Dissertation of the 2nd Cycle of Studies Leading to Master's Degree in Analytical, Clinical and Forensic Toxicology

TRACE ELEMENTS AND CARDIOVASCULAR DISEASES

Evidences from the comparative postmortem analysis of tissues

Anne Sophie Pereira Alves

Supervised by:

Prof. Doutor Agostinho Almeida

(Faculdade de Farmácia da Universidade do Porto)

Prof. Doutor Agostinho Santos

(Faculdade de Medicina da Universidade do Porto;

Instituto Nacional de Medicina Legal e Ciências Forenses)

September 2014

AUTHOR'S DECLARATION

ACKNOWLEDGMENTS

I would like to express my gratitude to all the people involved in this work and who helped me to make this work possible. I would like to thank in particular:

To my supervisor, Prof. Agostinho Almeida, for his guidance and support during the course of this work.

To my co-supervisor, Prof. Agostinho Santos, for his support.

To all forensic pathologist and technicians involved in sample collection at Instituto Nacional de Medicina Legal. I would like to refer a special thanks to Ricardo, Débora and Maria.

To Patricia Ramos for all the support, patience and sympathy demonstrated since the first day.

To Mariana, thank you for the support and understanding.

To all my friend, that even from distance supported me. Catarina, Marisa, Lis thank you for everything.

And last but not least to my parents without them none of this would be possible. Thank you for support and love

ABSTRACT

Cardiovascular diseases (CVD) are a major public health problem. Disruption of trace element homeostasis may lead to oxidative stress, and consequent DNA damage, lipid peroxidation, protein modification and other biochemical effects, causing CVD. The main goal of this work was to directly study the disease-related changes on some trace elements levels (Cu, Cd, Pb, Se and Zn) in different tissues: kidney (medulla and cortex) and aorta (with and without atheroma plaque).

Comparing the two **kidney** regions, higher levels were found in cortex, but the difference reached statistical significance only for Zn (166.1±100.2 *vs* 115.7±86.5 μg/g; p≤0.01) and Cd (72.57±45.43 *vs* 41.37±38.72 μg/g; p≤0.0001). For *kidney medulla*, a gender-related difference was found for Pb, with higher levels in men (0.32±0.23 μg/g) than women (0.16±0.10 μg/g; p≤0.01). It was also observed that smoking habits have an influence in some trace elements levels in this kidney structure. Higher levels of Zn (16671±112.1 *vs* 91.46±63.47 μg/g; p≤0.01), Cd (75.97±61.04 *vs* 27.78±20.24 μg/g; p≤0,001) and Pb (0.39±0.19 *vs* 0.21±0.20 μg/g; p≤0.05) were found in smokers. In *kidney cortex* no significant difference was found between genders for any of the five elements studied. Regarding smoking habits, only Cd showed a significant difference between smokers (110.1±37.74 μg/g) and non-smokers (51.06±27.62 μg/g; p≤0.0001). In both kidney regions, a slight tendency for an age-related increase was observed for the five elements. However, the Cd levels in renal cortex only increased up to 50 years old; then a subsequent decrease was observed.

In **aorta** without plaque no significant gender- or smoking habits-related differences were found for any of five elements. In aorta with plaque, smokers showed significantly decreased Zn levels: 78.44±32.25 μg/g *vs* 126.45±66.60 μg/g in non-smokers (p≤0.05).

When comparing both tissues (aorta with and without plaque), increased levels of Zn and decreased levels of Se were found in aorta with plaque (110.9±58.42 *vs* 89.74±28.45 μg/g; p≤0,05 and 3.85±2.50 *vs* 4.71±2.33 μg/g; p≤0,01, respectively). In aorta without plaque only Pb showed to be related (increased) with age. In aorta with plaque, an age-related tendency for decreased levels was observed for Cu, Cd and Se. On the contrary, Zn and Pb showed a tendency to increase with age.

Individuals with CVD showed significantly lower levels of Cu and Se in both tissues – Cu kidney: 5.61±3.82 vs 7.96±3.71 μg/g; Cu aorta: 3.18±1.32 *vs* 4.48±2.69 μg/g; Se kidney: 3.90±2.80 vs 5.48±5.35 μg/g; Se aorta 3.59±2.45 vs 4.46±2.14 μg/g). Interestingly, decreased levels of Cd and Pb were also observed.

Keywords: Trace elements; cardiovascular diseases; ICP-MS; postmortem analysis; kidney; aorta.

RESUMO

As doenças cardiovasculares (DCVs) são um sério problema de saúde pública. A perturbação da homeostase dos elementos vestigiais pode levar a stress oxidativo e consequente dano no ADN, peroxidação lipídica e modificação de proteínas, entre outros efeitos bioquímicos, causando DCV. O principal objetivo deste trabalho foi estudar diretamente as alterações, relacionadas com as DCVs, nos níveis de alguns elementos vestigiais (Cu, Cd, Pb, Se e Zn) em diferentes tecidos: rim (medula e córtex) e aorta (com e sem placa de ateroma).

Comparando as duas regiões do rim, foram encontrados níveis mais elevados no córtex, mas a diferença só foi estatisticamente significativa para Zn (166,1±100,2 *vs* 115,7±86,5 µg/g; p≤0,01) e Cd (72,57±45,43 *vs* 41,37±38,72 µg/g; p≤0,0001). Em relação à medula renal, foi observada uma diferença entre sexos para o Pb, com níveis mais elevados nos homens (0,32±0,23 µg/g, *vs* 0,16±0,10 µg/g nas mulheres; p≤0,01). Observou-se também que o tabagismo influencia os níveis de alguns elementos nesta região do rim. Nos fumadores foram encontrados níveis mais elevados de Zn (166.73±112.1 *vs* 91.74±63.47 µg/g; p≤0.01), Cd (75.97±61.04 *vs* 27.78±20.24 µg/g; p≤0,001) e Pb (0,39±0,19 vs 0,21±0,205 µg/g; p≤0.05). No córtex renal não foram encontradas diferenças significativas entre sexos para qualquer dos cinco elementos estudados. Em relação ao tabagismo, somente o Cd mostrou uma diferença significativa entre fumadores (110,1±37,74 µg/g) e não fumadores (51,06±27,62 µg/g; p≤0.0001). Em ambas as regiões do rim, foi observada uma ligeira tendência para um aumento relacionado com a idade nos níveis dos cinco elementos. No entanto, os níveis de Cd no córtex renal aumentaram somente até aos 50 anos de idade, diminuindo depois.

Na aorta sem placa não foram encontradas diferenças significativas entre sexos ou relacionadas com os hábitos tabágicos para qualquer dos cinco elementos. Na aorta com placa, os fumadores apresentaram uma diminuição significativa dos níveis de Zn: 78,44±32,25 vs 126,45±66,60 µg/g em não-fumadores (p≤0,05). Na comparação entre os tecidos (aorta com e sem placa), foi observado um aumento significativo dos níveis de Zn e uma diminuição dos níveis de Se na aorta com placa (110.9±58.42 *vs* 89.74±28.45 µg/g; p≤0.05 e 3.85±2.50 vs 4.71±2,34 µg/g; p≤0.01, respetivamente). Na aorta sem placa só o Pb mostrou estar relacionado (aumentado) com a idade. Na aorta com placa observou-se uma tendência para a diminuição com a idade dos níveis de Cu, Cd e Se. Pelo contrário, o Zn e o Pb mostraram uma tendência para aumentarem com a idade.

Os indivíduos com DCV apresentaram níveis significativamente mais baixos de Cu e Se em ambos os tecidos – Cu renal: 5.61±3.82 vs 7.96±3.71 µg/g; Cu aorta: 3.18±1.32 vs 4.48 ± 2.69 µg/g; Se renal: 3.90±2.80 vs 5.48±5.35 µg/g; Se aorta 3.19±1,60 vs 4.00±1.53 µg/g). Curiosamente, também foi observada uma diminuição dos níveis de Cd e Pb.

Palavras-chave: Elementos vestigiais; doenças cardiovasculares; ICP-MS; análise postmortem; rim; aorta.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS LIST

AAS – Atomic absorption spectrometry

AHA – American Heart Association

ATSDR – Agency for Toxic Substances and Disease Registry

BMI – Body mass index

CHD – Coronary heart disease

CRF – Cardiovascular risk factor

CVDs – Cardiovascular diseases

CRM – Certified reference material

DALY – Disability adjusted life years

DM – Diabetes mellitus

GFR – Glomerular filtration rate

GSH – Glutathione

GPx – Glutathione peroxidase

HDL – High density lipoprotein

HT – Hypertension

ICP-AES – Inductively coupled plasma-atomic emission spectrometry

ICP-MS – Inductively coupled plasma-mass spectrometry

INE – Instituto Nacional de Estatística

INMLCF – Instituto Nacional de Medicina Legal e Ciências Forenses

LDL – Low density lipoprotein

Mt – Metallothioneins

NF-kB – Nuclear factor-kB

ppb – Parts per billion

ppm – Parts per million

RENNDA – Registo Nacional de Não Dadores

ROS – Reactive oxygen species

CHAPTER 1

THEORETICAL BACKGROUND

1.1 INTRODUCTORY NOTE

The aging of population in developed countries resulted in an increase in longevity. In some developed countries, where these increase of longevity began in the early twentieth century, more than 15% of the population has 65 years or more, and 3-4% of the population is 80 years old or over. European countries have the highest proportion of elderly people worldwide: about 14% of people in Western Europe have ≥ 65 years. In this sense, Sweden may be considered the country with the highest rate of longevity in the world, with 18% of its population aged ≥ 65 years (1).

According to WHO (World Health Organization), the average life expectancy in the world will reach 73 years in 2025. This increase corresponds to a decline in infant mortality in general and the decline of fertility rates. Such acute changes in population structure decreased the time available to and increased the resources needed to expand the infrastructure of health, already overburdened, to face chronic diseases, increasingly prevalent in older adults, including cardiovascular diseases, that appear prominently (2).

Portugal is no exception to this scenario. According to INE (National Statistics Institute) the proportion of young people (population under 15 years old) decreased to 15% and the elderly (people aged 65 years or over) increased to 19% between 2001 and 2011. The population aged 65 years or over residing in Portugal will increase from 2.033 to 3.043 million between 2012 and 2060 (3).

Cardiovascular diseases (CVD) are a major public health problem worldwide and are responsible for almost half of all deaths in Europe (4). Studies indicate that over 80% of CVD mortality occurs in individuals over the age of 65.

Atherosclerosis is considered an inflammatory process, and there are clinical, epidemiological and experimental studies which evidence that oxidative stress is a key-factor on its development and progression (5).

In this context, several theories exist suggesting that substances endowed with antioxidant properties, particularly some trace elements (e.g., Cu, Se), could have a protective effect on the development of atherosclerotic disease.

However, despite their wide dissemination, this "antioxidant" hypothesis is far from an absolutely established stand and it has been difficult to study the specific impact of dietary intake of trace elements and the correlation of blood levels with cardiovascular morbidity and mortality. More specifically, the extent to which changes in blood levels are reflected in the tissues is not clear.

A potentially more informative approach to this issue would be by directly studying the levels of trace elements in organs and target tissues (6-8).

In this context, the main objective of the work we proposed to develop was to study the possible involvement of trace elements – both “essential” (Zn, Cu, Se) and "toxic" (Cd, Pb) in atherosclerosis and CVD.

Most of the existing studies on this topic are based on the analysis of body fluids such as blood and urine because they can be collected easily. In this work the direct analysis of tissues (kidney and aorta), collected during autopsy exams, was performed.

To this end, the most appropriate analytical procedures were used: a closed vessel acid digestion in a microwave oven (a process very efficient and that ensures maximum protection of the samples from loss and/or contamination), for samples solubilization; and ICP-MS (inductively coupled plasma-mass spectrometry), an analytical instrumental technique that is essentially characterized by its high sensitivity and multi-element analysis capability, for the analysis of the resulting solutions.

1.2 CARDIOVASCULAR DISEASES

CVD are the leading cause of morbidity and mortality in developed countries, accounting for over a third of all deaths (9). According to WHO (10), CVD are a group of diseases of the heart and blood vessels comprising:

1. CVD due to atherosclerosis:
    - Ischaemic heart disease or coronary artery disease (e.g. heart attack)
    - Cerebrovascular disease (e.g. stroke)
    - Diseases of the aorta and arteries, including hypertension and peripheral vascular disease
2. Other CVD
    - Congenital heart disease
    - Rheumatic heart disease
    - Cardiomyopathies
    - Cardiac arrhythmias

Myocardial infarctions ("heart attack") and stroke are usually acute events and are mainly caused by a blockage that stops blood from flowing to the heart or the brain. The most common cause is the formation of fatty deposits on the inner walls of blood vessels. Strokes may be caused by bleeding from a blood vessel in the brain (hemorrhagic stroke) or by a vessel blockade due to a blood clot (ischemic stroke).

Rheumatic heart disease is caused by damage to the heart muscle and heart valves from rheumatic fever. Malformations of heart structures present at birth are known as congenital heart defects. They may be caused by: (i) a close blood relation between parents (consanguinity); (ii) maternal infections (e.g. rubella); (iii) maternal abuse of alcohol and drugs (e.g. warfarin); and (iv) poor maternal nutrition (e.g. deficiency of folic acid). In some cases the cause remains unknown. Examples of congenital heart disease include holes in the septum of the heart, abnormal valves and abnormalities in heart chambers (10).

Other CVD, such as disorders of the electrical conduction system of the heart (e.g. cardiac arrhythmias), disorders of the heart muscle (e.g. cardiomyopathy) and heart valve diseases are less common than heart attacks and strokes.

The costs of CVD are one of the higher, in the different group of diagnosis. Thus, prevention and minimization of the various risk factors, may be the most effective means of preventing clinical events during the life (11).

The American Heart Association (AHA) has established a new concept, the "ideal cardiovascular health", which emphasizes seven positive behaviours and the factors that increase the likelihood of living free of CVD, and stroke in particular. This concept consists in:

1. Simultaneous presence of four healthy behaviours: a) abstinence from smoking in the last year; b) ideal body mass index (BMI); c) physical activity; d) a dietary pattern that promotes cardiovascular health;
2. Simultaneous presence of three factors: a) total cholesterol less than 200 mg/dL; b) arterial blood pressure lower than 120/80 mmHg; c) absence of diabetes mellitus;
3. No clinically established CVD (e.g., coronary artery disease, stroke).

1.2.1 Arteriosclerosis

Arteriosclerosis is a general term for the thickening and hardening of arteries. Due to the decreased elasticity of the arterial wall, systolic blood pressure increases and the diastolic blood pressure decreases. Arteriosclerosis is almost universally present in the elderly and it is more prevalent in males (12).

1.2.2 Atherosclerosis

Atherosclerosis is a type of arteriosclerosis. It is an inflammatory disease (13). Atherosclerotic lesions are often found in the aorta and major aortic branches. They are also common in the coronary arteries, where the condition is called "coronary artery disease" (also called coronary heart disease or ischemic heart disease).

The underlying pathology is characterized by a chronic inflammatory process of the arterial wall which disturbed the blood laminar flow, particularly at the branch points (14). On initiation of an atherogenic diet, rich in cholesterol and saturated fat, one of the first ultra-structural alterations is the accumulation of small lipoprotein particles in the intima. The binding of lipoproteins to proteoglycan in the intima captures and retains these particles, accounting for their prolonged residence time. Lipoprotein particles bound to proteoglycan seems to exhibit increased susceptibility to oxidative or other chemical modifications, considered by many to be an important component of the pathogenesis of early atherosclerosis (2).

The second morphologically definable event in the initiation of atheroma (a mass or plaque of degenerated thickened arterial intima, occurring in atherosclerosis) is leukocyte recruitment and accumulation. Under normal conditions, endothelial cells inhibit leukocyte adhesion to the vascular surface. However, early after initiation of hypercholesterolemia,

leukocytes adhere to the vascular surface and begin to accumulate lipids and transform into “foam cells”. Via secretion of cytokines, leukocytes penetrate the endothelial cell and enter the arterial wall (2).

Whereas the early events in atheroma initiation involve primarily altered endothelial function and recruitment and accumulation of leukocytes, the subsequent evolution of atheroma into more complex plaques involves migration of circulating monocytes, T cells and smooth muscle cells from the media, leading to an accumulation of these cells within the vascular intima. In addition, smooth muscle cell death may also participate in complication of the atherosclerotic plaque (2).

Plaques often develop areas of calcification as they evolve. Some subpopulations of smooth muscle cells may foster calcification by enhanced secretion of cytokines such as bone morphogenetic proteins, homologues of TGF-beta (2).

These lesions (atheromatous plaques) enlarge as cells and lipids accumulate and begin to bulge into the vessel lumen (Figure 1). When the process continues, there is thinning of the fibrous cap accompanied by fissuring of the endothelial surface of the plaque, which may rupture. With the rupture of the plaque, lipid fragments and cellular debris are released into the vessel lumen. These are exposed to thrombogenic agents on the endothelial surface, resulting in the formation of a thrombus. If the thrombus is large enough, and a coronary blood vessel or a cerebral blood vessel is blocked, this results in a heart attack or a stroke (10).

Atherosclerosis is clinically manifested in 10% of the population over 50 years, its development is slow and progressive, and significant arterial obstruction is needed, about 75% of the caliber of an artery, to lead to early ischemic symptoms (12).

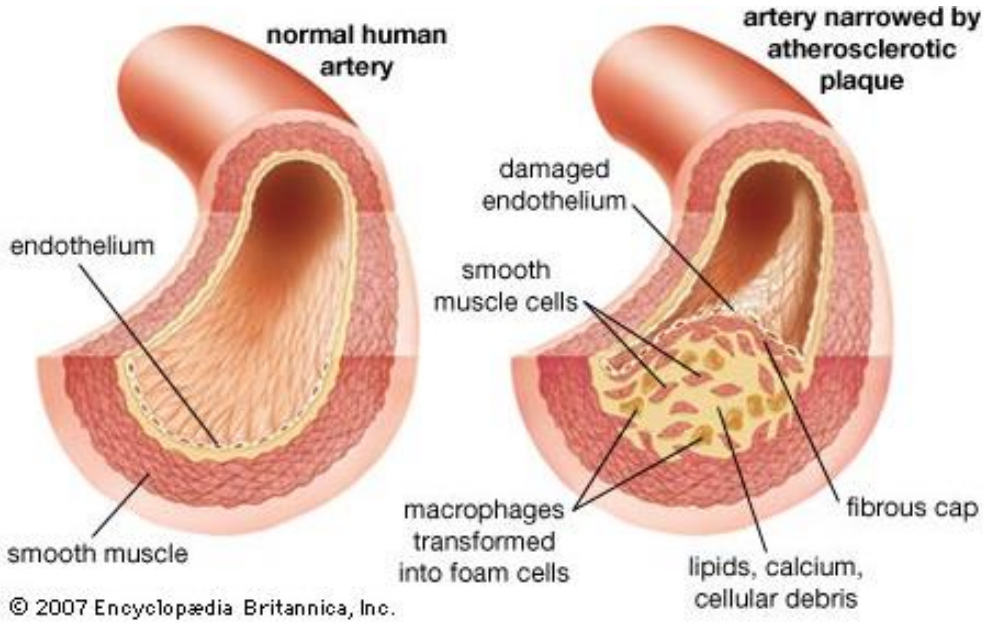


Figure 1 Normal artery (left) and an artery affected by atherosclerosis (right).

Certain drugs can reduce the risk associated with atherosclerosis. These include statins, which reduce the level of cholesterol and other fats in the blood, as well as anticoagulant drugs such as aspirin that prevent the formation of blood clots. In large arteries such as the aorta or carotid, the sections blocked by atheroma plaques may be surgically removed and replaced by synthetic materials. They can also be removed by atherectomy, in which fatty deposits are removed carefully using a blade inserted into the vessel through a catheter. In the case of completely obstructed coronary arteries, the lives of patients have been saved through coronary artery bypass surgery, in which sections of blood vessels from other parts of the body are used to divert blood flow around the blockages. Some occlusions can be opened by balloon angioplasty, in which a catheter is inserted into the site of the obstruction and a balloon is inflated in order to dilate the artery and flatten the deposits (atherosclerotic plaques) (15).

1.3 RISK FACTORS

Risk factor is a particular condition which is associated with increased probability of an individual to suffer or die from a disease. The knowledge of risk factors allows then to predict, determine the aetiology, diagnose or prevent the disease (16).

The rate of progression of atherosclerosis is influenced by cardiovascular risk factors. A classic classification of cardiovascular risk factors (CRF) is based on the possibility of the patient to modify them or not. The modifiable risk factors are those that can be changed through the adoption of new behaviours or lifestyles: hypertension (HT), diabetes mellitus (DM), hypercholesterolemia, dyslipidaemia, obesity, smoking, sedentary lifestyle, dietary habits, alcoholism, stress and others. The non-modifiable risk factors are those that do not depend on the will of the individual, so they cannot be changed, such as gender, age, previous personal history and family history. According to WHO, 75% of CVD can be attributed to modifiable risk factors (16).

Table 1 Modifiable and non-modifiable risk factors for cardiovascular disease.

Modifiable risk factors	Non-modifiable risk factors
Hypertension	Gender
Diabetes *mellitus*	Age
Hypercholesterolemia	Previous history
Dyslipidaemia	Family history
Obesity	
Smoking	
Sedentary	
Eating habits	
Alcoholism	
Stress	

1.3.1 Age

Of all the risk factors, age showed to have the strongest and most consistent association with CVD (17). Studies indicate that the incidence of stroke increases exponentially from 50-60 years and more than 80% of CVD mortality occurs in individuals older than 65 years (18, 19). As age advances, there is an increase in intimal thickening of arteries by diffuse accumulation of smooth muscle cells, connective tissue, decreased elastin and gradual increase in the stiffness of the vessels. The weakness of the arterial wall is recognized as a phenomenon that increases with age, especially after age 60 (17, 18).

1.3.2 Hypertension

Worldwide, approximately 62% of strokes and 49% of cases of coronary heart disease (CHD) are attributed to a higher blood pressure (> 115 mmHg systolic), a factor that accounts for more than 7 million deaths per year (2). According to the study by Lawes *et al.* (20), it is estimated that about 14% of deaths and 6% of disability-adjusted life years (DALY)[1] are caused by a non-optimal blood pressure.

When blood pressure rises, the resistance to the expulsion of the blood increases and the heart, to move the same volume of blood, is subject to a larger effort. To address this situation the heart has adaptation mechanisms, which is the cardiac hypertrophy. The heart is able to gradually increase the thickness of its walls, thereby increasing the force with which it contracts. However, there are limits, and permanently increased arterial resistance causes the heart to fail after a few years, exceeding its resilience and adaptability. Then, heart begins to dilate and stops working as efficiently, moving into a period of incapacity to promote adequate blood flow to the body's needs, thus resulting in heart failure. This mechanism is just one of the dangers of hypertension. The other one is the fact that hypertension functions as risk factors for atherosclerosis. Thus, excessive pressure in the arteries favors the deposition of fat in the walls and the consequent development of atherosclerosis. And the greater the values of blood pressure, especially the "minimum" (diastolic), the higher is the probability of appearance of lesions and their complications, the most common being the stroke and heart attacks, as mentioned above. If hypertension is associated with other factors (smoking habits, increased blood fats, diabetes, etc.) the risk increases much more (21).

Blood pressure levels have been shown to be positively and progressively related to the risk of stroke and coronary heart disease. In some age groups, the risk of CVD doubles for each increase of 20/10 mmHg on blood pressure, starting as low as 115/75 mmHg. In addition to coronary heart disease and cerebrovascular disease, uncontrolled blood pressure causes heart failure, renal impairment, peripheral vascular disease, damage to retinal blood vessels and visual impairment (10).

The stroke and heart attack risk of people with high cardiovascular risk and/or raised blood pressure can be reduced through non-pharmacological (e.g. low salt diet, physical activity) and pharmacological measures. These measures are particularly important for people with diabetes, as they are also particularly vulnerable to heart attacks and strokes

[1] In practice, the sum of years lost due to premature mortality and years lived with disability, adjusted for the severity of the disease.

(10). Policies to reduce salt consumption can shift the population distribution of blood pressure so that there is a reduction in cardiovascular risk.

1.3.3 Tobacco

Smoking is estimated to cause nearly 10% of CVD. There is a large body of evidence from prospective cohort studies regarding the beneficial effect of smoking cessation on coronary heart disease mortality. A 50-year follow-up of British doctors demonstrated that, among ex-smokers, the age of quitting has a major impact on survival prospects: those who quit between 35 and 44 years of age had the same survival rates as those who had never smoked (10).

Tobacco use is the leading preventable cause of death. Almost 6 million people die from tobacco use and exposure each year, accounting for 6% of all female and 12% of all male deaths in the world. “Second hand smoke” is also well established as a cause of coronary heart disease (2).

Tobacco is certainly incriminated as a causative factor for atherosclerosis. The combined action of nicotine and carbon monoxide results in the onset of atherosclerosis, especially coronary atherosclerosis. Nicotine causes increased heart work and carbon monoxide decreases the oxygen available, which leads to the possibility of arrhythmias and sudden cardiac death (21).

1.3.4 Dietary habits

There is a considerable body of evidence regarding the nutritional background of atherosclerosis in general and coronary heart disease in particular. High dietary intakes of saturated fat, cholesterol and salt, and low intake of fruits, vegetables and fish are linked to cardiovascular risk (10).

Worldwide, high levels of cholesterol cause about 56% of ischemic heart disease and 18% of stroke, a total of 4.4 million deaths annually. Changes in lifestyle that accompany urbanization clearly play an important role, since plasma cholesterol levels tend to be higher in the urban than in the rural population. These changes are largely due to the increased dietary consumption of fat, especially animal products and processed vegetable oils, and decreased physical activity (2).

The amount of dietary salt consumed is an important determinant of blood pressure levels and overall cardiovascular risk. In order to help the prevention of CVD, WHO recommends

a population salt intake of less than 5 grams/person/day. Adequate consumption of fruit and vegetables reduces the risk of CVD (10).

High consumption of saturated fats and trans-fatty acids is linked to heart disease; elimination of trans-fat and replacement of saturated with polyunsaturated vegetable oils lowers coronary heart disease risk (10).

The increase of cholesterol and lipid levels in blood contributes to the development of atherosclerosis and thus coronary disease. It is the increase in blood cholesterol that leads to the penetration and deposition in the inner layers of the arterial wall, this phenomenon being greater the higher is the concentration of cholesterol in blood (21).

Thus, the number of people who fall ill and die from coronary disease correlates perfectly with cholesterol values. Individuals with a cholesterol level higher than 300 mg/dL have a risk of developing CVD four times higher than individuals with cholesterol concentrations below 200 mg/dL (21).

A healthy diet can contribute to a healthy body weight, a desirable lipid profile and a desirable blood pressure (10).

1.3.5 Obesity

Obesity is a cardiovascular risk factor closely linked to diet and physical inactivity. Obesity results when there is an imbalance between energy intake in the diet and energy expenditure. Regular physical activity can prevent obesity by increasing the expended energy (10).

Worldwide, at least 2.8 million people die each year as a result of being overweight or obese, and an estimated 35.8 million (2.3%) of global DALYs are caused by overweight or obesity (10). Obesity is a major risk factor for CVD in adults (4).

Overweight and obesity are associated with numerous co-morbidities, such as CVD, type 2 diabetes and certain cancers. The rising prevalence of obesity represents a global public health issue, with an estimated 30% of CHD and ischaemic stroke and almost 60% of hypertensive disease in developed countries being attributable to increased BMI (4).

CHD is more frequent in obese people, and the risk increases with the degree of overweight. Obesity is often associated with high blood pressure, diabetes and increased levels of fats and uric acid in the blood. The very marked obesity also causes other cardiac problems. In particular, it requires a permanent increase of heart work that leads to hypertrophy, dilatation and onset of heart failure (21).

To achieve optimal health, the median BMI for adult populations should be in the range of 21-23 kg/m^2, while the goal for individuals should be to maintain a BMI in the range 18.5-24.9 kg/m^2.

1.3.6 Diabetes

Diabetes is a major risk factor for CVD. Diabetes is defined as having a fasting plasma glucose value ≥7.0 mmol/L (126 mg/dL). Impaired glucose tolerance and impaired fasting glycaemia are an important risk for future development of CVD (10).

DM affects approximately 180 million people worldwide, and it is estimated that this number could double by 2030. Most cases are in the age group of 45 to 64 years in developing countries, while in developed countries the prevalence is higher in individuals over 65 years (2).

Increased levels of glucose are also a factor responsible for atherosclerosis. Atherosclerosis is not only very common in diabetics as they have earlier and more extensive lesions than non-diabetics. Atherosclerotic disease in diabetics is often widespread, premature and severe, leading to stroke, disturbed circulation in the legs and changes in vision. It is believed that sugar acts directly or by favouring the formation of fat in the body and its penetration into the arterial wall (21).

Cardiovascular risk increases with raised glucose values. Furthermore, abnormal glucose regulation tends to occur together with other known cardiovascular risk factors such as central obesity, elevated blood pressure, low high density lipoprotein (HDL) cholesterol and high triglyceride levels (10).

1.3.7 Hyperlipidaemia

The lipoprotein profile includes: (i) low density lipoprotein (LDL) cholesterol, also called “bad” cholesterol); (ii) HDL, also called “good” cholesterol; and (iii) very low density lipoprotein cholesterol.

LDL cholesterol is deposited in the walls of arteries and causes atherosclerosis. In general, low LDL cholesterol levels are better for vascular health. HDL cholesterol protects against vascular disease by removing the LDL cholesterol out of the wall of arteries.

Excess calories are converted into triglycerides and stored into fat cells throughout the body. High triglycerides also increase the risk of atherosclerotic CVD (10).

1.3.8 Oral contraceptives

Oral contraceptives increase the risk of venous thrombosis, stroke and myocardial infarction. Although controversial, it has been accepted that the use of oral contraceptives is really another factor that increases the risk of atherosclerosis (21).

1.4 TRACE ELEMENTS

Most of the naturally occurring elements are present in very low concentrations in the human body, and they commonly known as “trace elements”. Most of them are metals, but a few are non-metals (e.g., Se) or metalloids (e.g., As).

Some trace elements (e.g., Cu, Zn, Se) are essential to the normal metabolic function, however they can become toxic at high levels of exposure. Others, such as Pb or Cd, only exert toxic effects in the human body, even at very low levels of exposure. In the toxicological literature/language, these particularly “toxic” elements are commonly referred as “heavy metals" (22).

An indication of the importance of “heavy metals” in the context of particularly toxic substances is their place in the ranking of the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), which lists the different substances according to their intrinsic of toxicity. The first, second, third and sixth in this list are Pb, Hg, As and Cd, respectively.

Apart from not having any known metabolic function, when toxic trace elements are present in the body they can disrupt normal cellular processes, leading to toxicity in a number of organs. They are relatively poorly absorbed into the body, but once absorbed they are slowly excreted and can accumulate, causing organ damage. Consequently, toxic trace elements toxicity is largely due to their accumulation in tissues. Their distribution in the body depends on its binding capacity to carrier molecules in the circulation. Metallothioneins (Mt) are small proteins rich in cysteine residues that display unique metal-binding properties and play a major role in the distribution and storage of metals in the body (23).

Disruption of metal ion homeostasis may lead to oxidative stress, a state where increased formation of reactive oxygen species (ROS) overwhelms body antioxidant protection and consequently induces DNA damage, lipid peroxidation, protein modification and other biochemical effects, all in the origin of numerous diseases, involving cancer, CVD, diabetes, atherosclerosis, neurological disorders (Alzheimer’s disease, Parkinson’s disease), chronic inflammation and others (24).

On a laboratory perspective (particularly an analytical perspective), the intrinsic stability of metals facilitates their detection and quantification in biological materials, although the clinical significance of the values obtained is not always very clear.

1.4.1 Cadmium

Cadmium is a "modern", very toxic metal. It occurs in the earth's crust at a concentration of 0.1-0.5 ppm and is commonly associated with Zn, Pb and Cu ores. It is also a natural constituent of seawater, with average levels between 5 and 110 ng/L. Natural emissions of Cd to the environment can result from volcanic eruptions, forest fires, generation of sea salt aerosols and other natural phenomena (25).

Cd main use is in plating and galvanizing due to its properties of corrosion resistance. It is also used in pigments for paints and plastics and as a cathode material for Ni-Cd batteries. Cd is a by-product of the mining and smelting of Pb and Zn, which are important sources of environmental pollution (26).

Cd from polluted soil and water can accumulate in plants and organisms, thus entering the food chain. Smoking greatly increases exposure to Cd, as tobacco leaves naturally accumulate high amounts of Cd. It has been estimated that tobacco smokers are exposed to 1.7 µg Cd per cigarette, and about 10% of this is inhaled when smoked (25).

With chronic exposure to Cd, approximately 50% of the accumulated dose is stored in the kidneys (27). After absorption, Cd is taken up by the hepatocytes, and then from the liver it circulates in the blood bound to Mt. At the kidney, the small molecular size of the Cd-Mt complex allows its easy filtration in the glomerular membrane and reabsorption by proximal tubular cells. Mt are then catabolised, releasing Cd ions in the tubular cells cytoplasm where they induce the synthesis of new Mt molecules (28). It then remains in the tubular cells and makes up for the major part of the Cd body burden. A number of studies have shown that this accumulation of Cd in kidneys causes tubular dysfunction and end-stage renal disease (29).

Cd is toxic to tubular cells and glomeruli, markedly impairing renal function, these lesions consisting in initial tubular cell necrosis and degeneration, progressing to an interstitial inflammation and fibrosis (26).

Chronic exposure to Cd has been associated with CVD. Increasing evidence supports that Cd may play a role in the development of a number of traditional CVD risk factors, including hypertension and chronic kidney disease, which could mediate in part the cardiovascular effects of Cd (30).

Also, experimental evidence suggests that Cd could directly induce atherosclerosis initiation and progression. Several mechanisms have been suggested to explain the role of Cd in promoting atherosclerosis.

First, Cd indirectly increases ROS production and interferes with antioxidant defence by binding to Mt (31).

Second, Cd reduces endothelial barrier function through disruption of endothelial cell–cell adhesions and by causing endothelial cell death, with subsequent attraction and activation of macrophages. Furthermore, due to Cd-caused necrotic death of endothelial cells and other cell types in the vessel wall, cellular remnants are released. All these phenomena cause cellular stress, resulting in the secretion of cytokines. A vicious circle of damage, stress and inflammation is then initiated (32).

Third, Cd may partly contribute to atherosclerosis formation through vasopressor mechanisms such as direct vasoconstrictor action, inhibition of vasodilator substances (such as nitric oxide), or activation of the sympathetic nervous system (31).

Other phenomena that have been observed in response to Cd exposure and initiate and promote atherosclerosis are hypertension, by salt retention and volume overload in the kidney (31). In rats it has been proven that Cd induces hypertension, but human studies have only yielded unconvincing and conflicting results (33).

However, most existing studies on this topic are simply based on the analysis of body fluids such as blood and urine, since they can be easily obtained (34-37).

1.4.2 Copper

Cu is widely distributed in nature and is an essential trace element. Cu deficiency is characterized by hypochromic microcytic anemia resulting from a defective synthesis of hemoglobin. Antioxidant enzymes such as catalase, peroxidase, cytochrome oxidase and others also require Cu. Cu sulfate is used medically as an emetic. It has also been used as an anthelmintic for its intense and caustic action. Cu sulfate mixed with lime has been used as a fungicide (26).

Approximately 55% to 75% of an oral dose of Cu is absorbed from the gastrointestinal tract, primarily in the duodenum. Intestinal Cu absorption can be reduced by zinc, iron, molybdate and fructose. The cellular transport and metabolism of Cu comprises a series of Cu-binding proteins and small peptides, such as albumin, ceruloplasmin (a sensitive marker of Cu levels in the body), glutathione, Mt and cytosolic Cu chaperons, which work in conjunction with Cu-ATPases to maintain Cu homeostasis. Cu levels are maintained mainly through control of biliary excretion, although Cu binding to hepatic Mt may act as a form of Cu storage. In mammals, little Cu is excreted into the urine, and the bile is the

major route of excretion. Bile secretion, enterohepatic recirculation and intestinal absorption all help to control Cu status (26).

Cu is an essential component of several important enzymes, including type A oxidases and type B monoamine oxidase. Cytochrome *c*-oxidase is probably one of the most important because it catalyses a key reaction in energy metabolism, and inherited mutational defects can result in severe pathology in humans (26).

Because Cu is a transition metal, it is capable of assuming different oxidation (or valence) states, and it is an active participant in redox reactions.

One of the most accepted explanations for Cu-induced cellular toxicity comes from the assumption that Cu ions are prone to participate in the formation of ROS. Cupric and cuprous ions can act in redox reactions. The cupric ion (Cu^{2+}), in the presence of biological reductants such as ascorbic acid or glutathione (GSH), can be reduced to the cuprous (Cu^{+}) form, which is capable of catalyzing the formation of the highly reactive hydroxyl radicals (•OH) through the decomposition of hydrogen peroxide (H_2O_2) via the Fenton's reaction:

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^{-}$$

Figure 2 Fenton reaction.

The hydroxyl radical is extremely reactive and can further react with practically any biological molecules (38).

A consequence of the generation of hydroxyl radicals is lipid peroxidation. Oxidation of LDL has several consequences, including the promotion of atherogenesis by increasing the transformation of macrophages into foam cells and by developing vasoconstrictor and pro-thrombotic properties. While the *in vivo* relevance of the initiation of LDL oxidation by Cu ions is still unclear, *in vitro* studies have clearly demonstrated the oxidation of LDL induced by Cu. *In vitro* studies of LDL oxidation are performed by incubating the reaction system with Cu ions. The samples possessing atherosclerotic lesions contained either Cu or Fe ions capable of catalysing the formation of free radicals. In addition to the studies on the oxidation of LDL, it is also known that HDL is susceptible to oxidation (38).

If Cu contributes to atherosclerosis or is simply a marker for other processes is a question that has yet to be solved, because the implications for prevention and treatment are very significant (39).

The kidneys accumulate Cu. Although primarily bound to Mt, when available, free Cu may participate in oxidation reactions (Figure 2), in a manner analogous to that of Fe. Thus, ROS are probably also responsible for the nephrotoxic effects of Cu (33).

One of the largest population-based studies on the influence of Cu in CVD, the NHANES II Mortality Study, that gathered results of previous prospective studies, has shown a strong association between the serum Cu concentration and the rate of mortality from heart disease coronary. Generally, an increase in the risk was observed when the concentration of Cu in the serum was increased. In a Dutch case control study of 10,532 people aged > 65 years, 62 subjects who died from CVD during the 6-9 years of follow-up were compared with 124 controls subjects (40). The relative risk of CVD among individuals with serum Cu concentration > 1.43 mg/L was 3.5 (CI 95%: 1.4-8.7), compared with subjects who had a serum Cu concentration in the interval 1.05 to 1.43 mg/L.

1.4.3 Lead

Lead occurs naturally in low concentrations in the earth's crust. The widespread occurrence of Pb in the environment is largely the result of anthropogenic activities. Pb has found wide use in pipes and plumbing, pigments and paints, gasoline additives, building materials and lead-acid batteries. The use of Pb in pipes, paints and gasoline additives resulted in substantial release of this metal into the environment and human exposure. More recently several measures to counter this were implemented. Because of its well-known toxicity to most living beings and because it was not demonstrated any biological need of Pb for life, the main discussion has been the determination of the dose from which it becomes toxic (26).

More than 90% of Pb in blood is found in red blood cells (26). It is shown that gastrointestinal absorption, the main route of Pb entry into the body, varies widely, depending on the chemical environment on the gastrointestinal lumen, age and Fe stores (nutritional status) of the subject. Certain components of the diet may act by increasing the solubility of Pb, such as ascorbic acid, amino acids, vitamin D, proteins, fats and lactose, thus increasing its absorption. No feedback mechanism exists limiting the absorption of Pb. The amount absorbed is excreted primarily in the urine. Feces contain predominantly the Pb that was not absorbed. Being a calcium-like element, Pb usually follows the movement of Ca in the body and can be affected by the physiological regulators of Ca metabolism. Because bone storages more than 90% of the total body burden, increased bone turnover, either by physiological (e.g., pregnancy or lactation) or pathologic (e.g., osteoporosis) causes leads to the release of Pb from bone. Pb can be

remobilized from bone by competing with Ca for transport and for binding sites and it is released, along with Ca, when bone is resorbed. The mechanisms by which both elements enter and leave the bone are similar and, through these mechanisms, bone Pb equilibrates with blood Pb (23).

The toxic effects of Pb can range from subtle biochemical effects to manifest clinical effects. Children are particularly sensitive to the toxic effects of Pb and the most critical effects are observed in the nervous system. For adults with excessive occupational exposure or even accidental exposure, the main concerns are peripheral neuropathy, chronic nephropathy and hypertension. Pb can directly affect blood pressure by changing the sensitivity of vascular smooth muscles to vasoactive stimuli or indirectly altering the neuroendocrine function of smooth muscle cells (26).

The most important manifestation of Pb toxicity on the cardiovascular system is hypertension. This is likely caused by altered calcium-activated changes in contractility of vascular smooth muscle cell secondary to decreased $Na^{+}K^{+}$-ATPase activity and stimulation of the Na^{+}-Ca^{2+} exchange pump. Pb may also effect vessels by altering neuroendocrine input or sensitivity to such stimuli or by increasing ROS that enhance nitric oxide inactivation (33).

Population-based studies on the cardiovascular effects of Pb have focused mainly on the association with hypertension, an important risk factor for morbidity and mortality from CVD (41). The relationship between the blood Pb level and blood pressure has been described as statistically significant (42). The hypertension induced by high doses of Pb can be partly explained by the nephrotoxic action of this metal (43). In individuals exposed to low concentrations of Pb, renal function is responsible for a persistent increase in blood pressure, with an inverse association between glomerular filtration rate (GFR) and the concentration of Pb in blood, which was observed in people with blood levels as low as 10 µg/dL (44) or even 5 µg/dL (45). It was also noticed that much other cardiovascular conditions, including CHD, stroke and peripheral artery disease were associated with exposure to Pb, but the exact role of Pb in CVD is not yet fully defined (46).

In animal experimental studies it has been shown that Pb exposure promotes atherosclerosis. Depending on the degree and duration of the exposure, cardiac and vascular complications may be potentially fatal. There are also indications that chronic exposure to Pb can affect lipid metabolism (47). Current evidence on oxidative stress induced by Pb has been mainly based on *in vitro* experiments (48) or in animal studies (49). Chronic exposure has also been associated with atherosclerosis and increased cardiovascular mortality in humans (50). Several epidemiological studies among workers

with high occupational exposure have reported an association between Pb exposure and markers of oxidative stress (51). Epidemiological studies have reported that low-level Pb exposure has an association with several diseases such as hypertension and peripheral arterial disease (52, 53).

1.4.4 Selenium

The trace element Se is a non-metallic essential nutrient of fundamental importance to human biology. The biological role of Se in mammals, including man, is mainly attributed to the presence of selenocysteine at each of the four catalytic sites of the enzyme glutathione peroxidase (GPx). This enzyme uses glutathione to reduce peroxides in the cell and, this way, protects the lipid membranes and possibly proteins and nucleic acids from damage caused by free radicals and other oxidative agents. The need for Se is related to the degree of oxidative activity and the supply of nutrients such as Zn, Cu, Mn, Fe and vitamin E (26).

The most extensively documented deficiency of Se in humans is Keshan disease, an endemic cardiomyopathy first discovered in Keshan County, People's Republic of China in 1935. The disease is clinically characterized by varying degrees of cardiomegaly and cardiac decompensation. The histopathologic exam of the myocardium shows degeneration and necrosis of myocardial fibers and their replacement by fibrosis and areas of healing.

Se deficiency is also one of the factors that influence the risk of hypertension and CVD. Furthermore, myocardial ischemia can also be worsened in case of Se deficiency (54).

Experimental studies suggest that Se may reduce CVD risk via several mechanisms (Table 2). For example, antioxidant defences may reduce vascular and tissue injury resulting from formation of ROS due to shear stress, hypoxia, hypertension, hyperlipidemia or diabetes. Systems related to Se may also decrease the oxidation of lipids and protect the vascular endothelium from damage due to oxidized LDL particles. In animal studies, Se consumption has shown to increase cardiomyocyte GPx activity, to improve cardiac recovery from ischemia-reperfusion and to reduce the size of myocardial infarction (55).

Table 2 Experimentally-observed effects of selenium that may reduce CVD risk. (55)

Systemic effects	Direct cardiovascular effects
Antioxidant defence against free radicals and reactive oxygen species	Increased myocardial antioxidant glutathione peroxidase activity
Decreased lipid peroxidation	Improved cardiac recovery from ischemia-reperfusion injury
Protection against vascular damage from oxidized LDL particles	Limitation of ischemia-induced and diabetes-induced ultrastructural damage
Antithrombotic effects from decreased plasma thromboxane A2	Reduction in myocardial infarct size
	Restoration of altered myocyte ion currents
	Reduced incidence of ischemia-induced ventricular arrhythmias

1.4.5 Zinc

Zn is an essential trace element that is vital in maintaining normal physiology and cellular functions. Normal Zn levels in plasma are in the range of 70 to 120 mg/dL. Low levels of Zn ("deficiency") are usually defined as Zn plasma levels lower than 60 mg/dL (56).

The absorption of Zn from the gastrointestinal tract is homeostatically regulated. About 20-30% of ingested Zn is absorbed into systemic blood circulation. Zn uptake from the intestinal lumen involves passive diffusion and a carrier-mediated process through specific Zn transporters, such as ZnT-1. Intestinal absorption of Zn can be reduced by dietary fiber, phytates, Ca and phosphorus, while amino acids, picolinic acid and prostaglandin E2 can enhance Zn absorption. Once absorbed, Zn is widely distributed throughout the body. The total content of Zn in the human body is in the range of 1.5-3 g. Most of this is found in muscle (60%), bone (30%), skin/hair (8%), liver (5%) and pancreas (3%). The highest concentrations of Zn are found in prostate, pancreas, liver and kidney. In plasma, Zn is mostly bound to albumin (60-80%), which represents the metabolically active pool of the element. The remainder is bound to α_2-macroglobulin and transferrin. Zn is excreted both by urine and feces. The concentration of Zn in plasma is not a sensitive indicator of Zn status and does not reflect Zn levels in the tissues and its effects at the various target sites. Zn ions are involved as inter- and intracellular messengers and the homeostasis of Zn has to be tightly controlled (26). Zn is an effective inducer of Mt synthesis and, when

Mt become saturated in intestinal cells, Zn absorption is decreased. Mt are also an important intracellular Zn storage (26).

Zn is found in more than 200 metalloenzymes, including acid phosphatase, alkaline phosphatase, alcohol dehydrogenase, carbonic anhydrase, superoxide dismutase and DNA and RNA polymerases. Zn contributes to gene expression and chelates with either cysteine or histidine in a tetrahedral configuration, forming looped structures know as *Zn fingers*, which bind to specific DNA regions. Other functions of Zn include membrane stabilization, vitamin A metabolism and the development and maintenance of the nervous system. Zn and Cu concentration generally have an inverse relationship in the serum, with elevated Zn concentrations resulting in decreased Cu concentrations (33).

It is important to note that, although Zn ions *per se* are redox inert, they have profound effects on redox state and, conversely, redox state has a profound effect on Zn metabolism. Increased oxidative stress can release Zn from its binding sites, where it performs coordination function, as reported in a number of proteins (Fig. 3). Zn ions released from proteins may potentially act on signal transduction pathways, modify mitochondrial metabolism, and can affect the redox status of the cell. Zn at various concentrations can increase the cell's antioxidant capacity or the release of toxic ROS (56).

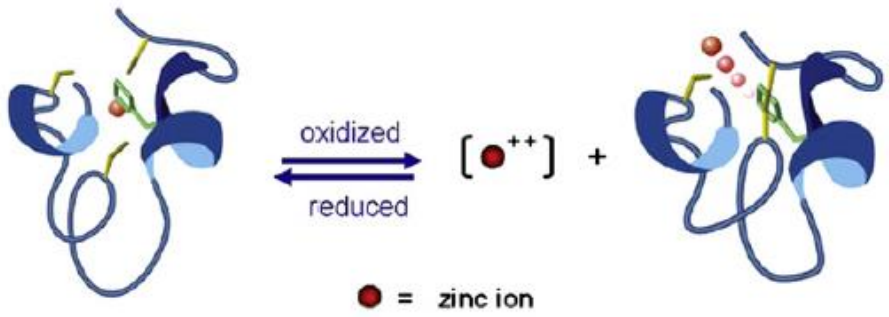


Figure 3 Chemical background for the flexibility of zinc ion in changing the redox environment (redox zinc switch). Zinc is coordinated in a reduced sulphur-containing protein domain and is released under oxidation.

Numerous studies have explored the association of Zn with cardiomyopathies, arrhythmias and coronary diseases. Several investigators have shown decreased blood Zn levels in patients with ischemia/myocardial infarction, congestive heart failure, conduction abnormalities and heart transplant, resulting in poor outcomes (56).

The 'response to injury' hypothesis of atherosclerosis by Ross and Glomset (60) states that atherosclerosis begins with endothelial cell injury. It is now known that endothelial cells undergo apoptosis, possibly as a result of increased oxidative stress from oxidized LDL. Evidence suggests that Zn may be protective by maintaining the integrity of endothelial cells and thus reducing vessel susceptibility to atherosclerosis. Therefore, deficiency of Zn may corollary promote endothelial cell injury. A mediator of inflammatory responses in many cells is nuclear factor-kB (NF-kB), a transcription factor that regulates the gene expression associated with apoptosis and inflammation. The NF-kB binding to DNA is dependent on Zn and, consequently, the NF-kB transcriptional activity is regulated by Zn. In endothelial cells the Zn ionophore pyrithione inhibits the regulatory activity of NF-kB, thereby inhibiting the inflammatory process responsible for the atherosclerotic process (56).

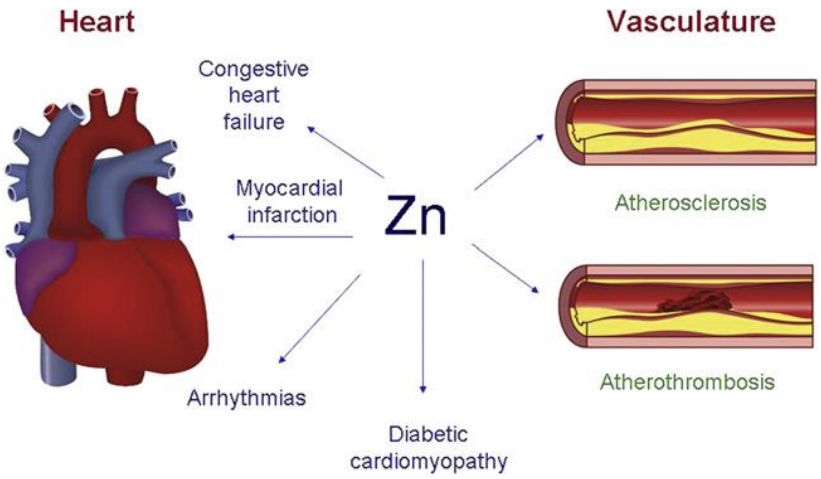


Figure 4 Role of zinc in cardiovascular diseases. (56)

Zn has not only an influence in the atherosclerotic process but also in other CVD like arrhythmias and myocardial infarction. In addition, Zn is involved in insulin signalling and the pathogenesis of diabetes, further indicating the potential role in diabetic cardiomyopathy. Zn supplementation also protects cardiomyocytes from acute redox stress and prevents inflammatory processes that are triggered during myocardial damage. Zn is also a wound-healing agent, and it is believe that it may support survival of cardiac stem cells that are essential components of cardiac healing.

Some studies seem to corroborate the hypothesis that Zn may have an anti-atherosclerotic role in both animals (57-59) and humans (60).

1.5 TRACE ELEMENT ANALYSIS

As previously discussed, there is considerable evidence supporting the hypothesis that atherosclerosis involves chronic inflammation and oxidative stress, although the origin of oxidation is still not fully established. Various substances, including transition metal ions have been suggested as sources of oxidizing species in atherosclerotic lesions (5). Heavy metals ions are shown to be present at high levels in some animal models and also in human atherosclerotic plaques, which are in agreement with the hypothesis that metal ions contribute to the formation of plaque as well as for their destabilization (8, 61, 62).

Many chemical elements occur in biological matrices at so low levels that they could not be detected at the beginning of the development of instrumental analysis (63). However, the extraordinary development of analytical techniques observed in last decades made possible a reliable quantification of these chemical species ("trace elements"), and contributed to the current understanding of the importance of these elements in human health and disease.

One of the first important contributions to metal analysis in clinical chemistry was the introduction of the flame photometer, based on atomic emission spectrometry, which significantly improved Na and K determination (64). Later, the development of atomic absorption spectrometry (AAS) with flame atomization allowed faster determinations of several important metals (e.g., Ca, Mg, Fe, Cu, Zn), with great accuracy and precision, in small sample volumes. However, the range of analyzable metals was low due to its poor sensitivity (high detection limits). This limitation was overtaken in the 1970s with the introduction of the graphite furnace as atomization system, which greatly improves the efficiency of the atomization process (65). The development of atomic absorption spectrophotometry with graphite furnace (or electrothermal) atomization allowed to decrease by about 10 to 100 times the limits of detection for most metallic elements, making possible its quantification at the parts per billion (ppb) level (66).

In practice, all spectroscopic elemental techniques, with different requirements regarding sample preparation and with an applicability dependent on the sensitivity and selectivity needed, can be used for the determination of trace elements in biological samples. Highlight for AAS, in its different atomization modes (flame atomization, electrothermal atomization and hydride generation), inductively coupled plasma-atomic emission spectrophotometry (ICP-AES), X-ray fluorescence and inductively coupled plasma-mass spectrometry (ICP-MS) (67).

In particular, ICP-MS is currently the “golden standard” for trace element analysis. The potential of ICP-MS in the determination of trace elements in various fluids (whole blood, plasma, serum, urine) and biological tissues is extensively illustrated in the literature (68). The combination of excellent metrological characteristics (very low limits of detection, typically in the order of 10^{-4}-10^{-5} g/L, wide dynamic range, of nine orders of magnitude, and high precision) with very rapid multi-elemental analysis capability is the basis of the high potential of this analytical technique. This last feature (the ability to fast multi-elemental analysis), in particular, makes ICP-MS a very important tool in the evaluation of the interactions and reciprocal effects of trace elements in the human body (67).

A mass spectrometer comprises three main components: (i) an ion source (the plasma, in ICP-MS), which converts sample components in ions (mainly mono-elemental and positive, monovalent) in the gas phase; (ii) one (or more) mass analyzer, which separates the ions (ionized analytes) based on their mass-to-charge ratio (*m/z*); and (iii) a detector that counts the number of ions of each *m/z* value, producing a mass spectrum.

The use of a high temperature plasma (6000-10000 ºC) as ion source for elemental mass spectrometry has been exploited over the last decades of the twentieth century, and the more successful combination was ICP-MS. After its commercial introduction in the early 1980s (69), ICP-MS has become the leading technique for trace element analysis, allowing the fast determination of more than 70% of the elements of the periodic table. This stems from the fact that, due to the high temperature of the plasma, ionization and subsequent atomization is highly effective, i.e., a complete fragmentation of molecules in its constituent atoms and subsequent ionization occurs in a very large extent (70).

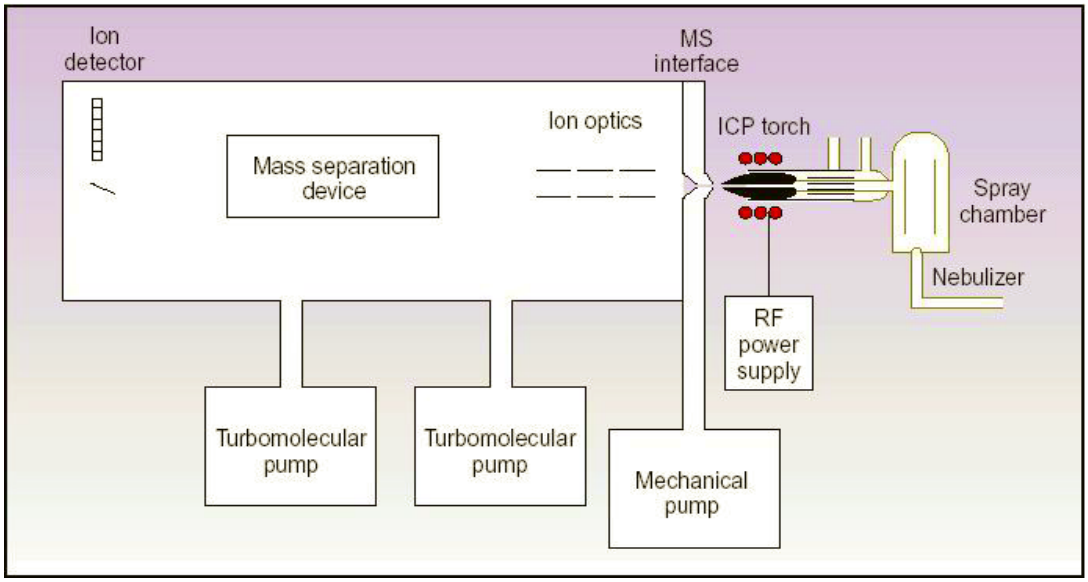


Figure 5 Diagram of ICP-MS instruments (71).

One of the major problems in the analysis of biological fluids and tissues is the possible contamination of the samples at all stages of the analytical procedure (from sampling and preservation to sample pre-treatment and final instrumental analysis). It is well known that even slight hemolysis can increase the concentration of Fe, Cu, Zn, Pb, Rb, Mn and Mg in serum from several tens to several hundred percent. With the use of needles for sampling, the blood may be contaminated with Al, Cr, Ni and Zn. Glassware is another source of contamination, and is preferable to use plastic materials. The use of anticoagulants itself (heparin, EDTA) can contaminate the samples at the stage of sampling (67).

The complex matrix of biological samples in most cases requires a step of sample preparation. Depending on the circumstances, sample preparation can range from a simple dilution of the sample to a thermal mineralization or an acid digestion in a microwave oven (67).

The ideal process for solubilization of samples should be able to solubilize the sample completely, leaving no residue, should be reasonably fast and reagents used should not interfere with the subsequent determination or should be easily removable. It must also be taken into account that the reagents used should be available in high purity grade (to not contaminate the sample), and losses of analyte(s) must be insignificant. Together with these criteria, it must be taken into account that the reactants/reaction products should not attack the containers where solubilisation procedure is carried-out, and this should be a safe procedure.

There are essentially two methods of solubilisation of samples: "dry ashing" (incineration, ashing; dry mineralization) and "wet ashing" (digestion; wet mineralization). The first is the classical method used to mineralize large amounts of samples with high organic content (e.g. biological samples, solid waste, sediments). The samples are heated at elevated temperatures (in a furnace) to destroy organic matter. The ashes are then treated as if they were a simple inorganic sample. This method has several limitations, such as the possibility of losing some of the more volatile elements, some ashes can be difficult to solubilize completely and there is a great potential for sample contamination. In the second case (wet digestion) the solubilisation is performed by using concentrated strong acids (e.g., $HClO_4$, HNO_3, HCl, H_3PO_4, H_2SO_4, HF) and/or other reagents such as H_2O_2, which lead to the decomposition of organic matter. This procedure can be done in two different ways: a) In open vessels – by simply bringing the mixture [acid(s) + sample] to boiling in a beaker covered with a watch glass; or b) in closed vessels – in a microwave oven, where microwave radiation provides the energy to heat the mixture. Although it can only be used for small sample masses (up to 1 g), the last one is a very efficient (because

it occurs at high pressures/temperatures) and safe solubilization process (it minimizes losses and contamination).

The efficiency of wet (acid) digestion in a microwave oven depends on the nature of the sample, the adjustment of heating time, pressure and temperature, the mass of the sample and the type of acids used. Among these parameters, the choice of acid(s) is very important. Several concentrated acids such as nitric acid, hydrochloric acid, sulfuric acid or a mixture of these acids have been widely used to solubilize samples (68). The microwave heating is very efficient as heat is developed within the mixtures [sample + reagent (s)] and is not "driven" through the vessel walls as it happens when the sample is put into a vessel over an electric plate. Microwave heating also produces a very uniform heating.

The acid digestion in closed vessels in a microwave oven has become the most important procedure for organic samples solubilization. As already highlighted, this procedure minimizes the amount of reagents required and, as a result, a reduced possibility of contamination (and the “sample blank” value). There is also a significant decrease in time spent on this task – the decomposition of the samples occurs at higher temperatures, which greatly increases the reaction rate. Importantly, the use of closed vessels makes it possible to avoid uncontrollable losses of trace elements, which inevitably occur when solubilisation is made in open vessels. This is a very important issue, especially when determining trace elements that form hydrides and/or volatile halides, such as As, B, Cr, Hg, Sb, Sn and Se (67).

CHAPTER 2

OBJECTIVES

2 OBJECTIVES

The link between trace element imbalances and CVD is not fully understood. Most of the existing studies on this subject are simply based on the analysis of body fluids (blood, serum, plasma, urine), since they can be easily obtained.

From a theoretical point of view, a potentially more conclusive approach could be the direct study of trace elements changes in the target organs and tissues, and this was the rational for the study performed.

The study focused on five trace elements: Zn, Cu, Se – three elements with a well-recognized “essentiality”, needed for very important physiological functions; Cd and Pb – two important "toxic" trace elements, whose role in atherosclerosis and CVD is long suspected.

Basically, we aimed to directly look, in the tissues themselves, for evidences of significant changes in trace elements levels that could be related to CVD.

For a better understanding of these changes, the age-related changes in “normal” (non-diseased individuals), the gender-related differences and the effect of smoking habits were also assessed.

CHAPTER 3

MATERIALS AND METHODS

3.1 LABORATORY WARE

Glass and metal materials were avoided in order to reduce the risk of contamination. Whenever possible, polypropylene made labware (pipette tips, volumetric flasks, tubes, auto sampler cups, etc.) was used.

All material was previously decontaminated with 10% (v/v) HNO_3 before being thoroughly rinsed with ultrapure water. Ultrapure water (resistivity > 18.2 MΩ.cm at 25 °C) was obtained by de-ionization with mixed bed ion-exchange resins and further purification in an Arium® pro B water purification system (Sartorius, Germany).

3.2 SUBJECTS

This study was conducted in kidney and aorta samples obtained from individuals (n=56) submitted to medico-legal autopsy exam at Instituto Nacional de Medicina Legal e Ciências Forenses, I.P. – Delegação do Norte (INMLCF) during the period of October 2013 to February 2014.

Samples were collected during the medico-legal autopsy by the cooperating forensic pathologists. Only individuals with a post-mortem interval (i.e., the time that has elapsed since the person has died) lower than 72 hours were considered for the study.

In order to fulfil all current legal regulations regarding human tissue collection for scientific research purposes, it was assured that the individual's name was not registered in the Portuguese "Registo Nacional de Não Dadores" (RENNDA) database.

Two groups of individuals were studied:

Group A – "Healthy" individuals (n=19). Clinicopathological criteria were used for case selection: absence of a cardiovascular disease history and atheromatous plaques causing less than 50% of luminal obstruction.

Group B – Individuals with evidence of "cardiovascular diseases" (n=23). Clinical condition was evaluated through the examination of medical files and a standard questionnaire to relatives. Cases of drowning, burns, severe sepsis or history of severe systemic disease were excluded from the study.

Note: In total, samples from 54 individuals were collected. However, it was not possible to include all the individuals in one of the above defined groups because it was not always possible to assess the degree of lumen obstruction caused by atheromatous plaques.

3.3 SAMPLE COLLECTION

Kidney tissue: renal cortex and renal medulla – For systematic reasons, the right kidney was always collected. Using a long-bladed knife, a longitudinal cut was made along the kidney, in order to expose the cortex and the medulla (Fig. 6). Then fragments of about 1 cm^3 (~1 g) were collected from the cortical and medullar zones of the kidney.

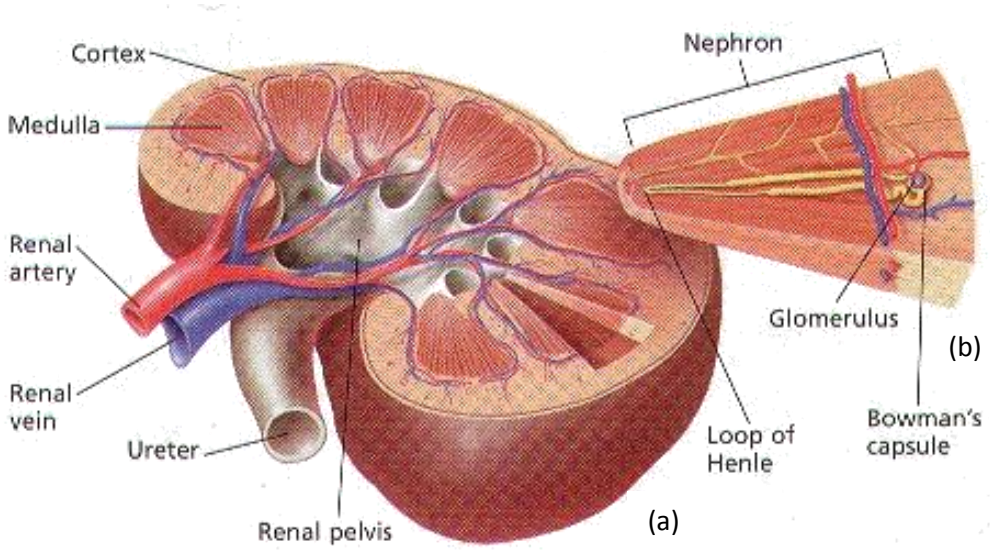


Figure 6 (a) Schematic representation of kidney anatomy; **(b)** Nephron structure (72)

Aorta tissue (with and without evidence of atherosclerotic plaques) – For systematic reasons, samples were always collected from the thoracic aorta. Using plastic knives, samples from aorta segments, both with and without evidence of atherosclerotic plaques, were collected (n=54).

In all cases, after samples removal from the corpse, blood residues were thoroughly cleansed with ultrapure water. Samples were then stored into decontaminated polypropylene tubes. After transport to laboratory in refrigerated conditions, samples were stored frozen (-20 ºC).

3.4 SAMPLE PREPARATION

Before analysis, samples were washed several times with ultrapure water and placed in a dry oven, at 110 ºC, until constant weight (approximately 24 hours). Then, between 100 and 500 mg of the dried samples were weighted into the Teflon microwave oven digestion vessels (previously decontaminated and washed with ultrapure water).

Samples were digested using 2.5 mL of concentrated (≥ 65%), high-purity HNO_3 (TraceSELECT®, Sigma-Aldrich, France) and 1.0 mL of concentrated (≥ 30%) high-purity H_2O_2 (TraceSELECT® Ultra, Sigma-Aldrich) in a MLS-1200 Mega microwave oven (Milestone, Italy) equipped with an HPR-1000/10 S rotor. The microwave oven program is shown in next Table.

Table 3 Microwave oven program for samples acid digestion.

Step	Power (W)	Time (min.)
1	250	1
2	0	2
3	250	5
4	400	5
5	600	5

After cooling, the vessels content was transferred into decontaminated polypropylene volumetric flasks and diluted to 50 mL with ultrapure water. The solutions were stored in decontaminated polypropylene tubes at 4 °C until analysis.

3.5 SAMPLE ANALYSIS

The analytical determination of Cd, Cu, Pb, Se and Zn was performed using a VG Elemental (Winsford, UK), PlasmaQuad 3 ICP-MS instrument, equipped with a Meinhard® type A pneumatic concentric nebulizer, a water-cooled quartz spray chamber with impact-bead, a standard quartz torch and nickel sample and skimmer cones. Both the spray chamber and the ICP-MS interface system were cooled to 12 ºC by circulating water. High purity (99.9999%) argon (Gasin, Portugal) was used as nebulizer and plasma gas. For sample introduction, a Gilson (Villiers le Bel, France) model M312 peristaltic pump was used. A 2% (v/v) HNO_3 solution was used for the washing of the sample introduction system.

The elemental isotopes (*m/z* ratios) ^{55}Mn, ^{65}Cu, ^{66}Zn, ^{111}Cd and ^{208}Pb (as analytical masses) and ^{45}Sc, ^{89}Y, ^{115}In, ^{159}Tb and ^{209}Bi (as internal standards) were monitored.

The limit of detection (LoD) and the limit of quantification (LoQ) were calculated as the concentration corresponding to 3 and 10 standard deviations of 10 repeated determinations of the blank signal, respectively. Expressed as µg/g in real samples (tissues), the LoQ achieved were: 0.59 (Cu); 1.01 (Zn); 0.735 (Se); 0.04 (Pb) and 0.11 (Cd).

A 10 µg/L tuning solution was prepared by dilution of a commercial multi-element aqueous solution (AccuTrace™, ICP-MS 200.8-TUN-1; AccuStandards, New Haven, CT, USA). The ICP-MS instrument was daily tuned for maximum signal sensitivity and stability using ^{115}In as the target isotope.

Internal standards (Sc, Y, In, Tb and Bi) solution was prepared by dilution of a commercial solution (AccuTrace™, ICP-MS 200.8-IS-1; AccuStandards). It was added to all the samples and standards solutions in order to obtain a 10 µg/L final concentration.

ICP-MS calibrating solutions were prepared by dilution of a commercial multi-element aqueous solution (AccuTrace™, ICP-MS 200.8-CAL1R-1; Isostandards Material, Madrid, Spain). Calibration curves were obtained with six solutions of element concentrations within the 1-200 µg/L range.

All the solutions were prepared with 2% (v/v) HNO_3.

3.6 ANALYTICAL QUALITY CONTROL

Since kidney and aorta tissue are not available as certified reference materials (CRM) for trace element analysis, DOLT-4 (dogfish liver) and DORM-3 (fish Protein), CRM available from the National Research Council (Canada) and independent standard solutions were used to validate and ensure accuracy of the analytical procedure. CRM were subjected to the same sample pre-treatment and were analysed within the concentration range of the analytes in the sample.

For contamination control during the microwave-assisted acid digestion procedure, a sample blank was performed in each digestion cycle (10 samples).

All the samples, after adequate dilution, were analyzed in duplicate (two determinations in the same analytical run). For results with relative standard deviation ≥ 10% two additional determinations were performed.

3.7 STATISTICAL ANALYSIS

Data statistical analysis was performed using GraphPad Prism 5 for Windows (San Diego, California, USA) statistical software package, version 5.04.

For each sample, element concentration (µg/g) was expressed as the mean±SD of the two replicate determinations.

Descriptive statistical parameters (mean, median, standard deviation, minimum and maximum) were calculated for each element in each sample type (kidney cortex and

kidney medulla; aorta with and without evidence of atherosclerotic plaques; atherosclerotic plaques).

For the statistics calculation, results that fell below the LoQ were assumed as the LoQ divided by the square root of 2, a commonly used procedure for data imputation in such cases (73).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 KIDNEY

4.1.1 Medulla

a) All the samples

Table 4 Concentration (µg/g) of elements in kidney medulla.

	Cu	**Zn**	**Se**	**Cd**	**Pb**
N	57	57	53	57	57
Mean	6.72	115.7	3.97	41.37	0.26
SD	3.98	86.48	2.18	38.72	0.21
Median	6.29	89.74	3.86	31.36	0.19
Minimum	1.28	16.93	0.81	4.47	0.04
Maximum	15.82	437.4	9.05	210.7	1.01

b) Men vs Women

In medulla, no significant difference was found between genders for Cd, Cu, Se and Zn (Tables 5 and 6). Most of the available literature is focused on the metal content of kidney cortex. Results for renal medulla are very scarce. Lower Zn and Se levels have been found in women blood serum (74), but apparently this is not reflected in the content of renal medulla tissue.

Table 5 Concentration (µg/g) of elements in kidney medulla in men.

Men	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	35	35	34	32	35
Mean	6.69	121.8	4.60	36.95	0.32
SD	4.19	97.15	5.78	23.79	0.23
Median	6.29	84.42	3.66	34.74	0.27
Minimum	1.28	16.93	0.81	4.47	0.05
Maximum	15.82	437.4	35.32	83.90	1.01

Table 6 Concentration (µg/g) of elements in kidney medulla in women.

Women	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	18	18	17	17	18
Mean	7.02	109.3	4.80	24.72	0.16
SD	3.82	68.30	2.88	14.10	0.10
Median	6.56	106.8	4.03	26.53	0.14
Minimum	1.47	21.31	1.55	5.57	0.04
Maximum	13.97	301.0	11.90	54.96	0.46

For Pb, a higher concentration was found in men (0.32±0.23 versus 0.16±0.10 in women; p≤0.01) (Figure 7).

In general, men have higher blood Pb levels than women. Pb in blood is bound to hemoglobin, inside the erythrocytes. Therefore, since men have higher hematocrit values (and higher haemoglobin values), it is expected that higher Pb levels are also found in men (75).

Our findings may also be due to a higher percentage of smokers in the men sub-group (35,5% vs 13,6% in the women sub-group), and smoking is an important source of human exposure to Pb (29).

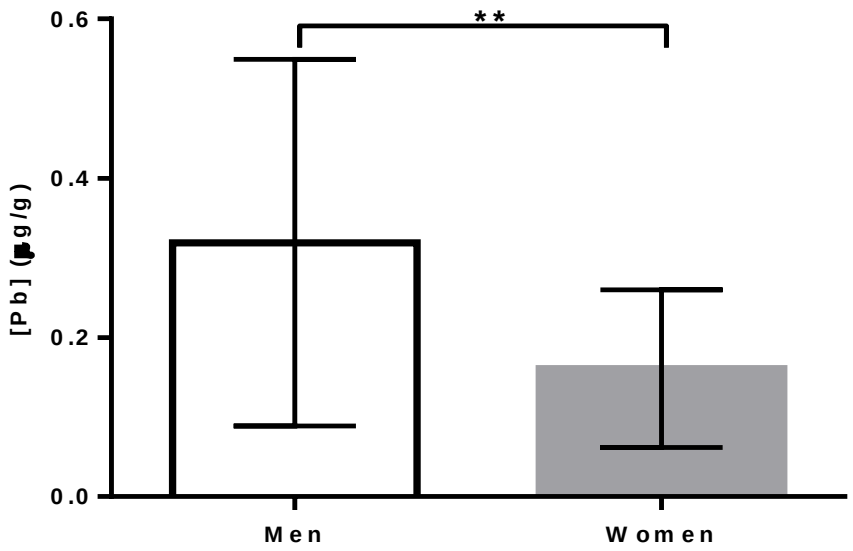


Figure 7 Lead levels (µg/g) in kidney medulla in men (n=35) and women (n=18), **p ≤0.01

c) Smokers vs Non-smokers

Results for Cu, Zn, Se, Cd and Pb in the renal medulla tissue of smokers and non-smokers are shown in next tables.

Table 7 Concentration (µg/g) of elements in kidney medulla of smokers.

Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	13	13	11	13	13
Mean	7.46	166.7	3.26	75.97	0.39
SD	4.71	112.1	1.77	61.04	0.19
Median	6.66	146.7	2.66	52.00	0.33
Minimum	1.28	24.53	0.81	7.03	0.14
Maximum	15.82	437.4	6.05	210.7	0.70

Table 8 Concentration (µg/g) of elements in kidney medulla of non-smokers.

Non-smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	28	28	28	28	28
Mean	6.16	91.46	4.06	27.78	0.21
SD	3.60	63.47	2.51	20.24	0.20
Median	5.83	76.15	3.61	20.87	0.16
Minimum	1.44	16.93	1.16	4.47	0.05
Maximum	13.97	301.0	11.90	80.29	1.01

Plants have evolved physiological mechanisms to gain access to essential elements from the soil. On the other hand, plants can accumulate toxic metals, which is an ability used by modern biotechnology to remove metals from contaminated soils. However, regarding *Nicotiana tabacum* farming for cigarette production, this ability of tobacco plant becomes a health problem. Toxic metals such as Cd, Pb, Hg and Ni are found in tobacco, and consequently in cigarette smoke (29).

Recent data suggests that smoking interferes with the metal homeostasis of the human body and plays a crucial role in the pathogenesis of a number of diseases. It was demonstrated that metals present in cigarette smoke are essential in the process that led to the injury of the vascular endothelium (76). And as already pointed out, the dysfunction

and disruption of vascular endothelium is the primary event in the genesis of atherosclerosis (29).

We found a significant difference between smokers and non-smokers regarding the kidney medulla levels of Cd, Zn (Figure 8) and Pb (Figure 9).

The average content of Zn in cigarettes has been estimated in 24 µg/g and about 70% is transferred to the smoke. Studies regarding the relationship between plasma Zn concentration and smoking habits found no differences in Zn levels between smokers and non-smokers (74, 77). In our study, however, a significantly higher Zn content in the renal medulla of smokers (166.7±112.1 µg/g versus 91.46±63.47 µg/g in non-smokers; p≤0.01) was found (Figure 7). It seems that excretion by the kidney tends to increase to remove the excess of Zn inhaled via smoke.

Smokers also showed significantly higher levels of Cd (75.97±61.04 µg/g vs 27.78±20.24 µg/g in non-smokers) (Figure 8). This is in accordance with Scott et al. (78), who also found increased levels of Cd in smokers kidney tissue.

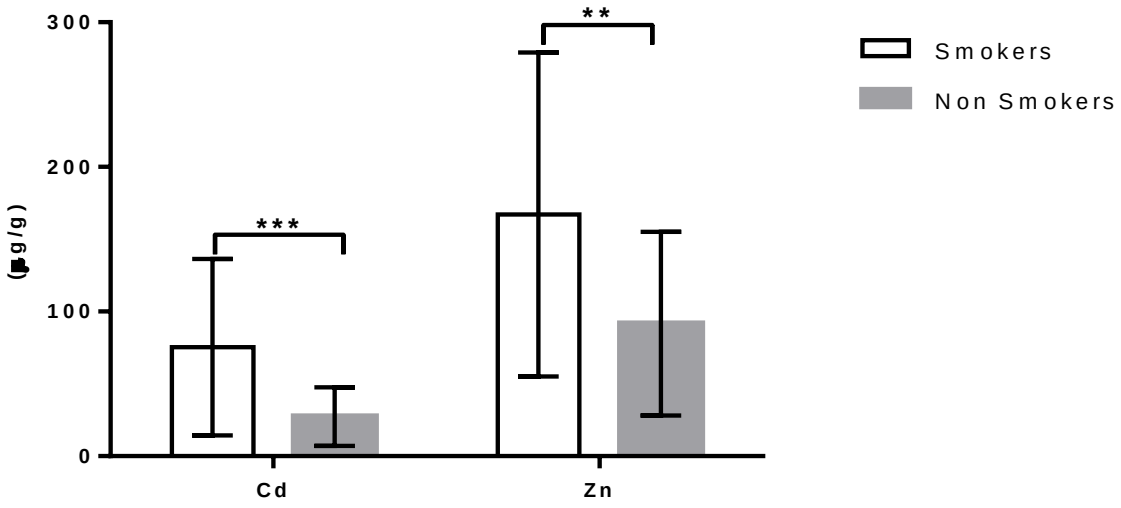


Figure 8 Cadmium and zinc levels (µg/g) in kidney medulla of smokers (n=13) and non-smokers (n=28); **p≤0.01, ***p≤0.001

The Pb content of a cigarette has been estimated around 1.2 µg, and about 6% of this total amount is thought to pass over to mainstream smoke, which is further inhaled by smokers (29). Navas-Acien *et al.* (53) and Mortada *et al.* (79) reported higher Pb levels in smokers blood. In our study, Pb was significantly higher in the smokers' renal medulla: 0.39±0.19 µg/g *versus* 0.21±0.20 µg/g in non-smokers (Figure 9).

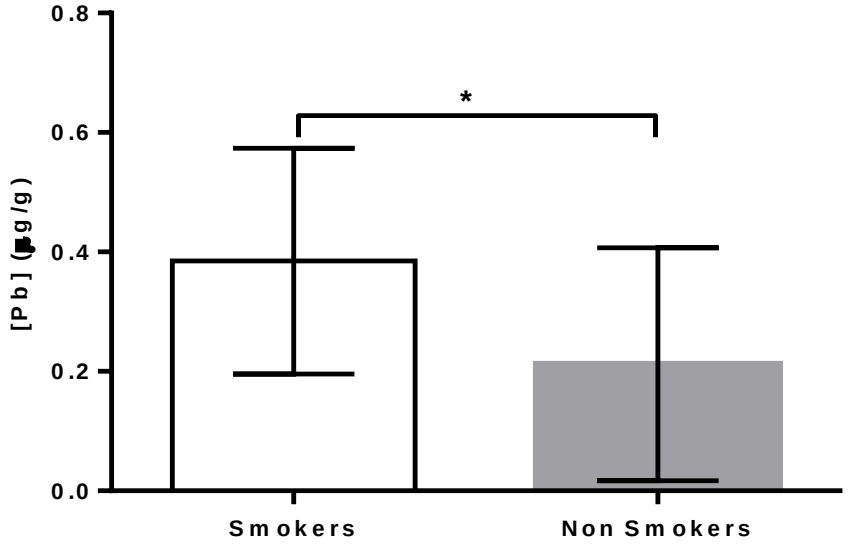
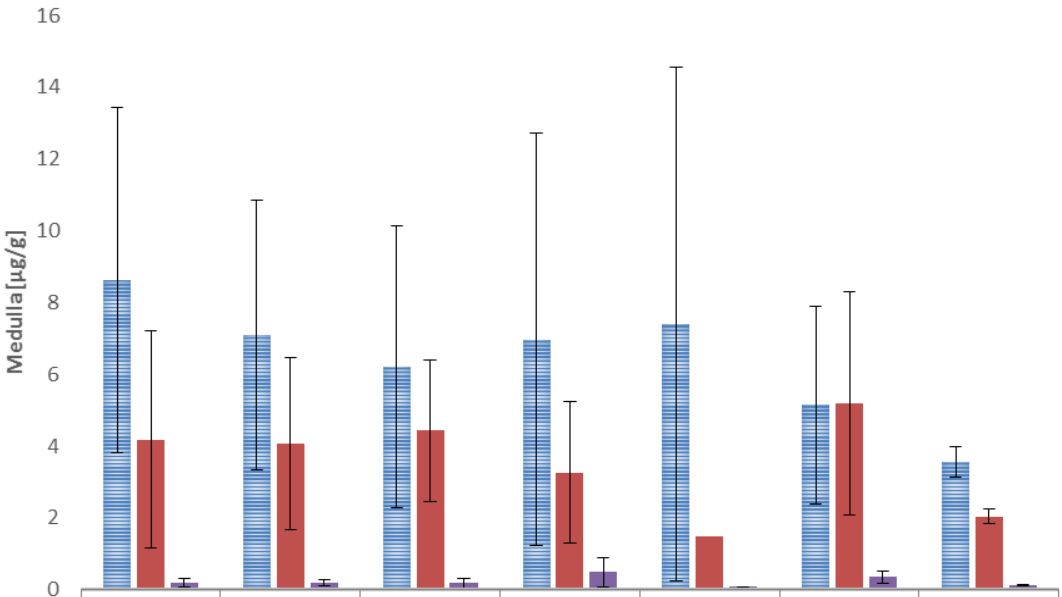


Figure 9 Lead levels (μg/g) in kidney medulla of smokers (n=13) and non-smokers (n=28); *p≤0.05

d) Differences by area of residence

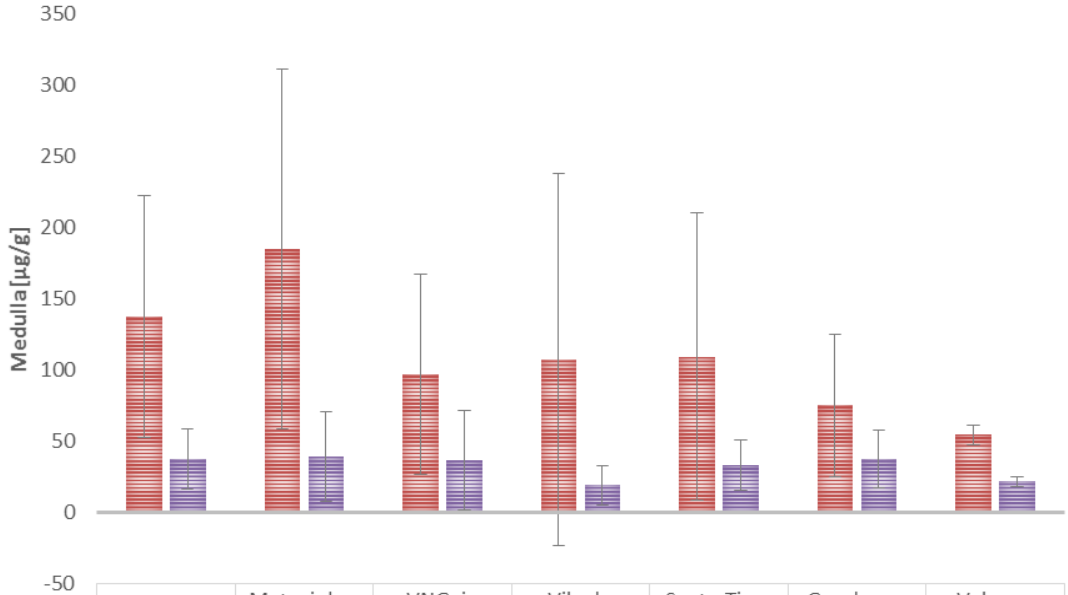
Because of the large inter-individual variability, different number of smokers and unequal distribution of subjects across the different geographical areas, it is not possible to draw significant conclusions about the influence of the area of residence in the trace element levels in kidney medulla.

However, individuals from Gondomar showed lower Cu levels in kidney medulla (without reaching statistical significance) (Figure 10) and Zn (p≤0.05) (Figure 11). Individuals from Santo Tirso and Vila do Conde showed lower concentrations of Cd (Figure 11).



	Porto (n=11)	Matosinhos (n=5)	VNGaia (n=10)	Vila do Conde (n=4)	Santo Tirso (n=2)	Gondomar (n=7)	Valongo (n=2)
Cu	8.611	7.097	6.213	6.960	7.393	5.143	3.551
Se	4.176	4.056	4.432	3.257	1.475	5.194	2.034
Pb	0.194	0.187	0.182	0.483	0.078	0.349	0.121

Figure 10 Average concentration (µg/g) of Cu, Se and Pb in renal medulla according to individuals' area of residence.



	Porto (n=11)	Matosinhos (n=5)	VNGaia (n=10)	Vila do Conde (n=4)	Santo Tirso (n=2)	Gondomar (n=7)	Valongo (n=2)
Zn	137.558	185.021	97.268	107.577	109.452	75.112	54.539
Cd	37.827	39.187	36.602	19.014	32.850	37.277	21.578

Figure 11 Average concentration (µg/g) of Zn and Cd in renal medulla according to individuals' area of residence.

4.1.2 Cortex

a) All the samples

Table 9 Concentration (µg/g) of the elements in kidney cortex.

	Cu	Zn	Se	Cd	Pb
N	56	56	51	56	56
Mean	7.52	166.1	4.83	72.57	0.34
SD	4.10	100.2	2.54	45.43	0.39
Median	7.61	144.9	4.84	67.97	0.26
Minimum	1.68	31.54	1.47	0.03	0.02
Maximum	16.63	409.5	12.30	198.5	2.56

b) Men vs Women

No significant difference was found between genders for any of the five analysed elements in kidney cortex (Tables 10 and 11).

These results are in close agreement with those found in other studies where no significant gender-related differences were also found regarding Pb, Cd, Cu, Se and Zn levels in the kidney (80-82).

Table 10 Concentration (µg/g) of elements in kidney cortex of men.

Men	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	33	33	29	33	32
Mean	7.20	168.05	4.34	78.54	0.35
SD	4.28	105.46	2.14	47.35	0.30
Median	5.68	139.54	4.06	75.42	0.28
Minimum	1.68	31.54	1.47	18.87	0.02
Maximum	16.63	409.48	9.88	198.49	1.20

Table 11 Concentration (µg/L) of elements in kidney cortex of women.

Women	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	20	20	19	20	20
Mean	8.25	171.7	5.69	68.92	0.25
SD	3.87	94.88	2.99	42.97	0.16
Median	8.79	154.0	4.90	60.83	0.25
Minimum	2.34	48.97	1.68	0.03	0.04
Maximum	12.90	403.0	12.30	179.5	0.63

c) Smokers vs Non-smokers

No significant differences were found between smokers and non-smokers regarding Cu, Zn, Se and Zn levels in kidney cortex (Tables 12 and 13).

Studies on the concentration of Cu in serum (77, 83) showed higher levels of this element in smokers. Since free Cu^{2+} ions are potent catalysts in Fenton reaction (Figure 2), these observations might indicate and increased risk of oxidative stress.

No clear evidence exists about the influence of smoking in Zn levels. Garcia *et al.* (80) didn't found a significant difference in Zn levels between smokers and non-smokers, but Blanusa *et al*. (84) found higher levels in smokers.

Table 12 Concentration (µg/g) of elements in kidney cortex of smokers.

Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	14	14	12	14	14
Mean	8.31	210.0	4.28	110.1	0.43
SD	4.65	103.9	1.87	37.74	0.40
Median	9.39	195.1	4.72	110.6	0.280
Minimum	1.98	66.59	1.87	48.09	0.02
Maximum	16.63	409.5	8.09	179.5	1.20

Table 13 Concentration (μg/g) of elements in kidney cortex of non-smokers.

Non Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	29	29	26	29	29
Mean	7.49	159.9	5.91	51.06	0.34
SD	3.85	103.6	2.98	27.62	0.46
Median	7.67	124.8	6.20	44.74	0.27
Minimum	2.15	31.54	1.68	0.03	0.04
Maximum	15.94	403.0	12.30	110.7	2.59

Cd showed significant higher levels in smokers (110.1±37.74 μg/g versus 51.06±27.62 μg/g in non-smokers) (Figure 12). This is in close agreement with other studies on the influence of smoking habits in the Cd content of kidney cortex (81, 82, 84).

Smoking is assumed to be the main source of human exposure to Cd. Although the amounts of Cd varied, the average content of this element has been found between 0.5 and 1.5 μg per cigarette (29). When the cigarette is smoked, Cd is transformed into CdO, which is then inhaled. It is estimated that approximately 10% of the Cd inhaled is deposited in the lungs, and 20-50% of this amount is transferred to the blood circulation.

Cd not only increases in the circulation, but also accumulates in kidney, mainly in the kidney cortex, where Mt chelate and immobilize it. As already stressed, studies have shown that Cd accumulation in kidney causes tubular dysfunction and renal failure, but there is also some evidence that the amount of Cd released with the smoke is not enough to cause kidney failure (29).

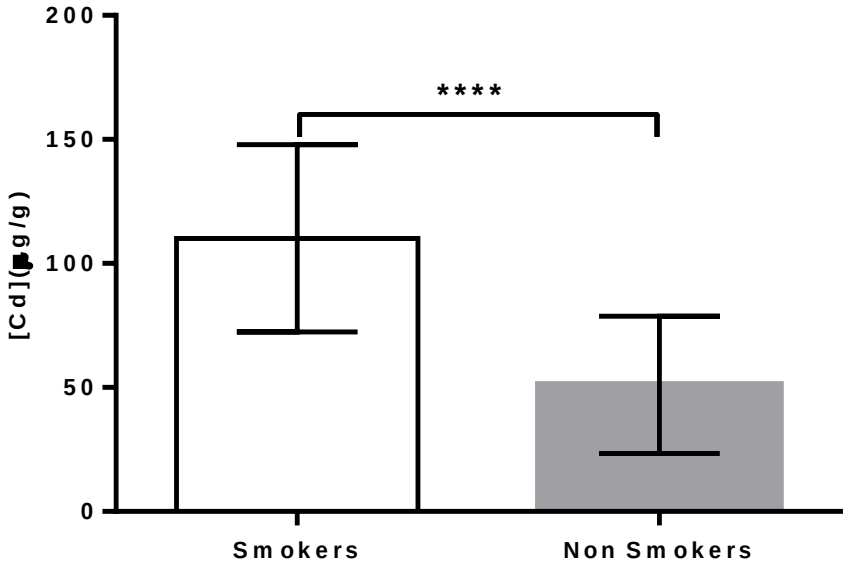
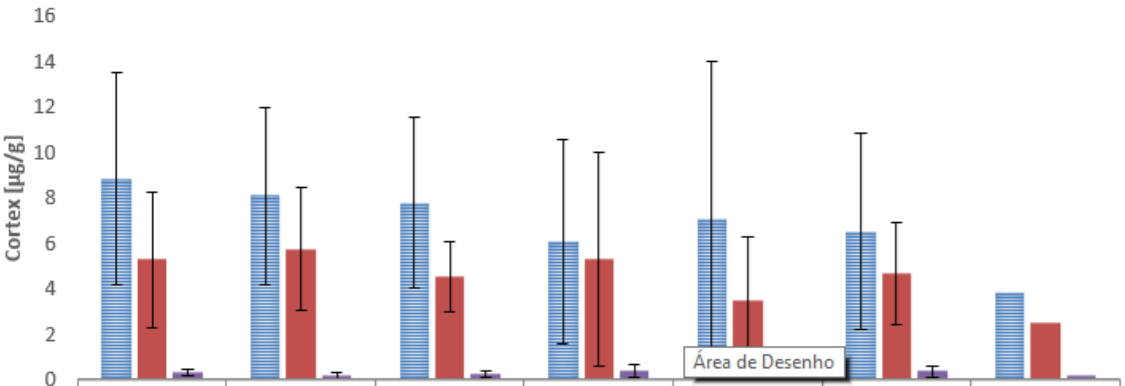


Figure 12 Cadmium levels (µg/g) in the kidney cortex of smokers (n=14) and non smokers (n=29); ****p≤0.0001

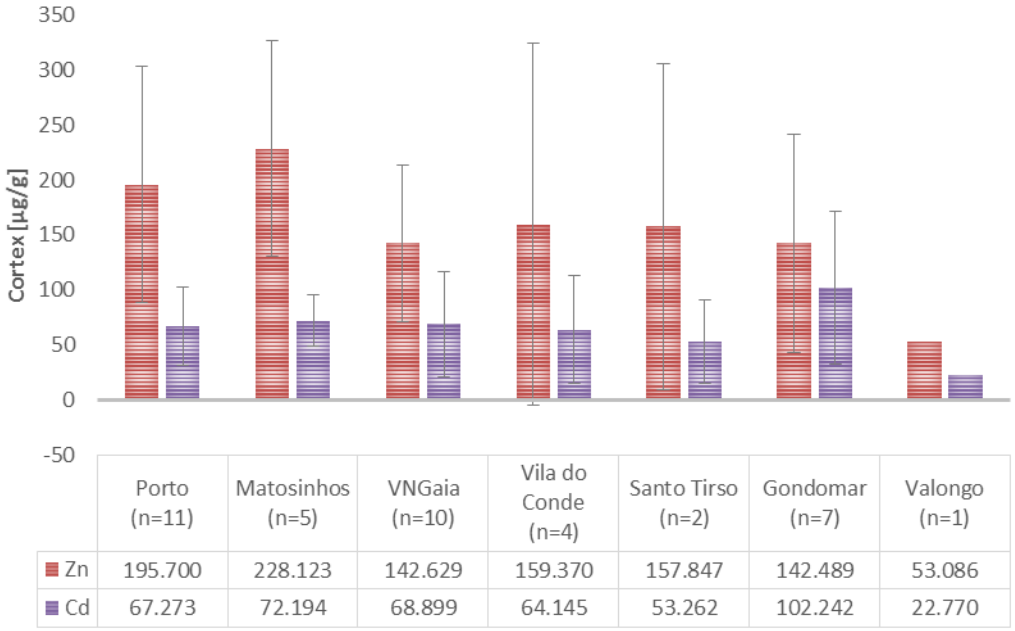
d) Differences by area of residence

No significant differences were found between areas of residence. Individuals from Matosinhos showed higher levels of Zn and individuals from Gondomar showed lower levels of Cd (Figure 13), but the difference reached no statistical significance.



	Porto (n=11)	Matosinhos (n=5)	VNGaia (n=10)	Vila do Conde (n=4)	Santo Tirso (n=2)	Gondomar (n=7)	Valongo (n=1)
Cu	8,797	8,058	7,753	6,058	7,059	6,470	3,812
Se	5,250	5,726	4,482	5,275	3,463	4,633	2,470
Pb	0,286	0,155	0,218	0,367	0,156	0,336	0,134

Figure 13 Average concentration (µg/g) of Cu, Se and Pb in renal cortex according to individual's area of residence.



	Porto (n=11)	Matosinhos (n=5)	VNGaia (n=10)	Vila do Conde (n=4)	Santo Tirso (n=2)	Gondomar (n=7)	Valongo (n=1)
Zn	195.700	228.123	142.629	159.370	157.847	142.489	53.086
Cd	67.273	72.194	68.899	64.145	53.262	102.242	22.770

Figure 14 Average concentration (µg/g) of Zn and Cd in cortex according to individual's area of residence.

4.1.3 Cortex vs Medulla

Although we have found a tendency for higher levels of all the studied trace elements in the kidney cortex, the difference was statistically significant only for Zn (166.1±100.2 µg/g in renal cortex and 115.7±86.48 in renal medulla; p≤0.01) and Cd (72.57±45.43 µg/g in cortex and 41.37±38.72 µg/g in medulla; p≤0.0001) (Figure 15).

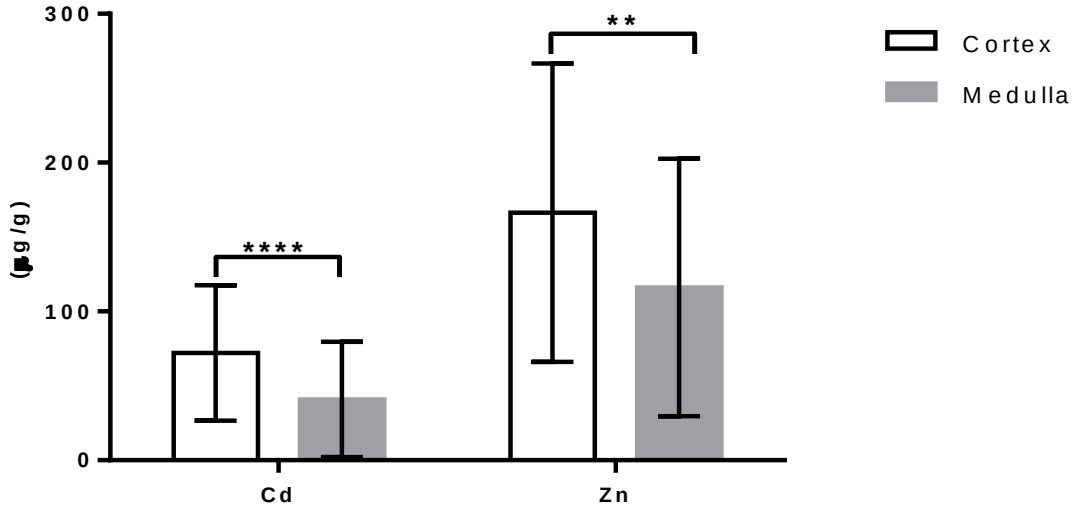


Figure 15 Cadmium and zinc levels (µg/g) in kidney cortex (n=56) and medulla (n=57); **p ≤0.01, ****p ≤0.0001

As previously mentioned, the renal filtration and reabsorption of Cd–Mt complexes occurs, respectively, at the glomerulus and proximal tubules, which are both nephron structures located in renal cortex (Figure 6). This may explain the higher levels of Cd found in kidney cortex.

On the other hand, it seems that Cd-Mt and Zn-Mt complexes are handled by the same renal mechanism (85), which explains the concomitant Zn accumulation in the kidney cortex.

4.2 AORTA TISSUE

4.2.1 Aorta tissue without plaque of atheroma

a) All samples

Table 14 Concentration (µg/g) of the elements in aorta tissue without plaque of atheroma

	Cu	Zn	Se	Cd	Pb
N	52	52	45	52	52
Mean	4.28	89.74	4.71	1.55	3.22
SD	1.21	28.45	2.33	2.72	3.58
Median	4.08	88.03	4.40	0.13	1.93
Minimum	1.83	21.22	1.51	0.03	0.06
Maximum	8.15	175.7	14.17	10.91	14.35

In aorta tissue without plaque of atheroma Zn was the element present at higher levels and Cd at the lower. The whole results are summarized in Table 14.

b) Men vs Women

No differences were found between genders (Tables 15 and 16).

Table 15 Concentration (µg/g) of the elements in aorta tissue without plaque of atheroma of men.

Men	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	28	28	23	28	28
Mean	4.15	86.54	5.00	1.36	2.92
SD	1.08	32.26	2.76	2.71	2.94
Median	3.99	83.58	4.68	0.08	1.96
Minimum	1.83	21.22	1.90	0.03	0.10
Maximum	5.69	175.7	14.17	10.09	12.42

Table 16 Concentration (µg/g) of the elements in aorta tissue without plaque of atheroma of women

Women	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	19	19	17	19	19
Mean	4.19	88.54	4.33	1.15	3.27
SD	1.14	19.28	1.62	2.00	3.87
Median	4.43	91.82	4.21	0.16	1.80
Minimum	2.25	44.64	1.51	0.03	0.06
Maximum	5.93	115.2	7.40	6.69	14.19

c) Smokers vs Non-smokers

No differences were also found between smokers and non-smokers (Tables 17 and 18).

Table 17 Concentration (µg/g) of the elements in aorta tissue without plaque of atheroma of smokers.

Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	13	13	11	13	13
Mean	4.36	85.37	5.29	2.02	2.40
SD	0.96	20.79	3.28	3.20	2.13
Median	4.01	86.64	4.53	0.17	2.09
Minimum	3.20	43.11	2.30	0.04	0.11
Maximum	5.93	116.51	14.17	10.09	7.70

Table 18 Concentration (µg/g) of the elements in aorta tissue without plaque of atheroma of non-smokers.

Non-Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	25	25	22	25	25
Mean	4.02	86.43	4.62	1.48	3.41
SD	1.14	26.18	1.94	2.34	3.75
Median	3.87	83.92	4.26	0.09	1.80
Minimum	2.04	21.22	1.51	0.03	0.06
Maximum	6.26	131.89	9.91	7.84	14.19

4.2.2 Aorta tissue with plaque of atheroma

a) All samples

Table 19 Concentration (µg/g) of the elements in aorta with plaque of atheroma.

	Cu	Zn	Se	Cd	Pb
N	41	41	36	40	41
Mean	3.74	111.0	3.85	1.22	2.95
SD	2.78	58.42	2.50	3.81	2.61
Median	3.23	94.52	3.65	0.08	2.47
Minimum	1.10	30.06	0.52	0.01	0.05
Maximum	14.69	350.2	13.52	23.28	10.81

b) Men vs Women

No differences were found between genders (Tables 20 and 21).

Table 20 Concentration (µg/g) of the elements in aorta with plaque of atheroma of men.

Men	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	22	22	18	22	22
Mean	3.79	105.5	3.41	1.64	2.56
SD	2.68	51.17	2.05	5.00	1.95
Median	3.26	89.21	2.95	0.08	2.00
Minimum	1.31	30.06	0.52	0.01	0.19
Maximum	14.24	217.6	10.13	23.28	6.95

Table 21 Concentration (µg/g) of the elements in aorta with plaque of atheroma of women.

Women	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	18	18	17	17	18
Mean	3.67	116.78	3.75	0.67	3.00
SD	3.05	68.58	1.77	1.43	2.75
Median	2.93	98.78	3.77	0.08	2.67
Minimum	1.10	39.67	1.60	0.01	0.05
Maximum	14.69	350.23	7.19	5.23	9.45

c) Smokers vs non smokers

Table 22 Concentration (µg/g) of the elements in aorta with plaque of atheroma of smokers.

Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	10	10	9	10	10
Mean	3.47	78.44	3.93	0.91	1.84
SD	1.03	32.25	1.62	1.63	1.69
Median	3.49	70.09	3.77	0.16	1.29
Minimum	1.75	39.67	1.81	0.01	0.09
Maximum	5.09	147.2	7.19	5.23	5.83

Table 23 Concentration (µg/g) of the elements in aorta with plaque of atheroma of non- smokers.

Non-Smokers	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	20	20	20	17	20
Mean	3.36	126.4	3.33	0.47	3.51
SD	2.94	66.60	1.56	1.29	2.67
Median	2.50	106.8	3.65	0.07	3.06
Minimum	1.10	40.88	0.52	0.01	0.10
Maximum	14.69	350.2	7.10	23.28	9.45

A significant difference was found for Zn levels in aorta with plaque. Smokers showed a lower Zn concentration: 78.44±32.25 µg/g vs 126.5±66.60 in non-smokers ($p \leq 0.05$) (Figure 16).

The mechanism through which cigarette smoke reduces Zn levels is the aorta with plaque of atheroma is thought to be the result of an increased blood Cd concentration, which induces the expression of Mt. Mt complexes not only bind Cd but also Zn, and later this complexes accumulate in the renal cortex (29).

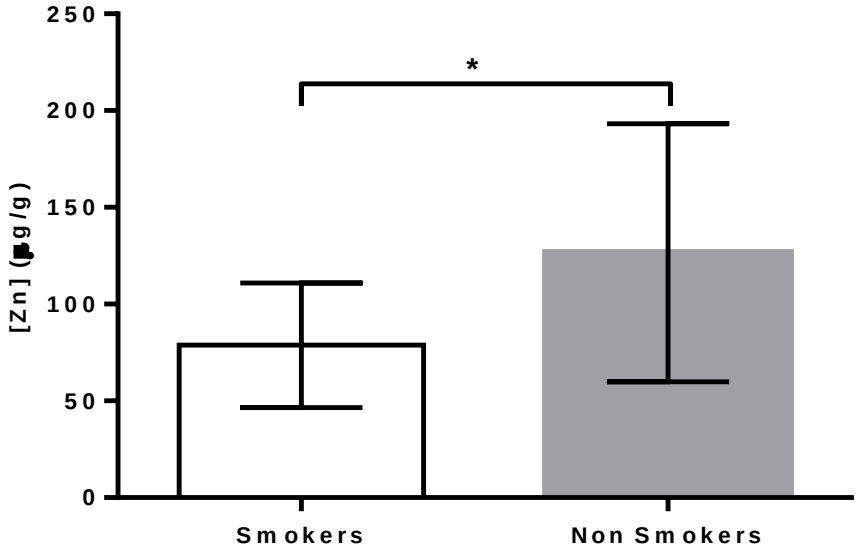
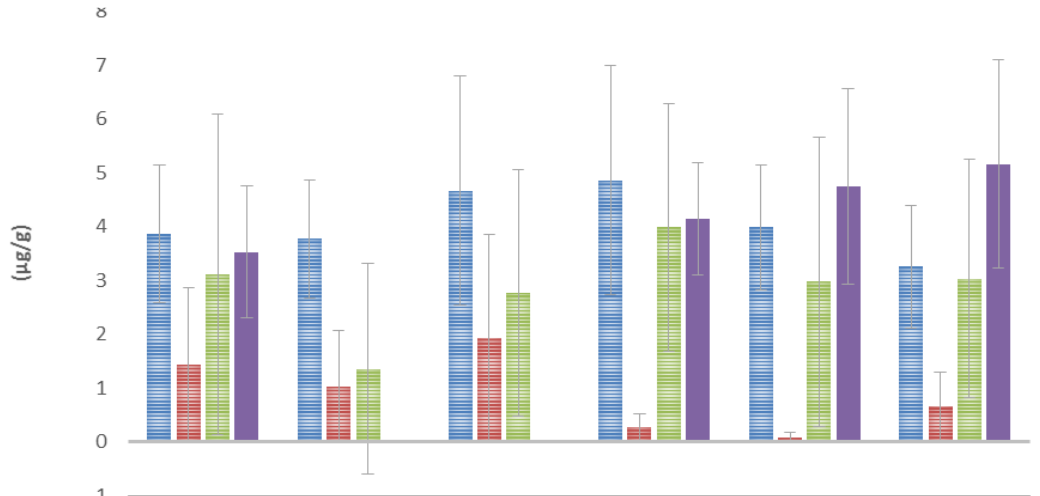


Figure 16 Zinc levels (µg/g) in aorta tissue with plaque **of atheroma** of **smokers (n=10) and non-smokers (n=20)**; *p≤0.05

d) Differences by area of residence

As also observed in renal medulla, individuals from Santo Tirso and Vila do Conde showed lower concentrations of Cd in aorta tissue (with and without plaque) (Figure 17).



	Porto (n=11)	Matosinhos (n=4)	VNGaia (n=11)	Vila do Conde (n=5)	Santo Tirso (n=2)	Gondomar (n=6)
Cu	3.860	3.763	4.662	4.855	3.980	3.252
Cd	1.434	1.032	1.929	0.262	0.082	0.649
Pb	3.105	1.354	2.767	3.979	2.968	3.029
Se	3.519			4.136	4.743	5.156

Figure 17 Average concentration (µg/g) of Cu, Cd, Pb and Se in aorta according to individuals' area of residence.

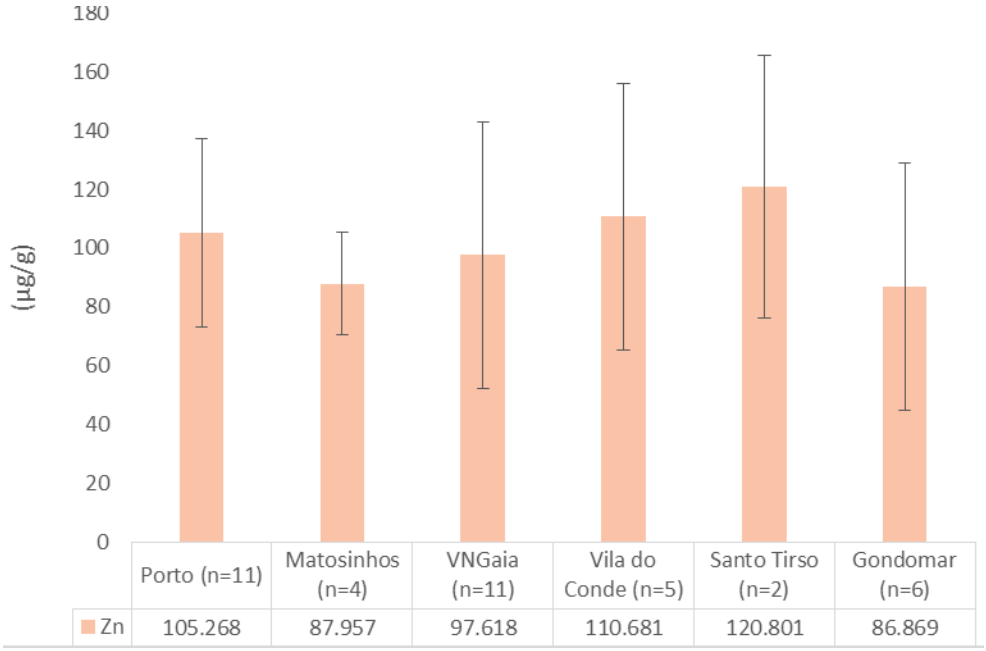


Figure 18 Average concentration (µg/g) of Zn in aorta according to individuals' area of residence.

4.2.3 Aorta tissue with and without plaque of atheroma

Significant differences were found between both aorta tissues (i.e., with and without plaque of atheroma) for Zn and Se.

For Zn, significantly higher concentrations were found in aorta tissue with plaque of atheroma: 111.0±58.42 µg/g vs 89.74±28.45 µg/g in aorta without plaque (Figure 19).

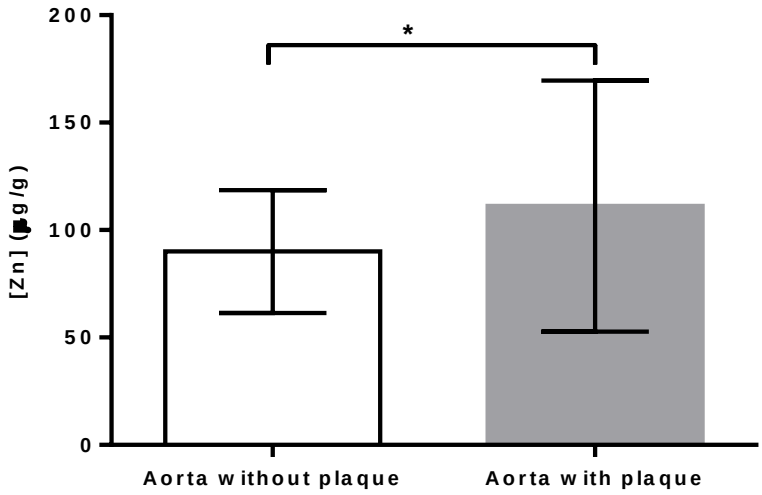


Figure 19 Zinc levels (µg/g) in aorta tissue without plaque of atheroma (n=52) and aorta tissue with plaque of atheroma (n= 41); *p≤0.05

Our results are in agreement with those obtained by Tohno *et al.* (86), who studied the human thoracic aorta and found a lack of correlation between Zn and some independent markers of protein oxidation (tyrosine nitration and thiol oxidation), suggesting that Zn is unlikely to protect against transition metals induced lipoprotein oxidation. Moreover, a highly significant correlation between Zn and Ca in all lesions was observed. It seems that high Zn levels may merely be an indicator of Ca accumulation and fibrosis.

Regarding Se, we found a significantly higher concentration of this element in the aorta tissue without plaque of atheroma (4.71±2.33 μg/g) when compared with the aorta tissue with plaque of atheroma (3.85±2.50 μg/g) (Figure 20).

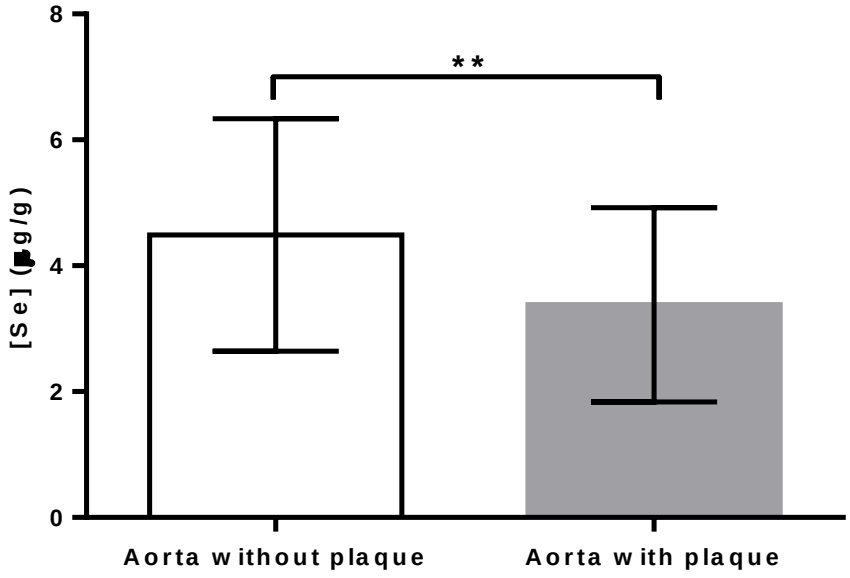


Figure 20 Selenium levels (μg/g) in aorta tissue without plaque of atheroma (n=45) and in aorta tissue with plaque of atheroma (n=36); **p≤0.01

The higher Se concentration in aorta without plaque seems to confirm that Se is a cardioprotector element (87).

In a study by Lubos *et al.* (88), the plasma Se concentration of individuals with acute coronary syndrome was inversely associated with cardiovascular mortality, independently of classical risk factors.

The exact mechanism through which low plasma Se levels act in CVD is not yet known. Some authors have pointed out that low Se concentrations in atherosclerosis may facilitate the formation of lipid hydroperoxides, which could attack vascular endothelium. Other authors have argued that Se modifies prostaglandin synthesis, improving

thromboxane levels in platelets and diminishing prostacyclin concentration in vascular endothelium. In these circumstances, Se, as an antioxidant agent, is closely related to prostaglandin metabolism, since it acts as a GPx cofactor. Another mechanism would be the protective action of Se against the toxicity of heavy metals (88).

4.3 AGE-RELATED CHANGES

4.3.1 Kidney

For Cd levels in renal cortex, an age-related increase up to 50 years old with a subsequent decrease was found (Figure 21). This behaviour has already been reported from previous studies (80). The decline in Cd levels after 50 years of age may be due to an age-related degeneration or Cd-induced damage of the kidney tissue. In medulla, no significant age-related changes in Cd levels were found.

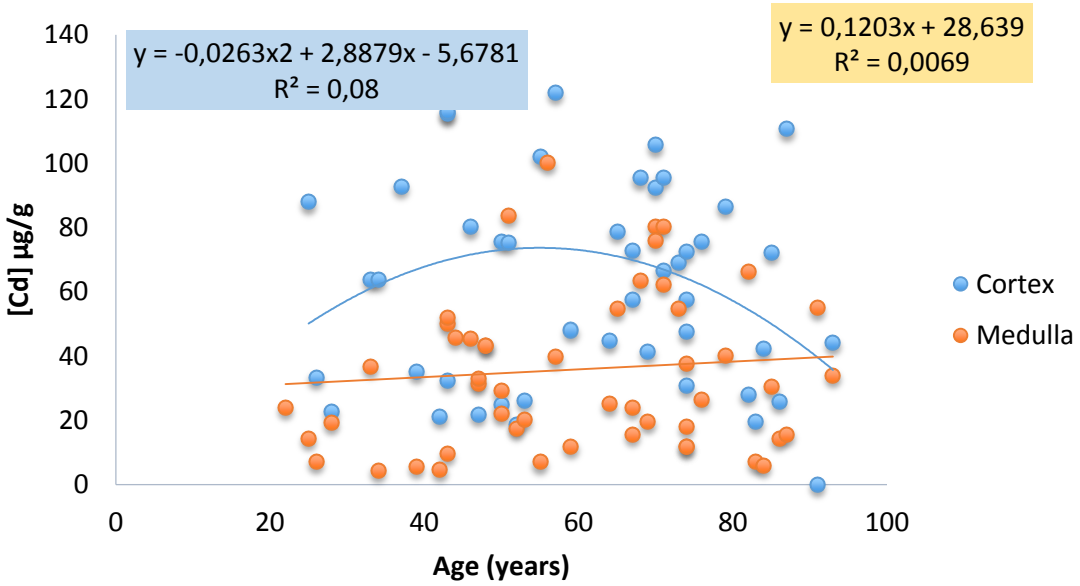


Figure 21 Relationship between Cd levels (µg/g) in kidney and age (years).

For Cu, Zn, Pb and Se linear regression analysis of metal content versus age produced positive slopes, suggesting a tendency for an increase in the elements levels with age (Figures 22-25). For Zn, this is in disagreement with Garcia *et al.* (79), who reported no age-related changes in kidney tissue levels. This is also in disagreement with the same authors (79) for Pb, as they reported a decrease in kidney Pb levels with age.

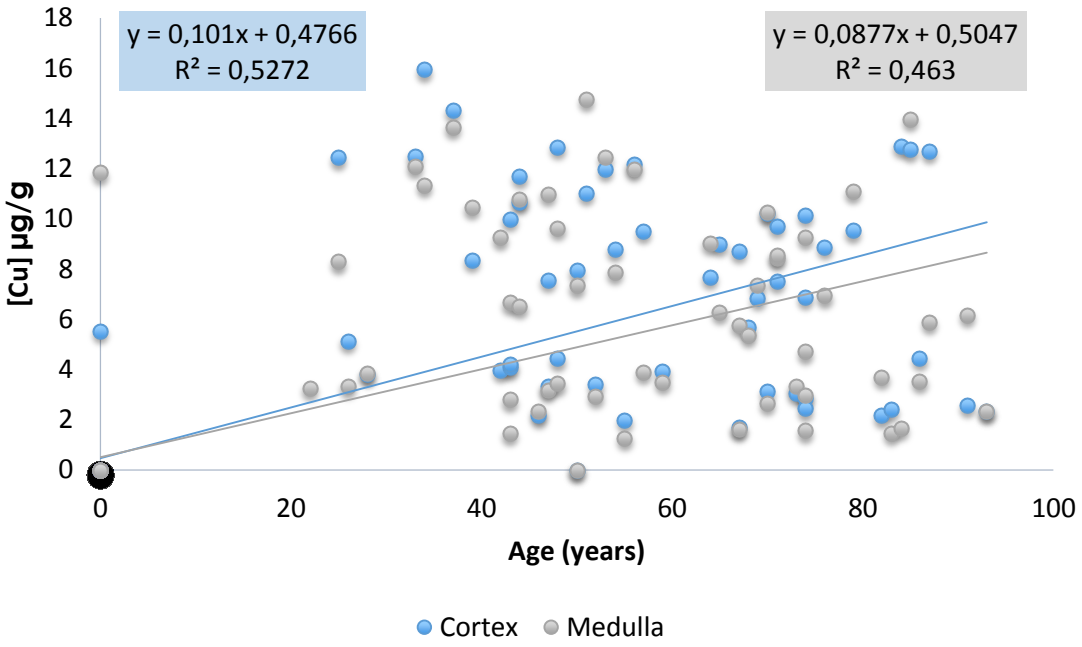


Figure 22 Relationship between Cu levels (µg/g) in kidney and age (years).

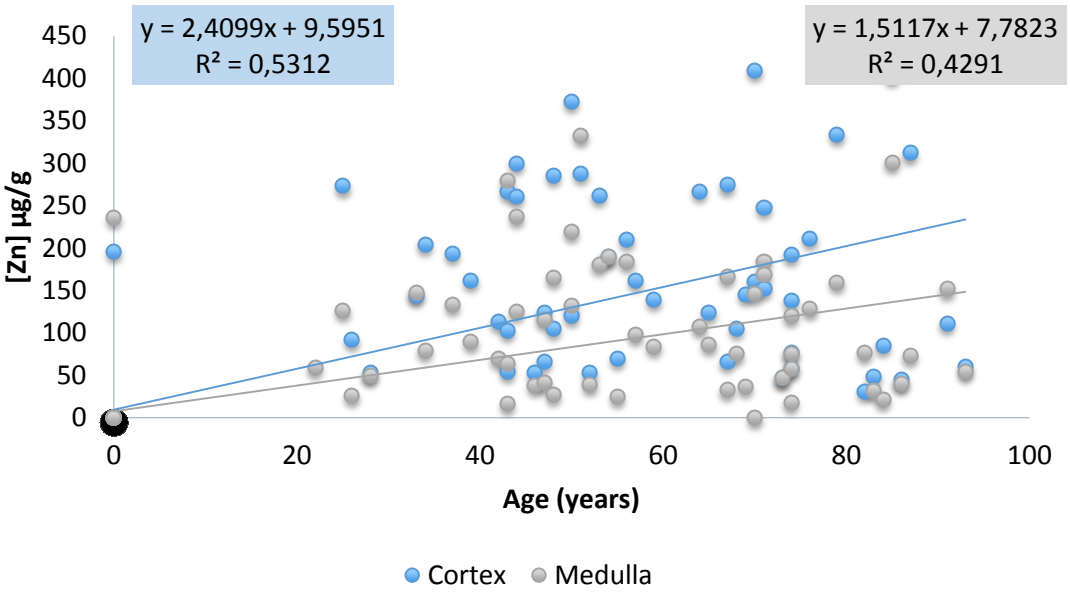


Figure 23 Relationship between Zn levels (µg/g) in kidney and age (years).

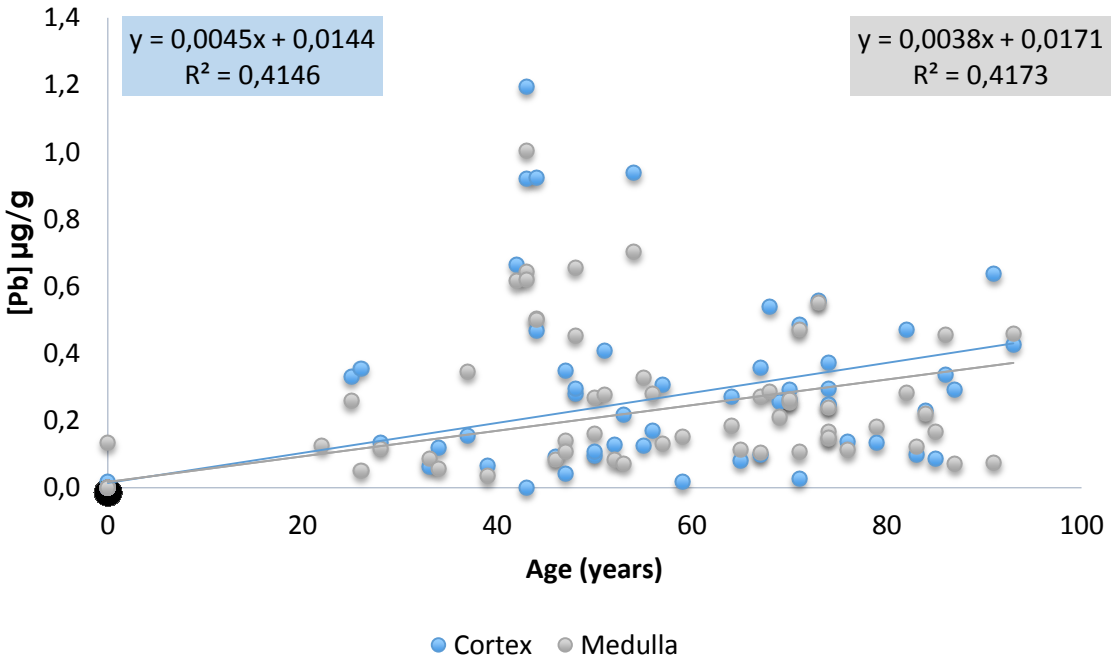


Figure 24 Relationship between Pb levels (µg/g) in kidney and age (years).

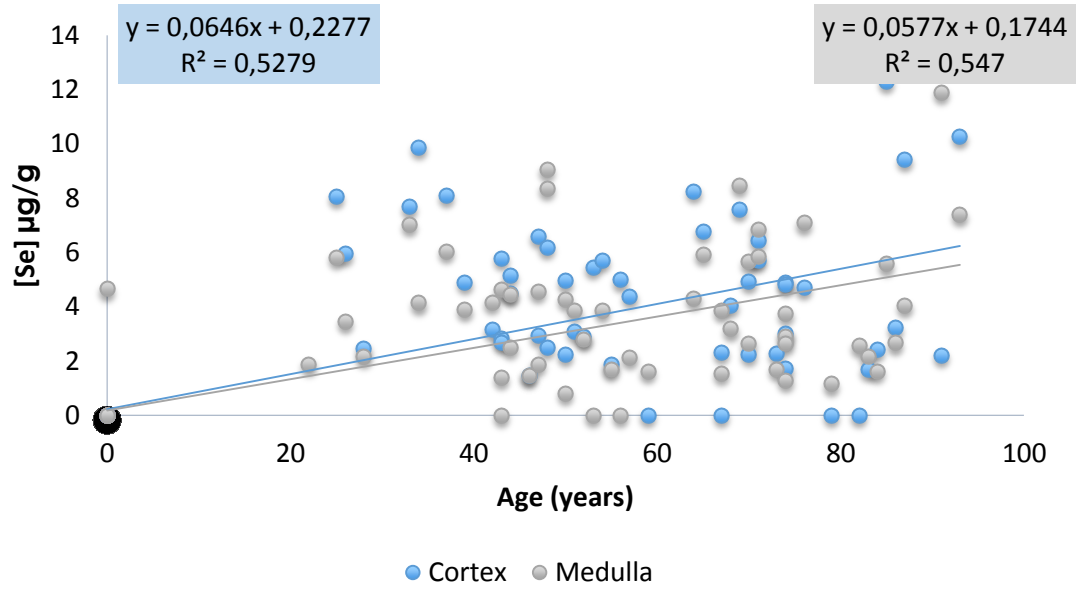


Figure 25 Relationship between Se levels (µg/g) in kidney and age (years).

4.3.2 Aorta

Except for Pb, which showed to increase with age (Figure 29), Cu, Zn, Cd and Se in aorta tissue without plaque of atheroma showed quite constant levels with the age increasing (Figures 26, 27, 28, 30).

For aorta tissue with plaque of atheroma linear, Cu, Cd and Se showed a tendency for a decrease with age (Figure 26, 28 and 30), while Zn and Pb showed a tendency to increase (Figures 27 and 29).

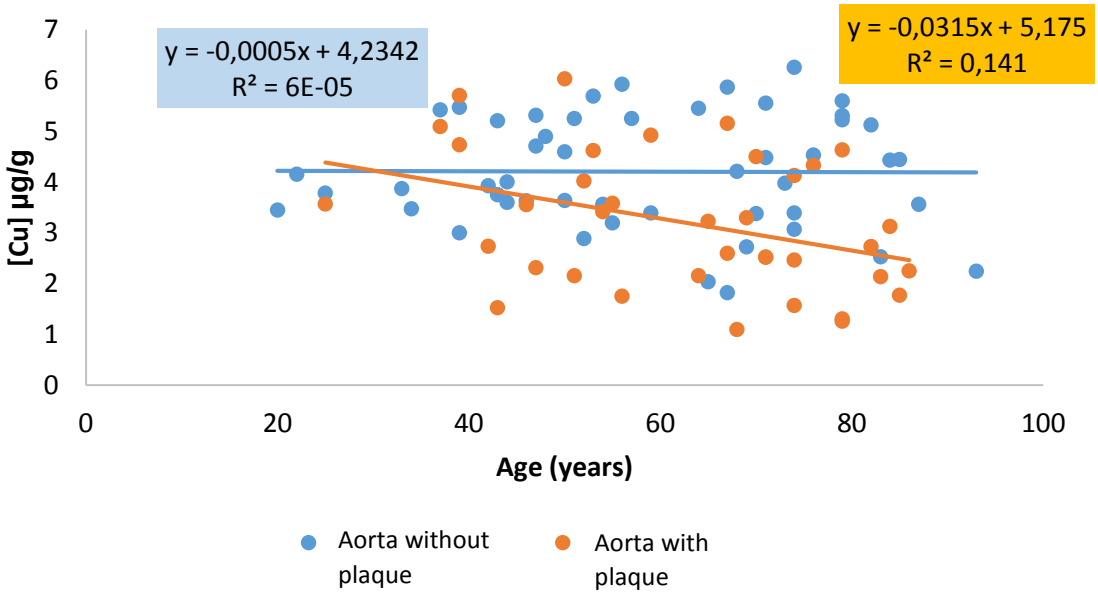


Figure 26 Relationship between Cu levels (µg/g) in aorta and age (years).

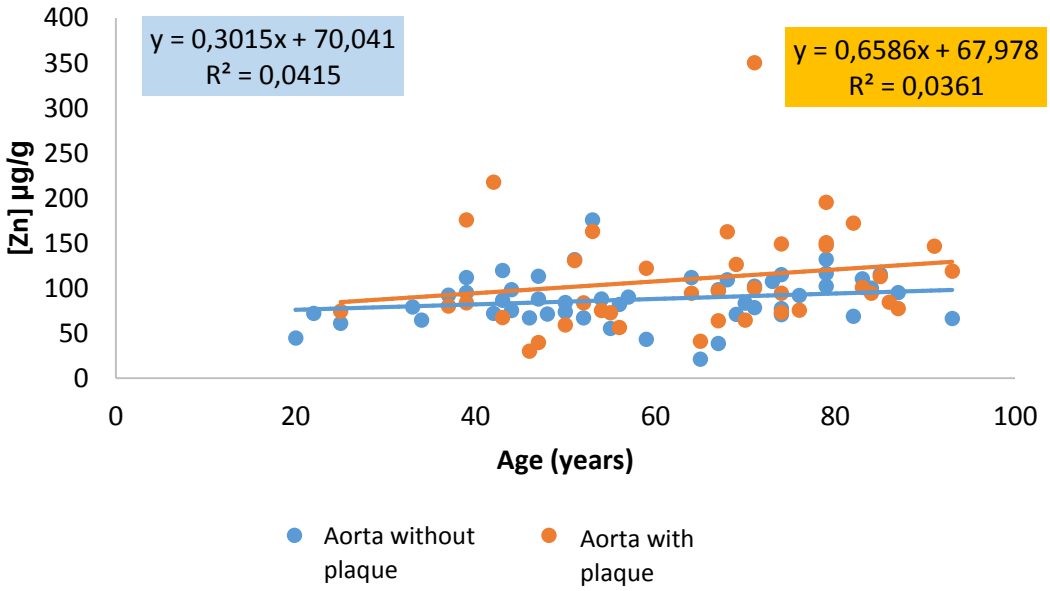


Figure 27 Relationship between Zn levels (µg/g) in aorta and age (years).

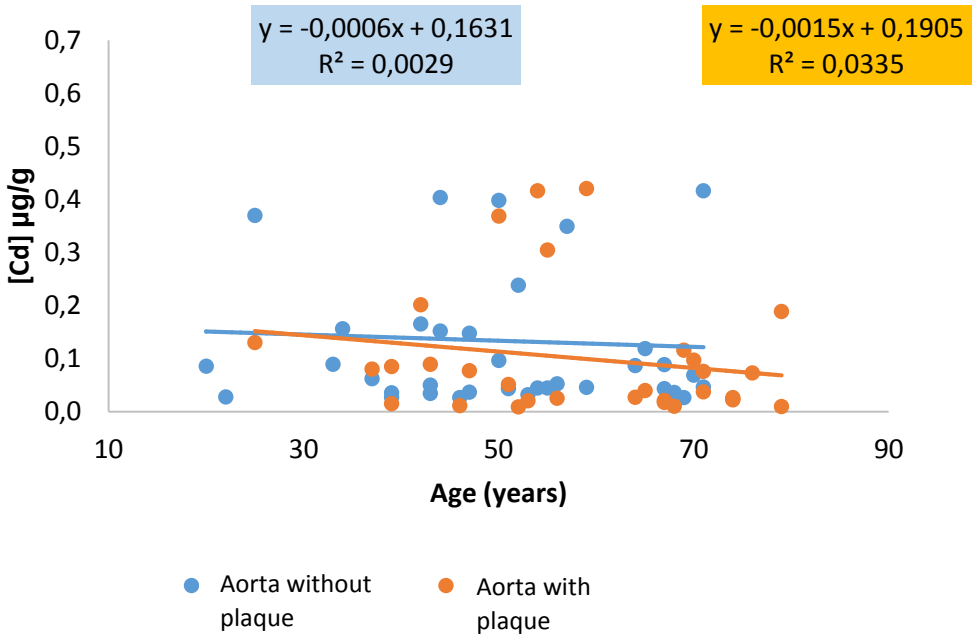


Figure 28 Relationship between Cd levels (µg/g) in aorta and age (years).

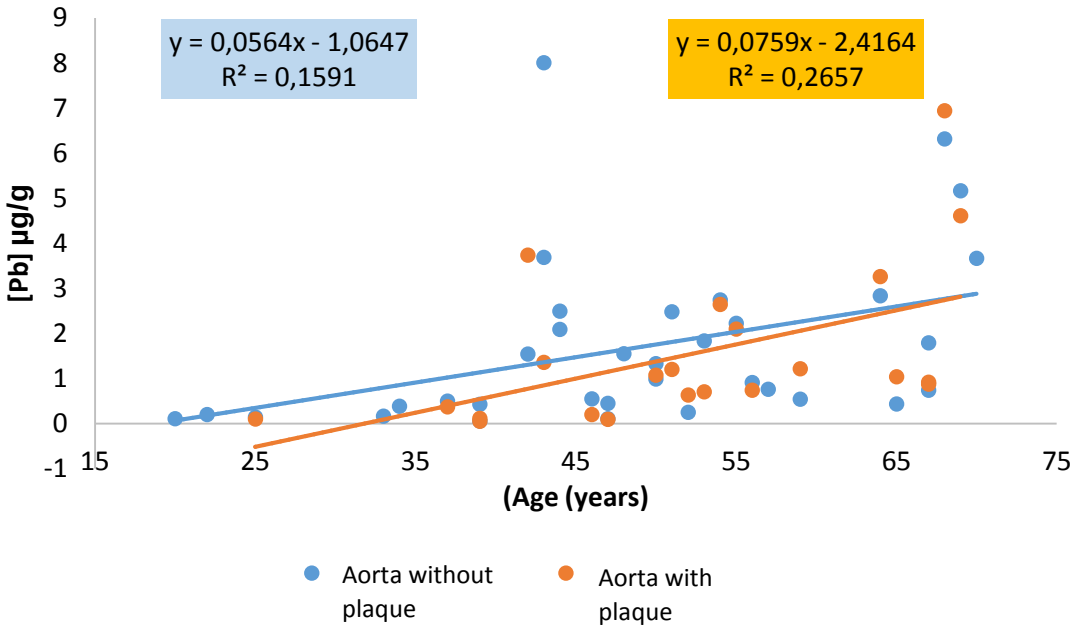


Figure 29 Relationship between Pb levels (µg/g) in aorta and age (years).

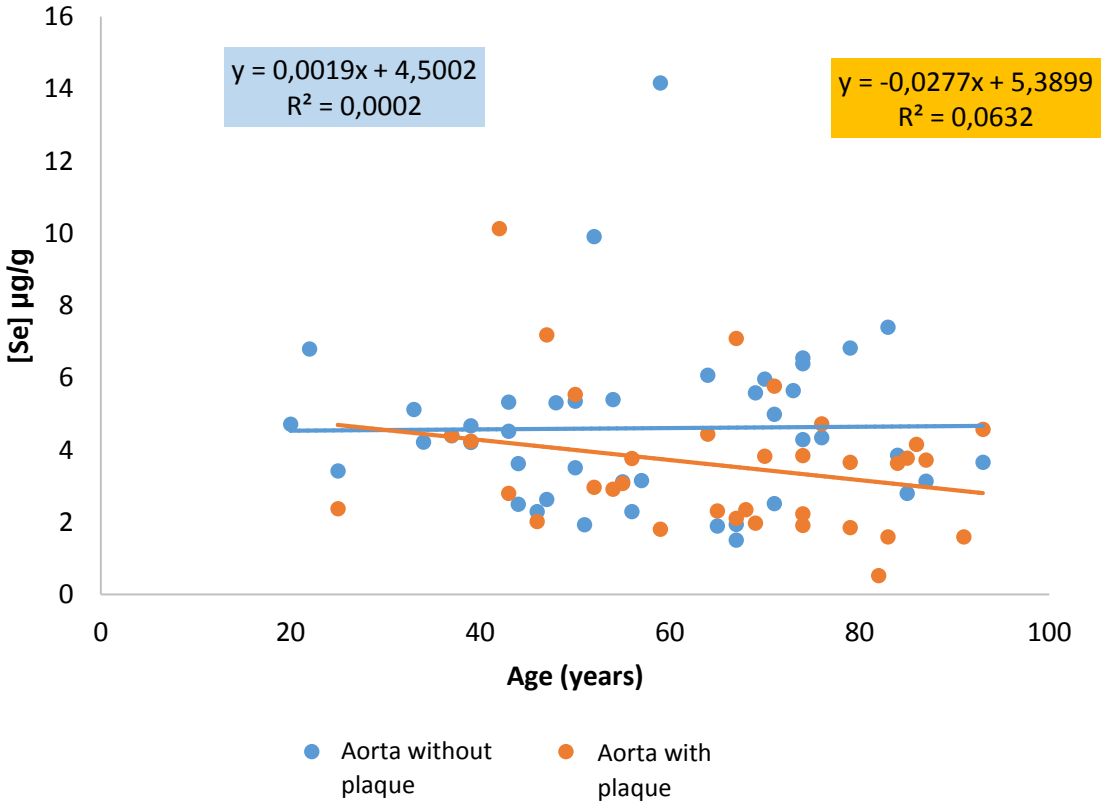


Figure 30 Relationship between Se levels (µg/g) in aorta and age (years).

4.4 HEALTHY INDIVIDUALS VS INDIVIDUALS WITH CVD

Next tables summarize the results obtained for “healthy” individuals and for individuals that fulfil the criteria for inclusion in the CVD group. Tables 24 and 25 regards the determination in kidney tissue (cortex and medulla results); table 26 and 27 regards the determination in aorta tissue (with and without atheroma plaque).

Table 24 Concentration (µg/g) of the elements in the kidney tissue of healthy individuals

Kidney	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	41	41	39	41	41
Mean	7.96	142.1	5.48	55.04	0.41
SD	3.71	81.60	5.35	44.32	0.45
Median	8.35	125.3	4.51	39.73	0.26
Minimum	1.44	16.93	1.39	4.47	0.04
Maximum	15.94	300.0	35.32	179.5	2.56

Table 25 Concentration (µg/g) of the elements in the kidney tissue of individuals with CVD.

Kidney	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	44	45	40	45	45
Mean	5.61	115.7	3.90	50.35	0.24
SD	3.82	88.42	2.80	41.51	0.16
Median	4.57	85.54	2.66	43.32	0.22
Minimum	1.28	18.46	0.83	0.03	0.02
Maximum	16.63	373.4	11.90	198.5	0.66

Table 26 Concentration (µg/g) of the elements in aorta tissue of healthy individuals.

Aorta	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	38	38	34	37	38
Mean	4.48	105.3	4.46	1.92	2.79
SD	2.69	56.56	2.14	4.13	3.06
Median	3.96	92.09	4.35	0.35	1.93
Minimum	1.10	24.54	1.34	0.01	0.05
Maximum	14.69	350.2	10.53	23.28	12.42

Table 27 Concentration (µg/g) of the elements in aorta tissue of individuals with CVD.

Aorta	**Cu**	**Zn**	**Se**	**Cd**	**Pb**
N	43	43	38	42	43
Mean	3.18	106.3	3.59	0.36	3.22
SD	1.32	69.01	2.45	0.95	2.83
Median	3.13	88.03	3.14	0.05	2.22
Minimum	1.26	21.22	0.47	0.01	0.10
Maximum	6.26	358.0	14.17	4.63	14.19

Individuals with CVD showed significant lower levels of Cu than healthy subjects in both the kidney (5.61±3.82 *vs* 7.96±3.71 µg/g; p<0.01) and aorta tissue (3.18±1.32 *vs* 4.48±2.69 µg/g; p≤0.01) (Figure 31). Which is in disagreement with the hypothesis that Cu is a risk factor for CVD (38).

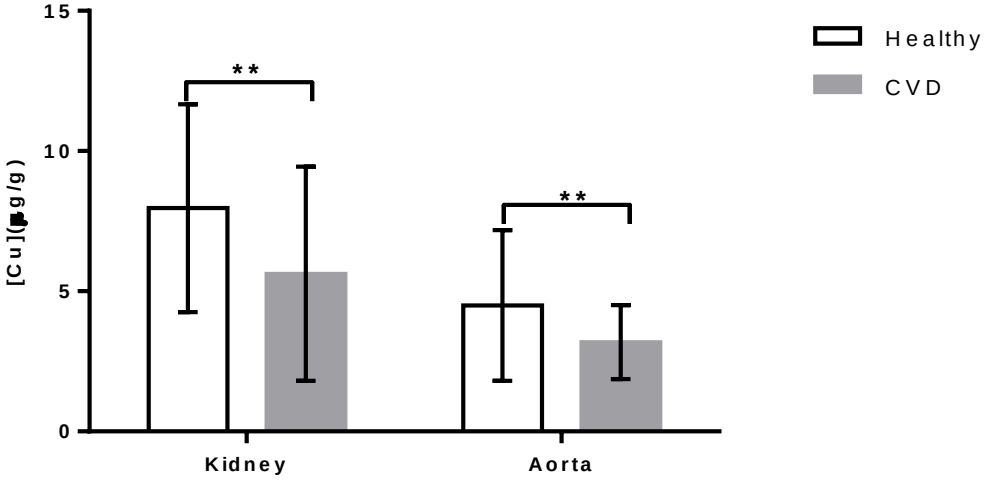


Figure 31 Copper levels (µg/g) in kidney and aorta tissue in healthy individuals (n=19) and individuals with CVD (n=23); **p≤0.01

A similar finding was observed for Cd and Pb. Individuals with CVD showed lower levels of Cd in aorta tissue (0.36±0.95 *vs* 1.92±4.13 µg/g; p≤0.05) (Figure 32) and lower levels of Pb in the kidney than healthy individuals (0.24±0.16 µg/g *vs* 0.41±0.45 µg/g; p≤0,05).

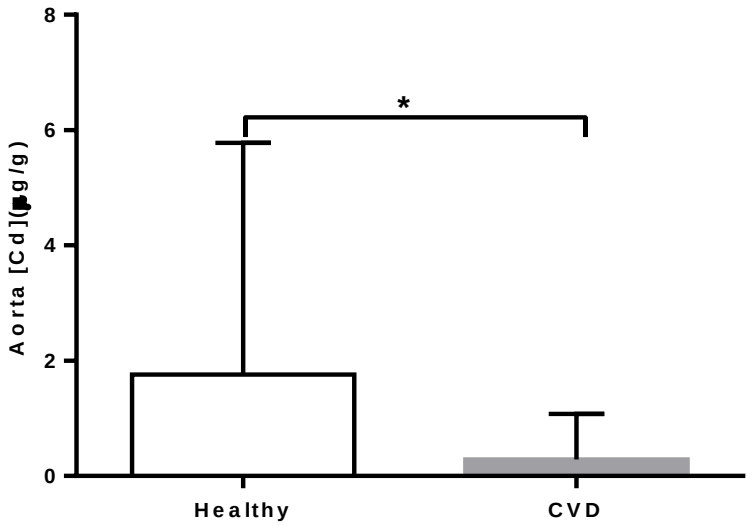


Figure 32 Cadmium levels (µg/g) in aorta tissue of healthy individuals (n=19) and individuals with CVD (n=23) *p≤0.05

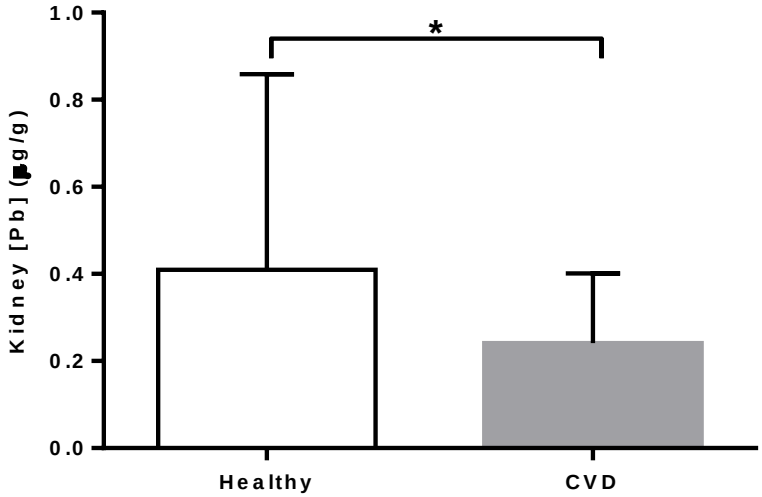


Figure 33 Lead levels (µg/g) in kidney in healthy individuals (n=19) and individuals with CVD (n=23); *p≤0.05

Selenium was the only exception to this general tendency. Individuals with CVD showed lower levels than healthy subjects in both the kidney (3.90±2.80 *vs* 5.48±5.35 µg/g; p≤0.05) and aorta tissue (3.59± 2.45 *vs* 4.46±2.14 µg/g; p≤0.05) (Figure 34), which in accordance with current evidence from prospective studies that supports a modest inverse association between Se levels and the risk of heart disease (54).

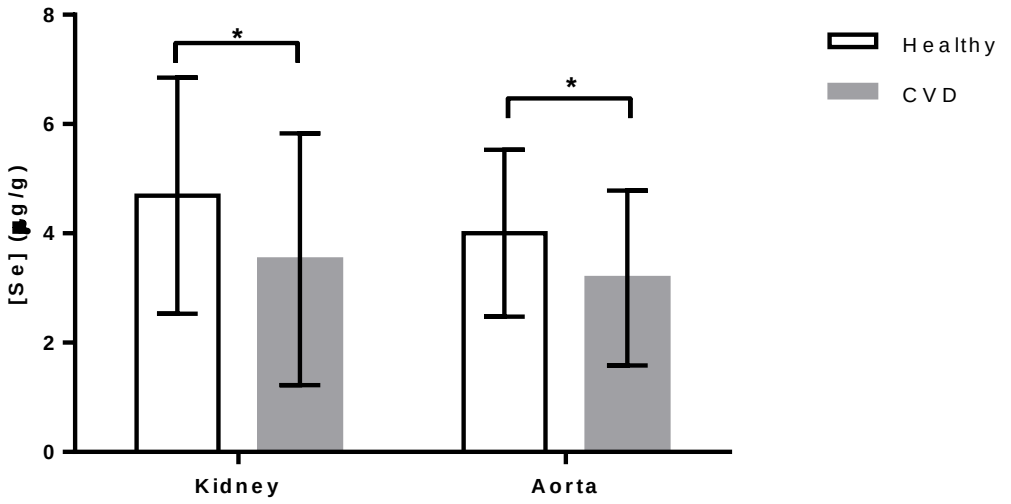


Figure 34 Selenium levels (µg/g) in kidney and aorta tissue from healthy individuals (n=19) and individuals with CVD (n=23); *p≤0.05

Zinc showed no difference between both groups (healthy individuals and individuals with CVD). There are numerous conflicting studies regarding Zn and CVD. Via nitric oxide pathway, Zn deficiency may cause hypertension, an important risk factor for atherosclerosis. In addition, the antioxidant action of Zn prevents oxidation of LDL and consequently stops the main mechanism of atherogenesis (85). However, increased levels of Zn may enhance atherosclerosis via increased oxidant species generation and decreased HDL levels (60).

Several studies corroborate the hypothesis that Zn deficiency may be a risk factor for atherosclerosis in animals (57, 58, 86). Recently, Islamoglu et al. (87) showed significantly decreased serum levels of Zn and Cu in human patients with atherosclerosis compared to the control group.

Although there are strong reasons to admit that trace element imbalances may be involved in the aetiology of CVD, epidemiological studies have been inconsistent.

CHAPTER 5

CONCLUSIONS and FUTURE RESEARCH

CONCLUSIONS AND FUTURE RESEARCH

This work aimed to study the link between trace element imbalances and CVD. A direct approach (i.e., the analysis of the "target" tissues themselves) was used, instead of the usually performed analysis of body fluids (blood, urine), which is a particular and distinctive feature of this work. It is widely assumed that this may be a potentially more conclusive approach.

The direct study of the changes of three important trace elements (Zn, Cu and Se) and two important "heavy metals" (Cd and Pb) in kidney tissue (cortex and medulla) and aorta wall (both with and without atheroma plaque) was performed.

Data obtained clearly show that:

- The two kidney regions (cortex and medulla) present significantly different levels of Zn and Cd (higher in cortex).
- In kidney medulla a gender-related difference was observed for Pb (higher values in men). Smoking habits leads to increased levels of Zn, Cd and Pb.
- In kidney cortex smoking habits showed to induce significantly increases levels of Cd.
- In both kidney regions a slight tendency for an age-related increase in trace elements levels was observed. However, Cd in kidney cortex showed an interesting behaviour: after a increase up to 50 years old, a subsequent decreased was observed.
- For aorta without plaque no gender or smoking habits-related differences were found for the five elements.
- In aorta with plaque, smokers showed significantly decreased Zn levels.
  Aorta with plaque showed higher levels of Zn and lower levels of Se than aorta without plaque.

The comparison between results from "healthy" individuals and individuals with CVD showed:

- Significantly lower levels of Se in both kidney and aorta tissues, indicating a potential cardio-protector role for Se.
- However Cu (in both kidney and aorta tissues), Cd (in aorta) and Pb (in the kidney) were also significantly lower in CVD, which is may be considered as unexpected findings, requiring further investigation.

The data presented should be considered as preliminary results. However, they seem to corroborate the hypothesis that trace elements play an important role in CVD and

important trace element imbalances may occur in tissues. A main question is whether these changes are a cause or a consequence of CVDs.

Further investigation of trace elements imbalances in body tissues and its correlation with CVD must be expanded to include more individuals, more elements and more tissues.

CHAPTER 6

REFERENCES

REFERENCES

1. World Health Organization. Epidemiology and prevention of Cardiovascular diseases in elderly people. Geneva: 1995 853.
2. Bonow R, Mann D, Zipes D, Libby P. Braunwald's Heart Disease A textbook of Cardiovascular Medicine. Ninth edition ed: Elsevier Saunders; 2012.
3. Institute NS. População residente em Portugal com tendência para diminuição e envelhecimento 2014. Available from: http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_destaques&DESTAQUESdest_boui=218629052&DESTAQUESmodo=2.
4. Lloyd LJ, Langley-Evans SC, McMullen S. Childhood obesity and adult cardiovascular disease risk: a systematic review. International Journal Of Obesity. 2010;34(1):18-28.
5. Stocker R, Keaney JF. Role of Oxidative Modifications in Atherosclerosis. Physiological Reviews. 2004;84(4):1381-478.
6. Minqin R, Watt F, Tan Kwong Huat B, Halliwell B. Trace elemental distributions in induced atherosclerotic lesions using nuclear microscopy. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms. 2003;210(0):336-42.
7. Pinheiro T, Pallon J, Fernandes R, Halpern M, Homman P, Malmqvist K. Nuclear microprobe applied to the study of coronary artery walls--a distinct look at atherogenesis. Cellular and molecular biology (Noisy-le-Grand, France). 1996;42(1):89.
8. Stadler N, Lindner RA, Davies MJ. Direct Detection and Quantification of Transition Metal Ions in Human Atherosclerotic Plaques: Evidence for the Presence of Elevated Levels of Iron and Copper. Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24(5):949-54.
9. Murray CJL, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. The Lancet. 1997;349(9063):1436-42.
10. Mendis S, Puska P, Norrving B. Global atlas on cardiovascular disease prevention and control: World Health Organization; 2011.
11. Zeng Q, Dong S-Y, Song Z-Y, Zheng Y-S, Wu H-Y, Mao L-N. Ideal cardiovascular health in Chinese urban population. International journal of cardiology. 2013;167(5):2311-7.
12. Correia R. Arteriosclerose vs aterosclerose 14/11/2010 [updated 12/1272010; cited 08/07/2013 08/07].
13. Ross R. Atherosclerosis — An Inflammatory Disease. New England Journal of Medicine. 1999;340(2):115-26.
14. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nature medicine. 2011;17(11):1410-22.
15. Encyclopædia Britannica I. Atherosclerosis 2013 [cited 2012 29/05/2013]. Available from: http://www.britannica.com/EBchecked/topic/40908/atherosclerosis.
16. Ribeiro S. Desigualdades socioeconómica na doença cardiovascular em Portugal. Lisboa: Universidade Nova de Lisboa; 2010.
17. Silva e Carvalho J. Colesterol, lipidos e doença vascular. Lisboa: Lidel; 2000.
18. Braunwald E*ea*. Harrison: Medicina interna. . Rio de Janeiro: McGraw-Hill; 2002.
19. Ferro J, Verdelho A. Epidemiologia, factores de risco e prevenção primária do AVC. Pathos. 2000;7:5-10.
20. Lawes CMM, Hoorn SV, Rodgers A. Global burden of blood-pressure-related disease, 2001. The Lancet.371(9623):1513-8.
21. Correia D. O coração saudável e doente: Editoral Caminho, SARL; 1981.
22. Duffus JH. " Heavy metals" a meaningless term?(IUPAC Technical Report). Pure and Applied Chemistry. 2002;74(5):793-807.
23. Alissa EM, Ferns GA. Heavy metal poisoning and cardiovascular disease. Journal of toxicology. 2011;2011:870125.

24. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011;283(2–3):65-87.
25. (ATSDR) AfTSaDR. Toxicological profile for cadmium. Atlanta, GA: US Department of Public Health and Human Services, Public Health Service. 2012.
26. Watkins JB, Klaassen CD, Acosta D. Casarett & Doull's essentials of toxicology: McGraw-Hill; 2010.
27. Johri N, Jacquillet G, Unwin R. Heavy metal poisoning: the effects of cadmium on the kidney. Biometals. 2010;23(5):783-92.
28. Faroon O, Ashizawa A, Wright S, Tucker P, Jenkins K, Ingerman L, et al. Toxicological Profile for Cadmium. Agency for Toxic Substances and Disease Registry Toxicological Profile. 2012.
29. Bernhard D, Rossmann A, Wick G. Metals in cigarette smoke. IUBMB Life. 2005;57(12):805-9.
30. Tellez-Plaza M, Jones MR, Dominguez-Lucas A, Guallar E, Navas-Acien A. Cadmium exposure and clinical cardiovascular disease: a systematic review. Current Atherosclerosis Reports. 2013;15(10):1-15.
31. Tellez-Plaza M, Navas-Acien A, Guallar E. Cadmium as a novel cardiovascular risk factor: supportive evidence and future directions. Nature Reviews Cardiology. 2010;7(7):41-6.
32. Messner B, Bernhard D. Cadmium and cardiovascular diseases: cell biology, pathophysiology, and epidemiological relevance. Biometals. 2010;23(5):811-22.
33. Hoffman RS. Goldfrank's manual of toxicologic emergencies: McGraw-Hill New York; 2007.
34. Tellez-Plaza M, Navas-Acien A, Crainiceanu CM, Guallar E. Cadmium exposure and hypertension in the 1999-2004 National Health and Nutrition Examination Survey (NHANES). Environmental health perspectives. 2008:51-6.
35. Swaddiwudhipong W, Mahasakpan P, Limpatanachote P, Krintratun S. Correlations of urinary cadmium with hypertension and diabetes in persons living in cadmium-contaminated villages in northwestern Thailand: A population study. Environmental Research. 2010;110(6):612-6.
36. Zhang W-L, Du Y, Zhai M-M, Shang Q. Cadmium exposure and its health effects: A 19-year follow-up study of a polluted area in China. Science of The Total Environment. 2014;470:224-8.
37. Peters JL, Perlstein TS, Perry MJ, McNeely E, Weuve J. Cadmium exposure in association with history of stroke and heart failure. Environmental Research. 2010;110(2):199-206.
38. Valko M, Morris H, Cronin M. Metals, toxicity and oxidative stress. Current Medicinal Chemistry. 2005;12(10):1161-208.
39. Ford ES. Serum copper concentration and coronary heart disease among US adults. American Journal of Epidemiology. 2000;151(12):1182-8.
40. KOK FJ, VAN DUIJN CM, HOFMAN A, VAN DER VOET GB, DE WOLFF FA, PAAYS CHC, et al. Serum copper and zinc and the risk of death from cancer and cardiovascular disease. American Journal of Epidemiology. 1988;128(2):352-9.
41. Schwartz J. Lead, blood pressure, and cardiovascular disease in men. Archives of Environmental Health: An International Journal. 1995;50(1):31-7.
42. Nawrot T, Thijs L, Den Hond E, Roels H, Staessen JA. An epidemiological re-appraisal of the association between blood pressure and blood lead: a meta-analysis. Journal of Human Hypertension. 2002;16(2):123-31.
43. Navas-Acien A, Tellez-Plaza M, Guallar E, Muntner P, Silbergeld E, Jaar B, et al. Blood cadmium and lead and chronic kidney disease in US adults: a joint analysis. American Journal of Epidemiology. 2009;170(9):1156-64.
44. Fadrowski Jj N-AAT-PMGEWVMFSL. Blood lead level and kidney function in us adolescents: The third national health and nutrition examination survey. Archives of Internal Medicine. 2010;170(1):75-82.
45. Ekong EB, Jaar B, Weaver V. Lead-related nephrotoxicity: a review of the epidemiologic evidence. Kidney International. 2006;70(12):2074-84.

46. Navas-Acien A, Guallar E, Silbergeld EK, Rothenberg SJ. Lead exposure and cardiovascular disease—a systematic review. Environmental Health Perspectives. 2007;115(3):472.
47. Skoczynska A, Skoczynska M. Low-Level Exposure to Lead as a Cardiovascular Risk Factor. 2012.
48. Ding Y, Gonick HC, Vaziri ND. Lead promotes hydroxyl radical generation and lipid peroxidation in cultured aortic endothelial cells. American Journal of Hypertension. 2000;13(5):552-5.
49. Fowler BA, Whittaker MH, Lipsky M, Wang G, Chen X-Q. Oxidative stress induced by lead, cadmium and arsenic mixtures: 30-day, 90-day, and 180-day drinking water studies in rats: an overview. Biometals. 2004;17(5):567-8.
50. Schober SE, Mirel LB, Graubard BI, Brody DJ, Flegal KM. Blood lead levels and death from all causes, cardiovascular disease, and cancer: results from the NHANES III mortality study. Environmental Health Perspectives. 2006;114(10):1538.
51. Kasperczyk S, Birkner E, Kasperczyk A, Kasperczyk J. Lipids, lipid peroxidation and 7-ketocholesterol in workers exposed to lead. Human and Experimental Toxicology. 2005;24(6):287-95.
52. Lee D-H, Lim J-S, Song K, Boo Y, Jacobs Jr DR. Graded associations of blood lead and urinary cadmium concentrations with oxidative-stress-related markers in the US population: results from the third National Health and Nutrition Examination Survey. Environmental Health Perspectives. 2006:350-4.
53. Navas-Acien A, Selvin E, Sharrett AR, Calderon-Aranda E, Silbergeld E, Guallar E. Lead, cadmium, smoking, and increased risk of peripheral arterial disease. Circulation. 2004;109(25):3196-201.
54. Oster O, Prellwitz W. Selenium and cardiovascular disease. Biological Trace Element Research. 1990;24(2-3):91-103.
55. Mozaffarian D. Fish, mercury, selenium and cardiovascular risk: current evidence and unanswered questions. International Journal of Environmental Research and Public Health. 2009;6(6):1894-916.
56. Little PJ, Bhattacharya R, Moreyra AE, Korichneva IL. Zinc and cardiovascular disease. Nutrition. 2010;26(11):1050-7.
57. Reiterer G, MacDonald R, Browning JD, Morrow J, Matveev SV, Daugherty A, et al. Zinc Deficiency Increases Plasma Lipids and Atherosclerotic Markers in LDL-Receptor–Deficient Mice. The Journal of Nutrition. 2005;135(9):2114-8.
58. Jenner A, Ren M, Rajendran R, Ning P, Huat BTK, Watt F, et al. Zinc supplementation inhibits lipid peroxidation and the development of atherosclerosis in rabbits fed a high cholesterol diet. Free Radical Biology and Medicine. 2007;42(4):559-66.
59. Ren M, Rajendran R, Ning P, Tan Kwong Huat B, Choon Nam O, Watt F, et al. Zinc supplementation decreases the development of atherosclerosis in rabbits. Free Radical Biology and Medicine. 2006;41(2):222-5.
60. Stadler N, Stanley N, Heeneman S, Vacata V, Daemen MJAP, Bannon PG, et al. Accumulation of Zinc in Human Atherosclerotic Lesions Correlates With Calcium Levels But Does Not Protect Against Protein Oxidation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2008;28(5):1024-30.
61. Stanley N, Stadler N, Woods AA, Bannon PG, Davies MJ. Concentrations of iron correlate with the extent of protein, but not lipid, oxidation in advanced human atherosclerotic lesions. Free Radical Biology and Medicine. 2006;40(9):1636-43.
62. Raman SV, Winner IIIMW, Tran T, Velayutham M, Simonetti OP, Baker PB, et al. In Vivo Atherosclerotic Plaque Characterization Using Magnetic Susceptibility Distinguishes Symptom-Producing Plaques. JACC: Cardiovascular Imaging. 2008;1(1):49-57.
63. Brown RJC, Milton MJT. Analytical techniques for trace element analysis: an overview. Trends in Analytical Chemistry. 2005;24(3):266-74.

64. Willis J. The early days of atomic absorption spectrometry in clinical chemistry. Spectrochimica Acta Part B: Atomic Spectroscopy. 1999;54(14):1971-5.
65. Parsons PJ, Barbosa Jr F. Atomic spectrometry and trends in clinical laboratory medicine. Spectrochimica Acta Part B: Atomic Spectroscopy. 2007;62(9):992-1003.
66. Todd AC, Parsons PJ, Carroll S, Geraghty C, Khan FA, Tang S, et al. Measurements of lead in human tibiae. A comparison between K-shell x-ray fluorescence and electrothermal atomic absorption spectrometry. Physics in Medicine and Biology. 2002;47(4):673.
67. Ivanenko N, Ganeev A, Solovyev N, Moskvin L. Determination of trace elements in biological fluids. Analytical Chemistry. 2011;66(9):784-99.
68. D'Ilio S, Violante N, Di Gregorio M, Senofonte O, Petrucci F. Simultaneous quantification of 17 trace elements in blood by dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) equipped with a high-efficiency sample introduction system. Analytica Chimica Acta. 2006;579(2):202-8.
69. Houk RS, Fassel VA, Flesch GD, Svec HJ, Gray AL, Taylor CE. Inductively coupled argon plasma as an ion source for mass spectrometric determination of trace elements. Analytical Chemistry. 1980;52(14):2283-9.
70. Becker S. Inorganic mass spectrometry: principles and applications: Wiley-Interscience; 2008.
71. Scientific T. From first principles: An introduction to the ICP-MS technique.
72. Schools MP. The Urinary System [cited 2014]. Available from: http://www.methuen.k12.ma.us/mnmelan/Excratory%20system.htm.
73. Succop PA, Clark S, Chen M, Galke W. Imputation of data values that are less than a detection limit. Journal of occupational and environmental hygiene. 2004;1(7):436-41.
74. Galan P, Viteri F, Bertrais S, Czernichow S, Faure H, Arnaud J, et al. Serum concentrations of β-carotene, vitamins C and E, zinc and selenium are influenced by sex, age, diet, smoking status, alcohol consumption and corpulence in a general French adult population. European Journal of Clinical Nutrition. 2005;59(10):1181-90.
75. Vahter M, Åkesson A, Lidén C, Ceccatelli S, Berglund M. Gender differences in the disposition and toxicity of metals. Environmental Research. 2007;104(1):85-95.
76. Bernhard D, Csordas A, Henderson B, Rossmann A, Kind M, Wick G. Cigarette smoke metal-catalyzed protein oxidation leads to vascular endothelial cell contraction by depolymerization of microtubules. The FASEB Journal. 2005;19(9):1096-107.
77. Kocyigit A, Erel O, Gur S. Effects of tobacco smoking on plasma selenium, zinc, copper and iron concentrations and related antioxidative enzyme activities. Clinical Biochemistry. 2001;34(8):629-33.
78. Scott R, Aughey E, Fell GS, Quinn MJ. Cadmium Concentrations in Human Kidneys from the UK. Human and Experimental Toxicology. 1987;6(2):111-20.
79. Mortada WI, Sobh MA, El-Defrawy MM. The exposure to cadmium, lead and mercury from smoking and its impact on renal integrity. Medical Science Monitor. 2004;10(3):116.
80. Garcia F, Ortega A, Domingo J, Corbella J. Accumulation of metals in autopsy tissues of subjects living in Tarragona County, Spain. Journal of Environmental Science and Health, Part A. 2001;36(9):1767-86.
81. Barregard L, Fabricius-Lagging E, Lundh T, Mölne J, Wallin M, Olausson M, et al. Cadmium, mercury, and lead in kidney cortex of living kidney donors: Impact of different exposure sources. Environmental research. 2010;110(1):47-54.
82. Barregård L, Svalander C, Schütz A, Westberg G, Sällsten G, Blohmé I, et al. Cadmium, mercury, and lead in kidney cortex of the general swedish population: a study of biopsies from living kidney donors. Environmental Health Perspectives. 1999;107(11):867.
83. Lapenna D, Mezzetti A, De Gioia S, Pierdomenico SD, Daniele F, Cuccurullo F. Plasma copper and lipid peroxidation in cigarette smokers. Free Radical Biology and Medicine. 1995;19(6):849-52.

84. Blanuša M, Kralj Z, Bunarević A. Interaction of cadmium, zinc and copper in relation to smoking habit, age and histopathological findings in human kidney cortex. Archives of Toxicology. 1985;58(2):115-7.
85. Dorian C, Klaassen CD. Protection by zinc-metallothionein (ZnMT) against cadmium-metallothionein-induced nephrotoxicity. Fundamental and Applied Toxicology. 1995;26(1):99-106.
86. Tohno S, Tohno Y, Minami T, Moriwake Y, Azuma C, Ohnishi Y. Elements of calcified sites in human thoracic aorta. Biological Trace Element Research. 2002;86(1):23-30.
87. Navarro-Alarcón M, López-Martínez MC. Essentiality of selenium in the human body: relationship with different diseases. Science of The Total Environment. 2000;249(1–3):347-71.
88. Lubos E, Sinning CR, Schnabel RB, Wild PS, Zeller T, Rupprecht HJ, et al. Serum selenium and prognosis in cardiovascular disease: results from the AtheroGene study. Atherosclerosis. 2010;209(1):271-7.

ATTACHMENT

Data of the studied subjects

Age Range	Cause of death	Gender	Age	Smoker	CVD	Residence
20-29	Traumatic Brain Injury	F	20	No	No	Maia
	Traumatic Brain Injury	M	22	No	No	Valongo
	Eating disorders	F	25	No	No	Matosinhos
	Traumatic Brain Injury	M	26	No	?	Gaia
	Traumatic Brain Injury	M	28	No	No	Valongo
30-39	Arrhythmia	F	33	No	No	Matosinhos
	Traumatic Brain Injury	M	34	No	No	Vila Nova de Famalicão
	Acute Myocardial Infarction	M	37	Former smoker	Yes	Porto
	Chest trauma by firearm	F	39	No	No	Gaia
	Suicide by stab	F	39	?	No	Gaia
40-49	Asphyxia from hanging	M	42	?	No	Vila do Conde
	Traumatic Brain Injury	M	43	Yes	No	Alfandega da Fé
	Left ventricular hypertrophy	M	43	No	No	Vila do Conde
	Alcohol intoxication associated with aspiration of vomit	M	44	Yes	No	Gondomar
	Traumatic Brain Injury	M	44	Yes	No	Felgueiras
	?	M	46	?	Yes	Santo Tirso
	Left hypertrophic cardiomyopathy	F	47	Yes	?	Vila do Conde
	Drug intoxication	F	47	No	No	Porto
	Acute Myocardial Infarction	M	48	Yes	Yes	Gondomar
50-59	Bronchopneumonia	M	50	No	Yes	?
	Acute Myocardial Infarction	M	50	Yes	Yes	Porto
	Asphyxia from hanging	M	51	?	?	Porto
	Asphyxiation due to aspiration of foreign body	M	52	No	?	Gaia
	Asphyxia from hanging	M	53	?	No	Santo Tirso
	Acute Myocardial Infarction	M	54	Yes	Yes	Gaia
	Hepatorrenal syndrome	M	55	Yes	Yes	Gondomar
	Pneumonia	F	56	Yes	No	Gaia
	Traumatic Brain Injury	F	57	?	No	Porto
	Acute Myocardial Infarction	M	59	Yes	Yes	Matosinhos
60-69	Traumatic Brain Injury	M	64	No	Yes	Mirandela
	Acute Myocardial Infarction	M	65	Yes	Yes	Gondomar
	Asphyxia from hanging	M	67	?	Yes	Gaia

	?	F	67	No	Yes	Matosinhos
	Acute Myocardial Infarction	M	68	No	Yes	Maia
	Pulmonary embolism	F	69	No	No	Gondomar
70-79	Traumatic Brain Injury	M	70	Former smoker	?	Matosinhos
	?	M	71	?	?	Gaia
	?	F	71	No	No	Gaia
	Pneumonia	M	73	No	No	Barcelos
	Sepsis	F	74	No	Yes	Porto
	Asphyxia from hanging	M	74	?	No	Gondomar
	Pulmonary embolism	F	74	No	Yes	Porto
	Asphyxia from hanging	F	76	?	?	Gaia
	Acute Myocardial Infarction	F	79	Yes	No	Vila do Conde
	?	M	79	Former smoker	?	Vila do Conde
	Traumatic injuries	M	79	No	Yes	Porto
80-89	Traumatic injuries	M	82	No	Yes	Gondomar
	Multiorgan failure due to pneumonia	F	83	No	Yes	Marco de Canaveses
	Stroke	F	84	No	Yes	Gaia
	Abdominal infection	F	85	No	?	Vila do Conde
	Traumatic Brain Injury	M	86	No	Yes	Chaves
	Traumatic Brain Injury	F	87	No	Yes	Porto
≥90	?	F	91	No	Yes	Porto
	Pulmonary embolism	F	93	No	Yes	Porto