures of all three metals for four weeks through the drinking water. The two mixture ratios were based on known reference values or environmental exposure ratios from the literature and levels adjusted to be within the range of single metal doses used. To investigate the potential mechanism of actions, blood pressure was measured after four weeks and compared to

plasma nitrotyrosine or reduced/oxidized glutathione levels in aorta, heart, kidney and liver as markers for oxidative stress. Kidney function was assessed via urinary and plasma creatinine levels (also creatinine clearance) and urinary kidney-injury molecule (KIM-1) to assess kidney damage. Finally, urinary 5-methyl-2'-deoxycytidine was used as a marker for global DNA methylation. While exposure to lead by itself increased oxidative stress and caused kidney damage, methylmercury by itself did not affect any of the potential mechanisms investigated. Inorganic mercury was the only metal, which showed an increase in global DNA methylation. In contrast, when administered as mixtures, lead no longer increased oxidative stress. More interestingly was the observation that kidney function, as indicated by creatinine levels, was affected differently between the two different metal mixtures. Moreover, only the mixture based on the reference value ratio was associated with increased blood pressure. Based on our results, the prominent mechanism of action associated with the development of hypertension seems to be oxidative stress and kidney damage for lead and reduced kidney function for metal ratios in mixtures.

5.4 Introduction

Cardiovascular diseases, such as heart attacks and stroke, present a major public health problem because these are responsible for the majority of deaths worldwide (Mendis et al., 2011). Although hypertension is considered to be the most important risk factor for this group of diseases, its etiology is not completely understood (Mendis et al., 2011). In addition to numerous life-style risk factors environmental pollutants, such as heavy metals, are gaining more attention as potential risk factors contributing to hypertension. The human population is exposed chronically to low levels of lead and mercury through the environment (Nawrot et al., 2002; Bautista et al., 2009; Roman et al., 2011; Park et al., 2013; Peters et al., 2012). Based on the scientific evidence, lead (Pb(II)) is now considered to have a causal relationship with hypertension (Nawrot et al., 2002; Navas-Acien et al., 2007; Glenn et al., 2003). The relationship between mercury exposure and hypertension is less clear. A positive association between mercury exposure and hypertension was found in some human studies (Bautista et al., 2009; Valera et al., 2009; Pedersen et al., 2005), while a negative association was found in another set of epidemiological studies (Vupputuri et al., 2005; Johansson et al., 2002; Mozaffarian et al., 2011; 2012; Valera et al., 2011a; Park et al., 2013).

Despite an increasing number of mechanistic studies, the underlying mechanism(s) by which exposures to Pb(II), inorganic mercury (Hg(II)), organic mercury (MeHg(I)) or their mixtures influence blood pressure is not understood. One leading hypothesis for mechanism of action of metals is oxidative stress. Exposure to either Pb(II) or Hg has been reported to cause increased production of reactive oxygen species (ROS) (Ercal et al., 2001), depletion of antioxidant defenses or both (Saeidnia and Abdollahi, 2013). In addition, studies have shown that Pb(II) exposure increased the

production of ROS, such as superoxide and this secondarily decreased the availability of nitric oxide (NO) leading to the production of the highly toxic peroxynitrite (Farmand et al., 2005;Ding et al., 2001;Vaziri et al., 2003;Vaziri, 2008). At the same time, Pb(II) exposure reduces the available amount of antioxidant glutathione (Kasperczyk et al., 2004). Similarly, Hg(II) and MeHg(I) have also been reported to decrease the availability of NO through increased ROS production (Wiggers et al., 2008; de Marco et al., 2009; Lemos et al., 2012). In particular MeHg(I) has a high affinity for the sulfhydryl groups of glutathione, resulting in inactivated glutathione and depleted antioxidant defenses (Ballatori, 2002). The well-known consequences of disturbances in ROS production, depleted antioxidants and NO inactivation are numerous, but include endothelial dysfunction, vasoconstriction and hypertension (Li and Foerstermann, 2000).

In addition to the central nervous system, the kidneys play a crucial role in blood pressure regulation (Wadei and Textor, 2012). Therefore, impaired kidney function due to exposure to Pb(II) and Hg is a second mechanism that may be important for the development of hypertension. Analyzing data from the Third National Health and Nutrition Examination Survey (NHANES) (Muntner et al., 2003) and the Normative Aging Study (Tsaih et al., 2004) showed positive associations between Pb body burdens and increased serum creatinine concentrations, a biomarker for impaired kidney function (Vlasakova et al., 2014). The kidneys are the target organ for the accumulation of Hg(II), making Hg(II) particularly nephrotoxic (Zalups, 2000). The proximal tubule of the nephron (Zalups, 2000; Massanyi et al., 2007) is the main target of Hg(II), resulting in increased serum creatinine levels (Shi et al., 2011).

Epigenetic mechanisms and in particular DNA methylation, which mainly occurs at the so called CpG-islands, are also increasingly recognized as playing an important role in the development of human diseases, including cardiovascular disease (Robertson, 2005; Shirodkar and Marsden, 2011). As Pb(II) and Hg are generally classified as neurotoxicants, the few studies available investigating the influence of DNA methylation focused on neurotoxicity, not cardiovascular effects, but similar mechanisms are likely to affect both systems. The major conclusion to be drawn from these initial studies are that Pb(II) and Hg (Hanna et al., 2012) both alter the methylation status of genes and thus influence important biochemical pathways. DNA hypermethylation due to Pb(II) exposure was observed in animal and human studies (Kovatsi et al., 2010; Schneider et al., 2013). Although mercury species showed increased DNA methylation in animals (Onishchenko et al., 2008; Pilsner et al., 2010), Hg(II) and MeHg(I) seem to affect DNA methylation differently (Goodrich et al., 2013a) in humans. While MeHg(I) indicated a trend to reduce DNA methylation of SEPP1, Hg(II) did not show an effect (Goodrich et al., 2013a). Despite the existence of studies indicating a link between Pb(II) and mercury exposure and adverse cardiovascular effects through changes in DNA methylation, little is known about the detailed mechanism.

Based on a previous study in our lab (Wildemann et al., 2015), we showed that exposures to Pb(II) or MeHg(I), but not Hg(II), each as single metals alone increased the blood pressure in rats. In contrast, in a follow-up study (Wildemann et al., 2014), we found that mixtures of these three metals had no effect on blood pressure. These results were puzzling since these initial studies showed that metal blood levels were similar or perhaps even higher after mixture exposures compared to the metals alone (Wildemann et al., 2014), indicating that altered bioaccumulation was not the mechanism responsible for loss of blood pressure effect of metal mixtures. Therefore, we hypothesized that oral exposure to Pb(II), Hg(II), MeHg(I) had different mechanisms of action compared to exposure to their mixtures; specifically, we hypothesized that blood pressure alterations after four weeks of exposure in rats occurred through three different mechanisms, namely oxidative stress, kidney function and damage and/or changes in DNA methylation. In order to investigate this hypothesis, we assessed oxidative stress by measuring plasma nitrotyrosine and oxidized/reduced glutathione in aorta, kidney, heart and liver. Metals, such as Pb(II) and Hg species, are well known to increase oxidative stress. Since peroxynitrite is formed when both nitric oxide (NO) and superoxide (O_2^-) are present in high levels, this can be used as a measure of oxidative stress. Peroxynitrite has a high affinity for covalently modifying many different macromolecules, but has a high affinity for tyrosine residues in proteins, thereby forming nitrotyrosine.

Thus, nitrotyrosine is a surrogate that can be used to indicate both oxidative stress and inactivation of the biological effect of nitric oxide in the cardiovascular system. Kidney function was evaluated with urinary and plasma creatinine, allowing creatinine clearance to be calculated, and kidney damage evaluated with urinary Kidney Injury Molecule 1 (KIM-1; biomarker for proximal tubule injury) (Vlasakova et al., 2014). Creatinine is freely filtrated, but not reabsorbed, by the kidneys and is therefore a good marker for glomerular filtration and kidney function. While plasma creatinine can be influenced by protein metabolism, the clearance of creatinine should remain constant in a healthy animal. While creatinine levels and clearance have known ranges for clinical norms, KIM-1 protein is a biomarker for proximal tubule injury in the kidney and is normally low in a healthy individual. Global changes in DNA methylation were measured using urinary 5-methyl-2'-deoxycytidine (Itoh et al., 1995). Methylation of DNA is a mechanism to regulate the expression of a gene, with hypermethylation of the promoter region for a given gene resulting in reduced gene expression. Global hyper- or hypomethylation is associated with diseases such as cancer, atherosclerosis and rheumatoid arthritis, but the effects of heavy metals on this epigenetic mechanism in cardiovascular toxicity are not fully understood (Ray et al., 2014). DNA methylation status can be represented as the amount of free 5-methyl-2'-deoxycytidine in urine. Methylation remains intact on deoxycytidine during normal turnover/repair of DNA and thus urinary levels are most closely linked to the global

level of methylation in the body. The patterns of change in these metal mechanisms of action were then compared to alterations in blood pressure after four weeks of exposure in male rats.

5.5 Materials and methods

5.5.1 Animals and exposures

Male rats (Wistar strain, 250-300 g) were obtained from Charles River Laboratories, Senneville, QC, Canada and were housed in single cages, at 22°C room temperature and a 12:12H-light dark cycle at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The animals were acclimatized for one week and had access to standard rat chow *ad libitum*. For the duration of four weeks, rats (n = 5-6/group) received either lead acetate (Pb(II)), mercury chloride (Hg(II)), mono-methylmercury chloride (MeHg(I)) or a mixture of all three metals through the drinking water (tap water with 0.2% nitric acid). Rats exposed to Pb(II) received either 1607 or 45000 µg Pb(II)/kg-bw/d, to Hg(II) either 357 or 4000 µg Hg(II)/kg-bw/d and to MeHg(I) either 7 or 357 µg MeHg(I)/kg-bw/d via the drinking water. Mixture ratios were based on published reference values or on published environmental exposure values (EFSA, 2012b; 2012a). For the reference values based ratio (R), the ratio was MeHg(I):Hg(II):Pb(II) = 1:3:7. In a previous study (Wildemann et al., 2015), a MeHg(I) dose of 290 µg/kg-bw/d showed significant adverse effects on blood pressure. As MeHg(I) is the most toxic of these three metals, this dose was used as starting dose, resulting in a ratio of MeHg(I):Hg(II):Pb(II) = 290:870:2030 µg/kg-bw/d. Similarly, the environmental exposure ratio (E) was established. The published values for environmental exposure resulted in a ratio of MeHg(I):Hg(II):Pb(II) = 1:2:17. Using 290 µg MeHg(I)/kg-bw/d as the starting dose leads to a ratio of MeHg(I):Hg(II):Pb(II) = 290:580:4930 µg/kg-bw/d (Table 5.1).

Table 5.1. Ratios for combined exposures.

	MeHg(I) (µg/kg-bw/d)	Hg(II) (µg/kg-bw/d)	Pb(II) (µg/kg-bw/d)
Reference values	0.23	0.57	1.5
Ratio	1	3	7
Environmental exposure values	0.039	0.08	0.68
Ratio	1	2	17
Experimental ratios			
Reference values (R)	290	870	2030
Environmental exposure values (E)	290	580	4930

Reference values and environmental exposure values were obtained from the published literature and used to define MeHg(I):Hg(II):Pb(II) ratios for mixtures. MeHg(I) is the most toxic of these metals and was hence used as the starting point. In a previous study, a MeHg(I) dose of 290 µg/kg-bw/d showed an increase in blood pressure (Wildemann et al., 2015).

The drinking water of the control rats (n = 6/group) consisted of tap water with 0.2% nitric acid. At the end of the exposure duration, the animals were euthanized. Blood, urine and tissues were collected, processed as appropriate and stored at -80°C until further analysis.

Chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).The Animal Research Ethics Board at the University of Saskatchewan approved all procedures in this experiment and were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC).

5.5.2 Blood pressure measurement

For blood pressure measurements after four weeks of exposure, rats were anesthetized (induction with 5% isoflurane and 1-3% for maintenance), injected with 3 ml saline (s.c.) and placed on a heating blanket (37°C). The systolic and diastolic blood pressure was measured by inserting a pre-calibrated (calibrated to a mercury column), saline-filled pressure catheter into the femoral artery. Values were recorded for 10 minutes using a PowerLab (ADInstruments, Colorado Springs, CO, USA). Average values from each rat were used for statistical analysis. Rats were then euthanized immediately after blood pressure and ECG recording followed by collection of blood and tissues.

5.5.3 Oxidative stress

Nitrotyrosine levels (an indicator of peroxynitrite formation) were measured in plasma samples (n = 5-6/group) with a commercially available ELISA based enzyme-linked immunosorbent assay (Nitrotyrosine ELISA Kit, Hycult biotech, The Netherlands). After collection, aorta, heart, kidney and liver tissues were homogenized, deproteinated and stored at -80°C until further processing. Samples were analyzed for their oxidized (GSSG) and the total GSH (which were then used to calculate

GSSG/GSH ratio) using a commercially available enzymatic assay (Glutathione Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). Based on the measured amounts of GSSG and total GSH, the ratio GSSH/total GSH was calculated.

5.5.4 Kidney function and damage

Creatinine levels were analyzed in urine and plasma samples (n = 5-6/group) using an assay based on a coupled enzyme reaction (Creatinine Assay Kit, Sigma-Aldrich, St. Louis, MO, USA) as indicators of kidney function. Based on the plasma and urine concentrations, the creatinine clearance (C_{CR} in µl/min) was calculated as the product of creatinine concentration in urine (U_{CR} in ng/µl) and urine flow (V in µl/min) divided by the creatinine plasma concentration (P_{CR} in ng/µl). Urine flow was not measured directly and was assumed to be 15 µl/min for all rats, based on published values (Wuthrich, 2000). Kidney Injury Molecule-1 (KIM-1) is a biomarker for injury to the proximal tubule of the kidney. Its concentration was measured in urine samples (n = 5-6/group) with a commercially available *in vitro* ELISA Immunosorbent Assay (KIM-1 Rat ELISA Kit, Abcam, Cambridge, England).

5.5.5 DNA methylation

The global DNA methylation status was measured in urine (n = 5-6/group) using a commercially available competitive enzyme immunoassay (DNA Methylation EIA Kit, Cayman Chemical Company, Ann Arbor, MI, USA).

5.5.6 Statistical analysis

The animals were either exposed to one metal (MeHg(I), Hg(II), Pb(II)) or a mixture of all three metals. Grubb's test was used to detect outliers. Values with a $p < 0.05$ were considered to be significantly different and removed. Results are presented as mean ± standard deviation (SD). Data were evaluated for normality and homogeneity of variance with Komogorov-Smirnow and Bartlett's tests. The data satisfied the requirements for parametric statistics. Differences were analyzed by one-way Analysis of Variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post-hoc test (GraphPadPrism, GraphPad Software, Inc., San Diego, CA, USA). A $p < 0.05$ was considered significant.

5.6 Results

5.6.1 Blood pressure

Among the numerous risk factors for cardiovascular diseases, hypertension is considered to be the most important. In the current study, blood pressure (Figure 5.1) was increased by single exposures

to Pb(II), Hg (II), MeHg(I) and the mixture based on the reference value ratio (R = MeHg(I):Hg(II):Pb(II) = 290:870:2030), but not by the mixture based on the environmental exposure ratio (E = MeHg(I):Hg(II):Pb(II) = 290:580:4930). The Pb(II) doses (1607 and 45000 µg/kg-bw/d) showed a significant increase of 20 mmHg in systolic blood pressure compared to the control group (Figure 5.1), an increase sufficiently large to make a normotensive individual hypertensive. Similarly, exposure to MeHg(I) raised the systolic blood pressure by 15 mmHg for the lower dose (7 µg MeHg(I)/kg-bw/d) and 27 mmHg for the higher MeHg(I) dose (357 µg/kg-bw/d) in comparison to the control group. In contrast, Hg(II) did not significantly affect systolic blood pressure at any dose. Differences were observed between the two mixtures. While the systolic blood pressure was also increased for the reference values ratio (R) compared to the controls, no change was observed for the environmental exposure ratio (E). Comparing single metal exposures to both mixtures, the systolic blood pressure was increased by the mixture based on the reference values ratio compared to the higher Hg(II) dose (4000 µg/kg-bw/d). The systolic blood pressure was decreased by the environmental exposure ratio in comparison to the higher MeHg(I) dose (357 µg/kg-bw/d) and the mixture based on the reference value ratio.

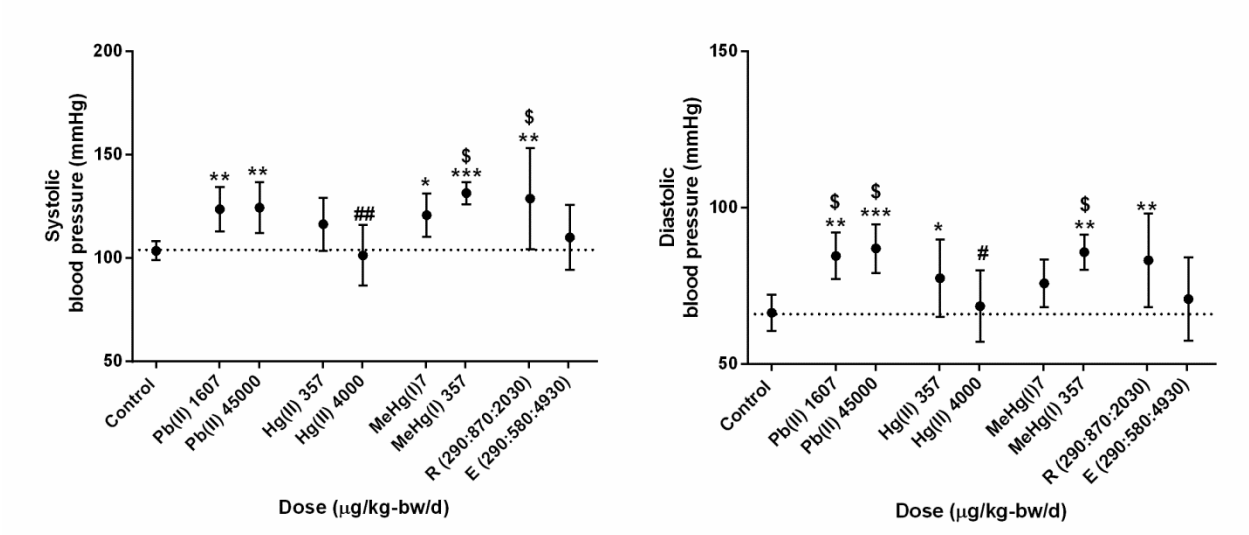

Fig. 5.1. Systolic and diastolic blood pressure in rats (n=5-6 rats/group) after 4 weeks of exposure to lead, inorganic mercury or methylmercury or a mixture of all three metals through the drinking water. Data are shown as mean ± standard error of mean. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A $p < 0.05$ was considered to be significant.

Changes in diastolic blood pressure after metal exposure showed a similar pattern of effect as systolic pressure changes (Figure 5.1). For both Pb(II) doses, diastolic pressure was increased

compared to the control group. The lower Hg(II) dose significantly raised the diastolic blood pressure by 11 mmHg in comparison to controls, while the higher Hg(II) dose was not different from the control group. Similarly, the lower MeHg(I) dose was not different from the control group, but the higher MeHg(I) dose raised the diastolic blood pressure by 20 mmHg. For the mixtures, the pattern for diastolic blood pressure was the same as for systolic blood pressure. The reference values ratio (R) increased the diastolic blood pressure, while the environmental exposure ratio (E) was not different from the control group. As seen with systolic blood pressure, the diastolic blood pressure was decreased for 4000 µg Hg(II)/kg-bw/d in comparison to the mixture based on the regulatory value ratio. The diastolic blood pressure for the mixture based on the environmental exposure ratio was reduced compared to both Pb(II) doses (1607 and 45000 µg/kg-bw/d) and the higher MeHg(I) dose (357 µg/kg-bw/d).

5.6.2 Oxidative stress

The Pb(II) dose of 45000 µg/kg-bw/d significantly increased the nitrotyrosine plasma levels by about 350% in comparison to the control group and the two mixtures (Figure 5.2). In contrast, both Hg(II) and both MeHg(I) doses and the two mixtures were not different from the control group. This indicates that Pb(II) has an exposure threshold for the production of oxidative stress and inactivation of nitric oxide, while Hg species and mixtures do not seem to affect this pathway.

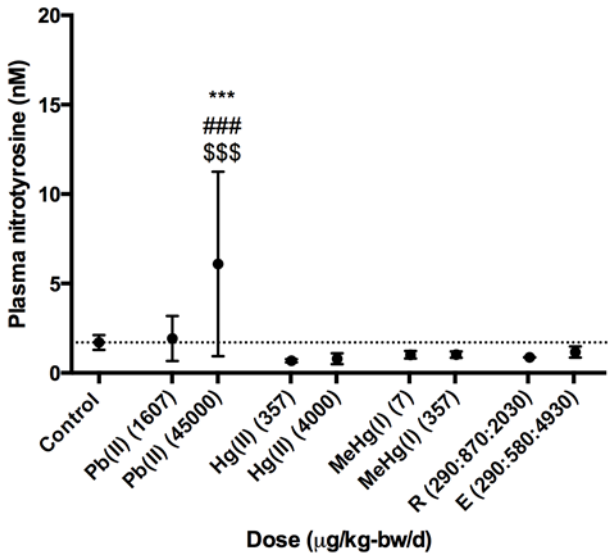

Fig. 5.2. Plasma nitrotyrosine in rats (n=3-6 rats/group) after 4 weeks of exposure to lead, inorganic mercury or methylmercury or a mixture of all three metals through the drinking water. Data are shown as mean ± standard deviation. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared

to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A p< 0.05 was considered to be significant.

Glutathione is an intracellular antioxidant system that counteracts oxidative stress. In liver tissue, none of the single or combined exposures increased significantly total GSH compared to control (Figure 5.3A). Significant changes were observed for GSSG for both Pb(II) doses compared to the two mixtures but not to the control group (Figure 5.3B). The ratio of GSSG to total GSH (Figure 5.3C) was significantly increased for the higher Pb(II) dose of 45000 µg/kg-bw/d and the lower Hg(II) dose of 357 µg/kg-bw/d compared to the control group and both mixtures.

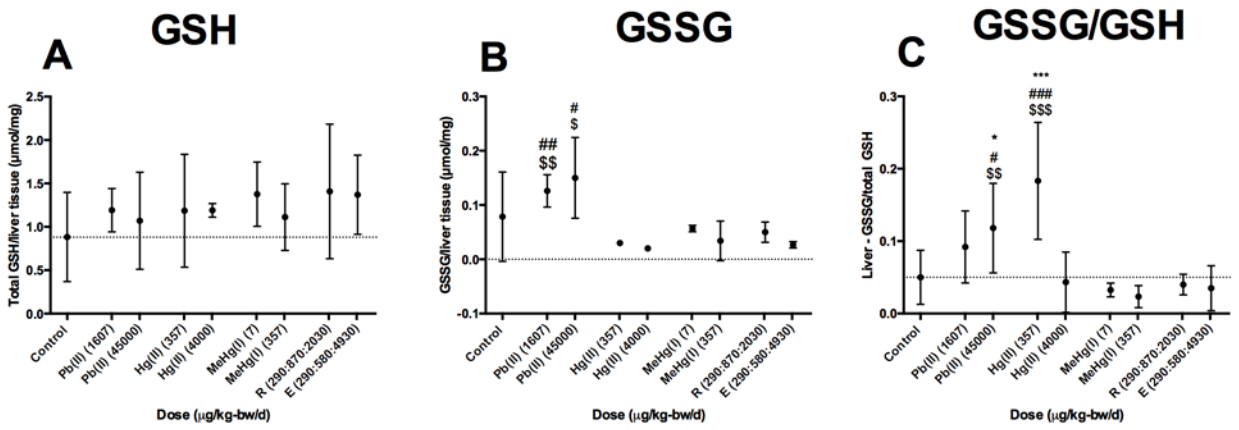

Fig. 5.3. Total GSH, GSSG and their ratio in liver tissue in rats (n=4-6 rats/group) after 4 weeks of exposure to lead, inorganic mercury or methylmercury or a mixture of all three metals through the drinking water. Data are shown as mean ± SD. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A p< 0.05 was considered to be significant.

No significant changes for total GSH, GSSG or their ratio was observed in aorta, heart or kidney (data not shown).

5.6.3 Kidney function and damage

Plasma creatinine levels were not affected by any of the single or combined exposures in comparison to the control group or to the mixtures (Figure 5.4A). In contrast to the plasma creatinine levels, urine creatinine levels were significantly elevated compared to control (Figure 5.4B) by about 200% for the lower Hg(II) dose and the higher dose of MeHg(I) and by about 280% for the environmental exposure ratio (E). Moreover, the environmental exposure ratio (E) was further increased in comparison to both Pb(II) doses, the higher Hg(II) dose, the lower MeHg(I) dose and

210% for the reference values ratio (R), highlighting differences in response to different metal mixtures. For the creatinine clearance, only the lower Pb(II) dose showed a significant increase compared to the control group and the mixtures (Figure 5.4C).

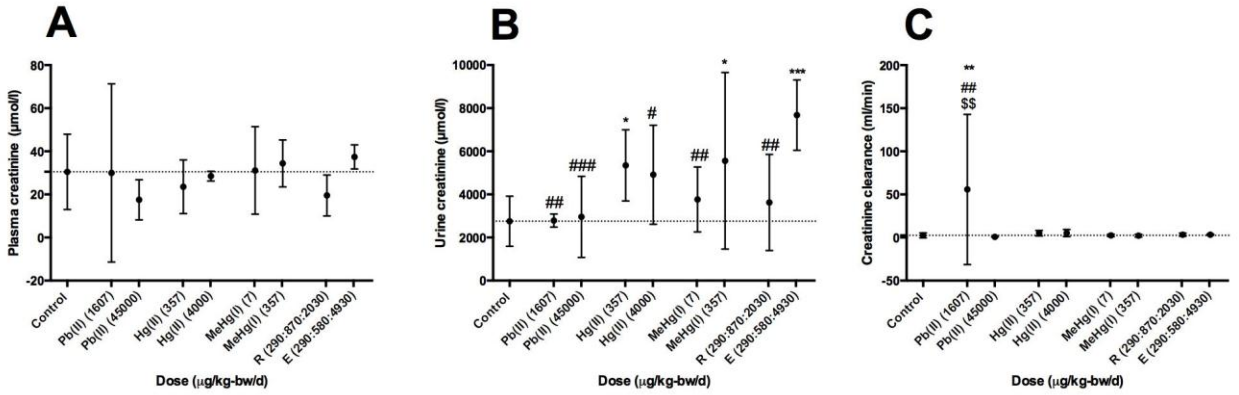

Fig. 5.4. Creatinine levels in plasma (A), in urine (B) and creatinine clearance (C) in rats (n=4-6 rats/group) after 4 weeks of exposure to lead, inorganic mercury or methylmercury or a mixture of all three metals through the drinking water. Data are shown as mean ± SD. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A $p< 0.05$ was considered to be significant.

The amount of KIM-1 in urine was significantly increased for the lower Pb(II) dose in comparison to the control group (Figure 5.5). No other significant differences among treatments were observed for urinary KIM-1.

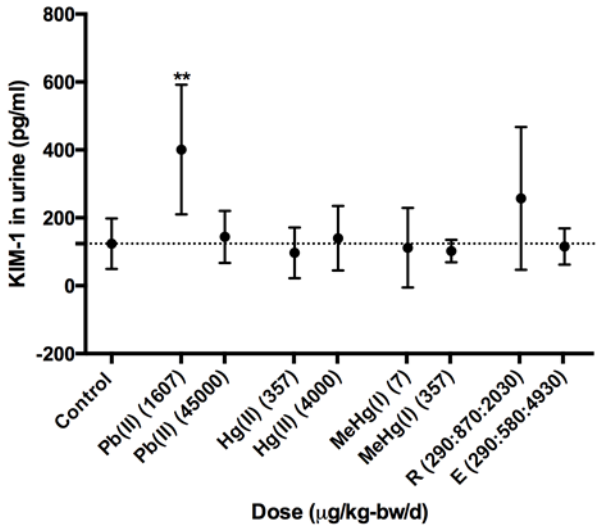

Fig. 5.5. KIM-1 levels in urine in rats (n=3-6 rats/group) after 4 weeks of exposure to lead, inorganic mercury or methylmercury or a mixture of all three metals through the drinking water. Data are shown as mean ± SD. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A $p < 0.05$ was considered to be significant.

5.6.4 DNA methylation

In our study, free urinary 5-methyl-2'-deoxycytidine (Figure 5.6) was significantly increased for both Hg(II) doses but not for any of the other exposures.

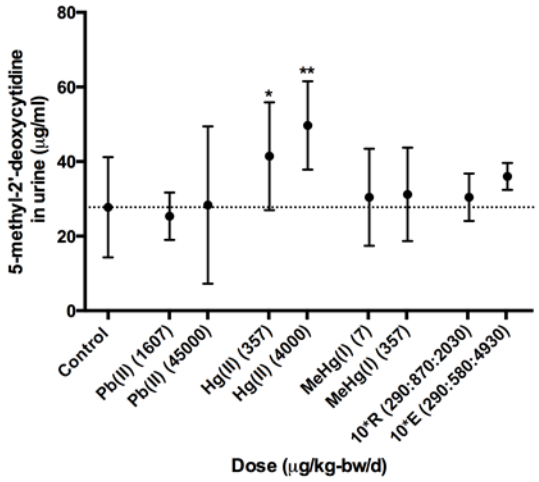

Fig. 5.6. Concentration of 5-methyl-2'-deoxycytidine was measured after four weeks of exposure in urine. Rats (n=5-6 rats/group) received either one metal Pb(II), Hg(II) or MeHg(I) or a mixture based on reference values (R) or environmental exposures (E) through the drinking water. Data are shown as mean ± SD. Horizontal line shows the mean value for the control group (dotted line). Single exposures were compared to control and mixtures in Fisher's LSD posteriori test after one-way ANOVA. * indicates significance versus control group, # significance versus R, $ significance versus E. A $p < 0.05$ was considered to be significant.

5.7 Discussion

In contrast to single metal exposure to Pb(II) and the lower Hg(II) dose, exposure to mixtures of all three metals did not affect oxidative stress production, indicating antagonism. While effects of metal mixtures on oxidative stress and kidney function were consistent with each other, mixture effects diverged for blood pressure depending on the mixture ratio. Although no comparable study with the same metal combination used in the current study is available, single and co-exposure to lead, cadmium and arsenic (Fowler et al., 2004) showed similar results on oxidative stress production depending on single metal exposure or combined exposures. Therefore, all three metals seem to affect the cardiovascular system through different mechanisms.

Pb(II) supports the development of hypertension through kidney injury and disturbing the ROS/antioxidant system, while MeHg(I) might act through a decrease of kidney function. Global DNA methylation was significantly altered by Hg(II), which did not elevate blood pressure, indicating that this mechanism of action is not associated with the development of hypertension.

5.7.1 Oxidative stress

Our data show that Pb(II) may cause hypertension through a combination of increasing oxidative stress production and kidney damage. Increased plasma nitrotyrosine levels are the result of an increased production of O_2^-, a sign for oxidative stress, as well as inactivation of nitric oxide, effects generally accepted to increase blood pressure (Vaziri and Khan, 2007). In agreement with our study, Ding et al (2001) showed an increase in nitrotyrosine in rats exposed to low levels of Pb(II) (100 ppm). The GSH/GSSG system is an internal antioxidant system and an increased GSSG level a sign for oxidative stress.

While none of the metal treatments increased GSSG levels in liver compared to the control group, any GSSG produced as a result of oxidative stress would be recovered to GSH via actions of the enzyme glutathione peroxidase (Bhabak and Mugesh, 2010). Previous studies have reported that levels of this enzyme were not altered after Pb(II) exposure (100 ppm) in rat aorta (Farmand et al., 2005) or in kidney, brain and left ventricle (100 ppm) (Vaziri et al., 2003), agreeing with results with GSSG in this study. In contrast, a human study of lead exposure in an occupational setting (Kasperczyk et al., 2004) showed an increase in glutathione peroxidase in erythrocytes (blood Pb level: 25-40 µg/dL). A reason for the discrepancy is likely due to differences between liver tissue and erythrocytes and the potentially longer exposure duration in humans than in experimental rats.

Mercury is another representative of a metal with the reported ability to induce oxidative stress. Previous studies reported that in aorta from rats (Wiggers et al., 2008; Lemos et al., 2012) and humans (de Marco et al., 2009) there was a decrease in NO availability. Also, MeHg(I) is reported to bind to GSH and inactivate this complex (Bridges et al., 2012). However, in the current study, MeHg(I) did not affect the GSH/GSSG system. Therefore, mechanisms other than those examined are likely linked to the ability of MeHg(II) to increase blood pressure such as alterations in autonomic neural function or renin-angiotensin system activation. Moreover, inorganic mercury did not seem to act through oxidative stress production with only disturbing the GSSG/GSH ratio at the lower Hg(II) dose. Taken together, we observed that Pb(II), but not MeHg(I) or Hg(II), disturbs the delicate balance between ROS and antioxidants which may be a mechanism for the development of hypertension.

5.7.2 Kidney function and damage

Our study found that none of the metals decreased plasma creatinine levels. In contrast, data from the Normative Aging Study (Kim et al., 1996) showed a significant relation between low level lead and increased serum creatinine, which is corroborated by findings from NHANES (Muntner et al., 2003; Fadrowski et al., 2010). This discrepancy might be due either to differences in exposure duration or species differences. A Canadian research group (Dutton et al., 2013; Zwicker et al., 2014) showed a positive association between urinary mercury from dental amalgam and urinary creatinine. We found links between urinary creatinine and the lower Hg(II) dose and the higher MeHg(I) dose. While not showing a consistent dose-related pattern, others have found that exposure for 60 days to Hg(II) or MeHg(I) increased serum creatinine for MeHg(I), but not for Hg(II) (Shi et al., 2011). We observed that urinary creatinine levels differed depending on the metal ratio in a mixture with normal levels for the mixture based on the reference value ratio and an increase for the mixture based on the environmental value ratio.

An opposite pattern was observed with urinary KIM-1. Albeit not significant the mixture based on the reference value ratio appeared to increase urinary KIM-1, while the mixture based on environmental value ratio was comparable to the control group values. This suggests that the proximal tubule could be a target depending on the metal ratio. As the mixtures reversed the effect of Pb(II) exposure alone, Hg species seem to antagonize the potential kidney injury through Pb(II). In summary, kidney damage due to Pb(II) exposure has the potential to be linked to changes in blood pressure, while MeHg(I) does not seem to inflict injury of the proximal tubule.

5.7.3 DNA methylation

In our study, exposure to Pb(II) and MeHg(I) did not alter global DNA methylation in urine but increased blood pressure. In contrast, Hg(II) did not increase blood pressure but significantly changed urinary global DNA methylation, indicating that global DNA methylation is not associated with blood pressure regulation. As only global DNA methylation was analyzed, it is impossible to relate the results to specific genes relevant for the homeostasis of the cardiovascular system in our animal experiment. Other studies using cell lines, have found that alterations in DNA methylation of genes involved in cardiovascular health are potentially associated with the development of cardiovascular diseases. In cells, Chan et al (2004) showed that DNA methylation is a key mechanism to steer the expression of the eNOS gene which is involved in NO metabolism. Kim et al (2010) go a step further and suggest DNA methylation as a biomarker to assess cardiovascular risk in humans based on their finding that increased DNA methylation in peripheral blood leukocytes has a direct association with the prevalence of cardiovascular diseases. Future studies examining epigenetic mechanisms with metals and a possible link to hypertension should examine methylation

of specific genes of interest to this system or possibly use leukocytes instead of urinary deoxynucleotides as marker.

5.8 Conclusions

Based on our knowledge, this is the first time that exposures to MeHg(I), Hg(II) or Pb(II) and their mixtures were compared regarding their ability for oxidative stress production, kidney function/damage and DNA methylation as potential mechanisms of action for the development of hypertension. We observed that each of the three metals had different effects on the three potential mechanisms of action. Pb(II) could be linked to blood pressure increase through oxidative stress and kidney damage. Although MeHg(I) increased blood pressure, it did not alter oxidative stress levels or impair kidney function. Neither of these two metals altered global DNA methylation in contrast to Hg(II), which showed no increased oxidative stress or adverse effect on the kidneys, which is in agreement with the only minor effect on blood pressure. Therefore, global DNA methylation appears not to be associated with the development of hypertension. Moreover, effects of mixture ratios differed but generally diminished blood pressure effects of single metals. However, effects on plasma and urinary creatinine levels indicate opposite effects on the kidney function between the two mixtures. Our results show lead and mercury species may act through different mechanisms of actions on blood pressure, but the mechanism of action is still unclear for all metals examined, particularly for methylmercury. Moreover, exposing rats to different mixtures produced different effects on both blood pressure and potential mechanisms, highlighting the difficulty in extrapolating single metal animal studies to human populations where exposures are generally to complex mixtures.

6 SYNTHESIS AND CONCLUSIONS

Cardiovascular diseases, such as heart attacks and stroke, are the major cause of death worldwide (Laslett et al., 2012). In addition to life-style and metabolic risk factors, environmental pollutants are increasingly discussed (Mendis et al., 2011). Lead and mercury species are ubiquitously present in the environment leading to chronic, low dose exposure in humans. While Pb(II) is clearly related to hypertension, additional cardiovascular end-points are poorly studied. Even less evidence exists for a relation between exposure to mercury species and adverse cardiovascular effects. Hence, the contribution of these metals to the prevalence of CVDs is not known.

The main goals of the presented research were to

1. Identify cardiovascular effects of single exposures to Pb(II), Hg(II) and MeHg(I),
2. Explore cardiovascular effects of combined exposures to Pb(II), Hg(II) and MeHg(I),
3. Investigate underlying mechanisms of action, e.g. oxidative stress, kidney damage and DNA methylation, for cardiovascular effects.

The research results provided answers to questions, such as: Are Pb(II), Hg(II) and MeHg(I) risk factors for the development of CVDs? Which is the most sensitive cardiovascular end-point? Do safe exposure thresholds exist? Do mixture effects deviate from additivity? Do these metals elicit cardiovascular toxicity through the same mechanisms?

6.1 Main findings

Blood pressure was affected by Pb(II) and MeHg(I) but not by Hg(II) exposure. Pb(II) showed a biphasic dose-response curve with a blood pressure decrease at doses ≤ 29 µg/kg-bw/d and increased blood pressure at doses ≥ 1607 µg/kg-bw/d (Figure 3.1). A dose of 357 µg Pb(II)/kg-bw/d yielded a BLL of 17 ± 7 µg/L (Table 3.2), which corresponds to a BLL in the general population. Considering a linear relation between Pb(II) exposure and BLL, it can be assumed that doses at which a decrease in blood pressure was observed are too low to be relevant for human exposure. However, increased blood pressure was observed at BLLs (Table 3.2), which can be found in the general population and in occupational settings. Hence, Pb(II) exposure in the human population can be considered as a risk factor for hypertension. The mercury species MeHg(I) and Hg(II) differ in their effects on blood pressure. While MeHg(I) increased blood pressure at even low doses (≥ 7 µg/kg-bw/d) (Figure 3.1), Hg(II) did not affect blood pressure but had a mortality rate of 100% at a dose of 4000 µg Hg(II)/kg-bw/d (Table 3.1). As a consequence, it will be important to specify the mercury species at exposure level. Furthermore, these findings might explain the inconclusive

results on mercury exposure and blood pressure from epidemiological studies which often do not differentiate between exposure to MeHg(I) and Hg(II).

The human population is generally exposed to mixtures of chemicals. In the case of Pb(II) and mercury, Pb(II) exposure occurs through food and drinking water (Health Canada, 2013), mercury vapor from dental amalgam fillings (Richardson and Allan, 1996), which is quickly oxidized to Hg(II) in the body and MeHg(I) through fish and seafood (EFSA, 2012a). In mixture toxicology, it is assumed that the mixture components do not interact, i.e. they are additive (U.S. EPA, 1986). For the mixture experiment, it was also assumed that exposure to MeHg(I), Hg(II) and Pb(II) occur based on fixed ratios. Therefore, literature values for published reference and environmental exposure values were used as base for combined exposures (Table 4.1). The results from the mixture experiment showed that the assumption of additivity is wrong. The adverse effects on blood pressure from exposures to Pb(II) and MeHg(I) alone were reversed and normal blood pressure levels observed (Figure 4.1) indicating antagonism. In contrast, combined exposures to Pb(II), Hg(II) and MeHg(I) adversely affected the cardiac electrical activity (synergism), which was not affected by single exposures (Figure 4.3). Therefore, it is not possible to predict the cardiovascular effects from combined exposures from single exposures to MeHg(I), Hg(II) and Pb(II). In addition to the cardiovascular system, the blood levels of Pb(II) and total mercury differed between single metal and combined metal exposure. While the BLL decreased compared to single Pb(II) exposure, the total mercury blood level was increased (Table 4.3). As the blood pressure was not increased with combined exposure, the blood levels of the metals are not a reliable marker for changes in blood pressure. Furthermore, differences based on the mixture ratios were observed. Exposures based on the reference values ratio increased BLL and total mercury blood levels more than the environmental exposure ratio (Table 4.3). A similar pattern was visible with cardiovascular end-points, such as blood pressure (Figure 4.1), heart rate (Figure 4.2) and electrical activity of the heart (figure 4.3), which indicates the importance of the metal ratio.

Epidemiological studies show a clear association between Pb(II) and blood pressure (Scinicariello et al., 2011; Cheng et al., 2001; Nash et al., 2003), while the same relation for mercury is unclear (Bautista et al., 2009; Mozaffarian et al., 2012). This type of studies usually focus on Pb(II) exposure or mercury exposure and therefore, neglecting potential mixture effects. The presented findings show that neither blood levels nor effects from single exposure are reliable predictors for effects from combined exposures, which is the reality in the human population. Thus, epidemiological studies need to be analyzed for combined exposures to Pb(II) and mercury species and the metal ratio be calculated.

The differences between the cardiovascular effects of single metal and combined exposure were surprising and could not be explained by the blood levels of Pb(II) and total mercury. As hypertension is the major risk factor for the development of CVDs, effects at cellular and molecular level were investigated to elucidate the mechanisms for blood pressure development. Blood pressure increase was observed with single exposures to Pb(II) or MeHg(I) and to the reference value ratio, but not for Hg(II) or the environmental exposure ratio (Figure 5.1). Metals, such as Pb(II) and mercury species are well known to increase oxidative stress (Ercal et al., 2001). While single exposure to Pb(II) raised the production of oxidative stress (Figures 5.2 and 5.3), no changes were observed with mercury. This indicates that oxidative stress is a mechanisms for blood pressure increase due to single metal exposure to Pb(II). Although exposure to the reference value ratio increased blood pressure, no change in oxidative stress production was observed indicating that metal mixtures act through a different mechanism than single metal exposure. Pb(II) and mercury are known systemic toxicants and have the ability to adversely affect e.g. the kidneys, which play an important role in blood pressure regulation. The kidney function might be affected by single exposure to mercury but the dose-response relation is not clear (Figure 5.4). As seen with cardiovascular end-points, the effects of the mixtures differed depending on the ratio. While only the mixture based on the environmental exposure ratio showed an adverse effect on the kidney function (Figure 5.4), the blood pressure increase was observed with the mixture based on the reference value ratio (Figure 5.1). With regard to kidney damage, only Pb(II) exposure showed an adverse effect (Figure 5.5) indicating a second mechanism for the development of hypertension (Figure 5.1). The mixtures differed again with respect to their effect on kidney damage but in the opposite way. While the mixture based on the reference value ratio showed a trend to negatively affect the kidney, the mixtures based on the environmental exposure ratio had no effect (Figure 5.5). A third potential mechanism for the alteration of blood pressure due to metal exposure is the change of the global DNA methylation. While Pb(II), MeHg(I) and the mixtures did not show an effect, Hg(II) raised the global DNA methylation (Figure 5.6). However, Hg(II) was the metal which did not affect blood pressure. Thus, it can be assumed that global DNA methylation might not be a mechanism for the development of hypertension.

6.2 Strengths and limitations

A broad range of cardiovascular effects were evaluated with high-resolution ultrasound and a catheter inserted into the femoral artery. Due to the high sensitivity of these methods, it was possible to measure even small changes in heart function, blood flow and blood pressure and thus, better characterize the cardiovascular effects of various Pb(II) and mercury exposures. A group of control animals was run with each experiment to assure the quality of the study. For the data analysis, the selection of animals for the control group was based on normotensive blood pressure values. For

single metal exposures, 7-8 different doses per metal were applied allowing the derivation of safe thresholds of exposure. For combined metal exposures, the selected ratios were based on published reference and environmental exposure values, which renders them particularly relevant for human exposure scenarios. The exposure scenarios of first single metal exposure and then combined metal exposures allowed for a unique comparison of cardiovascular effects between one metal and a metal mixture. As a consequence, it was also possible to compare the mechanisms for cardiovascular toxicity of single and combined metal exposures.

The few limitations of the presented research include the sub-chronic exposure duration versus chronic exposure in humans, the use of only male rats, tap water and the low number of animals per group. However, the listed strengths outnumber the study limitations and the applied high-sensitivity methods add confidence to the findings.

6.3 Future research

The presented research showed that exposure to Pb(II), Hg(II) and MeHg(I) and their mixtures can adversely affect the cardiovascular system and thus, impair public health. Future studies should investigate the influence of exposure duration on the cardiovascular system to better relate effects from short-term exposures in animals to long-term exposure in human adults. Epidemiological studies have shown gender differences in the development of hypertension (Sandberg and Ji, 2012), which were not addressed in this research. Therefore, it will be important to explore the impact of gender on blood pressure, heart function, blood flow and electrical activity of the heart to prevent cardiovascular deaths.

Epidemiological studies play a crucial role in characterization of cardiovascular effects and to establish reference values for safe levels of exposure. However, previous studies should be re-analyzed to consider mixture effects due to combined exposure to Pb(II) and mercury and mixture ratios be calculated. As blood pressure was not the most sensitive end-point for mixture effects, these studies should include a variety of cardiovascular end-points to better estimate the overall cardiovascular risk. Future human studies should include these factors in their experimental designs, which improve the understanding of the cardiovascular risk due to Pb(II) and mercury exposure.

Future studies should also explore the underlying mechanisms for cardiovascular toxicity. The research results showed that no single mechanism is responsible for the adverse effects and that the mechanism might differ depending on single or combined exposures. It is also not understood how epigenetic mechanisms, in particular DNA methylation, affect the cardiovascular system. Future studies should investigate which genes relevant for cardiovascular health are affected by Pb(II) or

mercury exposure. Once such changes are identified, it might be possible to identify biomarkers for increased cardiovascular risk.

A well-populated database for cardiovascular effects from exposures to Pb(II), Hg(II) and MeHg(I) and their mixtures will allow regulatory toxicologists in establishing safe exposure thresholds and provide a useful tool for predicting cardiovascular risks from metals with similar properties, e.g. cadmium. Overall, a detailed knowledge of the cardiovascular system at physiological, cellular and molecular levels due to exposure to Pb(II) and mercury will improve the protection of public health.

6.4 Conclusions

Lead and mercury species are traditionally known as neurotoxicants and most of the research has been carried out on their effects on the nervous system. Over the last decades, a growing number of studies indicate an association between exposure to lead or mercury species and adverse cardiovascular effects. However, their adverse effects on the cardiovascular system are poorly characterized and mixture effects are almost completely unknown. The presented research could broaden the understanding of the range of cardiovascular effects elicited by these metals but also that there are clear differences between each metal. The finding that mixtures of Pb(II), Hg(II) and MeHg(I) deviate from additivity raises the question if the general population is protected or not. Mechanisms for cardiovascular toxicity due to exposure to Pb(II), Hg(II) and MeHg(I) are partly understood, little is known about their mechanistic interactions or the influence of epigenetics. The research results were able to answer some questions but also raised many new questions, which will hopefully be addressed by future research projects.

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8 APPENDICES

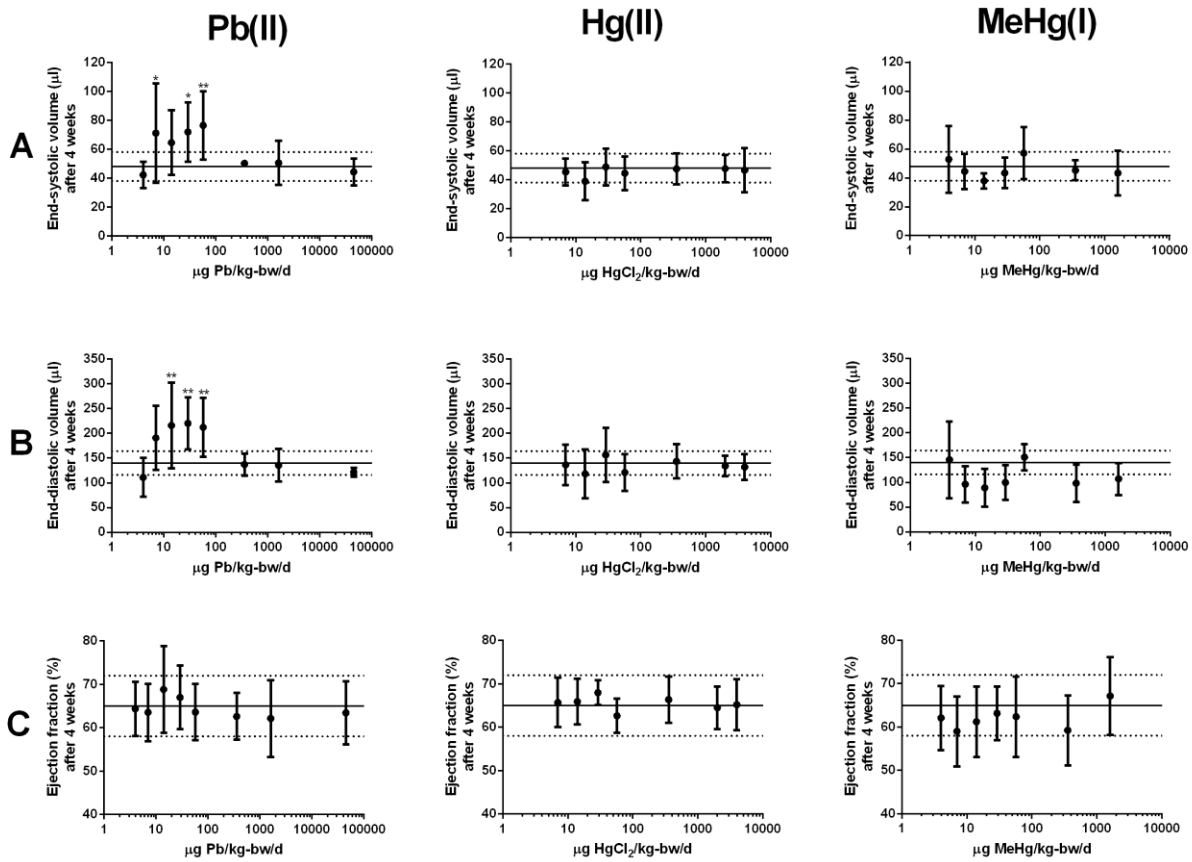
8.1 Appendix A: Supplemental data for study 1 (chapter 3)

Supplemental table S8.1. Baseline echocardiographic and ultrasound values for rats obtained 1-day prior to starting 4-week oral exposures to lead, inorganic mercury or methylmercury. Values for each individual rat were used to correct subsequent measurements after 4-weeks exposure to indicate change caused by treatment.

Experimental dose (µg/kg-bw/d)	Heart free wall thickness systole (mm) at baseline	Heart free wall thickness diastole (mm) at baseline	Carotid artery diameter (mm) at baseline	Carotid artery wall thickness at baseline	Peak carotid velocity (mm/s) at baseline
MeHg					
4	0.79 ± 0.1	0.70 ± 0.09	0.73 ± 0.07	0.49 ± 0.06	-740 ± 110
7	0.76 ± 0.05	0.66 ± 0.04	0.90 ± 0.04	0.45 ± 0.04	-685 ± 116
14	0.74 ± 0.11	0.71 ± 0.12	0.95 ± 0.12	0.46 ± 0.04	-591 ± 155
29	0.76 ± 0.09	0.69 ± 0.08	0.81 ± 0.04	0.47 ± 0.03	-853 ± 153
57	0.74 ± 0.08	0.71 ± 0.06	0.83 ± 0.18	0.46 ± 0.03	-772 ± 63
357	0.71 ± 0.08	0.71 ± 0.07	0.91 ± 0.08	0.46 ± 0.02	-595 ± 189
1607	0.73 ± 0.06	0.70 ± 0.08	0.88 ± 0.04	0.45 ± 0.02	-653 ± 146

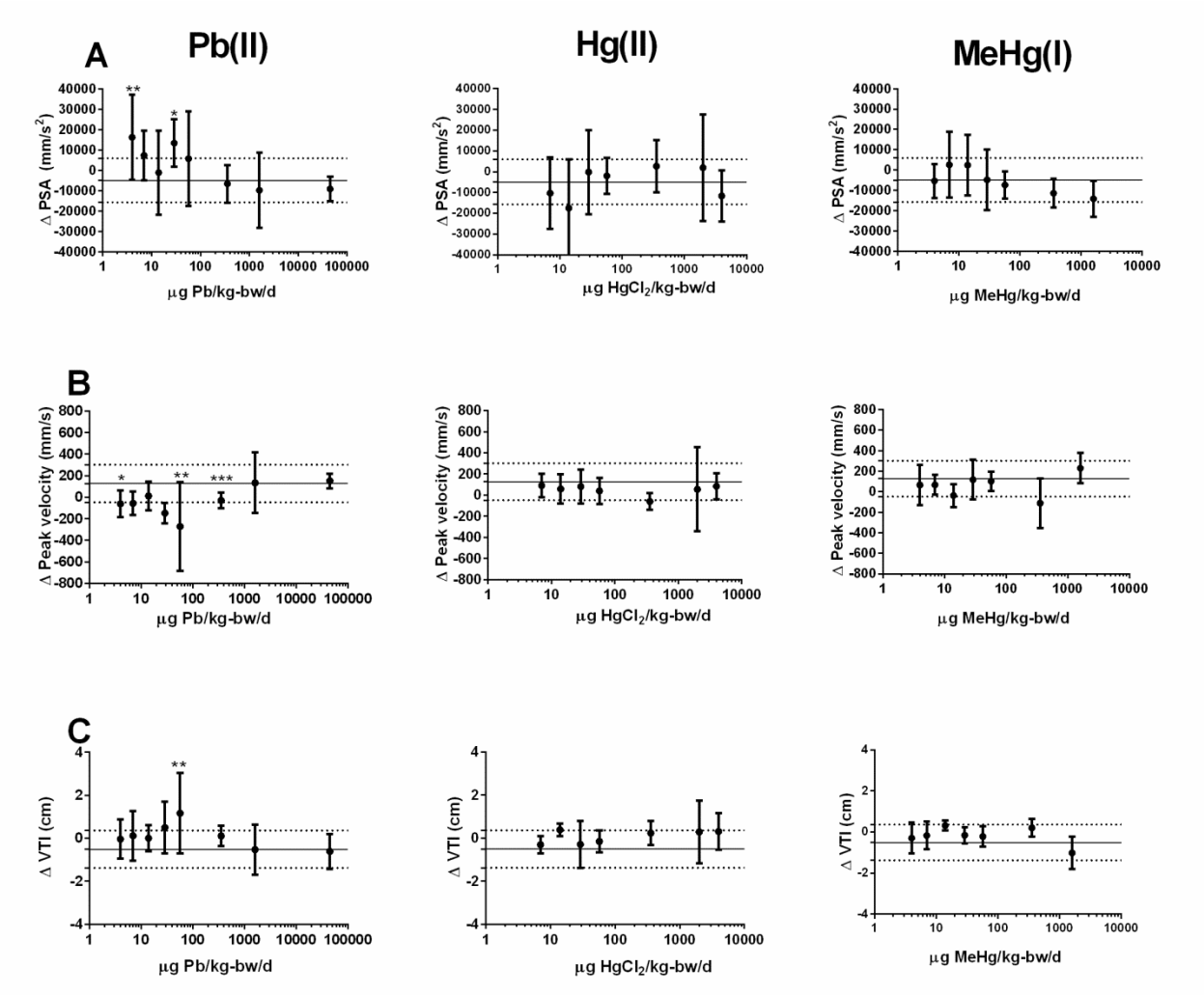
No significant differences among treatment groups were detected using 1-way ANOVA for any end-points at baseline.

Supplemental figure S8.1. End-diastolic volume (EDV), end-systolic volume (ESV) and ejection fraction determined using echocardiography in rats exposed to lead (left column), inorganic mercury (middle column) and methylmercury (right column) after 4 weeks of exposure via the drinking water: Results for EDV (A), ESV (B) and ejection fraction (C) are shown as mean ± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05, **p<0.01 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.

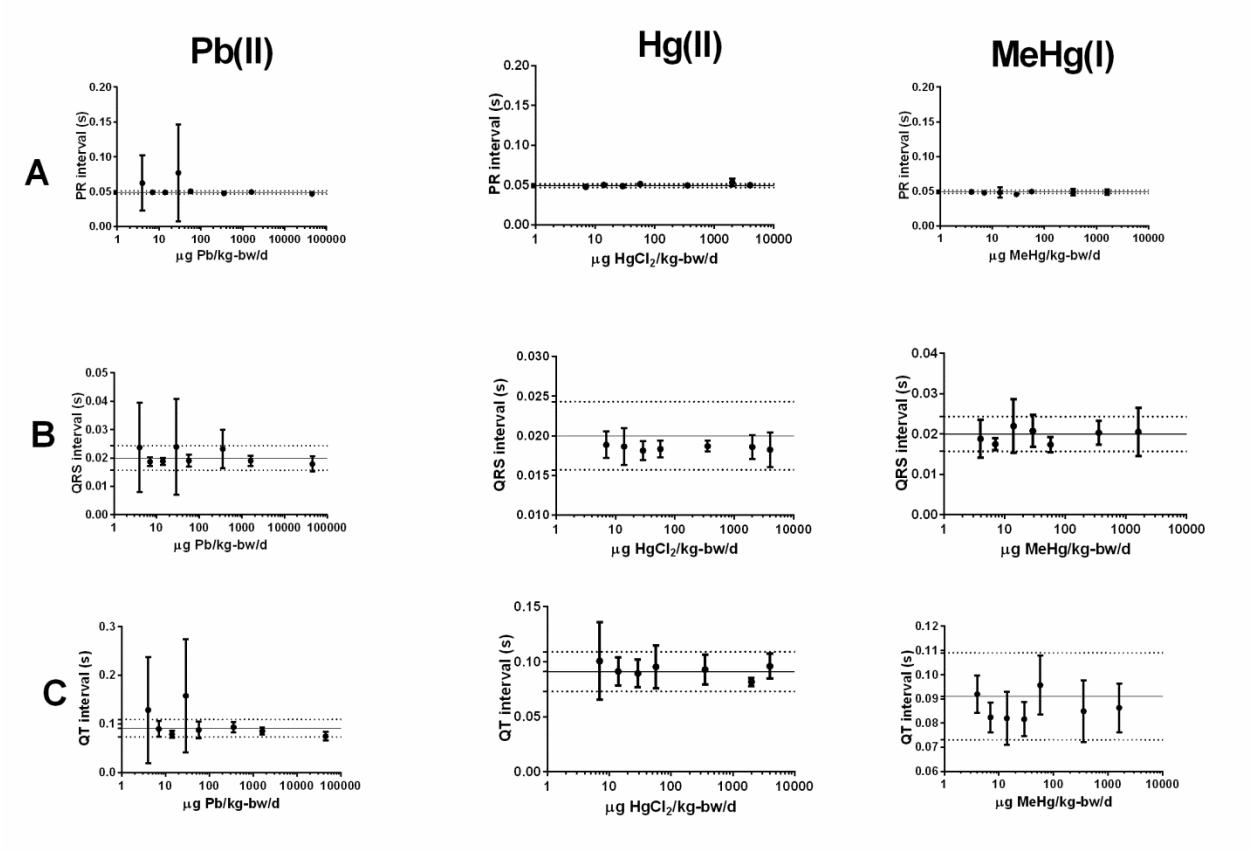


8.2 Appendix B: Supplemental data

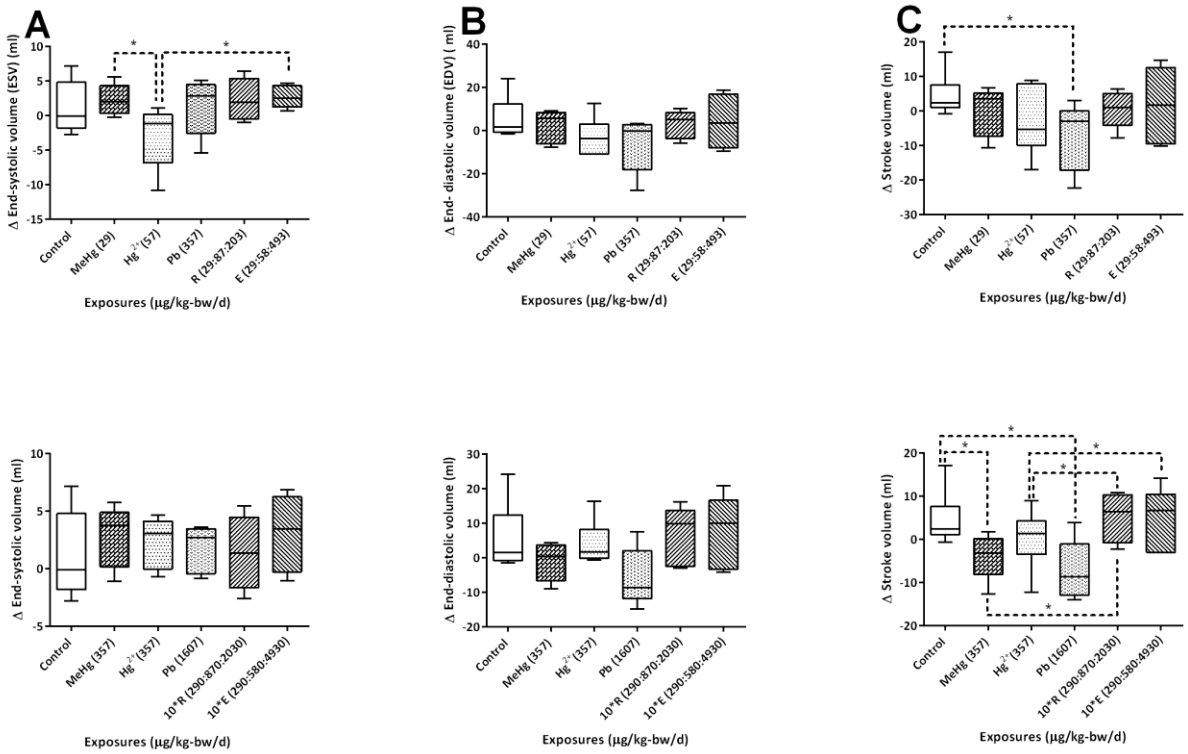
Peak systolic acceleration (PSA), peak velocity and velocity time interval (VTI) determined using echocardiography in rats exposed to lead (left column), inorganic mercury (middle column) and methylmercury (right column) at baseline and after four weeks of exposure via the drinking water: Results for PSA (A), peak velocity (B) and VTI (C) are shown as mean ± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05, **p<0.01 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.



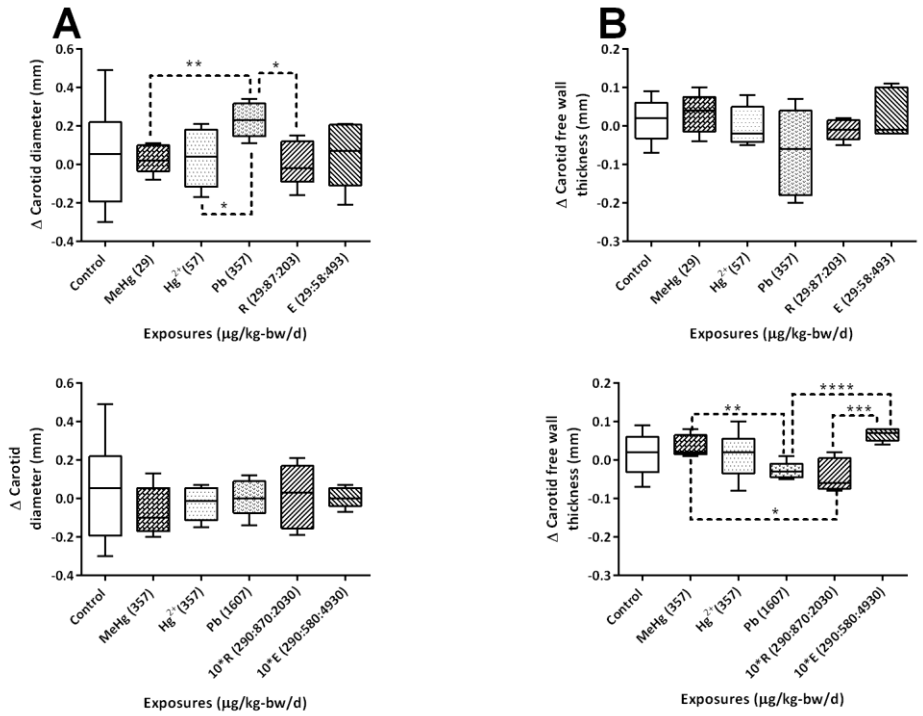
An electrocardiogram (ECG) was recorded in rats exposed to lead (left column), inorganic mercury (middle column) and methylmercury (right column) after four weeks of exposure via the drinking water. Results for PR interval (A), QRS interval (B) and QT interval (C) are shown as mean ± SD. For every graph, the mean value (solid horizontal line) for the control group ± its SD (dotted lines) are shown. *p<0.05, **p<0.01 compared to control in Fisher's LSD posteriori test after one-way ANOVA. n=5-6 rats/group.



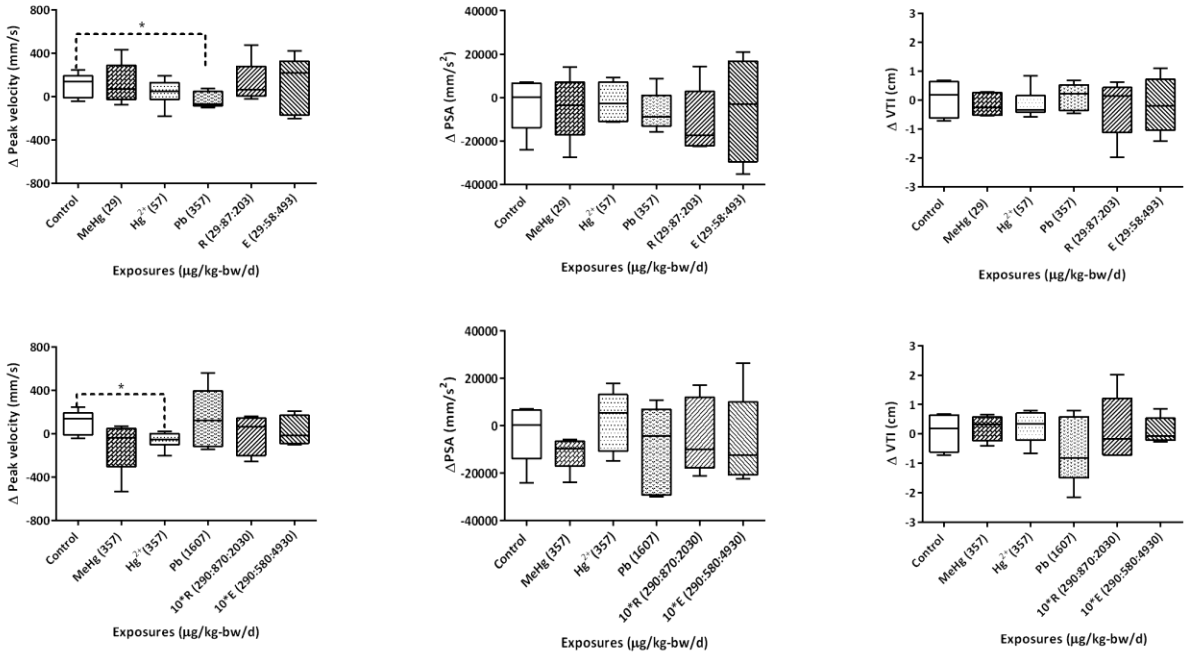
End-systolic volume (ESV), end-diastolic volume (EDV) and stroke volume. Results are shows as the difference between week-4 and baseline. Row A: ESV, EDV and stroke volume for one-metal exposure of methylmercury (29 μg/kg-bw/day), Hg(II) (57 μg/kg-bw/day), Pb(II) (357 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 29:87:203] and environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 29:58:493]; row B: ESV, EDV and stroke volume for one-metal exposure of methylmercury (357 μg/kg-bw/day), Hg(II) (357 μg/kg-bw/day), Pb(II) (1607 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 290:870:2030] and the environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 290:580:4930]; unpaired Student's t-test *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.001–0.0001, ****p<0.0001.



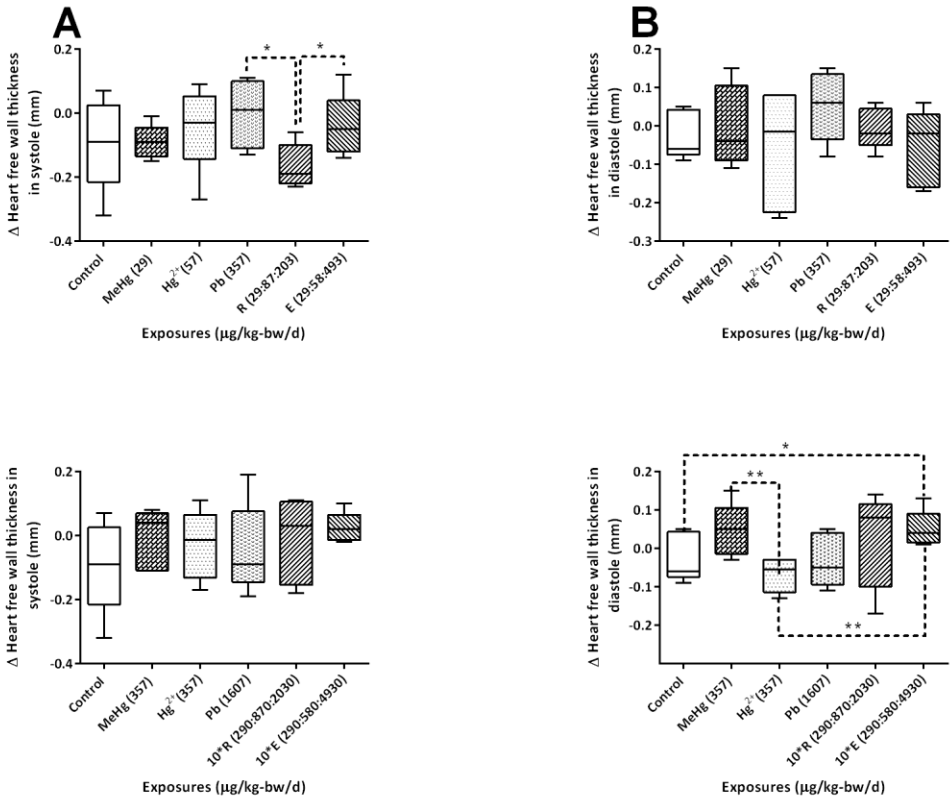
Carotid diameter and free wall thickness of the carotid. Results are shows as the difference between week-4 and baseline. Row A: carotid diameter and free wall thickness for one-metal exposure of methylmercury (29 μg/kg-bw/day), Hg(II) (57 μg/kg-bw/day), Pb(II) (357 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 29:87:203] and environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 29:58:493]; row B: carotid diameter and free wall thickness for one-metal exposure of methylmercury (357 μg/kg-bw/day), Hg(II) (357 μg/kg-bw/day), Pb(II) (1607 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 290:870:2030] and the environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 290:580:4930]; unpaired Student's t-test *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.001–0.0001, ****p<0.0001.



Peak velocity, peak systolic acceleration (PSA) and velocity time interval (VTI). Results are shows as the difference between week-4 and baseline. Row A: peak velocity, PSA and VTI for one-metal exposure of methylmercury (29 μg/kg-bw/day), Hg(II) (57 μg/kg-bw/day), Pb(II) (357 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 29:87:203] and environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 29:58:493]; row B: peak velocity, PSA and VTI for one-metal exposure of methylmercury (357 μg/kg-bw/day), Hg(II) (357 μg/kg-bw/day), Pb(II) (1607 μg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 290:870:2030] and the environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 290:580:4930]; unpaired Student's t-test *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.001–0.0001, ****p<0.0001.



Heart free wall thickness in systole and diastole. Results are shows as the difference between week-4 and baseline. Row A: heart free wall thickness in systole and diastole for one-metal exposure of methylmercury (29 µg/kg-bw/day), Hg(II) (57 µg/kg-bw/day), Pb(II) (357 µg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 29:87:203] and environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 29:58:493]; row B: heart free wall thickness in systole and diastole for one-metal exposure of methylmercury (357 µg/kg-bw/day), Hg(II) (357 µg/kg-bw/day), Pb(II) (1607 µg/kg-bw/day) and the reference value ratio [MeHg(I):Hg(II): Pb(II) = 290:870:2030] and the environmental exposure ratio [MeHg(I):Hg(II): Pb(II) = 290:580:4930]; unpaired Student's t-test *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.001–0.0001, ****p<0.0001.



8.3 Appendix C: Permissions

Wildemann, T.M., Mirhosseini, N.; Siciliano, S.D.; Weber, L.P. (2015) Cardiovascular responses to lead are biphasic, while methylmercury, but not inorganic mercury, monotonically increases blood pressure in rats, *Toxicology*, 328, 1-11 DOI 10.1016/j.tox.2014.11.009

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Wildemann, T.M.; Weber, L.P.; Siciliano, S.D. (2014) Combined exposure to lead, inorganic mercury and methylmercury shows deviation from additivity for cardiovascular toxicity in rats, *Journal of Applied Toxicology* (in press) DOI 10.1002/jat.3092

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