Investigating the effects of the heavy metals mercury, nickel and manganese, alone and in combination, on human erythrocytes and components of the coagulation system

By

Precious Busisiwe Maseko

10263269

Dissertation submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in the

FACULTY OF HEALTH SCIENCES

Department of Anatomy

University of Pretoria

2019

Investigating the effects of the heavy metals mercury, nickel and manganese, alone and in combination, on human erythrocytes and components of the coagulation system

by

PRECIOUS BUSISIWE MASEKO

SUPERVISOR: Prof. HM Oberholzer

CO-SUPERVISOR: Dr. H Taute

CO-SUPERVISOR: Dr. M van Rooy

DEGREE: MSc in Anatomy with specialization in Cell Biology

Abstract

Heavy metals are major pollutants across the globe. Heavy metals are used in various industries and the waste matter is discarded in a manner that allows them to enter the soil, rivers and other parts of the environment. The widespread usage of heavy metals within different sectors such as agricultural, domestic and industrial areas raises an alarming concern over their impact on the environment and human health because of the wide spread contamination. Some metals are known to pose a danger to human health, while others such as copper and chromium are considered essential metals to humans and animals. However, chronic exposure to heavy metals may result in various ailments such as cardiovascular diseases, neurological- and behavioural disorders, and those affecting the immune system and kidneys. Blood cells play a crucial role in cardiovascular health where erythrocytes are responsible for the transportation of oxygen in the body and platelets and fibrin networks play a crucial role in the coagulation system also known as haemostasis. Abnormalities in haemostasis can result in haemorrhage or thrombosis. Heavy metals have been reported to cause alterations in erythrocytes influencing their function, morphology and distribution across the body, thus contributing to the pathophysiology of cardiovascular diseases. The aim of this study was to investigate the effects that the metals mercury, nickel and manganese alone and in combination, have on erythrocyte morphology and other components of the coagulation system by using the haemolysis assay, scanning electron microscopy and confocal microscopy. The metals in the study where chosen based on the likelihood of being exposed to them in South Africa. In this study, human blood was exposed to the heavy metals mercury, nickel and manganese *ex vivo* at concentration ranges of 1x, 10x, 100x, 1000x and 10000x the World Health Organization safety level standards for each respective metal. The World Health Organization safety level standards for these metals are: mercury = 6µg/L; nickel = 20µg/L; manganese = 400 µg/L. Blood samples were obtained from healthy male donors after written informed consent was obtained. Exposure of mercury caused increased haemolysis compared to nickel and manganese alone and in combination. At the highest concentration of 10000x all metals including the double and triple combination caused increased haemolysis. Results indicate that the erythrocytes membrane integrity was compromised resulting in haemoglobin leaking. Results provided by ultrastructural analysis indicated that the heavy metals (mercury, nickel and manganese) significantly impact on the shape of erythrocytes and structure of platelets and fibrin networks altering the coagulation system. Scanning electron microscopy results showed that with an increase in heavy metal exposure the erythrocytes lose

their typical biconcave morphology and become echinocytic with a bulging appearance visible with increased membrane roughness. Platelet membrane spreading and presence of pseudopodia increases, and fibrin networks appeared unorganized with increased membrane roughness observed in all the single metal groups (Hg, Ni and Mn) and combinations (Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn). Confocal microscopy results showed that the membrane phospholipid, phosphatidylserine, was translocated from the internal membrane leaflet to the outer surface of the membranes of erythrocytes exposed to all three metals alone (Hg, Ni and Mn) and in combination (Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn), this could indicate eryptosis of erythrocytes. The morphological changes of erythrocytes, platelets and fibrin networks may cause weakened capacity for erythrocytes to carry oxygen effectively, impair the coagulation system resulting in thrombosis and additionally prevent the restoration of homeostasis of the body. The outcome of this study provides a better understanding of the effect of these metals on the coagulation system and the negative impact on human health.

Declaration

I, Precious Busisiwe Maseko, hereby declare that this dissertation entitled: "**Investigating the effects of the heavy metals mercury, nickel and manganese, alone and in combination, on human erythrocytes and components of the coagulation system**", which I hereby submit for the degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. I understand what plagiarism is and am aware of the University's policy in this regard. Where other people's work has been used (either from a printed source, internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements. I have not used work previously produced by another student or any other person to hand in as my own.

Ethics statement: The author, Precious Busisiwe Maseko, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

....

SIGNATURE **DATE**

Department of Anatomy, School of Medicine, Faculty of Health Sciences

University of Pretoria

South Africa

Acknowledgments

First and foremost, thank you to the Almighty God for giving me the strength and knowledge to get this far. It has been a difficult journey and it would not have been easy without his presence. The ability to complete this research experiment to its satisfactory standard would not be possible without the almighty's blessing.

I would like to dearly appreciate and give my biggest gratitude to my supervisor Prof Nanette Oberholzer for all her help throughout my research, without your guidance and assistance I wouldn't have made it. Thank you for your words of encouragement and support invaluable guidance, inspiration and suggestions in my quest for knowledge. Prof Oberholzer always gave me the freedom to do my experiments but always ensured I stuck to the objectives and gave feedback of how the research is coming along.

I would like to acknowledge my Co-supervisors Dr. Helena Taute from the department of Anatomy and Dr. Mia van Rooy from the department of Physiology for always being there to assist with my research project. Your knowledge and input are greatly appreciated. Thank you for all your valuable comments.

Thank you to every individual who helped me with my experimental work in the laboratory. To Prof P Soma, Dr C Grobbelaar and Dr. JG Roode thank you all for assisting with the withdrawing of blood from the volunteers. Thank you to Dr June Serem from the department of Anatomy, she helped me every time I encountered problems in the lab or needed clarity on the experimental work and the statistics. Thank you to Dr. C Venter for all the assistance you provided me with at the microscopy lab concerning sample preparation, viewing of samples and interpretation of results. To my fellow Cell biology student and friend Anwani Nekhumbe, thank you dearly for assisting me with all my samples I don't think I would have finished if you did not lend a helping hand. The male volunteers in my study were all keen and willing to help, thank you so much gentlemen. Lastly. Thank you to my family members The Maseko's and Mbele's and all those who kept encouraging me to never give up but to chase my dreams to the best of my ability. My sincere gratitude to everyone who contributed I truly appreciate your help and guidance.

Table of Contents

List of figures

List of tables

List of abbreviations, symbols and formulae

°C	Degrees Celsius
%	Percentage
kDa	Kilodalton
xg	Times gravity
u	Atomic mass
ω	Omega
U/ml	Units per millilitre
A	Absorbance
ADP	Adenosine phosphate
cAMP	Cyclic adenosine phosphate
ATP	Adenosine triphosphate
$CdCl_2$	Cadmium chloride
CH_3Hg^+	Methylmercury
CLSM	Confocal laser scanning microscope
Cr	Chromium
CSIR	Council of Scientific and Industrial Research
Cu	Copper
dH_2O	Distilled water
EtOH	Ethanol
FA	Formaldehyde
GA	Glutaraldehyde
FAO	Food and agriculture organization
GIT	Gastrointestinal tract
GFR	Glomerular filtration rate
GSH	Glutathione
Hg	Mercury
$Hg^{+/2+}$	Inorganic mercury/ mercuric ions
$HgCl_2$	Mercuric chloride

HMDS	Hexamethyldisilazane
iso	Isotonic
KCL	Potassium chloride
Mn	Manganese
MES	Minimum Emission Standards
NAC	N-acetyl;cysteine
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
$Na_2H_2PO_4$	Sodium dihydrogen phosphate.
Ni	Nickel
$NiSO_4$	Nickel Sulphate
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PGM	Platinum group minerals
PS	Phosphatidylserine
ROS	Reactive oxidative species
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy / microscope
-SH	Thiol group
SLS	Sodium lauryl sulphate
SOD	Superoxidase dismutase
TxA2	Thromboxane A_2
WHO	World Health Organization

1. INTRODUCTION

Heavy metals are extensively distributed on earth, in seawater, air and fresh water [1]. Many of the elements that are considered heavy metals on the periodic table are essential for human biological processes. These elements include copper (Cu), zinc (Zn), selenium (Se) and chromium (Cr) and they assist by partaking in the metabolism of the body. However, these metals may be toxic at high concentrations. Sources of heavy metals range from the diet we consume, to contaminated drinking water and air. The inability to destroy heavy metals poses a great danger as heavy metal exposure in occupational settings and the environment is mainly via inhalation and ingestion. Exposure to heavy metals compromises the health of an individual as it can lead to various ailments such as cardiovascular disease, neurological-, behavioural-, immune system- and kidney disorders [2]. The presence of heavy metals in the atmosphere, soil and water has adverse effects on all organisms including humans. In humans, heavy metals have gradually become a great concern due to their tendency to accumulate in food and their threat to human health. Heavy metals have a high degree of compactness and are toxic to humans as well as animals [3]. Examples of such metals are mercury (Hg), cadmium (Cd), lead (Pb), nickel (Ni), manganese (Mn) and chromium (Cr). Several metals are important to the natural occurring environment but their bio-toxic effects to human health pose a great danger. Most metals are used in industries where the waste is discarded into the environment. Microbial communities are important in the decomposition of soil organic matter, as they regulate the nutrients availability to plants. The presence of metals in the environment will result in a reduced microbial community and affect their decomposing ability [4-6]. Electroplating, plastics manufacturing, automobile exhausts, production of fertilizers and waste after mining are some of the sources of heavy metal pollutants [7]. Heavy metals seep into the underground waters, moving along water pathways and eventually pollute the soil [8].

Blood is composed of different cells i.e. erythrocytes, platelets and white blood cells. Erythrocytes are important in the functioning of the human body as they carry oxygen. Erythrocytes are round and have a biconcave shape [9] and execute an imperative role in the transportation of oxygen to the tissue and carbon dioxide from tissues to the lungs. A study conducted on the effect of iron overload on erythrocytes provides evidence that heavy metals cause changes in the morphology of erythrocytes [10]. Furthermore, erythrocytes together with platelets function with coagulation proteins such as fibrin to aid in the coagulation system. Platelets are involved in the development of blood clots within the coagulation system to prevent blood loss from a damaged blood vessel

[11]. The first step of clot formation requires platelets to be recruited to the site of blood vessel injury. Platelets will bind to exposed collagen with the help of von Willebrand factor and become activated. The binding of platelets will activate platelets to release pro-thrombotic factors such as platelet activating factor (PAF), serotonin as well as adenosine diphosphate (ADP) to further recruit more platelets. The platelet factors will enable a temporary vasoconstriction of the blood vessel and further activate more platelets to form a platelet plug [11]. Fibrinogen is then converted to fibrin by thrombin. Thrombin causes fibrin fibres to weave through the platelet plug and trap erythrocytes within a mesh, stabilizing the formed fibrin clot. Blood coagulation is an essential part of haemostasis and human survival is dependent on the ability of blood to clot effectively [12].

Effective haemostasis is not only dependent on the coagulation of blood but also on the removal of the clot once the injury has healed. A process known as fibrinolysis dissolves the fibrin mesh. A balance should be maintained between coagulation and fibrinolysis to ensure haemostasis [11].

In this study, the effects of the metals Hg, Ni and Mn, alone and in combination on the coagulation system were investigated by the haemolysis assay, scanning electron microscopy and confocal microscopy. In the literature review to follow, sources, states, exposure, health effects, metabolism and elimination of all metals are discussed as well as the role and function of erythrocytes, platelets and fibrin networks in the coagulation system and haemostasis.

2. LITERATURE REVIEW

The introduction of various metals into the environment has caused major problems in the environmental sector. The resistance to the degradation of heavy metals is concerning as they are difficult to completely remove from the environment once they enter in it, as a result they accumulate in plants, human tissue and animals. Metal toxicity has increased substantially over the past years and has become a major concern in the health sector [13]. The presence of metals in the environment produces several modifications of microbial communities and affects the activity of these microbes. Very few heavy metals are essential trace elements that are needed for nutritional purposes. Most heavy metals can at high concentrations be toxic to all forms of life, including humans, animals and microbes [6,14-15]. The exposure of heavy metals is not only limited to single metals, but it is often a combination of metals that are already present within the atmosphere. Wood & Wang [16] found that at high concentrations of heavy metals, the metals exercise inhibitory actions on microorganisms by dislodging vital metal ions or by altering the biological molecules active confirmations [16]. Nonetheless, at low concentrations metals such as Cobalt (Co), Cu, Ni, and Zn are necessary for microorganisms as they make available important cofactors for enzymes. Microbial communities arise due to availability of the heavy metal as well as the type of metal and microbial species [14,17-18].

The widespread use of metals in the environment has increased pollution drastically. Copper and Hg are among some of the metals that are used daily in occupational settings and released into the atmosphere. Numerous studies have been done on the potential risk factors associated with diseases due to consumption of fish contaminated with high concentrations of Hg. An association between increased Hg levels in the body and risk of cardiovascular diseases (CVD) has been established in epidemiological studies [19]. The World Health Organization (WHO) has defined a normal range for the consumption of heavy metals for the general population. This was done to safeguard the population from deficiency and toxicity of metals [20].

Mercury, Mn and Ni are used in the study and where chosen based on the likelihood of humans being exposed to these metals in South Africa. The above-mentioned metals are frequently reviewed by the WHO, an international body that has studied the dangers and effects these metals have on human health. Mercury emissions, due to the generation of power by Eskom, has been estimated to be 77% of the total Hg emitted in South Africa [21]. Limited information exists on the current status of Hg emissions in most African countries, although the emission of Hg continues to rise due the rapid economic growth in Africa. South Africa has the world's biggest Mn ore

deposits located in the North West and Northern Cape Provinces. Exposure levels of Mn differ worldwide, and South Arica has a level 25 times higher than the United States [22]. The level of Ni in drinking water within South Africa is very high compared to Europe. It is seven times higher than European, Dutch and WHO guidelines. This may be due to high levels of mining operations in South Africa [20]. Mining in South Africa dates back decades, especially in areas surrounding Johannesburg, Pretoria and the North-West (Rustenburg). The mining of Cu, Ni, gold (Au), platinum (Pt), and other minerals has in the past contributed to environmental pollution and it still continues to do so. Elevated concentrations of heavy metals in the soil have a negative impact on plants, by means of the uptake of heavy metals by the plants [23]. Kroondal is a town situated in the North-West province of South Africa consisting of established shallow, mechanized platinum group minerals (PGM) mines in the Western Limb of the Bushveld Complex. The area is surrounded by four mines and ore processing plants. These mines are very close to farms, settlements, and irrigation systems. Heavy metals from the mining industries leaches into the plants and has negatively impacted the people in the area who consume the food from the farm [24]. The transfer of metals through natural food chains extending to humans and animals can threaten their health [25].

2.1 Mercury

Mercury is a unique metal that has no biological importance. It is a heavy metal that is widely dispersed in the earth's surface [26]. Mercury has an atomic mass of 200.6 u, a melting point of -38.9°C and a boiling point of 356.6°C, making it a very volatile element [1]. It is liquid at room temperature and is 13.6 times heavier than water. Mercury is considered a major environmental toxicant throughout the world, although its unique physical properties have been used for Hg switches, thermostats, thermometers, fluorescent light bulbs and commercially in batteries [27-28]. Exposure to Hg in the general population is via three main sources: fish, dental amalgam and vaccines, although the uses of dental amalgam and Hg vaccines were discontinued some time ago. Human exposure to Hg usually results due to increased consumption of fish with a high Hg content. Dental amalgam is a dental filling material used to fill cavities caused by tooth decay. The use of liquid Hg mixture in dental amalgam was a major contributor to the exposure of Hg in the past. Each tooth filling used contained 750-1000 mg of Hg [29]. Thimerosal is a mercury-based preservative that has been used in the United States to prevent the growth of germs like fungi and bacteria in infants. It contains the ethyl mercury radical that exhibits similar toxicological effects as methylmercury (CH_3Hg^+) [30]. Each of the Hg sources has its characteristic form of Hg and the toxicology and clinical symptoms differ. Mercury occurs in numerous biochemical forms,

with intricate pharmacokinetics. Toxic properties of methylmercury have been manipulated for the use in antiseptic and diuretics [31]. A wide range of clinical manifestations such as pneumonitis, weakness, fatigue, anorexia, weight loss and gastrointestinal distress can be linked to Hg exposure [32].

2.1.1 States of mercury

Mercury is found in various forms namely elemental mercury (Hg°), inorganic mercuric salts (Hg^{+}) and mercury ions (Hg^{2+}). Each form possesses different properties and toxicity. Elemental mercury is very stable in the environment, volatile and water soluble [1]. Once Hg is in the aquatic environment it can be altered by bacteria into CH_3Hg^{+}. The methylation occurs in the upper muddy layers of the sea or lakes. Methylmercury is found in large concentrations in fish due to bioaccumulation and is the most toxic form of Hg [33].

2.1.2 Exposure to mercury

Mercury occurs naturally in the earth's surface, sea water, freshwater as well as the air. Mercury is released into the environment by human and volcanic activity and the weathering of rocks [31]. Further sources include the combustion of fossil fuels, the use of coal stoves for heating and cooking, waste burners and the mining of gold and other metals are the main causes of Hg in the ecosystem [31]. Humans are exposed to various forms and concentrations of Hg. The general population is exposed to Hg through inhaling Hg° released from dental amalgam fillings [32]. A number of countries have banned or limited the use of dental amalgams but some regions of the world including the United States are still making use of it. Upon inhalation of CH_3Hg^{+}, it accumulates in the erythrocytes and undergoes oxidation to Hg^{2+} by an enzyme called catalase [34]. The utilization of skin lightning cosmetic creams has increased in most African countries. In Nigeria, 77% of women use skin lightning products to enhance their beauty on a regular basis [35]. Mali, Senegal, South Africa and Togo follow with 59%, 35%, 27% and 25% of women using skin lightening products, respectively. These products contain excessive amounts of Hg which can result in adverse effects. Various regulatory bodies such as the European Union and African nations have banned the use of skin products containing Hg [35]. The increased consumption of fish contaminated with Hg is the most common route of exposure to Hg [32]. Orally absorbed CH_3Hg^{+} is distributed into erythrocytes and turned into Hg^{2+} through demethylation in the liver and the spleen. A study by McKelvey, 2007 [36] on adults who consumed a high fish diet demonstrated 3.7 times the Hg levels in their blood when compared to people who do not consume fish at all. This places this population at greater risk of cardiovascular diseases amongst others. The consumption of fish by humans is an essential part of our diet. Fish is a good source of omega-3

polyunsaturated fatty acids and a rich nutrient provider of vitamin D [37]. Although fish also provides ω-3 (n-3) fatty acids that assist in decreasing the risk of heart diseases, stroke, premature delivery and reduces the level of cholesterol,[38] the Hg content can have adverse effects if fish is consumed frequently [39]. This is especially true in areas where fish is a staple diet. A recent study indicated that fish sold in South Africa's retail stores contains levels of Hg that are above the recommended WHO guidelines [40]. The Council of Scientific and Industrial Research (CSIR) found that swordfish contains the highest level of Hg and the yellowtail contains a minimum amount of Hg (Figure 1) [40].

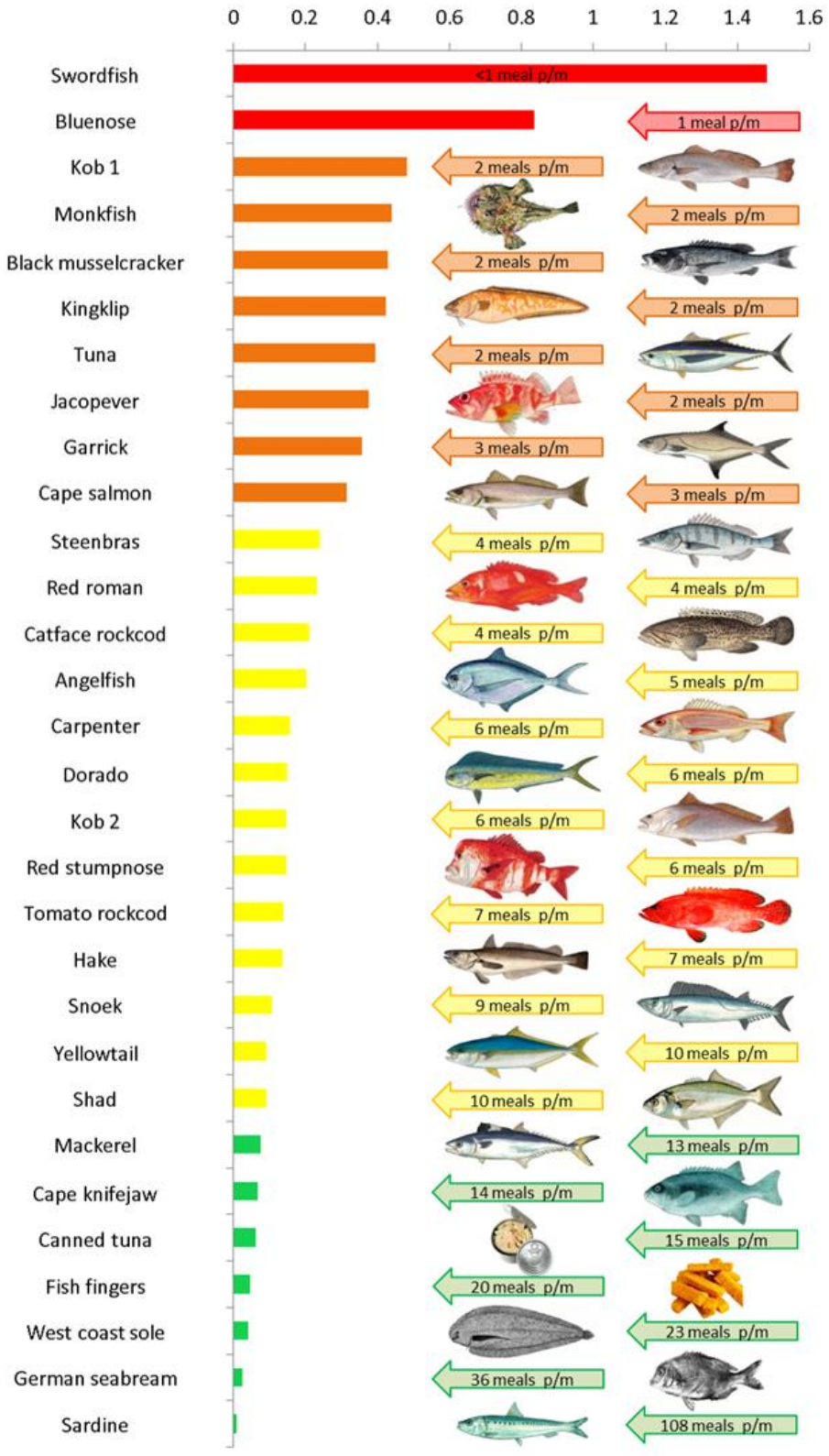


Figure 2.1: Average Hg concentration [mg/kg, wet weight per month (p/m)] Image adapted from [40]

South Africa has been reported to have the second highest emission of Hg in the world, it contributes 10% of the global Hg emissions. The emission of Hg is mainly due to coal combustion and gold mines in and around certain communities [41]. South Africa has also been identified to be amongst the countries with the highest elemental Hg within the environment [42]. Small-sale gold mining in most African countries such as Mali, Zimbabwe and Tanzania is a source of income, however can be dangerous due to exposure to Hg. Tanzanian children are often recruited as mine workers within the formal and informal mining sector [43]. The miners make use of their bare hands to mix the Hg into the ore. Gold-mercury amalgam is created and burned over an open flame resulting in the inhalation of Hg fumes [44].

2.1.3 Health effects of high levels of mercury

Increased exposure to Hg has adverse effects on human health. Various diseases involving the cardiovascular-, nervous- and immune systems and those causing renal failure result from the consumption of Hg. Inorganic Hg salts are harsh to the skin, eyes and gastrointestinal tracts (GIT) and may induce kidney toxicity if swallowed [1]. Populations with greater risk of adverse effects to Hg exposure include pregnant women, as exposure to CH_3Hg^+ can result in adverse effects of the foetus' nervous system and brain as well as individuals who are exposed regularly to Hg (chronic exposure) [31].

Aspects that determine if adverse health effects will ensue and what their severity will be include: 1) type of Hg consumed, 2) dosage, 3) the age at which a foetus, child or adult is exposed, 4) period of exposure 5) the route of exposure [45-46].

2.1.4 Metabolism of mercury

Absorption

The various routes of absorption of Hg include inhalation, ingestion and skin contact.

- Inhalation: Hg vapour is absorbed in the alveolar air due to rapid diffusion of Hg through the alveolar membrane [47].
- Ingestion: The absorption of liquid Hg^{2+} is very poor through the GIT.
- Skin: The absorption of Hg^{2+} occurs through the epidermis. It is possible that Hg can cross the skin barrier via sebaceous glands, hair follicles and sweat glands [48].

Transportation and distribution

The main organ that typically contains the highest content of Hg^{2+} is the kidney due to absorption via the GIT. Friberg, 1979 [1] conducted a study on mice, where it was shown that after a single

exposure of Hg vapour more Hg is reserved in the brain as opposed to intravenously injecting the same dosage of Hg. It was hypothesized that these findings are as a result of dissolved Hg vapour in the blood being transported to the brain. The distribution of Hg among the plasma proteins varies according to the dosage and time after administration [1]. Metallic Hg is corrosive and entrenched in body tissue, but ingested metallic Hg into the GIT is not soluble. The enzyme catalase is a mammalian intracellular protein that oxidises Hg^{2+} to Hg^{0} with the aid of certain bacteria found within the GIT and the oral cavity [49-50]. Bacterial resistance to toxic metals has shown increased antibiotic resistance [51]. The exposure to Hg^{2+} through dental amalgams is a health risk as dental amalgams contain 50% Hg [52], thus Hg in the oral cavity will get oxidized and cause adverse health effects.

Elimination

Mercury elimination in the body is through various routes of excretion via faeces, urine, salivary, lacrimal and sweat glands [1]. The largest portion of Hg^{2+} and Hg is excreted via the faeces and urine. The rate of excretion is dosage dependent [53].

2.2 Nickel

Nickel (Ni) is a naturally occurring element with a silver-white shiny metal appearance. It is the most common element on earth and occurs in the earth's crust. The majority of Ni is unattainable in the earth's core and the occurrence of Ni in the environment is very low. Stainless steel products have become common household items. Stainless steel often contains about 8-10 % nickel. Applications of Ni are Ni plating, colouring of ceramics, manufacturing of batteries and the conduction of heat and electricity as well as in the preparation of alloys. Nickel alloys are characterized by their ability to deform under tensile stress and force as well as its resistance to weathering and high temperatures. Nickel alloys are widely used for various applications, majority of the alloys involve corrosion resistance as well as heat resistance. Some of these alloys include; aircraft gas turbines, steam turbines, power plants and medical applications [54-55]. Nickel is used as a catalyst to enhance the rate of a chemical reaction [56-58].

2.2.1 States of Nickel

Nickel occurs in five natural isotopic forms Ni-58, Ni-60, Ni-61, Ni-62 [1], and Ni-64 with Ni-58 making up 67.8% of the natural abundance. Water has no effect on metallic Ni although diluted hydrochloric and sulfuric acid dissolves Ni at moderate speed compared to nitric acid that dissolves Ni at rapid speeds. Nitrate, chloride sulfate and acetate are Ni salts that are soluble in

water, however disulfides, subsulfides, sulfides, and oxides are virtually insoluble in water [59]. The versatility of Ni allows it to mix with a variety of metals.

2.2.2 Exposure to nickel

The concentrations of Ni in isolated regions range between 1 to 3 ng/m^3, while concentrations in urban and rural areas range from 5 to 35 ng/m^3. The inhalation of Ni in non-occupational settings is estimated to be between 0.1 to 0.4 µg/day [60]. Nickel is also found in groundwater and its concentrations are dependent on the pH, soil use and the deepness of sampling. Acid rain escalates the movement of Ni within the soil and therefore intensifies the concentrations of Ni in the ground [61]. In a study conducted by Schwenk [62], it was suspected that wells made of stainless-steel material in Arizona, United States were a source of increased Ni concentrations in groundwater. The discharge of Ni from Cr-Ni steel pipe into drinking water was reduced indicated by very low levels of Cr in the water weeks later. The seepage of Ni is not a result of the deterioration of pipes but rather inert discharge of Ni ions from pipes underground [62].

Biologically, food contains minor quantities of Ni. The level of Ni found in food ranges between 0.01 and 0.1 mg/kg [63-65]. Whole-wheat products, beans, seeds, nuts, wheat bran and cacao are among some of the food products that contain relatively high levels of Ni. Using stainless steel cooking utensils such as oven- and roasting pans increase levels of Ni found in cooked food [65]. A study conducted by Flint & Packirisamy [66], concluded that minor increases of Ni concentrations were detected in new stainless steel pans, if first used with acidic fruits. Nickel intake increases when people consume large quantities of vegetables from soils polluted with Ni. Smoking causes a high intake of Ni through the lungs. About 0.04 to 0.58 µg of Ni is released with a single cigarette [67]. Typically 2 to 23 µg of Ni would be inhaled by an individual who smokes 40 cigarettes a day [68]. The use of prosthetics or implants that are made from alloys containing Ni may cause patients to be exposed to Ni. Exposure of Ni to an unborn baby may occur through the transmission of Ni via the mother's blood to the foetus. Elevated use of Ni products consequently results in occupational and environmental pollution. In occupational settings, the exposure of Ni occurs due to Ni refining and welding.

2.2.3 Health effects of high levels of nickel

The exposure of children and adults to Ni has different side effects dependent on their sensitivity to Ni. The consumption of Ni in food, water and the environment results in adverse effects. An allergic reaction is a common health effect after exposure to Ni. Women are more sensitive to Ni with a population frequency of 10 to 20% compared to men which is only 2%. Direct contact with

jewellery containing Ni can cause sensitivity of the skin. The most life-threatening health effects caused by Ni include nasal and lung cancer with exposure of 10 mg of Ni [69], bronchitis, lung function problems, sinusitis, reduced body weight, haemoglobin and plasma phosphate. People working at Ni refineries are exposed to high concentrations of Ni as compared to the levels within the environment and are prone to these adverse effects [69] due to the inhalation of dust particles that contain Ni. High levels of soluble Ni compounds via oral exposure through the environment are very rare, although some foods may contain Ni at very low levels. An animal study in which rats were exposed to 500 to 1000 mg/kg of Ni in the diet for six weeks found that the exposure to Ni significantly reduced haemoglobin content, plasma protein concentration and body weight [70]. The levels used in the animal study were 1000 times more than the usual levels found in drinking water [70]. The consumption of Ni affected the GIT, growth and immune systems. Although the growth of rats decreased this was most likely not because of Ni intake but rather because of decreased food consumption. Nickel salts affect the immune system by suppressing the activity of natural killer cells and T-cell system in rats and mice [71]. An increase in new-born deaths as well as a decrease in new-born weight was documented in an animal study after subsequent exposure to Ni [71].

2.2.4 Metabolism of nickel

Absorption

Nickel can enter the body through inhalation as well as eating food or drinking water that contains Ni, which is then absorbed by the GIT. The respiratory tract is the major target organ for Ni absorption and toxicity. Fifty percent of a single Ni carbonyl dose that is inhaled is absorbed [67]. The amount of Ni that reaches the lungs is dependent on the size of the Ni particles inhaled. The larger the particles of Ni within the air the less likely it is to reach the lungs and consequently the alveoli. The absorption of Ni from the lungs to the body occurs when Ni easily dissolves in water. Human absorption of Ni in drinking water is 15-50% after an overnight fast compared to that in food at 15% [72]. Skin contact with Ni can cause an allergic reaction.

Transportation and distribution

Albumin is the main protein carrier of Ni in serum, but Ni also binds to α-2 macroglobulin and histadine [73]. A higher amount of Ni contained in drinking water will be transported to the stomach and intestines than if food is consumed containing the same amount of Ni. Nickel is mainly distributed in the GIT and kidneys [74].

Elimination

Nickel that is not absorbed in the GIT is eliminated through faeces, thus reflecting the daily dietary intake. Absorbed Ni is also eliminated in the urine. The excretion of Ni via sweat, saliva and removal in hair has been reported. The most common elimination route of Ni is through urine and the half-life depends on the type of Ni species tested. Soluble Ni compounds have a half-life of 11 to 39 hours in humans [75]. Certain Ni compounds are toxic and carcinogenic; this may be associated with the uptake, transport, distribution, and retention of Ni at a cellular level [76].

2.3 Manganese

Manganese is a common element in the earth's crust and is widely distributed throughout the planet's surface. The classification of Mn as a nutritionally essential metal is because it is an activator and cofactor for enzymes involved in phosphorylation, decarboxylation, hydrolysis and transamination [77]. Manganese plays an important role in the functioning of both humans and animals, as a requirement for cellular enzymes [such as Mn superoxide dismutase (SOD), pyruvate carboxylase] and serves to activate kinases, decarboxylases and transferases. Many alloys containing Mn are used in the production of steel, manufacturing of glass and in the production of making soda cans leaner and stronger [78-79]. Plant growth is largely dependent on Mn as it is involved in the absorption of nitrates in algae and green plants [80].

2.3.1 States of manganese

Manganese is not found as a pure element but is a component of over 100 minerals [79]. The most important Mn compounds contain Mn^{2+}, Mn^{4+} or Mn^{7+}. The chemical and physical properties of the various Mn compounds differ substantially. Environmental behaviour and fate, exposure and potential toxicity of each compound is characterised by the physical and chemical properties of each compound.

2.3.2 Exposure to manganese

Air erosion of soils or dust is the most common source of Mn in the atmosphere. The release of Mn in water streams mainly occurs as a result of rocks and soil corroding, extraction of valuable minerals, waste produced by industrial processes as well as the leakage of Mn by human activity, such as dry-cell batteries discarded in soils and landfills. The general population is exposed to Mn through inhalation, drinking water, skin contact, soil, and food containing Mn. The primary source of exposure of humans to Mn is the ingestion of Mn-containing food or nutritional supplements [79]. People living near or employees of mines and industrial areas using Mn as well as automobile exhausts are at greater risks of being exposed due to high levels of Mn dust.

Tobacco smokers and second hand smokers are exposed to Mn at levels greater than non-smokers [81]. Manganese is present in water that collects on the surface of the ground and soil. A minor risk exists of exposure to Mn through ingestion of seafood originating from waters containing Mn, especially if the levels of Mn in the seafood are tremendously high or seafood is eaten raw. The second most common exposure of Mn is through the ingestion of food within the general population [79-80]. Vegetables, nuts, grains and animal products contain Mn [82]. Table 2.1 below indicates the ranges of Mn concentrations in food.

Table 2.1: Average concentration (mg/kg) of Mn in food sources in America[80].

Types of food	**Ranges of mean concentrations (mg/kg)**
Nuts and nut products	18.21-46.83
Grains and grain products	0.42-40.70
Legumes	2.24-6.73
Fruits	0.20-10.38
Fruit juices and drinks	0.05-11.47
Vegetables and vegetable products	0.42-6.64
Desserts	0.04-7398
Infants foods	0.17-4.83
Meat, poultry, fish and eggs	0.10-3.99
Mixed dishes	0.69-2.98
Fats and sweeteners	0.04-1.45
Beverages (Including tea)	0.00-2.09
Soups	0.19-0.65
Milk and milk products	0.02-0.49

The Food and Nutrition Board of the Institute of Medicine recommends Mn ingestion of 2.3 mg/day for men and 1.8 mg/day for women. Table 2.2 shows the recommended concentration of Mn for different age groups. The WHO confirmed the findings of the regulation that 2 to 3 mg of Mn per day is acceptable for adults and 8 to 9 mg/day is a safe dosage [83]. The threshold limit value exposure levels of Mn in South Africa are 5.0mg/m^3, which is 25 times higher than the United States and significantly higher than the safe dosage indicated by the WHO, making it a very toxic metal for humans [84].

Table 2.2: Satisfactory consumption levels of Mn for different age groups in America [83].

Age groups	Range of mean concentrations (mg/day)
Birth to 6 months	0.003
7 months to 1-year	0.6
1-3years	1.2
4-13 years	1.5-1.9
Adolescents and adults	1.6-2.3

2.3.3 Health effects of manganese

A deficiency of Mn is quite rare as most foods contain Mn. Animal studies have been used to investigate the effects of Mn deficiency. Results have indicated skeletal abnormalities, impaired growth, reproductive deficits and defects in the metabolism of lipids and carbohydrates [85-86]. Defects of the nervous system have been studied extensively in humans exposed to high levels of Mn [79,87]. Manganism is a syndrome caused by excessive exposure to Mn dusts or fumes and has similar symptoms to Parkinson's disease. Motor functions are affected after continuous exposure to Mn at levels above the recommended levels. An inflammatory response in the lungs is caused by inhaling excessive Mn dust concentrations [specifically manganese dioxide (MnO_2) and manganese tetroxide (Mn_3O_4] and results in impaired lung function. Studies have shown that chronic ingestion of 1 to 2 mg of Mn in rabbits resulted in changes in the synthesis of haemoglobin as well as a decreased appetite for food. A study on mice and rats signified that the consumption of Mn could possibly interrupt reproductive maturation in male animals [79].

2.3.4 Metabolism of manganese

Absorption

The human body contains about 10 mg of Mn, mostly found in the liver, bones, and kidneys. The daily intake of Mn is 2 to 3 mg. Inhaled Mn is frequently transported straight to the brain before the liver metabolizes it. A number of studies corroborate that Mn is promptly taken up within the nasal cavity in presynaptic nerve endings of axonal projections leading from the olfactory and trigeminal nerves [88-90]. Ingested Mn from food and water enters the body via the digestive tract and is absorbed in the GIT. There are various studies done on the consumption of large quantities of drinking water containing high levels of Mn that may be associated with Manganism. A study conducted on adults in Greece found a high occurrence of neurological symptoms after consumption of water containing Mn at levels of 1.8 to 2.3 mg/l, while a study done in Germany

[91] found no evidence of neurological symptoms in people drinking water with Mn at 0.3 to 2.2 mg/l [92]. A contradiction is seen with the two studies although the Mn levels are similar.

Distribution and transportation

Manganese is distributed throughout the body in bones, the liver, kidneys, and pancreas and mostly accumulates in tissue rich in mitochondria. Manganese is transported by transferrin, which is a glycoprotein that also transports aluminium and is taken up by the extrahepatic tissue [93]. Iron and Mn interact during its transfer from the plasma to the brain, liver and kidneys in a synergistic manner [94].

Elimination

Manganese is eliminated from the body through bile. The ability of the body to excrete Mn is impaired when cirrhosis and portosystemic shunts makes it difficult for the liver to function to its maximum capacity, causing Mn accumulation in the blood and, eventually the brain [95].

The effects of these metals on the blood and specifically erythrocytes and platelets are very limited and was therefore chosen as the aim of this study.

2.4 Erythrocytes, platelets and fibrin network

Environmental studies based on heavy metals typically focus on the level of metals in the blood but less information exists on how the metals influence or cause change to clotting parameters, endothelial integrity and the structure of erythrocytes. Erythrocytes are essential health indicators as they play a part in tracking diseases and treatment [11]. Erythrocytes are highly susceptible to changes in the environment and this is evident in alterations seen in erythrocyte membranes. Erythrocytes that are in circulation are exposed to endogenous and exogenous sources of reactive oxidative species (ROS), which can easily cause injury to the blood vessels and reduce its ability to function properly [96]. Erythrocytes play a vital role in the transportation of oxygen molecules in the body. Morphological changes in the cells can weaken its ability to carry oxygen throughout the body. The large membrane variations and general shape of erythrocytes in normal physiology, are maintained by adenosine triphosphate (ATP) [97].

Erythrocytes are derived from a single cell type known as the pluripotent hematopoietic stem cell. The development of erythrocytes begins in the embryo and continues throughout an individual's life. Soon after erythrocytes enter the blood stream, they lose their nuclei [11]. Erythrocytes are the most abundant of the different cell types in the blood. A microliter (µL) of blood contains approximately 5 million erythrocytes in comparison to white blood cells and platelets, which range

between 4000 to 11000 and 150000 to 450000 platelets respectively[11]. The cytoskeleton of erythrocytes plays a vital role in the function and the maintenance of the surface area to volume ratio of the biconcave cell. As the erythrocytes travel through vasculature the cells undergo reversible deformation to enable it to pass through very small capillaries. The plasma membrane of erythrocytes is supported mechanically by spectrin tetramers connected by actin junctional complexes, forming a 2D six-fold triangular network anchored to the lipid bilayer [98]. Any disruption of the connections between the spectrin tetramers and the actin junctional complexes may result in shape changes of the erythrocytes (spherocytes, stomatocytes and echinocytes) such as those described in Table 2.3.

Table 2.3: Types of erythrocytes and their description [99].

Types of erythrocytes	**Morphological description**
Normal erythrocytes	Mature erythrocyte is small, round, and biconcave
Stomatocytes	Erythrocytes with a central linear slit or stoma. Seen as mouth shaped form in peripheral smear
Spherocytes	Erythrocytes with an absent biconcave shape and develops a spherical shape
Echinocytes	Erythrocytes with pointed projections

Platelets are produced by megakaryocytes in the bone marrow [99]. Platelets are small cell fragments that circulate within the blood and play an important role in the vascular integrity as well as regulation of haemostasis. Platelets are also intricately involved in the chronic inflammation often associated with disease pathologies [11]. The diameter of a mature platelet is 2 to 3 µm, with a life span of approximately 10 days. Two-thirds of the platelets circulate in the blood and a third is stored in the spleen. Platelets circulate in the blood stream but are only functional once there is damage to the walls of blood vessels [100]. Platelet activation results in platelet recruitment, adhesion t and the formation of a platelet plug through the release and functioning of signalling molecules.

The discoid structure of platelets as well as the protection of the cell from getting sheared within the bloodstream is maintained by the highly specialized cytoskeleton of platelets. The cytoskeleton consists of three major components: (1) the spectrin-based membrane skeleton, (2) the actin cytoskeleton, and (3) the marginal microtubule coil [100]. Platelets lack a nucleus but have a distinct mitochondria [101]. The plasma membrane of platelets is composed of a phospholipid

bilayer and is the site of various surface receptors and lipid rafts which assist in intracellular trafficking and signalling. Platelet markers include CD36, CD63, CD9, GPCR, IIbIIIa, and GLUT-3 [100]. These surface receptors trigger the release of alpha (α) granules which play a major role in coagulation, inflammation, wound healing and other platelet related diseases [102]. Of all these surface receptors the GPCR is reportedly the most crucial receptor as it enables the secretion of adenosine diphosphate (ADP) from dense granules. The secretion of ADP is crucial as ADP interacts with ADP receptors found on platelets (P2Y1, P2Y12 and P2X1) thus leading to further platelet activation. The phospholipids (e.g., phosphatidylserine and phosphatidylinositol) in platelets are arranged asymmetrically and are present in the inner layer of the plasma membrane, to enable the stability of the platelet when it is not activated. Upon platelet activation (Figure 2.2), the surface of the platelets exposes amino phospholipids by ATP-dependent flippases and scramblases to initiate the coagulation cascade [103]. The open canalicular system is an internal membrane structure found in platelets and remains open during the platelet release reaction and is connected to the plasma membrane [104]. The function of the open canalicular system serves to transport substances into the cell and channels the discharge of alpha granules products secreted during platelet release reaction.

Haemostasis is a process whereby blood is prevented from escaping from a damaged blood vessel through the functioning of platelets, erythrocytes and fibrinogen. Three mechanisms involved in haemostasis are summarized in Figure 2.2.

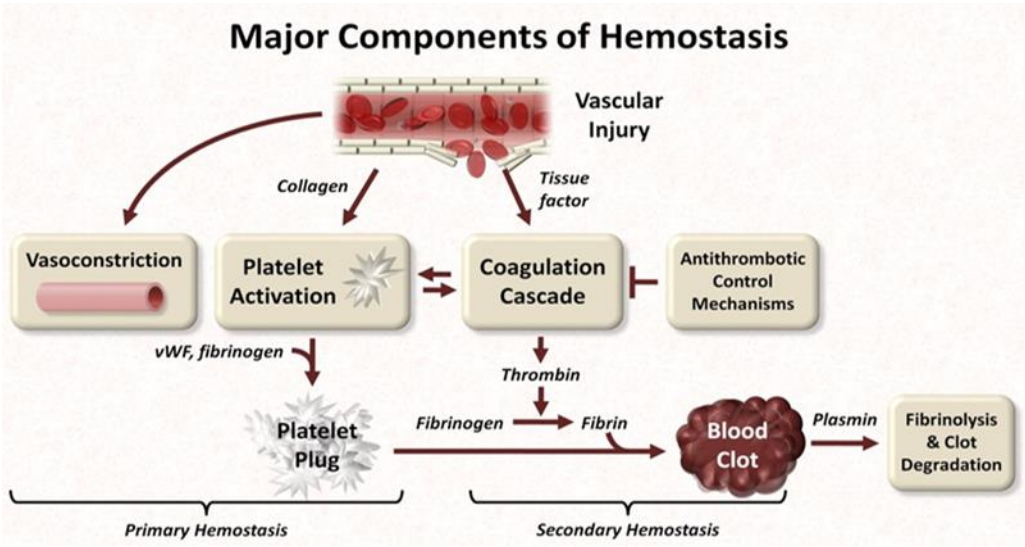


Figure 2.2: Mechanisms of haemostasis after damage to a blood vessel [105].

After injury to a blood vessel, vasoconstriction immediately takes place to prevent further loss of blood. Platelets adhere to the exposed subendothelial matrix and collagen, activating the platelet, forming a haemostatic plug to close the leak within the vessel. Platelets become activated and change their shape from being generally discoid and flattened in shape with no pseudopods to being activated with pseudopods. Cytokines are released the moment platelets adhere, recruiting more platelets which then initiate platelet plug formation [12]. The binding of a platelets causes the release of cytoplasmic granules such as serotonin, ADP and platelet activating factor (PAF) [11]. The PAF initiates the pathway that translocates platelet membrane phospholipids to release thromboxane A2. Adenosine triphosphate attracts more platelets to the site of injury, serotonin and thromboxane A2 assists in platelet aggregation, vasoconstriction and degranulation. As more cytoplasmic granulations are secreted, more and more platelets adhere together and create a platelet plug and the process continues in a positive feedback loop [10]. Coagulation is completed when the platelet plug is converted into a stable clot and plasma fibrinogen is converted into a solidified mass of fibrin. The stable fibrin clot prevents blood loss and allows healing to take place. Coagulation is divided into two pathways that will eventually merge into one (Figure 2.3). The intrinsic pathway begins when there is damage to a blood vessel. This pathway uses proteins already present in the plasma. Collagen activates factor XII to factor XIIa to begin the cascade. Factor XIIa will then enable factor XI to be converted into XIa, and then XIa will allow for the process of factor IX to be converted to factor IXa. Factor IXa enables factor X to be converted to factor Xa with the assistance from factor VIII, platelet membrane phospholipid and calcium iron. The extrinsic pathway begins when there is damage to the tissue outside the vessel also known as tissue thromboplastin. Tissue factors activate factor VII to begin the extrinsic pathway. The two pathways then combine to form a common pathway which begins with factor Xa. Factor Xa together with factor V, platelet membrane phospholipid and calcium ions enable the conversion of prothrombin into thrombin. Thrombin will then enable the conversion of factor XIII to factor XIIIa and the conversion of fibrinogen to fibrin. Fibrin production is a result of a pro-coagulation process that generates thrombin at a site of injury [106]. Finally, the fibrin fibres weave through the platelet plug and traps erythrocytes within the mesh. As the damaged blood vessel wall repairs itself, the clot then disintegrates when fibrin is broken into fragments by the enzyme plasmin in a process known as fibrinolysis [11].

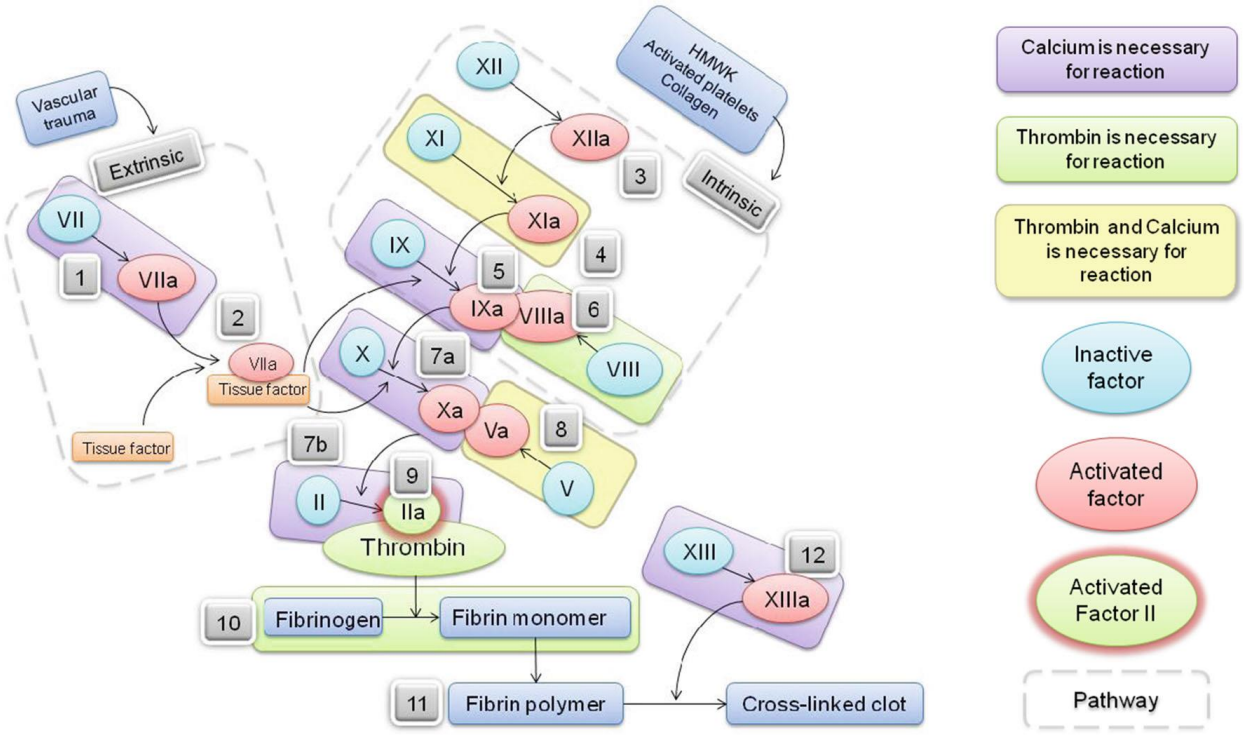


Figure 2.3: The coagulation cascade [107]

The formation of a fibrin network is important for haemostasis and current literature indicates that abnormal fibrin structure is related to thrombotic diseases, including myocardial infarctions and venous thromboembolism [108].

2.5 Eryptosis

Erythrocytes reach the end of their lifespan after a maximum of 120 days in circulation. Mature circulating erythrocytes may undergo senescence and get removed from the circulatory system by means of erythrophagocytosis or eryptosis [109]. Eryptosis is characterized by shrinkage, cell blebbing and membrane scrambling indicated by the exposure of PS on the external surface of the cell [110]. Morphological features of damaged erythrocytes are membrane alterations including spike formation and blebbing, size alterations including swelling, changes in shape including distortions, and haemolysis resulting in the loss of cell content [110]. One of the major triggers of eryptosis is the intracellular increase in calcium (Ca^{2+}) activity. Cytosolic increase of Ca^{2+} is due to entry of Ca^{2+} permeable unselective cation channels. The Ca^{2+} channels are activated by prostaglandin E_2 (PGE_2) and furthermore activated by isosmotic replacement of NaCl with sorbitol and by the substitution of extracellular Cl^- with gluconate, Br^-, I^- or SCN^- [110-111]. An increase in Ca^{2+} concentration causes an activation of Ca^{2+} - sensitive K^+ channels resulting in potassium chloride (KCl) and water exiting the cell, causing cell shrinkage [112]. Calcium stimulates cell membrane

scrambling thus leading to the exposure of PS at the cells' surface [110]. Oxidative stress, the presence of xenobiotic and endogenous substances, energy depletion and the ageing of the cells also play a role in eryptosis taking place. Eryptosis increases with an increase in age of erythrocytes, and this is due to increased sensitivity to oxidative stress [113]. In a study conducted on the impact of erythrocyte age on eryptosis, the study concluded that susceptibility to oxidation-induced PS exposure is increased in older erythrocytes as compared to younger cells [114]. Eryptosis can also be caused by various clinical conditions such as endogenous mediators and xenobiotics. Well known xenobiotics that are capable of inducing eryptosis includes, but are not limited to aluminium [115], lead [116] and cadmium [117]. Erythrocytes with exposed PS are quickly cleared from circulatory blood as PS binds to phagocytosing receptor cells thus leading to engulfment and degradation of the affected cells [114]. The loss of eryptotic erythrocytes should be directly proportional to erythropoiesis to prevent a decrease in the number of erythrocytes, if not this may lead to the development of anaemia [118].

2.6 Aims

The aim of the study was to investigate the effects of the heavy metals Hg, Ni and Mn alone and in combination on the integrity of the erythrocyte membrane as well as on the morphology of the components of the coagulation system at concentrations of 1x, 10x, 100x, 1000x and 10000x the WHO safety level standards for each respective metal.

2.7 Objectives

The objectives of this study were to:

- Investigate the effects of the metals Hg, Ni and Mn alone and in combination, on the integrity of the erythrocyte cell membrane by using the haemolysis assay.
- Determine morphologically if these heavy metals induce platelet activation and alter fibrin network formation and erythrocytes by using scanning electron microscopy.
- Investigate whether these metals alone and in combination induce eryptosis by using confocal laser scanning microscopy.

The results obtained from the experimental analysis of the above-mentioned methods are presented in the chapters to follow.

3. MATERIALS AND METHODS

3.1 Patient information

Ten healthy, male, human volunteers between the ages of 20 and 30, who were non-smokers and not taking any chronic medication and who did not have any inflammatory conditions were permitted to take part in the study. Any person on anticoagulants or any chronic medication, were excluded from the study. The donors were University of Pretoria staff members from the Basic Medical Sciences building. Donors were approached by means of email, telephone or a verbal request. Written informed consent was obtained from each person volunteering to participate. Men were chosen to avoid the implications that the female hormone estrogen has on the coagulation system [119].

3.2 Metal preparation

Mercury chloride powder ($HgCl_2$), hexamethyldisilazane (HMDS), isotonic phosphate buffer saline (isoPBS), manganese chloride ($MnCl_2$), nickel chloride ($NiCl_2$), human thrombin and all other reagents were obtained from Sigma-Aldrich (South Africa) unless otherwise specified. Stock solution concentrations were equal to X10 000 the WHO values for each metal respectively. Working solutions were made from the stock solutions. The concentration ranges of the various metals included: 1x, 10x, 100x, 1000x and 10000x the WHO safety level standards for each respective metal. The WHO safety level standards for these metals are: mercury = 6 µg/L; nickel = 20 µg/L; manganese = 400 µg/L. The final volume for all single, double and triple combinations was the same.

3.3 Blood collection

Blood was drawn from participants by a trained phlebotomist or medical doctor using a sterile needle inserted into a vacuum extraction blood tube containing 3.2% sodium citrate. A volume of 5 mL venous blood was collected. Each tube was labelled with the sample number and the date on which the blood was collected so as to maintain the donor's anonymity.

The objectives were achieved by using the experimental design presented in Figure 3.1.

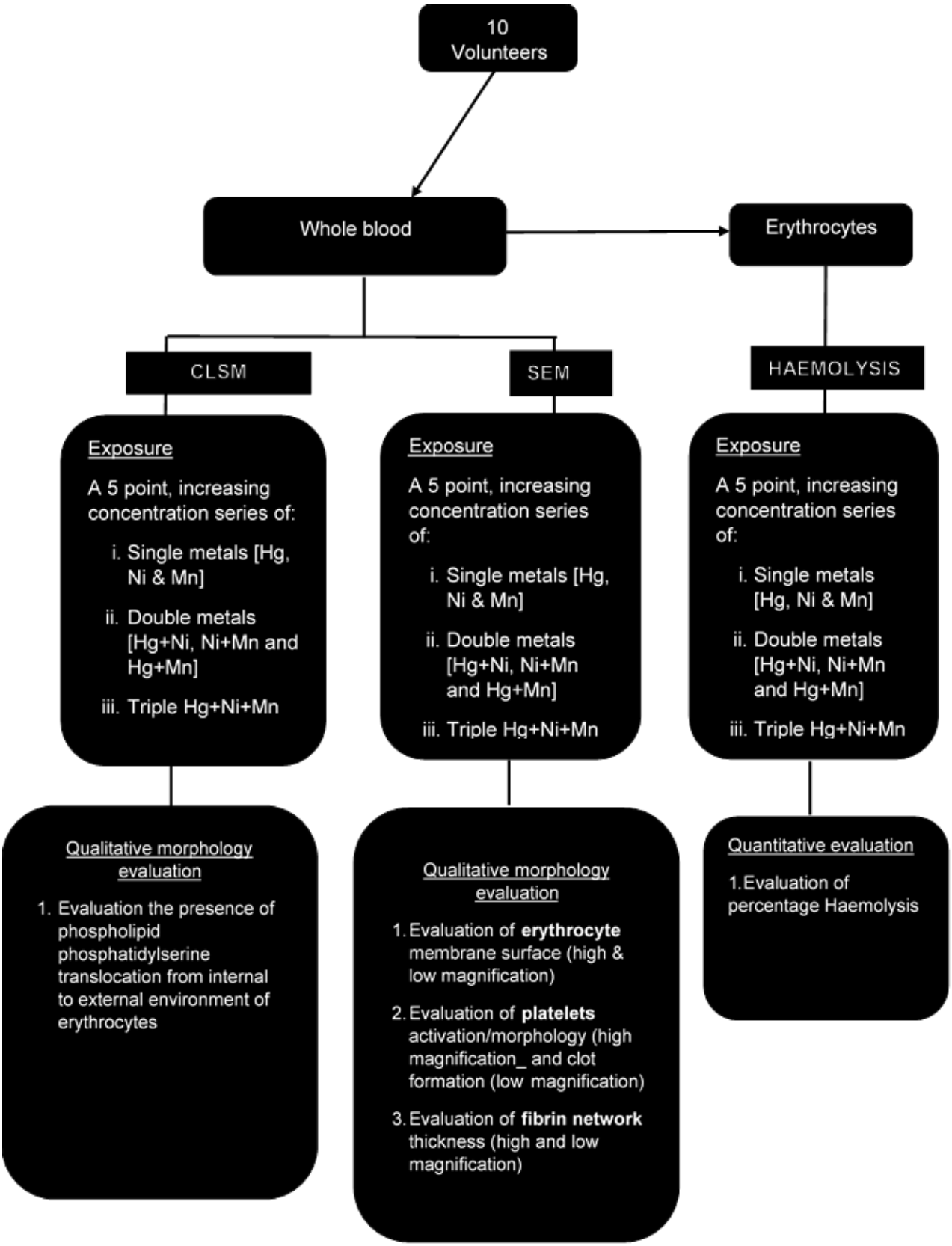


Figure 3.1: Flow diagram explaining the methodologies used for the different blood constituents being investigated.

3.4 Haemolysis assay

3.4.1 Principle of the assay

The absence of most cellular organelles and the presence of a lipid bilayer make erythrocytes ideal to study membrane alterations. Erythrocytes have a flattened discoid shape with a large surface area aiding gaseous exchange. The cells carry oxygen efficiently with the aid of a highly specialized protein called haemoglobin, which also gives the cells its red colour. Haemoglobin (Hb) is a molecule consisting of sub-units referred to as alpha and beta. The sub-units are essential in the binding of oxygen in the lungs and delivering the oxygen to tissues in various parts of the body. Damage to erythrocytes is evident by membrane alteration. Haemolysis is the rupturing of erythrocyte cell membrane, resulting in the release of free Hb into the plasma or extracellular matrix [120-121]. Haemolysis ultimately results in haemolytic anaemia because of a reduction in the number of erythrocytes. Haemolytic anaemia exists in two types: (1) intrinsic haemolytic anaemia which is an inherited disorder characterized by defective erythrocytes produced by the body, and (2) extrinsic haemolytic anaemia where the cells are destroyed by the spleen, toxins or infectious agents [122]. Various factors can account for haemolysis such as: shear stress, oxidative stress, drugs, membrane defects, temperature, bacterial contamination, and osmotic and pH changes [123].

The percentage of haemolysis is determined by quantifying the amount of Hb leaking out from the cell and into the surrounding medium. An increase in cell damage is directly proportional to the amount of Hb leaking out. Hemoglobin in the sample increases the spectrophotometric absorbance at a wavelength that is within the absorbance range of Hb. One of the main advantages of using the haemolysis assay is the biological significance of erythrocytes. The absence of cellular organelles makes it an exceptional model as it is not affected by mitochondrial production of reactive oxidative species and the generation of cellular energy through glycolysis pathway [124]. The cells offer enhanced signals of shielding capabilities of compounds being tested, as compared to other types of cells used in the screening of pharmaceutical compounds. The assay is fast, simple and reliable.

3.4.2. Sample preparation

Whole blood was centrifuged at 3000 xg for 10 minutes, after which the plasma and buffy coat was removed. The erythrocytes were washed with isoPBS (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) twice, by re-suspending the erythrocytes in isoPBS and

centrifuging between washes, at 1400 xg for 3 minutes. A 5% (v/v) blood suspension was made by diluting the packed erythrocytes in isoPBS. A positive control, 2% sodium dodecyl sulphate (SDS) solution was used to induce 100% haemolysis and a negative control, isoPBS, was used to represent 0% haemolysis. The 5% blood suspension was then exposed to the metals, alone and in combination. The concentration ranges of the various metals included: 1x, 10x, 100x, 1000x and 10000x the WHO safety level standards for each respective metal. All the exposed samples were incubated for 16 hours at 37°C. The samples were centrifuged at 1400 xg for 2 minutes after which equal volumes of the supernatant was transferred to a 96-well plate and the absorbance was read at 570 nm. The results were expressed as percentage haemolysis, using the following formula:

$$\%\ Haemolysis = \left(\frac{A_{sample} - A_{0\%}}{A_{100\%} - A_{0\%}}\right) * 100$$

Where $\mathbf{A_{\_sample}}$ is the absorbance of erythrocytes exposed to different solutions, $\mathbf{A_{0\%}}$ is the absorbance of erythrocytes exposed to isoPBS (0% haemolysis), and $\mathbf{A_{100\%}}$ is the absorbance of erythrocytes exposed to 2% SDS (100% haemolysis).

Analysis of combined effects was achieved using the model deviation ratio (MDR) method.

$$\boldsymbol{MDR = \frac{Ov}{Ev}}$$

Where **Ov** is the observed value (average % haemolysis) of the combination group, and **Ev** is the expected value (sum of the average % haemolysis of the single metal groups/number of groups).

Volume differences were considered by dividing the Ev by 2 for double metal combinations and 3 for the triple metal combinations. An additive effect is indicated where 0.5<MDR<2, antagonism where MDR <0.5 and synergism where MDR >2 [125-126].

3.4.3 Statistical analysis

Of primary interest in this study was to compare three heavy metals, alone and in combination, at five different concentrations using the haemolysis assay. A biostatistician was consulted to determine the suitable statistical tests (see Appendix 8.3).

By convention, the sample size when comparing the heavy metal combinations in an analysis of variance (ANOVA), for a two-factor study design (heavy metals combination × concentration), the aim is to have at least 30 degrees of freedom for the error term (residuals). In this study, from three experiments, there was 70 degrees of freedom where we considered treatment at seven levels (heavy metal combinations), concentration at five levels along with an interaction term for treatment by consideration.

Data followed Gaussian distribution and since the experiments were done under well-controlled conditions, both analytical and biological variation was expected to be low. Data summary was within treatment combination (heavy metal combination × concentration) for one assay. Data analysis employed an appropriate ANOVA for this two-factor study design with an interaction term. Stata Release 14 statistical software was employed and post hoc testing used the very flexible margins command in Stata. Testing was done at the 0.05 level of significance and the family-wise type I error was included.

3.5 Scanning electron microscopy

3.5.1 Principle of the assay

The scanning electron microscope (SEM) is an instrument employed to produce high-resolution images of samples. To investigate surface morphological changes seen in erythrocytes, platelets and fibrin networks, SEM was used. The region of interest on the sample is analysed with a focused beam of electrons that is scanned over the surface. This allows a researcher to study the surface of cells with extreme detail [127]

3.5.2 Sample preparation

The effect of the metals, alone and in combination, on the morphological changes to erythrocyte membranes together with platelets and fibrin networks were investigated using SEM. Whole blood of 90µL was exposed to 10µL of each metal, alone and in combination and then incubated for 10 minutes at room temperature. This exposure time was determined to be the optimal period for exposure through a time-based study comparing samples exposed for 10 minutes, 30 minutes and 16 hours. The standard SEM sample preparation procedures were then followed. Blood smears of 10µL were made on round glass cover slips, with and without the addition of human thrombin (20 U/mL). The addition of human thrombin was 5µl per cover slip. The cover slips were then dried for 10 minutes and washed in phosphate buffered saline (PBS) (0.075 M Na_2HPO_4, 0.2 M $NaH_2PO_4.H_2O$, and 0.2 M NaCl), for 20 minutes. The samples were fixed in a 2.5%

glutaraldehyde/formaldehyde (GA/FA) solution in 0.075M PBS for 30 minutes and then washed three times in PBS. The samples then underwent secondary fixation in 1% osmium tetroxide for 30 minutes and were washed again as explained in the previous step. The samples were dehydrated by using serial dehydration with 30%, 50%, 70% and 90% ethanol (EtOH), followed by three changes of absolute EtOH. The 100% EtOH was removed and 100% hexamethyldisilazane (HMDS) was then added for 30 minutes. The HMDS was discarded and 2 drops of HMDS were placed on the cover slips and the samples were air-dried. Once the samples were dry, the cover slips were mounted on aluminium stubs, coated with carbon and viewed with an Ultra Plus FEG SEM (Zeiss, Oberkochen, Germany).

To ensure repeatability and accuracy of morphological analysis, a micrograph was taken on low magnification (2000x) on three cover slips created for each participant. This ensured that an accurate depiction of erythrocytes, platelets, or fibrin network formation was captured. This was then followed by micrographs at higher magnification (10000x, 15000x and 20000x) to study individual erythrocytes or platelets.

Erythrocytes were characterised in three categories:

- Normal morphology (biconcave shape with uniform membrane)
- Slightly affected (loss of biconcave shape with some membrane alterations e.g. bleb formation
- Significantly affected (eryptotic cells)

Platelets were characterised in three categories:

- Initial activation (presence of pseudopodia)
- Moderate activation (presence of pseudopodia with some membrane spreading)
- Complete activation (platelet spreading with no platelet body visible)

3.6 Confocal laser scanning microscopy

3.6.1 Principle of assay

In eryptotic cells, phospholipid phosphatidylserine (PS) is flipped from the inner to the outer leaflet of the plasma membrane [128]. An enzyme called flippase retains PS within the cell. PS is exposed by the action of scramblase on the cell's surface in processes such as apoptosis and platelet activation. PS becomes exposed to the cell's external environment. Annexin V is a 35 to 36 kDa Ca^{2+} dependent phospholipid-binding protein. The protein has a high binding affinity to which PS

tends to bind [129]. The confocal laser scanning microscope (CLSM) was used to detect PS on the membrane surface of erythrocytes, which gave an indication of eryptosis taking pace. The conventional wide-field fluorescence microscope optics are unable to view thick cells due to bright fluorescent signals from objects outside the focal plane thus increasing the background and giving off low contrast images. Confocal microscopy solves this predicament by blocking all the signals coming from neighbouring surroundings either below or above. This is accomplished by lighting the specimen with a laser beam focused entirely on the specimen at one focal plane and by placing a pinhole aperture in the plane of the image in front of the electronic photon detector. The confocal microscope can produce images of high contrast and visually section through fluorescent objects of up to 10 to 50 µm [127].

3.6.2 Sample preparation

Whole blood was collected in citrate tubes. Whole blood of 900µL was exposed to 100µL of *iso*PBS and 100µL of metals dissolved in *iso*PBS. The positive control was Melittin (an apoptosis inducing agent) [130] to which the blood was exposed for four hours. After blood collection, 1 mL was transferred to a micro centrifuge tube and centrifuged at 3000 xg for 10 minutes at room temperature to isolate the erythrocytes. The supernatant was discarded (plasma, leukocytes and platelets) and the remaining erythrocyte pellet was washed twice with a 0.075 M PBS (pH 7.4) for 3 minutes at room temperature, where after it was washed once with Annexin V binding buffer for 3 minutes. A volume of 5 uL of the Annexin V probe was added to the blood and incubated for 90 minutes at room temperature protected from light. After incubation, the samples were washed twice with 0.075 M PBS (pH 7.4) and once with Annexin binding buffer for 3 minutes each, to remove unbound antibodies. A volume of 10µℓ of the prepared sample was mounted on a glass slide and covered with a coverslip. The samples were viewed with the Zeiss LSM 880 confocal laser-scanning microscope with Airyscan (Carl Zeiss Microsocpy, Oberkochen, Germany).

To visualize the erythrocytes, two different lasers were used with different filters and beam splitter overlaying the respective images to show which erythrocytes have a PS flip present on the membrane. To view the auto-fluorescence of all the cells present on the slide, the 405 nm laser was used to excite naturally occurring fluorescence found in erythrocytes, and a red colour was assigned to this fluorescent signal. In an unstained sample, the 405 lasers were used together with the 465 nm-505 nm Band pass (BP) and 525 nm long pass (LP) filters and the 488/405 nm beam splitters. These settings showed that all the RBCs present on the slide had auto-fluorescence, and this auto-fluorescence could therefore be used as a contrasting method against the Annexin-V binding, in the case where PS flip is present. To visualize Annexin-V binding the

488nm laser was used with the 495-550nm BP filters and the 488/405nm beam splitters, and showed a green fluorescence, indicating the presence of PS on the erythrocyte's membranes.

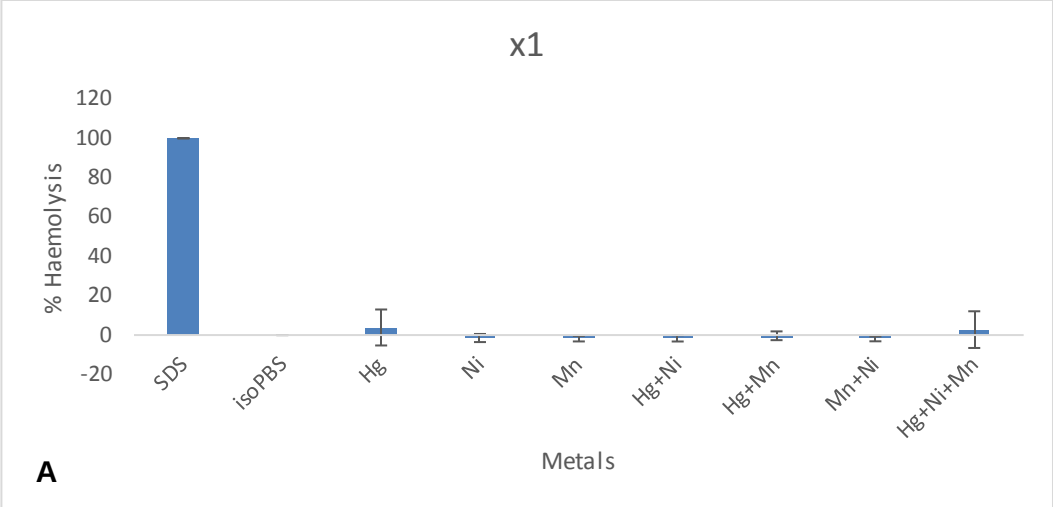
3.7 Ethical consideration

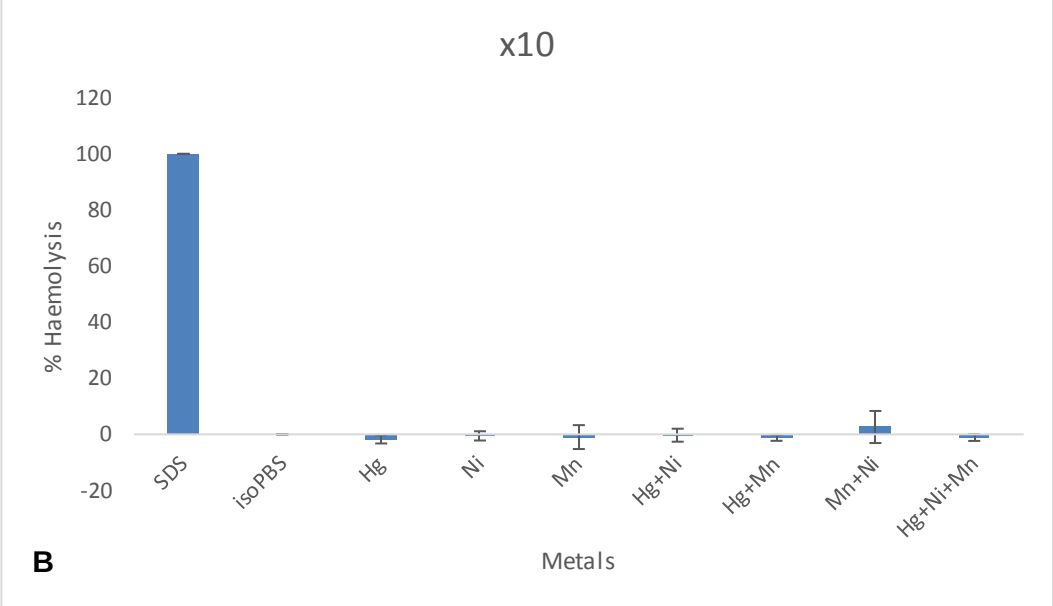
Ethical approval for this study was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Ethical clearance number 12/2018). Each volunteer who took part in this study was provided with a written participant informed consent (see appendix 8.1). The blood tubes were collected from each volunteer and labelled with a number and not with the volunteers' name to ensure anonymity.

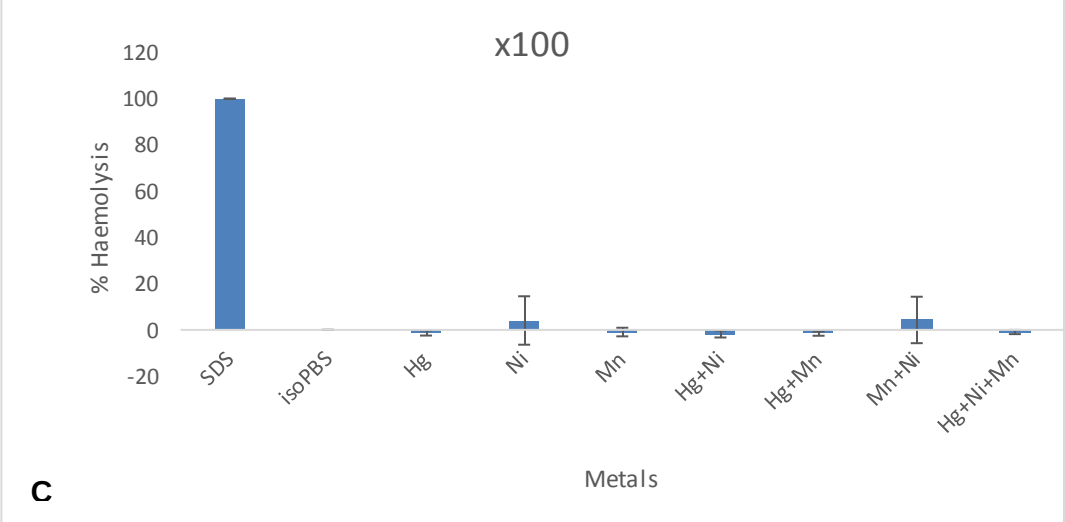
4. RESULTS

4.1 Haemolysis

The haemolysis assay was used to detect the haemoglobin content released from the erythrocytes as an indication of the effect of the heavy metals on erythrocyte membrane integrity. The haemolysis assay is a quantitative evaluator of percentage haemolysis. The metals used in the study alone and in double and triple combinations induced varying degrees of haemolysis at different concentrations between x1 - x10000 the WHO safety limit. The Hg (x1) p-value of <0.9731 and Hg+Ni+Mn (x1) p-value of <0.9969 induced haemolysis represented in Figure 4.1A, although it was statistically insignificant compared to isoPBS. The other groups did not indicate significant haemolysis. As indicated in Figure 4.1B, Ni+Mn (x10) showed minimal haemolysis with the other groups exhibiting no haemolysis. Figure 4.1C shows that Ni (x100) and Mn+Ni (x100) caused minimal percentage haemolysis. The remaining groups showed no haemolysis. As shown in Figure 4.1D Mn (x1000) and Ni+Mn (x1000) induced haemolysis. The rest of the groups showed no haemolysis. As shown in Figure 4.1E representing the 10000x concentration all the groups induced haemolysis. The percentage haemolysis for all groups was well above 30% except Ni which exhibited the lowest percentage haemolysis (Hg 68.1%, Ni 1.6%, Mn 30.4%, Hg+Ni 66.7%, Hg+Mn 33.4%, Ni+Mn 45.6% and Hg+Ni+Mn 58.9%). Hg, Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn were all significantly different to the negative control (isoPBS). Figure 4.1F shows a comparison of all the metals groups with the varying concentration ranges.


x1
120
100
80
60
40
20
0
-20
% Haemolysis
SDS
isoPBS
Hg
Ni
Mn
Hg+Ni
Hg+Mn
Mn+Ni
Hg+Ni+Mn
Metals
A


x10
120
100
80
60
40
20
0
-20
% Haemolysis
SDS
isoPBS
Hg
Ni
Mn
Hg+Ni
Hg+Mn
Mn+Ni
Hg+Ni+Mn
Metals
B


x100
120
100
80
60
40
20
0
-20
% Haemolysis
SDS
isoPBS
Hg
Ni
Mn
Hg+Ni
Hg+Mn
Mn+Ni
Hg+Ni+Mn
Metals
C

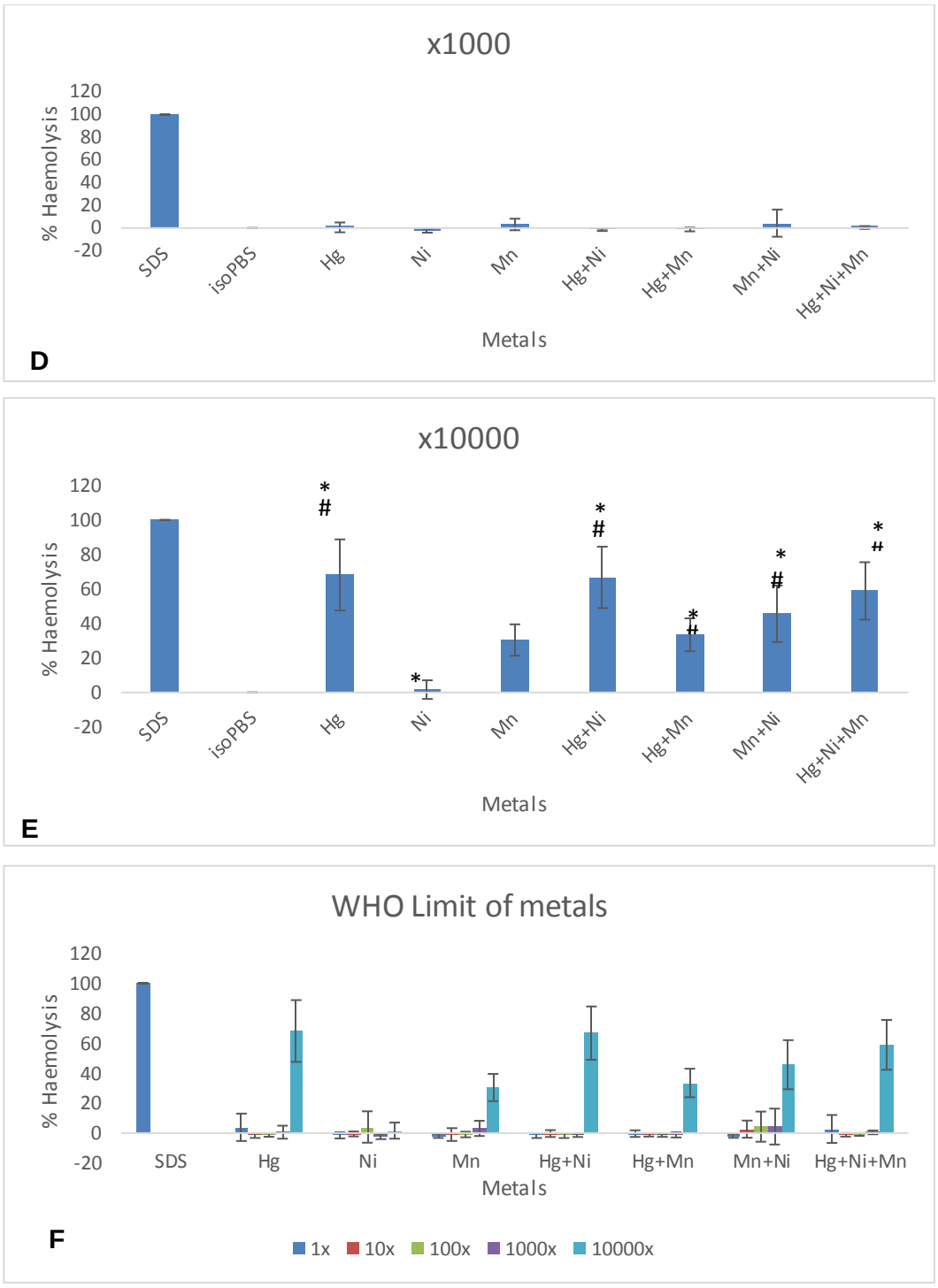


Figure 4.1: A comparison of heavy metal induced percentage haemolysis. The haemolytic effects of Hg, Ni and Mn at A) X1, B) X10, C) X100, D) X1000, E) X 10000 and F) overall representation of the haemolysis of all the metals the WHO safety level standards of each metal. The data is an average of 10 independent experiments expressed as the mean ± standard error of mean (SEM) compared to positive control (SDS) 100% haemolysis and negative control (NC) (isoPBS0) 0% haemolysis. # indicates significance compared to the NC and * indicates significance compared to other metal groups.

A metal is considered to be toxic when it changes to its ionized form. The bound and conjugated forms of the filtered metal are not toxic, but it is the divalent form of these metals released from the complexes that is accountable for toxicity of the cell [131]. The haemolysis assay only indicated the effect of the metal combination on the erythrocyte's membrane, resulting in the appearance of free haemoglobin in the plasma. The assay did not propose any information on the interaction between the double and triple combinations of metals containing Hg, Ni and Mn across the five point increasing concentrations. There are three types of interactions found amongst metal combinations. An antagonistic effect is when two or more metals produce contrasting effects. For example, when Hg has an increasing effect on the percentage haemolysis but Mn has a decreasing effect on haemolysis. An additive effect is when the combination of the metals produces the same percentage haemolysis compared to the single metal effect. A synergistic effect is when the combination of metals produces a greater haemolysis percentage compared to the individual metal. Table 4.1 provides a better understanding of what effect the double and triple combinations of metals had on each other when combined.

Table 4: Effect of metal combinations on haemolysis results

	Metals	Calculated	Expected	Effect
X1 WHO	Hg+Ni	$\frac{3.854+(-1.500)}{2}=1.17$	-1.67713287	Antagonistic
	Hg+Mn	$\frac{3.854+(-1.840)}{2}=1.0$	-0.35723247	Antagonistic
	Mn+Ni	$\frac{-1.500+(-1.840)}{2}=1.67$	-1.93274258	Antagonistic
	Hg+Ni+Mn	$\frac{3.854+(-1.500)+(-1.840)=0.171}{3}$	2.7683465	Antagonistic
X10 WHO	Hg+Ni	$\frac{-1.772+(-0.552)}{2}=-1.162$	-0.312	Synergistic
	Hg+Mn	$\frac{-1.772+(-0.979)}{2}=-1.375$	-1.252	Synergistic
	Mn+Ni	$\frac{-0.522+(-0.979)}{2}=-0.765$	2.616	Synergistic
	Hg+Ni+Mn	$\frac{-1.772+(-0.552)+(-0.979)}{3}=-1.101$	-1.164	Antagonistic
X100 WHO	Hg+Ni	$\frac{-1.341+4.055}{2}=1.357$	-1.789	Antagonistic
	Hg+Mn	$\frac{4.055+(-0.942)}{2}=-1.141$	-1.626	Antagonistic
	Mn+Ni	$\frac{4.055+(-0.942)}{2}=1.556$	4.285	Synergistic
	Hg+Ni+Mn	$\frac{-1.341+4.055+(-0.942)}{3}=-0.590$	-0.929	Antagonistic
X1000 WHO	Hg+Ni	$\frac{0.602+(-2.398)}{2}=-0.898$	-1.603	Antagonistic
	Hg+Mn	$\frac{0.602+3.139}{2}=1.870$	-1.200	Antagonistic
	Mn+Ni	$\frac{-2.398+3.139}{2}=0.370$	4.342	Synergistic
	Hg+Ni+Mn	$\frac{0.602+(-2.398)+3.139}{3}=0.447$	0.486	Additive
X10000 WHO	Hg+Ni	$\frac{68.132+1.660}{2}=69.792$	66.727	Synergistic
	Hg+Mn	$\frac{68.132+30.411}{2}=98.543$	33.445	Antagonistic
	Mn+Ni	$\frac{1.660+30.411}{2}=32.071$	45.638	Synergistic
	Hg+Ni+Mn	$\frac{68.132+1.660+30.411}{3}=100.203$	58.890	Synergistic

4.2 Scanning electron microscopy

4.2.1 Whole blood without thrombin

The effects of Hg, Ni and Mn alone and in combination on the morphology of erythrocytes were studied using SEM. Concentration ranges of x1, x10 and x100 the WHO safety limit were used. The images obtained are representative of erythrocytes acquired from blood smears with and without the addition of thrombin. The addition of thrombin induced fibrin formation. Figure 4.2 shows scanning electron micrographs of erythrocytes exposed to metal concentrations at x1 the WHO limit. Control erythrocytes are shown in Figure 4.2A, with a typical biconcave structure. In Figure 4.2B, Hg exposure caused the presence of spike-like nodules (indicative of echinocytes) indicated by the yellow arrows. Nickel exposure (Figure 4.2C) caused a bulging appearance of the erythrocytes (cell blebbing), which was also seen with Mn exposure (Figure 4.2D). Echinocytes were observed in the Hg+Ni and Hg+Mn exposed groups (Figure 4.2E and F). Hg+Ni+Mn exposure caused a loss of biconcave morphology in the erythrocytes as shown in Figure 4.2H.

Figure 4.3 shows erythrocytes exposed to metal concentrations at x10 the WHO limit. In the group exposed to Hg echinocytes were present. This was true for the Ni and Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn groups. Cell blebbing was observed with Mn exposure (Figure 4.3D.

Figure 4.4 shows erythrocytes exposed to metal concentrations x100 WHO limit. In the group exposed to Hg echinocytes were present with increased membrane roughness. This was true for Hg+Mn, Ni+Mn and Hg+Ni+Mn. The Ni exposed group also had echinocytes present with cell blebbing also seen for Mn (Figure 4.4C) and Hg+Ni (Figure 4.4 D).

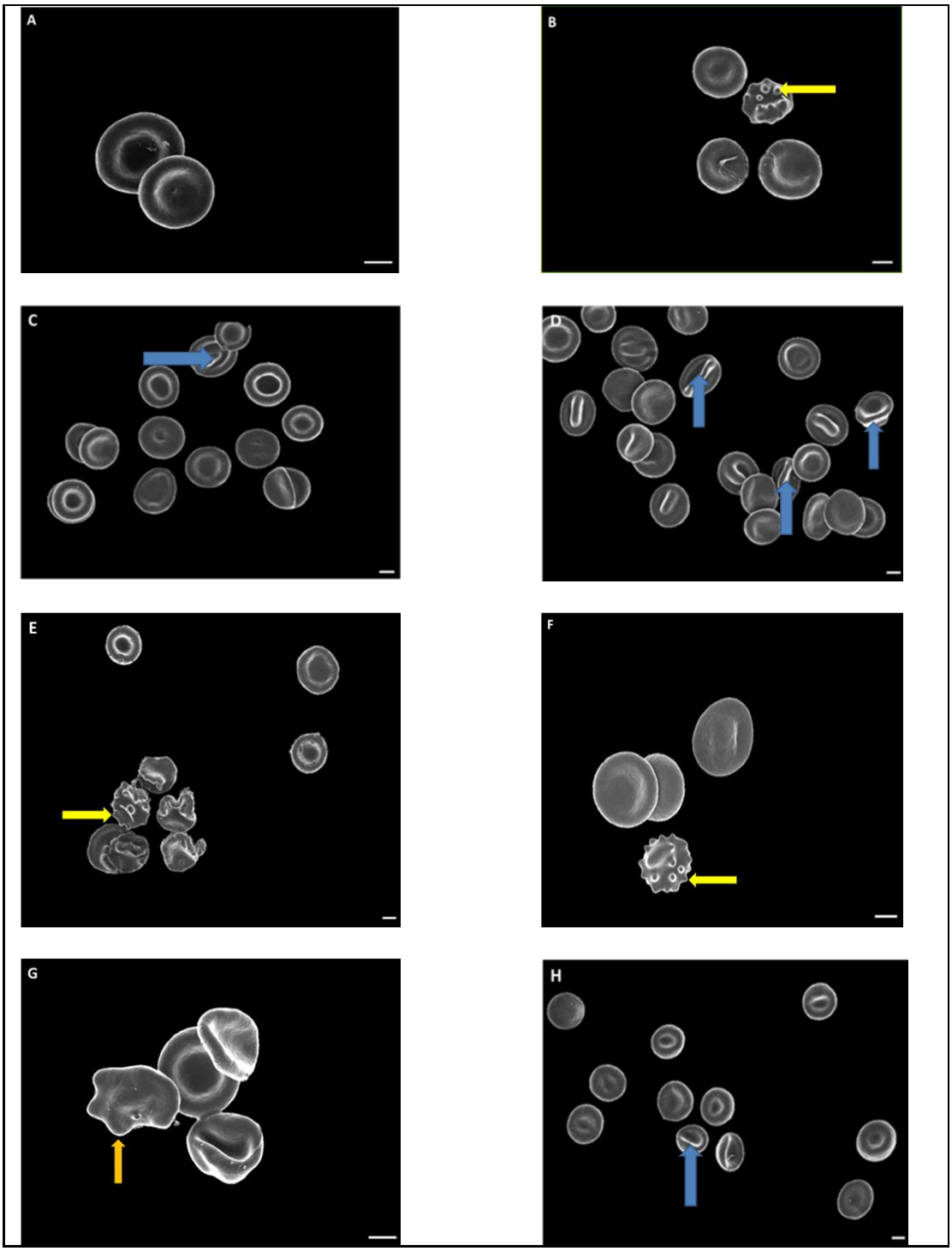


Figure 4.2: Scanning electron micrographs of whole blood without thrombin exposed to Hg, Ni and Mn, alone and in combination at x1 the WHO safety limit. (A): Control with normal biconcave erythrocytes. (B): Hg, (C): Ni, (D): Mn, (E):

Hg+Ni, (F): Hg+Mn, (G): Ni+Mn, (H): Hg+Ni+Mn. Scale bars=2µm. Blue arrows: bulging appearance, Yellow arrows: echinocytes (nodule like spikes).

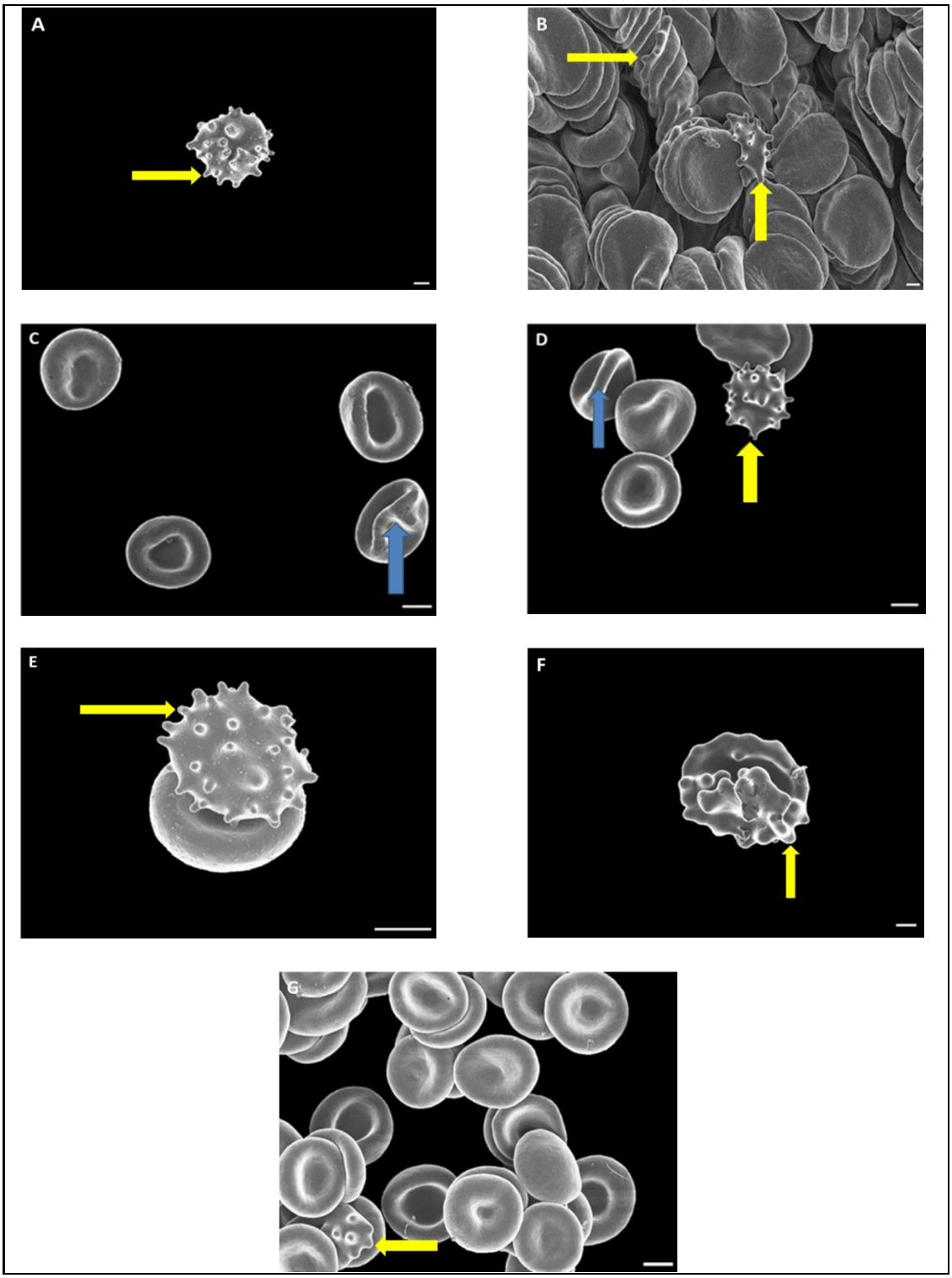


Figure 4.3: Scanning electron micrographs of whole blood without thrombin exposed to Hg, Ni and Mn, alone and in combination at x10 the WHO safety limit. (A): Hg (B): Ni, (C): Mn, (D): Hg+Ni, (E): Hg+Mn, (F): Ni+Mn, (G): Hg+Ni+Mn.

C, D, E & G Scale bars=2µm, A, B & F scale bar =1µm. Blue arrows bulging appearance, Yellow arrows echinocytes (nodule like spikes).

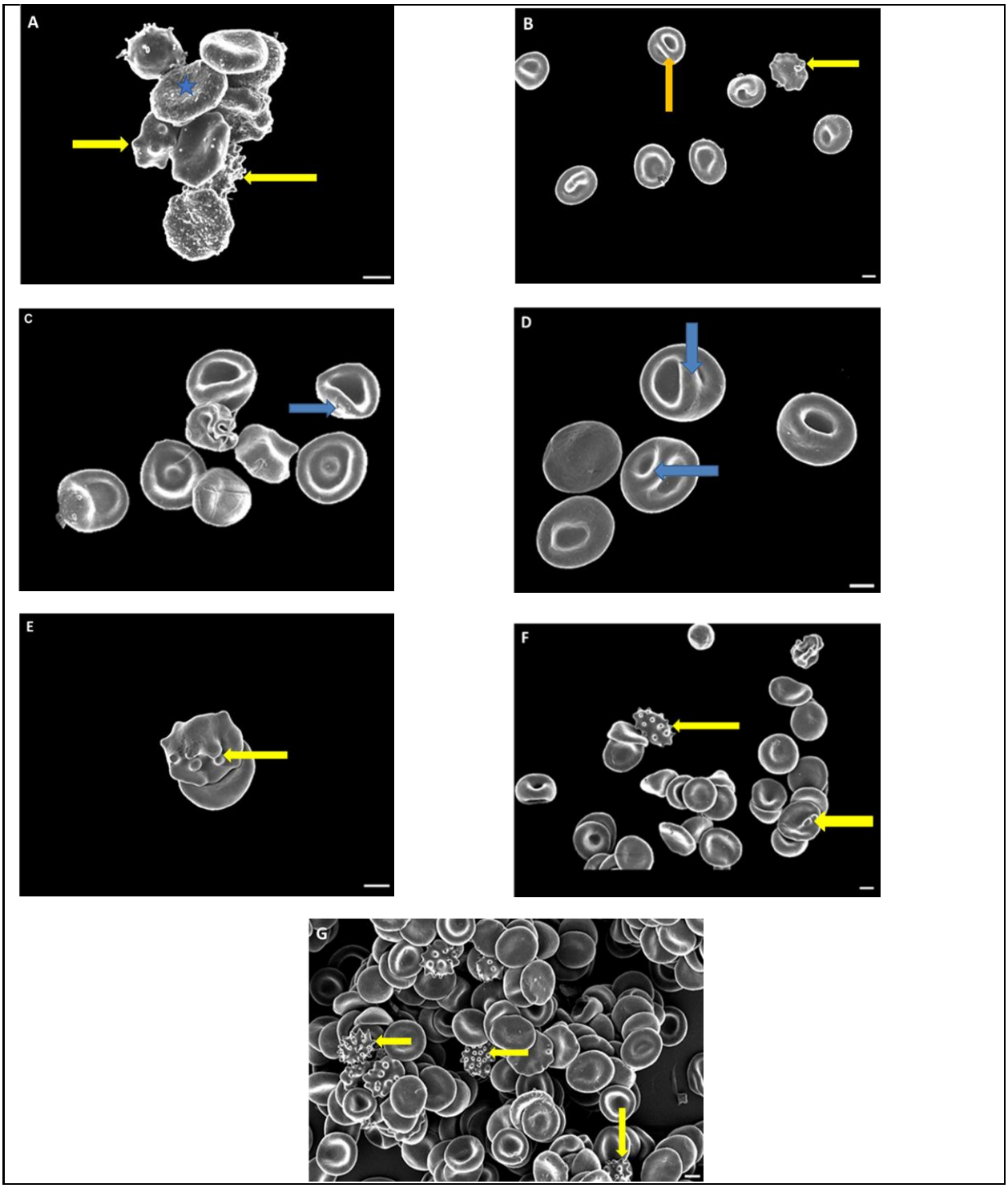


Figure 4.4: Scanning electron micrographs of whole blood without thrombin exposed to Hg, Ni and Mn, alone and in combination at x100 the WHO safety limit. (A): Hg (B): Ni, (C): Mn, (D): Hg+Ni, (E): Hg+Mn, (F): Ni+Mn, (G): Hg+Ni+Mn.

C, D, E & G Scale bar=2µm, A, B & F; scale bars =1µm. Blue arrows bulging appearance, Yellow arrows echinocytes (nodule like spikes).

Figure 4.5 shows scanning electron micrographs of platelets exposed to metal concentrations at x1 the WHO limit. Figure 4.5A shows the normal morphology of platelets with the presence of pseudopodia. Contact activation is expected during the preparation of the samples with some pseudopodia formation. Figure 4.5B to G shows activated platelets with the presence of pseudopodia to a greater degree than that caused by contact activation as seen in the control sample in Figure 4.5A. In Figure 4.5H the presence of pseudopodia and membrane spreading can be seen in the triple combination group. Figure 4.6 shows platelets exposed to metal concentrations x10 the WHO limit. In Figure 4.6A to D and F, platelets exposed to heavy metals became activated with increased pseudopodia and increased membrane spreading. Presence of pseudopods and platelet interaction are seen in Figure 4.6G. Figure 4.7 shows platelets exposed to metal concentrations at x100 the WHO limit. Figures 4.7 (A to E) showed presence of pseudopodia. Platelet interaction was seen in Figure 4.7F and membrane spreading was observed in Figure 4.7G.

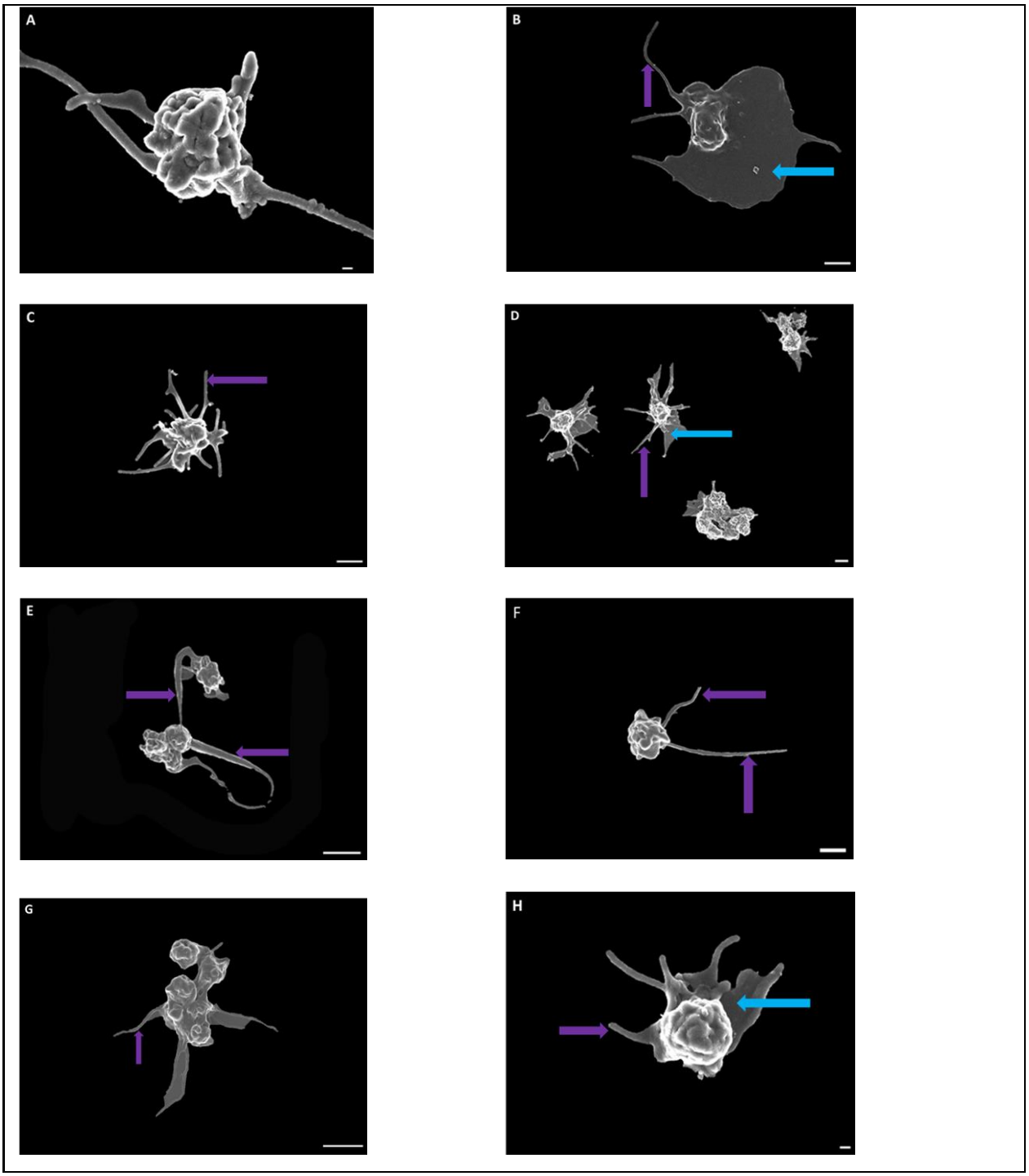


Figure 4.5: Scanning electron micrographs of control and x1 heavy metal exposed platelets. (A): Control, (B): Hg, (C): Ni, (D): Mn, (E) Hg+Ni, (F): Hg+Mn, (G): Ni+Mn and (H): Hg+Ni+Mn. A, C &D: Scale bars = 1µm; B: Scale bar=200 nm. Blue arrows= membrane spreading. Purple arrows= pseudopodia.

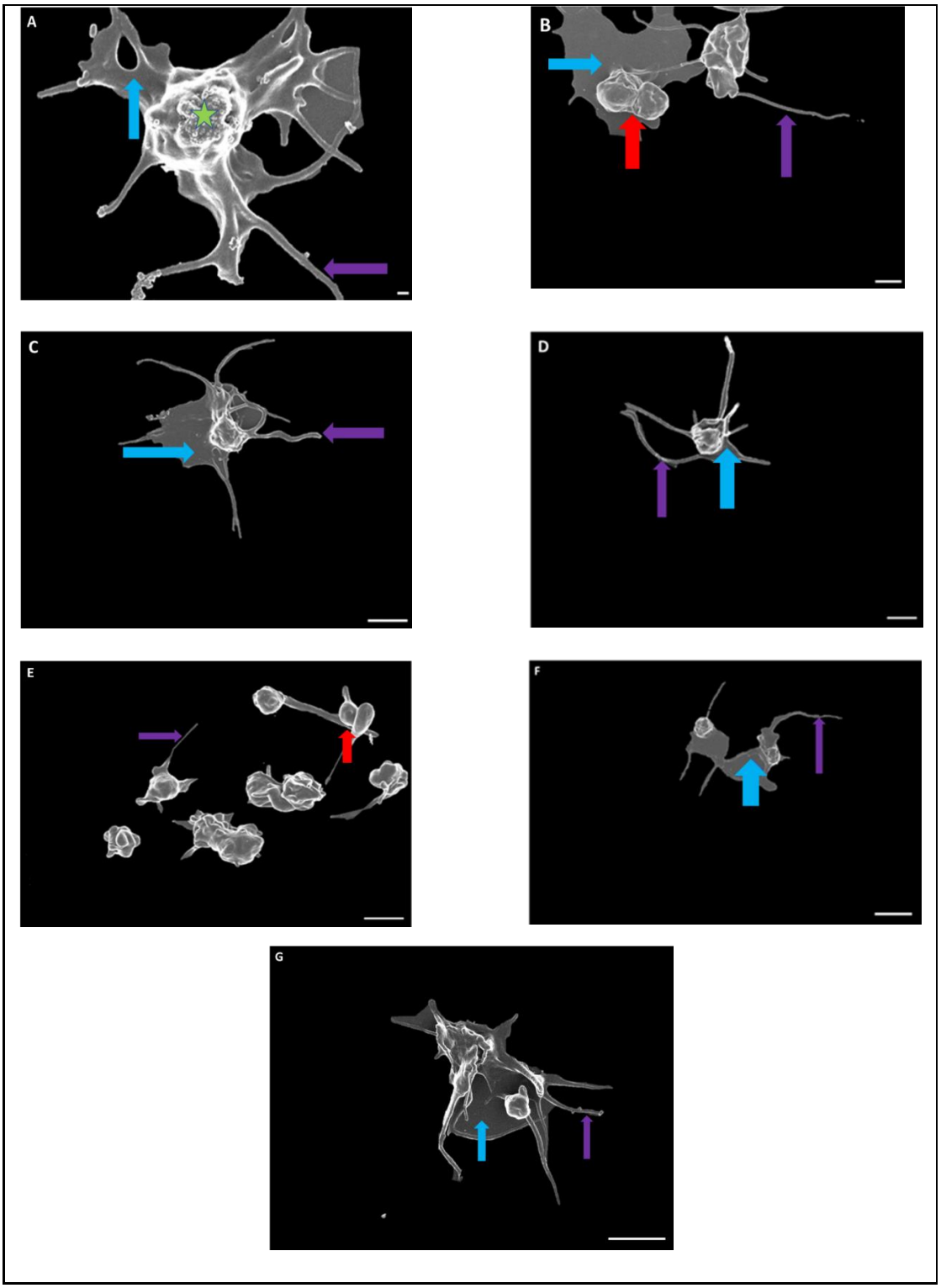


Figure 4.6: Scanning electron micrographs of platelets exposed to x10 heavy metal concentration. The platelets are activated thus membrane spreading seen. (A): Hg, (B): Ni, (C): Mn, (D): Hg+Ni, (E): Hg+Mn, (F): Ni+Mn, (G): Hg+Ni+Mn. A Scale bar= 200nm. B & D Scale bars= 1µm. C, F & G Scale bars= 2µm. Blue arrows= membrane spreading. Purple arrows= pseudopodia. Red arrows= platelet-platelet interaction. Green star= rough membrane surface.

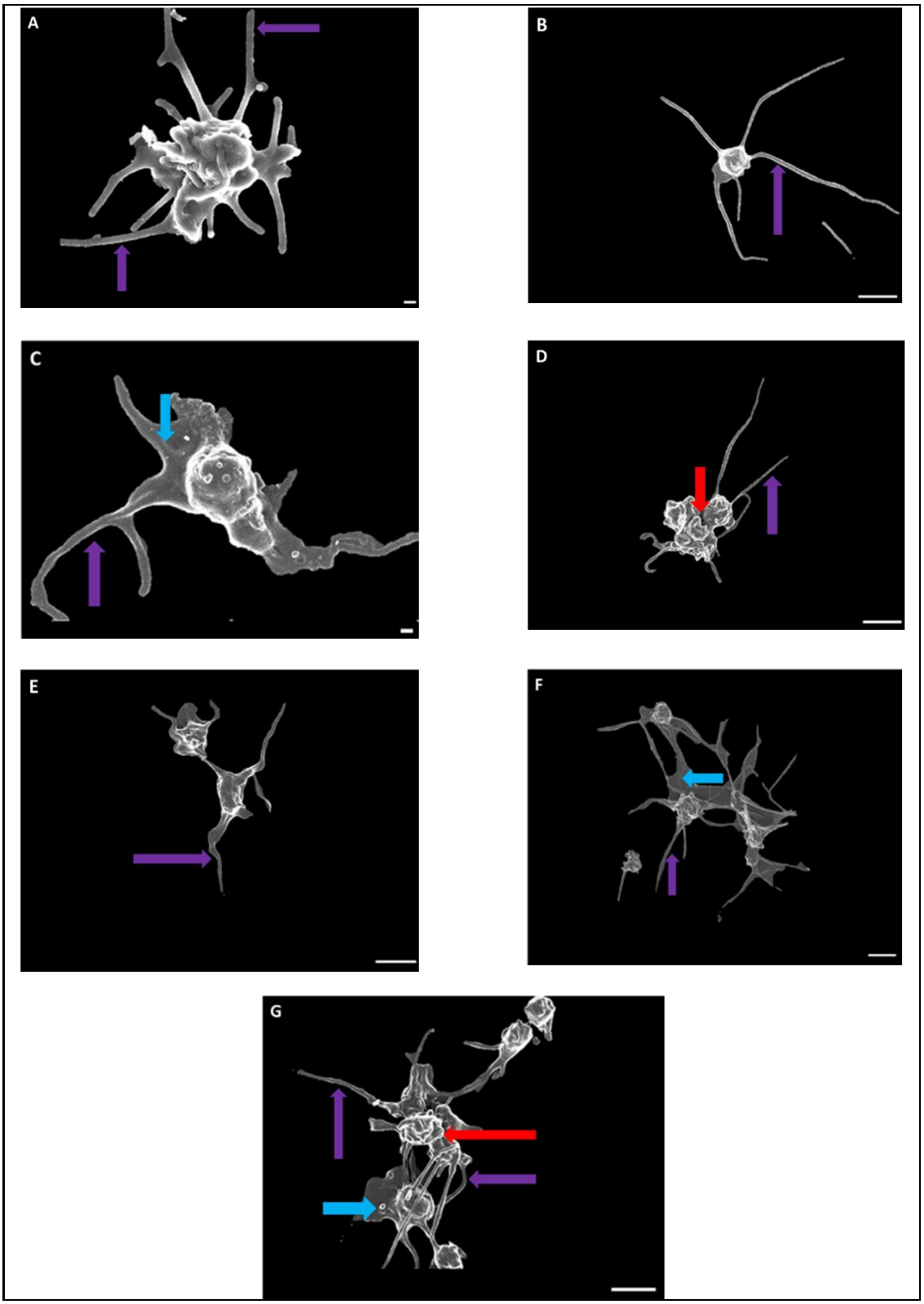


Figure 4.7: Scanning electron micrographs of platelets exposed to x100 heavy metal concentration. (A): Hg, (B): Ni, (C): Mn, (D): Hg+Ni, (E): Hg+Mn, (F): (Ni+Mn), (G): Hg+Ni+Mn. A & C Scale bars= 200 nm. B, D-G Scale bars= 2 µm. Blue arrows =membrane spreading. Purple arrows= pseudopodia. Red arrows= platelet-platelet interaction.

Figure 4.8 shows scanning electron micrographs of whole blood with thrombin exposed to the heavy metal concentration at x1 the WHO limit. Figure 4.8A represents the fibrin network of a control sample. Healthy fibrin clots should contain thin, thick and taut fibres. In Figure 4.8B and C thick, thin and less taut fibres and the presence of echinocytes was observed. Thick and less taut fibres are seen with Mn exposure, thin fibres are seen in the Hg+Ni (Figure 4.8E) and Ni+Mn (Figure 4.8G) exposed groups and Hg+Mn (Figure 4.8F) exposed group showed thick and thin fibres.

Figure 4.9 shows scanning electron micrographs of whole blood with thrombin exposed to heavy metal concentration at x10 the WHO limit. In Figure 4.9A fibrin fibres forming a mesh network are observed, thin and taut fibres (B), thick fibres (C), thin fibres (D) and a fibrin mesh network around the erythrocytes (F) were respectively seen in Figure 4.9 B, C, D and E. Figure 4.9F showed thick and less taut fibres and Figure 4.9G thick and thin fibres.

Figure 4.10 shows scanning electron micrographs of whole blood exposed to heavy metals at x100 the WHO safety limit. Figure 4.10A showed the presence of fibrin fibres forming a fibrin network mesh, Figure 4.10B thin, thick and less taut fibres were observed and a presence of fibrin fibres forming a fibrin network mesh were observed in Figure 4.10C. Thick fibres and fibrin fibres forming a fibrin network were observed in 4.10D. Presence of thin fibres and fibrin network forming a mesh can be observed in 4.10E. Fibrin network forming a mesh and thick fibres and presence of echinocytes were respectively seen in Figure 4.10F and G.

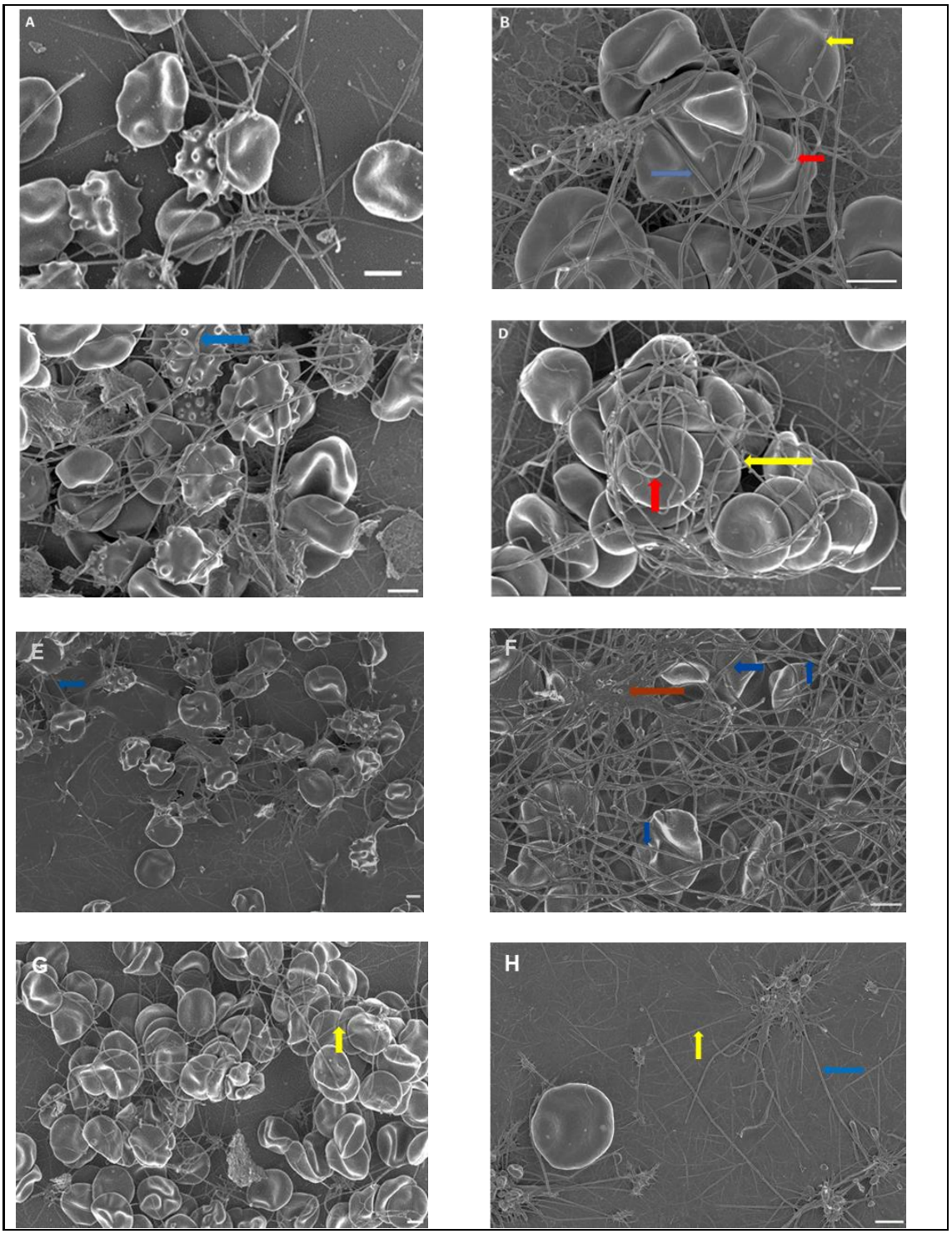


Figure 4.8: Scanning electron micrographs of whole blood with thrombin exposed to Hg, Ni and Mn, alone and in combination at concentrations at x1 showing fibrin network formation together with erythrocytes. (A): Control, (B): Hg. (C): Ni, (D): Mn, (E): Hg+Ni, (F): Hg+Mn, (G): Ni+Mn, (H): Hg+Ni+Mn. (A. C, D, F & H), Scale bars=2µm, (B, E & F)

scale bars=1µm. Brown arrows=fibrin mesh. Yellow arrows = Thin fibres. Blue arrows= Thick fibres. Red arrows= Less taut fibrin fibres.

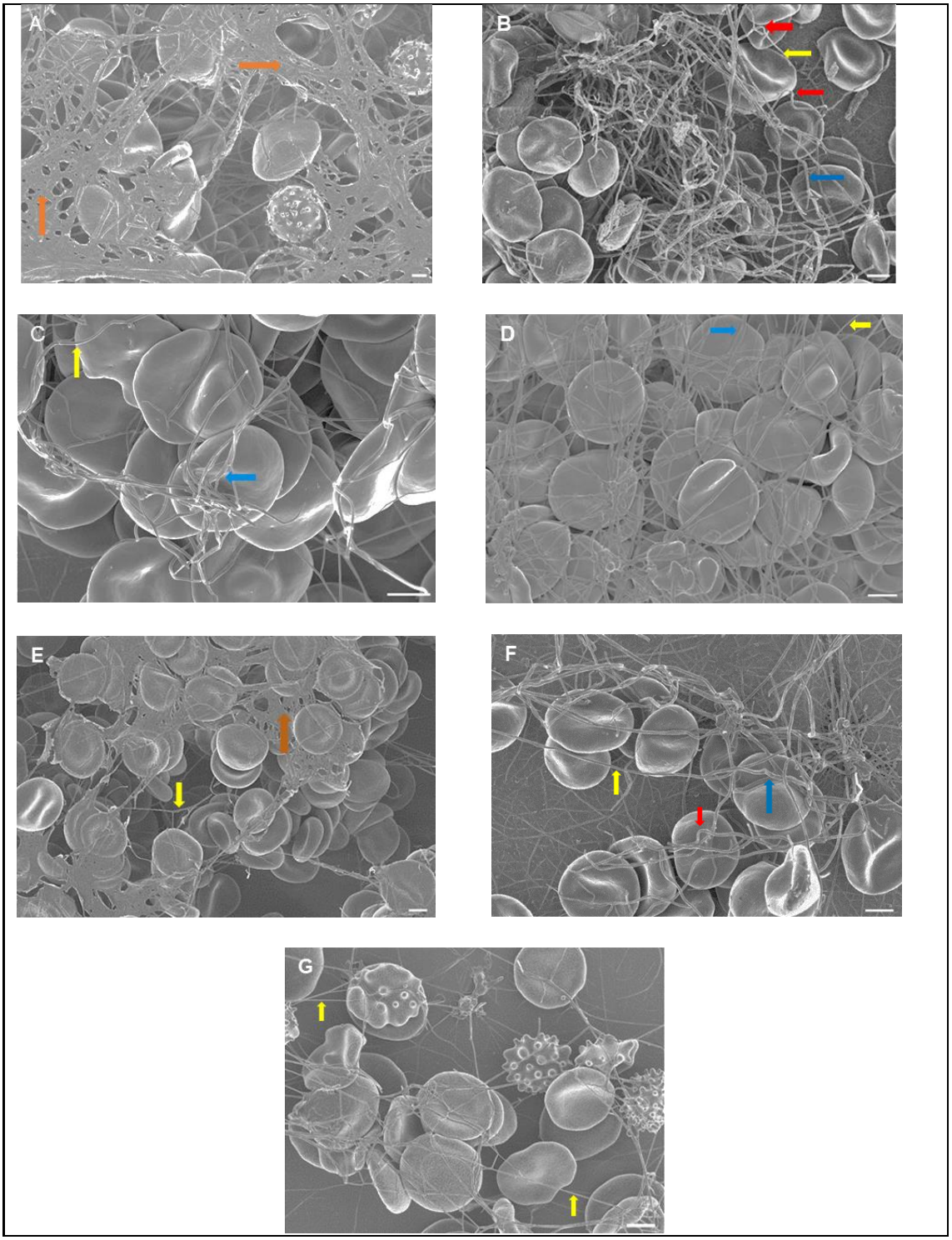


Figure 4.9: Scanning electron micrographs of whole blood with thrombin exposed to Hg, Ni and Mn alone and in combination at concentrations at x10 showing fibrin network formation together with erythrocytes. (A): Hg (B): Ni. (C):

Mn, (D): Hg+Ni, (E): Hg+Mn, (F): Mn+ Ni, (G): Hg+Ni+Mn. (C, D, E F & G), Scale bars=2µm, (A, B & E) scale bars=1µm. Brown arrows= fibrin mesh. Yellow arrows= Thin fibres. Blue arrows= Thick fibres. Red arrows= Less taut fibrin fibres.

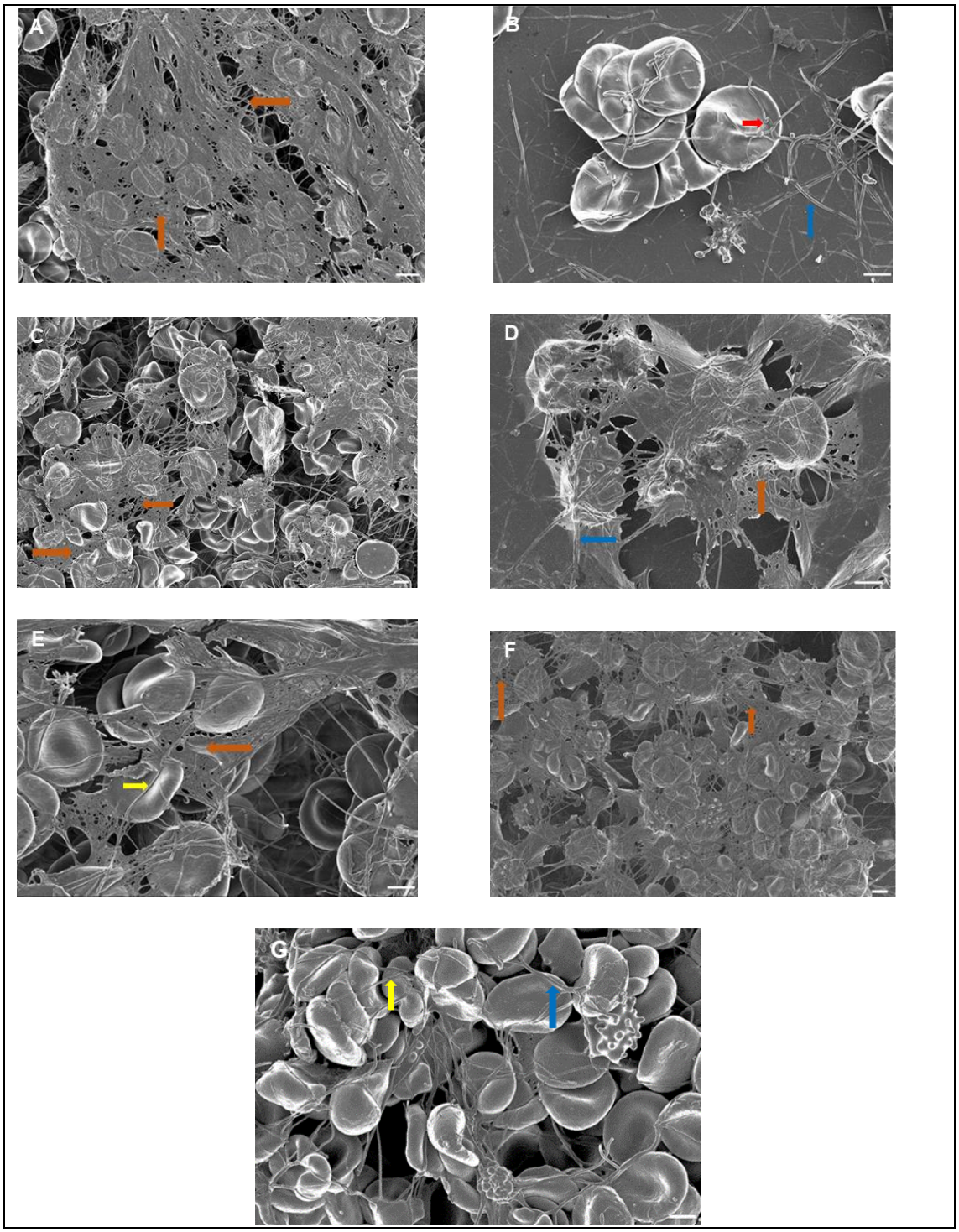


Figure 4.10: Scanning electron micrographs of whole blood with thrombin exposed to Hg, Ni and Mn alone and in combination at concentrations at x100 showing fibrin network formation together with erythrocytes. (A): Hg, (B): Ni. (C):

Mn, (D): Hg+Ni, (E): Hg+Mn, (F): Ni+Mn, (G): Hg+Ni+Mn. (B, D, E G), Scale bars=2µm, (A, C & F) scale bars=1µm. Brown arrows= Fibrin mesh. Yellow arrows = Thin fibres. Blue arrows= Thick fibres. Red arrows= Less taut fibrin fibres.

4.3 Confocal laser scanning microscopy

In the positive control, erythrocytes were exposed to Mellitin represented in Figure 4.11. Figure 4.11A represents the auto-fluorescence of the erythrocytes in the positive control. Figure 4.11B represents the positive Annexin V signal (indicated with green fluorescence) that was observed in the positive control. Figure 4.11C shows the transmission light image and Figure 4.11D shows the overlay images. In the negative control group, no positive Annexin V signal was observed as shown in Figure 4.12A. Figures 4.12 B to H are images of Annexin V positive signal that were obtained in all the metal exposed groups, single (Hg, Ni and Mn), double (Hg+Ni, Hg+Mn and Ni+Mn) and triple combination (Hg+Ni+Mn). Phosphatidylserine flip positive cells were scattered throughout the samples.

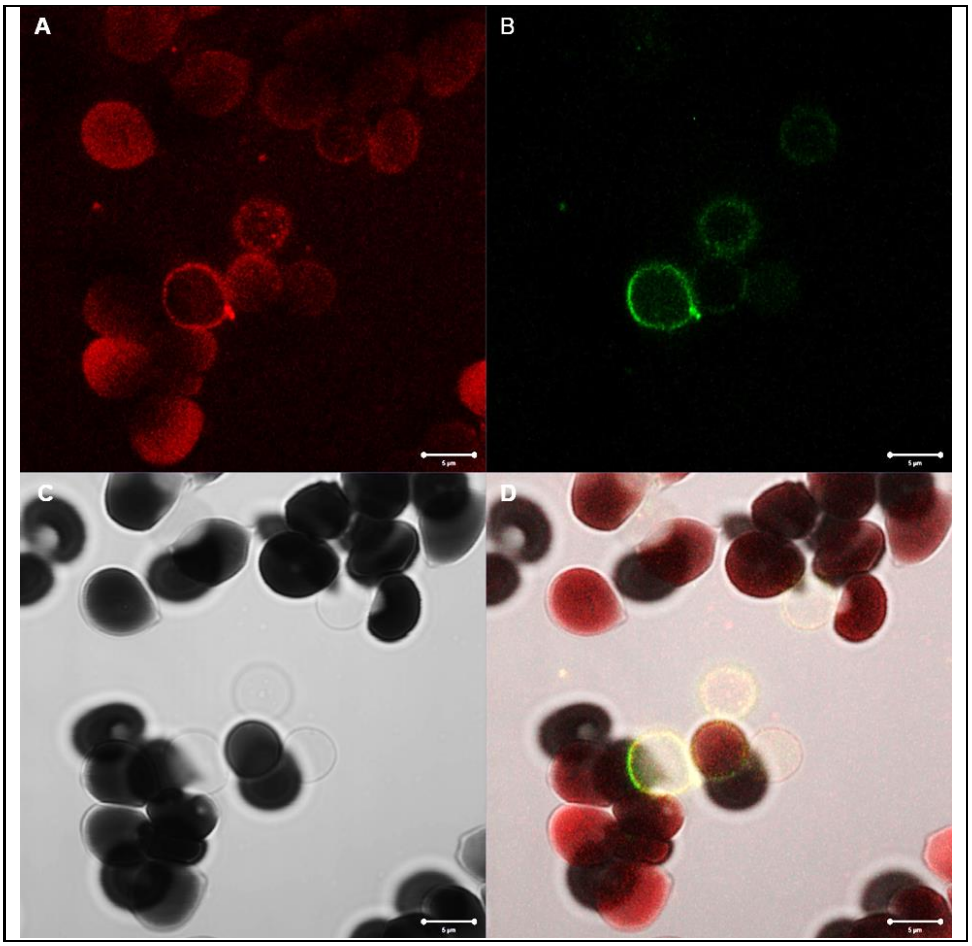


Figure 4.11: Confocal laser scanning microscope micrographs of the positive control (A-D). Figures A: auto-fluorescence of the erythrocytes, B: Annexin V signal obtained, C: transmission light and D showing the overlay of the images (transmission, fluorescence and annexin V) (Scale bars: 5µm).

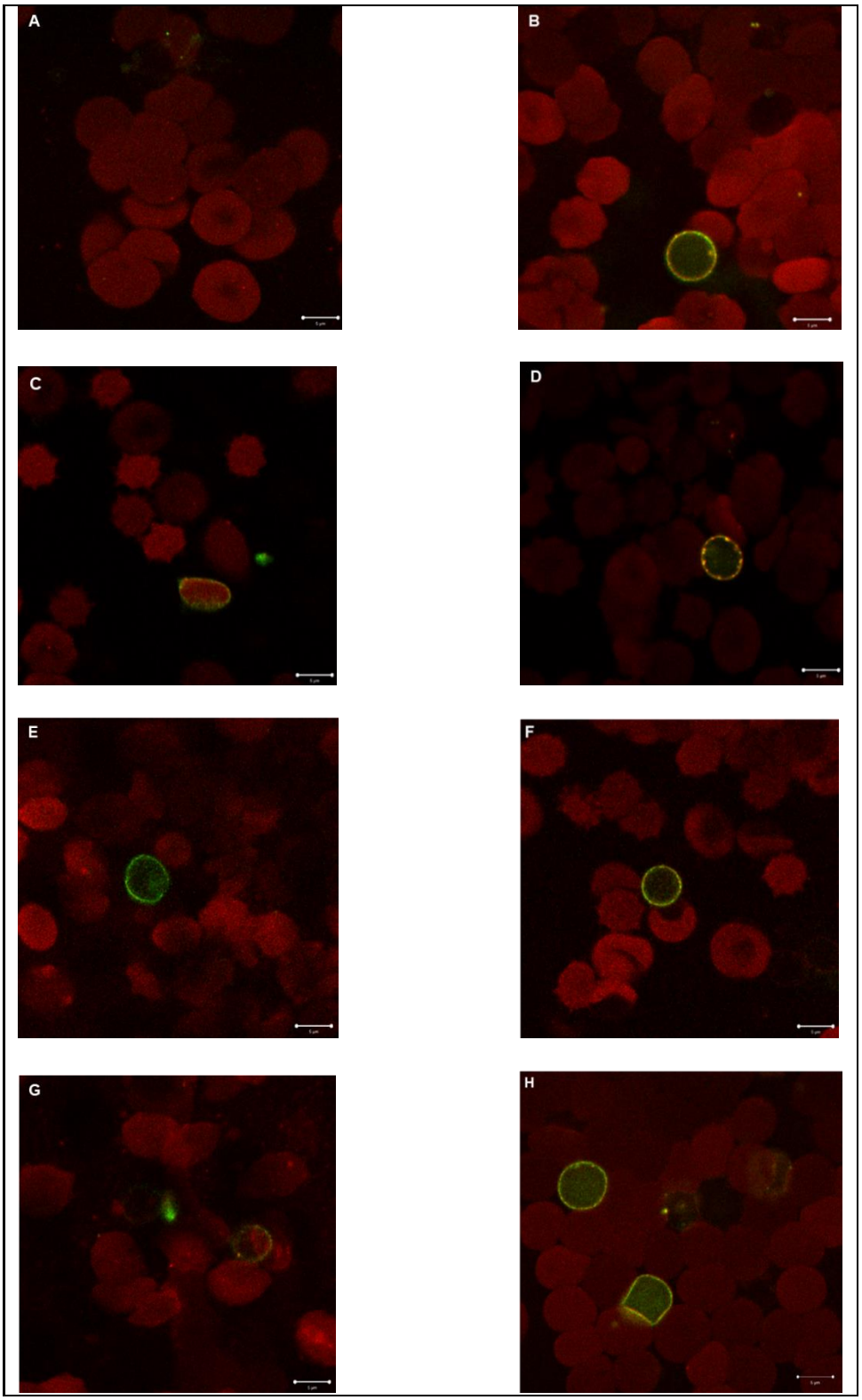


Figure 4.12: Phosphatidylserine exposure evaluation of erythrocytes subsequent to heavy metal exposure of x1 Hg, Ni and Mn alone and in combination using the confocal laser-scanning microscope. (A): Negative control. (B- H) indicates the Annexin V positive erythrocytes after exposure to Hg (B), Ni (C), Mn (D), Hg+Ni (E), Hg+Mn (F), Mn+Ni (G) and Hg+Ni+Mn (H) (Scale bars: 5µm).

5. DISCUSSION

5.1 Haemolysis assay

Water, soil and air are natural resources that humans and animals survive on. These three resources need to be monitored to control and lower pollution levels and degradation. These resources are essential for the wellbeing of humanity [132]. The distribution of metals throughout the natural environment and resources is increasingly becoming a concern. Several studies pertaining to the accumulation of metals in agriculture, water and living systems has been done [133-134] and found that a high level of metal content was found in livestock, the environment and agricultural soil. International agencies need to be concerned about heavy metal pollution and its consequence on livestock and products. Global modernization has seen several new industries and urban areas rapidly developing. Anthropogenic activities are also on the rise which increases the application of heavy metals. The production, processing as well as disposal of certain products that contain heavy metals is released into the immediate environment, which affects the soil, water and air quality. South Africa and the world at large have seen pressure coming from civil societies and governmental organizations to reduce air pollution [135] and, the banning or phasing out of products and processes that rely on Hg [136-137]. As a result, various companies are introducing innovative or upgraded air pollution control measures. Pollution of the environment is largely associated with numerous human health and environmental impacts including heavy metal poisoning, respiratory diseases and increased levels of acidity in lakes, affecting the quality of water and aquatic biodiversity. Although the quality of the water in urban and affluent communities is tightly regulated, the same cannot be guaranteed for rural settlements. Citizens that live in rural settlements do not have access to clean water and therefore make use of river water for washing, drinking and agricultural purposes. The same water they are making use of is possibly contaminated with metals, making them vulnerable to health complications. The use of wastewater contaminated with metals for irrigation, does not only contaminate the soil, but also affects the quality and safety of food [138]. Heavy metals easily accumulate in the edible parts of leafy vegetables, as compared to grain or fruit crops [139].

In a study conducted by Barbier, 2004 [140], the effect of cadmium chloride ($CdCl_2$) on rats was investigated and was found to cause hypokaliuria, hyperphosphaturia and hypercalciuria without any changes to the glomerular filtration rate (GFR) [140]. In contrast to a similar study, a single 20-fold lower dosage of Hg^{2+} and Pb^{2+} caused impairment to the kidney tubules and glomerulus with

damage characterized by the reduction of the GFR glycosuria, proteinuria as well as impediment of the tubular system [141]. These findings indicate that the level of toxicity to the kidney differs among heavy metals. Mercury and Pb^{2+} are far more hazardous compared to Cd^{2+} since they caused irreparable kidney function deficiency at low dosages compared to Cd^{2+}. The liver is another organ that is affected by exposure to heavy metals. Lee, 2017 [142] conducted a study on 560 aged individuals (60 years or older) where the effect of blood Hg concentrations on the liver were studied. The study concluded that Hg levels in the blood where associated with the impairment of the liver [142]. The application of hepatotoxicity due to Hg needs more clarification; various literature sources propose that Hg generates reactive oxygen species (ROS) and weakens the components of antioxidant systems which causes hepatotoxicity [143]. From the various studies conducted on the effects of Hg in the blood, it is evident that the metal is very toxic and causes impairment or damage to organs in the human body.

In this study an *ex-vivo* model was used to study the effects of Hg, Ni and Mn alone and in combination.

Mercury is among several metals considered to be toxic [144]. Figure 4.2A at x1 the WHO safety limit shows that Hg did cause haemolysis. At low concentrations of Hg, it was observed that the metal caused rupturing of the membrane surface of erythrocytes thus resulting in haemolysis. Mercury is a very toxic metal even at its lowest concentration. It is thus very important for guidelines to be set with regards to the consumption of Hg. The WHO and the Joint Food and agriculture organization (FAO) expert committee on Food Additives (JECFA) established a safety intake of 1.6µg/kg bodyweight per week for CH_3Hg^+. The same value is recommended for pregnant mothers to protect the developing foetus from neurotoxic effects [145]. Figure 4.2B (x10 Mn+Ni), 4.2C (x100 Ni and Mn+Ni) and 4.2D (x1000 Hg, Mn and Mn+Ni), showed minimal or no haemolysis respectively. With increasing concentrations of the WHO safety limit, it is expected that there will be an increase in the percentage of haemolysis, however, in the results above a different trend is observed. With an increase in the WHO safety limit (x10, x100 and x1000) of Hg, haemolysis does not occur. At concentrations of x10 000 the WHO safety limit Hg caused 68.132% haemolysis. These findings show a correlation with a study conducted by Bhakdi, 1984 [146], where it was observed that at low concentrations, the amount of toxin binding to either rabbit or human erythrocytes was below the detection limit set by the system and no haemolysis was observed. However, at increased toxin levels human erythrocytes lysed and the percentage haemolysis increased. The concentration and incubation time (90min) were far less than in this study (16 hours) nonetheless at higher concentrations of the toxins haemolytic effects were

observed [146]. In a similar study conducted by Janse van Rensburg *et al* in 2018 [147], the haemolysis percentage of Hg at x10 000 the WHO safety limit was 75.20% [147]. These findings correlate with the current study. There is a direct association between the concentration of Hg and percentage haemolysis of erythrocytes. Mercury plays a role in the propagation of oxidative stress thus resulting in the manifestation of cardiovascular diseases. The presence of Hg in erythrocytes inhibits SOD, catalase and glutathione peroxidase thus causing an increase in oxidative stress. This is achieved by Hg inhibiting antioxidant pathway by the development of ROS that bind to N-acetyl cysteine (NAC) and glutathione (GSH) as a result of Hg having a high binding affinity to the thiol groups of NAC and GSH. A decrease in the content of GSH and other thiol containing redox molecules is due to the increase of Hg binding to GSH. Not only does this cause ROS accumulation but also causes enzyme inhibition activity and related biochemical pathways [148]. A relationship exists between increased Hg levels and the risk of developing cardiovascular diseases, pulmonary embolism, hypertension and the obstruction of vessels as seen in various epidemiological studies [19]. In a study conducted by Akagi, 1995 [149] reports of people living in areas surrounding active gold mining exhibited extremely high levels of blood Hg which were about 150 µg/L [149]. These levels are well above the WHO safety levels of Hg, thus emphasizing the concern that Hg is an environmental pollutant with adverse effects.

The total amount of electricity generated by Eskom's coal-fires power stations in South Africa in 2015 was just below 93%. The main fuel used in the generation of electricity is coal, which contains mostly ash, carbon, minimal sulphur and trace amounts of Hg. Sulphur combined with Hg can be released into the atmosphere during the combustion of coal [150]. It is therefore important to control and limit the emission of Hg into the environment as 43% of coal is used in the generation of electricity in South Africa [21]. In a study conducted by Eskom employees by Garnham *et al.*, 2016 [151] it was discovered that the amount of Hg emitted from Eskom's coal powered stations in 2015 was between 16.8 and 22.6 tons. Six of Eskom's power stations produced on average just under 82% of the total Hg emitted in 2015 [151]. Studies done prior to Garnham *et.al* in 2016 provided results that vary greatly. The results ranged from 9.8 tons in 2000 [41] to 83 tons in 2004 [152] of emitted Hg. The emission of Hg by Eskom's power stations will continue if remedial action is not taken immediately. With increased human population, there is a need to generate more electricity to power homes and businesses, which means increased anthropogenic activities. It is estimated that Hg emissions will be reduced to between 6% and 13% over the next coming years if Eskom implements emission reduction plans [151]. Mpumalanga province is home to twelve coal fired power plants and has a total capacity of over 32 gigawatts owned and operated

by Eskom [153]. Reports indicated that Witbank has the world's most polluted air due to Eskom's emission of coal. The air quality regulations in South Africa are very poor and several reports have been issued in accordance to weak Minimum Emission Standards (MES) but nothing is being done to rectify this error. The exposure of Hg in South African communities is largely unknown. A study conducted by Dalvie and Ehrlich, 2006 [154] in the community of Cape Town, was based on the effect of Hg inhalation. The community is close to a waste site and fossil fuel burning operations. The urine samples of the community were compared to a control group which is further from the waste site and fossil fuel-burning operation. The median concentrations of Hg in both groups was less than the WHO guideline of 6 µg/L, however a statistically significant difference ($p<0.05$) was found amongst the two groups, with the exposed group exhibiting greater concentrations as compared to the control [154]. Mercury has a high chemical bonding affinity for sulfhydryl groups, there is some interest in the interactions occurring between mercuric ions and thiols of proteins, amino acids and peptides.

In erythrocytes, Hg binds to the sulfhydryl groups on the haemoglobin molecule and to glutathione [155]. Mercuric Hg has equal distribution to the plasma and the erythrocytes upon entry into the blood stream. Once in the blood stream, Hg reacts with the thiol group (-SH) of the globin protein. The thiol group depletes intracellular thiols such as glutathione, affecting the cellular integrity causing the formation of oxidative species. The presence of free radicals and products of peroxidation cause damage to the cell membrane and leaves the cell vulnerable to diseases [156]. Glutathione peroxidase, SOD, ubiquinol and catalase are among some of the antioxidants that assist in limiting the formation of free radicals within the cell [157]. Similar findings are seen with a study done by Durak, 2010 [156] which found that pre-treatment of erythrocytes with vitamin C and vitamin E (antioxidants) at concentrations that are similar to $HgCl_2$ used in this study can decrease $HgCl_2$ induced oxidative stress by reducing lipid peroxidation, thereby altering antioxidant defence systems in erythrocytes. Vitamin C and E increased the levels of SOD, catalase and peroxidase. The response was concentration dependent [156]. Dietary supplements of vitamin C and E may be beneficial in populations that are occupationally and accidentally exposed to $HgCl_2$ poisoning [158].

The global production of Ni keeps growing over the years and continues to benefit the economy but however, this all comes with a significant price to pay which is costing the environment. Nickel compounds may be released into the environment at high amounts at all stages of production [159]. Industries that make use of Ni have always faced environmental challenges such as air emissions, toxic effluents and waste. Airborne Ni compounds and $NiCl_2$ salts are carcinogenic to humans [160]. Exposure of Ni in occupational settings is still common [161]. The Ni plating industry and the coin

industry makes use of high concentrations of Ni sulphate and $NiCl_2$ and if in contact with the skin it results in hand eczema [162].

At x1 and x10 the WHO safety limit intake of Ni, no haemolysis was observed in the current study. At x100 the WHO safety limit haemolysis was observed. In a study conducted by Nielsen, 1999 [76], patients with eczema on their hands and Ni allergies submerged a finger into low Ni concentrations. The results showed extensive increase in the formation of vesicles and increase in blood flow with a comparison to a group that submerged their fingers in water. The Ni concentrations further caused inflammation and skin changes on patients who had sodium lauryl sulphate (SLS) treatment on the forearm skin [76].The WHO safety limit of Ni is 20µg/L. A study conducted by Sunderman, 1988 [163], found that thirty-two workers drank water polluted with $NiCl_2$ and Ni sulphate (1.63g/l) at a Ni electroplating factory and experienced nausea, short breath, abdominal pain, headaches, diarrhoea, coughs. These symptoms and signs of toxicity lasted for two days with uneventful recoveries for all workers [163]. Weischer, 1980 [164] has reported that oral administration of $NiCl_2$ in male rats at concentrations of 2.5-5.0 and 10 µg/ml in drinking water as well as inhalation exposure to NiO aerosols (0.2; 0.4 and 0.8 mg/m^3) throughout 28-days caused a dosage dependent increase in urea (nitrogen containing substance), a reduction in serum creatine and an excess of glucose in the bloodstream [164]. A study by Das, 2008 [165] established that intraperitoneal administrative exposure of Ni sulphate in rats resulted in a significant reduction of the erythrocyte count, haemoglobin concentrations and haematocrit value (PVC) when compared to the untreated control. An increase in clot formation time and a decrease in leukocyte and platelet count was also reported. A decrease in platelet and leukocyte count can manifest to Ni-induced anaemia (non-regenerative anaemia) which arises as a result from damaged haematopoietic stem cells. Nickel sulphate ($NiSO_4$) has the possibility of reducing different kinds of blood cells in rats and to inhibit bone marrow activity [165]. At concentrations of x1000 the WHO safety limit, no haemolysis was observed in the current study. At x10000 the WHO safety limit the haemolysis observed was minimal at (1.66%). In this study we observed that Ni caused minimal or no haemolysis at its lowest and highest concentrations respectively. From the literature review, noticeable effects of Ni- induced toxicity are seen with concentrations above 1000 mg [74]. A mechanism of heavy metal toxicity via transferal of electrons usually involves the cross-linking of protein groups. Nickel generates free radicals straight from molecular oxygen to generate superoxide anion. The anions produced can bind to protons in a reaction thus producing hydrogen peroxide during the reaction [165]. Multiple studies making use of cultured human peripheral blood lymphocytes suggest that oxidative species in humans can be induced by Ni [166-167]. An *in vitro*

experiment using lymphocytes treated with $NiCl_2$ to evaluate oxidative effects of acute Ni exposure was studied. An increase in the generation of lipid peroxidation and hydrogen peroxide was observed. Factors such as intracellular ROS, lipid peroxidation and hydroxyl radicals and the possible effects of antioxidants were scrutinized. Hydroxyl radical levels were much higher in the treated group than in the control. Partial reduction of $NiCl_2$ induced elevation of oxidants was due to catalase, while SOD enhanced the oxidants levels. The accelerated production of hydroxyl radicals might play a role in oxidative damage in lymphocytes exposed to $NiCl_2$ [167]. Furthermore, antioxidants such as GSH, catalase and mannitol were capable of providing protection against Ni induced oxidative stress [167]. The intrinsic antioxidants defence system of the body can ensure that potential lethal oxygen species from free radicals are kept under control within physiological conditions. Excessive production of these oxygen species might overpower the cellular antioxidant defence system, thus resulting in oxidative stress [165]. Literature also suggests that antioxidants can reduce some dangers of Ni [168].

The WHO safety limit of Mn is 400 µg/L. The tolerable daily intake (TDI) is 60 µg/kg of body weight, based on the upper range value of Mn intake of 11000 µg/day [169-170]. As much as excessive consumption of Mn can lead to toxicity, deficiency of Mn can result in adverse effects. Figure 4.1 A, B and C show minimal or no haemolysis of x1, x10 and x100 the WHO safety limit of Mn. Manganese metalloenzyme SOD prevents lipid peroxidation. Lipid peroxidation is the process by which lipid oxidative degradation occurs. Radicals scavenge electrons from lipids within the cell, resulting in cellular damage. Manganese dependent enzymes prevents the process of lipid peroxidation by superoxide radicals [171-172]. Various studies on rats have been done to obtain an understanding of what impact Mn has on rats. In an animal study conducted by Zidenburg-Cherr, 1983 [173], the study found that Mn deficient rats had low Mn SOD activity in the liver and the heart [173]. A study done by Brock, 1994 [174], indicated that a Mn deficient diet fed to rats caused a decrease in the plasma urea concentration that was related with a decrease in arginase activity and an increase in plasma ammonia concentrations [174]. Nutritional Mn plays a protective role against lipid peroxidation of the mitochondrial membrane heart tissue of Sprague-Dawley rats [175]. Rats fed a low Mn diet had decreased SOD activity in the heart [176]. Deficiency of Mn also causes decreased pancreatic insulin synthesis [177]. Recent studies indicate that 5 mg/kg of Mn in the diet is suitable for normal growth and development and it is within the WHO safety limit [178]. Findings that 5 mg/kg of Mn in the diet is suitable for normal growth and development indicate contradictory data about dietary Mn requirements regarding rats. The national requirements of laboratory animals (NRC 1995) [179] estimated the Mn dietary requirement to be at

10 mg/kg due to findings indicating that diverse breeds of rats react differently to Mn consumption [179]. Overexposure of Mn can cause toxicity. Extrapyramidal dysfunction and neuropsychiatric symptoms are clinical symptoms associated with Mn intoxication. Neurotoxicity studies of Mn are well documented and recognised [180-181], however, the consequence of cardiovascular system related to Mn has minimal information. A study done by Kobert, 1883 [182] proposed that Mn salts can cause decreased blood pressure and it is validated by experiments done on animal models compromising of rats, dogs and cats [182]. A decline in myocardial contraction is associated with chronic exposure to Mn. Subsequent to venous inoculation of 0.5-5 mg of $MnCl_2$/kg, the P-R and Q-T intervals on an electrocardiography (ECG) machine were elongated and the QRS wave was broad. The results were due to weakened myocardial contraction due to Mn toxicity directly affecting the mitochondrial function [183]. In the same study, when the rats were fed 1000 mg of Mn orally for eight weeks, the mitochondria swelled up and vacuoles in cardiac myocytes were seen by electron microscopy. Authors of the research suggested that Mn exposure may impair the myocyte cytoplasmic membrane, cause vacuoles and increase mitochondrial membrane permeability. A dysfunctional mitochondrion contributes to a decline in the contraction of the heart [183].

Figure 4D and E (x1000 and x10000 the WHO safety limit) shows haemolysis. Manganese can cause vasodilation of the vessels, thus causing decreased blood pressure following high administration of Mn [184-185]. In a study using dogs, where 10 mg/kg of $MnCl_2$ was administered daily for four days through the inferior vena cava, the blood pressure dropped significantly accompanied by reflex tachycardia [185]. Effects of Mn exposure in occupational settings suggest that chronic Mn exposure from a Mn ferroalloy company affect the normal functioning of the heart. The geometric mean concentration of airborne Mn (as MnO_2) in the working environment was 0.07 mg/m$^{3.}$ The heart rates of the workers were rapid, and the P-R intervals on an ECG were seen to be brief in the workers smelters group of females than the female controls. The QRS waves and T waves were elevated and broader respectively in both females and males smelting workers than in controls [183,186]. In another study workers exposed to Mn dust, the airborne Mn concentration was 0.13 mg/m^3 in the working environment. The data obtained did not show any relationship between the ECG changes and the level of Mn exposure but however, several workers exposed to Mn dust suffered arterial and vein ailments [183]. With a geometric mean of 1.96 mg/m^3 of Mn in the environment, leading symptoms documented were light-headedness, headaches, memory loss, tiredness and sleeping disorders. Results from the ECG did not show abnormality among exposed workers in comparison with the control subject [183]. Employees

exposed to Mn dust particles did not show any alterations with regards to the heart function, but nonetheless their diastolic pressure was significantly lower than the control group [187]. The diastolic pressure among Mn-exposed workers indicated an indirectly proportional relationship to age and time. Diastolic pressure in the age group 20-30 years was higher compared to the other age groups and was also higher in female employees exposed to Mn in comparison to the male employees. From the results obtained in that study it was indicated that youthful and female employees were more vulnerable to Mn stimulated arterial and venous dilation outcomes [187]. Research done in various groups indicated that Mn exposure resulted in vasodilation, thus causing a decrease in diastolic pressure [183].

It is rare that metal toxicity is restricted to one metal. Most toxicity studies of heavy metals are focused on one metal at a time. Metals from industrial areas may be emitted into the air one at a time but if there are various companies in a surrounding area that emit different metals, those metals will be found in combination within the atmosphere. Heavy metal exposure to humans through food, air and water usually encompasses a mixture of numerous heavy metals at different concentrations. Interactions between metals may possibly decrease or increase the degree of toxicity. This is dependent on the nature of the individual metal.

In this study Hg, Ni and Mn were combined to observe what effects the double combination of these heavy metals have on erythrocytes. The combinations of the metals were Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn at five point increasing concentrations of the WHO safety limit. A synergistic effect is of great concern because this results in the combination of metals being toxic to a greater extent than the metal on its own. The Hg+Ni double combination at x1-x1000 resulted in no haemolysis. As shown in Table 4, the effects of the double combination was antagonistic at x1, x100 and x1000 but was synergistic at x10. At x1 the WHO safety limit the single metal Hg caused haemolysis, but Ni caused no haemolysis. These findings together with that shown in Table 4 indicate that the two metals have an antagonistic effect on each other. The same observations are seen at x10, x100 and x1000. At x10000 the WHO safety limit, the combination of the two metals result in haemolysis and caused a synergistic effect. A study on synergistic effect of Ni and Hg on fatty acid composition in the muscle of fish conducted by Senthamilselvan, 2016 [188] found that at high concentrations of 0.8, 2.0 and 4.0 mg/L of Hg alone, Ni alone and Hg+Ni combination caused a decrease in fatty acid composition [188]. Similar findings were also observed in a study done by Kawamoto, 2007 [189] and Konar, 2010 [190]. Nutritional benefits of fish are found in high quality protein and high content of two ω-3-polyunsaturated fatty acids. The exposure of fish to Hg and Ni alone and in combination resulted in damage of the positive effects

of ω-3 fatty acids present in fish and could lead to heart disease [191]. A decrease in monosaturated and polyunsaturated fatty acid was due to metals interrupting the prostaglandin biosynthesis pathway. The modification of the metabolic pathway by the metals might play a big role in reducing the levels of polyunsaturated fatty acids [192]. The transfer of heavy metals from aquatic species to humans is through their food chain. Heavy metals accumulate in the edible portions of fish such as muscle and skin. Physiological and biochemical responses occur when aquatic species absorb toxins [193].

Mercury is a toxic and inhibitory metal in nature whilst Mn is an enhancer of growth at recommended WHO safety limits. The double combination of Hg+Mn yields no haemolysis at x1-x1000 concentrations respectively. The interaction between Hg+Mn is antagonistic at x1, x100 and x1000 but additive for x10 the WHO safety limit. At concentrations of Mn above the WHO safety limit alone or in combination can be toxic. In a study done by Pathak, 1987 [194], Hg and Mn interaction studies on barley germination and phytotoxicity were studied. At x10000 the WHO safety limit, it is observed that haemolysis occurs. At x10000 WHO safety limit of each metal respectively causes haemolysis. However, the combination of the two metals causes an antagonistic effect rather than a synergistic effect. Manganese reduces the toxic effect of Hg when in combination.

The results obtained from the combination of Mn+Ni showed that at x1 the WHO safety limit, there was no haemolysis observed and the interaction between the two metals was antagonistic. At x10-x10000 the WHO safety limit, haemolysis was observed and the interaction between the metals at the various concentrations respectively yielded a synergistic effect. Nickel interrelates with at least 13 other essential elements found in plants animals and organisms such as Zn, Na, P, Mn, K, Fe, Cu, Co, among others. The pathogenic effects of Ni may be due to the interloping with metabolism of important metals like Mn, Fe, Zn, Ca or Mg [74]. In a study of calves administered Ni supplementation (0.45-0.57 mg/kg) for a maximum of 140 days, Ni caused a reduction in Mn concentrations in the muscles of calves [195]. Limited information exists about the interaction of Ni and Mn on animal studies or human studies. One of the most important interactions of Ni is with Fe. A study based on the beneficial effect of Ni on haematopoiesis in moderately iron (Fe)-deficient rats was due to physiologic and/or pharmacologic mechanisms. The rats were administered dietary Ni at concentrations of 5, 10, 20 or 50 mg/kg and it was observed that these concentrations favoured haematopoiesis and increased the Fe content of rats who were Fe deficient. At nutritional Ni concentrations (0.3, 50, or 100 mg/kg) the Fe concentrations were not significantly influenced in the plasma, femur, kidney, spleen and the liver. Simultaneously, Zn

concentrations in the femur of rats were drastically reduced after administration of 100 mg/kg of Ni [74]. It was reported that the interaction between Fe and Ni in rats caused changes in haemoglobin levels, plasma alkaline phosphate activity, haematocrit, plasma phospholipid level, liver lipid extracts yellow pigment, liver Cu concentrations, and possibly liver Mn and Ni concentration. Nickel deficiency developed quickly and severely in the rats with minimal Fe dietary levels. The results obtained from rats fed minimal iron (Fe), indicated that Ni deficiency impairs the absorption of Fe. The deficiency of Fe causes more harm to Ni-supplemented than to Ni deficient rats. Growth is drastically supressed and pre-natal mortality was observed in Ni-supplemented rats [74]. The same effect can be deduced from this study, metal combination of Ni with Hg or Mn at x1, x100 and x1000 indicated an antagonistic effect. Nickel has both synergistic and antagonistic effects when it interacts with other elements.

The interaction of the triple combination (Hg+Ni+Mn) of the metals only caused haemolysis at x1 and x10000 the WHO safety limit. At x10, x100 and x1000 there was no significant haemolysis observed. A dosage dependent increase in haemolysis was not observed. The interaction at x1 and x10000 was both synergistic. The percentage haemolysis at x10000 the WHO safety limit was at 58%. To our knowledge, there is no literature available on the effect of Hg, Ni and Mn in combination on humans or animals.

The presence of heavy metals alters coagulation factors by causing endothelial cells to undergo oxidative stress. Vascular endothelium contains epithelial cells that are specialized and line the luminal surface of all blood vessels. Endothelial cells undergo damage or death if exposed to sufficiently high concentrations of heavy metals [196]. Damage to endothelial cells causes loss of endothelial barrier integrity and therefore oedema. An increase in haemolysis affects the coagulation system; haemolysed erythrocytes can cause possible increase in fibrinogen, factor VII and factor V. Intravascular coagulation also causes an increased release in thrombo-plastic properties by the haemolysed erythrocytes [196]. The effect of heavy metals on the erythrocytes should not be limited to the amount of haemoglobin released by the cell but also what effect the metals have on the morphology of the cells. The coagulation system consists of erythrocytes, platelets and fibrin network. The morphological effects of the metals on components of the coagulation system were studied by using scanning electron microscopy.

5.2 Scanning Electron Microscopy

In this study, scanning electron microscopy was used the investigate the morphological changes caused by the exposure of whole blood to the heavy metals alone and in combination in order to determine the outcomes of these metals on elements of the coagulation system. Erythrocytes are the most abundant cell type in the blood. Erythrocytes have a discoid shape and lack a nucleus and mitochondria, making them ideal cells to study membrane changes [98]. Erythrocyte function involves the transportation of oxygen in the body. Erythrocytes are roughly 1.7 to 2.2 µm thick and. 7.5 to 8.7 µm in diameter, and circulate through the heart approximately 170 000 times withstanding osmotic swelling, deformability while passing through small blood vessels and shrinkage while travelling through the lungs and kidneys [197]. The biconcave shape of erythrocytes and its ability to deform is a vital feature of the cells' biological function. The disruption of this important characteristic may be due to acquired pathological conditions, external stress and genetics [197]. The membrane of erythrocytes is flexible with a high surface-to-volume ratio which consists of three layers, the carbohydrate-rich glycocalyx on the exterior, the lipid bilayer that contains transmembrane proteins and the membrane skeleton consisting of a structural network of proteins located on the inner surface of the lipid bilayer. The lipid bilayer has minimal shear resistance and is accountable for the changes in the shape of the cell, elasticity and toughness, and assists in reclaiming the discoid shape [198]. The cell's health state is specified by the rough membrane surface [200-201]. Erythrocytes are extremely sensitive cells and aid in being a component of the body's health indicator [98]. The distribution of heavy metals causes adverse effects on blood homeostasis thus resulting in changes and damage to erythrocytes [147]. The normal functioning of erythrocytes is primarily dependent on the cell's intact membrane.

Adenosine Triphosphate (ATP) is essential in maintaining the discoid shape of erythrocytes. Gov, 2005 [97] conducted a study to determine the function of ATP in erythrocytes and found that the depletion of ATP causes morphological changes of the cells from biconcave to echinocyte shape [97]. Alterations in ATP levels in *in vitro* laboratory experimental conditions stimulated erythrocyte shape changes and increased membrane fluctuations. Changes of ATP *in vivo* could possibly result from infections, trauma, hereditary abnormalities associated with erythrocytes, cancer as well as age related diseases. In a study conducted by Park, 2010 [201] where phase microscopy indicated a direct cause and effect of reduced ATP reduction thus resulting in an increase on the magnitude of erythrocytes membrane instability. The fluctuations of erythrocytes are directly associated to the cytoskeleton network and membrane bilayer. The results obtained suggested that crucial binding between the spectrin network and the bilayer is actively controlled by ATP.

ATP provides necessary energy for the binding of such a dynamic process therefore making ATP important in maintaining the discoid shape of erythrocytes. Park, 2010 [201] also observed morphological changes of erythrocytes from normal discocyte shape to echinocytes due to the absence of ATP. The morphological transformation is reversible with restoration in normal ATP levels [201]. Damage to erythrocytes or the membrane prevents it from functioning adequately leading to destruction of the cell by means of haemolysis or eryptosis. Exposure of toxins, including heavy metals, to the membrane of erythrocytes alters the morphology and functioning of the cell [10,146,130]. To facilitate this fluidity, dynamic cytoskeleton remodelling of the spectrin network was shown [202]. There is a percentage of abnormal erythrocytes in a healthy individual. In a study done using light microscopy in 1977, 55% of the erythrocytes observed were deformable with a bowl-shaped structure, 44% had a rigid discocyte shape and 1% were echinocytes and knizocytes [203]. Erythrocytes can undergo various shape changes such as discocyte, echinocytes, spherocytes, codocyte, knizocyte and stomatocytes [204-205]. Erythrocytes exposed to Hg (x1, x10 and x100) alone and in combination caused the erythrocytes to change from the normal biconcave shape to echinocytes (spike like nodules). In a study conducted by Lim, 2010 [206] exposure of Hg at 0.25 µM to erythrocytes altered the biconcave shape into echinocytes and further into spherocytes depending on the length of exposure. Erythrocytes change into spherocytes by extensive loss of membrane surface due to the shape buffering capacity being lost thus resulting in a new default shape and formation of vesicles [206] Mercury concentrations as low as millimolars have been reported to stimulate haemolysis and shape changes. Suwalsky, 2000 [207], found that echinocytes can further change their shape to spherocytes due to longer exposure of Hg^{2+} to the cells [207]. Similar results are seen in Figure 4.2B where erythrocytes have lost their biconcave morphology. In a study done by Maheshwari, 2016 [208] on the effect of $HgCl_2$ on fish Channa Punctatus, it was found that substantial changes in the morphology of erythrocytes was observed. The effects of Hg were duration and dosage dependant. The changes observed on the morphology of erythrocytes are indicative of poor health status of the fish. The erythrocytes were round and swollen and alterations in morphology are associated with heavy metal pollution and pesticides. As a result of Hg having higher affinity for sulfhydryl groups, the binding of the two leads to their inactivation. Thus, a decrease of antioxidant activity of the membrane. Cells with depleted ATP and increased Ca^{2+} generate echinocytes but this can however be reversed by the restitution of suitable ATP and Ca^{2+} levels which will have a result of discocyte erythrocytes. Observation from this study also proposes that $HgCl_2$ has echinocyte formation properties [208]. Exposure of erythrocytes to Ni caused cells to change from the typical biconcave morphology to cell blebbing (bulging appearance). With increase in concentration more cell blebbing was

observed. A study done by De Luca, 2007 [208] indicated that exposure of $NiCl_2$ to erythrocytes caused morphological changes. The erythrocytes appeared spherical and echinocytes were also observed [209]. Manganese caused shape changes to erythrocytes. In a study conducted by Chandel, 2016 [210] rats treated with Mn had distorted erythrocytes and it was dose-dependent, therefore suggesting that the metal lead to both quality and quantity decline in erythrocytes [210]. From the results obtained in the current study, Mn alone and in combination (x1, x10 and x100) caused changes to the erythrocytes. The cells changed from the normal biconcave shape to cell blebbing (bulging appearance), eryptosis also occurred as cell blebbing is a characteristic of eryptosis. There was an increase in deformation of erythrocytes with an increase in concentration. The interaction of the three metals (Hg+Ni+Mn) had increased deformation of erythrocytes as seen in Figures 4.2, 4.3 and 4.4 of the combination of metals. All erythrocytes exposed to the metals were distinctively different from the control thus suggesting that heavy metal exposure does cause morphological changes. Increase in heavy metal concentration causes more damage to the cells. The presence of echinocytes was more noticeable in the x100 triple combination of metals.

Platelets have no nuclei and are composed of fragments of the cytoplasm derived from megakaryocytes [211]. Their function is to regulate hemostasis as well as clotting of blood in the circulatory system [11]. Platelets play a role in innate immunity as well as regulating tumour growth and movement of leukocytes from the capillaries to the surrounding tissue in the vessel [212]. The crucial functions of the platelets signify its natural functions and flexibility in circulation. The restructuring of the platelet's cytoskeleton is an essential factor in the complex mechanisms found in hemostasis and thrombus formation. The regulation of platelet shape changes is primarily due to the cytoskeleton. Actin is the main cytoskeletal component in platelets [213-214]. Platelet aggregating agents include adenosine diphosphate (ADP), thrombin and collagen which is the main cause of thrombosis in humans and animals. Calcium and cyclic adenosine monophosphate (cAMP) are responsible for modulating the levels of platelet reactivity as a reaction to various agonists. Platelet function is mainly regulated by cAMP and the increase of intracellular cAMP by any agent is accountable for inhibiting platelet aggregation as indicated by prostaglandins [215]. Upon platelet activation, the platelets change shape and spread and cluster together to form a very tight clot. Furthermore, platelets are entangled in fibrin fibre mesh, along with erythrocytes [11,215]. In the establishment of an enduring platelet plug at the site of damage, it is necessary that both the initiation and propagation of platelets be activated. Bare collagen and vWF typically provide initiation in the wall of the vessel and by the generation of thrombin as soon as the

formation of tissue factor/VIIa complexes are completed [11]. The spreading of platelets takes place once more platelets are employed to form a plug by secreting thromboxane A_2 (TxA2), ADP and the α-granule product. Platelet dense granules actively secrete ADP and damaged erythrocytes and endothelial cells passively. Upon stimulation with ADP, platelets begin to change their shape and phospholipase C activity and cytosolic Ca^{2+} increases. ADP represses the production of cAMP, a result that is important for the occurrence of platelet activation [216-217]. Various other proteins are also involved in the restructuring of the cytoskeleton. Platelets from whole blood exposed to Hg x1, x10 and x100 were seen to differ in morphology to the control platelets (Figure 4.5). An increase in pseudopods and membrane spreading was observed as well as the presence of a rough membrane surface. These findings are similar to those observed by Janse van Rensburg, 2017 [147] where erythrocytes exposed to Hg at varying concentrations appeared to have the same morphology as seen in this study [147]. Platelets from whole blood exposed to Ni became over activated with an increase in pseudopodia. With increased exposure of metals, multiple pseudopodia were observed, and platelets aggregated to form a clot.

Tobacco is an agricultural crop used to make cigarettes. Tobacco, cigarette paper, filters, and cigarette smoke consist of various heavy metals such as Co, Hg, Pb, Cr, Cd, Al, Ni and Zn [218]. In a study conducted by Pretorius, 2013 [219] the results showed that platelets from smokers are highly activated compared to the control [219]. The same conclusion was also reached by a study by Padmavathi, 2010 [220] that smoking induces alterations in platelet membrane fluidity and NA^+/K^+-ATPase activity and directly causes platelets to aggregate, increase in adhesion and increased pseudopods [220]. Manganese exposure caused the platelets to have increased pseudopodia, membrane spreading and activation. The combination of metals at varying concentrations (Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn) also showed platelet changes, formation of increased pseudopodia, membrane spreading, highly activated platelets and platelet interaction. Interaction of the activated platelets and shown in Figure 4.6 B & E and Figure 4.7D &G. The changes observed in platelets are due to the generation of reactive oxygen and nitrogen species, they are correlated with an increase in lipid peroxidation and carbonyl groups [221]. Critical signal regulating platelet activity has been suggested to be caused by ROS. A burst of H_2O_2 is associated with platelet aggregation mediated by fibrinogen binding to integrin aIIbb3 (GPIIb/ IIIa) independent of ADP secreted from dense granules [222-224]. The activation of platelets and morphological changes such as membrane spreading, pseudopods and platelet aggregation are a good indication of the coagulation cascade being initiated and activated. Activation of platelets was observed in all

experimental groups with Hg, Ni and Mn causing more changes to platelet morphology. All metals caused platelet activation.

One of the most important structural components of coagulation is fibrin fibres. The collaboration of fibrin and thrombin plays a major role in blood clotting. Thrombin enables the conversion of factor XIII to factor XIIIa and the conversion of fibrinogen to fibrin [11,225]. The fibrin fibres form horizontal and vertical cross-linkages as seen from the images obtained in Figure 4.8, 4.9 and 4.10. The addition of thrombin to the whole blood entraps the erythrocytes within a mesh of fibres (Figure 4.9A). It is important to note that the erythrocytes of healthy individuals keep their biconcave shape and do not fold around the fibrin fibres. Nonetheless, for the duration of systemic inflammation and conditions linked with the enhancement of eryptosis, the erythrocytes fold around the fibres losing their shape. This demonstrates vulnerability of the membrane. The cells of healthy individuals will slightly deform when fibrin is formed around and over the cells but still maintain the discoid shape. Typical characteristics of fibrin network usually encompasses particularly major thick fibres with a few thin fibres dispersed amongst them [226-227]. Figure 4.8F exhibits fibres and fibre aggregates thus increasing clot formation [228]. The presence of heavy metals causes the production of ROS which result in the indirect activation of platelets because the ROS scavenge nitric oxide [229]. In a study conducted to determine the effect of fibrin structure on fibrinolysis it was determined that fibrin structure contributes to the regulation of the fibrinolytic rate. As the fibrin fibre decreases in size, the fibrinolytic rate also decreases [230]. The structure of fibrin was altered by changing the ratio of thrombin to fibrinogen. Thin fibrin causes decreased fibrinolytic rate [230].

The construction of the fibrin network has been shown to influence the process of fibrinolysis, and the breakdown process of fibrin fibres. In a study conducted by Collet, 2000 [231] fibrin fibres that are highly compacted and composed of thin fibrin fibres breaks down more slowly than the fibres with a loose fibrin fibre formation that is composed of thicker fibrin fibres [231]. From the results obtained, erythrocytes become entrapped within the fibrin mesh together with the platelets as seen in Figure 4.8A. Thick and thin fibrin fibres are seen around the cells. Fibrin network forms a mesh structure in and around the erythrocytes. An increase in fibre thickness and fibre aggregation increases the formation of a clot [228]. In Figure 4.10A, C, D, E and F, fibrin network forming a mesh was observed. In general, the scanning electron micrographs of the whole blood with added thrombin showed that the different metal groups at different concentrations can form well defined clots. Fibrin fibres that are less organised with less taut fibres are present when the clot breaks down to repair the site of injury.

5.3 Confocal Laser Microscopy

Injury or damage to the erythrocytes results in the removal by premature suicidal death or by a process known as eryptosis. Studies pertaining to eryptosis have focussed on determining if membrane changes have occurred, specifically whether Ca^{2+} leakage into the cell changes to ceramide, a PS flip and cell shrinkage have occurred. Eryptosis is characterized by cell shrinkage, cell blebbing and membrane scrambling resulting in the exposure of PS from the internal membrane of the cell surface. Eryptosis is triggered by a wide variety of causes such as oxidative stress, the presence of xenobiotic and endogenous substances, energy depletion, hyperosmolarity, increase in Ca^{2+}, antibiotics, ageing of the cells, inflammatory diseases such as Parkinson's disease, type two diabetes, increase in temperature and heavy metal exposure [232]. Heavy metals account for most of the causes of eryptosis. The impact of eryptosis increases with an increase in age of erythrocytes, and this is due to increased sensitivity to oxidative stress [112]. In a study conducted on the impact of erythrocyte age on eryptosis, the study concluded that susceptibility to oxidation-induced PS exposure is increased in older erythrocytes than younger cells. An advantage of eryptosis is eryptosis-mediated removal of damaged erythrocytes from the circulatory system [113]. Stressors, including oxidative stress, cause injury to erythrocytes thus triggering eryptosis. Two signalling pathways are responsible for triggering eryptosis: (a) the production of phospholipase E (2) activation of Ca^{2+} permeable cation channels, and (b): phospholipase A (2) -mediated release of platelet-activating factor (PAF) activates a sphingomyelinase, leading to formation of ceramide [112]. Increased cytosolic Ca^{2+} activity and enhanced ceramide levels lead to membrane scrambling with subsequent PS exposure from the inner membrane to the outer cell membrane [233]. The exposed PS at the cell surface binds to receptors of phagocytes resulting in the cell being engulfed and degraded [234]. The cell is cleared from the circulatory system rapidly [235]. The number of erythrocytes will remain constant as long as stimulus of erythropoiesis is significantly greater than eryptosis [234]. Erythrocytes may make use of eryptosis to escape haemolysis; however excessive eryptosis may lead to anaemia [112]. Eryptosis is an essential protective mechanism in certain cases as it provides the cells with another form of cell death excluding haemolysis. The haemolysis of incapacitated and impaired erythrocytes causes a release of haemoglobin and other cell contents into the bloodstream. Eryptosis is therefore an efficient way for erythrocyte cell death as it averts haemolysis and the difficulties concerned with haemolysis. In a study conducted by Lim, 2010 [34] Hg^{2+} induced the exposure of PS on erythrocytes. Exposure of cells to Hg^{2+} facilitated the decrease of protein thiol, which caused an increase in Ca^{2+} and depleting ATP and causing flippase inhibition. Increased

levels of intracellular Ca^{2+} causes scramblase activation to increase, thus consequently resulting in augmented attachment of erythrocytes to endothelial cells, exposure of PS to the extracellular surface of erythrocytes and accelerated thrombin generation. The depletion of thiol by Hg^{2+} can be clarified by the resilient thiol-binding affinity of nucleophilic Hg^{2+}. Thrombosis and hemostasis through procoagulant activation can be possibly contributed by erythrocytes via the exposure of PS and micro-vesicle generation. The exposure of PS is seen in figure 4.12 B, E and F. In all three figures Hg alone and in combination to Ni or Mn causes PS flip and exposure onto the cell's membrane surface. A study on employees occupationally exposed to mercuric vapour exhibited very high blood coagulation levels together with exacerbated thrombin generation levels [236]. Mercury induced procoagulation activation of erythrocytes might possibly influence the increase of cardiovascular diseases in human population. Zwaal, 1977 [237] conducted a study that concluded that modifications of erythrocyte membrane such as the exposure of PS and microvesicles formation is reported to cause erythrocytes to be procoagulant thus enabling erythrocytes to partake in thrombosis [237-238]. In a study conducted by Jang, 2011 [236] Pb^{2+} caused an increased PS exposure and micro-vesicle generation in erythrocytes through the depletion of ATP. The results obtained from the confocal laser scanning microscope in the current study showed that exposure of Ni to erythrocytes caused a PS flip. Hg, Ni and Mn alone and in combination caused changes in erythrocyte morphology. Although all three metals have different mechanisms of causing toxicity, the result thereof is comparable to eryptosis.

6. CONCLUSION

South Africa is one of the fastest emerging countries in Africa and in the world with industrialization at the top of its list. Energy generation from coal and mining activity is the greatest contributor to the economy. Metal pollution unavoidably occurs thus causing adverse effects to the environment and human health. Human exposure to heavy metals is often to a combination of metals rather than single metals. From the study it can be concluded that the presence of heavy metals impacts on the integrity of erythrocytes and causes membrane damage. Mercury is a very toxic metal and it caused increased haemolysis as compared to Ni, Mn, double and triple combinations. Low concentrations of all the metals had minimal or no haemolytic effect. With an increase in metal concentration, increased haemolysis was observed especially at x100 and x10 000 with Hg. Variability was observed throughout all five different concentrations and metals but overall it was observed that the presence of heavy metals on the membrane integrity of erythrocytes caused some level of toxicity. Mercury had the highest level of toxicity. Heavy metals induced haemolysis as a result of the cells undergoing oxidative stress. The seven experimental groups with whole blood exposed to heavy metals resulted in morphological changes of erythrocytes. Heavy metals impact on the morphology of erythrocytes, platelets and fibrin network. Exposure of heavy metals to the cells increased the formation of echinocytes and further caused increased roughness on the membrane surface. Platelets became over activated with increased pseudopodia formation and membrane spreading. An increase in membrane spreading and pseudopodia formation is a clear sign for increased platelet activation, that can intensify the probability of clot formation [34]. The membrane surface had a necrotic appearance as a result of heavy metal exposure. Thick and thin fibres were observed. Fibre thickness and platelet aggregation form a coagulant state of blood and results in the formation of a thrombus which accounts for cardiovascular diseases. Annexin V positive signal was observed from all the metals at x10 WHO safety limit. Heavy metals are environmental pollutants, toxins and oxidative stress promoters that are associated with a number of diseases that are disadvantageous to our health [1,3,239]. This study gives us a better understanding of the impact of Hg, Ni and Mn in our bodies as well as on the coagulation system.

Future perspectives and limitations

Additional techniques can be implemented to give a better understanding and provide future prospective of the effects heavy metals have on the erythrocytes, platelets and fibrin network. The Thromboelastography method can be utilized to quantitatively evaluate whole blood clotting parameters. The use of confocal microscopy together with an ultrastructural method such as SEM is a complete validation of eryptosis. Eryptosis can also be determined using flow cytometry. The use of both confocal laser microscopy and flow cytometry can also validate how eryptosis affects erythrocytes. Clot lysing is just as important as clot formation. A clot lysing technique can be added to the study to determine how a clot is lysed and the estimated time it takes for a clot to break down before thrombosis occurs. An increase in sample size might also increase the statistical relevance as well as give a better representation of the population.

Future studies could include the use of blood from people who reside near mines, Eskom coal power plants in Witbank and workers who are occupationally exposed to metals. It would be interesting to observe the possible changes to the components of their coagulation system. In future a larger sample size can be used to give a better representation of the population. The effect on women could be studied and compared to the results of men. The effect of other toxic metals can be investigated and compared to the effect of the metals used in the study.

7. REFERENCES

1. Friberg L NG, Vouk VB. Handbook on the toxicology of metals. The Netherlands: Elsevier/North-Holland Biomedical Press; 1979.

2. Organization WH. Trace elements in human nutrition: Report of a WHO expert committee [meeting held in geneva from 9 to 17 april 1973]. 1973.

3. Fergusson JE. Heavy elements: Chemistry, environmental impact and health effects: Pergamon; 1990.

4. Jansen E, Michels M, Van Til M, Doelman P. Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. Biology Fertilizing Soils 1994; 40:515-24.

5. Hiraki M. Populations of cd-tolerant microorganisms in soils polluted with heavy metals. Soil science and plant nutrition. 1994; 40(3):515-24.

6. Starzecka A, Bednarz T. Comparison of development and metabolic activity of algae and bacteria in soils under the influence of short-and long-term contamination with metallurgic industrial dusts. Algological Studies/Archiv für Hydrobiologie, Supplement Volumes. 1993:71-88.

7. Zouboulis A, Loukidou M, Matis K. Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. Process Biochemistry. 2004; 39(8):909-16.

8. Tadiboyina R, Ptsrk PR. Trace analysis of heavy metals in ground waters of vijayawada industrial area. International Journal of Environmental and Science Education. 2016; 11(10):3215-29.

9. Smith J. Erythrocyte membrane: Structure, function, and pathophysiology. Veterinary pathology. 1987; 24(6):471-6.

10. Pretorius E, Vermeulen N, Bester J, du Plooy JL, Gericke GS. The effect of iron overload on red blood cell morphology. The Lancet. 2014; 383(9918):722.

11. Silverton DU OW, Garrison CW, Silverthorn AC, Johnson BR. Human physiology: An intergrated approach. 6 ed. San Francisco: Pearson/ Benjamin Cummings; 2010.

12. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: Initiation, maintenance, and regulation. Biochemistry. 1991; 30(43):10363-70.

13. El Baz S, Baz M, Barakate M, Hassani L, El Gharmali A, Imziln B. Resistance to and accumulation of heavy metals by antinobacteria isolated from abandoned mining areas. The Scientific World Journal. 2015; 2015:1-14

14. Coblenz A, Wolf K. The role of glutathione biosynthesis in heavy metal resistance in the fission yeast schizosaccharomyces pombe. FEMS microbiology reviews. 1994; 14(4):303-8.

15. Li F, Tan T. Monitoring bod in the presence of heavy metal ions using a poly (4-vinylpyridine)-coated microbial sensor. Biosensors and Bioelectronics. 1994; 9(6):445-55.

16. Wood JM, Wang H-K. Microbial resistance to heavy metals. Environmental science & technology. 1983; 17(12):582A-90A.

17. Gadd GM. Metals and microorganisms: A problem of definition. FEMS Microbiology Letters. 1992; 100(1-3):197-203.

18. Eiland F. The effects of application of sewage sludge on microorganisms in soil [microbial biomass, microbial activity, enzymatic activity, sewage sludge, heavy metals]. Journal for plant breeding (Denmark). 1981.

19. Virtanen JK, Rissanen TH, Voutilainen S, Tuomainen T-P. Mercury as a risk factor for cardiovascular diseases. The Journal of nutritional biochemistry. 2007; 18(2):75-85.

20. Mamba BB, Rietveld LC, Verberk JQJC. South africa drinking water standards under the microscope. Water Wheel. 2008; 7:24-7.

21. Pirrone N, Aas W, Cinnirella S, Ebinghaus R, Hedgecock IM, Pacyna J, et al. Toward the next generation of air quality monitoring: Mercury. Atmospheric Environment. 2013; 80:599-611.

22. Coombs WM. Managanese-the silent poison. Occupational Health South Africa, 2005.

23. Singo NK. An assessment of heavy metal pollution near an old copper mine dump in musina, south africa. 2013.

24. McKay D. Strong rand pummels debt-laden sibanye-stillwater. finweek. 2018; 2018(9):34-8.

25. Peralta-Videa JR, Lopez ML, Narayan M, Saupe G, Gardea-Torresdey J. The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. The international journal of biochemistry & cell biology. 2009; 41(8-9):1665-77.

26. E C [Internet] Mercury and the environment: Sources of mercury. [updated 2016 February]. Available from: http://www.ec.gc.ca//mercuremercury/default.asp?long=En&n=2C1BBBB.

27. Mahaffey KR, Mergler D. Blood levels of total and organic mercury in residents of the upper st. Lawrence river basin, quebec: Association with age, gender, and fish consumption. Environmental research. 1998; 77(2):104-14.

28. Salonen JT, Seppänen K, Lakka TA, Salonen R, Kaplan GA. Mercury accumulation and accelerated progression of carotid atherosclerosis: A population-based prospective 4-year follow-up study in men in eastern finland. Atherosclerosis. 2000; 148(2):265-73.

29. Skinner EW, Phillips RW. The science of dental materials. 6 ed. Philadelphia: W.B. Saunders Co; 1969.

30. Organization WH. Exposure to mercury: A major public health concern. In: WHO, editor. Geneva: WHO Document Production Services; 2007.

31. Byczkowski JZ. Methyl mercury toxicity: pharmacokinetics and toxicodynamic aspects. Reviews Food Nutrition Toxicity. 2005; 3:61-84.

32. Berlin M, Zalups RK. Mercury. Chapter 33 in:(eds). Nordberg gf, fowler ba, nordberg m, friberg l. Handbook on the toxicology of metals 3rd ed. ISBN 978-0-12-3694213-3. Academic Press Publishers, Elsevier. 943pp, 2007.

33. Organization WH. Inorganic mercury. Environmental Health Criteria. 1991; 118.

34. Lim K-M, Kim S, Noh J-Y, Kim K, Jang W-H, Bae O-N, et al. Low-level mercury can enhance procoagulant activity of erythrocytes: A new contributing factor for mercury-related thrombotic disease. Environmental health perspectives. 2010; 118(7):928.

35. Ladizinski B, Mistry N, Kundu RV. Widespread use of toxic skin lightening compounds: Medical and psychosocial aspects. Dermatologic Clinics. 2011; 29(1):111-23.

36. McKelvey W, Gwynn RC, Jeffery N, Kass D, Thorpe LE, Garg RK, et al. A biomonitoring study of lead, cadmium, and mercury in the blood of new york city adults. Environmental Health Perspectives. 2007; 115(10):1435.

37. Dahl L, Bjørkkjaer T, Graff IE, Malde MK, Klementsen B. Fish--more than just omega 3. Journal for the Norwegian Medical Association: journal for medicine, new series. 2006; 126(3):309-11.

38. Daviglus M, Sheeshka J, Murkin E. Health benefits from eating fish. Comments on Toxicology. 2002; 8(4-6):345-74.

39. Rissanen T, Voutilainen S, Nyyssönen K, Lakka TA, Salonen JT. Fish oil–derived fatty acids, docosahexaenoic acid and docosapentaenoic acid, and the risk of acute coronary events: The kuopio ischaemic heart disease risk factor study. Circulation. 2000; 102(22):2677-9.

40. 24 H [Internet] See: Potentially dangerous mercury levels in sa retail fish. 2016 [updated 1 November 2016]. Available from: https://www.health24.com/Lifestyle/Environmental-health/News/see-potentially-dangerous-mercury-levels-in-sa-retail-fish-20161101.

41. Pacyna EG, Pacyna JM, Steenhuisen F, Wilson S. Global anthropogenic mercury emission inventory for 2000. Atmospheric environment. 2006; 40(22):4048-63.

42. Assessment UGM. Sources, emissions, releases and environmental transport. UNEP Chemicals Branch, Geneva, Switzerland. 2013; 42.

43. Purcell A [Internet] Child labor: Mining in tanzania. 2013 [updated 2013 September]. Available from: https://borgenproject.org.

44. Bento AL. All that is gold does not glitter: Mercury exposure to children in artisanal and small scale gold mines and the inadequacy of the minamata convention. J. Int'l Bus. & L. 2016; 16:283.

45. Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. heavy meatl toxicity and the environment. In Molecular, clinical and environmental toxicology. 2012: 133-64

46. (WHO) WHO [Internet] Health and mercury. 2017 [updated March 2017]. Available from: http://www.who.int/mediacentre/factsheets/fs361/en/.

47. Berlin M, Fazackerley J, Nordberg G, Kand M. The uptake of mercury in the brains of mammals exposed to mercury vapor and to mercuric salts. Archives of Environmental Health: An International Journal. 1969; 18(5):719-29.

48. Chan TY. Inorganic mercury poisoning associated with skin-lightening cosmetic products. Clinical toxicology. 2011; 49(10):886-91.

49. Heintze U, Edwardsson S, Dérand T, Birkhed D. Methylation of mercury from dental amalgam and mercuric chloride by oral streptococci in vitro. European Journal of Oral Sciences. 1983; 91(2):150-2.

50. Rowland I, Grasso P, Davies M. The methylation of mercuric chloride by human intestinal bacteria. Experientia. 1975; 31(9):1064-5.

51. Chen S, Li X, Sun G, Zhang Y, Su J, Ye J. Heavy metal induced antibiotic resistance in bacterium lsjc7. International journal of molecular sciences. 2015; 16(10):23390-404.

52. Varkey IM, Shetty R, Hegde A. Mercury exposure levels in children with dental amalgam fillings. International journal of clinical pediatric dentistry. 2014; 7(3):180.

53. Krzyzanowski M, Cohen A. Update of WHO air quality guidelines. Air Quality, Atmosphere and Health. 2008; 1(1):7-13

54. Morgan L, Flint G. Nickel alloys and coatings: Release of nickel. Nickel and the skin: immunology and toxicology. CRC, Boca Raton. 1989:45-54.

55. Haudrech P, Foussereau J, Mantout B, Baroux B. Nickel release from nickel-plated metals and stainless steels. Contact dermatitis. 1994; 31(4):249-55.

56. Xi H, Frenkel K, Klein C, Costa M. Nickel induces increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence. Toxicology and applied pharmacology. 1993; 120(1):29-36.

57. Rahim MA, Hameed RA, Khalil M. Nickel as a catalyst for the electro-oxidation of methanol in alkaline medium. Journal of Power Sources. 2004; 134(2):160-9.

58. Adkins H, Cramer HI. The use of nickel as a catalyst for hydrogenation. Journal of the American Chemical Society. 1930; 52(11):4349-58.

59. Yamada M, Takahashi S, Sato H, Kondo T, Kikuchi T, Furuya K, et al. Solubility of nickel oxide particles in various solutions and rat alveolar macrophages. Biological trace element research. 1993; 36(1):89-98.

60. Bennett B. Environmental nickel pathways to man. IARC scientific publications. 1984; (53):487-95.

61. IPCS O. Environmental health criteria n 108: Nickel. World Health Organisation, International Programme on Chemical Safety. http://www. inchem. org/documents/ehc/ehc/ehc108. htm. 1991.

62. Schwenk W. Studies on the nickel release of stainless chromium-nickel steels in drinking water. GWF water sewage. 1992; 133(6):281-6.

63. Booth J. Nickel in the diet and its role in allergic dermatitis. Journal of human nutrition and dietetics. 1990; 3(4):233-43.

64. Jorhem L, Sundström B. Levels of lead, cadmium, zinc, copper, nickel, chromium, manganese, and cobalt in foods on the swedish market, 1983–1990. Journal of Food Composition and Analysis. 1993; 6(3):223-41.

65. Dabeka RW, McKenzie AD. Survey of lead, cadmium, fluoride, nickel, and cobalt in food composites and estimation of dietary intakes of these elements by canadians in 1986-1988. Journal of AOAC International. 1995; 78(4):897-909.

66. Flint G, Packirisamy S. Systemic nickel: The contribution made by stainless-steel cooking utensils. Contact Dermatitis. 1995; 32(4):218-24.

67. Andersen A, Berge S, Engeland A, Norseth T. Exposure to nickel compounds and smoking in relation to incidence of lung and nasal cancer among nickel refinery workers. Occupational and environmental medicine. 1996; 53(10):708-13.

68. International Agency for Reasearch on Cancer. Working Group on the Evaluation of Carcinogenic Risks to Humans, World Health Organization, International Agency for Research on Cancer. Tobacco smoke and involuntary smoking. Iarc; 2004.

69. Brimblecombe P. Long-term changes in elemental deposition at the earth's surface. Environmental pollution. 1994; 83(1-2):81-5.

70. Whanger P. Effects of dietary nickel on enzyme activities and mineral contents in rats. Toxicology and applied pharmacology. 1973; 25(3):323-31.

71. Smialowicz RJ, Rogers RR, Rowe DG, Riddle MM, Luebke RW. The effects of nickel on immune function in the rat. Toxicology. 1987; 44(3):271-81.

72. Sunderman Jr FW, Hopfer SM, Sweeney KR, Marcus AH, Most BM, Creason J. Nickel absorption and kinetics in human volunteers. Proceedings of the Society for Experimental Biology and Medicine. 1989; 191(1):5-11.

73. Sunderman Jr FW. Biological monitoring of nickel in humans. Scandinavian journal of work, environment & health. 1993:19: 34-8.

74. Samal L MC. Significance of nickel in livestock health and products. International Journal for Agro Veterinary and Medical Science 2011; 5(3):349-61.

75. Sunderman JR FW, Aitio A, Morgan LG, Norseth T. Biological monitoring of nickel. Toxicology and industrial health. 1986; 2(1):17-78.

76. Nielsen GD, Søderberg U, Jørgensen PJ, Templeton DM, Rasmussen SN, Andersen KE, et al. Absorption and retention of nickel from drinking water in relation to food intake and nickel sensitivity. Toxicology and applied pharmacology. 1999; 154(1):67-75.

77. Goyer R. Toxic effects of metals. In: Klassen cd (ed). Casarette and doulls' toxicology: The basic sciences of poisons. New York: Mc Graw-Hill; 1996.

78. Howe P, Malcolm H, Dobson S. Mangenese and its compounds: environmental aspects. World Health Organization. 2014.

79. Agency for Toxic Substances and Disease Registry. Toxicological profile for manganese. Atlanta, GA, United States: Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry; 2000.

80. Millaleo R, Reyes-Díaz M, Ivanov A, Mora M, Alberdi M. Manganese as essential and toxic element for plants: Transport, accumulation and resistance mechanisms. Journal of soil science and plant nutrition. 2010; 10(4):470-81.

81. Agency for Toxic Substances and Disease Registry. Public health statement: Manganese. Sciences. 2012 Contract No.: CAS # 7439-96-5.

82. Copper I. Dietary reference intakes for vitamin a vitamin k, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc: Washington, DC: The National Academies Press; 2001.

83. Organization WH. Trace elements in human nutrition: Report of a who WHO expert committee [meeting held in geneva from 9 to 17 april 1973]. 1973.

84. Coombs WM. Manganese –the silent poison. Occupational Health South Africa, 2005; 11(3): 1-3.

85. Sathawara N, Parikh D, Agarwal Y. Essential heavy metals in environmental samples from western india. Bulletin of environmental contamination and toxicology. 2004; 73(4):756-61.

86. Underwood E. Trace elements in human and animal nutrition: Elsevier; 2012.

87. Frisbie SH, Mitchell EJ, Dustin H, Maynard DM, Sarkar B. World health organization discontinues its drinking-water guideline for manganese. Environmental health perspectives. 2012; 120(6):775.

88. Fechter LD, Johnson DL, Lynch RA. The relationship of particle size to olfactory nerve uptake of a non-soluble form of manganese into brain. Neurotoxicology. 2002; 23(2):177-83.
89. Tjälve H, Henriksson J. Uptake of metals in the brain via olfactory pathways. Neurotoxicology. 1999; 20(2-3):181-95.
90. Vitarella D, Wong BA, Moss OR, Dorman DC. Pharmacokinetics of inhaled manganese phosphate in male sprague–dawley rats following subacute (14-day) exposure. Toxicology and applied pharmacology. 2000; 163(3):279-85.
91. Kondakis XG, Makris N, Leotsinidis M, Prinou M, Papapetropoulos T. Possible health effects of high manganese concentration in drinking water. Archives of Environmental Health: An International Journal. 1989; 44(3):175-8.
92. Vieregge P, Heinzow B, Korf G, Teichert H-M, Schleifenbaum P, Mösinger H-U. Long term exposure to manganese in rural well water has no neurological effects. Canadian journal of neurological sciences. 1995; 22(4):286-9.
93. Blaurock-Busch E, Amin OR, Rabah T. Heavy metals and trace elements in hair and urine of a sample of arab children with autistic spectrum disorder. Maedica. 2011; 6(4):247.
94. Chua AC, Morgan EH. Effects of iron deficiency and iron overload on manganese uptake and deposition in the brain and other organs of the rat. Biological trace element research. 1996; 55(1-2):39-54.
95. Rovira A, Alonso J, Cordoba J. MR imaging findings in hepatic encephalopathy. American Journal of Neuroradiology. 2008; 29(9):1612-21.
96. Mohanty J, Nagababu E, Rifkind JM. Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. Frontiers in physiology. 2014; 5:84.
97. Gov N, Safran S. Red blood cell membrane fluctuations and shape controlled by atp-induced cytoskeletal defects. Biophysical journal. 2005; 88(3):1859-74.
98. Li H, Lykotrafitis G. Erythrocyte membrane model with explicit description of the lipid bilayer and the spectrin network. Biophysical journal. 2014; 107(3):642-53.
99. Lefrancais E O-MG, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. Nature. 2017; 544(7648):105-9.
100. Ghoshal K, Bhattacharyya M. Overview of platelet physiology: Its hemostatic and nonhemostatic role in disease pathogenesis. The Scientific World Journal. 2014; 2014: 1-17.
101. Zharikov S, Shiva S. Platelets mitochondrial function: From regulation of thrombosis to biomaker of disease. Biochemical Society Transactions. 2013; 14(1):118-23.

102. Blair P, Flaumenhaft R. Platelet α-granules: Basic biology and clinical correlates. Blood reviews. 2009; 23(4):177-89.

103. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thrombosis and haemostasis. 2002; 88(08):186-93.

104. Behnke O, Forer A. From megakaryocytes to platelets: Platelet morphogenesis takes place in the bloodstream. European Journal of Haematology. 1998; 60(61):3-23.

105. Thombocytes. [Internet]. [Cited May 2016]. Available from: http://www.thrombocyte.com/hemostasis-definition/.

106. Smith EB. Fibrinogen, fibrin and fibrin degradation products in relation to atherosclerosis. Clinics in haematology. 1986; 15(2):355-70.

107. Swanepoel AC, Nielsen VG, Pretorius E. Viscoelasticity and ultrastructure in coagulation and inflammation: two diverse techniques, one conclusion. Inflammation. 2015;38(4):1707-26.

108. Undas A, Ariëns RA. Fribrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(12): 88-99.

109. Biondi C, Cotorruelo C, Ensinck A, Borrás SG, Racca L, Racca A. Senescent erythrocytes: Factors affecting the aging of red blood cells. Immunological investigations. 2002; 31(1):41-50.

110. Lang K, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F, et al. Cation channels trigger apoptotic death of erythrocytes. Cell death and differentiation. 2003; 10(2):249.

111. Föller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, et al. Trpc6 contributes to the ca^{2+} leak of human erythrocytes. Cellular Physiology and Biochemistry. 2008; 21(1-3):183-92.

112. Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM. Role of ca2+-activated k+ channels in human erythrocyte apoptosis. American Journal of Physiology-Cell Physiology. 2003; 285(6):C1553-C60.

113. Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Föller M. Eryptosis, a window to systemic disease. Cellular Physiology and Biochemistry. 2008; 22(5-6):373-80.

114. Ghashghaeinia M, Cluitmans JC, Akel A, Dreischer P, Toulany M, Köberle M, et al. The impact of erythrocyte age on eryptosis. British journal of haematology. 2012; 157(5):606-14.

115. Niemoeller OM, Föller M, Lang C, Huber SM, Lang F. Retinoic acid induced suicidal erythrocyte death. Cellular Physiology and Biochemistry. 2008; 21(1-3):193-202.

116. Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM, et al. Stimulation of erythrocyte phosphatidylserine exposure by lead ions. American Journal of Physiology-Cell Physiology. 2005; 288(2):C396-C402.

117. Sopjani M, Föller M, Dreischer P, Lang F. Stimulation of eryptosis by cadmium ions. Cellular Physiology and Biochemistry. 2008; 22(1-4):245-52.

118. Macciò A, Madeddu C. Management of anemia of inflammation in the elderly. Anemia. 2012; 2012:1-20

119. Bonnar J. Coagulation effects of oral contraception. American journal of obstetrics and gynecology. 1987; 157(4):1042-8.

120. Lemery L. Oh, no! It's hemolyzed! What, why, who, how? . Advance for Medical Laboratory Professionals 1998:24-5.

121. Page TC, Light WR, McKay CB, Hellums JD. Oxygen transport by erythrocyte/hemoglobin solution mixtures in anin VitroCapillary as a model of hemoglobin-based oxygen carrier performance. Microvascular research. 1998; 55(1):54-64.

122. Bain BJ. Blood cells: A practical guide: John Wiley & Sons; 2014.

123. Kohlstadt I. Advancing medicine with food and nutrients: CRC Press; 2012.

124. Blasa M, Candiracci M, Accorsi A, Piacentini MP, Piatti E. Honey flavonoids as protection agents against oxidative damage to human red blood cells. Food Chemistry. 2007; 104(4):1635-40.

125. Belden JB GRLM. How well can we predict the toxicity of pesticide mixtures to aquatic life? Integrated Environmental Assessment and Management. 2007; 3:364 – 72.

126. N. C. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. Public Library of Science ONE. 2014; 9(5):1-12.

127. Murphy DB, Davidson MW. Phase contrast microscopy and darkfield microscopy. Fundamentals of Light Microscopy and Electronic Imaging, Second Edition. 2001:115-33.

128. Bevers EM, Williamson PL. Getting to the outer leaflet: Physiology of phosphatidylserine exposure at the plasma membrane. Physiological reviews. 2016; 96(2):605-45.

129. Tait JF, Gibson D. Phospholipid binding of annexin v: Effects of calcium and membrane phosphatidylserine content. Archives of biochemistry and biophysics. 1992; 298(1):187-91.

130. Venter C, Oberholzer HM, Bester J, Van Rooy M-J, Bester MJ. Ultrastructural, confocal and viscoelastic characteristics of whole blood and plasma after exposure to cadmium and chromium alone and in combination: An *ex vivo* study. Cellular Physiology and Biochemistry. 2017; 43(3):1288-300.

131. Barbier Olivier, Jacquillet Grégory, Tauc Michel, Cougnon Marc, Philippe. P. Effect of heavy metals on, and handling by, the kidney. Nephron Physiology. 2005; 99(4):105-10.

132. Lal R. Tragedy of the global commons: Soil, water and air. Climate change, intercropping, pest control and beneficial microorganisms: Springer; 2009. 9-11.

133. Alves LR, dos Reis AR, Gratão PL. Heavy metals in agricultural soils: From plants to our daily life. Científica. 2016; 44(3):346-61.

134. Rajaganapathy V, Xavier F, Sreekumar D, Mandal P. Heavy metal contamination in soil, water and fodder and their presence in livestock and products: A review. Journal of Environmental Science and Technology. 2011; 4(3):234-49.

135. Governments U. International organisations, and ngos move to protect lives and the climate from dangerous air pollution [press release]. Paris, france: United nations environment programme (22 may 2015).

136. Selin NE. Mercury rising: Is global action needed to protect human health and the environment? Environment: Science and Policy for Sustainable Development. 2005; 47(1):22-35.

137. Carnie T [Internet] World treaty to ban and reduce mercury poison comes into force. South Africa: News 24; 2017 [updated 2019-03-12; cited 2017 2017-08-17]. Available from: https://www.news24.com/Green/News/world-treaty-to-ban-and-reduce-mercury-poison-comes-into-force-20170817.

138. Muchuweti M, Birkett J, Chinyanga E, Zvauya R, Scrimshaw MD, Lester J. Heavy metal content of vegetables irrigated with mixtures of wastewater and sewage sludge in zimbabwe: Implications for human health. Agriculture, Ecosystems & Environment. 2006; 112(1):41-8.

139. Eslami A, Khaniki GJ, Nurani M, Mehrasbi M, Peyda M, Azimi R. Heavy metals in edible green vegetables grown along the sites of the Zanjanrood river in Zanjan, Iran. Journal of Biological Sciences. 2007; 7(6):943-8.

140. Barbier O, Jacquillet G, Tauc M, Poujeol P, Cougnon M. Acute study of interaction between cadmium, calcium and zinc transport along the rat nephron *in vivo*. American Journal of Physiology-Renal Physiology. 2004.

141. Barbier O, Jacquillet G, Tauc M, Cougnon M, Poujeol P. Effect of heavy metals on, and handling by, the kidney. Nephron Physiology. 2005; 99(4):105-10.

142. Lee MR, Lim YH, Lee BE, Hong YC. Blood mercury concentrations are associated with decline in liver function in an elderly population: A panel study. Environmental Health. 2017; 16(1):17.

143. Hussain S, Atkinson A, Thompson S, Khan A. Accumulation of mercury and its effect on antioxidant enzymes in brain, liver, and kidneys of mice. Journal of Environmental Science & Health Part B. 1999; 34(4):645-60.

144. Clarkson TW, Magos L. The toxicology of mercury and its chemical compounds. Critical reviews in toxicology. 2006; 36(8):609-62.

145. Joint Food Additives WHO Expert Committee on Food additives. Safety evaluation of certain food additives. 2006.

146. Bhakdi S, Muhly M, Füssle R. Correlation between toxin binding and hemolytic activity in membrane damage by staphylococcal alpha-toxin. Infection and immunity. 1984; 46(2):318-23.

147. Janse van Rensburg M. The effects of copper manganese and mercury alone and in combinations in an *ex vivo* model of coagulation: University of Pretoria. 2018.

148. Becker A, Soliman KF. The role of intracellular glutathione in inorganic mercury-induced toxicity in neuroblastoma cells. Neurochemical research. 2009; 34(9):1677-84.

149. Akagi H. Human exposure to mercury due to gold mining in the Tapajos river basin, Amazon, Brazil. Speciation of mercury in human hair, blood and urine. Water, Air, & Soil Pollution. 1995.

150. Miller GT. Sustaining the earth: An integrated approach. 8 ed. Canada: Thomson; 2007.

151. Garnham BL, Langerman KE. Mercury emissions from South Africa's coal-fired power stations. Clean Air Journal. 2016; 26(2):14-20.

152. Dabrowski JM, Ashton PJ, Murray K, Leaner JJ, Mason RP. Anthropogenic mercury emissions in South Africa: Coal combustion in power plants. Atmospheric environment. 2008; 42(27):6620-6.

153. Global conctruction review [Internet] Coal makes Mpumalanga, South Africa the most polluted place in the world: Greenpeace. 2018 [cited 2018 29 October]. Available from: http://www.globalconstructionreview.com/news/coal-makes-mpumalanga-south-africa-most-polluted-p/.

154. Dalvie MA, Ehrlich R. Community mercury levels in the vicinity of peri-urban waste disposal sites and fossil fuel burning operations. Environment international. 2006; 32(4):493-9.

155. Gatti R, Belletti S, Uggeri J, Vettori MV, Mutti A, Scandroglio R, et al. Methylmercury cytotoxicity in PC12 cells is mediated by primary glutathione depletion independent of excess reactive oxygen species generation. Toxicology. 2004; 204(2-3):175-85.

156. Durak D, Kalender S, Uzun FG, Demýr F, Kalender Y. Mercury chloride-induced oxidative stress in human erythrocytes and the effect of vitamins C and E *in vitro*. African Journal of Biotechnology. 2010; 9(4):488-495

157. Shi H, Noguchi N, Niki E. Comparative study on dynamics of antioxidative action of α-tocopheryl hydroquinone, ubiquinol, and α-tocopherol against lipid peroxidation. Free Radical Biology and Medicine. 1999; 27(3-4):334-46.

158. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy reviews. 2010; 4(8):118.

159. Costa M. Mechanisms of nickel genotoxicity and carcinogenicity. Toxicology of metals. 1996; 1:245-51.

160. Grimsrud TK, Andersen A. Evidence of carcinogenicity in humans of water-soluble nickel salts. Journal of Occupational Medicine and Toxicology. 2010; 5(1):1-7

161. Staton I, Ma R, Evans N, Hutchinson R, McLeod C, Gawkrodger D. Dermal nickel exposure associated with coin handling and in various occupational settings: Assessment using a newly developed finger immersion method. British Journal of Dermatology. 2006; 154(4):658-64.

162. Wojciechowska M, Czajkowski R, Kowaliszyn B, Żbikowska-Gotz M, Bartuzi Z. Analysis of skin patch test results and metalloproteinase-2 levels in a patient with contact dermatitis. Advances in Dermatology and Allergology. 2015; 32(3):154-161.

163. Sunderman Jr FW, Dingle B, Hopfer SM, Swift T. Acute nickel toxicity in electroplating workers who accidently ingested a solution of nickel sulfate and nickel chloride. American journal of industrial medicine. 1988; 14(3):257-66.

164. Weischer C, Kördel W, Hochrainer D. Effects of $NiCl_2$ and NiO in Wrats after oral uptake and inhalation exposure respectively. Central Journal of Bacteriology, Microbiology and Hygiene 1 Dept. Originals B, Hygiene.1980; 171(4-5):336-51.

165. Das K, Das S, Dhundasi S. Nickel, its adverse health effects & oxidative stress. Indian Journal of Medical Research. 2008; 128(4):412-26.

166. M'Bemba-Meka P, Lemieux N, Chakrabarti SK. Role of oxidative stress and intracellular calcium in nickel carbonate hydroxide-induced sister-chromatid exchange, and alterations in replication index and mitotic index in cultured human peripheral blood lymphocytes. Archives of toxicology. 2007; 81(2):89-99.

167. M'Bemba-Meka P, Lemieux N, SK. C. Role ofoxidative stress, mitochondrial membrane potential, and calcium homeostasis in nickel sulphate-induced human lymphocyte death *in vitro*. Chemico-Biological Interactions. 2005; 156:69-80.

168. Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress part i: Mechanisms involved in metal-induced oxidative damage. Current topics in medicinal chemistry. 2001; 1(6):529-39.

169. Ljung K, Vahter M. Time to re-evaluate the guideline value for manganese in drinking water? Environmental health perspectives. 2007; 115(11):1533-8.

170. Schroeder HA, Balassa JJ, Tipton IH. Essential trace metals in man: Manganese: A study in homeostasis. Journal of chronic diseases. 1966; 19(5):545-71.

171. Munusamy S, MacMillan-Crow LA. Mitochondrial superoxide plays a crucial role in the development of mitochondrial dysfunction during high glucose exposure in rat renal proximal tubular cells. Free Radical Biology and Medicine. 2009; 46(8):1149-57.
172. Azadmanesh J, Borgstahl G. A review of the catalytic mechanism of human manganese superoxide dismutase. Antioxidants. 2018; 7(2):1-25.
173. Zidenberg-Cherr S, Keen CL, Lönnerdal B, Hurley LS. Superoxide dismutase activity and lipid peroxidation in the rat: Developmental correlations affected by manganese deficiency. The Journal of nutrition. 1983; 113(12):2498-504.
174. Brock AA, Chapman SA, Ulman EA, Wu G. Dietary manganese deficiency decreases rat hepatic arginase activity. The Journal of nutrition. 1994; 124(3):340-4.
175. Malecki EA, Greger J. Manganese protects against heart mitochondrial lipid peroxidation in rats fed high levels of polyunsaturated fatty acids. The Journal of nutrition. 1996; 126(1):27-33.
176. Finley JW, Davis CD. Manganese absorption and retention in rats is affected by the type of dietary fat. Biological trace element research. 2001; 82(1-3):143-58.
177. Baly DL, Curry DL, Keen CL, Hurley LS. Dynamics of insulin and glucagon release in rats: Influence of dietary manganese. Endocrinology. 1985; 116(5):1734-40.
178. Baly DL, Keen CL, Hurley LS. Effects of manganese deficiency on pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity and carbohydrate homeostasis in adult rats. Biological trace element research. 1986; 11(1):201-212.
179. Council NR. 1995. Nutrient requirements of laboratory animals: 4th Revised Ed: The National Academies Press.
180. Dorman DC, Struve MF, Vitarella D, Byerly FL, Goetz J, Miller R. Neurotoxicity of manganese chloride in neonatal and adult cd rats following subchronic (21-day) high-dose oral exposure. Journal of Applied Toxicology: An International Journal. 2000; 20(3):179-87.
181. Dobson AW, Erikson KM, Aschner M. Manganese neurotoxicity. Annals of the New York Academy of Sciences. 2004; 1012(1):115-28.
182. Kobert R. Zur pharmakologie des mangans und eisens. Naunyn-Schmiedeberg's Archives of Pharmacology. 1883; 16(5):361-92.
183. Jiang Y, Zheng W. Cardiovascular toxicities upon managanese exposure. Cardiovascular toxicology. 2005; 5(4):345-54.
184. Charash B, Placek E, Sos TA, Kligfield P. Dose-related effects of manganese on the canine electrocardiogram. Journal of electrocardiology. 1982; 15(2):149-52.
185. Vander EL, Colet J-M, Muller RN. Spectroscopic and metabolic effects of $MnCl_2$ and MnDPDP on the isolated and perfused rat heart. Investigative radiology. 1997; 32(10):581-8.

186. Yueming J, Jipei L, Yan T. Effects of low level manganese exposure on workers' cardiovascular functions. Industrial Health And Occupational Diseases-Beijing-. 2000; 26(1):28-30.

187. Jiang Y, Zheng W. Cardiovascular toxicities upon managanese exposure. Cardiovascular toxicology. 2005; 5(4):341-3.

188. Senthamilselvan D, Chezhian A, Suresh E. Synergistic effect of nickel and mercury on fatty acid composition in the muscle of fish lates calcarifer. J Fish Aquat Sci. 2016; 11(1):77-84.

189. Kawamoto S, Kawamura T, Miyazaki Y, Hosoya T. Effects of atorvastatin on hyperlipidemia in kidney disease patients. Nihon Jinzo Gakkai Shi. 2007; 49(1):41-8.

190. Konar V, Aydogmus C, Orun I, Kandemir S. The effects of cadmium on fatty acid composition in the muscle and skin of juvenile rainbow trout (Oncorhynchus mykiss, Walbaum 1792). Journal of Animal and Veterinary Advances. 2010; 9(7):1191-6.

191. Chan HM, Egeland GM. Fish consumption, mercury exposure, and heart diseases. Nutrition Reviews. 2004; 62(2):68-72

192. Choi JH, Chang HW, Rhee SJ. Effect of green tea catechin on arachidonic acid cascade in chronic cadmium-poisoned rats. Asia Pacific journal of clinical nutrition. 2002; 11(4):292-7.

193. Nwanunike MO. The effects of bioaccumulation of heavy metals on fish fin over two years. Journal of Fisheries and Livestock Production. 2016; 4(2): 1-7

194. Pathak S, Mukhiya Y, Singh V. Mercury, manganese interaction studies on barley germination and phyto toxicity. Indian Journal of Plant Physiology. 1987; 30(1):13-19.

195. Spears JW, Harvey R, Samsell LJ. Effects of dietary nickel and protein on growth, nitrogen metabolism and tissue concentrations of nickel, iron, zinc, manganese and copper in calves. The Journal of nutrition. 1986; 116(10):1873-82.

196. Mannucci P, Lobina G, Caocci L, Dioguardi N. Effect on blood coagulation of massive intravascular haemolysis. Blood. 1969; 33(2):207-13.

197. Diez-Silva M, Dao M, Han J, Lim C-T, Suresh S. Shape and biomechanical characteristics of human red blood cells in health and disease. MRS bulletin. 2010; 35(5):382-8.

198. Pretorius E. The adaptability of red blood cells. Cardiovascular diabetology. 2013; 12(1):1-7

199. Antonio PD, Lasalvia M, Perna G, Capozzi V. Scale-independent roughness value of cell membranes studied by means of afm technique. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2012; 1818(12):3141-8.

200. Buys AV, Van Rooy M-J, Soma P, Van Papendorp D, Lipinski B, Pretorius E. Changes in red blood cell membrane structure in type 2 diabetes: A scanning electron and atomic force microscopy study. Cardiovascular diabetology. 2013; 12(1):1-7

201. Park Y, Best CA, Auth T, Gov NS, Safran SA, Popescu G, et al. Metabolic remodeling of the human red blood cell membrane. Proceedings of the National Academy of Sciences. 2010; 107(4):1289-94.

202. Li J, Lykotrafitis G, Dao M, and Suresh S, proc. Cytoskeletal dynamics of human erythrocyte. Preceeding of the National Acadamey of Sciences. 2007; 104(12):4937-42

203. Swanepoel AC, Pretorius E. Scanning electron microscopy analysis of erythrocytes in thromboembolic ischemic stroke. International journal of laboratory hematology. 2012; 34(2):185-91.

204. Tatsuzo F, Takashi S, Akira T, Motoko W, Yasunori K. Shape changes of human erythrocytes induced by various amphipathic drugs acting on the membrane of the intact cells. Biochemical pharmacology. 1979; 28(5):613-20.

205. Irie T, Otagiri M, Sunada M, Uekama K, Ohtani Y, Yamada Y, et al. Cyclodextrin-induced hemolysis and shape changes of human erythrocytes *in vitro*. Journal of Pharmacobio-dynamics. 1982; 5(9):741-4.

206. Lim KM, Kim S, Noh JY, Kim K, Jang WH, Bae ON, et al. Low-level mercury can enhance procoagulant activity of erythrocytes: A new contributing factor for mercury-related thrombotic disease. Environmental health perspectives. 2010; 118(7):928-35.

207. Suwalsky M, Ungerer B, Villena F, Cuevas F, Sotomayor CP. $HgCl_2$ disrupts the structure of the human erythrocyte membrane and model phospholipid bilayers. Journal of Inorganic Biochemistry. 2000; 81(4):267-73.

208. Maheshwari S, Dua A. Structural analysis of the erythrocytes of channa punctatus (bloch) exposed to mercuric chloride using scanning electron microscopy. Turkish Journal of Fisheries and Aquatic Sciences. 2016; 16(4):865-71.

209. De Luca G, Gugliotta T, Parisi G, Romano P, Geraci A, Romano O, et al. Effects of nickel on human and fish red blood cells. Bioscience reports. 2007; 27(4-5):265-73.

210. Chandel M, Jain GC. Manganese induced hematological alteration in wistar rats. Journal of Environmental and Occupational Science. 2016; 5(4):77-81.

211. Machlus KR, Thon JN, Italiano Jr JE. Interpreting the developmental dance of the megakaryocyte: A review of the cellular and molecular processes mediating platelet formation. British journal of haematology. 2014; 165(2):227-36.

212. Semple JW, Freedman J. Platelets and innate immunity. Cellular and Molecular Life Sciences. 2010; 67(4):499-511.

213. Tran R, Myers DR, Ciciliano J, Trybus Hardy EL, Sakurai Y, Ahn B, et al. Biomechanics of haemostasis and thrombosis in health and disease: From the macro-to molecular scale. Journal of cellular and molecular medicine. 2013; 17(5):579-96.
214. Cerecedo D. Platelet cytoskeleton and its hemostatic role. Blood Coagulation & Fibrinolysis. 2013; 24(8):798-808.
215. Fox JE. The platelet cytoskeleton. Thrombosis and haemostasis. 1993; 70(6):0884-93.
216. Siess W. Molecular mechanisms of platelet activation. Physiological reviews. 1989; 69(1):58-178.
217. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein iib. Iiia complex during platelet activation. Journal of Biological Chemistry. 1985; 260(20):11107-14.
218. Engida AM, Chandravanshi BS. Assessment of heavy metals in tobacco of cigarettes commonly sold in ethiopia. Chemistry International. 2017; 3(3):213-9.
219. Pretorius E, du Plooy JN, Soma P, Keyser I, Buys AV. Smoking and fluidity of erythrocyte membranes: A high resolution scanning electron and atomic force microscopy investigation. NiO. 2013; 35:42-6.
220. Padmavathi P, Reddy VD, Maturu P, Varadacharyulu N. Smoking-induced alterations in platelet membrane fluidity and Na+/K+-ATPase activity in chronic cigarette smokers. Journal of atherosclerosis and thrombosis. 2010; 17(6):619-27.
221. Wachowicz B, Olas B, Zbikowska H, Buczyński A. Generation of reactive oxygen species in blood platelets. Platelets. 2002; 13(3):175-82.
222. Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Hydrogen peroxide is involved in collagen-induced platelet activation. Blood. 1998; 91(2):484-90.
223. Hedin H, Fowler C. Further studies of the effects of diamide and hydrogen peroxide on calcium signaling in the human platelet. Methods and findings in experimental and clinical pharmacology. 1999; 21(5):321-6.
224. Iuliano L, Pedersen JZ, Pratico D, Rotilio G, Violi F. Role of hydroxyl radicals in the activation of human platelets. European journal of biochemistry. 1994; 221(2):695-704.
225. Brass LF. Thrombin and platelet activation. Chest. 2003; 124(3):18-25.
226. Pretorius E, Oberholzer HM, Vieira WA, Smit E. Ultrastructure of platelets and fibrin networks of asthmatic mice exposed to selenium and withania somnifera. Anatomical science international. 2009; 84(3):210-7.

227. Pretorius E, Vieira WA, Oberholzer HM, Auer RE. Comparative scanning electron microscopy of platelets and fibrin networks of human and differents animals. International Journal of Morphology. 2009; 27(1):69-77.

228. Van Rooy M-J, Duim W, Ehlers R, Buys AV, Pretorius E. Platelet hyperactivity and fibrin clot structure in transient ischemic attack individuals in the presence of metabolic syndrome: A microscopy and thromboelastography® study. Cardiovascular diabetology. 2015; 14(1):1-13

229. Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, et al. gp91phox-dependent expression of platelet CD40 ligand. Circulation. 2004; 110(10):1326-9.

230. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. Journal of Biological Chemistry. 1992; 267(34):24259-63.

231. Collet J, Park D, Lesty C, Soria J, Soria C, Montalescot G, et al. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: Dynamic and structural approaches by confocal microscopy. Arteriosclerosis, thrombosis, and vascular biology. 2000; 20(5):1354-61.

232. Pretorius E, du Plooy JN, Bester J. A comprehensive review on eryptosis. Cellular Physiology and Biochemistry. 2016; 39:1977-2000.

233. Lang E, Lang F. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. Seminars in cell and developmental biology; 2015: Elsevier.

234. Lang E, Qadri SM, Lang F. Killing me softly–suicidal erythrocyte death. The international journal of biochemistry & cell biology. 2012; 44(8):1236-43.

235. Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, et al. Enhanced programmed cell death of iron-deficient erythrocytes. The FASEB journal. 2006; 20(2):368-70.

236. Jang W-H, Lim K-M, Kim K, Noh J-Y, Kang S, Chang Y-K, et al. Low level of lead can induce phosphatidylserine exposure and erythrophagocytosis: A new mechanism underlying lead-associated anemia. Toxicological sciences. 2011; 122(1):177-84.

237. Zwaal R, Comfurius P, Van Deenen L. Membrane asymmetry and blood coagulation. Nature. 1977; 268(5618):358.

238. Comfurius P, Zwaal R. The enzymatic synthesis of phosphatidylserine and purification by cm-cellulose column chromatography. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1977; 488(1):36-42.

239. Farina M, Avila DS, Da Rocha JBT, Aschner M. Metals, oxidative stress and neurodegeneration: A focus on iron, manganese and mercury. Neurochemistry international. 2013; 62(5):575-94.

8. APPENDIX

8.1 Ethics letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

13/02/2018

Approval Certificate
New Application

Ethics Reference No: 12/2018

Title: Investigating the effects of the heavy metals mercury, nickel and manganese, alone and in combination, on human erythrocytes and components of the coagulation system

Dear Precious Busisiwe Maseko

The **New Application** as supported by documents specified in your cover letter dated 2/02/2018 for your research received on the 2/02/2018, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 13/02/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**12/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60 / 61, 31 Bophelo Road, Gezina, Pretoria

8.2 Consent Form

PICD Nr

PATIENT OR PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: Effects of heavy metals mercury, nickel and manganese alone and in combination on an ex-vivo human red blood cells and the blood clotting system

SPONSOR: University of Pretoria

Principal Investigators: Busisiwe Precious Maseko (Principle investigator), Prof. HM Oberholzer (Supervisor)

Institution: University of Pretoria

DAYTIME AND AFTER-HOURS TELEPHONE NUMBER(S):
Daytime numbers: Ms Maseko 076 373 7301, Prof Oberholzer 012-319-2533
Afterhours: Ms Maseko 076 373 7301

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

				:
dd	**mm**	**y**		**Time**

Dear Mr ..... date of consent procedure …..../…..../……..

1) INTRODUCTION

You are invited to volunteer for a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

You are invited to take part in a research study. The aim of this study is to investigate the effects of heavy metals mercury, nickel and manganese alone and in combination on red blood cells and the blood clotting system. People in South Africa may be exposed to these heavy metals through mining and industrial activities. By doing this we wish to find out at which level these metals are toxic as well as how they possibly worsen diseases related to red blood cells and blood clotting.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves answering some questions with regard to your health status.

Question	**Answer**	
	Yes	**No**
Are you 20 years or older?		
Are you taking any medication?		

If yes, which medication/s are you taking?	

If you are older than 20, are not taking any medication we would like to collect two 5 ml tubes of blood. The tubes of blood will not be labelled with your name but a number will be assigned. This is done to ensure that no one knows it is you. In the laboratory, the blood components will be separated from each other and the red blood cells will be exposed to different concentrations of each metal, alone and in combination. The effect on the cells and the blood clotting system will then be measured. Any blood left over after these measurements will be destroyed as biohazardous material.

4) RISK AND DISCOMFORT INVOLVED.

The only possible risk and discomfort involved is the taking of blood from a vein. Small bruising or mild soreness at the puncture site may be experienced for several days. Any risk is minimized using pre-packaged sterilized equipment and careful attention to proper technique. The University of Pretoria has limited insurance for research related injuries.

5) POSSIBLE BENEFITS OF THIS STUDY.

Although you will not benefit directly from the study, the results of the study will tell us at what level mercury, nickel and manganese poses a threat to human health.

6) I understand that if I do not want to participate in this study, I will still receive standard treatment for my illness.

7) I may at any time withdraw from this study.

8) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

9) INFORMATION If I have any questions concerning this study, I should contact:

Prof HM Oberholzer tel: 012-319-2533 or cell: 072 373 3569

10) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that volunteers remain unidentifiable.

11) CONSENT TO PARTICIPATE IN THIS STUDY.

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

.....	.....
Patient Name	Date
.....	.....
Patient signature	Date
.....	.....
Investigator's name	Date
.....	.....
Investigator's signature	Date
.....	.....
Witness name and signature	Date

VERBAL PATIENT INFORMED CONSENT (applicable when patients cannot read or write)

I, the undersigned, Dr……..…, have read and have explained fully to the patient, named …….... and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

I hereby certify that the patient has agreed to participate in this study.

Patient's Name_____

(Please print)

Patient's Signature_____ Date _____

Investigator's Name_____

(Please print)

Investigator's Signature _____ Date _____

Witness's Name _____

Witness's Signature_____ Date _____

(Please print)

(Witness - sign that he/she has witnessed the process of informed consent)

8.3 Declaration of originality

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

The Department of Anatomy places great emphasis upon integrity and ethical conduct in thepreparation of all written work submitted for academic evaluation.

While academic staff teaches you about referencing techniques and how to avoid plagiarism, you toohave a responsibility in this regard. If you are at any stage uncertain as to what is required, you shouldspeak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article ora website) without acknowledging the source and pass it off as your own. In effect, you are stealingsomething that belongs to someone else. This is not only the case when you copy work word-for-word(verbatim), but also when you submit someone else's work in a slightly altered form (paraphrase) oruse a line of argument without acknowledging it. You are not allowed to use work previously producedby another student. You are also not allowed to let anybody copy your work with the intention ofpassing if off as his/her work.

Students who commit plagiarism will not be given any credit for plagiarised work. The matter may alsobe referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a seriouscontravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student ofthe Department of Anatomy. No written work will be accepted unless the declaration has beencompleted and attached.

Full names of student: Precious Busisiwe Maseko

Student number: 10263269

Topic of work: Investigating the effects of the heavy metals mercury, nickel and manganese, alone and in combination on human erythrocytes and components of the coagulation system

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.

2. I declare that this Dissertation (e.g. essay, report, project, assignment,dissertation, thesis, etc.) is my own original work. Where other people's work has been used(either from a printed source, Internet or any other source), this has been properlyacknowledged and referenced in accordance with departmental requirements.

3. I have not used work previously produced by another student or any other person to hand inas my own.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing itoff as his or her own wok.

SIGNATURE: