Universidade de Aveiro Departamento de Biologia

2015

Ana Catarina Duarte Alves

Mercury levels in parturient and newborns from Aveiro region, Portugal

Níveis de mercúrio em parturientes e recém-nascidos da região de Aveiro, Portugal

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Equiparada a Investigadora Auxiliar do Departamento de Biologia e CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro, e co-orientação da Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos, Investigadora em Pós-Doutoramento do Departamento de Biologia e CESAM da Universidade de Aveiro.

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“ Recomeça... se puderes, sem angústia e sem pressa e os passos que deres, nesse caminho duro do futuro, dá-os em liberdade, enquanto não alcances não descanses, de nenhum fruto queiras só metade.”

Miguel Torga

o júri

Presidente

Prof. Doutor Carlos Miguel Miguez Barroso
Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Doutor João Paulo Fernandes Teixeira
Investigador Auxiliar do Departamento de Saúde Ambiental, Instituto Nacional de Saúde Dr. Ricardo Jorge, Porto

Doutora Susana Patrícia Mendes Loureiro
Equiparada a Investigadora Auxiliar do Departamento de Biologia e CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro

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palavras-chave

mercúrio, gravidez, biomonitorização, biomarcadores de exposição, unidade materno-fetal-placentária, cabelo do escalpe.

resumo

Químicos ambientais, como o mercúrio (Hg), em mulheres grávidas podem atravessar a placenta e desencadear efeitos teratogénicos. Em Portugal, a exposição pré-natal continua pouco documentada. A região portuguesa de Aveiro enfrentou uma contaminação ambiental devido à indústria de cloro, localizada em Estarreja. Efluentes ricos em Hg foram libertados durante 5 décadas na ria de Aveiro e consequentemente, os solos urbanos e agrícolas circundantes, os sedimentos e a biota foram negativamente afetados. Dada a importância do contexto regional e geográfico, esta tese teve como objectivos: (i) avaliar o grau de exposição a Hg em parturientes e recém-nascidos do distrito de Aveiro, Portugal; (ii) usar material biológico não invasivo, descartado após parto, para realizar um estudo de biomonitorização; (iii) melhorar o conhecimento acerca da distribuição e retenção de Hg ao longo da unidade materno-fetal-placentária; (iv) relacionar os níveis de Hg com potenciais factores de risco incluindo o estilo de vida materno, hábitos, dieta e características demográficas; e (v) investigar a distribuição dos níveis de Hg ao longo do distrito de Aveiro.

Este estudo foi realizado em 50 pares mãe-recém-nascido do distrito de Aveiro. Uma correlação positiva foi observada entre os níveis de Hg no cabelo, tecidos placentários e cordão umbilical. Portanto, a viabilidade da utilização da placenta para avaliar a exposição intra-uterina a Hg foi confirmada, bem como outros marcadores não-invasivos e biológicos, como o teor em Hg no tecido do cordão e no cabelo do escalpe. Os nossos resultados detetaram a ocorrência de valores altos de Hg no cabelo materno, de acordo com a US EPA e a OMS, e elevados níveis de Hg nos tecidos placentários comparativamente com investigações anteriores de outros países Europeus. Os níveis mais elevados de Hg foram encontrados na membrana amniótica, a qual parece desempenhar um papel na eliminação de metais tóxicos do feto através da reabsorção do líquido amniótico. Exceptuando o grau de literacia, nenhum outro factor de risco foi positivamente relacionado com os níveis de Hg.

Por último, as 50 parturientes estudadas foram agrupadas pela sua residência atual, sita em nove concelhos do distrito de Aveiro. Albergaria-a-Velha e Águeda foram os concelhos com os níveis mais elevados no cabelo e no cordão umbilical. Para além disso, estes resultados mostraram que a exposição materna pode subestimar, em alguns casos, o grau de exposição pré-natal a Hg.

keywords

mercury, pregnancy, biomonitoring, biomarkers of exposure, maternal-fetal-placental unit, scalp hair.

abstract

Environmental chemicals such as mercury (Hg), in pregnant women can cross the placenta and trigger teratogenic effects. In Portugal, prenatal exposure to Hg is still poorly documented. The Portuguese region of Aveiro faced an environmental Hg contamination due to the activities from chlor-alkali industry, located in Estarreja. Effluents rich in Hg were released during 5 decades to the Ria de Aveiro lagoon system and consequently the surrounding urban and agricultural soils, sediments, and biota were negatively affected.

Given the importance of regional geographic context, this thesis aimed: (i) to assess Hg exposure in parturient and newborns from Aveiro district, Portugal; (ii) to use non-invasive biological material discarded after birth to perform a biomonitoring study; (iii) to improve the knowledge about the distribution and retention of Hg over the placental-fetal unit; (iv) to relate Hg levels with potential risk factors including maternal lifestyle, habits, diet and demographic characteristics; and (v) to investigate the distribution of Hg levels along the Aveiro district.

This study was performed in 50 mother-newborn pairs from Aveiro district. A strong positive correlation was found between Hg levels in hair, placental and cord tissues. Therefore, the feasibility of using the placenta to assess intrauterine exposure to Hg was confirmed as well as other non-invasive and biological markers like Hg level in cord tissue and scalp hair.

Our results detected the occurrence of high Hg levels in maternal hair according to US EPA and WHO, and higher Hg contents in placental tissues compared to previous reports from other European countries. The highest Hg levels were observed in amniotic membrane which seems to play a role in the elimination of toxic metals from the fetus by reabsorption from amniotic fluid. Further research should be carried out to get further knowledge on the ability of the amniotic membrane to retain and accumulate Hg and other metals. Apart from the level of education, no other risk factors were positively correlated with Hg levels.

Lastly, the 50 parturient studied were grouped per their actual residence, located in nine different counties from Aveiro district. Albergaria-a-Velha and Águeda were the counties with higher Hg levels in hair and umbilical cord. In addition, these results showed that maternal exposure may underestimate, in some cases, the degree of prenatal exposure to Hg.

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1. General Introduction

1. Introduction

It is widely known that people are daily exposed to a number of chemicals due to environmental contamination, habits and lifestyle. One of those chemicals is mercury (Hg), which it is a naturally occurring and non-essential element. Mercury has some unique chemical properties that have made it useful for several purposes throughout the history of mankind.

1.2 Mercury and its compounds

Mercury occurs in three oxidation states (0, +1 and +2) and may be found in three main chemical species: elemental mercury (Hg^0), mercurous ion (Hg_2^{2+}) and the mercuric ion (Hg^{2+}) **[1]**. When ions combine with other elements such as chlorine, sulfur or oxygen inorganic salts are formed (mercuric chloride, mercuric sulphide and mercuric oxide). Mercury can also be found in organic forms such as monoalkyl or dialkyl compounds (methylmercury (MeHg), dimethylmercury, ethylmercury and phenylmercury) **[2].**

Elemental and inorgancic Hg may occur naturally in the environment:

- mineral;
- deposits;
- volcanoes;
- forest fires;
- oceanic emission;
- crust degassing.

It also may be released to the environment through different anthropogenic activities/sources:

- agricultural industry (fungicides, seed preservatives);
- mining and mineral processing;
- combustion of fossil fuels;
- pharmaceuticals;
- pulp and paper preservatives;

- skin-lightning creams;
- catalysts in industrial processes;
- thermometers and batteries;
- amalgams;
- chlorine and caustic soda production.

Methylmercury has the capacity to bioaccumulate in organisms and to biomagnify through the food chain. Indeed, the diet is the main source of MeHg for humans and biota **[2].**

1.3 Environmental fate of mercury

Elemental Hg is the predominant form of Hg in the atmosphere released by natural and anthropogenic sources **[3].**

1.3.1 Soil

In the atmosphere by reaction with ozone and OH radicals Hg is oxidized to Hg^{2+} and deposited in soils. A portion of this oxidized Hg is reduced again to the elemental form and returns to the atmosphere in vapor form. The remaining Hg^{2+} which is not immediately reduced and evaporated can accumulate in the vegetation or instead it may be incorporated into a soil Hg pool where it is slowly transformed and released to the atmosphere, during a process that can take centuries or millennia **[3]**.

1.3.2 Aquatic systems and sediments

In water, the main Hg chemical forms present are elemental, complexes of Hg^{2+} with inorganic and organic ligands, and organic forms, predominantly MeHg and dimethylmercury. In fact, MeHg is typically less than 5% in estuarine and marine waters but the same form can reach more than 30% in fresh water systems **[4].** In the ocean and fresh water sediments as also in the water column Hg can be biologically methylated by sulphate-reducing bacteria and iron-reducing bacteria **[5]**. On the other hand, an abiotic methylation may occur when suitable methyl donors like humic matter are

available **[6]**. This process turns Hg into species more liposoluble and consequently toxic to living organisms including humans **[7]**. Methylmercury bioaccumulates and increases up in the aquatic food web. Therefore the highest concentrations are found in large and old predatory fish, such as sharks, swordfish, tuna and pike **[8]**.

1.4 Toxicokinetics

All Hg compounds are considered toxic to humans. Their toxicity depends on the oxidation state, binding elements, routes of exposure, duration and level of exposure. The metabolism of Hg species involves an oxidation/reduction cycle **[9]**.

1.4.1 Elemental mercury

Elemental Hg exposure from air is readily taken up through the lungs due to its volatility with a retention rate higher than 70% in the human body **[10]**. As it is highly lipophilic, inhaled Hg^0 crosses easily the alveolar membranes into the circulatory system. This feature also allows Hg crossing the main barriers including the blood-brain barrier and the placenta. In blood Hg^0 is oxidized to Hg^{2+} partly under the influence of catalase and hydrogen peroxide which influence brain uptake of Hg **[11]**.

1.4.2 Inorganic mercury compounds

The major target to accumulation of inorganic Hg is the liver and the kidney from where it is excreted. Rahola *et al.* **[12]** and Hattula and Rahola **[13]** described the kinetics of Hg^{2+} in humans and observed that no more than 16% of the initial dose was absorbed with a body half-time of about 41 days. Excretion via feces also occurs in a less extent and it involves the formation of GSH complexes prior to secretion into bile. Regarding the head region, no significant deposition of Hg has been observed at least for 58 days. On the other hand, in an experimental study in rats it was shown that there was an irregular distribution of mer

curic chloride in the nervous system. It was observed more mercuric chloride in the neurons compared to the glial cells, and an accumulation in lysosomes. It was also observed the presence of mercuric chloride in the motor neurons and their absence in the sensory neurons **[14]**. According to another study, after guinea-pigs skin topically

application of mercuric chloride it was observed that 8% of this compound can be absorbed in 5 h **[15]**.

In **Figure 1.1** it is shown the resume of the main routes of exposure to elemental and inorganic Hg: inhalation, skin absorption and ingestion. Through the lungs, skin and gastro-intestinal (GI)-tract Hg runs into blood and is (re)distributed by functional organic systems. There Hg suffers detoxification processes in order to facilitate the excretion or instead Hg may accumulate (e.g., Central Nervous System – CNS and kidney) **[16]**.

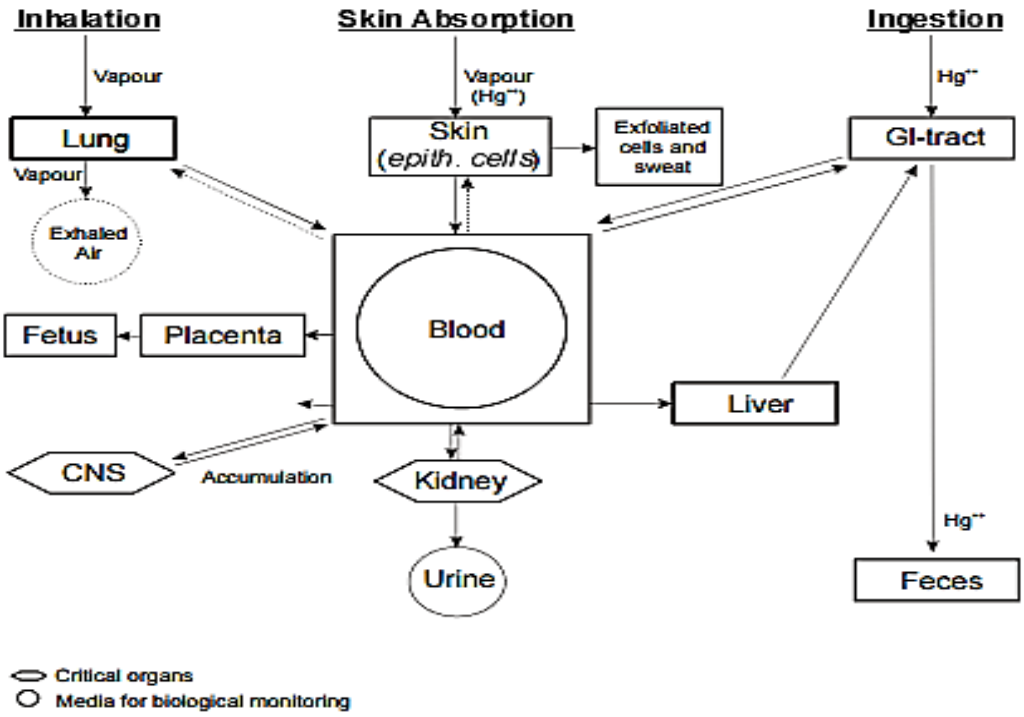

Figure 1.1- Routes of elemental and inorganic mercury exposure to humans & toxicokinetics. GI-tract, Gastrointestinal-tract; CNS, Central Nervous System. In: Elinder *et al.* **[16].**

1.4.3 Organic mercury

The dialkyl (e.g. dimethylmercury) compounds are very volatile being readily absorbed both through the respiratory airways and intact skin, and are highly toxic even at very low exposure. The dialkyl mercury compounds have an effect on the environmental distribution of MeHg as they are highly volatile, insoluble in water and do not bind to sulfhydryl (SH) groups **[17]**. Methylmercury is absorbed by inhalation with a retention rate about 80% after vapor exposure. Other routes of exposure include skin absorption and the ingestion of contaminated food with MeHg, such as fish, where it can be potentially 100% absorbed at the intestine **[18]**. Methylmercury is accumulated to a large extent in erythrocytes with a retention rate higher than 90% where it is bound to the cysteinyl residues of hemoglobin. In humans the erythrocytes to plasma ratio is about 20. In plasma 99% of MeHg is bound to albumin which is a free sulfhydryl group in a terminal cysteinyl residue **[19]**. After absorption into the blood the distribution to tissue is slow and equilibrium is reached within 30h to three days with about 5 and 10% ending up in blood and brain respectively **[20]**. The uptake into the brain is slower than for other organs probably due to the binding of MeHg to the erythrocytes which retards its entry into the brain. On the other hand the brain has a stronger affinity for MeHg and the brain concentration has been shown to be 3–6 times higher that found in the blood. About 20% of the MeHg present in brain is hydrophilic and can be found mainly as MeHg–GSH complexes. Throughout the rest of the body, MeHg is rather consistently distributed although some concentration dependent effects can be seen in the liver and the kidney **[20]**. Besides MeHg is also incorporated in hair during the hair follicle formation and it is positively related with the concentration of MeHg in blood **[21]**.

Maternal MeHg transfer to the offspring may occur in early and later stages of development **[22]**. Over the pregnancy, MeHg crosses the placenta and accumulates in the fetus at concentrations higher than in the mother **[23]**. For example, cord blood MeHg concentrations are higher than those found in maternal blood at delivery. This can be explained by the differences in hemoglobin content once it is the primary binding protein for MeHg in erythrocytes **[23]**. In postnatal period, infants are exposed to MeHg during the breastfeeding once it is also capable to cross the mammary gland **[24]**.

The exact mechanisms by which MeHg crosses the main powerful barriers are not fully understood. It has been hypothetised due to MeHg structural similarities to methionine, MeHg-L-cysteine may cross membranes via specific aminoacid transporters **[25]**. Moreover the transport across the cell membranes into cells is believed to occur by

MeHg complex with cysteine and the exit from cells by a glutathione complex via endogenous glutathione carriers **[1]**. Methylmercury is demethylated to Hg^{2+} in the presence of reactive oxygen species (ROS) as hydroxyl radical. This process may occur in liver, intestinal tract, the spleen, phagocytic cells, and kidney and slowly in the brain **[26]**. Methylmercury has a human body half-life of about 70-80 days, with about 90% being excreted through feces as Hg^{2+} **[27]**. Similarly, via the biliary route MeHg is eliminated after the conjugation with liver gluthatione-S-transferases (GST) and further eliminated by feces **[28]**. Intestinal demethylation contributes significantly to increase fecal excretion. Methylmercury is partly converted by the intestinal microflora to Hg^{2+} which is not reabsorbed via enterohepatic circulation to the same degree as MeHg **[29]**.

In **Figure 1.2** it is shown the resume of the main routes of exposure to organic Hg: inhalation, skin absorption and ingestion. Along the gastrointestinal (GI)-tract MeHg suffers a detoxification process namely demethylation in order to facilitate the excretion. Methylmercury also may accumulate mainly in CNS in adults, fetus during pregnancy and child during breastfeeding. Exhaled hair, epithelial cells, sweat, urine, blood, hair (child and adults) and feces can be used to perform biological monitoring concerning MeHg exposure **[16]**.

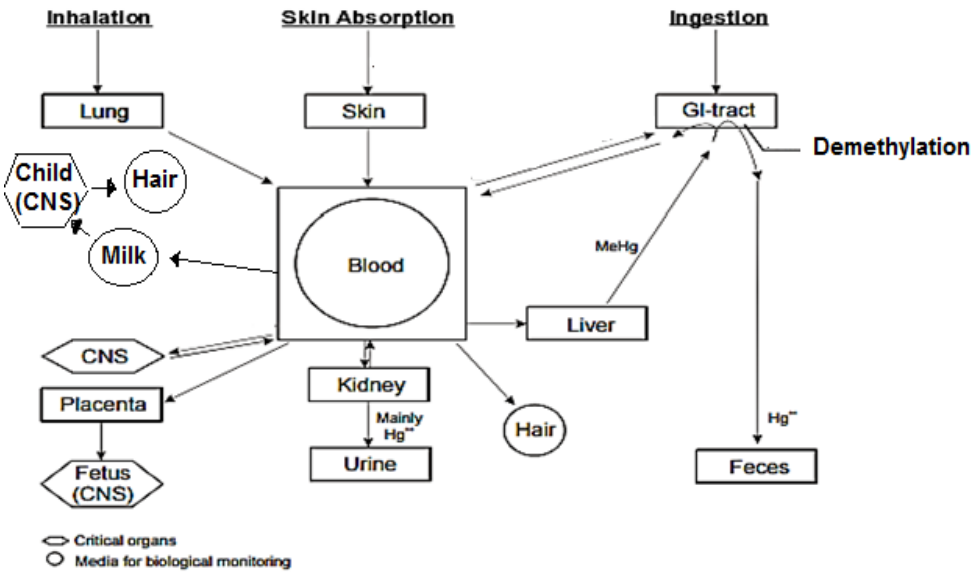

Figure 1.2- Routes of methylmercury exposure to humans & toxicokinetics. **GI-tract, Gastrointestinal-tract; CNS, Central Nervous System.** Adapted from Elinder *et al.***[16]**.

1.5 Mechanisms of action

1.5.1 Mercury, GSH system and ROS production

Glutathione is the major endogen antioxidant in mammals and its role as antioxidant is linked to enzymes that catalyze the synthesis of GSH and the interaction reactions of GSH with xenobiotics **[30]**. (GPx) uses GSH reduced to detoxify organic hydroperoxides which prevent the peroxidative damage in biomolecules **[31]**. In the reaction thiol-peroxidase GSH is oxidized to glutathione disulfide (GSSG) which is newly reduced by glutathione reductase (GR) enzyme with NADPH as cofactor **[32]**. Glutathione peroxidase and GR participate in detoxification of peroxides and in the reduction of GSSG. The activity of these enzymes and the maintenance of GSH/GSSH ratio are essential to the cells protection against oxidative damage. Mercury may interfere with cellular mechanisms and may be responsible for a variability of toxic effects in cells. Mercury has a great affinity to bind to proteins and non-proteins with thiol groups and glutathione is a tripeptide which contains cysteine **[33]**. The formation of Hg-GSH complexes decrease GSH activity as antioxidant leading to an increase of ROS. Mercury also affects the electron transport chain by stimulating the complex IV where the final receptor of electrons is the molecular oxygen. This may induce an electrons leakage into the molecular oxygen increasing the formation of superoxide radical and hydrogen peroxide (H_2O_2) **[34].**

1.5.2 Mercury and genotoxicity

One of the mechanisms that induce genotoxicity after Hg exposure is oxidative stress due to the action of ROS increased by the metal. ROS are highly reactive chemical species that may cause DNA damage **[35]**. Firstly, direct action of these species on nucleic acids may generate genetic mutations **[36]**. Secondly, ROS may induce conformational changes in proteins responsible for the formation and preservation of DNA such as repair enzymes, DNA-polymerases, and even tubulin and kinesin motor proteins, responsible for mitotic spindle and chromosomal segregation **[35, 36, 37, 38]**. Another mechanism of genotoxicity induced by Hg is the direct interaction between mercury compounds and DNA molecules. Yi *et al.* **[39]** tested several Hg compounds (methylmercury, ethylmercury, phenylmercury and inorganic mercury) and its interaction with DNA. It was observed higher affinity and interaction with DNA and a

fastest binding rate of formation of stable complexes especially between MeHg and DNA. This result was especially interesting once organometallic species have the capacity to easily cross nuclear membranes to reach the DNA helix. All the bases interacted directly with Hg species with predominance of guanine and cytosine-MeHg and thymine-Hg^{2+} bindings **[39]**.

1.6 Mercury poisoning

Mercury poisoning is known as mercurialism (or hydrargyria) and acrodynia. In the first half of the 20th century acrodynia also known as pink disease was relatively common among small children. It was found a pink discoloration of hands and legs with desquamation of the skin caused by teething powder containing mercurous chloride **[40]**.

Amalgam is an alloy of Hg and it is an excellent and versatile dental restorative material. It has been used in dentistry since 150 years ago due to its low cost, ease of application, strength, durability, and bacteriostatic effects. Dental amalgams are composed by 50% Hg^0 mixed with other metals such as silver, copper and zinc. This operation is handmade before the amalgam is used causing exposure to the dental personnel (occupational exposure). In the patient the release of Hg from fillings is mainly determined by chewing and the temperature of food items **[2, 9]**.

Methylmercury is linked with two major human disasters of massive Hg poisoning. The first took place in Japan (1956-1968) where a chemical factory released Hg as byproduct of their acetaldehyde production into the Minamata Bay. About 200,000 persons were exposed through the consumption of contaminated fish and shellfish. Adults developed sensory disturbances, ataxia, dysarthria, constriction of the visual field as well as psychiatric disorders. Besides newborns were affected during development from their mothers who were exposed through ingestion of contaminated food. In newborns disturbances in mental and motor developments were observed **[41]**. The second disaster happened in rural Iraq (1971-1972). About 40,000 people were exposed through homemade bread prepared from seed grain treated with MeHg as fungicide. The symptoms were similar to those observed in adults from Minamata Bay **[27]**.

1.7 Health effects

Factors that determine whether health effects occur from Hg exposure and their severity include **[42]:**

- the mercury species;
- the dose;
- the age or developmental stage of the person exposed;
- the duration of exposure;
- the route of exposure (inhalation, ingestion or dermal contact).

1.7.1 Neurotoxicity

The nervous system is the most sensitive to Hg effects compared to all functional systems that constitute the human body. Besides no other metal can affect central nervous system (CNS) as Hg does **[42, 43]**. Elemental and organic mercury can cross the blood-brain barrier and accumulate in the CNS **[43]**.

The chronic exposure to Hg^0 induces damage mainly in CNS. The first non-specific signals include: insomnia, low memory, loss of the appetite and tremor which sometimes lead to wrong diagnostics, such as psychiatric disorders **[44]**. A further exposure takes to the worsening of the patient's situation characterized by a triad of symptoms: severe tremors, gingivitis and erethism. The last comprises a wide spectrum of perturbations of personality that can result in dramatic changes in individual behavior such as delirium, hallucinations, excessive shyness and angry outbursts **[43].** The neurobehavioral changes caused by Hg vapor are classified in four groups: **1)** disorders of the motor system; **2)** deterioration of the intellectual capacity; **3)** change in the emotional state; and **4)** peripheral neurotoxicity **[44]**.

The chronic exposure to MeHg leads to different results in adult and developing CNS. The intoxication in adults is characterized by the existence of a latency period between the exposure and the development of symptoms. Paresthesia is a sensation of numbness or tingling and it is the first symptom to appear at the lowest dose of exposure. The clinical condition can after progress to cerebral ataxia, dysarthria, constriction of the visual field and hearing loss **[1]**.These symptoms are caused by modifications in the structure and biochemical features in neurons and astrocytes **[45]**. Besides, MeHg is associated with cell loss and reduced brain size **[46]**. Pathological

exams in intoxicated patients by MeHg showed that the cerebral cortex was the most affected zone and that its granule cells were sensitive to this compound **[47]**.

The neurotoxic effects caused by MeHg in developing CNS drive to serious disturbances in mental and motor development. Later exposed children may develop great difficulties in chewing, swallowing, speaking, crawling or any other activity involving coordinated or involuntary movements **[41]**. In fact, damages caused by MeHg to the CNS of human and other species/organisms' fetus are linked to a decrease in the number of neuronal cells and change in their cytoarchitecture. Both interfere with cellular events such as division, migration, differentiation and death that regulate the neuronal development **[48]**.

Neurotoxicity induced by MeHg is assigned by three main mechanisms: induction of oxidative stress by the increase of ROS, changes in intracellular calcium levels and interaction with thiol groups of several molecules **[47]**. Methylmercury binds covalently to thiol groups causing the inhibition of enzymes and inactivating non-enzymatic molecules such as GSH. Methylmercury can induce oxidative damage by direct interaction with nucleophilic groups of proteins even in the absence of significant changes in GSH levels and GSH/GSSH ratio **[46]**. The complex MeHg-Cys can penetrate in CNS and there MeHg breaks the mitochondrial electron transport chain leading to an increase of ROS such as hydrogen peroxide and superoxid ion ($O_2^{\bullet -}$).

The loss of glutamate (GLU) homeostasis in CNS is also a result in the neurotoxicity caused by MeHg **[47].** Glutamate is the main and abundant excitatory neurotransmitter of the mammal CNS and its release at the synaptic cleft is the key event to stop the signal transmission **[49]**. Methylmercury inhibits the entry of GLU into the astrocyte increasing the release of GLU from the pre-synaptic neuron. When GLU occurs at high concentrations in the synaptic cleft it acts like a toxin that overactivates the N-methyl-D-aspartate receptors (NMDARs). This event leads to an increase of Ca^{2+} flux into the post-synaptic neurons causing the activation of cell death pathways. Alternatively, Ca^{2+} internalized by mitochondria may cause mitochondrial dysfunction and increase the ROS production that diminishes the entry of GLU into the astrocytes **[47]**.

Methylmercury also may increase the release of other neurotransmitters as acetylcholine, dopamine, serotonin and norepinephrine **[50]**.

These events may be related with neurodegenerative disorders like Alzheimer, Parkinson, Huntington diseases, Amyotrophic lateral sclerosis **[51]** and autism **[52]**.

1.7.2 Nephrotoxicity

The kidneys, considered the main excretory organs of Hg, are also the major target for Hg accumulation **[53]**. Mercury, mainly in elemental and inorganic forms, can induce nephrotoxicity by the increase of ROS. This increase of ROS leads to the interruption of protein synthesis and enzymatic inactivation, cellular membrane damage and transport dysfunction **[54]**. The exposure to Hg^0 causes glomerular and tubular alterations as high molecular weight proteinuria and urinary enzyme excretion, respectively. Mercury also induces diuresis, glycosuria and albuminuria. The proximal tubule as a part of the nephron (cell unit) and is divided into two sections, pars convoluta and pars recta. The last has two segments, the cortical (S2) and the medullar (S3), are where Hg tends to accumulate **[55]**. When Hg ions get into the epithelial cell of proximal tube it triggers an alteration in the cell membrane permeability to the Ca^{2+} and consequent mitochondrial dysfunction. Moreover the renal failure after the Hg exposure may be caused by the decreased of renal reabsorption of calcium and chloride leading to an insufficient filtration **[56]**.

1.7.3 Cardiotoxicity

Hypertension, atherosclerosis, coronary arterial disease, acute myocardial infarction and sudden cardiac death from cardiac failure are some effects caused by Hg exposure. Moreover at the vascular level Hg may induce oxidative stress, inflammation, stroke, endothelial dysfunction, dyslipidemia and mitochondrial dysfunction **[56]**. The interaction between Hg and selenium and lipid peroxidation works as intermediate steps among the Hg exposure and the cardiovascular diseases. Although fish makes part of a healthy diet because of its rich proteins and poor saturated fats contents, high Hg levels in fish can delete the cardioprotective effects namely the selenium and polyunsaturated fatty acids (n-3 PUFAs) **[57]**. Different populations ingest different types and sources of fish therefore the risk of cardiovascular effects must be taken in consideration in terms of Hg levels and n-3 PUFAS. Besides it is also very important to consider that Hg binds to selenium decreasing its protective potential against cardiovascular effects **[56, 57]**.

1.7.4 Immunotoxicity

Mercury can affect the immune system through immunostimulation or immunosuppression and it depends on Hg compound **[58]**. Inorganic Hg induces immunostimulation causing the proliferation of adult T cells and stimulates autoimmunity **[59]**. Gardner *et al.* **[60]** showed that people exposed to elemental and inorganic Hg at work had an increase of cytokines, which are small secreted proteins released by cells that have a specific effect on the interactions and communications between cells, and antinuclear antibodies, that target "normal" proteins within the nucleus of a cell. Modulation of the cytokines and antibodies responses by Hg can affect the individual susceptibility to autoimmune diseases, allergies and infectious diseases **[60].** On the other hand MeHg acts firstly as an immunosuppressive before its conversion into inorganic form **[49]**.

1.7.5 Diabetes Mellitus

Diabetes Mellitus is known by hyperglycemia due to an insufficiency in the insulin secretion by pancreatic β-cells or receptor dysfunction. The β-cell is one of four major types of cells present in the islets of Langerhans. When the pancreatic antioxidant defense is weak it turns the pancreatic β-cells susceptible to ROS, which can be generated by Hg exposure. These species will react within pancreatic cells destroying them or turn those dysfunctional **[61]**.

1.7.6 Teratogenicity

During pregnancy, MeHg and Hg^0 are capable to cross the placental barrier and reach the fetus **[2]**. But, Hg^0 is less effective in targeting the fetal brain than MeHg since it is first oxidized to Hg^{2+} in the fetal liver **[9]**. As a consequence, Hg levels following exposure to Hg vapor are lower in the brain of the fetus that in the brain of the mother **[9]**. The developing brain is sensitive to MeHg and the fetus develops symptoms when the mother has no manifestation of mercury poisoning **[62]**. Also, the lack of maturity of the blood-brain barrier of the fetus makes it even more susceptible to Hg **[2]**. The Minamata and Iraq epidemics revealed that after exposure to high levels of MeHg in utero there was a severe distortion of the fetal brain architecture which was caused by difficulty of neuronal migration **[63]**. Consequences include microcephaly, cerebral palsy, blindness and ataxia with most severe cases ending in fetal death before or shortly after

birth **[62]**. Although children may appear physically normal, delayed neurodevelopment and serious retardation may occur **[2]**. Since both MeHg and Hg^0 are also secreted in breast milk, children in breastfeeding age might be exposed to harmful concentrations of MeHg or Hg^0 **[63]**. Overall, in utero developing fetus and early childhood are considered the most vulnerable life stages concerning exposure to Hg compounds **[2].**

1.8 Biomarkers of Hg exposure

Based on epidemiologic and toxicological studies, several guidance levels have been established to indicate levels of exposure to Hg that are related to risk in humans. These levels can be helpful to take decisions concerning the need for medical interventions or exposure reductions. **Table 1.1** gives an overview of these published guidance levels for Hg in blood, urine and scalp hair. In **Table 1.2** it is shown the range mean of Hg concentrations in biological samples of the European population since 2000 according to European Food Safety Authority (EFSA) **[67].** Other specimens such as placenta, umbilical cord tissue, human breast milk, sweat, nails and toenails are used in biological monitoring studies as biomarkers of Hg exposure.

Table 1.1- Guidance levels for mercury concentrations in blood, urine and scalp hair.

	Blood (µg/L)	Hair (µg/g)	Urine (µg/L)	Urine (µg/g Crea)	Source
Human bio-monitoring threshold limits	5-10	1[64]-2[65]	-	5	US EPA, 1997 **[64]** WHO, 2008 **[65]**
HBM II*	15	-	25	20	Schulz et al. (2007) **[66]**

*HBM II - The concentration above which there is increased risk of adverse health effects in susceptible individuals in the general population.

Table 1.2- Range of mean concentrations of total mercury in biological samples from the European population [67].

Matrix (unit)	Adults and eldery	Children
Cord blood (µg/L)	-	0.86-13.9
Blood (µg/L)	0.20-4.85	0.12-0.94
Hair (mg/Kg)	0.17-1.45	0.14-1.99

1.9 Mercury in placenta as a biomarker of Hg exposure

The choice of a specimen for monitoring pollutants affecting human health depends on the criteria chosen and also on the necessity of having an invasive procedure, like sampling blood. In context of health-related biomonitoring, placenta has not received as much attention as it deserves as a specimen. It is a unique specimen requiring non-invasive procedure and offers possibilities for real- and long-time monitoring. Besides, placenta can be defined as a dual purpose specimen for evaluating the pollutant burden exerted on the mother as well as on the fetus **[68]**.

1.9.1 Placenta

The placenta is a remarkable discoid organ between the mother and fetus and plays a key role in ensuring a successful pregnancy. During its relatively short lifespan, the placenta undergoes rapid growth, differentiation and maturation. The placenta forms an interface between the mother and fetus, performing its main function of facilitating the exchange of gases, nutrients and metabolic wastes **[69]**. It produces hormones and growth factors which are needed for the healthy development over the pregnancy. In turn, these hormones and growth factors will support the balanced physiological condition in the uterus for the continuation of the gestation period. They also affect the physiological changes of the maternal body, to adapt to and sustain the pregnancy. The trophoblast cells are a major component in the placenta and are fetal epithelial cells that form an interface between mother and offspring **[68]**. The human trophoblast differentiates along two pathways **[70]** (**Figure 1.3**): *the villous trophoblast pathway*: the mononucleated cytotrophoblast fuses into multinucleated syncytiotrophoblast forming the syncytial layer that covers the placental villous tree. These cells are directly involved in the exchange of gases, nutrients and waste across the materno-fetal interface; and *the*

extravillous trophoblast pathway: cytotrophoblast from the cell column of the anchoring villi exit the cell cycle and shift from a proliferative phase into a migratory and invasive phenotype. These invasive cells are denominated extravillous cytotrophoblast and can be further subdivided into other types of cells with distinct functions.

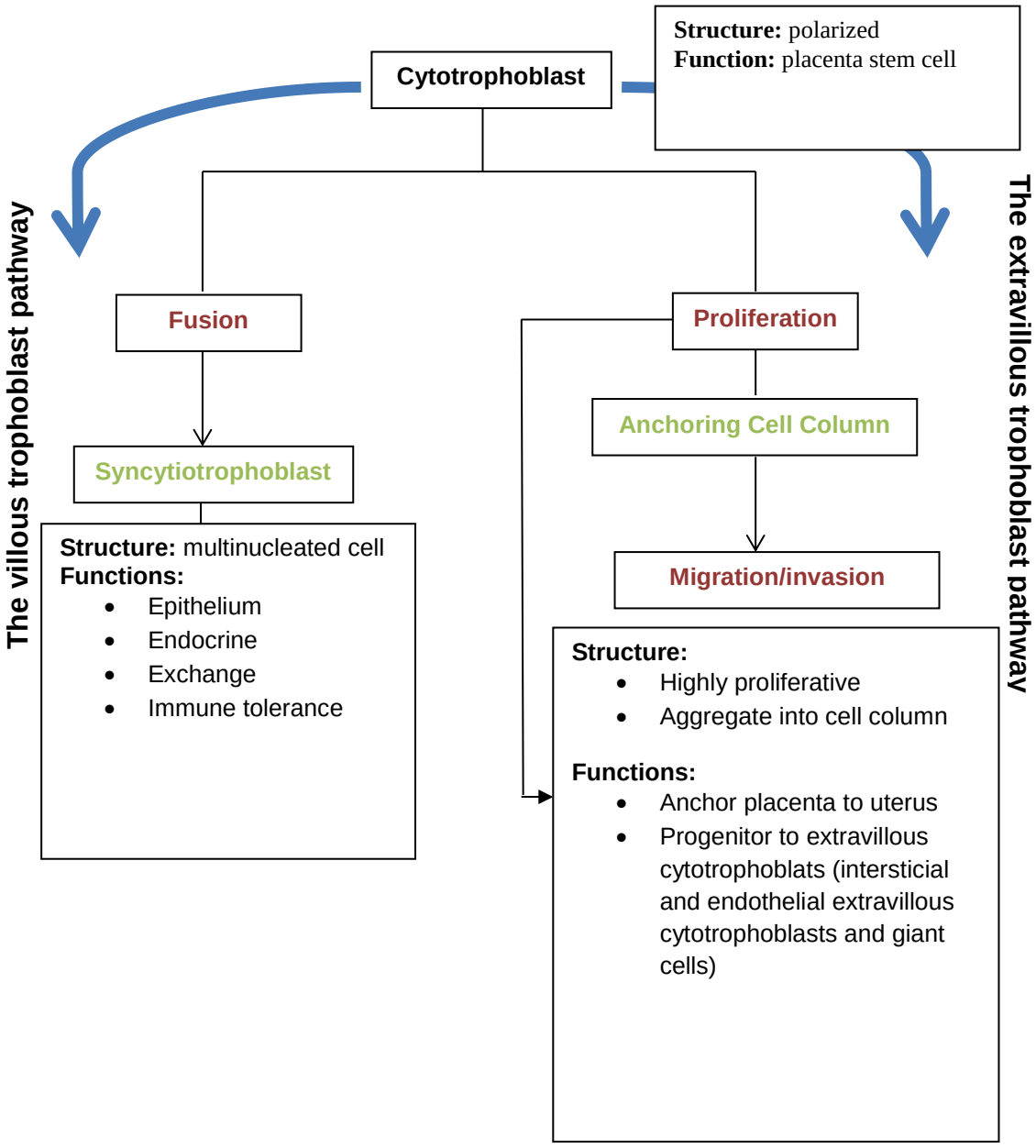

Figure 1.3- Trophoblast pathways of differentiation. Adapted from Huppertz *et al.* **[71].**

1.9.2 Structure of the mature placenta and fetal membranes

One of the most specific features of human embryonic development is the intimate relationship between the embryo and the mother. To survive and grow during intrauterine life, the embryo must keep a parasitic relationship with the body of the mother for acquiring oxygen and nutrients and eliminating wastes. Besides, it must avoid being rejected as a foreign body by the immune system of its maternal host. These exacting requirements are supported by the placenta and extraembryonic membranes that surround the embryo and work as the interface between the embryo and the mother **[71]**.

The mature placenta consists of a fetal and a maternal component. The fetal component is the chorionic plate and the chorionic villi arise from that part. The fetal membranes (chorion and amnion) also derive from fetal tissue. The maternal component is represented by the decidua basalis that is covered by a cytotrophoblastic layer derived from the fetal surface. The full-term human placenta is a circular discoid organ with a diameter of 15-25 cm, a central thickness of 3 cm and an average weight of 500-600 g (**Figure 1.4**).

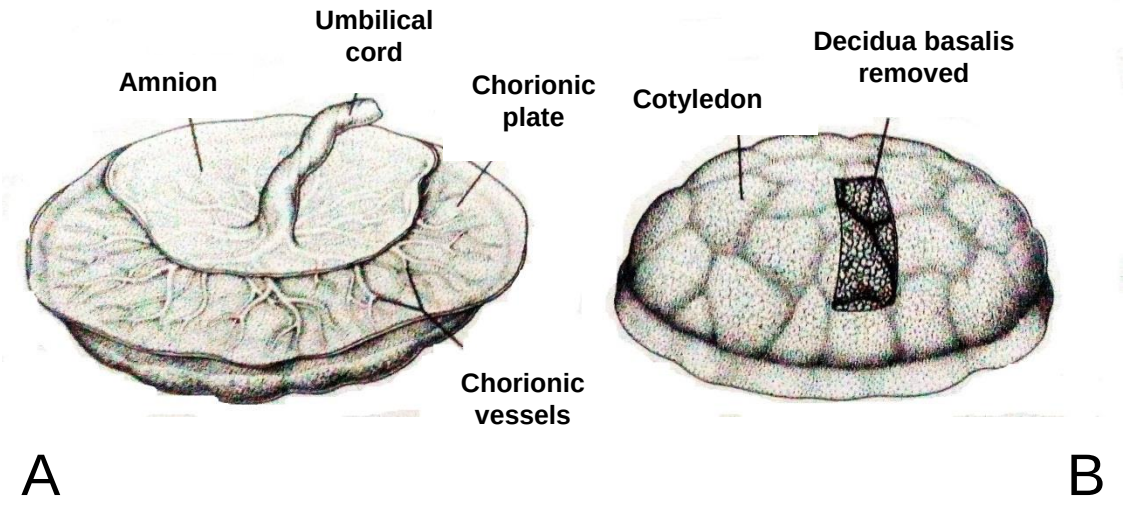

Figure 1.4- Human placenta at delivery - external anatomies. (A) Fetal surface. The chorionic plate and umbilical cord are covered by the amnion. (B) Maternal surface showing the cotyledons. In: Sadler and Langman **[72].**

Placenta

- **Chorionic plate:** the side of the placenta facing the amniotic cavity is the fetal surface also called the chorionic plate. This surface appears shiny due to the avascular and intact epithelium of the amnion that covers the chorionic plate **[73]**. The amniotic mesenchyme is only weakly attached to the chorionic mesenchyme and can easily be removed from the delivered placenta. From the fetal surface of the placenta the umbilical cord connects to the fetus **[71]**.
- **Chorionic villi:** The sub-branches of the blood vessels from the umbilical cord form the chorionic villous trees. The chorionic villi take the fetal blood to the fetal-maternal interface. The fetal blood flows from the umbilical arteries to the villi and then returns via the umbilical vein. The chorionic villi are bathed with the maternal blood, which flows directly into the intervillous space. The fetal-maternal interface of a mature human placenta is hemochorial with the mono-layered barrier of the syncytiotrophoblast, and the fetal endothelium separating the fetal and maternal blood. The declining of cytotrophoblast cells increase from minor to major villi and even if they remain in the major villi they do not participate in the transfer between fetal and maternal circulations **[72]**.
- **Decidual basalis:** also called maternal surface or basal plate is an artificial surface which emerged from the separation of the placenta from the uterine wall during the delivery. The major components of this structure are fetal extravillous trophoblast and all kinds of maternal cells of the uterine decidua (decidual stroma cells, natural killer cells, macrophages and other immune cells) **[71]**. It is composed by a system of flat grooves, which divide this part of the placenta into different lobes or cotyledons. Each of these cotyledons contains one or several chorionic villous trees, the principal functioning units of the blood circulation throughout the placenta **[73]**.

- **Umbilical cord:** it contains one vein and two arteries which coil around the vein in a helical configuration. The role of the vein is to supply nutrient-rich oxygenated blood to the fetus from the placenta. In turn arteries take the nutrient-depleted deoxygenated blood back to the placenta. The umbilical cord is connected to the fetus at the abdominal area which later becomes the umbilicus. Once inside the fetus the umbilical cord vein divides itself into two branches. One of them joins the hepatic portal vein taking the blood directly to the liver and the other directs the majority of blood to the fetal heart. The umbilical cord arteries split from the fetal internal iliac artery the main artery in the pelvic area **[73]**.

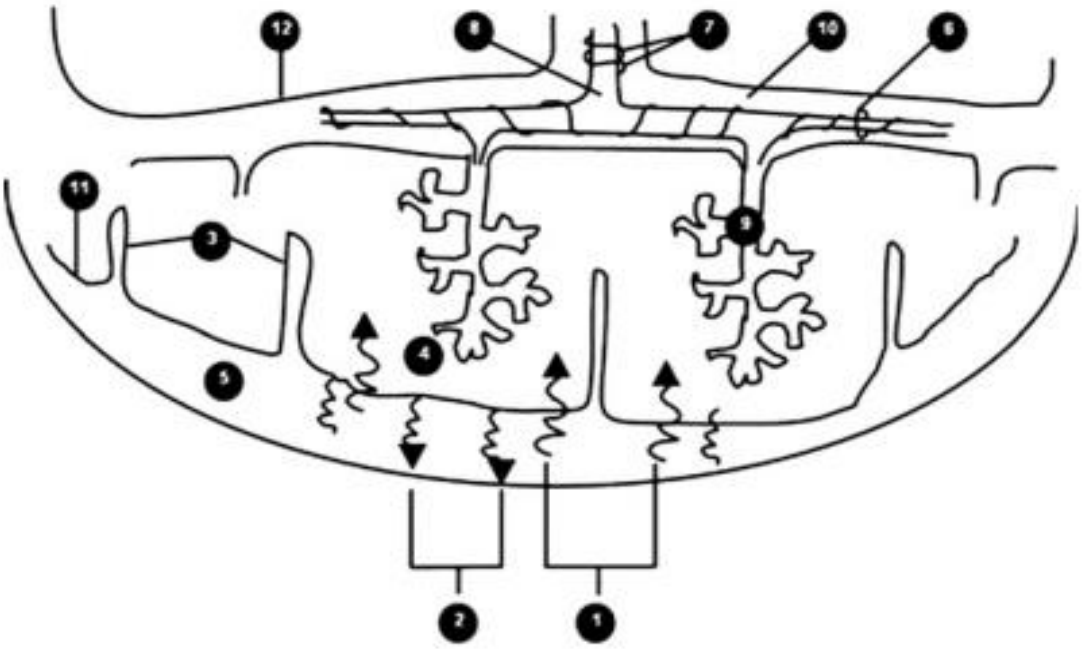

Figure 1.5- Human placenta at delivery – internal anatomies. (1) Endometrial arteries (maternal circulation), (2) endometrial veins (maternal circulation), (3) placental septa, (4) intervillous space, (5) decidua basalis, (6) chorionic plate, (7) umbilical arteries (fetal circulation), (8) umbilical veins (fetal circulation), (9) villous tree, (10) syncytiotrophoblast, (11) cytotrophoblast, and (12) amniotic membrane In: Pathak *et al.* **[74].**

Fetal membranes

The fetal membranes or chorioamniotic membrane is a thin membrane that surrounds the developing fetus and forms the amniotic cavity. This membrane is composed of two layers: chorion (outer layer) and the amnion (the inner layer). Both chorion and amnion constitute the amniotic sac filled with amniotic fluid, providing and protecting the fetal environment. At delivery, the amnion is only weakly attached to the chorion and can easily be removed from the delivered placenta **[71, 75]**.

- **Chorion:** the chorion is a more opaque membrane that exists between the developing fetus and maternal tissue. It consists of trophoblastic chorionic and mesenchymal tissues **[75].**
- **Amnion:** the amnion or amniotic membrane is a translucent structure adjacent to the amniotic fluid, which provides nutrients to the amniotic membrane cells. It has no nerves, muscles or lymph vessels and represents the innermost layer of the sac that encloses the fetus. The major components of the amniotic membrane are cells and the extracellular matrix. Collagen and proteoglycan molecules are, along with elastin, fibronectin and laminin, the major components of the amniotic membrane extracellular matrix **[76]**.

1.9.3 The placental barrier

Between the maternal and fetal circulations remains a physical barrier formed by the placental syncyotrophoblast. The syncyotrophoblast layer has two different faces: an apical membrane (microvillous brushborder) that faces maternal blood and a basal membrane that faces fetal circulation (**Figure 1.6**). Together they increase the selectivity of lipid membranes leaving only the essential components to the fetus. On the maternal surface the syncyotrophoblast is covered in abundant microvilli which provide a large surface area for substrate exchange **[77]**.

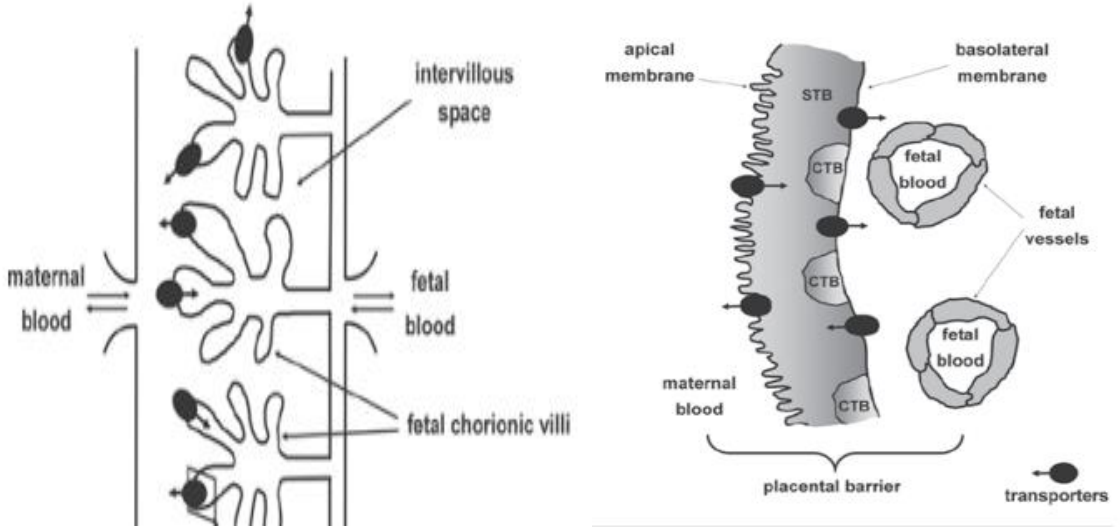

Figure 1.6- Placental barrier. CTB, cytotrophoblast; STB, syncytiotrophoblast. In: Staud *et al.* **[78]**

1.9.4 Blood circulation

The placental circulation (**Figure 1.7**) is a system of close relationships among fetal weight, placental and uterine size and umbilical blood flows during the pregnancy **[79]**. The exchange of substances between fetus and mother takes place at the placenta barrier **[79]** (**Figure 1.6**). This barrier allows water, oxygen, other nutritive substances and hormones to pass from mother to fetus and some of products of excretion from fetus to mother **[81]**. The oxygenated blood flows to the fetus via the single umbilical vein and deoxygenated blood flowing from the fetus back to the placenta via the two umbilical arteries **[82]**. Then the arterial blood flows direct to the mother lacuna into several cavities called sinuses. These placental sinuses contain villi. After the exchange of substances with maternal blood in the intervillous spaces, blood flows back through the villous blood vessels, which converge into the vein of the umbilical cord **[71]**. Then the blood flowing in the umbilical vein goes directly into the fetal liver and heart **[83]**.

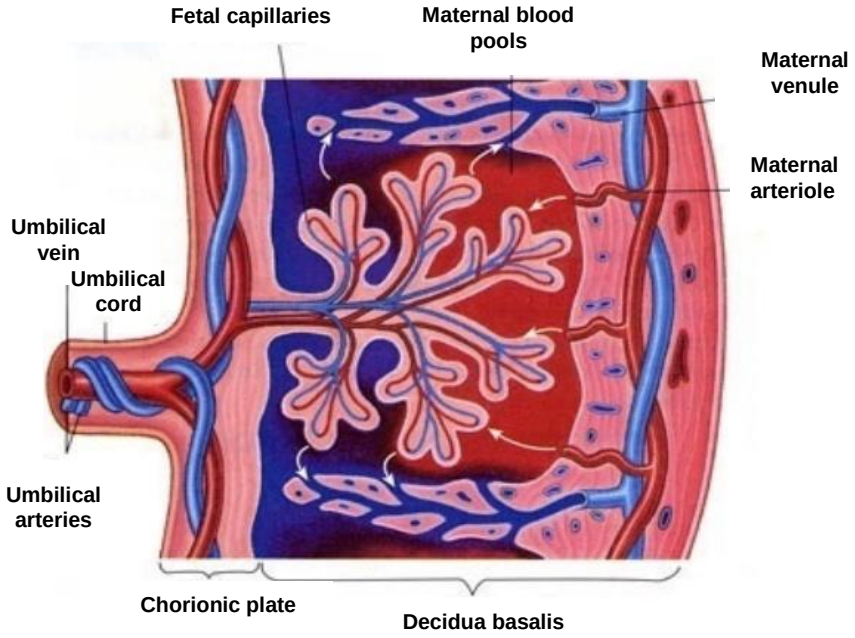

Figure 1.7- Placental circulation. Blue represents venous blood and red corresponds to arterial blood. Adapted from Saunders **[80].**

1.9.5 Placental transfer

The transport of substances between the placenta and the maternal blood is assisted by the great surface area of the placenta, which expands from 5 m^2 at 28 weeks to almost 11 m^2 at term **[84]**. The transfer of gases, nutrients, waste products and toxic substances across the placenta is bidirectional (**Figure 1.8**).

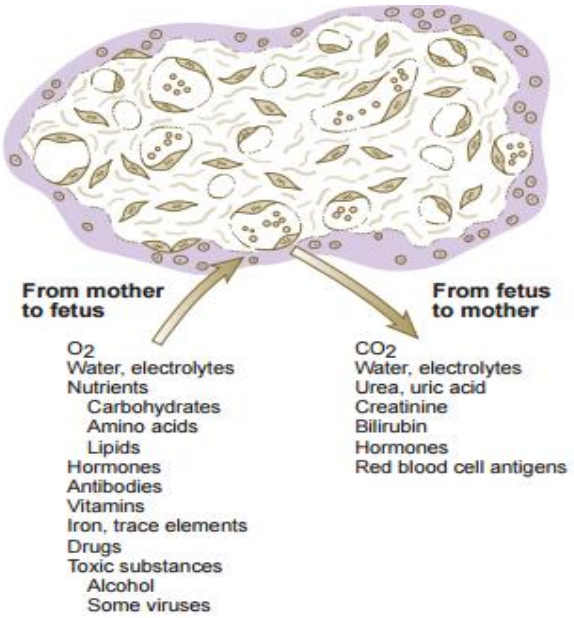

Figure 1.8- Exchange of substances across the placental barrier between the fetal and maternal circulation**.** *In*: Carlson **[84]**.

The transport of the substances through the placenta depends on the physiochemical and structural properties of the compound as well as the physical characteristics. The weight, ionization and lipid-solubility of the component determine its transfer rate through the placenta. Molecules with a weight up to 600 Da, non-ionized and lipid soluble will have a fast diffusion. On the other hand, larger, ionized and hydrophilic compounds will cross the placenta more slowly because the membrane limit their transfer. Also the transfer rate will depend on the factors that regulate maternal and fetal blood flows. The physical properties of the placental-fetal unit factors include the osmotic pressure between mother and fetal compartments, the thickness of the endothelio-syncytial membrane, the surface area of the exchange membrane, the maternal blood flow and the hydrostatic pressure in the intervillous space and the blood pressure in fetal capillaries **[85]**. There are two pathways of transference across the placenta: paracellular and transcellular. The first is based in the permeability of the placenta to inert hydrophilic solutes that do not enter in cells. But, placental barrier includes a layer of continuous trophoblast syncytium (syncyotrophoblast) (**Figure 1.6**). The transcellular route consists in transtrophoblastic channels. From them molecules pass through the plasma membranes of the cells that constitute the barrier. This

pathway is available for substances such as lipophilic molecules, very small hydrophilic molecules and membrane carriers and channels **[86]**.

The transfer of compounds can occur by five kinds of mechanisms **[84, 87]**:

(i) **Passive diffusion.** This process is a transfer without the use of energy. It depends on the compound characteristics and protein binding capacities. Oxygen, gases, urea and free fatty acids are some examples;

(ii) **Facilitated diffusion.** This process occurs mediated down a concentration gradient without energy-costs. The transmembrane proteins embedded in the plasma membrane facilitate the rate of transport. Glucose, hormones and nucleosides are some examples;

(iii) **Active transport.** This process consists in the movement of a substance against a chemical or electrical gradient with energy costs. Competition between related compounds may occur. Amino acids are an example;

(iv) **Pinocytosis.** In this process the compound is invaginated into the cell membrane being after transferred to the opposite site as a vesicle. The transfer of drugs is an example;

(v) **Aquaporins.** Several aquaporins (AQPs) are expressed in placenta and fetal membranes (AQP1, 3, 8, and 9). Water is transferred through both the paracellular and transcellular routes, and its transfer may be facilitated by integral membrane water channel proteins (ie, AQPs) **[85]**.

1.9.6 Transport of mercury across the placenta

Placenta cells have proteins which are involved in transport, retaining and detoxification of toxicants. However, accidental exposures of pregnant women have made evident that placenta cannot prevent the passage of teratogens such as mercury as it was described along this chapter. The chemical form of Hg determines its cellular uptake. Mechanisms of Hg transport through the placenta are not fully understood **[86]**. However, it is believed that Hg is transported and accumulated in the placenta by molecular and ionic mimicry. The mechanisms that mediate placental uptake of Hg are also poorly understood. However, after an extent review, Bridges and Zalups **[87]** proposed mechanisms by which Hg targets many type of cells including placenta cells. Inorganic Hg (Hg^{2+}) enters and accumulates in the placenta at low concentrations.

Inorganic Hg (Hg^{2+}) as a thiol-conjugate may mimic a structurally similar amino acid and perhaps utilized as a substrate by one or more amino acid transporters. Methylmercury crosses the placenta readily and accumulates in the fetus and placenta. The uptake of MeHg seems to be mediated by the system L in a conjugate form (CH_3Hg-S-Cys). System L is a sodium-independent transporter, which mediate the transport of neutral amino acids and it has been identified in the placenta. Besides it is an important participant in the transfer of nutrients from the maternal to the fetal circulation. Also other protein carriers have been identified in the placenta such as multidrug resistance-associated proteins (MRPs), organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs) and zinc transporters. One or more of them may play a role in the uptake and/or efflux of MeHg complexes. MRPs are known to participate in the detoxification suggesting these carriers may be responsible by the efflux of MeHg from the fetal circulation back to maternal circulation **[87]** Lastly, it is believed that Hg^0 may be transported by passive diffusion **[96]**.

1.9.7 European mercury levels in human placenta, umbilical cord and fetal membranes

Several studies have been focused on the study and importance of the placental transfer of essential and nonessential metals. Esteban-Vasallo *et al.* **[88]** discussed in their review the use of human placenta to evaluate biomarkers of exposure to toxic metals. They have concluded that the use of placental tissue specimens to assess toxic metal exposure is not fully explored **[88]**. **Table 1.3** resumes some European studies that used biologic material such as the placenta, umbilical cord and maternal hair regarding maternal-fetal transfer of Hg. To our knowledge, biomonitoring studies concerning Hg exposure during pregnancy have not been performed in Portugal.

Table 1.3- Total mercury levels in biological material related with pregnancy in Europe (1978 to 2013). Data are expressed in ng/g wet weight.

Country	Biologic material	Mean±SD	Min-Máx	Ref.
Belgium	Placenta	15.3±14.1	1.1-103.2	Roels *et al.* (1978) **[89]**
Italy	Placenta	12.7±9.0		Capelli *et al.* (1986) **[90]**
Czech Republic	Placenta	2.2±1.0		Truska et al. (1989) **[91]**
Spain	Placenta	5.4±3.1	2.3-14.3	Soria *et al.* (1992) **[92]**
	Maternal hair	2.9±3.4 (µg/g)	0.15-20.0	
Germany	Placenta	13 (median)		Scaal *et al.* (1998) **[93]**
Ukrain	Placenta	<2.2	2.2-45.8	Zadorozhnaja *et al.* (2000) **[94]**
Austria	Placenta	1.9 (median)	0.1-11.7	Gundacker *et al.* (2010) **[95]**
	Maternal hair	184 (median) (µg/g)	53-773	
Denmark (Faroe Islands)	Placenta	87 (median)		Needham *et al.* (2011) **[96]**
	Umbilical cord	85 (median)		
Poland	Placenta	-	4-104	
	Umbilical cord	-	3-64	Kozikowska *et al.* (2013) **[97]**

1.10 Aveiro region, Portugal

1.10.1 Pollution in the Ria de Aveiro

The Ria de Aveiro is a coastal lagoon on the NW coast of Portugal connected to the sea by a single channel. It generates a complex system characterized by narrow channels and extensive intertidal zones. This lagoon has been of several pollutants namely through agricultural activities, households-waste waters and industry **[98, 99]**.

During approximately five decades (1950-1994), Ria de Aveiro received continuous discharges of Hg, mainly from a chlor-alkali plant, located in a chemical-complex industry nearby Estarreja. These Hg rich effluents dispersed in the system, mainly in the Estarreja Channel and in the Laranjo Bay due to its semi enclosed characteristics **[99].** Consequently the surrounding urban and agricultural soils, sediments and biota (macrophytes, macrofauna and fish) were negatively affected. The Ria is regularly used by fishermen to catch fish both for their own consumption and for

selling in local markets. Since Hg can easily accumulate and biomagnify in aquatic biota and enter the food web **[53],** Ria and its surroundings have been considered a case-study in different abiotic and biotic compartments **[99]**. Pereira *et al.* **[99]** made a review that describes Hg levels in these compartments in the Aveiro region until 2008. These studies included the water column, sediments, primary producers, fish (muscle), mollusks and crustaceans (**Table 1.4** – fish, mollusks and crustaceans used for human consumption). According to the EU legislation the maximum amount of Hg that can be found in fish for human consumption is 0.5 µg/g for non-predatory fish, crustaceans and mollusks and at 1 µg/g for predatory species (e.g. shark, swordfish, tuna and black-scabbard fish) **[101]**.

Table 1.4- Mercury concentrations in fish, mollusks and crustaceans for human consumption in Ria de Aveiro and its surroundings (until 2008). (Mean±SD µg/g wet weight)

	Laranjo	**Ria**	**Ria nearshore**	**REF.**
Fish				
Dicentrarchus labrax	0.03-1.7	occasionally > 0.5	-	**[99]**
Trigla lucerna	-	-	0.043±0.01	
Dicologoglossa cuneata	-	-	0.12±0.04	
Liza aurata	<0.4	<0.1	-	**[100]**
Mollusks				**[99]**
Scobicularia plana	0.37±0.26	0.03±0.01	-	
Donax vittatus	-	-	0.085±0.0006	
Spisula solida		-	0.014±0.005	
Crustaceans				
Carcinus maenas	0.33±0.17	0.09±0.01	-	

Other food sources may have a little contribution for the total body burden in MeHg. In some particular polluted areas, vegetables and cereals such as rice can accumulate considerable amounts of mercury in the eatable part **[67, 102, 103]**. In 2009, human hair samples from Aveiro's residents had been collected and analyzed by gender. Women represented the group with the highest Hg concentrations ranging from 0.090 to 4.2 µg/g **[103]**. But in general, it was concluded that the Hg levels found in local population (mean: 0.5 µg/g) were considered within normal limits according to WHO guidelines. In the same study different species of fish from those represented in Table 1.5 and also for human consumption were purchased in market, fisherman and local

supermarkets. These species included *Chelon labrosus*, *Platichthys flesus*, *Scophthalmus rhombus*, *Solea solea*, *Trachurus trachurus*, *Sparus aurata*, *Scomber scombrus*, *Trisopterus luscus* and *Alosa fallax*. Fish from Ria de Aveiro showed a Hg mean concentration of 0.12±0.13 µg/g dry weight, higher than oceanic fish (0.080±0.063 µg/g dry weight). Regarding vegetables purchased in farmer's market and collected directly from agricultural fields, the species *Spinacia oleracea* (spinach), *Latuca sativa* (lettuce), *Nasturtium officinale* (watercress), *Brassica napus* (rapeseed) and *Brassica oleracea acephala* (collards) were analysed. Products from market had Hg levels of 0.069±0.039 µg/g dry weight while vegetables collected direct from the field showed the highest concentrations ranging from 0.020 to 0.25 (0.10±0.078 µg/g dry weight) **[103]**. According to the safety guidelines, authors concluded that food was not contaminated by Hg and should not was responsible for major human exposure to the metal despite it still remain in the environment. However, in September 2011 **[104]**, soils were collected again in the Aveiro region and showed Hg levels ranging from 0.03–13.65 µg/g and a mean of 0.15 µg/g, which is higher than European reference values (0.037 µg/g dry weight) **[105]**. At this time, scalp hair was not collected, which could transposed exposure to bioaccumulation**.**

1.11 Motivation, objectives and thesis layout

The health authorities have been concerned about the risk associated with Hg exposure, mainly due to its teratogenic effects and ability to cause irreversible neurological damage in humans. Besides, several studies have been focused on exposure assessment regarding potential risk factors and safety of local public health. In Portugal there is a lack of information about Hg exposure mainly during pregnancy and its effects during this critical window of development. Aveiro, more specifically the Ria de Aveiro, faced a severe Hg contamination due to industrial activities. Since that, a focused research has been made in order to assess to Hg levels that remain in lagoon and its surroundings as well as the occurrence of adverse effects in soils, water and biota.

Nowadays, Hg does not seem to represent a risk for Aveiro's residents taking into account the Hg levels assessed previously in scalp hair. But, it is important to remember that humans are not only exposed to Hg by fish, vegetables and/or fruit. Lifestyle, occupational exposure as well as other food sources, may contribute to the increase of Hg levels in human body **[65, 67]**.

According to our knowledge, prenatal exposure to this metal has been not assessed in Aveiro region so far. As it was described along this chapter, Hg has the capability to cross the placental barrier and to reach the fetus. Another research gap is the distribution and retention of Hg over the maternofetal-placental unit. Placenta, which is considered a non-invasive matrix, is constituted by different compartments that are the anatomophysiological bases which support bidirectional exchange of compounds between mother and fetus.

In this context, this dissertation includes two additional chapters:

- Chapter II entitled “Mercury levels in parturient and newborns from Aveiro region, Portugal” has four goals: (i) to assess Hg exposure in parturient and newborns from Aveiro district, Portugal; (ii) to use non-invasive biological material discarded after birth to perform a biomonitoring study; (iii) to improve the knowledge about the distribution and retention of Hg over the placental-fetal unit; and (iv) to relate Hg levels with potential risk factors including maternal lifestyle, habits, diet and demographic characteristics. To achieve this, and with the approval of the Ethic Committee of the Hospital Infante D. Pedro (HIDP, Aveiro, Portugal) (see Annex I), a cross-sectional study was performed in a total of 50 mothers hospitalized for delivery at HIDP. After signing an informed consent (Annex II), mothers were asked to fill a questionnaire regarding socio-demographic factors, lifestyle and diet habits (Annex III) and samples of biological material were collected and preserved for further analysis of Hg. Thus, this chapter presents the main results of this study and it is structured as a scientific paper to be submitted to an international peer review journal.
- The work presented in chapter II was performed in 50 mother-newborn pairs, randomly selected, from 9 different counties that belong to the Aveiro district: Aveiro, Águeda, Ílhavo, Oliveira do Bairro, Estarreja, Murtosa, Vagos, Ovar and Albergaria-a-Velha. Since it is known that Estarreja and surroundings were the areas more affected by Hg release due to the chlor-alkali industry, Hg analyses in biological media used in chapter II were also used to compare Hg exposure both in mothers and newborns from each county. Therefore, chapter III entitled “General remarks” beyond to describe the major conclusions of the entire work, it also includes these results which are presented in two representative graphs without resorting to statistical analysis.

Overall, both chapters support a better knowledge about Hg levels in Portuguese women and their newborns including comparisons with similar European reports.

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2. Mercury levels in parturient and newborns from Aveiro region, Portugal

Mercury levels in parturient and newborns from Aveiro region, Portugal

Ana Catarina Alves, Marta S. Monteiro, Cátia S. A. Santos, Luísa Machado, Amadeu M.V.M. Soares & Susana Loureiro

Department of Biology, Centro de Estudos do Ambiente e do MAR (CESAM), University of Aveiro, Portugal

Abstract. Mercury is a well-known teratogen since the outbreak of methylmercury poisoning in Japan and Iraq. The Portuguese region of Aveiro faced some decades ago an environmental Hg contamination due to the activities from a chlor-alkali plant, and so far there are no research concerning prenatal exposure to Hg in this area. The first objective of this study was to assess maternal and fetal exposure to Hg in the Aveiro region using non-invasive biological material. Total Hg (THg) measurements were performed by atomic absorption spectrometry after thermal decomposition of sample. Maternal hair had THg levels with a mean value of 0.90 μg g^{-1}, close to the U.S EPA limit but lower than the WHO limits. Mean values of THg levels in decidua basalis, chorionic plate and umbilical cord were similar. Amniotic membrane had the highest THg levels with a median value 33.65 ng g^{-1}, reaching a maximum value of 134.10 ng g^{-1}. The results of Hg found in our study were lower compared with values of previous European reports. A strong positive relationship was observed between THg levels in all matrices analyzed ($p<0.001$). Besides, no significant associations were found between Hg levels and anthropometric data of newborns. The second objective was to investigate the potential influence variables that contributed to Hg exposure during pregnancy. The consumption of fish rich in selenium and bottled water were negatively related to Hg levels. Finally, the third objective was to improve the knowledge about the Hg retention over the maternal-fetal-placental unit. In this study it was observed that Hg is capable to cross placenta but it also can accumulate in placental tissues. Amniotic membrane seemed to play a role in the Hg detoxification but further research is needed in order to explore this process/mechanism on metals retention capabilities.

Keywords: pregnancy, mercury, hair, umbilical cord, decidua basalis, chorionic plate, amniotic membrane

2.1 Introduction

Mercury (Hg) is ubiquitous and has been considered by World Health Organization (WHO) as one of the ten chemicals of major public health concern **[1]**. Environmental contamination by Hg is caused mainly by anthropogenic activities such as industrial, pharmaceutical and agricultural activities **[2]**. This metal exists in three forms: elemental, inorganic and organic. Humans can be exposed to elemental and inorganic Hg mainly due to dental amalgams and skin-lightening creams, respectively **[3]**. Fish and seafood consumption has been classified as the major sources of exposure to organic Hg in humans due to its capacity to bioaccumulate and biomagnify along the food chain **[4]**. Exposure to Hg can result in several toxic effects depending on its chemical form and route of exposure. Each chemical form acts according to its specific toxicological profile. For example, inorganic Hg salts can cause kidney damage while organic Hg compounds can cross the blood-brain barrier and produce neurological damage. On the other hand elemental Hg represents a threat for both renal and nervous systems **[3, 5]**.

Mercury is also a well-known teratogen **[6]**. Placenta was early considered one of the most powerful barriers avoiding the transfer of harmful substances to the offspring. However, during the outbreak of methylmercury (MeHg) poisoning in Japan and Iraq, it was proven the contrary **[7, 8, 9]**. Further, prenatal exposure to others forms of Hg were observed in mammals. Elemental Hg seems to be able to penetrate the placental barrier and accumulate in the fetus similarly to MeHg **[10]**. In turn inorganic Hg tended to be retained in placenta **[10]**. Since then several biomonitoring studies have been made worldwide concerning both maternal and fetal exposure to Hg **[**reviewed in **11]**. Mercury has been linked to miscarriage, spontaneous abortions, stillbirth, and low birth weights **[10]**. Prenatal exposure to Hg can inhibit fetal brain development leading to a delayed growth, neural tube defects and craniofacial malformations. In the latter stages of development, children may develop cerebral palsy and psychomotor retardation **[10, 12, 13]**.

Placenta is a temporary organ that makes the physical and functional connection between the mother and the developing embryo/fetus. The main functions of placenta are: (i) to provide oxygen, water and nutrients that are essential to the fetus; (ii) to remove carbon dioxide and other waste products; (iii) to metabolize several chemical substances; (iv) to release metabolic products into maternal and/or fetal circulations; (v)

to protect the fetus against xenobiotics, infections and maternal diseases; and (vi) to release hormones from both the maternal and fetal circulations, ensuring a well succeed gestation **[14]**. Placenta is remarkably capable to perform these functions without mixing maternal and fetal blood **[14, 15]**. The full-term human placenta is constituted by a decidua basalis which is the maternal surface; a chorionic plate that corresponds to a fetal surface; and lastly by an amniotic membrane.

Biological markers (biomarkers) of exposure have been defined as an exogenous substance or its metabolite that can be measured in human cells, tissues or fluids. In human health studies, while questionnaires offer an historical description of the exposure, biomarkers of exposure estimate the internal dose of the exposure **[16]**. Taking into account its complex structure, placenta can be considered as a dual purpose specimen for monitoring both fetal and maternal exposure to potentially harmful agents like Hg **[15, 17]**. Mercury level in cord tissue is also a well validated biomarker of exposure **[18]**. It has been well correlated with Hg concentrations in cord blood when expressed in dry weight of tissue. Since the Hg disaster in Minamata, the level of Hg in this tissue has been used as a fetal biomarker of exposure to Hg showing better results than those obtained with maternal hair **[19, 20]**. On the other hand, very little is known about the capacity of amniotic membrane to retain Hg. In 1967, Suzuki and colleagues **[21]** injected three different Hg compounds in pregnant mice and used the placenta and amniotic membrane as biological matrices. Interestingly, they found higher Hg content in amniotic membrane when compared with placenta. They proposed that water solubility of the mercury compound present may play an important role to determine the extent of retention **[21]**. It is important to refer that amniotic membrane is in permanent contact with amniotic fluid, which provides and receives fetal substances.

Scalp hair is also classified as a valuable matrix to assess Hg exposure **[18]**. It has the capacity to incorporate circulating Hg, preferably MeHg, through the follicle during growth **[22]**. In humans, the rate of hair growth is approximately one centimeter per month which allows hair to capture temporal exposure history **[23]**. Several studies have evaluated Hg level in scalp hair of pregnant women making the linkage to fish consumption **[22, 24, 25, 26, 27]**. The reference dose (RfD) of U.S. Environmental Protection Agency (USEPA) for Hg corresponds to 1.0 μg g^{-1} for people who have low fish consumption **[28]** while WHO adopted 2.0 μg g^{-1} as normal Hg levels in scalp hair **[29]**.

The Portuguese region of Aveiro faced an environmental Hg contamination due to the activities from a chlor-alkali plant. Effluents rich in Hg were released from 1950 till

1994 to the Ria de Aveiro lagoon system and consequently the surrounding urban and agricultural soils, sediments, and biota were negatively affected **[30]**. High Hg levels have been thereafter detected in fish, shellfish and vegetables used in the diet of local population **[30, 31, 32, 33].** In 2009, more than one decade after the cessation of Hg releases, scalp hair was collected from women and men, with an age mean of 41, near to the chemical plant. Women had the higher Hg values compared to men. In the total studied population, including both women and men, 42% presented Hg concentrations between 1–2 μg g^{-1} and 22% exceeded 2 μg g^{-1} **[33]**. However, according to our knowledge there are no studies regarding maternal and prenatal exposure to Hg in this region.

Therefore, the objectives of this study were (i) to assess maternal and fetal exposure to Hg in Aveiro region using standard (hair and dried cord tissue) and potential (placenta and fetal membranes) non-invasive biological material; (ii) to investigate the potential influence variables (sociodemographic factors, food habits and lifestyle) that contribute to maternal and fetal exposure to Hg during pregnancy in the Aveiro district; and (iii) to improve the knowledge about the Hg retention over the maternal-fetal-placental unit.

2.2 Materials and methods

2.2.1 Study population

A cross-sectional study was performed in eligible women hospitalized for delivery at Infante D. Pedro Hospital located in Aveiro, between October 2014 and April 2015. Inclusion/exclusion criteria were set to limit the presence of confounding variables within the study population. Thus, an entry criterion for inclusion of pregnant women in the study was to be resident in the Aveiro district (central—north Atlantic coast of Portugal) whereas multigestational pregnancies were excluded. In total, 50 women agreed to participate in the study by signing an informed consent form. This study was previously approved by the Ethic Committee of the Hospital.

2.2.2 Data collection

Sociodemographic, diet and lifestyle information during pregnancy was gathered on participants from face-to-face interview by hospital staff (medical doctors) within the first

24h after delivery. A detailed questionnaire was filled including: maternal age (years), time of residence (years), place of residence (rural or urban), maternal education (university; secondary; primary), parity (0; +1), prenatal care (dental filling (yes; no); vitamin supplements (yes; no); medication (yes; no); vaccination (yes; no)); occupational exposure (work status (unemployed; employed); exposure to chemicals (yes; no)); cosmetics usage (never; less than 1 time per week; more than 1 time per week; daily); hair dye (yes; no); eating habits: vegetables, fruits, fish and shellfish (never; 1 to 3 times per month; 1 to 3 times per week; 4 to 6 times per week; daily); water consumption: drinking at and outside home and for cooking (municipal; private well; bottled). Clinical information of newborns (head circumference; birth length; birth weight) was also obtained by review of medical records.

2.2.3 Sampling

A total of 50 placental-cord tissues were collected and coded. After collection all samples were immediately kept in saline solution 0.9% and maintained at 4ºC. Within 12 hours maximum the samples were transported from the hospital to the University of Aveiro facilities (500 meters distance) for further processing. Each placental tissue was partitioned in three different parts: decidua basalis (from the maternal surface), chorionic plate (from the fetal surface) and amniotic membrane. Small tissue pieces (minimum 6) were randomly collected from maternal and fetal surfaces of placenta while amniotic membrane was just easily detached from the chorion and cut into small sections. Each umbilical cord was also cut into small parts.

All replicates were weighted, coded and stored at -20ºC. All the cutting and weighting procedures were performed by using clean stainless steel scissors and tweezers. All the samples were then freeze-dried during 72 h. Decidua basalis and chorionic plate dried tissues were homogenized and transformed into powder with mortars and pestles. Cord and amniotic membrane dried tissues were cut into very small pieces. Lastly, samples were stored at room temperature in a desiccator with silica, protected from light, until Hg analysis.

Maternal hair samples *were cut* from the *scalp* at the *occipital region* using clean stainless steel scissors. Segments of 3.5-4 cm were collected since they give information related to the Hg exposure during the third trimester of pregnancy. However, because the hair follicle grows out of the skin surface after about 3 weeks, Hg concentrations will only reflect the Hg exposure from 3 weeks before

[23]. Samples were then kept in clean microtubes of 2 ml and identified appropriately. In the laboratory, each hair sample was cut into small pieces and washed according to the standard procedure recommended by the International Atomic Energy Agency: wash in acetone, three times in water, and once more in acetone **[34]**. The samples were then dried overnight at 35°C and stored at room temperature.

2.2.4 Determination of total mercury levels

Total Hg (THg) measurements were performed by atomic absorption spectrometry after thermal decomposition of the sample using the Advanced Mercury Analyser (AMA-254, LECO). This technique of quantification is based on a pyrolysis process of the sample using a combustion tube heated at 750 °C under an oxygen atmosphere. Volatilized mercury Hg(0) is trapped in a gold amalgamator and subsequently detected and quantified by atomic absorption spectrometry at 254 nm. The detection limit established was 0.01 ng Hg **[35]**. The number of technical replicates differed due to the homogenization processes of the samples. Total Hg levels were measured in decidua basalis and chorionic plate in three technical replicates while in cord and amniotic tissues a larger number (between 6 and 9) were used.

Whenever the hair mass was insufficient to achieve three technical replicates, duplicates were made. Analytical quality of the procedure was controlled using reference material ERM-BB184 (Bovine muscle) and ERM-DB001 (Human hair) (European Reference Materials, European Commission - Joint Research Centre, Institute for Reference Materials and Measurements) containing 0.0018±0.0010 µg g^{-1} (indicative value) and 0.365±0.028 µg g-1 (certified value) of THg respectively; and TORT-2 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Council of Canada) containing 0.27±0.06 µg g−1 of THg.

2.2.5 Statistical analysis

Shapiro-Wilk test was used to determine data normality. Data obtained from Hg analyses and medical records did not fit a normal distribution and therefore non-parametric tests were performed. Spearman correlation analysis were conducted between [THg] in maternal hair, decidua basalis, chorionic plate, umbilical cord and amniotic membrane. Anthropometric data of newborns was also correlated with [THg] in biological tissues by Spearman correlation analysis. A Mann Whitney U test was applied to calculate the differences of [THg] in matrices according to the newborn gender (male

and female). All the statistical analysis were performed using SPSS software version 22.0 for Windows (version 22.0. Amonk, NY. IBM Corp). A p-value <0.05 was set as the level of statistical significance.

To determine which factors related to the [THg] found in biological samples, the relationship between [THg] and potential influence factors included in questionnaires were investigated using Redundancy Analysis (RDA). Forward selection was used in RDA to select the subset of variables that provided the highest explanatory model. The significance of each variable was calculated by 499 permutations in Monte Carlo test and variables with p<0.05 were included in the constrained ordination model. These analyses were performed using the CANOCO 4.5 software.

2.3 Results

In this study, 50 mother-newborn pairs were evaluated. Demographic and clinical characteristics are described in Table 2.1. Mean age (±standard deviation) of the 50 parturient was 30.6 (±5.9) years and 50% had a secondary education. They lived in the same area of residence in average 17.8 years: 40% in urban dwellings and 36% in rural places. Parity results were almost equally distributed (48% and 52%, respectively). Anthropometric data of newborns (birth length, birth weight and head circumference) were similar for both boys (N=26) and girls (N=24).

Table 2.1- Demographic and clinical characteristics of 50 mother-newborn pairs from Aveiro region.

	Mean±SD or %	N
Maternal demographic characteristics		
Age (years)	30.6±5.9	50
Education level (%)		
Primary incomplete	4	2
Primary complete	10	5
Secondary	50	25
University	36	18
Time of residence (years)	17.8±12.4	
Place of residence (%)		
Urban	40	20
Rural	36	18
Intermediate	24	12
Parity (%)		
0	48	24
+1	52	26
Birth outcomes		
Gender (%)		
Male	52	26
Female	48	24
Birth length (cm)		
Male	49.31±1.61	26
Female	49.48±1.34	24
Birth weight (g)		
Male	3261.69±412.51	26
Female	3201.56±317.48	24
Head circumference (cm)		
Male	34.56±1.13	26
Female	34.29±0.76	24

Maternal hair [THg] ranged from 0.13 to 3.56 μg g^{-1} with a mean value of 0.90 μg g^{-1} (Table 2.2). According to the RfD established by US EPA, 32% of all individuals analyzed were above 1.0 μg g^{-1} and 6% were higher than normal Hg levels considered by WHO (2.0 μg g^{-1}) (Figure 1).

Median values of [THg] in decidua basalis, chorionic plate and umbilical cord were similar (27.55, 26.80 and 27.30 ng g^{-1}, respectively). Amniotic membrane had the highest [THg] with a median value 33.65 ng g^{-1} and a maximum range of 134.10 ng g^{-1} (Table 2.2).

Table 2.2 – Total mercury levels in maternal hair, decidua basalis, chorionic plate, umbilical cord and amniotic membrane.

	Mean	Median	SD	Minimum	Maximum
Maternal hair[1*]	0.90	0.72	0.64	0.13	3.56
Decidual basalis[2]	32.84	27.55	18.34	3.0	84.10
Chorionic plate[2]	30.18	26.80	16.81	2.7	84.10
Amniotic membrane[2]	42.35	33.65	29.07	6.0	134.10
Umbilical cord[2*]	30.67	27.30	16.67	3.6	76.3

[1] units expressed by µg g^{-1} fresh weight; relative to the third trimester of pregnancy
[2] units expressed by ng g^{-1} dry weight
*standard biomarkers of Hg exposure **[18]**
SD: standard deviation

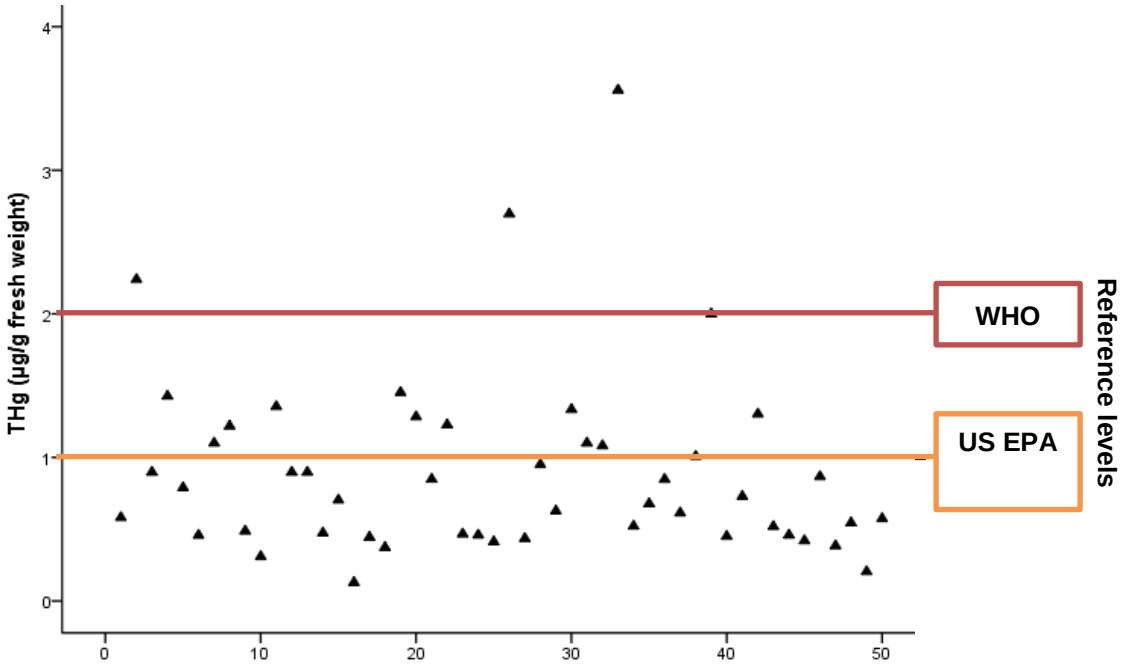

Figure 2.1- Total mercury concentration distribution among hair of 50 mothers from Aveiro region. Two lines were set according to US EPA (1 µg $g^{-}1$) and WHO (2 µg $g^{-}1$) Hg reference levels **[26, 27]**.

A strong positive relationship was observed between [THg] in all matrices analyzed with a *p* value <0.001 (Table 2.3). Decidua basalis and chorionic plate showed a spearman correlation coefficient close to 1 (0.965). However, the relationships found between maternal hair, cord and placental tissues were lower compared with those found among placental and cord tissues.

Table 2.3 – Spearman correlation coefficients between total mercury content in maternal hair, decidua basalis, chorionic plate, amniotic membrane and umbilical cord.

	Maternal Hair	Decidua basalis	Chorionic plate	Amniotic membrane	Umbilical cord
Maternal hair	**1.000**	0.656	0.698	0.592	0.669
Decidua basalis		**1.000**	0.965	0.837	0.908
Chorionic plate			**1.000**	0.823	0.912
Amniotic membrane				**1.000**	0.839
Umbilical cord					**1.000**

p<0.001

Table 2.4 describes the characteristics of mothers regarding prenatal care, lifestyle, and work status; diet and water consumption during the gestation period. The highlights of prenatal care were medication (86% yes) and vitamin supplements intake (74% yes). Hair dye was not a habit over the pregnancy (58% with no application) in opposite to cosmetics usage (70% with application).

Over half of the women studied were employed (N=28) and 26% of these working mothers were exposed to chemicals during the labor time.

Dairy products, fresh vegetables and fruit were also part of the maternal daily diet. In general, cod, sole and hake were the fish mostly consumed by mothers (1 to 3 times per week) followed by sardines, mackerel, salmon, mullet and canned fish (1 to 3 times per month). Mothers preferred to drink bottled water during pregnancy and use municipal water for cooking.

Table 2.4 – Lifestyle, diet and water consumption characteristics of mothers during pregnancy.

Prenatal care & lifestyle	%	**N**
Dental filling		
Yes	30	15
No	70	35
Medication		
Yes	86	43
No	14	7
Vitamin supplements		
Yes	74	37
No	13	13
Vaccination		
Yes	28	14
No	72	36
Cosmetics usage		
Daily	26	13
> 1 time per week	20	10
< 1 time per week	24	12
Never	30	15
Hair dye		
Yes	42	21
No	58	29
Working status		
Working	56	28
Unemployed	38	19
Student	6	3
Exposure to chemicals at work		
Yes	26	7
No	74	21
Living near to industrial activities		
Yes	30	15
No	70	35
Diet	mode	
Fresh vegetables	Daily	27
Canned vegetables	1 to 3 times per week	18
Fresh fruit	Daily	35
Canned fruit	1 to 3 times per month	16
Organic food or own crops	Never	16
Vegetarian food	Never	43
Poultry	1 to 3 times per week	29
Meat (beef, pork, lamb, ham, bacon, hamburger)	1 to 3 times per week	28
White fish (cod, sole, hake)	1 to 3 times per week	22
Swordfish and/or tuna	Never	20
Other fish (sardines, mackerel, salmon, mullet)	1 to 3 times per month	19
Canned fish (sardines. tuna, mackerel, roes, anchovies)	1 to 3 times per month	23
Shellfish	Never	28
Water consumption	mode	
Drinking water at home	Bottled	37
Cooking water at home	Municipal	41
Drinking water outside home	Bottled	43

According to the Redundancy Analysis, three variables were correlated with THg levels founded in matrices: education, bottled water and other fish (Table 2.5). The first RDA axis, representing 99.9% of media-potential influence factors variance, was positively related to Education's level, and negatively related with other fish (sardines, mackerel, salmon, mullet) intake and drinking bottled water outside home (Figure 2.2).

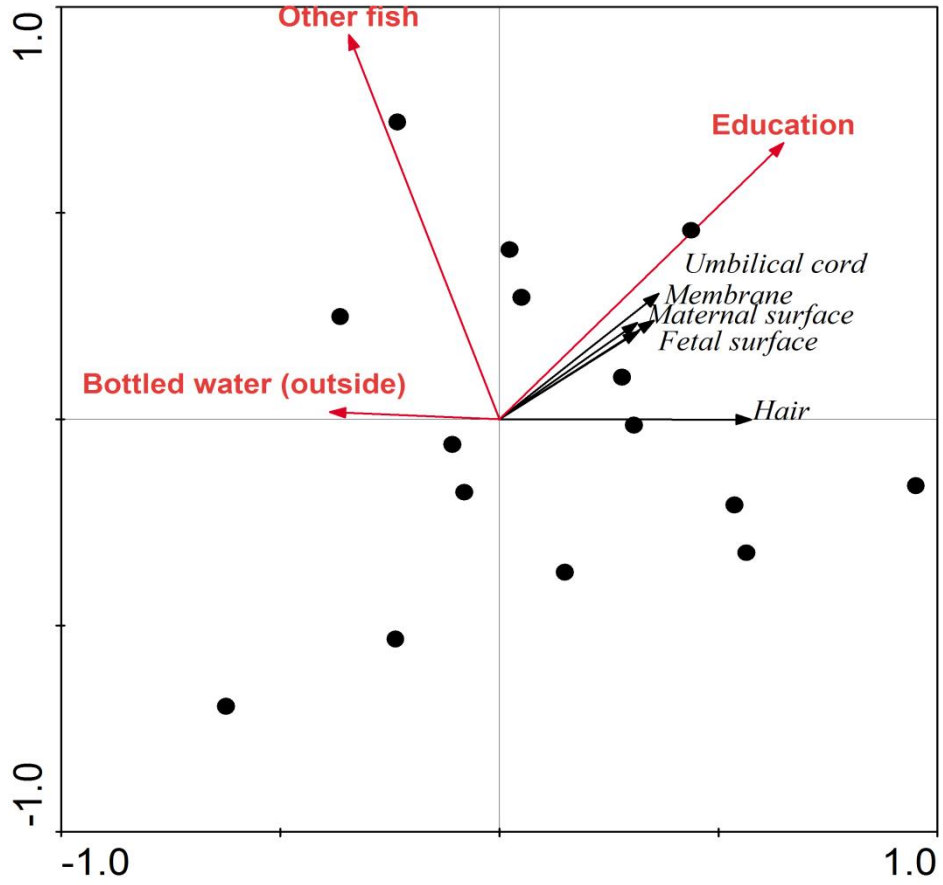

Figure 2.2- Biplots based on Redundancy Analysis (RDA) representing the correlation between biological data and significant potential influence variables: other fish (sardines, mackerel, salmon, mullet), education's level and drinking water outside home (bottled).

Table 2.5 - Significance of potential influence variables (maternal demographic characteristics, lifestyle, eating habits and water consumption) included in the Redundancy Analysis (RDA) model. Variables are shown by order of importance.

Variable	F	p-value
Other fish (sardines, mackerel, salmon, mullet)	8.002	0.006
Education's level	7.721	0.020
Drinking water outside home (Bottled)	4.503	0.046

No significant correlations (p>0.05) were found between [THg] in placental, cord tissues, maternal hair and data on anthropometric parameters (head circumference, birth length and birth weight) of newborns (Table 2.6). However, birth weight and head circumference showed a negative relation with [THg] both in placental and cord tissues.

Total Hg content in placental and cord tissues was not dependent (p>0.05) on newborn gender (Figure 2.3).

Table 2.6 – Spearman correlation coefficients between total mercury content in placental and cord tissues and neonatal anthropometric data. Correlation coefficients (p value).

	Maternal Hair	Decidua basalis	Chorionic plate	Amniotic membrane	Cord tissue
Head circumference	0.107(0.46)	-0.129(0.37)	-0.090(0.53)	-0.053(0.71)	-0.04(0.77)
Birth length	0.129(0.37)	-0.022(0.88)	-0.059(0.68)	0.000(0.99)	0.026(0.86)
Birth weight	0.042(0.77)	-0.210(0.14)	-0.223(0.12)	-0.123(0.38)	-0.206(0.15)

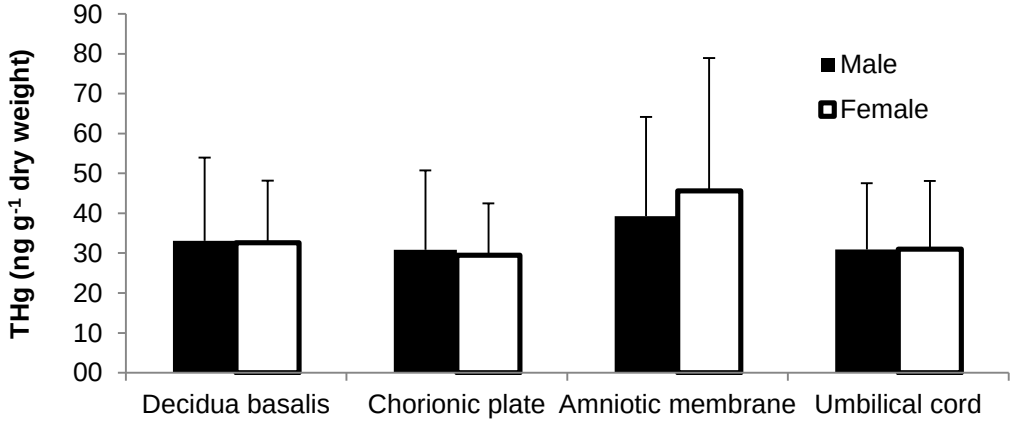

Figure 2.3- Mean concentration ± standard deviation of THg in placental and cord tissues by gender of newborns. Mann Whitney U test; p>0.05.

2.4.1 Mercury in maternal hair

In this study performed in 50 parturient and their newborns from Aveiro region (Portugal), Hg was detected in all hair, cord and placental samples. Maternal hair is considered an important biological matrix for reflecting Hg exposure, especially MeHg **[18]**. Fish intake is highly required throughout pregnancy because of its content in omega-3 fatty acid (omega-3). This essential nutrient is necessary to maintain maternal reserves which are used in physiological processes; and it is critical for fetal synaptogenesis and development of photoreceptors during the third trimester **[36, 37]**. Predatory fish such as swordfish and tuna, as well as shellfish, have been considered food items to be avoided by pregnant women due to their MeHg content **[38]**. But, as it was described in Table 2.4, the modal frequency for this kind of fish food consumption was "never". Besides, any category of fish considered in questionnaires was positively related with THg levels found not only in the hair, but in all tissues (Figure 2.2 and Table 2.5). In this study, maternal hair had a THg average of 0.90 μg g^{-1}, which is very close to the limit established by U.S EPA (1 μg g^{-1}) **[27]**. In addition, a total of 38% of the studied volunteers had THg concentrations in hair above this safe limit of which 6% were above of normal limit considered by WHO (2 μg g^{-1}) **[28]**. Our results are in accordance with a recent study performed also in the central—south of Portugal **[39]**, where Hg concentrations in maternal scalp hair from pregnant woman ranged from 0.07 to 5.3 μg g^{-1} and 7% had values above 2 μg g^{-1}. Even so, according to WHO and UNEP (2008) **[18],** Hg concentration in maternal hair associated with teratogenic effects in the fetus is 10 μg g^{-1} which is considerably higher than the ones obtained in the present study. On the other hand, Schoeman and colleagues set the concentration 0.3 μg g^{-1} as the Lowest Observable Adverse Effect Hair Concentrations (LOAEHC), looking at adverse effects on fetal brain development, related to Hg in maternal hair **[40]**. Regarding this, 50% of the parturient studied were recorded above this value.

2.4.2 Mercury in placental umbilical cord tissues

Our results showed a strong relationship between the Hg levels obtained in maternal hair, cord and placental tissues (decidua basalis, chorionic plate and amniotic membrane) ($p<0.001$) (Table 2.3). Therefore, the correlations support the use of all

specimens included in the present study. Besides, Hg was detected in all umbilical cord samples which proves its usefulness as a specimen to assess to fetal exposure to Hg **[41]**.

Table 2.7 – Total mercury levels in placenta collected in different epidemiological studies performed in Europe (from 1978 to 2013). Data are expressed in ng/g wet weight.

Country	Mean±SD	Min-Máx	Ref.
Belgium	15.3±14.1	1.1-103.2	Roels *et al.* (1978) **[42]**
Italy	12.7±9.0		Capelli *et al.* (1986) **[43]**
Czech Republic	2.2±1.0		Truska et al. (1989) **[44]**
Spain	5.4±3.1	2.3-14.3	Soria *et al.* (1992) **[45]**
Germany	13 (median)		Scaal *et al.* (1998) **[46]**
Ukrain	-	2.2-45.8	Zadorozhnaja *et al.* (2000) **[47]**
Austria	1.9 (median)	0.1-11.7	Gundacker *et al.* (2010) **[48]**
Denmark (Faroe Islands)	87 (median)		Needham *et al.* (2011) **[49]**
Poland	-	4-104	Kozikowska *et al.* (2013) **[50]**

Table 2.7 describes European results since 1978, recording THg levels (expressed in wet weight) in placenta without amniotic membrane or specifying which surface (maternal or fetal) was measured. In order to compare them with the results obtained in this work, which was expressed in dry weight, we divided Hg levels found in decidua basalis and chorionic plate (Table 2.2) by 6.0 according to an extent review made by Esteban-Vasallo *et al.* **[11]** in mammals. Thus, decidua basalis and chorionic plate had a mean (median) value of 5.47(4.59) and 5.03(4.47) ng g^{-1} wet weight, respectively. In view of this, our results were lower than placental Hg levels found in Belgium, Italy, Germany and Denmark; and higher than for the Czech Republic. On the other hand, Spain reported a mean value very similar to those found at the present work (5.4 ng g^{-1} wet weight – Table 2.7).

To our knowledge, there are no studies so far that included the simultaneous quantification of Hg in maternal hair, decidua basalis, chorionic plate, amniotic membrane and cord tissue. However, Soria *et al.* have already reported in 1992 **[45]** a

strong correlation between Hg in maternal hair and decidua basalis (cotyledons) and Neddham *et al.* in 2011 **[49]** observed the same between Hg levels found in maternal hair, cord tissue and chorionic plate. These results are in line with the correlation coefficients obtained in the present study, where the Hg levels in the different placental tissues analyzed presented a strong correlation with maternal hair levels.

2.4.3 Mercury in amniotic membrane

The major finding in the present study was the Hg levels found in the amniotic membrane. To our knowledge only three studies were made using metal level in amniotic membrane as biological marker of metal exposure. The first was carried out in pregnant mice **[21]**, where rats were injected with inorganic Hg and two different organic Hg compounds at the 14^{th} day of gestation. Four days after, the placentas, amniotic membranes, amniotic fluids, livers, kidneys and uteri were harvested. They found different patterns of accumulation and retention of Hg according to the chemistry of compound. Inorganic Hg showed a major tendency to accumulate in placenta and amniotic membrane while MeHg was found preferably in fetal liver. Suzuki and colleagues suggested the water solubility of Hg compounds as explanation to their findings **[21]**. Once amniotic fluid is mostly constituted by water and receives waste products from fetus **[51]**, excreted inorganic Hg may be catch and retained by amniotic membrane.

Afterwards, Yoshida **[10]** reconfirmed results achieved by Suzuki *et al.* **[22]** about the different Hg capabilities to be retained or transferred across placenta. A second study was performed in humans, concerning prenatal exposure to cadmium (Cd) and lead (Pb), where a higher accumulation of metals was found in the amniotic membrane followed by the amniotic fluid **[52]**. Their results lead to the hypothesis that fetal membranes may participate in the elimination of toxic metals from the fetus by reabsorption from amniotic fluid **[52]**. A third study, also performed in humans, contradicted these findings as higher Hg and Pb contents were found in placenta compared to those in the amniotic membrane **[53]**. In the present study, although our results are in agreement with Suzuki *et al.* **[21]**, we have no information about what Hg species/form was present in amniotic membrane and no data was collected on Hg concentrations in amniotic fluid. Further research is needed in order to explore the role of amniotic membrane in Hg detoxification. Larger size samples and Hg speciation analyses may be helpful to confirm or reject previous findings **[10, 21, 53].**

It is known that in early stages of pregnancy, amniotic fluid derives from maternal plasma and passes through the fetal membranes as amnion. Throughout the gestation, when placental and vessels develop, water and solute from maternal plasma are transported across the placenta to the fetus and then to the amniotic fluid **[54]**. It is also known that placenta has two pathways of transference that are permeable to both inert hydrophilic and lipophilic solutes. Water, the major component of amniotic fluid, is transported by both ways. This transport may be facilitated by aquaporins (AQPs) which exist not only in placenta but also in amniotic membrane, chorion, decidua parietalis, ovary and uterus **[51, 55]**. One of those aquaporins is the type 1 that contains a reactive cysteine residue and is one of the major AQPs both in the placenta and fetal membranes **[55, 56]**. In 2010, Hirano *et al.* described the molecular mechanisms of how mercury inhibits water permeation through AQP-1 and concluded that when Hg binds to cysteine residue in Cys-SHg^+ form, it decreases water permeability **[56]**. Therefore, it seems plausible the proposals launched by Suzuki, T *et al.* **[21]** and Korpela, H. *et al.* **[52]** as amniotic membrane may have a role in metals detoxification. Recently, glutathione S-transferases (GSTs) were identified in amniotic membrane **[57]**. One of them was GSTP1 and its function is the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles **[57]**. Mercury is usually eliminated as glutathione (GSH) conjugates due to highly affinity with thiol groups **[51]**. On one hand this function may be related to the presence of GSTP1 and, on the other hand Hg may be retained by AQP1. However, caution must be taken looking at this second hypothesis involving AQP1, once the retention of Hg may lead to a decrease of amniotic fluid volume that can be harmful to fetal development **[54]**.

In the present work, the levels of THg found in amniotic membrane from placenta supporting female newborns were higher than those from males. Although these differences were not statistically significant, this trend observed is in accordance with the findings of Zadrozna *et al.* **[53].**

2.4.4 Factors associated with Hg levels

According to the multivariate analysis (Figure 2.2, Table 2.5) drinking bottled water and fish intake such as sardines, mackerel, salmon and mullet were negatively related with Hg levels found in all matrices. On one hand, this type of fish is considered low in Hg by EFSA **[38]** compared with predatory fish like swordfish and tuna. On the other hand, these species are not only rich in omega-3 fatty acids, but also in selenium (Se).

Mercury's binding affinity to Se is a million times higher than for sulfur, so Se may act as a detoxification pathway in human body. Besides, this element may also protect against toxic effects of Hg due to antioxidant effects of selenoprotein systems **[58]**. Determine the speciation of Hg may be useful to better discriminate which risk factors contributed to Hg levels found in matrices. Regarding the research carried out by Yoshida **[10]** and Suzuki *et al*. **[21]** and results described in Table 2.2, Hg possibly occurred in different forms/species in the analyzed tissues.

2.4.5 Newborns anthropometry and Hg levels

The relation of Hg levels quantified in the different biological matrices and the newborns anthropometry presented in the present study (Table 2.1) is similar to those obtained with infants from Austria **[48]** and Poland **[49]**. As in the present study, no significant relationships were found in these studies between Hg levels in placenta and infant's measurements, despite the occurrence of a negative tendency between birth weight and Hg content both in placenta and cord tissue **[49]**.

2.5 Conclusions

For the first time, a cross-sectional study concerning Hg exposure was performed in the Aveiro region (North central Portugal), two decades after the Hg leakage into the Ria de Aveiro. Our results detected the occurrence of high Hg levels in maternal hair according to US EPA and WHO, and higher Hg contents in placental tissues compared to previous reports from other European countries. However, these concentrations were not related with fish intake or other risk factors described throughout the literature. Moreover, newborns anthropometry did not appear to be influenced by Hg levels. In this study it was also demonstrated a higher Hg retention in human amniotic membrane. In order to understand the role of the amniotic membrane in the placental-fetal accumulation of Hg, further research should be done with a larger sample size and Hg compound specification. The feasibility of using the placenta to assess intrauterine exposure to Hg was confirmed as well as other non-invasive and biological markers like Hg level in cord tissue and scalp hair. So far, only two cross-sectional studies were carried out in mainland Portuguese populations concerning prenatal exposure to toxic metals **[39, 59]**. Therefore, further assessments to metals exposure are required in Portugal in order to prevent possible adverse effects in offspring.

Acknowledgement

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3. General Remarks

Human biomonitoring studies are important to assess exposure to existing and emerging environmental substances, and the results can help make informed decisions on health protection **[1]**. One of the goals of this study was to assess mercury (Hg) exposure in parturient and their newborns from Aveiro region (Portugal). This goal was well succeed. In general, Hg levels found in maternal hair (mean Hg level: 0.90 μg g-1) were lower but close to safe limits established by health organizations. Besides, no significant associations were found between Hg levels in biological media and anthropometry of newborns.

Biomarkers have shown to be essential tools to study the relationship between health and environmental exposure **[3]**. Maternal hair and cord tissue were two of the biological matrices used in this work. They were chosen as matrices to assess Hg exposure for two reasons: firstly, they are recommended for that purpose **[2]** and both fitted in another aim of the study– to use non-invasive material. Secondly, in addition to the existence of standard protocols about hair collection and laboratory proceedings, WHO and U.S. EPA have set Hg reference levels for scalp hair, which gave us the capacity to determine the degree of exposure for this population.

Placenta was other matrix used in this study in order to evaluate the retention and distribution of Hg over the maternal-fetal-placental unit. We found a very strong association between all placental tissues and umbilical cord. These findings are very interesting because placenta (and amniotic membrane attached), is a temporary organ that is usually discarded after birth which favors major ethical issues. Moreover, this non-invasive matrix not only can tell us about the Hg transfer from mother to fetus as it can also provide different biologic markers of Hg exposure. Decidua basalis containing maternal blood **[4]** may represent parental exposure and chorionic plate containing fetal blood **[4]** may report the fetal exposure. Between these compartments there is a barrier – placental barrier, that avoid the mixture of both maternal and fetal blood **[4]** and through which Hg targets fetal blood for the first time (Figure 3.1, equation 1). In turn, while umbilical cord may well represent Hg transfer towards to fetal tissues (Figure 3.1, equation 2), amniotic membrane may function as the final Hg target after fetal excretion into amniotic fluid (Figure 3.1, equation 3).

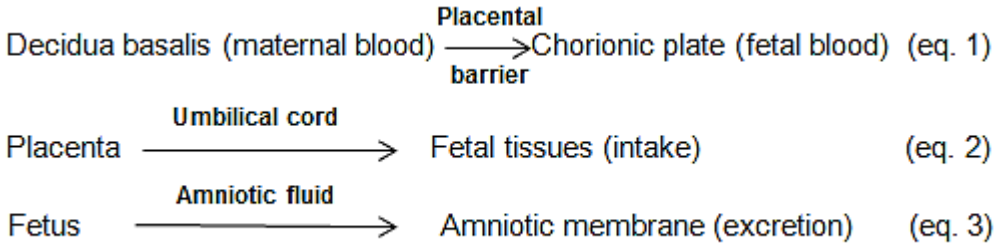

Figure 3.1- Tiered approach of Hg pathways across maternal-fetal-placental unit.

Further research should be carried out about the ability of the amniotic membrane to retain and accumulate Hg and other metals. Several authors pointed out this tissue as a protection to the fetus **[5, 6]**. But since it is known that Hg inhibits aquaporins functions **[7]** and amniotic fluid is refilled from fetal membranes in early stages of pregnancy **[8]**, some knowledge gaps should be filled in. Understand how amniotic membrane deals with Hg exposure in the different stages of pregnancy, should therefore be considered. Besides, aquaporins also exist in the placental barrier, and later amniotic fluid is refilled from there **[8]**. Taking into account this, further investigation should be also conducted aiming to make the linkage between these occurrences and possible adverse effects during pregnancy that may affect the fetal development.

Another objective of this research was to investigate how Hg levels were distributed along the Aveiro district. This work was performed in 50 mother-newborn pairs, randomly selected, from 9 different counties that belong to the Aveiro region: Ovar, Murtosa, Estarreja, Aveiro, Albergaria-a-Velha, Ilhavo, Vagos, Oliveira do Bairro and Agueda (Figure 3.2).

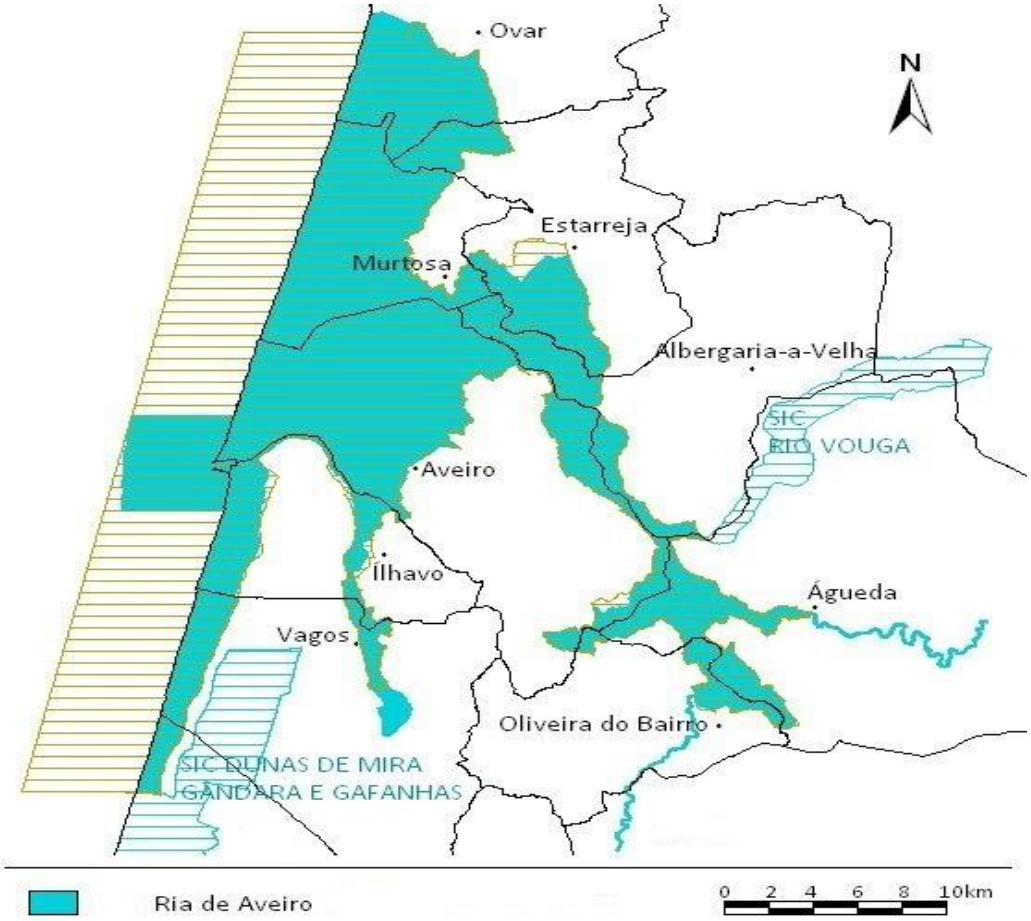

Figure 3.2 – Arrangement of the 9 counties along Aveiro distrcit that represented this study. Adapted from **[9].**

Looking at individual Hg analysis among volunteers, we found Hg levels above 2 µg g^{-1} (set value by WHO) **[10]** in three hair samples and thirteen were above 1 µg g^{-1} (set value by U.S. EPA) **[11]**. Together, they made up a total of 32% of mothers that represented the Aveiro region in this study. According to previous studies, we know that Estarreja and surroundings were the places more contaminated by Hg during (1950-1994) and even after the continuous release of this metal from a chlor-alkali plant **[12]**.

But it seems there are other counties which may deserve attention as it is shown in Figure 3.3. Oliveira do Bairro, Águeda, Albergaria-a-Velha and Vagos were the locations where higher Hg levels in maternal hair were found (>1 µg g^{-1}).

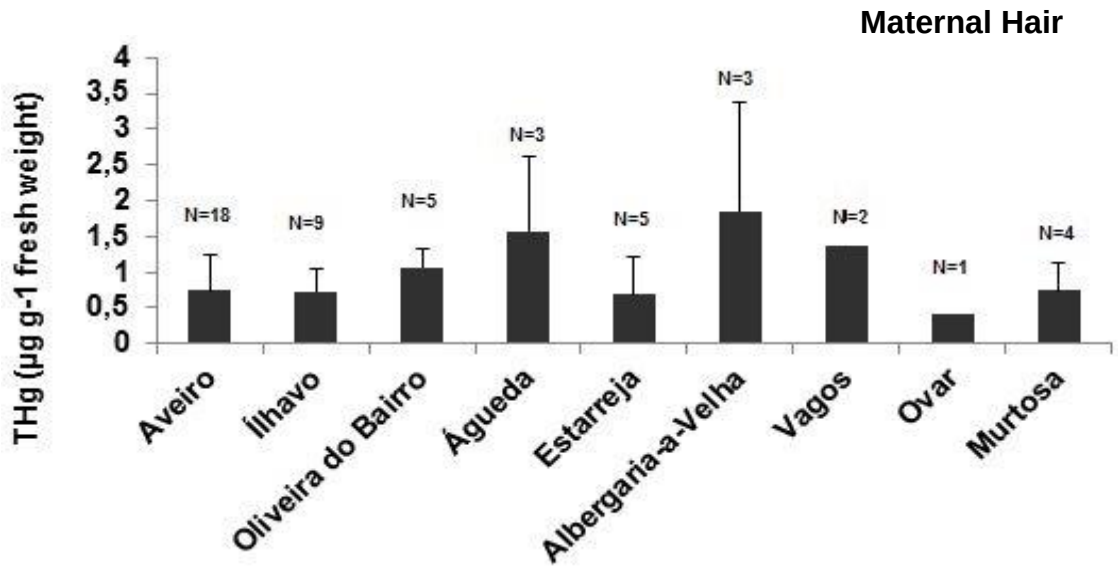

Figure 3.3 - Mercury levels in the maternal hair by county of residence in the Aveiro district of the 50 parturients sampled in this study. Bars represent mean and standard deviation. Vagos data is only represented by the mean value and no standard. deviation was included due to the low n (n=2).

In Figure 3.4 it is presented the distribution of Hg levels found in the umbilical cord tissue of the 50 newborns by county of residence of the mothers. Once again, we found Oliveira do Bairro, Águeda and Albergaria-a-Velha as the counties with highest Hg values. Besides, in what concerns to biomarkers of transplacental Hg transfer, Estarreja and Murtosa joined this group of counties presenting the highest Hg levels.

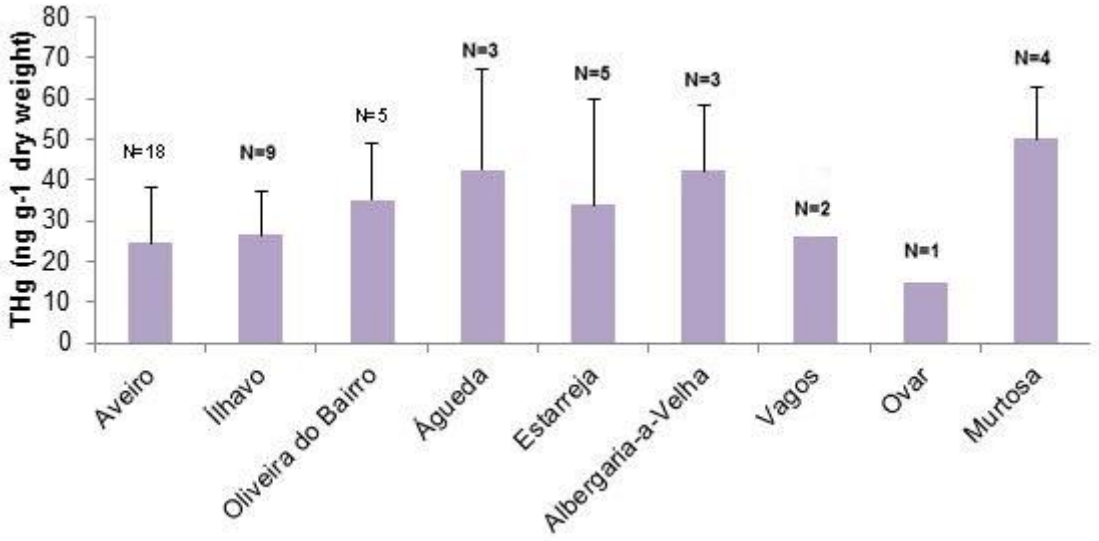

Figure 3.4 - Mercury levels in the umbilical cord of the 50 newborns by county of residence (Aveiro district) of the mother. Bars represent mean and standard deviation. Vagos data is only represented by the mean value and no standard. deviation was included due to the low n (n=2).

Taking into account these results, further longitudinal studies concerning Hg exposure are required and a larger sample size should be considered. Women with higher Hg levels in hair may be sporadic cases or instead, they may represent a case study in future research in this area; other aspect is the use of hair as unique biomarker in studies regarding Hg exposure during pregnancy. Since 80% of Hg found in hair is methylmercury **[13]**, care must be taken specially, when the intention is to express results in total Hg levels. The extrapolation of this results relating with outcomes during pregnancy, may be underestimated. The quantification of Hg in the umbilical cord might give more assertive information regarding direct fetal exposure during pregnancy and might complement the information given by maternal hair levels.

For the first time in Portugal, a biomonitoring study was performed concerning prenatal exposure to Hg using also the placenta as a biomarker for dual purpose of exposure assessment. Nonetheless, Portugal still has limited information about intrauterine exposure to environmental contaminants. Further research should be done, either longitudinal or cross-sectional, in order to prevent negative outcomes in Portuguese populations and offspring.

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Annexes

I. Approval of the Ethic Committee of the Hospital Infante D. Pedro (HIDP, Aveiro, Portugal

EXMO CONSELHO DE ADMINISTRAÇÃO

DO

CENTRO HOSPITALAR DO BAIXO VOUGA EPE

A Comissão de Ética reuniu com a ausência justificada da Dr.ª Filomena e do Padre João Gonçalves, no dia 17 de Setembro de 2014, pelas 10.30 horas, no novo espaço cedido pelo Conselho de Administração.
Analisou um pedido formulado para realização de um estudo dos hábitos tabágico e stressores ambientais em parturientes e recém nascidos para um tese de mestrado em Toxicologia e Ecotoxilogia pela aluna Ana Catarina Alves com o titulo "Avaliação da exposição a mercúrio em mães e recém nascidos da região de Aveiro", a ser realizado no Serviço de Obstetrícia tendo a doutora Suzana Loureiro como investigadora auxiliar, que em reunião da Comissão deu as explicações a questões formuladas.
O pedido obedece aos princípios éticos, pelo que a Comissão, por unanimidade dá o seu parecer favorável à petição formulada.

Respeitosos cumprimentos

Aveiro 17 de Setembro de 2014

Pela Comissão de Ética

Amorim Figueiredo

(Presidente)

II. Informed consent

CONSENTIMENTO INFORMADO, ESCLARECIDO E LIVRE PARA PARTICIPAÇÃO EM ESTUDOS DE INVESTIGAÇÃO NOS TERMOS DA NORMA Nº 015/2013 DA Direção-Geral da Saúde (de acordo com a Declaração de Helsínquia e a Convenção de Oviedo)

Título do estudo: Estudo sobre os hábitos tabágicos e stressores ambientais em parturientes e recém-nascidos.

Enquadramento: Estudo integrado entre a Universidade de Aveiro e o Instituto Nacional de Saúde Doutor Ricardo Jorge, e envolverá o estudo de mestrado da aluna Ana Catarina Alves "Avaliação da exposição a mercúrio e recém-nascidos da região de Aveiro", no âmbito do mestrado em Toxicologia e Ecotoxicologia.

Condições e financiamento: carácter voluntário da participação e ausência de prejuízos, assistenciais ou outros, caso não queira participar; o estudo mereceu Parecer favorável da Comissão de Ética do Centro Hospitalar do Baixo Vouga.

Confidencialidade e anonimato: este estudo é confidencial e de uso exclusivo dos dados recolhidos para o presente estudo; não serão registados dados de identificação.

Contacto da investigadora responsável – Susana Loureiro, Investigadora da Universidade de Aveiro, Departamento de Biologia. Email: sloureiro@ua.pt; tel.234370779

Contacto do médico responsável-_____

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorrecto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Assinatura/s/ e número/s de cédula profissional de quem pede consentimento: ……

Declaro ter lido e compreendido este documento, bem como as informações verbais qye me foram fornecidas pela/s pessoa/s que assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária, forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pela investigadora e equipa médica.

Nome: ……..

Assinatura: ……..

Data: ……/ …… / …….

ESTE DOCUMENTO É COMPOSTO DE 2 PÁGINAS E FEITO EM DUPLICADO: UMA VIA PARA OS INVESTIGADORES, OUTRA PARA A PESSOA QUE CONSENTE.

III. Questionnaire

dbio universidade de aveiro departamento de biologia

UAlg UNIVERSIDADE DO ALGARVE

Hospital Infante D. Pedro - Aveiro

QUESTIONÁRIO N.º |_____|

FICHA DE PRÉ-INCLUSÃO

Entrevistador: |_____| **Data da entrevista:** |_____|

DADOS PESSOAIS DE IDENTIFICAÇÃO

Data de nascimento: |_____|

Naturalidade: |_____| **Nacionalidade:** |_____|

Assinou a Declaração de Consentimento de Participação Informado?

|__| Sim |__| Não

RESIDÊNCIA ACTUAL

Distrito: |_____| **Concelho:** |_____|

Freguesia: |_____|

DADOS SÓCIO-DEMOGRÁFICOS

Q1. Há quantos anos vive no seu município/concelho atual? |___| anos

Q4. Em que zona do município/concelho vive?

☐ Zona urbana ☐ Zona rural

☐ Zona intermédia ☐ NS/NR

Q5. Que nível de estudos finalizou?

☐ Não sabe ler nem escrever ☐ Estudos secundários

☐ Sem estudos ou estudos primários incompletos ☐ Estudos Universitários

☐ Estudos primários ☐ Outros

Q6. Altura: |____| cm

Q7. Peso antes da gravidez: |_____| kg

Q8. Peso no final da gravidez: |_____| kg

Q9. Comprimento do recém-nascido: |_____| cm

Q10. Peso do recém-nascido: |_____| kg

Q11. Sexo do recém-nascido ☐ F ☐ M

Q12. Data do parto: |_____|

QUESTIONÁRIO DE GINECOLOGIA E OBSTETRÍCIA

Q13. Que idade tinha quando teve a sua primeira menstruação? |____| anos

Q14. Usou algum tipo de contracetivo?

☐ Sim ☐ Não (passar à questão 12)

Q14.1. Que tipo de contracetivo usou?

☐ Hormonal (pílula ou DIU hormonal) ☐ Não hormonal

Q14.2. Durante quanto tempo os utilizou? (incluindo o tempo em que houve interrupções)

☐ < 1 ano ☐ 6 a 10 anos

☐ 1 a 2 anos ☐ 11 a 15 anos

☐ 2 a 5 anos ☐ > 15 anos

Q15. Durante a gravidez, realizou alguma restauração dental?

☐ Sim ☐ Não (passar à questão 14)

Q15.1. Se sim, que tipo de enchimento dental foi utilizado?

☐ Massa ☐ Metálico

Q15.2. Se sim, durante que semana de gestação foi realizado o procedimento?

☐ Semana 0 a 13 ☐ Semana 14 a 26 ☐ Semana 27 a 42

Q16. Durante a gravidez tomou alguma medicação?

☐ Sim ☐ Não

Q16.1. Nessa medicação, estavam incluídos suplementos vitamínicos?

☐ Sim ☐ Não

Q17. Durante a gravidez, realizou algum tipo de vacinação?

☐ Sim ☐ Não

Se sim, qual? _____ Em que semana de gravidez?_____

Q18. Recebeu alguma transfusão de sangue durante a gravidez?

☐ Sim ☐ Não

Q19. Já tinha engravidado anteriormente?

☐ Sim ☐ Não

N.º	Duração da gravidez (semanas)	Sexo	Peso (g)	Comprimento (cm)	Problemas/ Patologias	Aborto	Malformação Neonatal	Gravidez extra-uterina
1								
2								
3								
4								
5								

QUESTIONÁRIO DE EXPOSIÇÃO AMBIENTAL

Q20. Qual a sua ocupação atual? |_____|

Q21. Onde desempenha a sua ocupação atual? |_____|

Q22. Há quanto tempo tem a sua ocupação atual? |_____| anos

Q23. Qual era a sua ocupação anterior? |_____|

Q24. Durante quanto tempo desempenhou a sua ocupação anterior? |_____| anos

Q25. Alguma vez esteve exposta, com o seu consentimento, a produtos químicos no seu trabalho?

☐ Sim ☐ Não

Q26. Por favor indique se esteve pessoalmente em contacto com algum dos seguintes produtos no trabalho:

Produtos	Nº de horas por semana
Pesticidas	
Tintas e pigmentos	
Produtos de limpeza incluindo desinfetantes	
Sprays para o cabelo (lacas)	
Fumos de tubos de escape (diesel, gasolina)	
Fumos de fábricas	
Fumo de tabaco	

Q27. Qual a idade da sua casa:

☐ < 5 anos ☐ > 29 anos

☐ 5 a 14 anos ☐ NS/NR

☐ 15 a 19 anos

Q28. Como descreveria a sua casa?

☐ Vivenda unifamiliar afastada de outras casas ☐ Apartamento

☐ Vivenda unifamiliar junto a uma ou mais casas ☐ Outro

Q29. Com que frequência passam carros na rua da sua casa?

☐ Continuamente ☐ Pouco

☐ Com muita frequência ☐ Praticamente nada

Q30. Com que frequência passam veículos pesados na rua da sua casa, à exceção da recolha de lixo?

☐ Continuamente ☐ Pouco

☐ Com muita frequência ☐ Praticamente nada

Q31. Fez obras na sua casa nos últimos 6 meses?

☐ Sim ☐ Não

Q32. Utiliza inseticidas ou outros produtos para afugentar mosquitos, baratas, moscas, formigas, etc, na sua casa?

☐ Sim ☐ Não

Q33. A sua casa fica perto de atividades industriais? (garagens, oficinas, fábricas, etc.)

☐ Sim ☐ Não

QUESTIONÁRIO DE HÁBITOS DE CONSUMO E ESTILO DE VIDA

COSMÉTICOS E OUTROS

Q34. Usou algum cosmético, maquilhagem, durante a gravidez? (rímel, contorno de olhos, base, cremes, loções, pomadas, etc.)

☐ Sim ☐ Não

Q34.1. Se sim, com que frequência?

☐ Diariamente ☐ > 1 vez por semana ☐ < 1 vez por semana

Q35. Durante a gravidez, tingiu, pintou ou fez madeixas no cabelo?

☐ Sim ☐ Não

CONSUMO DE ÁGUA

Q36. Qual a principal origem da água que bebeu em sua casa, durante a gravidez?

☐ Água municipal ☐ Água engarrafada

☐ Poço privado ☐ Outra; Qual: |_____|

Q37. Qual a principal origem da água que utilizou para cozinhar, durante a gravidez?

☐ Água municipal ☐ Água engarrafada

☐ Poço privado ☐ Outra; Qual: |_____|

Q38. Qual a principal origem da água que consumiu fora de casa, durante a gravidez?

☐ Água municipal ☐ Água engarrafada
☐ Poço privado ☐ Outra; Qual: |_____|

HÁBITOS ALIMENTARES

Q39. Com que frequência ingeriu algum dos seguintes alimentos durante a gravidez?

	Nunca	1 a 3 vezes por mês	1 a 3 vezes por semana	4 a 6 vezes por semana	Todos os dias
Lacticínios					
Chá					
Café					
Queijos frescos					
Queijos envelhecidos					
Ovos					
Vegetais verdes frescos (alface, tomate, feijão verde, bróculos, espinafres, agriões, couves, courgetes, abóbora, cenouras, etc)					
Vegetais em conserva (polpa de tomate, pimentos, etc)					
Fruta fresca ou sumos naturais					
Frutas em conserva (pêssegos, ananás, manga) ou sumos de fruta em embalagens tetra pack tipo néctar)					
Iced-teas, refrigerantes, etc					
Alimentos biológicos ou de colheita própria					
Tofu, miso, soja (ex: hambúrgueres, salsichas vegetarianas)					
Aves (frango, pato, perú)					
Carne (vaca, porco, borrego, presunto, bacon, hambúrguer)					
Fígado, paté de fígado, rim, coração, salsichas (lata)					
Peixe branco fresco ou congelado (bacalhau, linguado, pescada)					
Peixe espada (branco ou preto) e/ou atum					
Outro peixe fresco ou congelado (sardinha, cavalas, salmão, salmonete)					
Peixe em conserva (sardinhas, atum, cavalas, ovas, biqueirões)					
Marisco fresco ou congelado					

PERCEPÇÃO SOBRE O MEIO-AMBIENTE

Q40. Quais os problemas ambientais de relevo no local onde vive:

|_____|