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THE INFLUENCE OF AGE ON THE TOXICITY INDUCED BY MITOXANTRONE: *IN VIVO* STUDIES WITH MICE

Rita Guedes

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Trabalho realizado sob a orientação de:

Professora Doutora Vera Marisa Costa

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Author's declaration

Under the terms of the Decree-Law nº 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation. Under the terms of the referred Decree-Law, is hereby declared that the following communications were prepared in the scope of this dissertation.

The results presented in this dissertation are part of the following scientific communications:

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Abstract

Cancer is a huge health problem, affecting millions of people worldwide. To fight cancer, several anticancer treatments had been developed over the years. Nowadays, novel types of therapy are emerging; nevertheless, chemotherapy remains the most widely treatment used. Mitoxantrone (MTX) is an anticancer drug commonly used in chemotherapy for the treatment of several types of cancer, such as breast and prostate cancers, leukemia, lymphoma and multiple sclerosis. However, despite its benefits, MTX can cause several negative side effects, and cardiotoxicity is one of its major problems. MTX possesses pharmacological similarities with anthracyclines; however, its mechanisms of toxicity differ and the ones regarding MTX remain not properly clarified at this point. MTX metabolites may be, in part, related with its cardiotoxicity mechanisms. Additionally, it is known that several risk factors could aggravate the incidence of MTX toxicity, such as the administered doses, gender, age at diagnosis, pre-existent comorbidities, previous or concomitant treatment with other cytotoxic drugs and radiotherapy.

With this in mind, the present work aimed to study the toxicity induced by MTX in different aged CD-1 male mice (infants, adults, elderly) and the possible age-related differences on the metabolic profile of this chemotherapeutic drug. The animals received a cumulative dose of 6 mg/kg of MTX through 6 intraperitoneal injections, twice a week, to mimic the multiple therapeutic cycles performed in human patients. After the last administration, the mice were kept in a drug-free period until sacrifice, to allow the development of toxicity. General welfare, animal weight and food and water consumptions were recorded through the all experiment. The sacrifice occurred 7 days after the last administration in adult and elderly populations and 17 days later in the infants. The organs (brain, heart, kidneys, spleen, liver) and blood of the animals were collected. The heart tissues were analyzed through light microscopy. Plasma was used to biochemical determinations: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total creatine-kinase (CK) and creatine-kinase MB (CK-MB). Also, the mice plasma samples were injected in a high-performance liquid chromatography with a diode array detector (HPLC-DAD) system in order to detected MTX and one of its metabolites (naphtoquinoxaline, NAPHT).

Significant body weight loss was observed in the adult population treated with MTX, when compared to controls. Also, differences in food and water consumptions reached statistical significance in the infant and adult populations treated with MTX, in comparison with the respective controls. Regarding the differences in the organ weight/ brain weight ratios from the different age groups, only the adults and elderly treated with MTX presented

significant decreases comparing to controls: all the organs were significantly affected in the adult population, while in the elderly only the spleen seemed to be significantly affected. Concerning the biochemical determinations, ALT plasma levels were significantly increased in the MTX-treated infant and adult populations and, subsequently, the AST/ ALT ratios were affected. Regarding the histological observations in the heart tissue, all the MTX-treated populations presented cardiac lesions, as so the control elderly mice. The adult mice presented the highest damage.

To assess the metabolic profile of MTX in the plasma, using an HPLC-DAD system, the chromatographic conditions were set to a flow rate of 0.6 mL/min at the wavelengths 254 nm and 658 nm, with a running time of 30 minutes. The mobile phase consisted of acetonitrile: 0.5% formic acid, at pH 3.0. To determine MTX and its metabolite (NAPHT), the chromatographic conditions differed in the proportion of acetonitrile: formic acid in the mobile phase [20:80 (v/v) for the chemotherapeutic drug and 30:70 (v/v) for the metabolite]. Neither MTX or its metabolite were detected in the treated mice plasma samples, regardless of age.

In conclusion, the adult population seemed to be especially at risk when administered with a cumulative dose of 6 mg/kg of MTX. The pharmacokinetic features of the chemotherapeutic drug and its metabolite in plasma (rapid elimination) may have influenced their detection, since MTX and NAPHT may not be already detectable at the time of the analysis, or the concentrations of MTX and/ or NAPHT in plasma could be under the limit of detection (LOD) of the method. This way, further work needs to be conducted in order to better understand the results obtained.

Keywords: mitoxantrone; toxicity; age; metabolites.

Resumo

O cancro é um grande problema de saúde, afetando milhões de pessoas em todo o mundo. Para combater o cancro, várias terapêuticas anticancerígenas têm sido desenvolvidas ao longo dos anos. Recentemente, novas terapêuticas têm surgido; no entanto, a quimioterapia continua a ser a mais usada. A mitoxantrona (MTX) é um fármaco anticancerígeno comumente utilizado na quimioterapia para o tratamento de vários tipos de cancro, tais como o cancro da mama e da próstata, leucemia, linfoma e esclerose múltipla. No entanto, apesar dos seus benefícios, a MTX pode causar vários efeitos adversos, e a cardiotoxicidade é um desses problemas. A MTX possui similaridades farmacológicas com as antraciclinas; no entanto, os seus mecanismos de toxicidade diferem e os relativos à MTX permanecem por esclarecer, de forma adequada, até ao momento. Os seus metabolitos podem estar, em parte, relacionados com os seus mecanismos de cardiotoxicidade. Adicionalmente, sabe-se que vários fatores de risco podem agravar a incidência da toxicidade da MTX, tais como as doses administradas, género, idade no momento do diagnóstico, comorbidades pré-existentes, tratamento prévio ou concomitante com outros fármacos citotóxicos e radioterapia.

Com base nestas considerações, o presente trabalho teve como objetivo o estudo da toxicidade induzida pela MTX em ratinhos machos CD-1 de diferentes idades (infantis, adultos, idosos) e as possíveis diferenças relacionadas com a idade e o perfil metabólico deste fármaco. Os animais receberam uma dose cumulativa de 6 mg/kg de MTX através de 6 injeções intraperitoneais, duas vezes por semana, de modo a mimetizar os múltiplos ciclos terapêuticos realizados nos doentes humanos. Após a última administração, os ratinhos foram mantidos sem qualquer administração de fármaco até ao sacrifício, para permitir o desenvolvimento de toxicidade. O bem-estar geral, o peso dos animais e os consumos de comida e água foram registados durante toda a experiência. Os sacrifícios foram realizados 7 dias após a última administração nas populações adulta e idosa e 17 dias depois nos infantis. Os órgãos (cérebro, coração, rins, baço, fígado) e sangue foram recolhidos. Os tecidos do coração foram analisados por microscopia. O plasma foi utilizado para determinações bioquímicas: níveis de alanina aminotransferase (ALT), aspartato aminotransferase (AST), creatina-quinase total (CK) e creatina-quinase MB (CK-MB). Para além disso, o plasma foi analisado num sistema de cromatografia líquida de alta eficiência com um detetor de fotodiodos (HPLC-DAD) de modo a detetar a MTX e um dos seus metabolitos (naftoquinoxalina, NAPHT).

Foi observada uma diminuição do peso corporal da população adulta tratada com MTX, quando comparada com os controlos. Os consumos de comida e água também diminuíram

nas populações infantil e adulta tratadas com MTX, em comparação com os respetivos controlos. Relativamente às razões peso do órgão/ peso do cérebro dos diferentes grupos etários, somente os adultos e os idosos tratados com MTX apresentaram mudanças significativas comparativamente com os controlos: todos os órgãos tiveram essa razão diminuída na população adulta, enquanto nos idosos apenas o baço pareceu ser significativamente afetado. No que concerne às determinações bioquímicas, apenas os níveis plasmáticos de ALT estavam significativamente aumentados nos infantis e adultos tratados com MTX e, subsequentemente, a razão AST/ ALT foi afetada. Relativamente à histologia do tecido cardíaco, todas as populações tratadas com MTX apresentaram lesões cardíacas, bem como os ratinhos idosos controlo. Os ratinhos adultos foram aqueles que apresentaram maior dano.

Para determinar o perfil metabólico da MTX no plasma, usando um sistema HPLC-DAD, as condições cromatográficas foram ajustadas para um fluxo de 0,6 mL/min a comprimentos de onda de 254 nm e 658 nm e um tempo de análise de 30 minutos. A fase móvel consistiu em acetonitrilo: ácido fórmico 0,5% a pH 3,0. Para determinar a MTX e o seu metabolito (NAPHT), as condições cromatográficas diferiram na proporção de acetonitrilo: ácido fórmico na fase móvel [20:80 (v/v) para o fármaco quimioterapêutico e 30:70 (v/v) para o metabolito]. Nem a MTX nem o seu metabolito foram detetados no plasma dos ratinhos tratados com MTX, independentemente dos seus grupos etários.

Em conclusão, a população adulta pareceu estar especialmente em risco quando administrada com uma dose cumulativa de 6 mg/kg de MTX. As caraterísticas farmacocinéticas do fármaco quimioterapêutico e do seu metabolito no plasma (rápida eliminação) poderão ter influenciado essa deteção, uma vez que a MTX e a NAPHT poderão já não ser detetadas no momento da análise, ou as concentrações de MTX e/ ou NAPHT no plasma podem encontrar-se abaixo do limite de deteção (LOD) do método. Deste modo, estudos posteriores serão necessários de modo a entender melhor os resultados obtidos.

Palavras-chave: mitoxantrona; toxicidade; idade; metabolitos.

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Abbreviations

ALT – alanine aminotransferase

AMT – ametantrone

ANOVA – analysis of variance

AST – aspartate aminotransferase

ATP – adenosine triphosphate

CK – creatine kinase (total)

CK-MB – creatine kinase – muscle/ brain

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

GSH – glutathione

GSSG – glutathione disulfide

HF – heart failure

HPLC – high performance liquid chromatography

HPLC-DAD – high performance liquid chromatography – diode array detector

HPLC-UV – high performance liquid chromatography – ultraviolet detector

LC-MS – liquid chromatography mass spectrometry

LOD – limit of detection

LOQ – limit of quantification

LVD – left ventricular dysfunction

LVEF – left ventricular ejection fraction

MTX – mitoxantrone

NaCl – sodium chloride

NADH – reduced nicotinamide adenine dinucleotide

NADPH – reduced nicotinamide adenine dinucleotide phosphate

NAPHT – naphtoquinoxaline

ORBEA – organ responsible for animal welfare

PBS – phosphate buffered saline

RNA – ribonucleic acid

RNS – reactive nitrogen species

ROS – reactive oxygen species

CHAPTER 1.

Introduction

1. INTRODUCTION

1.1. The problematic of cancer

Cancer is a major problem of concern, being the second leading cause of death worldwide (WHO, 2018). Global data from 2015 shows that, among the 17.5 million cases recorded, cancer was responsible for 8.7 million deaths, being tracheal, bronchus and lung (TBL), colorectal, and stomach cancers the most lethal (Fitzmaurice et al., 2017). Cancer incidence has been increasing over the years; however, low developed countries stand out as the most affected especially due to socioeconomic factors (Fitzmaurice et al., 2017). This way, these countries represent approximately 70% of the deaths resulting from cancer (data from 2015) (WHO, 2018).

To fight cancer, several anticancer treatments have been used and developed over the years, being chemotherapy, radiotherapy, targeted therapies, or their combination the most commons (Yeh and Bickford, 2009). The choice of the adequate anticancer treatments depends on factors like the type of cancer and its characteristics, personal medical history, among others (WHO, 2018). The improvement on aspects on cancer prevention and its treatment has led to an increase in the number of cancer survivors over the last decades (Harlan and Warren, 2015). However, the increase in the survival rate of the cancer patients leads to long-term effects of these anticancer treatments (Minotti, 2010). For systematic purposes, only chemotherapy will be approached regarding the long-term side effects.

1.2. Anticancer drugs' toxicity

Chemotherapy is the most widely used anticancer treatment and its benefits regarding the decreases in the morbidity and mortality rates are well known (Shih et al., 2015). Chemotherapy works by stopping or slowing down the cancer cells from growing (NIH, 2015a). Since these cells usually grow and divided more quickly than most healthy cells, they are more easily recognized as targets by these anticancer drugs (NIH, 2015a). However, since conventional chemotherapeutic drugs are not selective, they can also affect normal cells, resulting in several negative side effects, like fatigue, nausea, hair loss, or even cardiotoxicity and neurotoxicity (Chen et al., 2007; NIH, 2015a). The conventional chemotherapeutic drugs act through different mechanisms (**Table 1**) so, they can cause side effects through different ways (American_Cancer_Society, 2016).

Cardiotoxicity is one of the major side effects induced by the chemotherapeutic treatment, and can be manifested as acute/subacute or chronic cardiotoxicity: the first type is observed at the beginning of the treatment up to two weeks after it ended (short-term), is rare and not dose-related, while chronic toxicity can occur within one year or more after treatment ended (long-term) and it is dose-related (Adão et al., 2012; Albini et al., 2010; Seiter, 2005). The heart has limited potential for regeneration, therefore, cardiotoxicity induced by the chemotherapeutic drugs can lead to serious long-term adverse effects (Reis-Mendes et al., 2015). Cardiac manifestations induced by these agents can vary from electrocardiographic changes, arrhythmias, heart failure (HF) and left ventricular dysfunction (LVD), with consequent cardiomyopathy and, ultimately, death (Albini et al., 2010; Reis-Mendes et al., 2015). Considering this wide range of possible cardiotoxic effects, the drugs used in anticancer therapy can be divided in two groups: type I agents, that cause dose-related cardiotoxicity and irreversible lesions, or type II agents, that lead to cardiotoxicity that is usually reversible and not dose-related. This reversibility factor has allowed their use for years or subsequent reintroduction after the recovery of cardiac injury (Adão et al., 2012).

For systematic purposes, anthracyclines, for their wide use, and mitoxantrone (MTX), the drug of study in this dissertation, will be the anticancer drugs highlighted in the present section.

Table 1. Conventional chemotherapeutic drugs and their mechanisms of action.

Cardiotoxicity agents		Mechanism of action	References
TOPOISOMERASE II INHIBITORS	Anthracyclines: Doxorubicin Epirubicin Idarubicin Daunorubicin	Deoxyribonucleic acid (DNA) intercalating agents with subsequent inhibition of the topoisomerase type II, formation of free radicals (lipid peroxidation and DNA damage), induction of apoptosis, direct membrane effects	(Beretta and Zunino, 2008; Gewirtz, 1999)
	Anthraquinones: MTX	DNA intercalating agents with subsequent inhibition of the topoisomerase type II (inhibition of DNA and ribonucleic acid (RNA) replication), cytocidal effect	(Alberts et al., 1985; DrugBank, 2018)
ALKYLATING AND METALATING AGENTS	Cyclophosphamide Ifosfamide Mitomycin Platinum agents: Cisplatin	Alkylation of DNA (DNA damage): inhibition of the replication process, fragmentation, cross-linking strands, mispairing of the nucleotides (causes mutations)	(Hall and Tilby, 1992; Siddik, 2002)

Cardiotoxicity agents		Mechanism of action	References
ANTIMICROTUBULE AGENTS	Taxanes: Paclitaxel Docetaxel	Disruption of the microtubule function with consequent inhibition of the cell division (mitotic inhibition)	(American_Cancer_Society, 2016; Canadian_Cancer_Society, 2017; Fitzpatrick and de Wit, 2014)
ANTIMICROTUBULE AGENTS	Vinca alkaloids: Vincristine Vinblastine Vinorelbine	Inhibition of tubulin polymerization (prevention of the mitotic spindle formation) and consequently blocking or slowing down the mitosis process	(American_Cancer_Society, 2016; Jordan, 2002)
ANTIMETABOLITES	5-Fluorouracil and its oral prodrug, capecitabine Gemcitabine	Interference in the synthesis of the DNA constituents: depletion of nucleotides and consequent inhibition of DNA replication	(American_Cancer_Society, 2016; Lansiaux, 2011)

1.2.1. Anthracyclines

Anthracyclines are topoisomerase II inhibitors that were first extracted from *Streptomyces peucetius*, including doxorubicin and daunorubicin. These two chemotherapeutic drugs differ only by a single hydroxyl group (Seiter, 2005). On the other hand, epirubicin and idarubicin are semisynthetic derivatives from doxorubicin and daunorubicin, respectively: epirubicin is the 4-epimer of doxorubicin (only differs in the configuration of the 4'-C atom) while in idarubicin the methoxy group from the aglycone of the daunorubicin molecule has been replaced with a hydrogen (Gerson et al., 2018; Launchbury and Habboubi, 1993; Seiter, 2005). The purpose of the synthesis of these two analogs was to find new anthracyclines with better chemotherapeutic activity and less side effects (Minotti et al., 2004). The chemical structures of these four anthracyclines are represented in **Figure 1**: the basic structure consists of a tetracyclic aglycone linked to an amino sugar. These anticancer drugs have shown to be very effective in the treatment of many forms of cancer such as leukemias, lymphomas and solid tumors (breast, stomach and lung cancers, for example) (FDA, 2012).

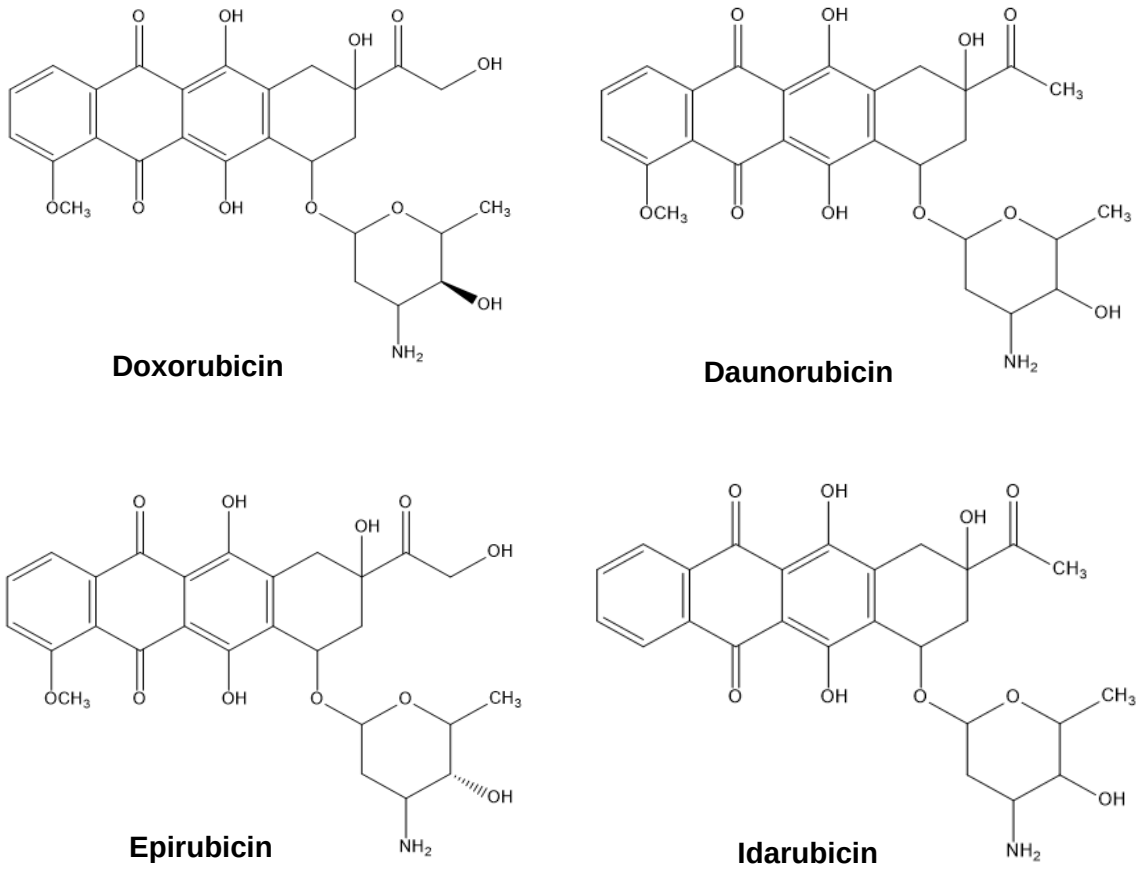


Figure 1. Chemical structures of doxorubicin, daunorubicin and their analogs: epirubicin and idarubicin, respectively.

1.2.1.1. Mechanism of action

Anthracyclines exert their pharmacologic action by intercalating the DNA chain (Beretta and Zunino, 2008). This results from the insertion of their planar tetracyclic chromophore between adjacent base pairs. So, DNA is recognized by these chemotherapeutic drugs as their primary target and the stabilization of the drug-DNA intercalation complex is made by electrostatic ionic interactions between the phosphate groups of the DNA molecule and the positively charged amino group of the drugs. This way, the DNA binding is a key factor for the anticancer activity of anthracyclines, such as the inhibition of the topoisomerase type II (Beretta and Zunino, 2008). This action leads to the inhibition of DNA and RNA syntheses (Gewirtz, 1999). Moreover, the action of anthracyclines lends to the formation of free radicals: the quinone structure of these drugs allows them to act as electron acceptors in reactions mediated by oxoreductive enzymes, such as cytochrome P450 reductase and reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase. The free radicals may induce DNA damage, lipid peroxidation, apoptosis and interact with molecular oxygen, forming reactive oxygen species (ROS), resulting in oxidative stress (Gewirtz, 1999). Additionally, the free radical formation can induce direct effects on the membrane of the cells (Beretta and Zunino, 2008).

1.2.1.2. Pharmacokinetics

Anthracyclines are commonly administered by intravenous infusion and are rapidly distributed through the body, accumulating primarily in the liver, kidneys, heart, lymph nodes and muscle (PDR, 2018a). The plasma protein binding differs according to the type of anthracycline and can go from approximately 75 - 77% to 97% (PDR, 2018a; b; c; d).

These chemotherapeutic drugs suffer hepatic metabolism, that consists in several enzymatic steps that differ according to the type of drug (Reis-Mendes et al., 2015). In a general way, anthracyclines can suffer metabolization through the reduction of the C-13 carbonyl group, which leads to the formation of C-13-alcohol metabolites via NADH-dependent aldo/ ketoses or carbonyl reductases enzymes, such as doxorubicinol and daunorubicinol (from doxorubicin and daunorubicin, respectively). Additionally, the anthracyclines can form a semiquinone radical that can, subsequently, suffer deglycosylation, that consists in the removal of the sugar from the molecules, producing aglycones. From this point, the anthracycline aglycones can be demethylated and conjugated with glucuronide or sulfate molecules (Reis-Mendes et al., 2015). The major

known metabolites of doxorubicin, daunorubicin, idarubicin and epirubicin are doxorubicinol, daunorubicinol, epirubicinol and idarubicinol, respectively (PDR, 2018a; b; c; d).

The excretion process is mainly biliary (Reis-Mendes et al., 2015). In the case of doxorubicin, the terminal half-life is between 20 and 48 hours and approximately 40% of the administered dose is found in the bile. In urine, only a small amount of the parent drug and its metabolites is present (5 – 12%, within 5 days) (PDR, 2018b). Daunorubicin presents a terminal half-life of 45 – 55 hours and, in the case of daunorubicinol, this value diminishes to 23 – 40 hours (PDR, 2018a). Similarly to doxorubicin, daunorubicin and its metabolites are primarily excreted in the bile and only a small amount is eliminated in the urine (10 – 15%) (PDR, 2018a). Regarding their analogs, the terminal half-lives are approximately 30 and 45 hours for epirubicin and idarubicin, respectively, and similar results to the two other previous anthracyclines, regarding their excretions, are observed (PDR, 2018c; d).

1.2.1.3. Anthracyclines toxicity

Anthracyclines are chemotherapeutic agents widely used; however, since they are not selective, can also affect normal cells and induce some negative side effects. These effects can include alopecia, hyperpigmentation (skin and nails), nausea, vomiting, mucositis and other types of gastrointestinal toxicity, increased liver function tests, myelosuppression, hematological toxicity (neutropenia, anemia and thrombocytopenia), neurotoxicity, cardiotoxicity and secondary leukemia (Seiter, 2005).

Cardiotoxicity is the main side effect of the treatment with anthracyclines, which is known since the late 1970's, so it has been extensively studied (Al-Ismail and Whittaker, 1979). In a study aimed to determine the risk of the incidence of cardiotoxicity associated with doxorubicin, 534 patients with breast cancer were analyzed (Buzdar et al., 1985). Doxorubicin cumulative doses varied according to the disease stage of the patients: patients with stage II or III had a cumulative dose limited to 300 mg/m^2 and patients in stage IV limited to 450 mg/m^2. The development of doxorubicin-associated HF was observed in 10 patients (1.87% of total patients) (Buzdar et al., 1985). In another study, the occurrence of subclinical late cardiomyopathy after successful doxorubicin treatment in adult patients with Hodgkin's or non-Hodgkin's lymphomas was analyzed (Hequet et al., 2004). The cohort included 141 patients who received different doses of doxorubicin that ranged between 250 mg/m^2 and 550 mg/m^2. Clinical cardiomyopathy was considered when signs and symptoms

of HF were observed. On the other hand, the criteria established for subclinical cardiomyopathy included the absence of clinical symptoms related with HF but with the presence of echocardiographic signs of cardiomyopathy, namely LVD with fractional shortening < 25% or two of the following alternative criteria: decrease of fractional shortening, decrease of ejection fraction or abnormal wall motion. Among the 141 patients, clinical cardiomyopathy developed in only one patient (0.71% of total patients). Regarding subclinical cardiomyopathy, 39 patients had fractional shortening < 25% and 29 patients had two of the symptoms used for the alternative criteria for subclinical cardiomyopathy (27.7% and 20.6% of total patients, respectively) (Hequet et al., 2004). These two studies clearly demonstrate the cardiotoxic potential of anthracycline therapy.

Nevertheless, despite the fact that approximately fifty years have passed since the discovery of its cardiotoxic effects, the exact mechanisms by which anthracyclines exerts cardiotoxicity remain unclear (Lefrak et al., 1973; McGowan et al., 2017). It is known that anthracyclines can induce the formation of ROS (Lipshultz et al., 2008). The semiquinone that can be formed as part of the anthracyclines' metabolism can donate an electron to oxygen, giving origin to superoxide anions (Lipshultz et al., 2008). This happens because the quinone structure of anthracyclines acts as an electron acceptor in reactions mediated by oxoreductive enzymes, such as cytochrome P450 reductase and NADH dehydrogenase (one-electron reduction) (Giantris et al., 1998). The superoxide anions can follow two pathways: direct subcellular damage or being further converted in hydrogen peroxide and hydroxyl radical (Lipshultz et al., 2008). This later is highly reactive and toxic and can react with lipids, proteins and nucleic acids, leading to lipid peroxidation and DNA damage. The heart is particularly susceptible to this mechanism because it has low levels of free radical scavenging system (catalase and glutathione peroxidase, for example), meaning that it has poor antioxidant defenses (Lipshultz et al., 2008). This way, reactive species accumulate, eventually leading to cell death (apoptosis) (Giantris et al., 1998). Originally, this mechanism was proposed as the basis for the anthracyclines-induced cardiotoxicity; however, some other hypotheses have been investigated, such as the interaction of the anthracyclines with iron (Puma et al., 2008). The semiquinone free radical reacts with Fe^{2+}, forming a complex, and its regenerated to its parental quinone form by reduction of the molecular oxygen to superoxide anion radical and hydrogen peroxide, meaning that ROS are formed (Reis-Mendes et al., 2015). The superoxide anion radical can further react with nitric oxide and subsequently forming reactive nitrogen species (RNS). In the end, all these reactions can lead to oxidative stress and damage of the cardiac mitochondrial DNA (Reis-Mendes et al., 2015). Additionally, the ROS and RNS generated can interact with the calcium release channels, resulting in myocytes damage from calcium overload (Giantris et al., 1998; Menna

et al., 2008). Moreover, cardiolipin, an important component of the inner mitochondrial membrane, has high affinity for anthracyclines, favoring the high intracellular levels of these drugs (Lipshultz et al., 2008). This way, when anthracyclines bind to cardiolipin, it leads to energy metabolism impairment and oxidative stress (Lipshultz et al., 2008).

Cytotoxicity induced by anthracyclines metabolites is also a reality (Giantris et al., 1998; Lipshultz et al., 2008; Menna et al., 2008). The alcohol metabolites can accumulate in the cardiomyocytes, resulting in dysregulation of the calcium and iron homeostasis and energy impairment with consequent oxidative stress. Anthracyclines are capable of accumulating in the heart at much higher concentrations than their extracellular concentrations; however, their metabolites are more polar than the parental drugs and, therefore, can remain in the cells more time, being less eliminated from the cardiomyocytes (Giantris et al., 1998; Lipshultz et al., 2008; Menna et al., 2008). Additionally, it has already been observed that the aglycones formed in the metabolism of anthracyclines can also be responsible for cardiotoxicity (Sokolove, 1994). These are more lipophilic than the parental drug and intercalate easily in the membranes of the cardiac mitochondria, causing mitochondrial dysfunction by modifying sulfhydryl groups and inducing calcium-independent oxidation of the mitochondrial NADH, which could lead to the production of superoxide anions and, ultimately, to oxidative stress. It can also affect the cellular respiration and cause energy metabolism impairment by diverting electrons from the normal pathways, since the electrons were redirect from NADH to oxygen (Sokolove, 1994).

Anthracyclines can also induce a decrease in the adenosine triphosphate (ATP) production by disrupting both cardiac specific gene expression of enzymes that are critical for energy production and structural gene products, such as cardiac troponins and creatine kinase (CK) (Lipshultz et al., 2008). The depletion in ATP levels reduces the ability of the cardiomyocytes to contract with efficiency, which can lead to cell death, in severe cases (Lipshultz et al., 2008). These are the most extensively explored mechanisms for anthracyclines-induced cardiotoxicity; however, other hypotheses have been considered, such as disturbances in the myocardial adrenergic function, release of vasoactive amines and the elaboration of proinflammatory cytokines (Giantris et al., 1998).

1.2.2. Mitoxantrone

MTX is a synthetic derivative of doxorubicin with a wide spectrum of action (NIH, 2015b). Since anthracyclines are difficult to synthesized and using them can bring serious adverse

effects, further research was conducted in order to produce several bis-substituted aminoanthraquinones derivatives (Ehninger et al., 1990). This way, MTX was first synthesized in 1979 and, since then, has been used for the treatment of leukemias, lymphomas, prostrate and breast cancers and, more recently, multiple sclerosis (Murdock et al., 1979; Rossato et al., 2013a). The purpose of its synthesis was to create a molecule with the same or improved benefits as anthracyclines' anticancer properties, but with none or reduced cardiotoxic effects (Minotti, 2010). This way, MTX maintains the same planar ring structure as anthracyclines, that allows the intercalation with DNA, but with an amino-containing group instead of the amino sugar (**Figure 2**). The base-idea was that the cardiotoxicity associated with anthracyclines might depend on the presence of the amino sugar (Henderson et al., 1989).

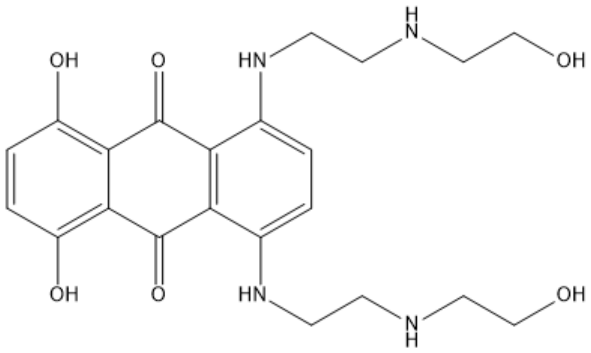


Figure 2. Chemical structure of MTX.

1.2.2.1. Mechanism of action

Despite the structural similarities with doxorubicin, MTX's mechanism of action is not exactly the same (Alberts et al., 1985). It is known that MTX is a DNA intercalating agent and a topoisomerase II inhibitor, which is an enzyme responsible for the uncoil and repairing of damaged DNA (Alberts et al., 1985; DrugBank, 2018; Drugs.com, 2017). The interaction of this chemotherapeutic drug with DNA involves two binding sites: a strong binding, through intercalation with the planar electron-rich chromophore of DNA, and a weaker one, by electrostatic interaction of its basic side chains (amino group) with the phosphate moiety of the DNA molecule (anionic exterior of the DNA helix) (Ehninger et al., 1990; Shenkenberg and Von Hoff, 1986; White and Durr, 1985). MTX is also responsible for the formation of crosslinks and strand breaks in the macromolecule (Shenkenberg and Von Hoff, 1986). Additionally, *in vitro* studies have demonstrated that MTX inhibits DNA and RNA replication

and affects the cells at various stages of their cycles. Mid-to-late-G1 and mid-to-late-G2 phases seemed to be the most affected, which precludes the cells from entering the mitosis process. Nonetheless, this cell killing mechanism is not cell-cycle specific since MTX acts in proliferating and non-proliferating cells (Ehninger et al., 1990; Shenkenberg and Von Hoff, 1986). At high concentrations, MTX can also be responsible for the inhibition of the biosynthesis of prostaglandin E2 and calcium release. Since prostaglandins are important in the metastization of cancers and in their hypercalcemia, this aspect is also significant (Ehninger et al., 1990).

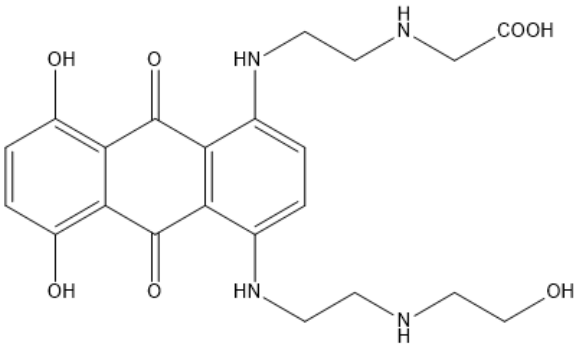
1.2.2.2. Pharmacokinetics

MTX is poorly absorbed orally, so it is commonly administered by intravenous infusion, at doses ranging from 12 to 14 mg/m^2, monthly or every three weeks, depending on the type of cancer (BC_Cancer, 2017; NIH, 2015b). The intravenous route of administration has shown to provide a rapid and extensive tissue distribution of the drug (BC_Cancer, 2017; Reis-Mendes et al., 2015; Rossato et al., 2013a). This tissue distribution is proportional to the tissue blood flow, meaning that in highly perfused tissues MTX is found in higher concentrations than in the less perfused ones (Batra et al., 1986). So, MTX concentrations are higher in the liver, heart, kidney, lung, spleen, pancreas, bone marrow, thyroid and blood components (PDR, 2018e). Regarding the brain, the amounts that could cross the blood-brain barrier are not in an appreciable extend (BC_Cancer, 2017). MTX presents a plasma protein binding of 78%, which is not affected by the presence of other drugs (Batra et al., 1986).

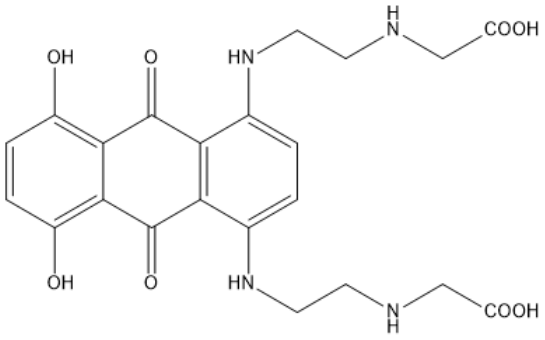
MTX's metabolism is hepatic and polar metabolites are formed; however, these pathways have not yet been clearly fully clarified (BC_Cancer, 2017; PDR, 2018e). It is known that MTX is resistant to reductive enzymatic activation; however, it can undergo enzymatic oxidation (Costa et al., 2013). The formation of its metabolites is still under investigation if it happens through one or two-electron reductions (Reis-Mendes et al., 2015). Regarding the one-electron reduction, the reaction is catalyzed by reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome *c* and NADH cytochrome b5 reductases, originating the semiquinone free radical (Reis-Mendes et al., 2015). Usually, the semiquinones are immediately re-oxidized, leading to the formation of ROS. However, MTX is not a good substrate for this type of reaction with NADPH cytochrome *c* reductase, and NADH reductase has low potential of reduction. So, the preferred pathway seems to be the two-electron reduction, which acts directly in the quinone

to form the hydroquinone (Reis-Mendes et al., 2015). In fact, some studies have already demonstrated the oxidation of MTX via two-electron reduction catalyzed by DT-diaphorase (Fisher et al., 1992; Li et al., 1995).

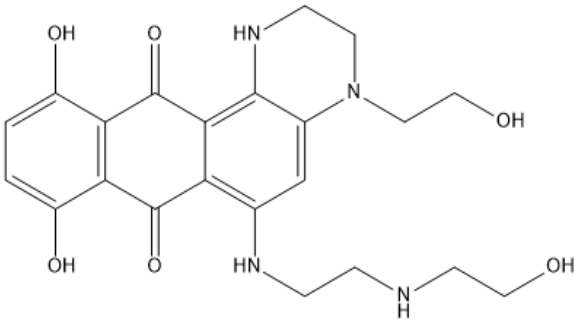
The main MTX metabolites found in human urine are the mono- and dicarboxylic acids, that result from the oxidation of the terminal hydroxyl groups of the side chains (Hrynchak et al., 2017; Reis-Mendes et al., 2015). Other metabolites were already identified in liver S9 fractions (supernatant fraction obtained from the liver homogenate centrifugation) isolated from rats, including the glucuronide conjugates of the previous acids, glutathione conjugates, acetoxy ester derivatives and a naphtoquinoxaline metabolite (8,11-dihydroxy-4-(2-hydroxyethyl)-6-[[2-[(2-hydroxyethyl)-amino]-ethyl]-amino]-1,2,3,4,7,12-hexahydronaphtho-[2,3-f]-quinoxaline-7,12-dione, NAPHT) (Hrynchak et al., 2017; Rossato et al., 2013a). Some studies stated that this latter metabolite plays an important part in the pharmacological anticancer activity of MTX and that may have anticancer properties (Mewes et al., 1993; Reis-Mendes et al., 2017). Studies with *in vitro* models, such as neonatal rat heart cells and differentiated H9c2 cells, also verified that this cyclic metabolite may be more cardio-safe than MTX (Reis-Mendes et al., 2017; Shipp et al., 1993). The chemical structures of some of the known metabolites of MTX are represented in **Figure 3**.



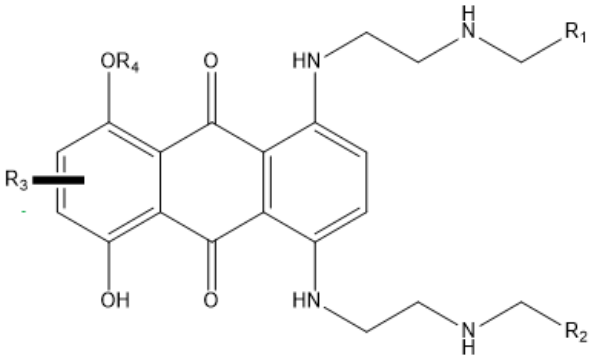
Monocarboxylic acid of MTX



Dicarboxylic acid of MTX



Naphtoquinoxaline



Other metabolites of MTX

Compounds	R_1	R_2	R_3	R_4
Glucuronide metabolite of MTX	CH_2OH-	CH_2OH-	H-	Glucuronide-
Glutathione metabolite of MTX	CH_2OH-	CH_2OH-	H-	Glutathione-
Glutathione metabolite of MTX (2)	CH_2OH-	CH_2OH-	Glutathione-	H-
Cysteine derivatives of MTX	CH_2OH-	CH_2OH-	Cysteine-	H-

Figure 3. Chemical structure of some MTX metabolites.

In general, MTX presents a clearance of 10.9 – 37.4 L/hour/m^2 (BC_Cancer, 2017). Nevertheless, it is known that MTX clearance is reduced in patients with hepatic dysfunction since the drug is predominantly handled by the liver (Batra et al., 1986). This means that a dose reduction is needed in patients with hepatic impairments and that should not be administered at all in patients with significant hepatic dysfunction (Batra et al., 1986; Rossato et al., 2013a).

Regarding its excretion, MTX presents a slow elimination phase with a half-life of 5 - 18 days (Reis-Mendes et al., 2015). The drug is excreted through the hepatobiliary (13 - 25%) and renal (6 – 11%) routes, unchanged (65%) or in the metabolized forms (BC_Cancer, 2017). In the first 5 days after administration, only 20 – 32% of the drug is excreted (Reis-Mendes et al., 2015).

1.2.2.3. Mitoxantrone toxicity

As already stated, MTX was synthetized in order to have same or improved benefits as anthracyclines' anticancer properties, but with none or reduced cardiotoxic effects (Minotti, 2010). However, cardiotoxicity related with MTX therapy has also shown to be a reality, being one of its main adverse effects (Aapro et al., 1983). Additionally, other negative effects associated with MTX treatment had also been reported, such as alopecia, nausea, vomiting, diarrhea, mucositis, hematological toxicity (neutropenia, thrombocytopenia, myelosuppression), abdominal pain, hepatic toxicity (< 1%) and secondary leukemia (in multiple sclerosis patients) (NIH, 2015b; Seiter, 2005).

Concerning the cardiac manifestations, they can vary from arrhythmias, electrocardiographic changes, HF and cardiomyopathy (BC_Cancer, 2017). The cardiotoxicity of MTX has been discovered as early as the 1980's (Cornbleet et al., 1984). Ninety-nine women with advanced breast cancer were treated with 14 mg/m^2 of MTX, every three weeks. Data from 60 patients who underwent serial electrocardiograms was analyzed and significant changes were recorded in 8 of them (13.3% of total patients), who had received cumulative doses of 112 mg/m^2, 182 mg/m^2, 238 mg/m^2 and 266 mg/m^2. Additionally, ventricular ejection fraction measurements were performed in 43 patients, where 4 of them (9.30% of the 43 patients) developed clinically significant evidences of cardiotoxicity after receiving cumulative doses of 174 – 256 mg/m^2 (Cornbleet et al., 1984). A more recent study also proven the cardiotoxic effect of MTX (Fleischer et al., 2014). Fleisher *et al.* studied the MTX-induced cardiotoxicity in 639 patients with multiple sclerosis, who received doses of 12 mg/m^2 of MTX, every 3 months. Cardiac dysfunctions were observed in 26 patients (4.07% of total patients): decrease in the left ventricular ejection fraction (LVEF) to < 50%, reduction of LVEF of ≥ 10% from the baseline value, arrhythmias, systolic dysfunction, diastolic relaxation dysfunction and pericardial effusion (Fleischer et al., 2014).

These studies demonstrate the MTX's potential to induce cardiotoxicity. However, similar to what happen with anthracyclines, the exact mechanism by which MTX exerts cardiotoxicity is not clearly defined. Despite MTX and anthracyclines share some clinical aspects of cardiotoxicity, the mechanisms responsible for it are not the same (Costa et al., 2013). Contrary to anthracyclines, MTX has little ability to form free radicals, which could result in decreased cardiotoxicity in comparison with anthracyclines (Dobson et al., 2008; Pai and Nahata, 2000). In a study that used an H2c9 embryonic heart rat-derivative cell line incubated with MTX, it was observed that this anticancer drug triggered the production of ROS, however in smaller amounts than the ones observed with doxorubicin (Corna et al., 2004). The possibility of ROS production is, however, possible since MTX's chemical structure contains a quinone group that can suffer oxidative activation and subsequently forming ROS (Li et al., 1995; Mewes et al., 1993). Nevertheless, since MTX is highly lipophilic, it can easily enter in the healthy organs, such the heart, where is highly accumulated, which can counterbalance its apparent lower ability to produce ROS through its high cardiac accumulation (Costa et al., 2013).

Mitochondrial dysfunction (aberrant mitochondria, interference on mitochondrial functionality, hyperpolarization of the mitochondrial membrane potential) seems to be the most widely accepted mechanism of MTX-induced toxicity (Albini et al., 2010; Rossato et

al., 2014). MTX can be responsible for late depletion of cardiac ATP levels (interference in the bioenergetics of the heart), resulting in an increased formation of reactive species and subsequent oxidative stress (Albini et al., 2010; Costa et al., 2013; Rossato et al., 2014). This aspect was verified in a study that used H2c9 cells incubated with MTX, where the increase in ROS' levels was only verified after a significant decrease in the ATP intracellular levels (Rossato et al., 2013b). Nevertheless, these oxidative alterations are a late event, which could be explained by the reduced ability of MTX to enter in the redox cycle (Costa et al., 2013; Rossato et al., 2013b). This energetic imbalance observed with MTX can lead to impairment in the calcium regulation cellular mechanisms, since they are ATP driven. This way, cytosolic calcium levels increase after MTX incubation, which can lead to the hyperpolarization of the mitochondrial membrane potential and, ultimately, to cell death (Rossato et al., 2013b). It is important to note that, once they suffered significant decreases in the ATP levels, the cardiac cells cannot recover its energetic levels even after cessation of MTX exposure, as already verified in a study performed by Shipp *et al.* using cultured neonatal rat cardiac cells incubated with the chemotherapeutic drug (Shipp et al., 1993). In this study, myocyte cultures were exposed to MTX (0.05 to 5 µg/ mL) for 3 hours and after that were washed out and incubated in a drug-free medium for more 72 hours. The intracellular levels of MTX were determined in several time-points, during the drug-free period. The authors observed that the ATP levels remained unchanged immediately after treatment and slowly decreased in a time-dependent manner over the next 72 hours, reaching its maximum of suppression at this point (Shipp et al., 1993).

Additionally, a decrease in the ATP-synthase expression and, especially, in its activity has been observed, which can contribute to the late ROS production (Rossato et al., 2013b). In its turn, this ROS generation can be responsible for decreases in the total glutathione (GSH) levels and increases in its oxidized form (oxidized glutathione), GSSH (Costa et al., 2013; Rossato et al., 2013b). This has consequences at the mitochondrial level since GSH is mainly concentrated in the mitochondria and exerts activity as its internal intracellular antioxidant buffer. With the GSH levels reduced, the heart is more prone to suffer oxidative stress (Rossato et al., 2013b).

On the other hand, the metabolism of MTX can also have an important role on its toxicity (Costa et al., 2013). As previous mentioned, MTX can suffer enzymatic oxidation, either by enzymatic activation through microsomal or peroxidase enzymes or in the presence of high concentrations of H_2O_2 (Costa et al., 2013). This way, despite the redox metabolites of MTX can only generate low amounts of ROS, they can efficiently bind to intracellular nucleophiles, such as GSH, leading to oxidative stress (Costa et al., 2013). Also, since the

heart forms high amounts of H_2O_2, even in non-stressful situations, the reaction with GSH is favored (Costa et al., 2013). Additionally, it was already observed that NAPHT can cause ATP depletion, such as its parental compound (Shipp et al., 1993). It has been assumed that this decrease in ATP levels is related with the hydroquinone ring present both in MTX and NAPHT, since ametantrone (AMT), an anthraquinone that does not possess this ring (**Figure 4**), did not demonstrated significant effects on the ATP levels, meaning that this ring may potentiate their cytotoxic activity (Ahn and Sok, 1996; Shipp et al., 1993).

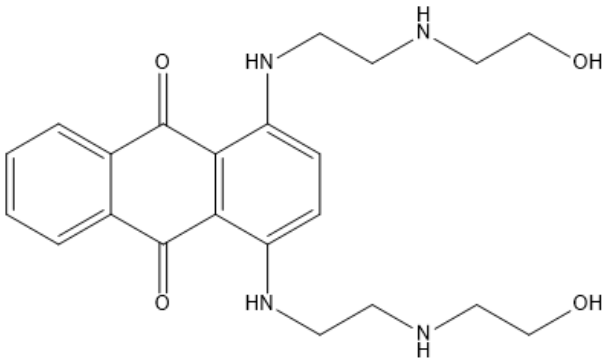


Figure 4. Chemical structure of AMT.

In conclusion, although MTX has some similarities with the mechanisms of toxicity of anthracyclines, they differ in some aspects: MTX interferes with the bioenergetics of the heart while the anthracyclines are a more redox-interfering drug (Damiani et al., 2016).

1.3. Risk factors for the toxicity induced by anthracyclines and mitoxantrone

As mentioned before, despite the great benefits associated with anticancer therapy, it can induce several negative effects. Concerning the anthracyclines and MTX, the heart has been stated as the main target of the toxicity induced by these chemotherapeutic agents, since it has limited potential for regeneration, as previously mentioned, which can lead to serious long-term effects (Reis-Mendes et al., 2015). The severity of the effects on the cardiovascular system depends on many factors that vary according to the type of therapy, the patients' genetic predisposition and if some or several risk factors are present (Adão et al., 2012).

Some of the risk factors that have been studied to influence the cardiotoxicity induced by anthracyclines and MTX are the dose, route of administration, dosing schedule, age, gender, pre-existing cardiovascular diseases/ comorbidities, previous or concomitant treatment with other cardiotoxic drugs and radiotherapy. Nevertheless, it is important to keep in mind that these are not the only risk factors that may be involved in the development of cardiotoxicity. In **Table 2**, the most studied risk factors for the toxicity induced by anthracyclines and MTX therapies are presented. For systematic purposes, it will only be analyzed with further detail the dose and age as risk factors for the cardiotoxicity induced by these chemotherapeutic drugs, since dose is the most studied acknowledged risk factor and age is the factor studied in this dissertation.

Table 2. Some risk factors for the toxicity induced by anthracyclines and MTX therapies. Adapted from Adão *et al.*, 2012, Reis-Mendes *et al.*, 2016, Watts *et al.*, 1991 and Lipshultz *et al.*, 2013.

Risk factors	Increased risk of toxicity
Cumulative dose	The higher the cumulative dose, greater the risk
Age	Pediatric and elderly populations
Gender	Female
Pre-existing comorbidities	Cardiovascular diseases, hypertension
Route of administration	Rapid intravenous administration
Combination therapy	Previous or concomitant administration of other drugs, such as trastuzumab or cyclophosphamide, for example
Radiotherapy	Previous or concomitant
Electrolyte disturbances	Hypocalcemia, hypomagnesemia

1.3.1. Dose

The different administered doses and its effects on the development of the cardiotoxicity induced by anticancer therapy has been extensively evaluated and dose has been considered the most important risk factor related with cardiotoxicity. Whenever dose is

evaluated as a risk factor, one may refer to the cumulative dose or to a single high dose. The “total amount of drug given to a patient over time” is defined as cumulative dose while a single dose is the “amount of drug taken at one time” (NIH, 2015a). With this in mind, it has been observed that cumulative dose can be a risk factor for the cardiotoxicity induced by anthracyclines and MTX. Taking the example of the study performed by Krischer *et al.*, 6493 children undergoing chemotherapy with doxorubicin for the treatment of leukemia, lymphoma, brain tumor, among others, were evaluated. Patients received different cumulative doses: 479 patients were administered with cumulative doses of 1-99 mg/m^2, 2045 with 100-199 mg/m^2, 1049 with 200-299 mg/m^2, 1613 with 300-399 mg/m^2, 1055 with 400-499 mg/m^2 and 252 received ≥ 500 mg/m^2. It was observed that 106 patients (1.63% of total patients) developed cardiac events and that this incidence was more notorious with higher cumulative doses: 1.56% of the patients who received cumulative doses < 300 mg/m^2 developed cardiotoxicity, in comparison with the 2.80% of the patients who received ≥ 500 mg/m^2. Through univariate and multivariate analyses, it was verified that high cumulative doses (≥ 550 mg/m^2) were statistically significant and responsible for higher cardiotoxicity (Krischer et al., 1997).

Studies with other anthracyclines have also been conducted and it has been observed that cardiotoxicity arises at different cumulative doses than doxorubicin. Taking the example of epirubicin, it is known that it presents a faster metabolization than doxorubicin and, therefore, a faster elimination (Minotti, 2010; Reis-Mendes et al., 2015). The metabolization is mainly by conjugation with glucuronide acid, so higher doses need to be used to have similar therapeutic effects (Minotti, 2010; Reis-Mendes et al., 2015). Despite epirubicin-induced cardiotoxicity occurs at higher cumulative doses, it has the same clinical meaning as the cumulative doses used with doxorubicin therapy, as stated in the study performed by Perez *et al.* (Perez et al., 1991). The authors compared these two anthracyclines as single agents in treatment against advanced breast cancer and observed that doxorubicin and epirubicin’s doses of 60 mg/m^2 and 90 mg/m^2, respectively, produced equivalent toxicities (Perez et al., 1991). Additionally, the cardiac events were similar in both drugs and the response rates (47% for doxorubicin and 49% for epirubicin) and the probability of survival (12 months for doxorubicin and 10 months for epirubicin) were not significantly different. So, the higher total dose of epirubicin used for anticancer treatment has no advantages over the dose of doxorubicin for the treatment of breast cancer (Perez et al., 1991).

Likewise, it has been observed an exponential increase, with increasing cumulative doses, of the risk of developing cardiotoxicity with the remaining anthracyclines (Adão et

al., 2012; Reis-Mendes et al., 2015). This way, in order to assist the health professionals in making decisions such whether to administer more drug or not during patient's lifetime, maximum recommended cumulative doses were established (**Table 3**) (Adão et al., 2012; Reis-Mendes et al., 2015). The values differ among the chemotherapeutic drugs due to their different pharmacokinetics, as explained before.

Table 3. Recommended maximum cumulative doses for the most commonly used anthracyclines. Adapted from Adão et al., 2013 and Reis-Mendes et al., 2015.

Anthracyclines	Maximum cumulative doses
Doxorubicin	400-550 mg/m^2
Epirubicin	900-1000 mg/m^2
Daunorubicin	550-800 mg/m^2
Idarubicin	150-225 mg/m^2

Regarding MTX, cumulative dose has also been studied as a potential risk factor for its induced-cardiotoxicity. Taking the example of Rivera *et al.*, 509 patients with multiple sclerosis, who received 12 mg/m^2 of MTX every 3 months, until reaching a cumulative dose of 140 mg/m^2 (unless discontinuation or interruption of therapy or the appearance of side effects that prevented further treatment) as part of their treatment, were evaluated (Rivera et al., 2013). The authors observed that, among the 97 patients who developed cardiotoxicity (19.1% of total patients), 6.19% of them received ≤ 35 mg/m^2, 19.6% received 36-75 mg/m^2, 34% received 76-105 mg/m^2 and 40.2% received > 105 mg/m^2 of MTX. This way, the observed cardiotoxicity between patients who received ≤ 75 mg/m^2 and the ones who received > 75 mg/m^2 reached statistical significance. Additionally, it was verified that the mean cumulative dose was higher (93.9 mg/m^2) than in the ones who did not develop cardiac events (63.7 mg/m^2). So, MTX higher cumulative doses showed to induce higher cardiotoxicity (Rivera et al., 2013). Similarly to anthracyclines, a recommended maximum cumulative dose for MTX was established to 100 – 140 mg/m^2 (Minotti, 2010). Since MTX is more lipophilic than anthracyclines, it has a higher ability to accumulate in tissues, therefore lower doses are required for its anticancer clinical use (Minotti, 2010).

Nevertheless, despite the established recommended maximum cumulative doses, cardiotoxicity of both anthracyclines and MTX has been described even at lower cumulative

doses (Fleischer et al., 2014; Lipshultz et al., 2005). This aspect makes it hard to deal with the idea of a “safe” cumulative dose (Lipshultz et al., 2005; Von Hoff et al., 1977).

1.3.2. Age

Alongside with the cumulative dose, patients’ age at treatment has also shown to be a major factor for cardiotoxicity development after treatment with anthracyclines and MTX. Starting again with anthracyclines, elderly patients (≥ 60 years) and children are particularly at risk of developing cardiac events when are being treated with these chemotherapeutic drugs (Minotti, 2010; Praga et al., 1979; Von Hoff et al., 1979). Concerning the latter, the susceptibility to cardiotoxicity is higher in the younger infants, namely the ones with less than 4 years old (Lipshultz et al., 1991; Minotti, 2010).

Concerning the elderly, patients with ≥ 65 years were often excluded from clinical trials and, when included, only the healthiest of them received anthracycline therapy and in lower cumulative doses than the younger patients (Pinder et al., 2007). This happened because clinicians usually are more concerned about toxicity and efficacy of treatment in advanced age, overall higher incidence of comorbidities, lower life expectancy, socioeconomic status, lack of social support, patient reluctance, among others, which may also contribute to lower compliance. This way, elderly people are generally underrepresented in clinical trials (Jensen et al., 2006). Nevertheless, this factor has been taken into consideration and it is already possible to find more studies reporting the increased risk of elderly people to develop cardiotoxicity after anticancer treatment. For example, Swain *et al.* evaluated the incidence of HF in 630 patients with breast or lung cancers, who had received doxorubicin as their treatment: 50 mg/m^2 every 3 weeks (Swain et al., 2003). LVEF was assessed at baseline and after doxorubicin at cumulative doses of 150 mg/m^2, 300 mg/m^2, 400 mg/m^2, 500 mg/m^2 and at every dose increment of 50 mg/m^2 thereafter. Patients’ age ranged between 23 and 82 years old: 458 patients had ≤ 65 years and 172 had > 65 years (172 patients). Cardiotoxicity was observed in 32 patients that had received ≥ 300 mg/m^2 (cumulative dose): 22 had ≤ 65 years old (4.80% of the younger patients) and 10 had > 65 years old (5.81% of the older patients). There were no significant differences between the two groups of age at lower cumulative doses of doxorubicin; however, after cumulative doses > 400 mg/m^2, the differences reached statistical significance, where the incidence of HF was increasingly higher in older patients (age > 65 years), as can be seen in **Figure 5** (Swain et al., 2003).

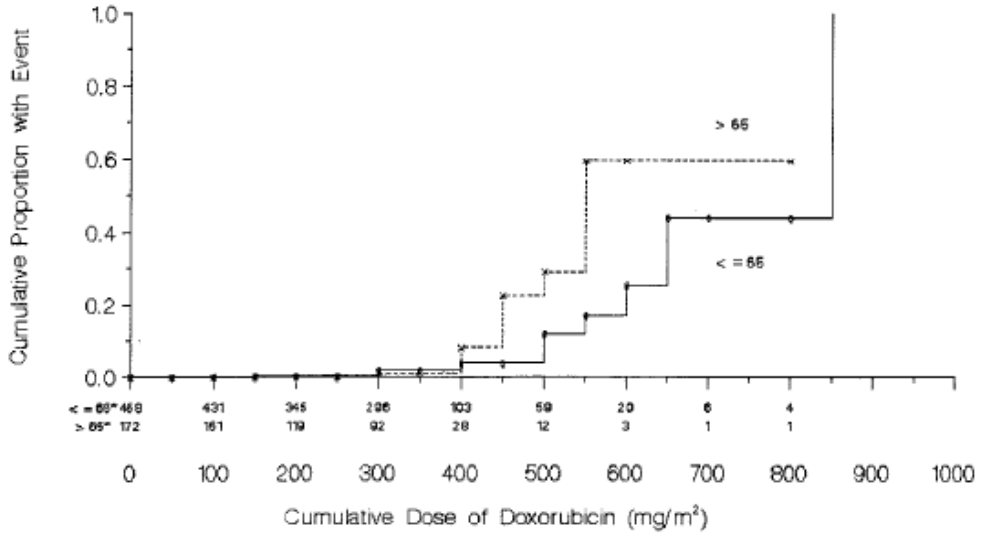


Figure 5. Risk of doxorubicin-induced cardiotoxicity at a given cumulative dose according to patients' age. Taken from Swain *et al.*, 2003.

Regarding children, treatment with anthracyclines significantly improved the disease-free survival of cancer in children but associated cardiotoxicity is still a reality (Krischer et al., 1997). In one study, Von Hoff *et al.* evaluated the risk factors for daunorubicin-induced cardiotoxicity, namely the patients' age. In order to do so, they analyzed 5613 patients with acute lymphocytic and acute myelocytic leukemias who received cumulative doses of daunorubicin up to 2000 mg/m^2 as their treatment. Cardiotoxicity was observed in 110 patients (1.96% of total patients): 65 presented HF and 45 electrocardiographic changes as a single manifestation. In order to evaluate the results, the authors divided the patients in two groups concerning the cardiac event they presented: the patients with signs and symptoms of HF and the ones with electrocardiographic changes (single manifestation) most likely related with daunorubicin. After this division, patients were separated according their age: 2861 children (< 15 years) and 2752 adults (≥ 15 years). Children with HF were analyzed separately from those with electrocardiographic changes, and the same happened with the older group. Concerning the group of patients who presented electrocardiographic changes as a single manifestation, 14 were children (0.49% of total children) and 31 were adults (1.13% of total adults). These differences were not statistically significant regarding patients age. However, these results are not conclusive because patients were not monitored closely with electrocardiograms during or immediately after the administration of daunorubicin (electrocardiograms were obtained at random times), and the electrocardiographic changes could be transient, due to disease progress or

complications and not to the daunorubicin treatment itself. Regarding the 65 patients with HF, 46 were children (1.61% of total children) and 19 were adults (0.69% of total adults), which corresponds to 70.8% and 29.2% of the reported cases of cardiomyopathy, respectively. Afterwards, the authors made a graphic representation of the incidence of HF (%) *versus* the total administered dose for the children and adults' groups (**Figure 6**) and verified that the differences between the slopes for these two groups were statistically significant and were increasingly higher with increasing doses, which means that children demonstrated to be more susceptible to daunorubicin-induced cardiotoxicity than adults, at equivalent doses (Von Hoff et al., 1977).

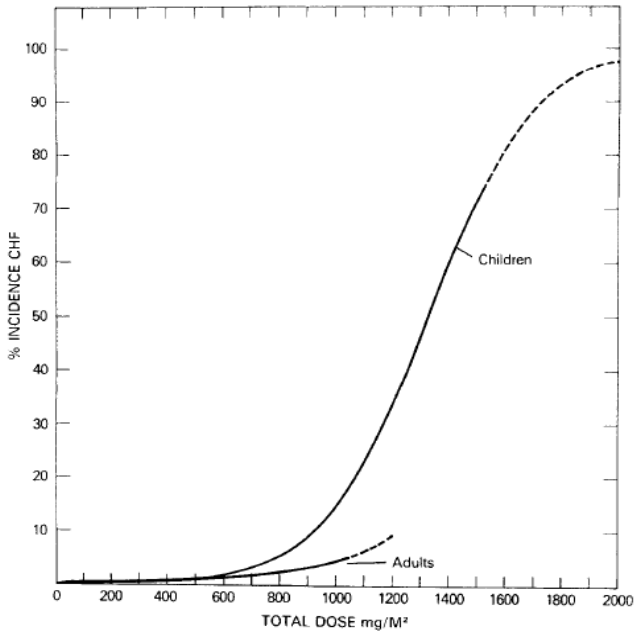


Figure 6. Incidence of HF (%) *per* total administered cumulative dose of daunorubicin in children and adults. The dashed lines represent the extrapolation of the curves. The curve concerning adults could not be further extended due to the small number of patients receiving higher doses. Taken from Von Hoff *et al.*, 1977.

In another study, Lipshultz *et al.* evaluated the late cardiotoxicity (1 to 15 years after successful treatment) induced by doxorubicin therapy in childhood cancer (Lipshultz et al., 1991). In order to do so, they analyzed 115 children with acute lymphoblastic leukemia who received different chemotherapeutic regimens: 18 patients received a single dose of doxorubicin (45 mg/m^2) and 97 received multiple doses (cumulative dose ranged from 228 to 550 mg/m^2). At the time of cancer diagnosis, patients' age ranged between 7 months and 19.1 years: 38 had < 4 years, 26 were 4 to 7 years, 18 had between 8 and 11 years and 15 had ≥ 10 years old. Cardiotoxicity developed in different forms. Left ventricular contractility was decreased in 22 patients: 12 had < 4 years (31.6% of the patients within this age range),

4 had between 4 and 7 years (15.4% of the patients within this age range), 2 had between 8 and 11 years (11.1% of the patients within this age range) and 4 had ≥ 10 years old (26.7% of the patients within this age range). On the other hand, increased afterload, which was attributable to reduced left ventricular wall thickness and that contributed to myocardial deterioration, was the most common abnormality found and it was observed in 57 children: 30 had < 4 years (78.9% of the patients within this age range), 11 had 4 to 7 years (42.3% of the patients within this age range), 8 had between 8 and 11 years (44.4% of the patients within this age range) and 8 had ≥ 10 years old (53.3% of the patients within this age range). Patients with reduced contractility presented severe myocardium injury; however, the authors could not evaluate if it was due to the acute doxorubicin toxicity, to the excess afterload or to a combination of both. Through an univariate analysis, age < 4 years at the time of treatment was found to be a risk factor for the incidence of late cardiac abnormalities and it was a significant predictor for increased afterload. Through a multivariate analysis, age at the time of treatment remained a significant risk factor for increased afterload. When the authors restricted the multivariate analysis to the patients who received doses of at least 228 mg/m^2 of doxorubicin, age < 4 years at the time of treatment was still considered a significant factor for predicting increased afterload. This cardiac event contributed to myocardial deterioration in children treated with doxorubicin and it affected especially the younger patients, possibly because they need higher proportions of myocardial growth to reach adult proportions (Lipshultz et al., 1991).

Regarding MTX, age has also been studied as a potential risk factor for the toxicity induced by this chemotherapeutic drug. However, this factor is not so well described with MTX. Considering the studies available, the elderly population seemed to be at increased risk for developing toxic effects induced by MTX-treatment, namely cardiotoxicity. For example, in a study performed by Rigacci *et al.*, 145 patients with non-Hodgkin lymphoma received a chemotherapeutic regimen that contained MTX, at a cumulative dose of 9 mg/m^2 (Rigacci et al., 2003). All patients had more than 64 years old. Several toxic side effects related with the treatment were observed, including cardiotoxicity in 2 patients (1.38% of total patients), who end up dying (Rigacci et al., 2003). In another study, Rivera *et al.* evaluated the long-term safety of MTX in patients with multiple sclerosis (Rivera et al., 2013). The cohort included 509 patients who received 12 mg/m^2 of MTX, every 3 months, until reaching a cumulative dose of 140 mg/m^2 (unless discontinuation or interruption of therapy or the appearance of side effects that prevented further treatment). Patients' age ranged between 19 and 68 years old. Cardiotoxicity related with the treatment was observed in 97 patients (19.1% of total patients) and a tendency for an increased incidence of cardiac effects was observed with increasing age (Rivera et al., 2013). So, MTX-induced

cardiotoxicity in the elderly population has demonstrated to be a reality; however, this aspect needs to be further investigated.

Concerning the childhood cancers, the 5-year survival rate increased from 30% to 80% in the last 40 years (Chen et al., 2011). Nevertheless, the incidence of late toxicity induced by MTX therapy in these patients is a reality, which makes them at a high risk of mortality when reaching adulthood. In fact, data regarding the United States population demonstrated that the lifespan of childhood cancer survivors may be shortened in average 10 years, when compared with the general population (Chen et al., 2011). Additionally, it has been verified that despite the childhood cancer representing less than 2% of the total number of cancers, it is the main cause of death in children older than 1 year (Vassal et al., 2013). Cardiotoxicity remains one of the most important side effects for childhood cancer survivors (Chen et al., 2011). A systematic review performed by van Dalen *et al.* showed that the incidence of symptomatic cardiotoxicity induced by MTX-treatment, namely HF, can reach up to 6.7% among children, and that asymptomatic cardiotoxicity can reach 80% (van Dalen et al., 2004).

Several studies had already demonstrated the toxicity induced in children after MTX therapy. For example, Pratt *et al.* evaluated 98 patients with several advanced malignant solid tumors who received a cumulative dose of 18 mg/m^2 of MTX, every 3 weeks, has part of their treatment (Pratt et al., 1986). All patients had less than 25 years at the time of the diagnosis of their childhood malignant solid tumor. Cardiotoxic effects were observed: 3 patients had their therapy discontinued due to the presence of abnormal LVEF or multigated acquisition scans and another 2 died from cardiomyopathy induced by the treatment (Pratt et al., 1986). In another study, 66 pediatric patients with acute myeloid leukemia were evaluated (Dahl et al., 2000). Their chemotherapeutic regimen contained MTX: 6 mg/m^2 for 5 days. Children had ages comprehended between 1.4 months and 20 years-old. When assessing the toxic response of the treatment, the authors observed several side effects, such as cardiotoxicity. Regarding the cardiac events, 15 patients had decreased LVEF or shortening fraction, in which 3 of them developed HF. Also, a 19-month-old boy who presented elevated creatinine serum levels also developed HF and end up dying (Dahl et al., 2000). A study conducted by Tan *et al.* evaluated 34 children with acute myeloid leukemia, who received MTX as part of their chemotherapeutical regimen (cumulative doses not specified) (Tan et al., 2007). Patients' age varied from 2 months and 15 years. The side effects due to MTX treatment expressed in several ways, such as cardiotoxicity: 2 patients developed HF and another 5 presented asymptomatic LV systolic disfunction (abnormalities in the fractional shortening) (Tan et al., 2007). As it can be seen, MTX-

induced toxicity in childhood cancer is a reality and it has been extensively reported. Nevertheless, there is a lack of studies making the comparison of the side effects of this chemotherapeutic drug between different aged groups, as it is seen in the case of the elderly population.

1.4. Mitoxantrone toxicity in experimental models

Experimental models have been used to study the toxic effects induced by MTX therapy and its mechanisms of toxicity. Whether they are cells (*in vitro*) or animals' models (*in vivo*), both are essential for biological researches. Focusing on *in vivo* studies, it is important to keep in mind several aspects, such as the route, volume and frequency of administration, the dosing schedule and the possible adverse effects (Turner et al., 2011). It is possible to find in the literature several reports of the toxic effects induced by MTX treatment in animal models. Concerning the potential cardiotoxic effects induced by MTX therapy, one study reported the chronic cardiotoxicity in an animal model, where female BALB/c mice were used and submitted to a weekly injection of 0.2 mg/kg of MTX, over a 12-week period (cumulative dose of 2.4 mg/kg) (Alderton et al., 1992). The animals were sacrificed one week after the last injection. Through electron microscopy, the authors observed that MTX was cardiotoxic in this animal model, inducing morphologic changes to the heart: mitochondrial swelling, vacuolation and swelling of the cisternae of the sarcoplasmic reticulum, among others (Alderton et al., 1992). In a report by Viglione *et al.*, the possible acute cardiac effects of MTX were assessed in rats (Viglione et al., 1993). In order to do so, rat atria from male Wistar rats were isolated and exposed to different concentrations of MTX (10^{-6} – 10^{-4} M). The authors observed that, with the highest concentration of MTX (10^{-4} M), the spontaneous frequency of rat atria was significantly decreased (negative chronotropic effect) after 120 minutes of incubation. This means that the effect promoted by MTX (decreased spontaneous rate in rat atria) was concentration-related and time-dependent. Concerning the positive chronotropic effect, significant effects were observed with MTX concentrations of 10^{-5} M after 120 minutes of incubation. The effects were even more accentuated after exposing the same concentration of MTX for 150 minutes. With this study, the authors verified that this chemotherapeutic drug could induce acute cardiac effects (Viglione et al., 1993).

In a more recent study, Rossato *et al.* evaluated the mitochondrial effects induced by MTX in male Wistar rats treated 2.5 mg/kg of MTX, at days 0, 10 and 20, for 3 cycles (cumulative dose of 7.5 mg/kg) (Rossato et al., 2014). The rats were sacrificed in two

different moments: one group 22 days after the beginning of the experiment and another one on day 48, in order to evaluate the early and late cardiotoxicity of MTX, respectively. The authors observed a decrease in the total-CK and creatine kinase-MB (CK-MB) plasma levels in the first euthanized group. In the second one, it was observed an increase in the lactate plasma levels. Both groups presented increased cardiac relative mass and microscopic changes. Also, MTX treatment induced mitochondrial effects in both groups: in the early-sacrificed rats, the activity of the mitochondrial complexes was increased, while in the late-sacrificed rats this activity was decreased and accompanied with a reduction in the ATP content. Additionally, there were no significant changes in the water and food intakes; however, it was observed a reduction in the relative body weight gain of both groups, meaning that this effect was a side effect of the treatment with MTX (Rossato et al., 2014). In another study, male CD-1 mice were injected with a cumulative dose of 9.0 mg/kg of MTX, administered through six intraperitoneal injections, twice a week (Dores-Sousa et al., 2015). The animals were sacrificed 24 hours or 20 days after the last administration, in order to evaluate the early and late cardiotoxicity, respectively. Also, the authors had the purpose to evaluate age as a risk factor for the MTX-induced toxicity, so infant (3-week old) and adult (8 – 10-week old) mice were used. Concerning the group of mice evaluated for early cardiotoxicity, significant decreases in plasma CK-MB levels were verified in the infant mice. Through histology, the authors observed significant damages in both age groups; however, the damages were higher in the adults. Also, adult mice presented higher values of cardiac glutathione disulfide (GSSG) and protein carbonylation. However, the levels of cardiac noradrenaline were lower than the observed in the infant population. Regarding the group of mice sacrificed 20 days after the last administration, it was verified that only 2 infant mice survived (no adult reached the end of this protocol). These two mice presented high plasma aspartate aminotransferase/ alanine aminotransferase (AST/ ALT) and GSH/ GSSG ratios, in comparison with the control group. This way, this study demonstrated that adult mice were more susceptible to the cardiotoxicity induced by MTX treatment. Infant mice seemed to be more resilient to the cardiac damage; however, cardiotoxicity in this age group also showed to be a reality (Dores-Sousa et al., 2015).

CHAPTER 2.

Aims

2. AIMS OF THE STUDY

Chemotherapeutic treatment with MTX is an asset; however, the toxicity of this drug towards the human system has been described over the years, especially cardiotoxicity. Nevertheless, the exact mechanisms by which MTX exerts its toxicity are not yet fully understood.

Additionally, it is known that the presence of some factors could aggravate the toxic effects induced by this chemotherapeutic drug, such as patients' age at diagnosis. Some studies had already demonstrated the susceptibility of infant and elderly populations to MTX-therapy; nevertheless, there is not yet a study that compares the possible negative effects of this treatment in the overall population stratified by ages or the differences of pharmacokinetics among differently aged population.

With this in mind, the main purpose of this work was to evaluate the effects of the MTX treatment and to assess the possible differences on the metabolic profile of MTX, in three different aged CD-1 male mice (infants, adults, elderly). The chemotherapeutic regimen consisted of multiple administrations, at different time points, in order to mimic the human anticancer therapy. After the last administration, the animals were kept in a drug-free period over one or two weeks, approximately, to evaluate the late overall toxicity and cardiotoxicity. To achieve the purposed objectives, the heart tissue was analyzed through light microscopy and the plasma samples were used for biochemical determinations (plasma biomarkers levels) and analyzed by high performance liquid chromatography (HPLC) for determinations of MTX and NAPHT, one of its metabolites.

CHAPTER 3.

Material and Methods

3. MATERIAL AND METHODS

3.1. Chemicals

Sodium chloride (NaCl) and dimethyl sulfoxide (DMSO) were purchased from VWR Chemicals (Leuven, Belgium), phosphate-buffered saline from Biochrom (Cambridge, UK) and formic acid from Merck (Darmstadt, Germany). Acetonitrile and methanol, both HPLC gradient grades, were obtained from Fisher Scientific (Loughborough, UK), while MTX dihydrochloride (≥ 97% purity), L-ascorbic acid, citric acid and sulfosalicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). AMT was purchased from DC Chemicals (Shangai, China). NAPHT was synthetized at the Laboratory of Organic and Pharmaceutical Chemistry (Chemistry Department) of the Faculty of Pharmacy of the University of Porto, according to the method described in Reis-Mendes *et al.* (Reis-Mendes et al., 2015). Isoflurane (Isoflo® 100% p/p) was obtained from Abbott Animal Health (North Chicago, IL, USA) and the ABX Pentra reagents were purchased from HORIBA (Kyoto, Japan).

3.2. Animals

Male CD-1 mice (*Mus Musculus*) were created and kept in the vivarium of Abel Salazar Institute of Biomedical Sciences (ICBAS), where they were always accompanied by experienced veterinarians. The animals were housed in IVC Sealsafe plus mouse Green Mouse 500 cages, in a maximum of three mice *per* cage, in a temperature (22 ± 2ºC) and humidity-controlled environment. Also, a 12-hour light-dark cycle was followed. Food and water were provided *ad libitum*. The cages were changed once a week and enriched with rolls and paper so that the mice could build their nests, accommodate themselves and maintain their social behaviors. General welfare was observed through the all experiment and a “pain scoring system”, that was elaborated according to the drug at use and approved by the Organ Responsible for Animal Welfare (ORBEA) of ICBAS and by the Portuguese General Directorate of Food and Veterinary (DGAV) (**Table 4**), was followed (Lloyd and Wolfensohn, 1999). If the sum of the values was between 6 and 9 points, the general welfare of the animals was assessed several times a day. If the values were between 9 and 12 points, an urgent intervention was recommended in order to avoid further suffering. The experiments considered the general welfare of the animals (minimized stress and suffering) and were approved by the ORBEA of ICBAS, University of Porto (process n. º 140) and the

Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (process n. º 021322 of 2016/10/26).

Table 4. General distress scoring sheet implemented for the evaluation of the general welfare of mice during all the experiment, based on Lloyd and Wolfensohn, 1999.

Criteria for the animals' supervision		Scoring
General State	Animal alert, active, normal posture and coat	0
	Some apathy, immobility, decreased grooming	1
	Apathy, chromodacryreia (CDR), loss of grooming	2
	Immobility, isolation, abnormal posture, piloerection	3
	Animal in decubitus, little reactive	4
Corporal Conditions	Normal growth (infants)/ normal weight (adults/ elderly)	0
	Do not grow up according to the curve (infants)/ maintain weight (adults/ elderly)	1
	Growth under the curve (juvenile)/ ↓ weight ~ 5%/ 48h (adults/ elderly)	2
	No growth (infants)/ ↓ weight ~ 10%/ 24h (adults/ elderly), dehydration ~ 5%	3
	↓ Weight (infants)/ ↓ weight > 15%/ 24h (adults/ elderly), dehydration ~ 8%	4
Clinical Parameters	Normal temperature (**T**), mucous membranes (**M**), respiratory frequency (**RF**)/ cardiac frequency (**CF**)	0
	T; **M**; **RF** or **CF** with little alterations	1
	Irregular **T**; **M**; **RF** or **CF** or with little alterations	2
	T > or < 2ºC; pale/ cianosated **M**; irregular **RF** or **CF** (↓ or ↑)	3
	T > or > 4ºC; very altered **M**; **RF** or **CF** very altered (↓ or ↓, abdominal or noisy respiration, orthopneic position, etc.)	4
Intraperitoneal administration		
Response	Without vocalization or general discomfort (**GD**)/ without abdominal pain (**AP**)	0
	Do not vocalize; **GD** (transitory immobilization, local irritation); **AP** < 5 minutes	1

Intraperitoneal administration		
Response	Vocalizes; evident **GD**; complete/ reversed with analgesia **AP** < 10 minutes	2
	Vocalizes; evident **GD**; partial/ reversed with analgesia **AP** > 10 minutes	3

In order to fulfil the purpose of this work and, therefore, evaluate age as a risk factor for the toxicity induced by MTX therapy, mice with different ages where used: 4 weeks, 3.5 months and approximately 1.5 years, at the beginning of the administrations. According to the literature, these age groups correspond to an infant, adult and elderly human populations, respectively (Dutta and Sengupta, 2016). With approximately 4 weeks, the mice were in the beginning of the puberty phase and, in this development phase, 1 human year corresponds to 3.65 mice days, which means that, in the beginning of the administrations, the age of the infant mice corresponded to approximately 8 human years. Regarding the adult mice, it is known that they reach sexual maturity at an average age of 10 weeks, while in humans this phase corresponds to an average age of 20 years. In this phase, 2.60 mice days correspond to 1 human year. Since the mice had 3.5 months (approximately 15 weeks) at the beginning of the administrations, they had clearly reached the adulthood phase (40 human years). Regarding the elderly population, the mice reach reproductive senescence at an age of 10 – 15 months and, in this phase, 8.82 mice days are equivalent to 1 human year. Since the mice had 1.5 years (18 months) at the beginning of the administrations, which corresponds to 61 human years, these animals were a good model to portrait the human elderly population (Dutta and Sengupta, 2016).

3.3. Study design

As mentioned above, three different age groups were studied in order to assess age as a risk factor for MTX-induced toxicity and subsequent metabolic profile. There were 12 infant mice and 10 elderly. Concerning the adults, two trials were made: the first one with 14 animals (assay 1) and the second with 20 (assay 2). In each one of the age groups, the animals were equally divided in two groups according to the drug that they received: control or MTX group. Concerning the MTX group, the animals were submitted to a total of six intraperitoneal injections of 1.0 mg/kg of MTX, twice a week, receiving a cumulative dose of 6.0 mg/kg. The MTX dihydrochloride solution was prepared in sterile NaCl 0.9% (saline

solution) in a concentration of 0.1 mg/mL. Regarding the control group, mice received six intraperitoneal injections of saline (NaCl 0.9%), also twice a week, in equivalent volume as the MTX-treated mice. The injections were given in the beginning of the afternoon because MTX has proven to be better tolerated at this point due to the circadian rhythm differences in the clinical toxicity of this drug (Lévi et al., 1994). Even though the intravenous route is being used in humans, it has a high risk of extravasation of the drug, which leads to tissue necrosis. Therefore, the intraperitoneal one was chosen since the administration is easier (large absorption area) and causes less pain to the mice. Moreover, a previous study shown that similar toxicity of MTX to the one observed in humans can be seen by this route (Dores-Sousa et al., 2015). The injections were alternated on each side of the abdomen in order to avoid extra injury and pain. After the last administration, the animals were kept in a drug-free period until sacrifice to allow the development of toxicity since it has been observed that in humans the adverse effects of MTX administration may occur years after therapy ended. The animals' weight, food and water consumptions and general welfare were recorded through the whole experiment. The consumptions *per* animal were calculated based on the body weight, since the animals were maintained in a social environment.

This schedule of administration was chosen in order to mimic the human anticancer therapy (multiple administrations at separated time-points) and it permitted to evaluate the toxicity induced by a cumulative dose, over time, instead of an acute response to the drug. An allometric scaling was used in order to ensure that the administered cumulative dose in mice does not exceed the maximum recommended cumulative dose for human MTX therapy (Curry et al., 2010; Hayes, 2001). In practical terms, the conversion of a specific animal dose (in mg/kg) to a human dose (in mg/kg) is made applying a formula that includes the body weight of both animal and human, in the same units (**Figure 7**) (Hayes, 2001).

$$\text{Human dose (mg/kg)} = \text{animal dose (mg/kg)} \times \left(\frac{\text{animal body weight}}{\text{human body weight}}\right)^{1/4}$$

Figure 7. Equation to convert the dose administered in animal models into human dose, both in mg/kg. Taken from Hayes, 2011.

Then, to convert the mg/kg in mg/m^2, it is only necessary to multiply the value obtained in the previous equation for 37, that corresponds to the conversion factor between mice and human, to calculate the equivalent dose among the units previous mentioned (**Table 5**) (Curry et al., 2010).

Table 5. Equivalent surface area dosage conversion factors. Taken from Curry et al., 2010.

Species	Body weight (kg)	Body surface area (kg m^{-2})	Factor* (k_m)	Approximate human dose equivalent
Mouse	0.02	0.0067	3.0	1/12
Rat	0.100	0.0192	5.2	1/7
Dog	8.0	0.400	20	1/2
Monkey	2.5	0.217	11.5	1/3
Human	60	1.62	37	N/A

In **Table 6**, is presented the data for the conversion of a cumulative dose of 6 mg/kg of MTX in mice to the equivalent cumulative dose in humans, through the implementation of the calculations previous mentioned. The weights presented for animals and humans were an estimative from the general body weight of the infant, adult and elderly populations.

Table 6. Application of the allometric scaling for converting a cumulative dose in mice (6 mg/kg) to its equivalent in humans (mg/m^2). General infant population's weight was considered 0.020 kg and 50.0 kg for mice and humans, respectively. The general adult population's weight was considered 0.045 kg and 70.0 kg for mice and humans, respectively. Likewise, the general elderly population's weight was considered 0.060 kg and 70.0 kg for mice and humans, respectively.

Animal dose (mg/kg)	Animal body weight (kg)	Human body weight (kg)	Human dose (mg/kg)	Human dose (mg/m^2)
6	0.020	50.0	0.849	31.4
	0.045	70.0	0.955	35.3
	0.060	70.0	1.03	38.0

3.4. Sacrifice

The animals were sacrificed 17 or 7 days after the last administration (infants and adults/ elderly, respectively). At this point, the infants, adults and elderly had 2 months, 4 months and 19 months, respectively, which means that the younger mice had already reached puberty (approximately 15 human years) (Dutta and Sengupta, 2016). The criteria used for sacrifice was the presence of diarrhea and ascites, loss of weight or general distress, according to the pain scoring system (9 – 12 points) (**Table 4**). This way, the infants were sacrificed at a later time point than the other two age groups. The mice were anesthetized

by isoflurane inhalation and were sacrificed by exsanguination, since it is an easy and quick technique to use and recommended for rodents. The abdomen was opened and blood was collected through the inferior vena cava with heparinized needles, for ethylenediaminetetraacetic acid (EDTA)-containing tubes (anticoagulant), as recommended for the biochemical analyses and MTX preservation (WHO, 2002). The tubes with blood were put under constant agitation until centrifugation at 910 *g* and 4ºC during 10 minutes (slow deceleration). Then, the supernatant (plasma) was carefully removed and separated into two tubes, one of them containing 10% L-ascorbic acid solution in 0.1M citrate buffer (pH 3.0), at a 5% concentration, in order to prevent the oxidative degradation of MTX (Hu et al., 1990). All plasma samples were stored at -20ºC until analysis. The ones with ascorbic acid were used for HPLC determination of the metabolic profile of MTX while the ones without it were used for biochemical determinations: ALT, AST, CK-MB and total-CK. After sacrifice, the brain, heart, kidneys, spleen and liver were also collected and weighted.

3.5. Measurement of the biochemical parameters

The plasma biomarkers mentioned above (ALT, AST, CK-MB, total-CK) were determined through enzymatic assays in the apparatus ABX Pentra 400 with ABX Pentra reagents, according to the manufacturer's instructions. The determinations were performed by Drª Laura Pereira, at the Clinical Analysis Laboratory of the Faculty of Pharmacy of the University of Porto.

3.6. Histological cardiac evaluation

The histological processing of the hearts by light microscopy was performed by Master Ana Reis-Mendes, at the Laboratory of Biochemistry and Experimental Morphology of the Faculty of Sport of the University of Porto, as described by Dores-Sousa *et al.* (Dores-Sousa et al., 2015).

3.7. HPLC-DAD determination

3.7.1. Mobile phase selection

To choose the appropriate mobile phase for MTX detection, several tests were made. Based on the literature, several isocratic mobile phases were tested, including acetonitrile: ammonium formate 160 mM, with hexanosulfonic acid 35 mM (29:71, v/v), adjusted to pH 2.7 with formic acid, based on a study performed by Johnson *et al.*, methanol: citric acid 50 mM (10:90, v/v), with octanesulfonic acid 0.46 mM, pH 3.0, and acetonitrile: ammonium formate 50 mM (10:90, v/v), pH 3.0 (Johnson et al., 2004). Another mobile phase composed by acetonitrile: ammonium acetate (25:75, v/v), adjusted to pH 4.0 with acetic acid, was tested, being ammonium acetate prepared at concentrations of 0.2 M, 0.1 M, 0.05 M and 0.01 M (Peng et al., 1982). Later, an isocratic mobile phase composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, was also tested (Rossato et al., 2013a).

3.7.2. Column selection

The first tests were conducted in an HPLC with an ultraviolet detector (HPLC-UV) system (Gilson, Middleton, WI, USA), using a Spherisorb RP-18 ODS2 column (250 x 4.6 mm, 5 µm; reversed-phase; Waters, Milford, MA, USA). Then, to analyze the mice plasma samples, an HPLC with a diode array detector (HPLC-DAD) system (Shimadzu corporation, Kyoto, Japan) was used. In order to reproduce the results previously found, it was necessary to change the column. A Nucleosil C18 ODS2 (250 x 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany), a Kinetex EVO C18 100Å (150 x 4.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) and a mediterranea sea_{18} (250 x 4.6 mm, 5 µm; Teknokroma, Barcelona, Spain), all reversed-phase columns, were tested.

3.7.3. Plasma sample analysis

Before analyzing the mice plasma samples, it was necessary to establish the best conditions for MTX and NAPHT detection. Human plasma samples were spiked with different concentrations of MTX standard solutions and calibration curves were drawn in plasma and phosphate buffered saline (PBS, pH 7.2 - 7.4). The limit of detection (LOD) and the limit of quantification (LOQ) were also determined according to the formulas in **Figure 8** and **Figure 9**, which are defined as the lowest concentration of an analyte present in a

sample that can be detected, but not necessarily quantified as an exact value, and as the lowest amount of analyte present in a sample which can be quantified with acceptable precision and accuracy, respectively (Ferreira et al., 2017; ICH, 1996). Besides the retention time, the retention factor (κ) of the compounds will also be referred, which measures the capacity of the column to retain an analyte of the sample (**Figure 10**) (Sousa et al., 2004). AMT was used as internal standard, since it has a similar structure to the parent drug (**Figure 4**, compared with **Figure 2**) and it was already reported in the literature (Johnson et al., 2004). A NAPHT standard solution was also prepared and analyzed.

$$LOD = \frac{3.3\sigma}{S}$$

Figure 8. Formula for the calculation of the limit of detection (LOD), where σ is the standard deviation of the lowest concentration detected and S is the slope of the analyte calibration curve.

$$LOQ = \frac{10\sigma}{S}$$

Figure 9. Formula for the calculation of the limit of quantification (LOQ), where σ is the standard deviation of the lowest concentration detected and S is the slope of the analyte calibration curve.

$$\kappa = \frac{t_r - t_0}{t_0}$$

Figure 10. Formula for the calculation of the retention factor, where t_r is the retention time of the analyte and t_0 is the time that the analyte is retained in the mobile phase.

Before analysis, plasma samples were thawed at room temperature and spiked with AMT (sample concentration of 8.8 µg/mL). Protein precipitation was achieved by the addition of 10 µL of a 50% aqueous solution of sulfosalicylic acid, followed by vortex mixing and centrifugation at 13000 rpm, 4ºC, during 10 minutes (Slørdal et al., 1993). Mice plasma samples were analyzed on the Shimadzu HPLC-DAD system, as previously mentioned, and MTX, NAPHT and the internal standard were detected at 254 nm and 658 nm, since these two wavelengths had already demonstrated to properly detect MTX in plasma and tissues (Hu et al., 1990; Rentsch et al., 1996). The mediterranea sea_{18} column (25 x 0.46 cm, 5 µm, reversed-phase; Teknokroma, Barcelona, Spain) was selected and the HPLC separations were accomplished using the isocratic mobile phase composed of acetonitrile: 0.5% formic

acid, adjusted to pH 3.0 (20:80, v/v), at a flow rate of 0.6 mL/min. The injection volume was 20 µL using a fixed loop. Additionally, in order to detect NAPHT the same conditions were used, however the proportion of acetonitrile: formic acid was changed to 30:70 (v/v). The analyses were completed after 30 minutes. Data were acquired using Shimadzu LCMSsolution ver.3 software.

3.8. Statistical analysis

The results are presented as mean ± standard deviation (SD). The possible changes in the animals' weight and the food and water consumptions were performed by two-way analysis of variance (2way ANOVA) Sidak *post hoc* test. When two groups and one time point were analyzed, the outliers were identified using the ROUT method (Q = 1%), in first place, and then, statistical analysis was performed by Student's t-test when the distribution was normal (parametric analysis performed by unpaired t-test with Welch's correlation) or by Mann-Whitney test when the distribution was not normal (non-parametric analysis). Statistical significance was considered with p values < 0.05. To perform the statistical analysis, GraphPad Prism 6 software program (San Diego, CA, USA) was used.

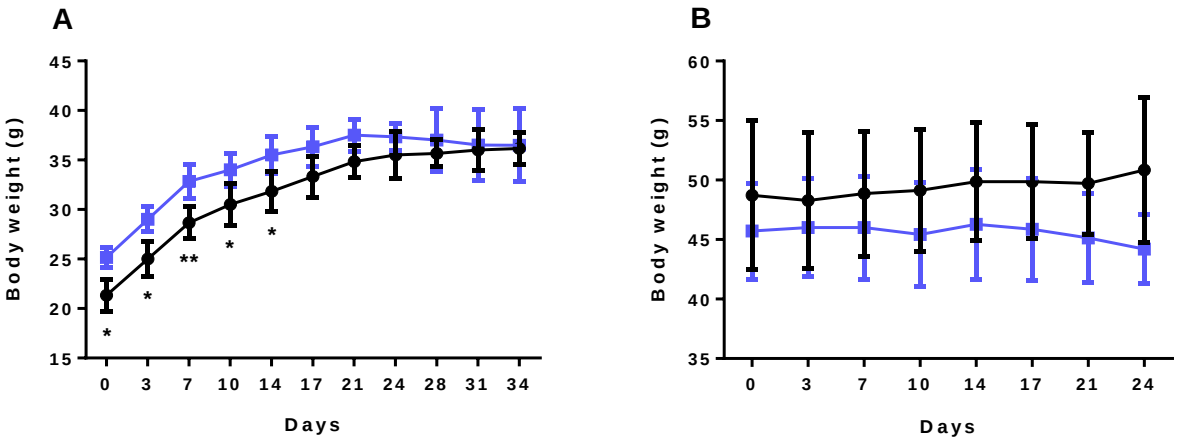
CHAPTER 4.
Results

4. RESULTS

4.1. General welfare and body weight gain

None of the animals was sacrificed before the designated time, since they did not reach critical scores. However, it is also important to highlight some observations taken at the moment of sacrifice in some MTX-treated mice, such as the presence of ascites, a swollen stomach and with much air, dark and swollen intestines, in the infants. In the MTX-treated adult population, some of them also presented ascites, a swollen stomach and a reduced liver. Regarding the elderly, some liver changes were observed in the MTX-treated animals, such as whitish liver and an abnormal structure.

Initial significant differences in the body weight gain between the MTX-treated and the control groups were seen in the infant population (**Figure 11A**). These initial differences were due to the random distribution, for each cage, of the mice belonging to different litters. Concerning the adults, two trials were made, as previous mentioned. In the assay 1 there were no significant differences in the body weight gain between the MTX and the control groups, although a tendency for lower weight occurred in the last days of the protocol (**Figure 11B**). In fact, in the assay 2, where a higher number of animals was analyzed, there was a significant body weight decrease in the MTX-treated group when compared to control, in the last days of the protocol (**Figure 11C**). Regarding the elderly population, the body weight of the animals was constant through the all experiment, that is, MTX and control groups did not show statistically significant differences in body weight (**Figure 11D**). This way, these results demonstrated that treatment with MTX at a cumulative dose of 6 mg/kg caused significant weight loss only in the adult mice.



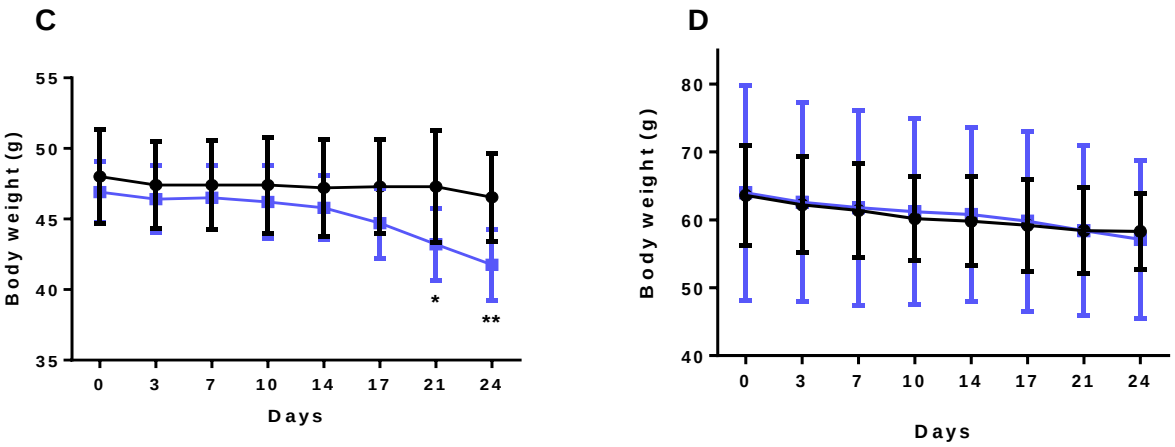


Figure 11. Body weight of the animals in the MTX (cumulative dose of 6 mg/kg) and in the control groups (NaCl 0.9%), through all the experiment, in three different aged groups. In the **infant** population (**A**), there were six mice in the MTX-treated group and another six in the control group. Two studies were done in the **adult** population: the first one (**B**) with seven mice in each group, and the second (**C**) with 10 in each one. In the **elderly** population (**D**), there were five MTX-treated mice and another five controls. The blue squares (■) represent the MTX-treated mice while the black circles (●) correspond to the control group. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by 2way ANOVA Sidak *post hoc* test: *p < 0.05, **p < 0.01, *vs* control.

4.2. Food and water consumptions

The food and water consumptions were determined by day and standardized for the animals' weight, since is not possible to determine the accurate consumption rates of each mice. However, with this determination it is assumed that the consumption rates were proportional to the animals' weight and that a weight loss is due to a lower intake.

Concerning food consumption, a decrease in the intake of the infant population treated with MTX throughout the experiment was observed, reaching statistical significance when the protocol was ending, when compared with the control group (**Figure 12A**). Concerning the adults from assay 1, there was a tendency for lower food consumption in the MTX-treated group, comparing with the controls (**Figure 12B**). Meanwhile, in assay 2, where the number of the animals in the study was higher, a significant difference at the end of the protocol between the food consumption of the MTX and the control groups was observed (**Figure 12C**). In the elderly population, the food intake of both groups seemed to be more constant and no significance differences were detected between them (**Figure 12D**). With these results, it was possible to observe that infant and adult mice ate significantly less, when compared to the control group, when administered with a cumulative dose of 6 mg/kg of MTX.

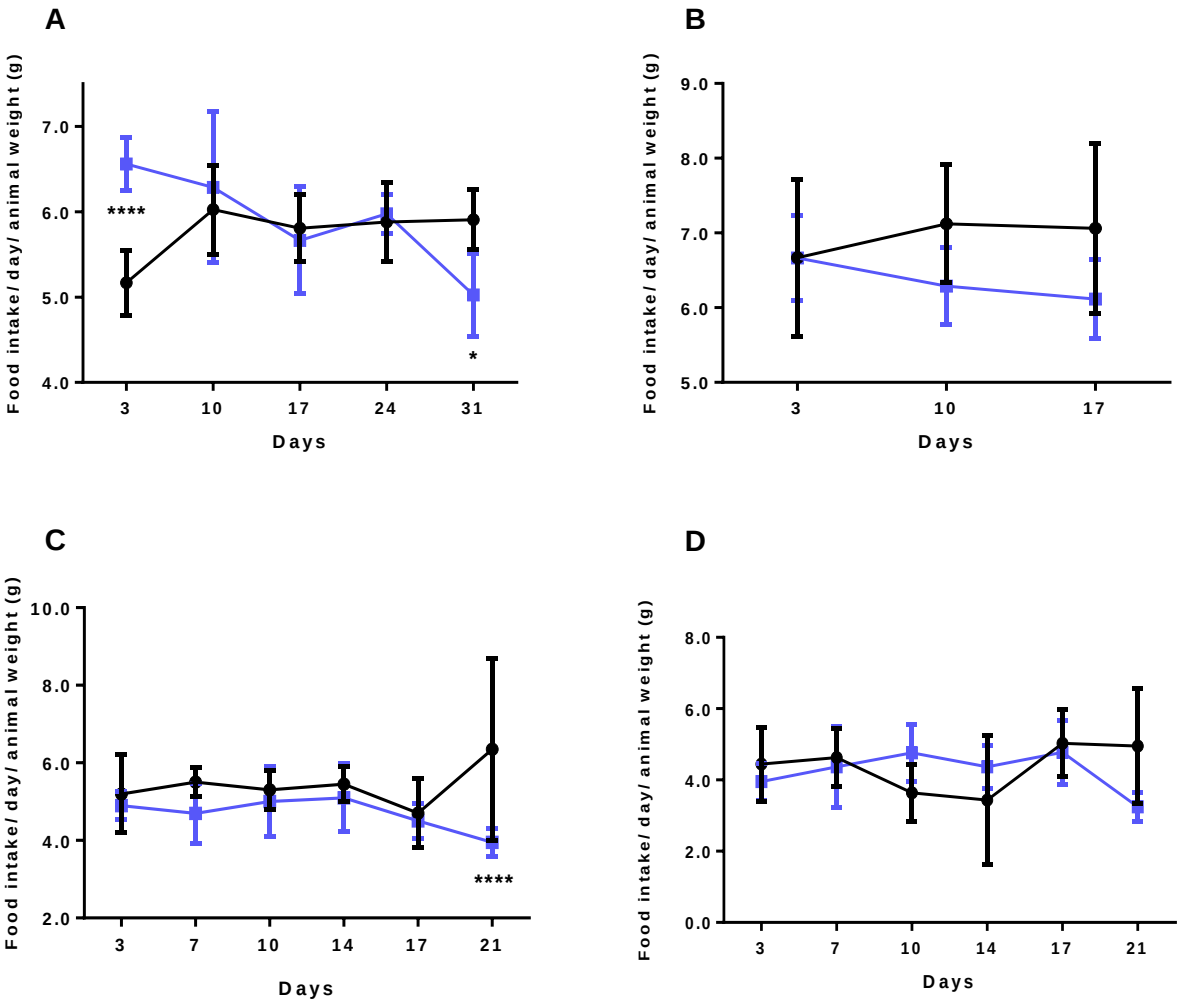


Figure 12. Food consumption of the animals in the MTX (cumulative dose of 6 mg/kg) and in the control groups (NaCl 0.9%), through all the experiment, in three different aged groups. In the **infant** population (**A**), there were six mice in the MTX-treated group and another six in the control group. Two studies were done in the **adult** population: the first one (**B**) with seven mice in each group, and the second (**C**) with 10 in each one. In the **elderly** population (**D**), there were five MTX-treated mice and another five controls. The blue squares (■) represent the MTX-treated mice while the black circles (●) correspond to the control group. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by 2way ANOVA Sidak *post hoc* test: *p < 0.05, ****p < 0.0001, *vs* control.

Similarly to what happened in food consumption, water intake in the MTX-treated infant mice decreased, being statistical significant in the last days of the protocol, when compared with the control group (**Figure 13A**). Regarding the adults, in the assay 1 a tendency to a decreased water intake in the last days of the protocol by the MTX group was observed, when comparing with the control one (**Figure 13B**). Meanwhile, when the number of analyzed mice was increased, this tendency was exacerbated and the differences between the MTX and the control groups were more notorious, reaching statistical significance

(**Figure 13C**). In the elderly population, the water consumption was practically constant when comparing the MTX-treated and the control mice (**Figure 13D**).

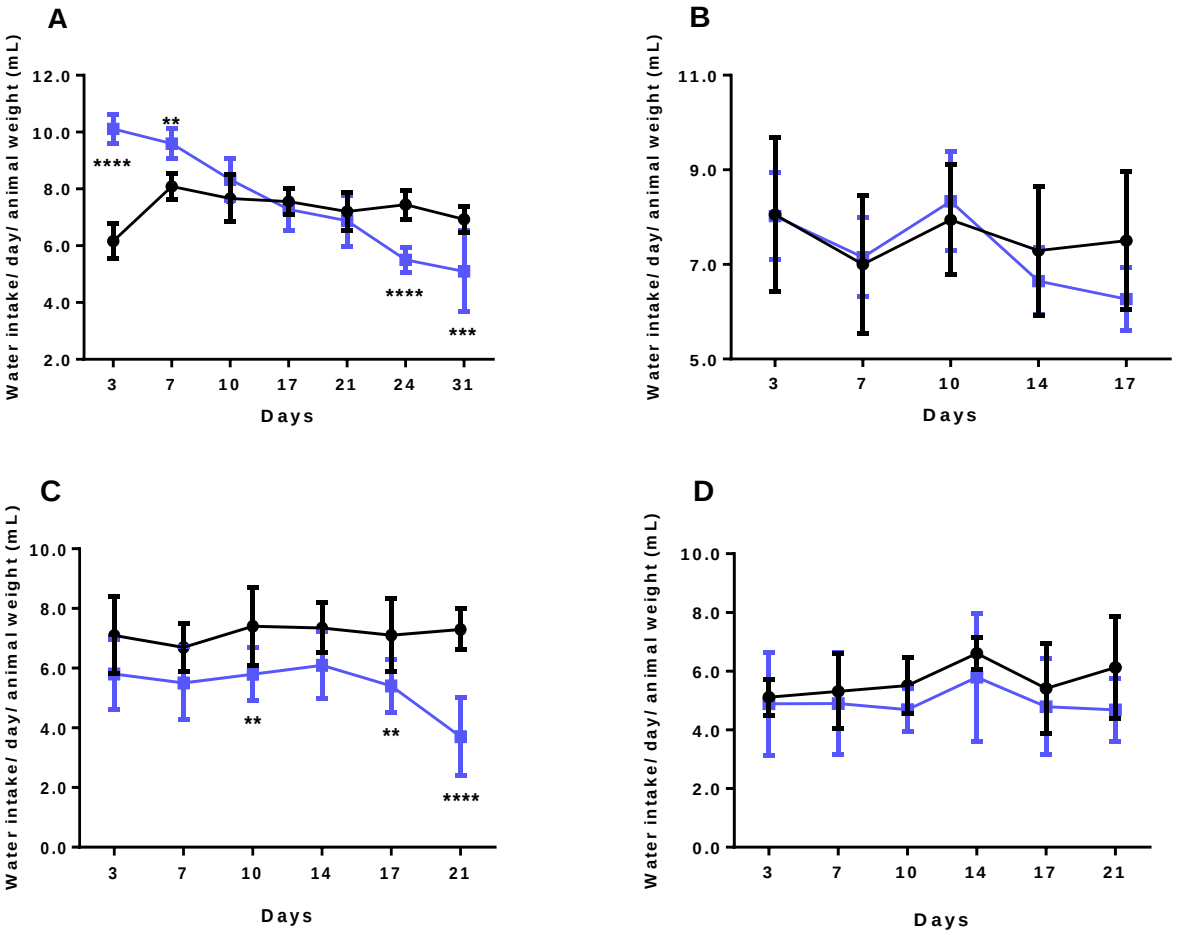


Figure 13. Water consumption of the animals in the MTX (cumulative dose of 6 mg/kg) and in the control groups (NaCl 0.9%), through all the experiment, in three different aged groups. In the **infant** population (**A**), there were six mice in the MTX-treated group and another six in the control group. Two studies were done in the **adult** population: the first one (**B**) with seven mice in each group, and the second (**C**) with 10 in each one. In the **elderly** population (**D**), there were five MTX-treated mice and another five controls. The blue squares (■) represent the MTX-treated mice while the black circles (●) correspond to the control group. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by 2way ANOVA Sidak *post hoc* test: **p < 0.01, ***p < 0.001, ****p < 0.0001, *vs* control.

4.3. Organ weight/ brain weight ratios

The ratio between organ weight/ brain weight was used for the assessment of changes in the weight of the remaining organs collected at sacrifice (heart, kidneys, spleen and liver). In the infant population, none of the organ weight/ brain weight ratios of the MTX-treated

mice significantly changed, when compared with the controls (**Figure 14**). So, the weight of the organs of the treated infant mice was not affected when administered with a cumulative dose of 6 mg/kg of MTX.

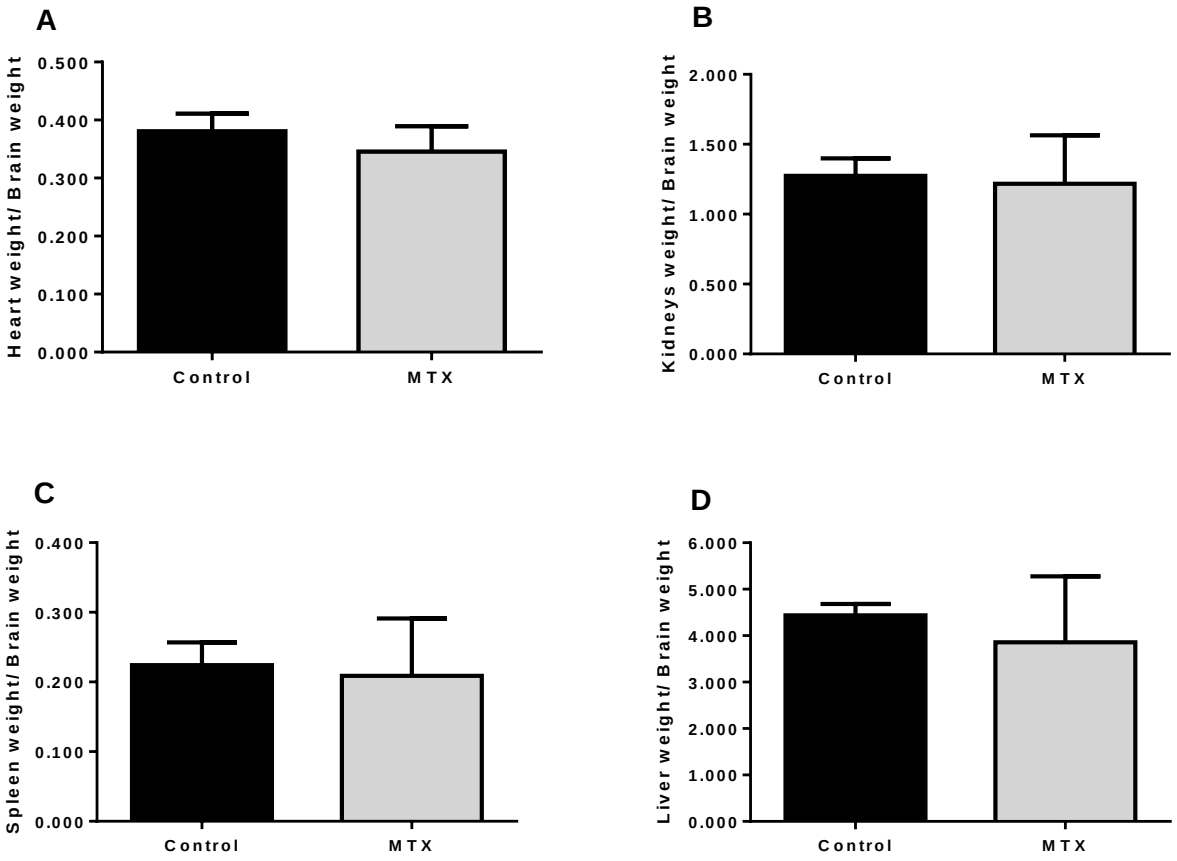


Figure 14. Ratios between organ weight (heart-**A**, kidneys-**B**, spleen-**C**, liver-**D**) and brain weight of twelve **infant** CD-1 mice: six from the MTX group (cumulative dose of 6 mg/kg) and six from the control group (NaCl 0.9%). The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Mann-Whitney test (non-parametric analysis).

Concerning the adult mice from assay 1, a tendency for decreased heart weight/ brain weight ratio of the MTX-treated group, comparing with the control one, was observed (p = 0.07); however, it did not achieve statistical significance. Meanwhile, the ratios kidneys weight/ brain weight and liver weight/ brain weight of the MTX-treated mice were significantly reduced in comparison to the control group (**Figure 15A**, **15C**, **15E** and **15G**). When the number of animals analyzed increased, the differences between MTX-treated and control groups were exacerbated and it was observed a significant decrease in all organ weight/ brain weight ratios (**Figure 15B**, **15D**, **15F** and **15H**).

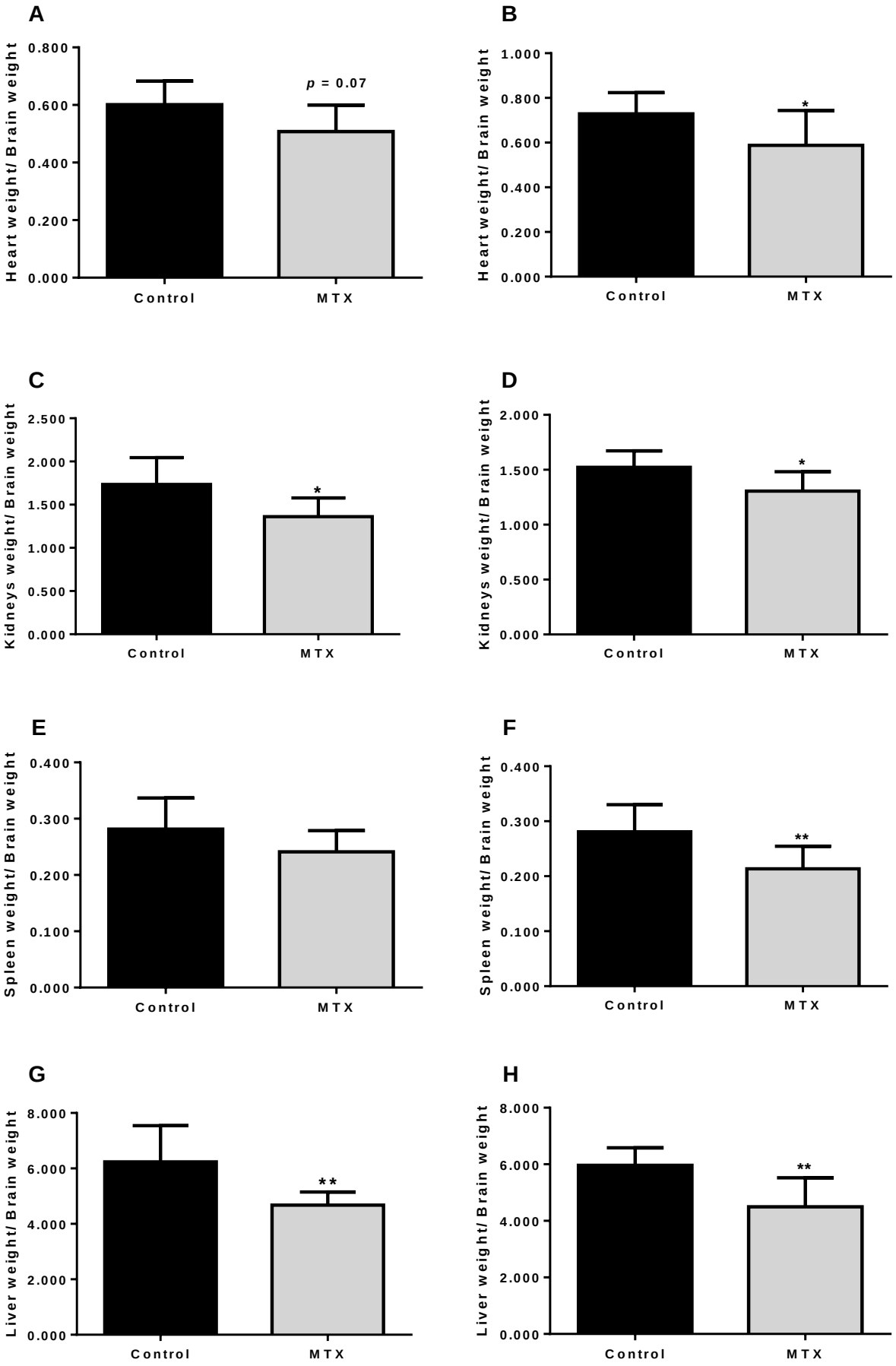

A
Heart weight/ Brain weight
0.800
0.600
0.400
0.200
0.000
p = 0.07
Control
MTX
B
Heart weight/ Brain weight
1.000
0.800
0.600
0.400
0.200
0.000
*
Control
MTX
C
Kidneys weight/ Brain weight
2.500
2.000
1.500
1.000
0.500
0.000
*
Control
MTX
D
Kidneys weight/ Brain weight
2.000
1.500
1.000
0.500
0.000
*
Control
MTX
E
Spleen weight/ Brain weight
0.400
0.300
0.200
0.100
0.000
Control
MTX
F
Spleen weight/ Brain weight
0.400
0.300
0.200
0.100
0.000
**
Control
MTX
G
Liver weight/ Brain weight
8.000
6.000
4.000
2.000
0.000
**
Control
MTX
H
Liver weight/ Brain weight
8.000
6.000
4.000
2.000
0.000
**
Control
MTX

Figure 15. Ratios between organ weight and brain weight of **adult** CD-1 mice. Two studies were done in the adult population: the first one (**A, C, E, G**) with seven mice in the MTX-treated group (cumulative dose of 6 mg/kg) and another seven in the control group (NaCl 0.9%), and the second one (**B, D, F, H**) with ten mice in each group. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Student's t-test when the distribution was normal (**B, D, F, H**) or by Mann-Whitney test when the distribution was not normal (**A, C, E, G**): *p < 0.05, **p < 0.01, *vs* control.

Regarding the elderly population, there was a tendency for reduced liver weight/ brain weight ratio of the MTX-treated mice, in comparison with controls (p = 0.06); however, significant differences were only seen in the spleen weight/ brain weight ratio (**Figure 16**). So, when administered with a cumulative dose of 6 mg/kg of MTX, the spleen of the elderly ones was specially affected.

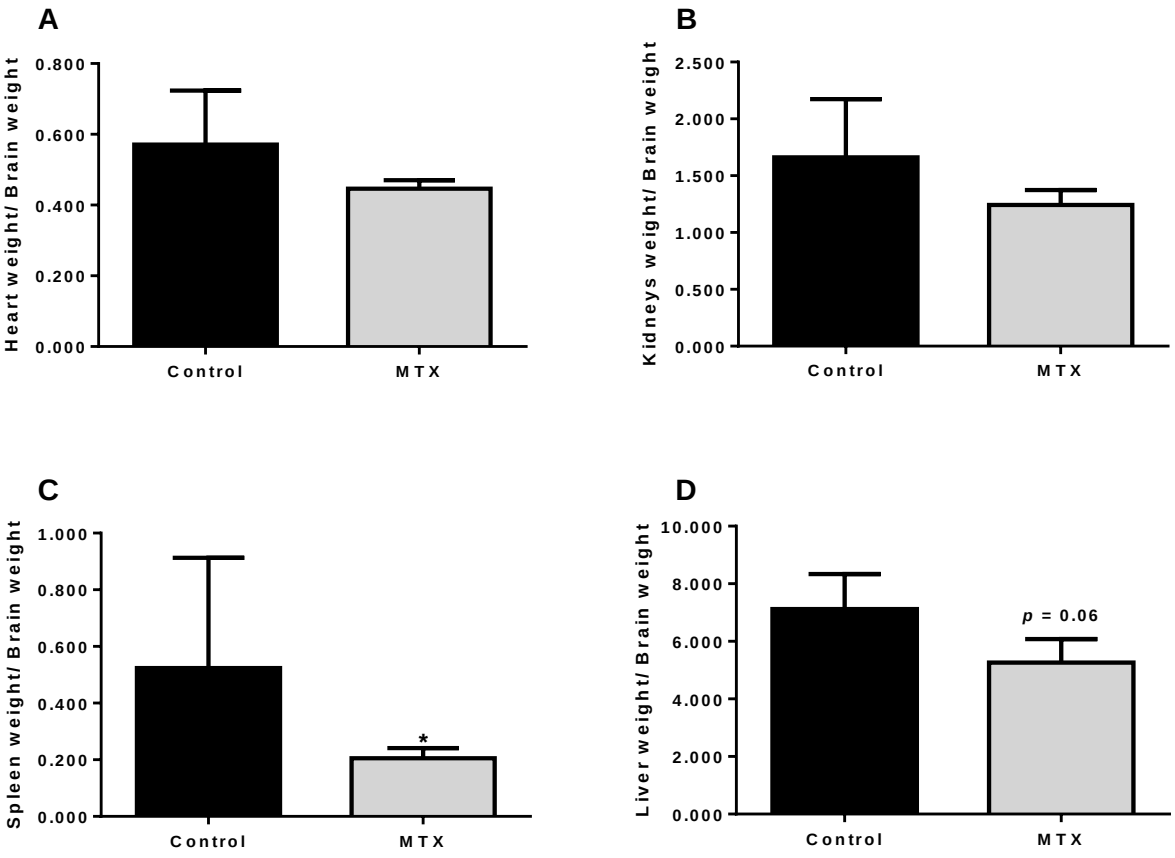


Figure 16. Ratios between organ weight (heart-**A**, kidneys-**B**, spleen-**C**, liver-**D**) and brain weight of ten **elderly** CD-1 mice: five from the MTX group (cumulative dose of 6 mg/kg) and five from the control group (NaCl 0.9%). The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Mann-Whitney test (non-parametric analysis): *p < 0.05, *vs* control.

4.4. Plasma biomarkers levels

Plasma biomarkers levels were found significant altered in some MTX-treated groups, in comparison with controls. In the infant population, ALT levels were significantly elevated while the AST/ ALT ratio was found significant decreased, when compared with the control group (**Figure 17**). Total-CK and CK-MB levels were not determined due to insufficient amount of the gathered plasma.

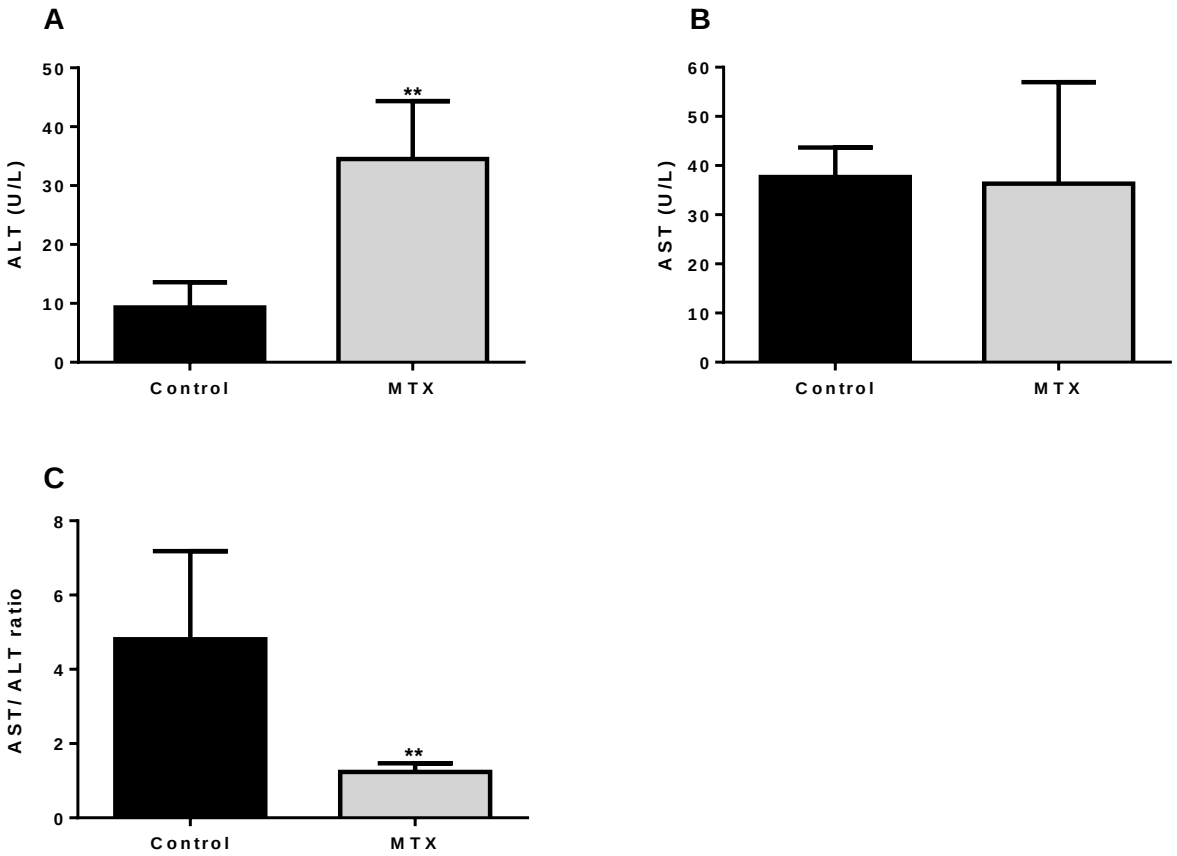
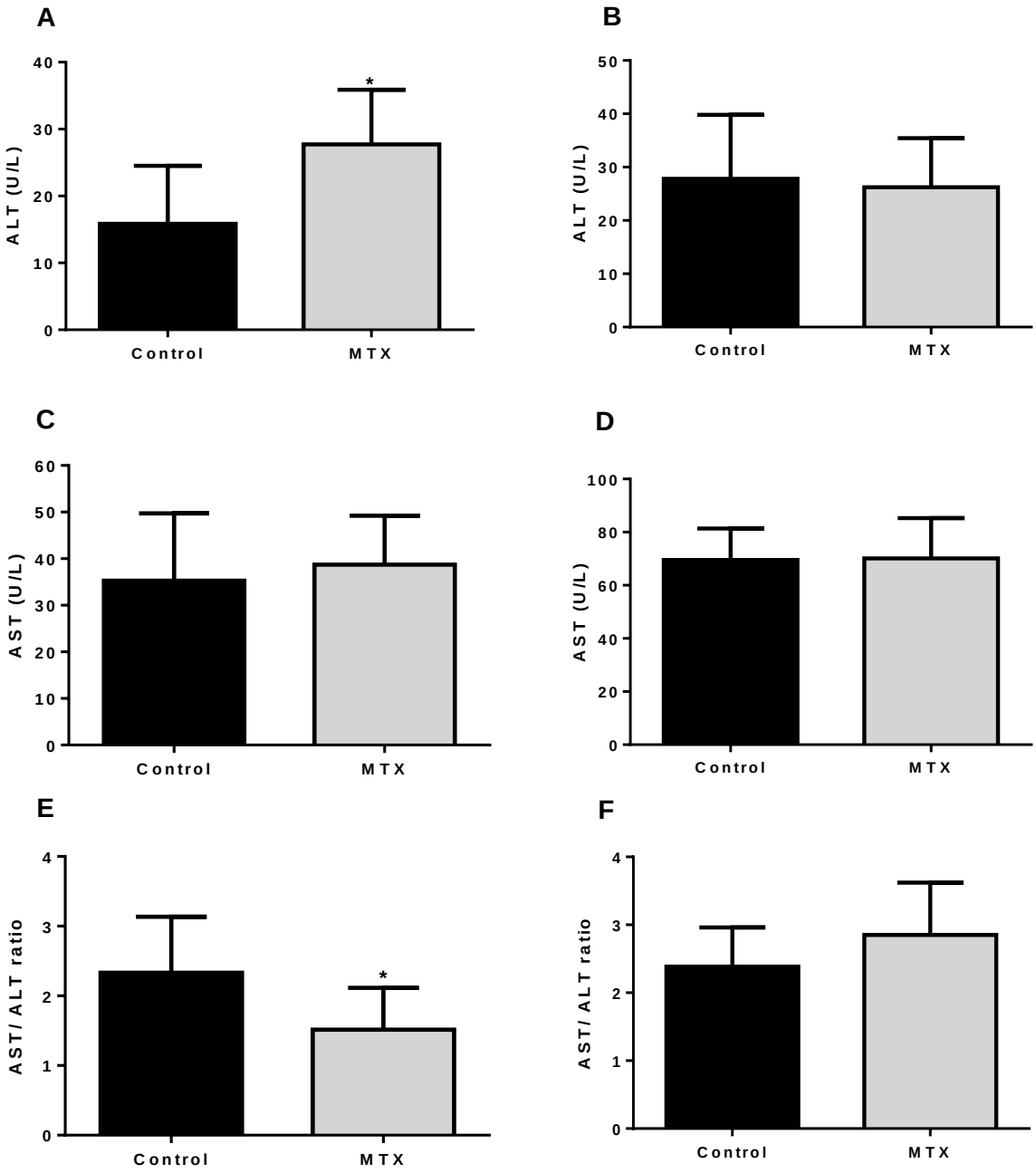


Figure 17. Plasma levels of alanine aminotransferase - ALT (**A**), aspartate aminotransferase - AST (**B**) and their ratio (**C**) of twelve **infant** CD-1 mice: six from the MTX group (cumulative dose of 6 mg/kg) and six from the control group (NaCl 0.9%). For the determination of ALT levels, it was only analyzed the plasma from five mice, due to insufficient amounts of the gathered plasma from the remaining mouse. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Mann-Whitney test (non-parametric analysis): **$p < 0.01$, *vs* control.

Concerning the first group of MTX-treated adults, ALT levels were found significantly increased while the AST/ ALT ratio was significant reduced, when compared with the levels from the control group (**Figure 18A**, **18C** and 1**8E**). Once again, total-CK and CK-MB levels were not determined due to insufficient amount of the gathered plasma. On the other hand,

in the assay 2, there were no significant changes in the plasma aminotransferases levels, when compared with controls. In this trial, no significant differences were observed between the MTX-treated and the control groups in total-CK and CK-MB levels (**Figure 18B**, **18D**, **18F**, **18G** and **18H**).



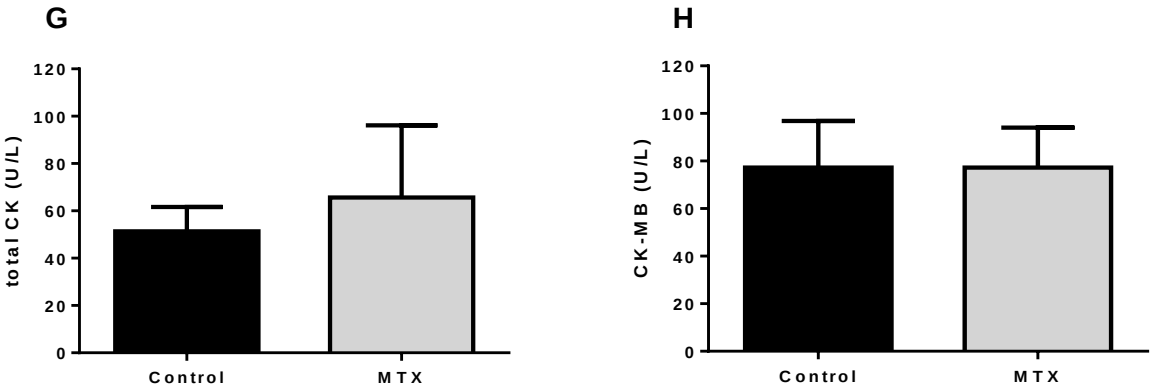
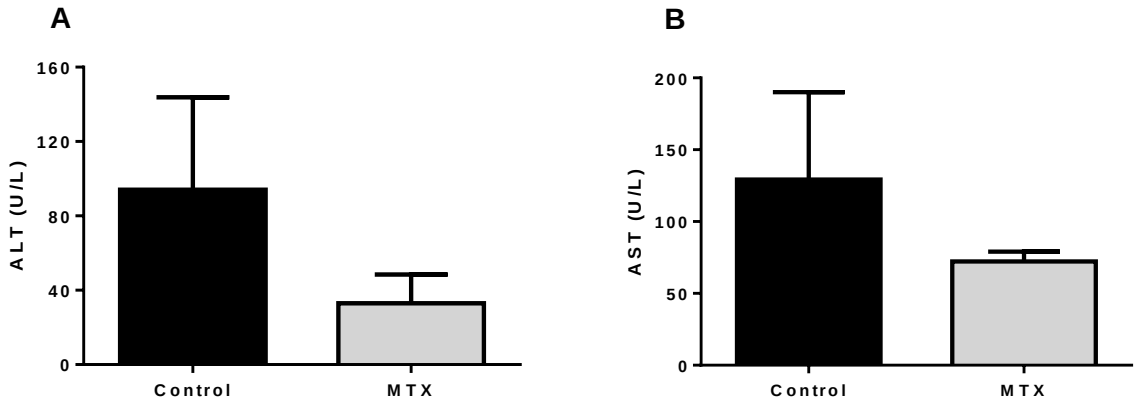


Figure 18. Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and their ratios, total creatine kinase (total-CK) and creatine kinase MB (CK-MB) of **adult** CD-1 mice. Two studies were done in the adult population: the first one (**A, C, E**) with seven mice in the MTX-treated group (cumulative dose of 6 mg/kg) and another seven in the control group (NaCl 0.9%), and the second one (**B, D, F, G, H**) with nine MTX-treated mice and ten controls. Total-CK and CK-MB plasma levels were only determined for the second trial. The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Student's t-test when the distribution was normal (**B, D, F, G, H**) or by Mann-Whitney test when the distribution was not normal (**A, C, E**): *p < 0.05, *vs* control.

Regarding the elderly population, none of the plasma biomarkers levels of the MTX-treated group significantly differ from the ones of the control group (**Figure 19**). This way, when treated with MTX at a cumulative dose of 6 mg/kg, the plasma levels of ALT, AST, total-CK and CK-MB from elderly mice were not significantly affected, when compared to control values.



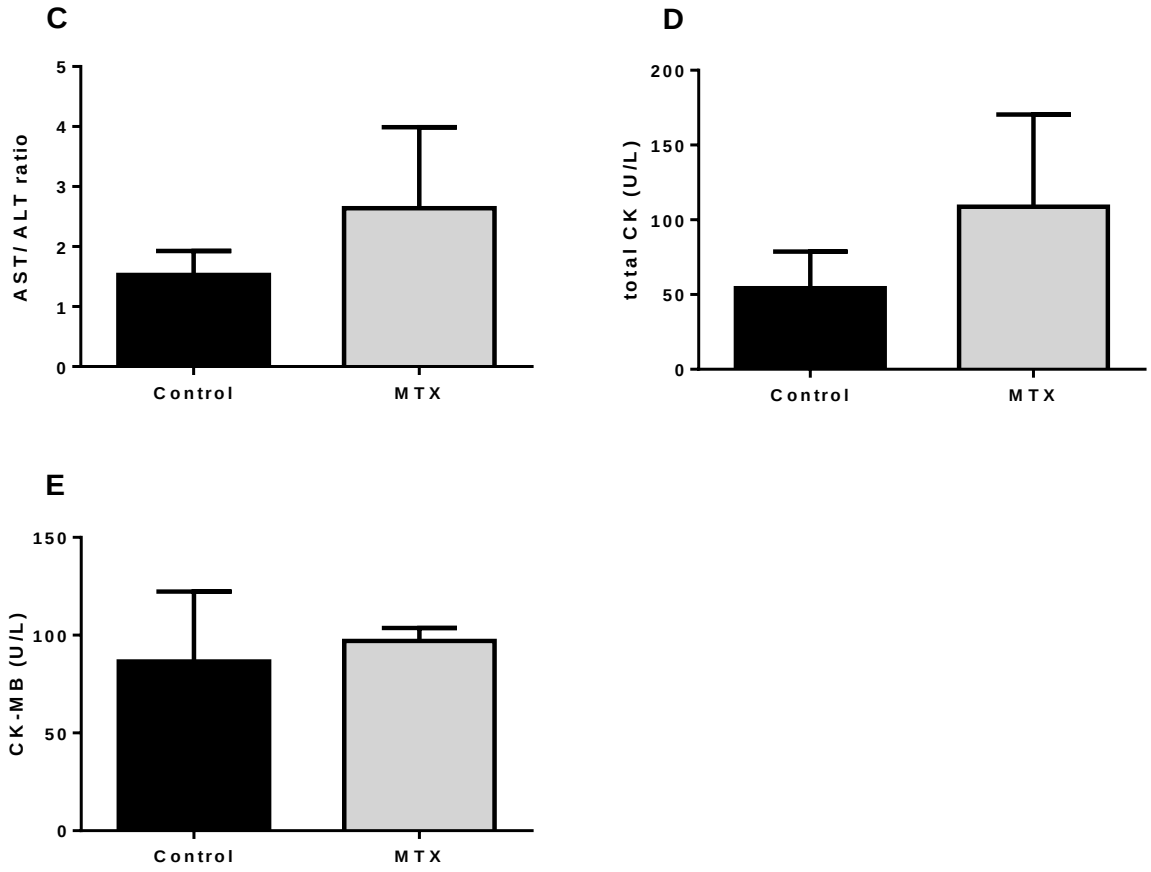


Figure 19. Plasma levels of alanine aminotransferase - ALT (**A**), aspartate aminotransferase - AST (**B**) and their ratio (**C**), total creatine kinase – total-CK (**D**) and creatine kinase MB - CK-MB (**E**) of eight **elderly** CD-1 mice: four from the MTX group (cumulative dose of 6 mg/kg) and four from the control group (NaCl 0.9%). The results are presented as mean ± standard deviation (SD) and statistical comparisons were performed by Mann-Whitney test (non-parametric analysis).

4.5. Histological cardiac evaluation

The histological cardiac evaluation of the MTX-treated mice and respective controls was conducted through light microscopy and then compared with control mice, in each age group. The infant control mice presented a normal histology (**Figure 20A**). Structural alterations in the cardiomyocytes of the MTX-treated mice were observed in all age groups. In the MTX-treated infant population, cellular edema, necrotic zones, inflammatory infiltrations, vacuolization and large and uncondensed nucleus were observed (**Figure 20B** and **Figure 20C**). The images presented herein are representative of the all infant population, since similar results were obtained.

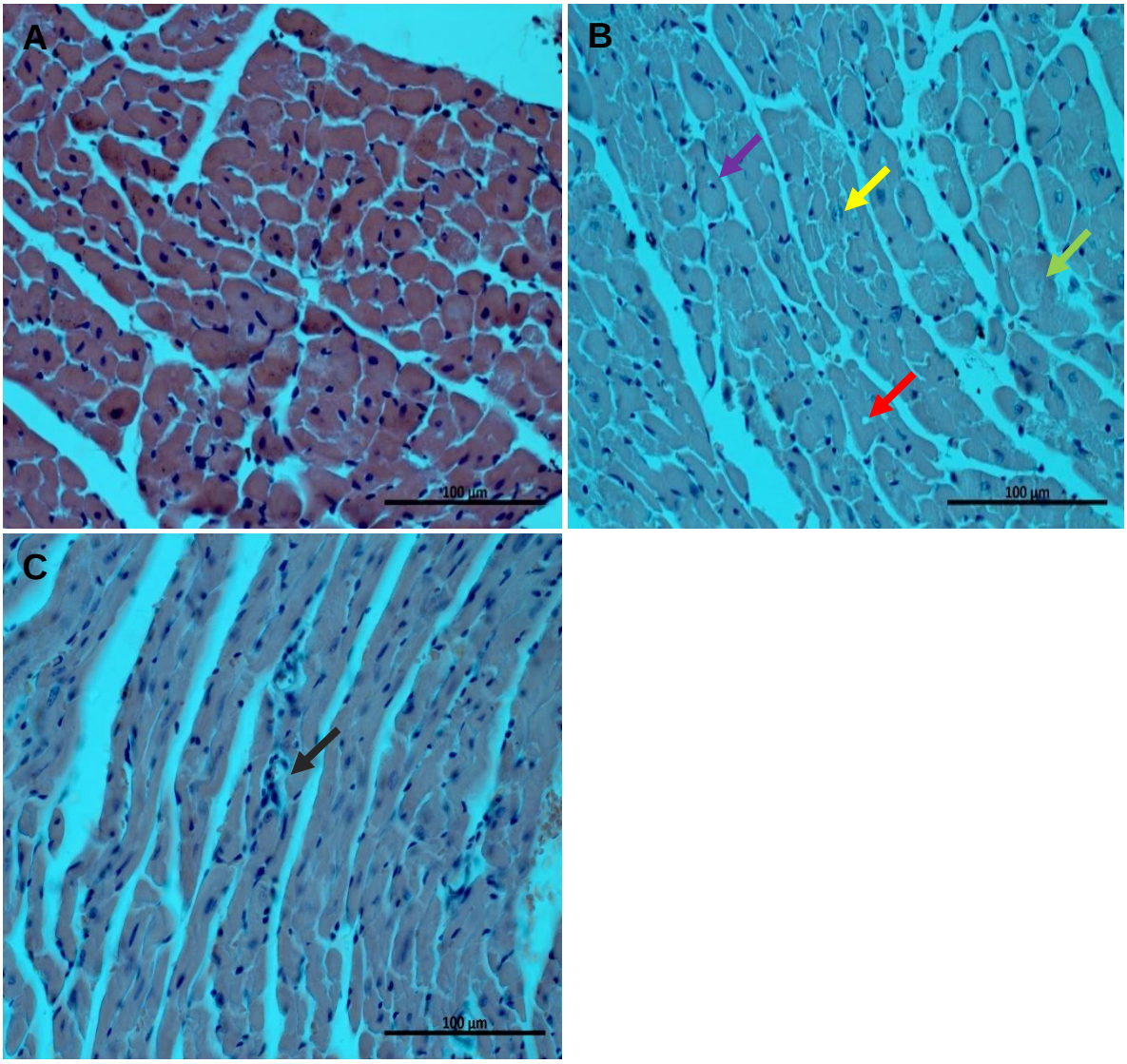


Figure 20. Light microscopy representative images of the heart of **control infant** (**A**) or **MTX-treated infant** (**B, C**) mice after a cumulative dose of 6 mg/kg of MTX. The control group presented a normal morphology and structure of the heart. The MTX-treated infant mice presented cellular edema (purple arrow), necrotic zones (green arrow), inflammatory infiltrations (black arrow), vacuolization (red arrow) and large and uncondensed nucleus (yellow arrow).

Similarly, the MTX-treated adults also presented the same structural alterations (cellular edema, necrotic zones, inflammatory infiltrations, vacuolization, large and uncondensed nucleus), with additional vascular congestion (**Figure 21B** and **Figure 21C**). The adult control mice presented a normal histology (**Figure 21A**). The following images are representative of the remaining adult population.

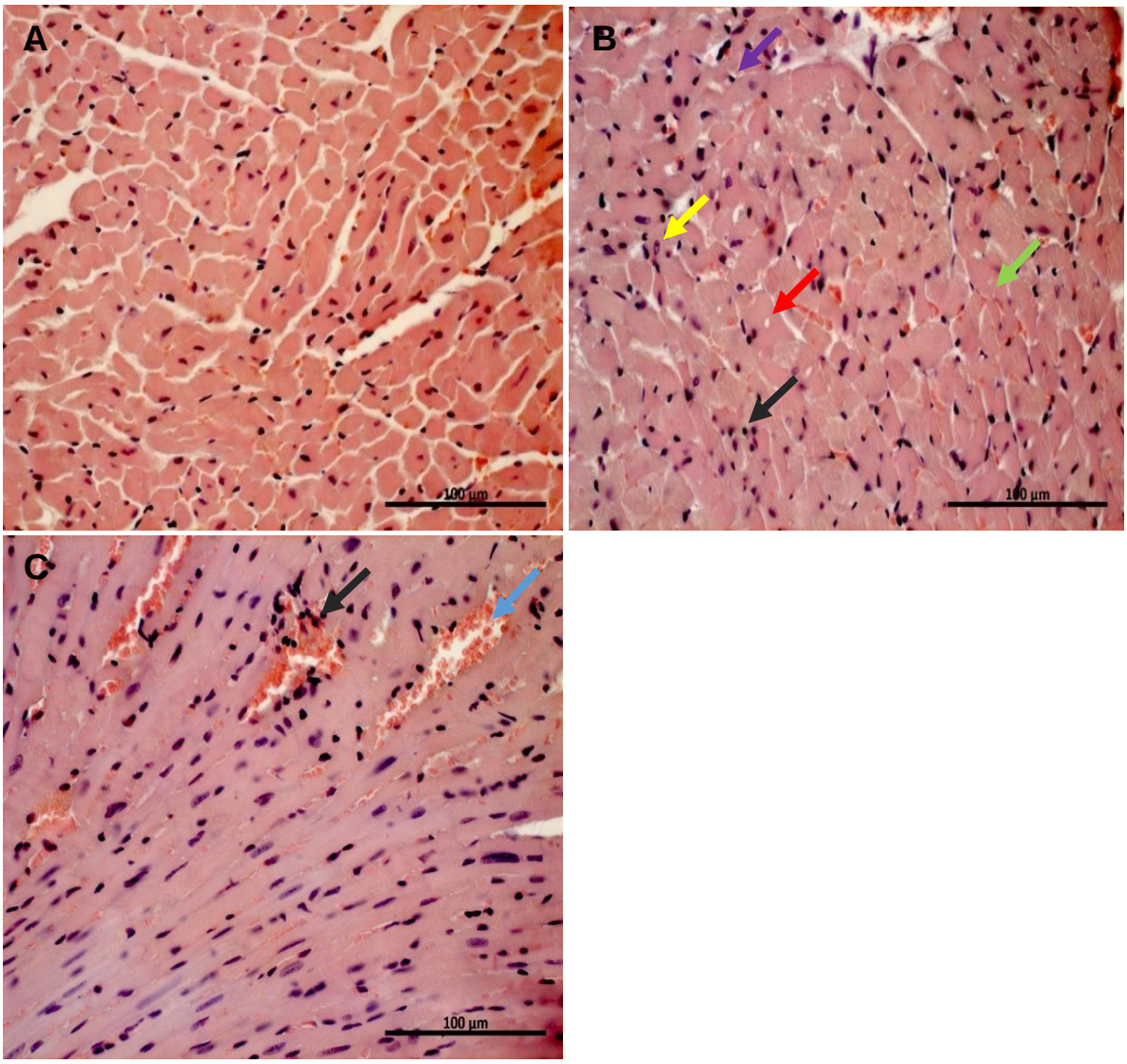


Figure 21. Light microscopy representative images of the heart of **control adult** (**A**) or **MTX-treated adult** (**B, C**) mice after a cumulative dose of 6 mg/kg of MTX. The control group presented a normal morphology and structure of the heart. The MTX-treated adult mice presented cellular edema (purple arrow), necrotic zones (green arrow), inflammatory infiltrations (black arrow), vacuolization (red arrow), vascular congestion (blue arrow) and large and uncondensed nucleus (yellow arrow).

Regarding the elderly population, both control and MTX-treated mice presented microscopical cardiac lesions. In the control elderly mice, the existence of necrotic zones, inflammatory infiltrations, vacuolization, large and uncondensed nucleus and a higher amount of loose connective tissue around the vessel walls and macrophages, in comparison with younger controls, were seen (**Figure 22A**). In the MTX-treated elderly mice, the same structural changes as the controls were observed, but cellular edema and

vascular congestion were also seen (**Figure 22B** and **Figure 22C**). The images present below are representative of all elderly population.

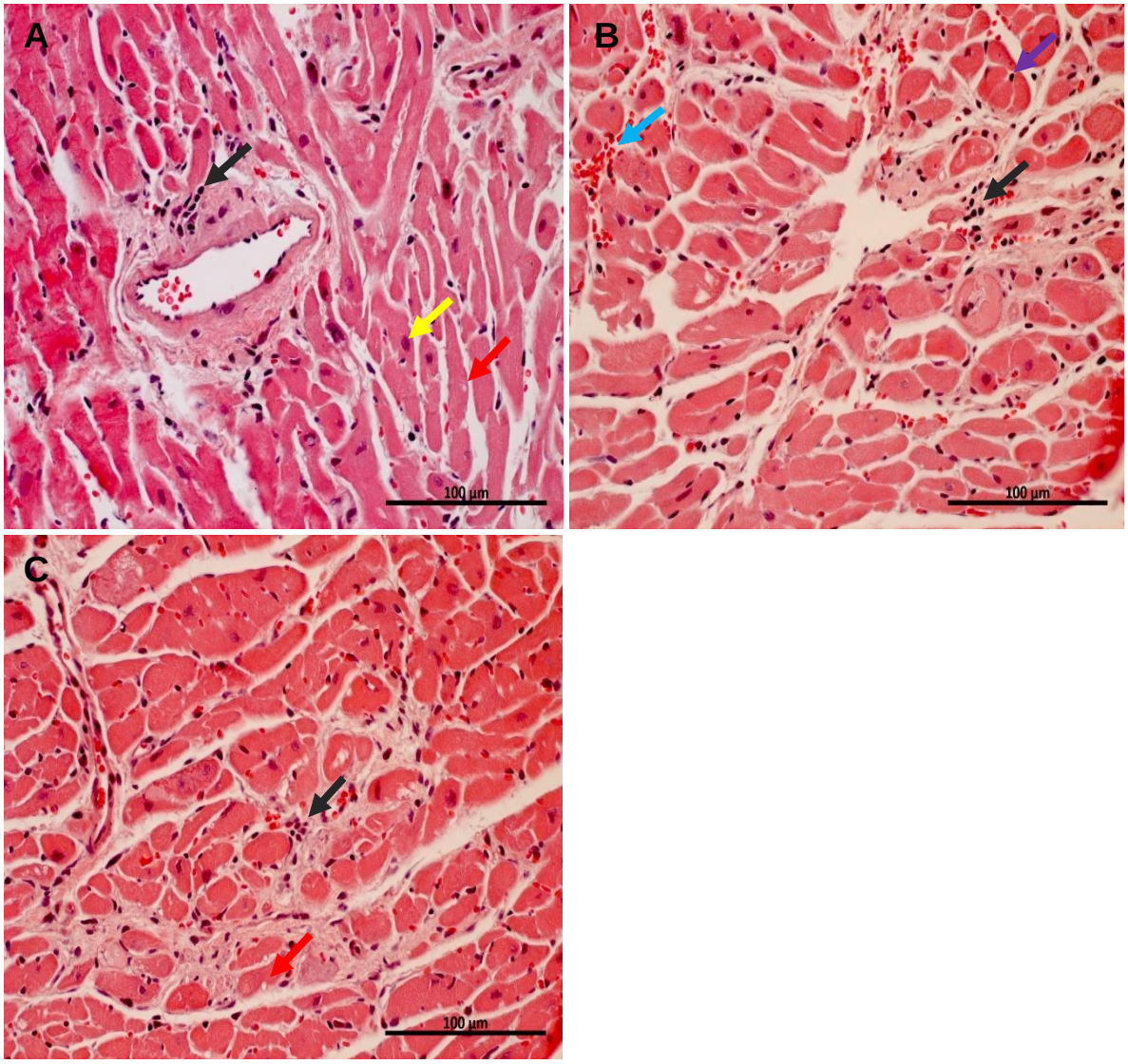


Figure 22. Light microscopy representative images of the heart of **control elderly** (**A**) or **MTX-treated elderly** (**B, C**) mice after a cumulative dose of 6 mg/kg of MTX. The control group did not present a normal morphology and structure of the heart: presence of necrotic zones (data not shown), inflammatory infiltrations (black arrow), vacuolization (red arrow), large and uncondensed nucleus (yellow arrow) and a higher amount of loose connective tissue around the vessel walls and macrophages. The cardiomyocytes of the MTX-treated elderly mice presented the same cardiac lesions as the controls and even cellular edema (purple arrow) and vascular congestion (blue arrow).

4.6. HPLC-DAD analysis

4.6.1. Mobile phase selection

Several isocratic mobile phases were tested in order to find the most appropriate for the MTX detection. The first experiments were conducted using acetonitrile: ammonium formate 160 mM, with hexanosulfonic acid 35 mM (29:71, v/v), adjusted to pH 2.7 with formic acid, methanol: citric acid 50 mM (10:90, v/v), with octanesulfonic acid 0.46 mM, at pH 3.0, and acetonitrile: ammonium formate 50 mM (10:90, v/v), at pH 3.0, conditions previously described to analyze MTX (Johnson et al., 2004). It was used a flow rate of 1.0 mL/min, a running time of 40 minutes and a wavelength of 254 nm. However, none of these mobile phases seemed suitable, since it was not possible to detect a standard solution of MTX at a concentration of 130 µM in these conditions (data not shown). Then, another mobile phase was tested, composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid. With this mobile phase, at a flow rate of 1.0 mL/min, during 40 minutes and at 254 nm, it was possible to detect the compound (data not shown). Since the equipment's pressure was close to its recommended limit and in order to obtain a better peak separation, the flow rate was diminished to 0.6 mL/min. Maintaining the wavelength and reducing the running time to 15 minutes, the detection of a standard solution of MTX at a concentration of 130 µM was accomplished at a retention time of 7.65 minutes (κ = 1.97) (**Figure 23**).

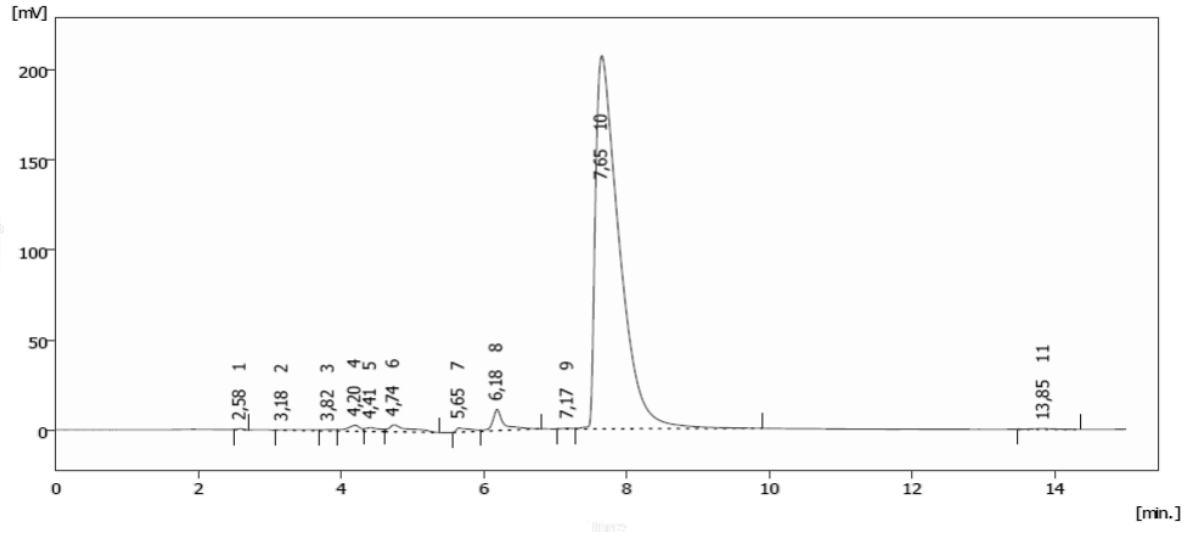


Figure 23. Chromatographic profile of a standard solution of MTX 130 µM using an HPLC-UV system with a Spherisorb RP-18 ODS2 column. The mobile phase was composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, and was kept at a flow rate of 0.6 mL/min. Wavelength was set to 254 nm and the running time to 15 minutes. The band 10 of the chromatogram corresponds to the anticancer drug, which had a retention time of 7.65 minutes (κ = 1.97).

Afterwards, the same mobile phase (acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid) was tested in an HPLC-DAD system (Shimadzu corporation, Kyoto, Japan), which permitted to analyzed the samples using two different wavelengths. Using a flow rate of 0.6 mL/min, a running time of 15 minutes and wavelengths of 254 nm and 658 nm, the MTX detection was accomplished at a retention time of 7.63 minutes (κ = 1.16) (**Figure 24**). The UV-Vis spectrum of MTX is presented in **Figure 25** and maximal wavelengths at 610 nm and 661 nm can be noted, as previously described (Rossato et al., 2013a).

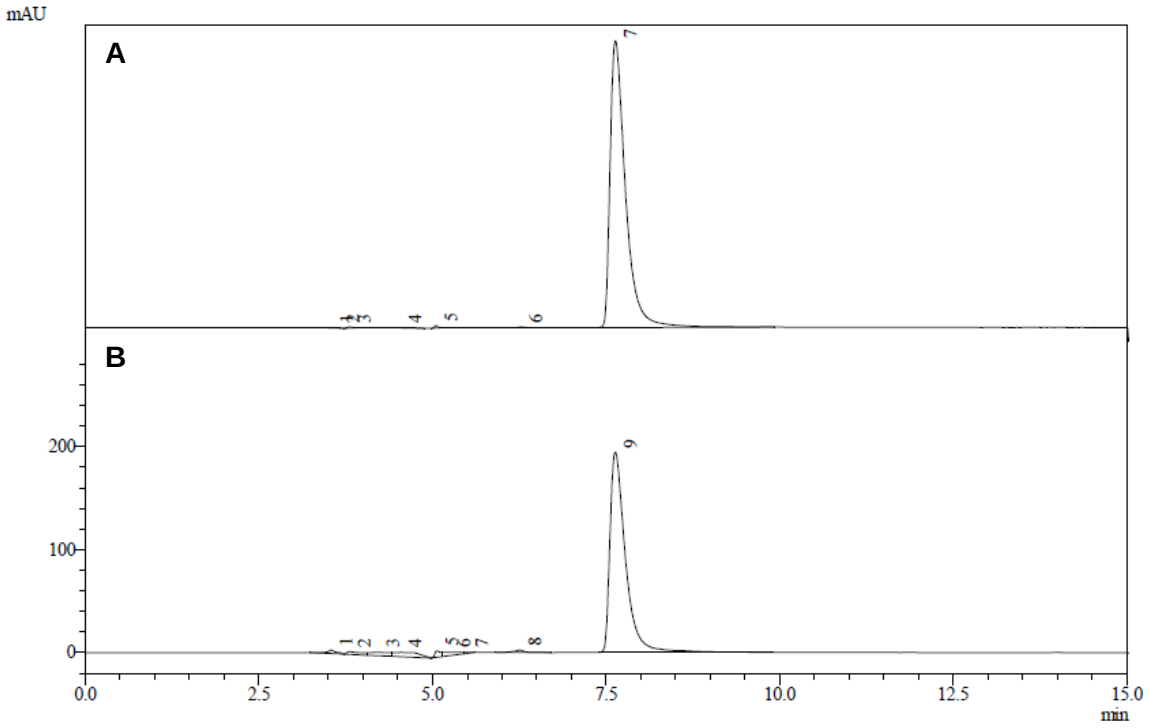


Figure 24. Chromatographic profile of a standard solution of MTX 130 µM using an HPLC-DAD system. The mobile phase was composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 658 nm (**A**) and 254 nm (**B**) and the running time to 15 minutes. The band 7 and the band 9 of the chromatograms **A** and **B**, respectively, correspond to the anticancer drug, which had a retention time of 7.63 minutes (κ = 1.16).

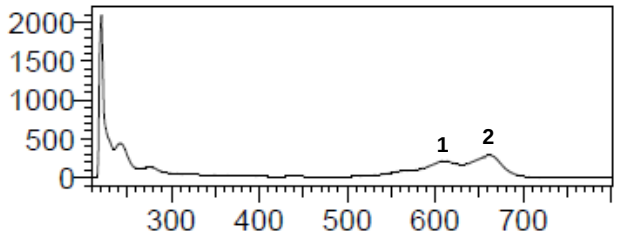


Figure 25. UV-Vis spectrum profile of MTX (standard solution of 130 µM). Peaks **1** and **2** correspond to 610 nm and 661 nm, respectively.

Then, with the possibility of analyzing the mice plasma samples with a liquid chromatography mass spectrometry system (LC-MS) in mind, other concentrations of ammonium acetate were tested (0.1M, 0.05M and 0.01M), since the recommended maximum concentration for this solvent in the mobile phase is 0.1M (Shimadzu, 2012). It was observed that, maintaining the same chromatographic conditions as before, only with the highest concentration the retention time of the anticancer drug was suitable, since with the other two analysis, MTX eluted with or very close to the solvent front (**Figure 26**).

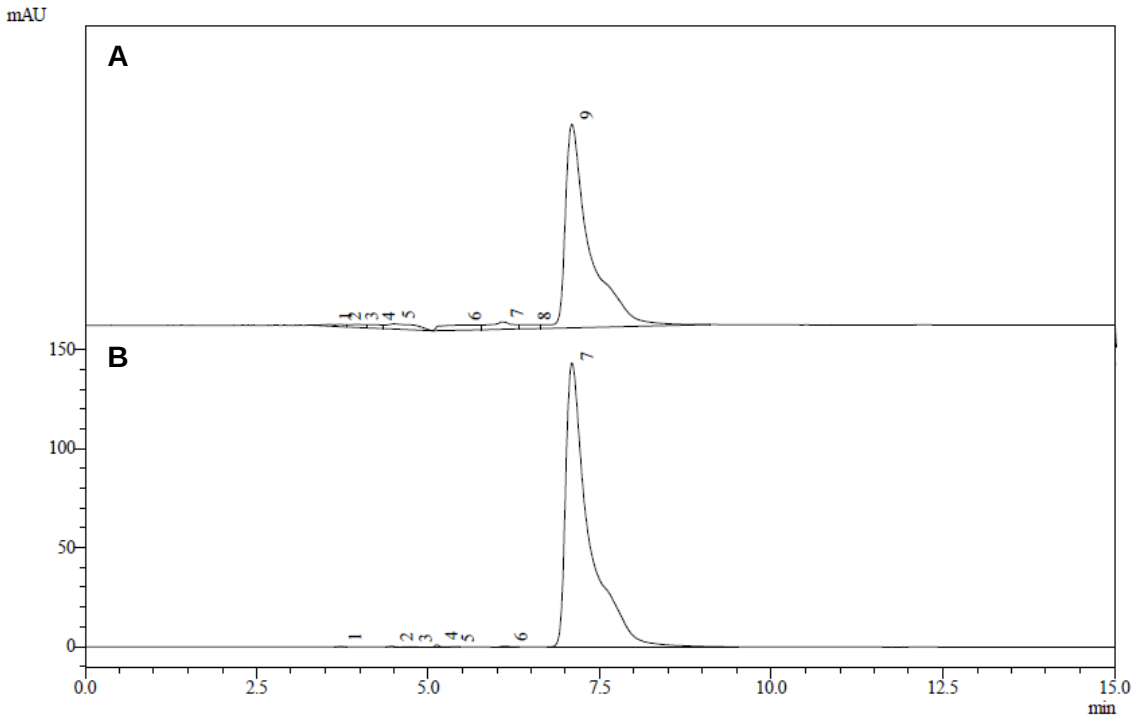


Figure 26. Chromatographic profile of a standard solution of MTX 130 µM using an HPLC-DAD system. The mobile phase was composed by acetonitrile: ammonium acetate 0.1 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 15 minutes. The band 9 and the band 7 of the chromatograms **A** and **B**, respectively, correspond to the anticancer drug, which had a retention time of 7.09 minutes (κ = 0.97).

Following, a mobile phase composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0 was also tested and, at a flow rate of 0.6 mL/min, a running time of 30 minutes and wavelengths of 254 nm and 658 nm, MTX (standard solution, 130 µM) was detected at a retention time of 16.57 minutes (κ = 3.49) (**Figure 27**). This mobile phase was selected to analyze the mice plasma samples.

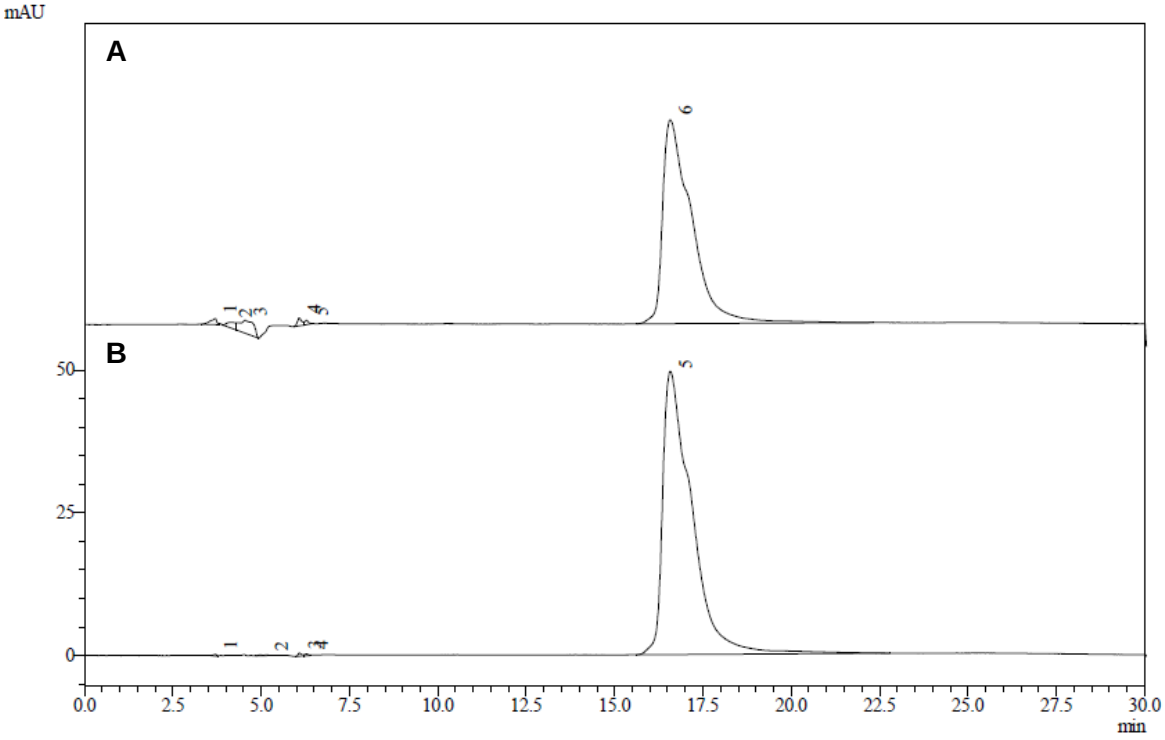


Figure 27. Chromatographic profile of a standard solution of MTX 130 µM using an HPLC-DAD system. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 6 and the band 5 of the chromatograms **A** and **B**, respectively, correspond to the anticancer drug, which had a retention time of 16.57 minutes (κ = 3.49).

4.6.2. Column selection

As already mentioned, the first tests were performed using a Spherisorb RP-18 ODS2 column (250 x 4.6 mm, 5 µm; reversed-phase; Waters, Milford, MA, USA). When another equipment was used [HPLC-DAD system (Shimadzu corporation, Kyoto, Japan)], the conditions previously found had to be replicated. This way, it was necessary to find an adequate column. It was tested a Nucleosil C18 ODS2 (250 x 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany), a Kinetex EVO C18 100Å (150 x 4.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) and a mediterranea sea_{18} (250 x 4.6 mm, 5 µm; Teknokroma, Barcelona, Spain), all in reversed-phase conditions. With the first one, MTX was not detected (data not shown). With the second one MTX eluted with the solvent front and presented entrainment (**Figure 28**). Only with the mediterranea sea_{18} MTX eluted properly (**Figure 29**).

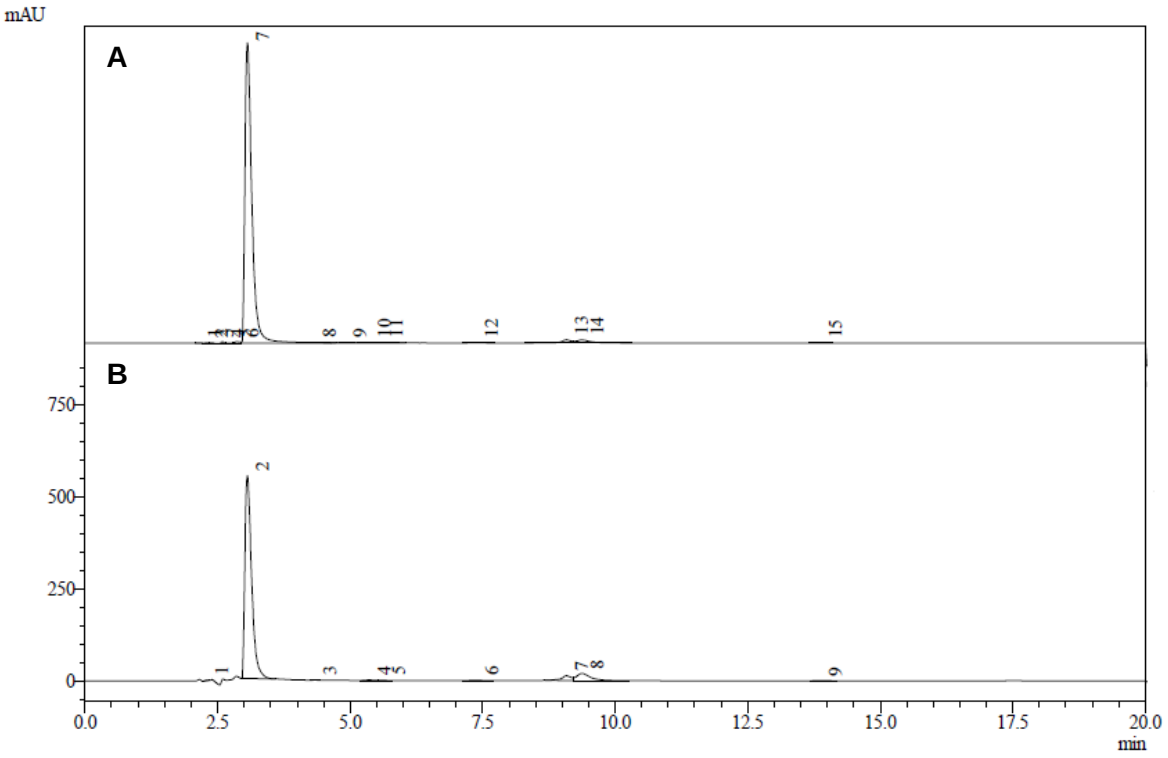


Figure 28. Chromatographic profile of a standard solution of MTX 260 µM using an HPLC-DAD system and a Kinetex EVO C18 100Å column. The mobile phase was composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 658 nm (**A**) and 254 nm (**B**) and the running time to 20 minutes. The band 7 and the band 2 of the chromatograms **A** and **B**, respectively, correspond to the anticancer drug, which had a retention time of 3.06 minutes (κ = 0.39). However, the following bands after these two are traces of MTX, which continued to be detected due to entrainment of the compound.

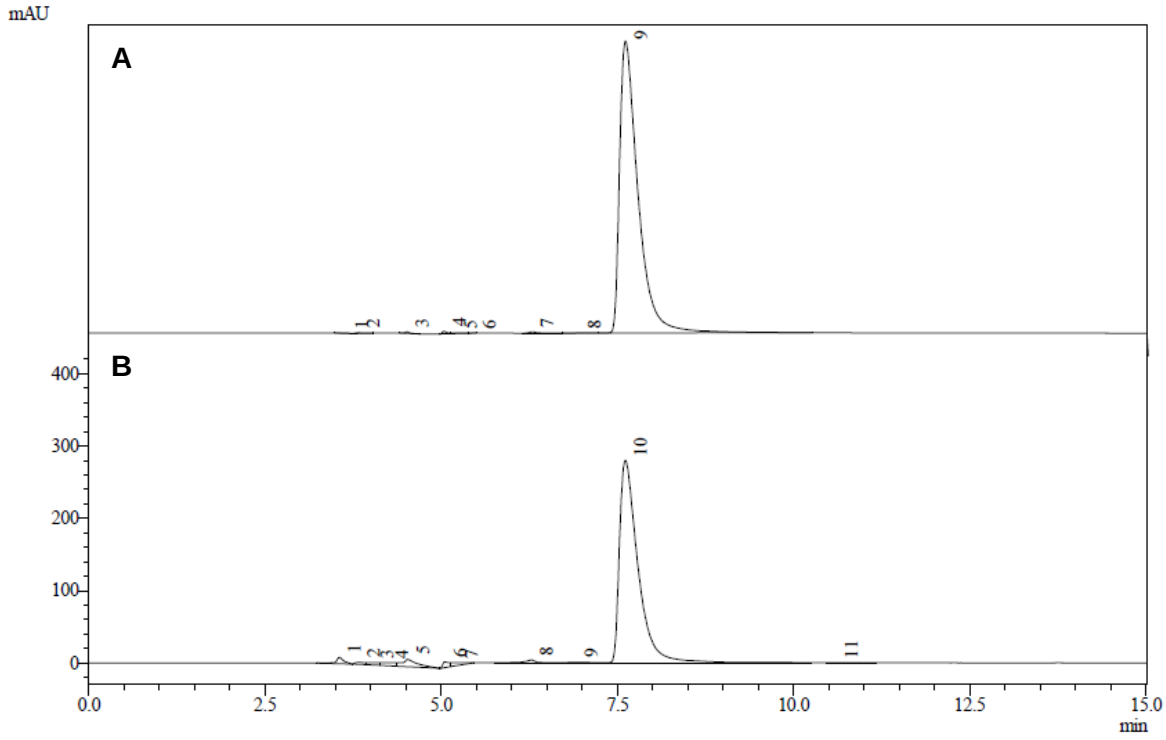


Figure 29. Chromatographic profile of a standard solution of MTX 260 µM using an HPLC-DAD system and a mediterranea sea$_{18}$ column. The mobile phase was composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 658 nm (**A**) and 254 nm (**B**) and the running time to 15 minutes. The band 9 and the band 10 of the chromatograms **A** and **B**, respectively, correspond to the anticancer drug, which had a retention time of 7.61 minutes (κ = 1.11).

4.6.3. Plasma sample analysis

Before the analysis of the mice plasma samples, human plasma or PBS were spiked with standard solutions of MTX, at different concentrations, and calibration curves were drawn. For systematic purposes, only the data regarding the calibration curve in plasma will be presented, since it was chosen as the most appropriate matrix if necessary to interpolate with the results obtained with the mice plasma samples (**Figure 32**). Since MTX has a strong carry-over effect, several precautions had to be considered while drawing the calibration curves: approximately 1 minute after each injection, the injector was washed out with human plasma, PBS, methanol and mobile phase and, between injections, PBS was also injected, in order to prevent the contamination of the following injections with MTX and to assure that MTX residues were not present, respectively.

Nevertheless, before any calibration curve being drawn, it was necessary to choose the appropriate internal standard. This way, AMT was used, since it has a similar structure to

MTX and it was already reported in the literature (Johnson et al., 2004). Then, it was necessary to verify if it could be detected using the same chromatographic conditions as MTX: at a flow rate of 0.6 mL/min, a running time of 20 min and wavelengths set to 254 nm and 658 nm, AMT (in PBS, 8.8 µg/mL), presented a retention time of 10.21 minutes (κ = 1.75) (**Figure 30**). Additionally, it was possible to verify that the UV-Vis spectra of AMT and MTX were similar (**Figure 31**, when comparing with **Figure 25**).

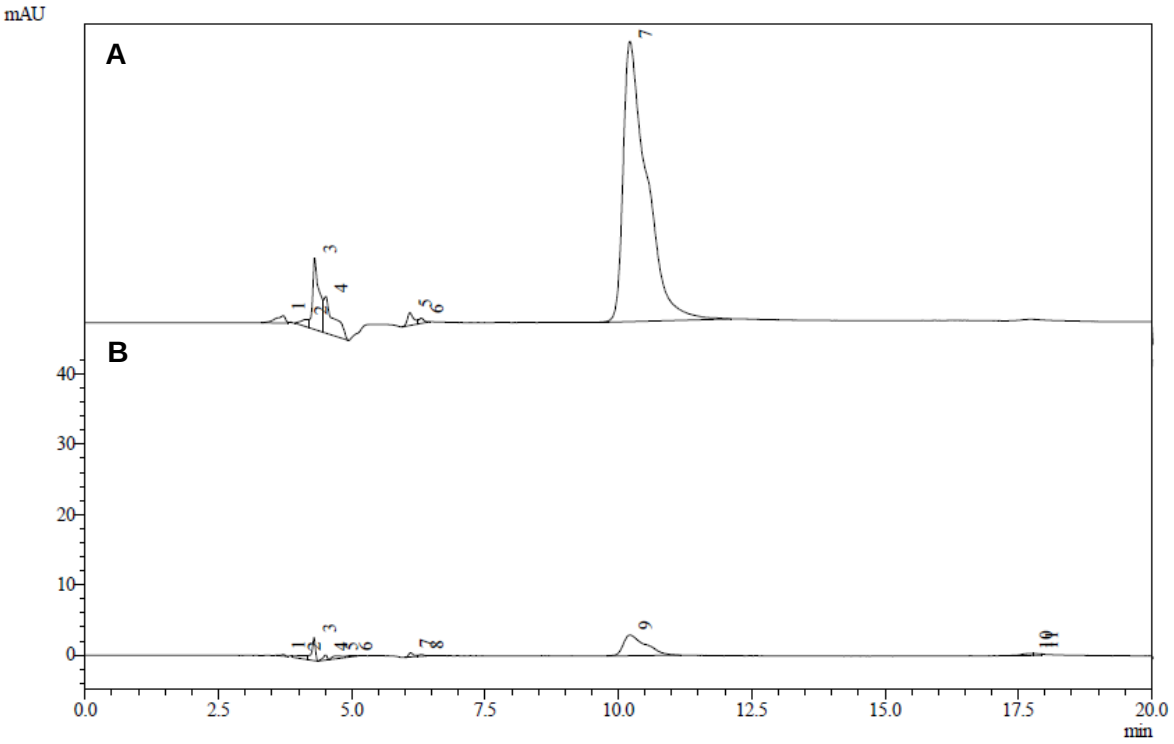


Figure 30. Chromatographic profile of a standard solution of AMT 8.8 µg/mL (in PBS) using an HPLC-DAD system and a mediterranea sea$_{18}$ column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 20 minutes. The band 7 and the band 9 of the chromatograms **A** and **B**, respectively, correspond to the compound, which had a retention time of 10.21 minutes (κ = 1.75).

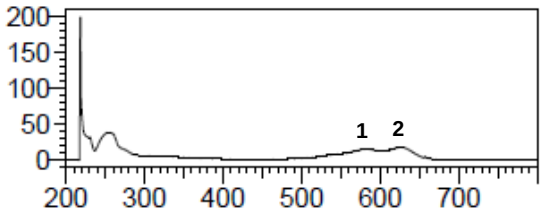


Figure 31. UV-Vis spectrum profile of AMT (standard solution of 8.8 µg/mL, in PBS). Peaks **1** and **2** correspond to 584 nm and 626 nm, respectively.

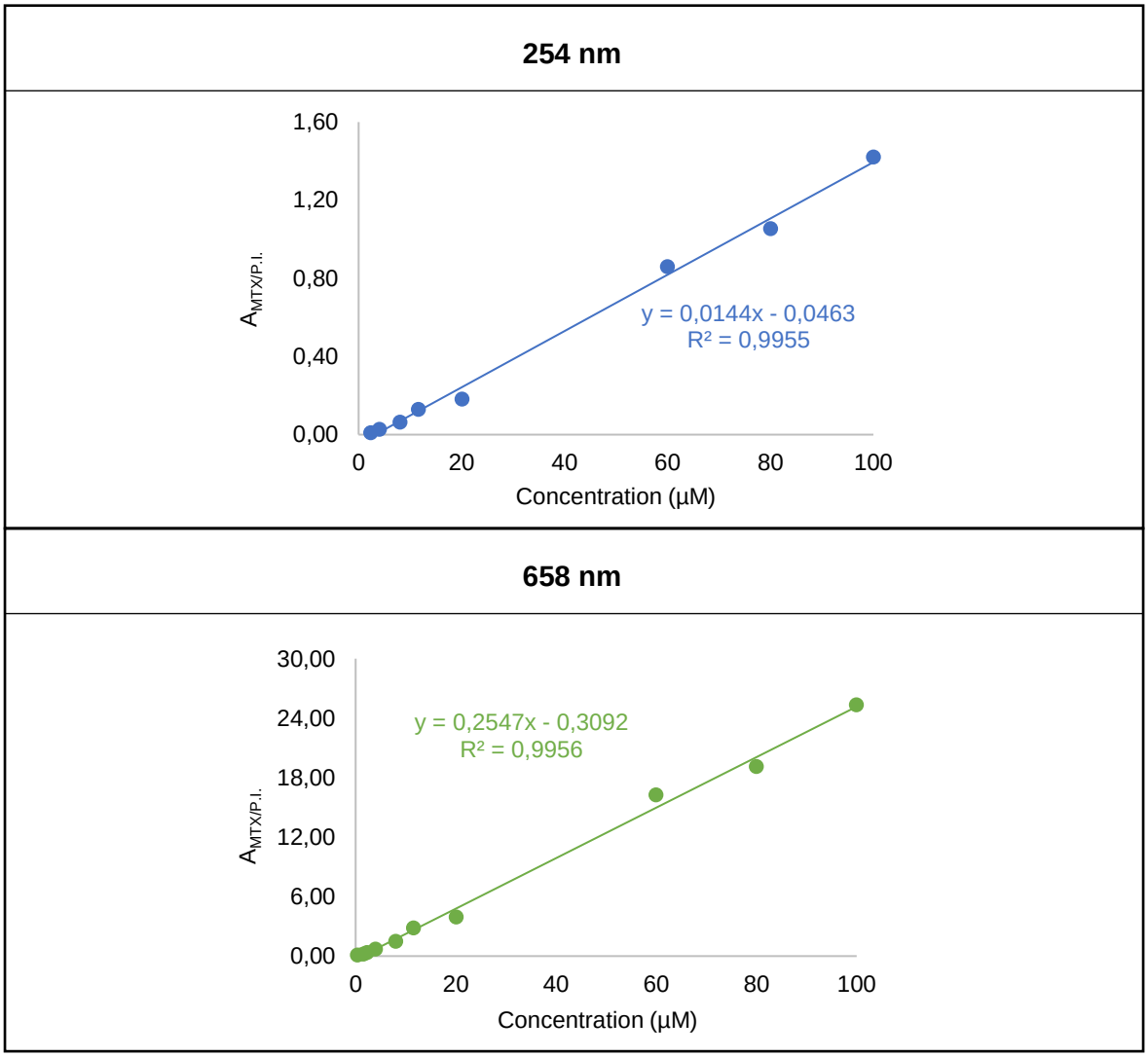


Figure 32. UV-Vis calibration curves in **plasma** with standard solutions of MTX at different concentrations and with AMT as the internal standard, at 254 nm and 658 nm. Each sample was injected in duplicate and the calibration curves were drawn using the mean value of the correspondent responses.

After the calibration curves being drawn, the LOD and LOQ were determined according to the formula in **Figure 8** and **Figure 9**, respectively, for both wavelengths: the LOD was 260 nM and 370 nM at 254 nm and 658 nm, respectively; the LOQ was 780 nM and 1.12 µM at 254 nm and 658 nm, respectively.

Afterwards, a standard solution of available NAPHT was prepared and analyzed, since this metabolite could be present/ detected in the MTX-treated mice plasma samples. No calibration curves were drawn for this compound so, AMT was not assessed if it was also a good internal standard for this compound. The flow rate was set to 0.6 mL/min and wavelengths of 254 nm and 658 nm were used. The mobile phase investigated was the same used for MTX detection: acetonitrile: 0.5% formic acid (20:80, v/v), at pH 3.0. However, it was observed that in these conditions NAPHT could not elute, not even when the running time was increased to 120 minutes. Therefore, different proportions of acetonitrile: 0.5% formic acid were investigated: (50:50, v/v), (40:60, v/v) and (30:70, v/v). It was found that a proportion of acetonitrile: 0.5% formic acid (30:70, v/v) was the best one to analyzed the MTX metabolite, using the same chromatographic conditions as for MTX. The analyses were completed after 30 minutes (**Figure 33**). In these chromatographic conditions, the MTX and AMT did not elute properly so, two injections *per* sample had to be made (elute with the solvent front). It was also possible to observe that NAPHT (standard solution, 520 µM) has a similar UV-Vis spectrum profile as MTX (**Figure 34**, compared with **Figure 25**).

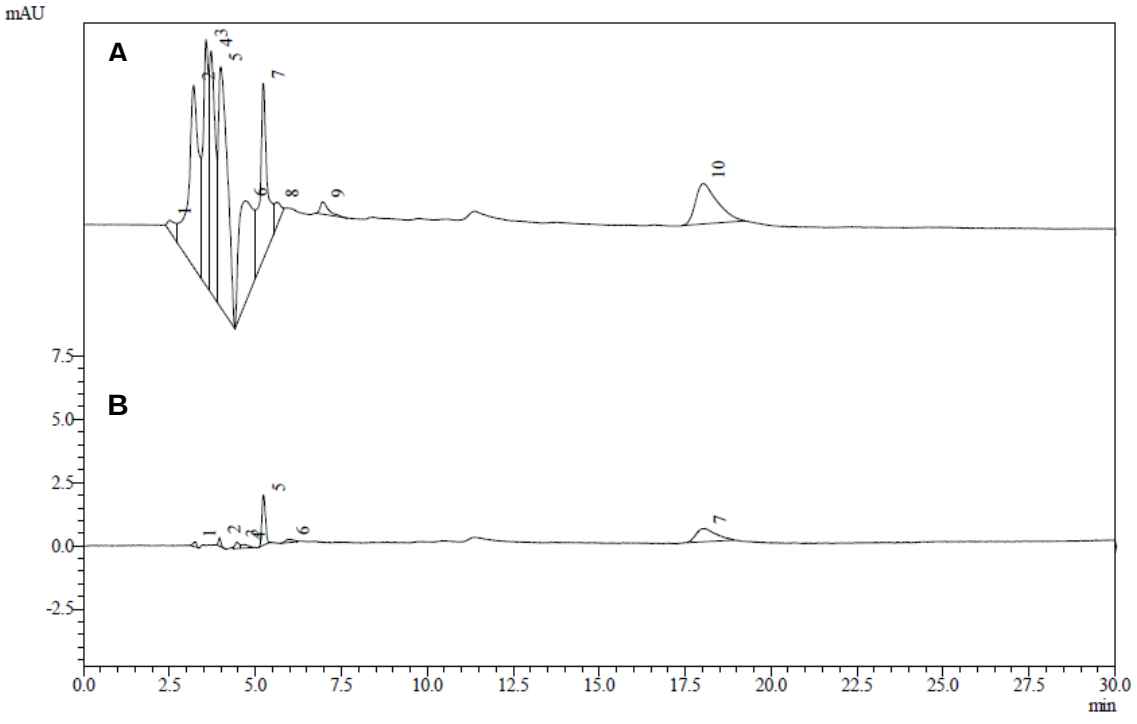


Figure 33. Chromatographic profile of a standard solution of NAPHT 520 µM using an HPLC-DAD system. The mobile phase was composed by acetonitrile: 0.5% formic acid (30:70, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 10 and the band 7 of the chromatograms **A** and **B**, respectively, correspond to the metabolite, which had a retention time of 18.05 minutes (κ = 5.33).

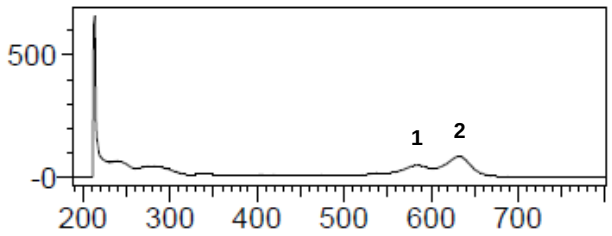


Figure 34. UV-Vis spectrum profile of NAPHT (standard solution of 520 µM). Peaks **1** and **2** correspond to 584 nm and 633 nm, respectively.

4.6.3.1. Mice plasma samples

Summing up the results previously found, the mice plasma samples were analyzed in an HPLC-DAD system (Shimadzu corporation, Kyoto, Japan), using a mediterranea sea_{18} (250 x 4.6 mm, 5 µm; Teknokroma, Barcelona, Spain) column and a mobile phase composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0. The flow rate was kept at 0.6 mL/min, the running time to 30 minutes and the wavelengths to 254 nm and 658 nm. Additionally, in order to trying to detect NAPHT, the same chromatographic conditions were used except for the acetonitrile: 0.5% formic acid proportion, which was set to 30:70, v/v. To prevent the contamination of the following injections with MTX, a cleaning procedure was adopted: approximately 1 minute after each injection, the injector was washed out with human plasma, PBS, methanol and mobile phase and, between injections, PBS was also injected. It is important to keep in mind the retention times of the chemotherapeutic drug and its metabolite, as well of the internal standard previously observed in these chromatographic conditions: 10.21 minutes, 16.57 minutes and 18.05 minutes for AMT, MTX and NAPHT, respectively, and that this method presented a LOD for MTX of 260 nM and 370 nM at 254 nm and 658 nm, respectively.

The results from MTX and NAPHT detections will be presented separately since they correspond to two different chromatographic runs. Starting with MTX, when analyzing the mice plasma samples from the infant-treated mice, it was verified that the chemotherapeutic drug could not be detected, being only observed the presence of the internal standard. For a better understanding of which chromatographic peaks could correspond to components naturally present in the plasma or not, the gathered plasma samples from the infant control mice were also analyzed. Therefore, two chromatograms will be presented to illustrate the analyses: one from an infant control mouse and other from a MTX-treated one (**Figure 35**

and **Figure 36**, respectively). The remaining chromatograms will not be shown since were similar to the ones presented herein.

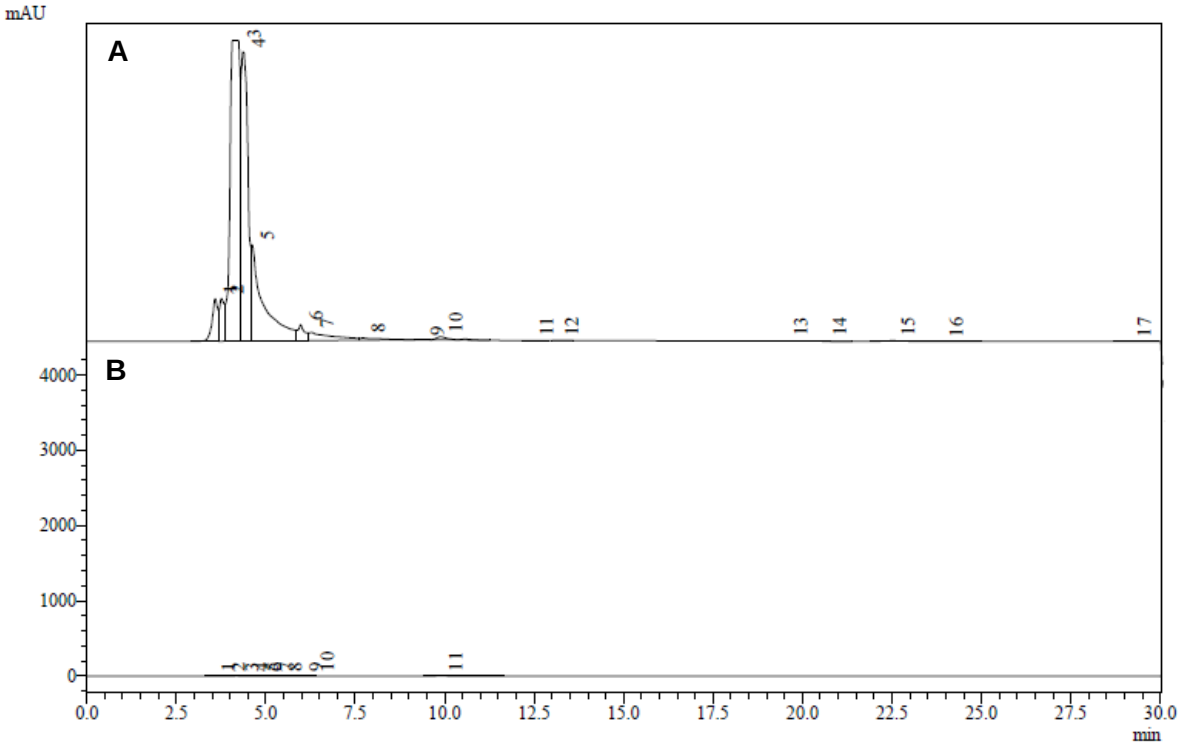


Figure 35. Chromatogram of a plasma sample from a **control infant** mouse, representative of the three analyzed control-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 10 and the band 11 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 9.88 minutes ($\kappa = 1.78$).

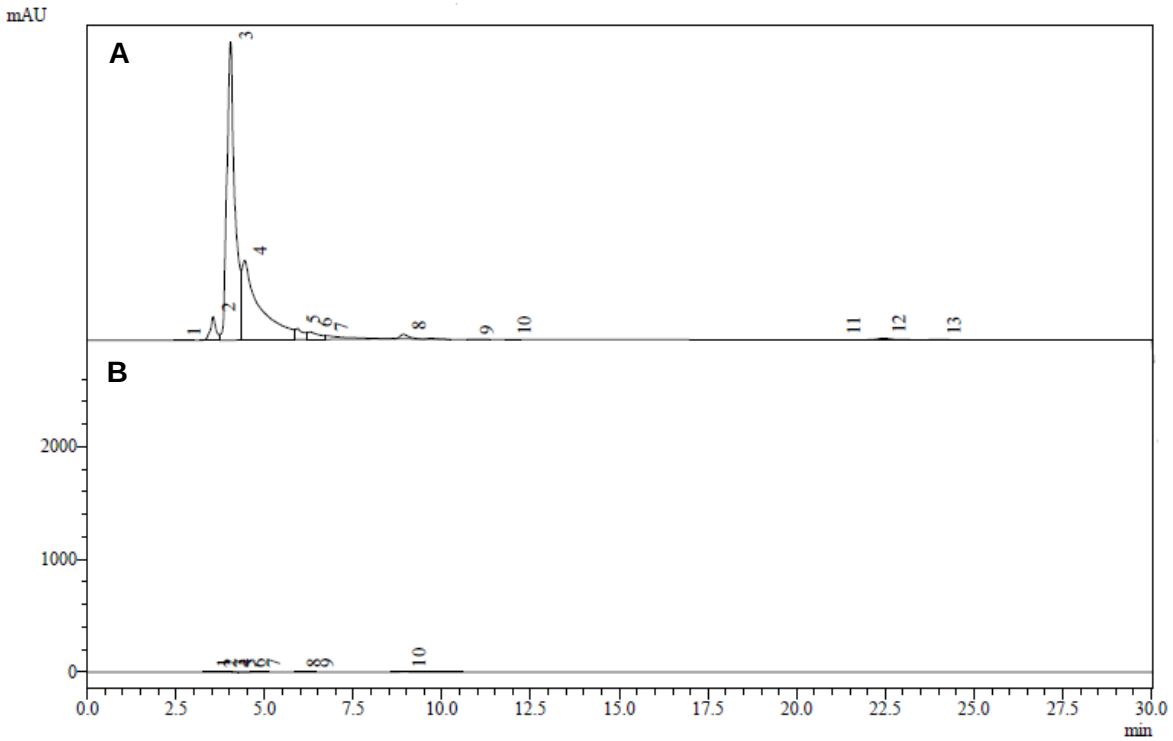


Figure 36. Chromatogram of a plasma sample from a **MTX-treated infant** mouse, representative of the six analyzed treated-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 8 and the band 10 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 8.91 minutes (κ = 1.97). MTX was not detected.

Regarding the adult population, similar results to the ones obtained with the infant mice were observed: MTX could not be detected in the plasma of these animals, being only verified the presence of AMT (**Figure 38**). Also, the results were similar between the two adult mice assays. The plasma samples from adult control mice were also analyzed and no interfering peaks were observed (**Figure 37**).

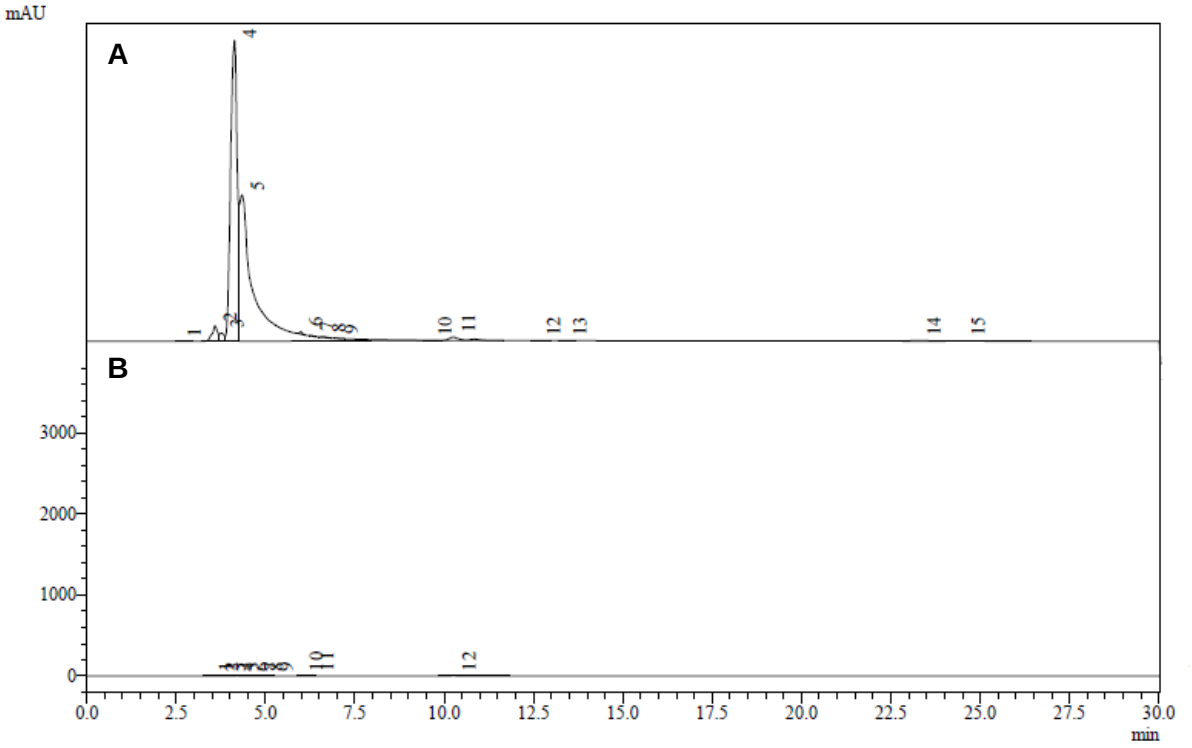


Figure 37. Chromatogram of a plasma sample from a **control adult** mouse, representative of the nine analyzed control-mice plasma samples: three from assay 1 and six from assay 2. An HPLC-DAD system was used, with a mediterranea sea18 column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 11 and the band 12 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 10.25 minutes (κ = 2.36).

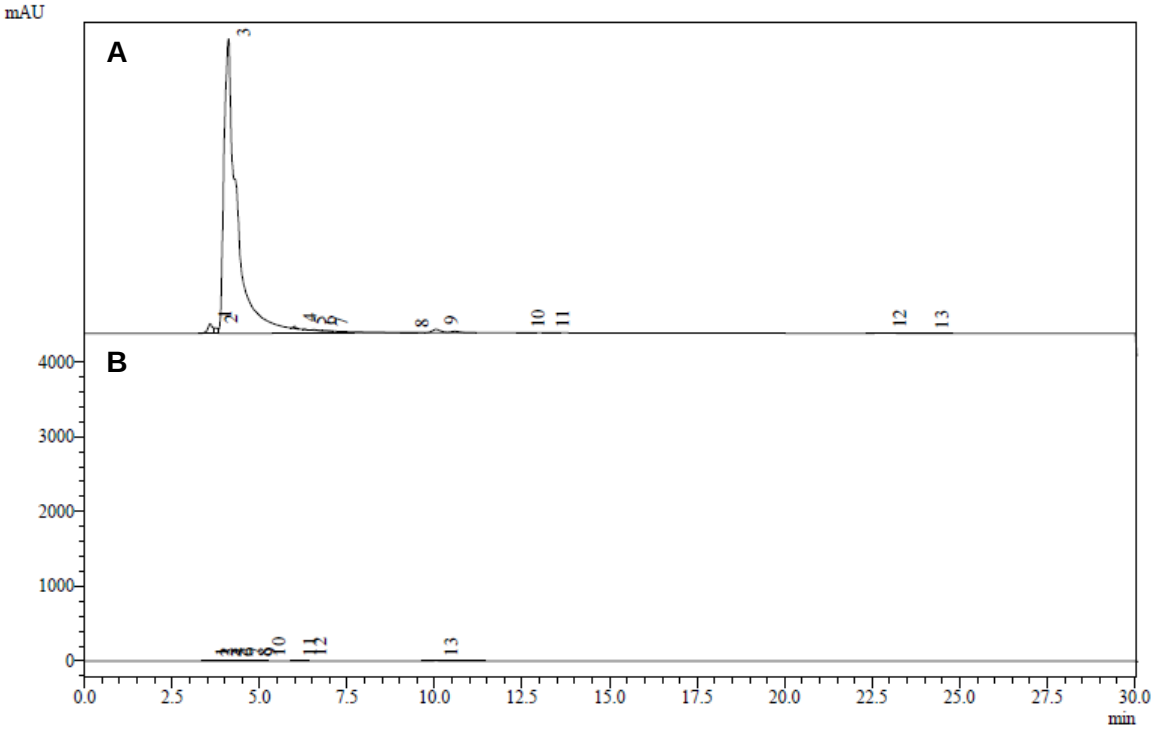


Figure 38. Chromatogram of a plasma sample from a **MTX-treated adult** mouse, representative of the seventeen analyzed treated-mice plasma samples: seven from assay 1 and ten from assay 2. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 9 and the band 13 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 10.04 minutes (κ = 1.83). MTX was not detected.

Likewise, the results observed in the MTX-treated elderly mice were similar to the ones from the other two mentioned age groups, that is, it was not possible to verify the presence of MTX in the plasma samples from these animals, being only detected the internal standard. The plasma samples from the control elderly mice were also analyzed and, once again, only the results from one mouse from each group (control and MTX groups) are shown, being representative of the all analyzed population (**Figure 39** and **Figure 40**).

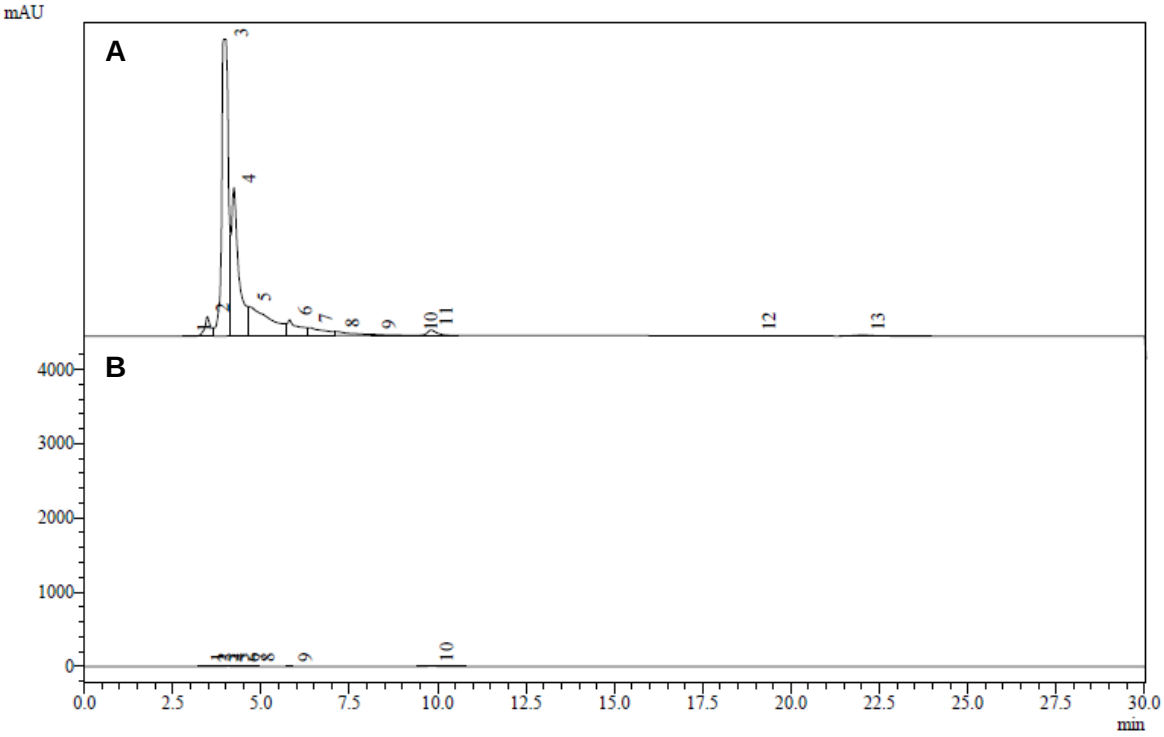


Figure 39. Chromatogram of a plasma sample from a **control elderly** mouse, representative of the three analyzed control-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea18 column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 11 and the band 10 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 10.09 minutes (κ = 2.10).

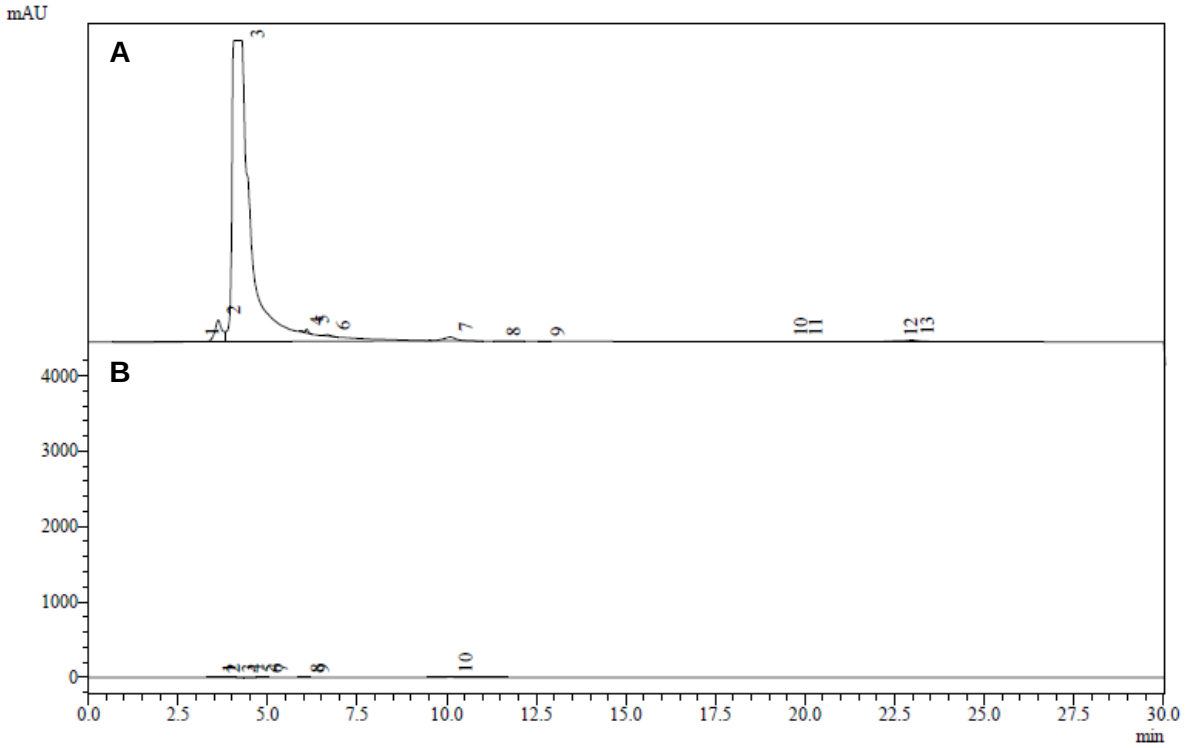


Figure 40. Chromatogram of a plasma sample from a **MTX-treated elderly** mouse, representative of the four analyzed treated-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 7 and the band 10 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 9.81 minutes (κ = 2.11). MTX was not detected.

Concerning the attempts to find NAPHT, only the plasma samples from adult mice (assay 2) were analyzed. Similar to what happened with MTX, it was not possible to detect the presence of this metabolite in the plasma samples from the analyzed MTX-treated adult mice. So, it was only possible to observe the presence of the internal standard. The plasma samples from the adult control mice (assay 2) were also analyzed. Herein, are presented two illustrative chromatograms: one from a control mouse and other from a MTX-treated one (**Figure 41** and **Figure 42**, respectively). The remaining chromatograms from the other analyzed adult mice will not be presented since similar results were obtained. The presented chromatograms are representative of the all analyzed mice, regarding each group (MTX and control groups).

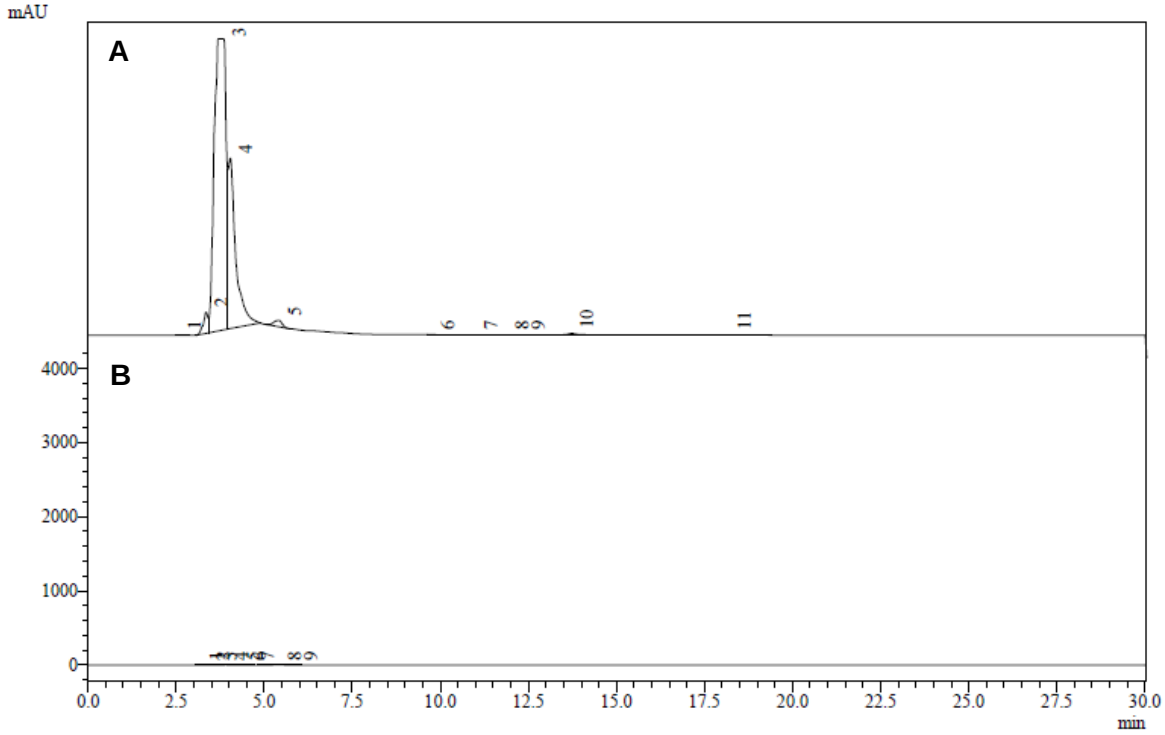


Figure 41. Chromatogram of a plasma sample from a **control adult** mouse (assay 2), representative of the two analyzed control-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (30:70, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 5 and the band 8 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 5.43 minutes (κ = 0.87). NAPHT was not detected.

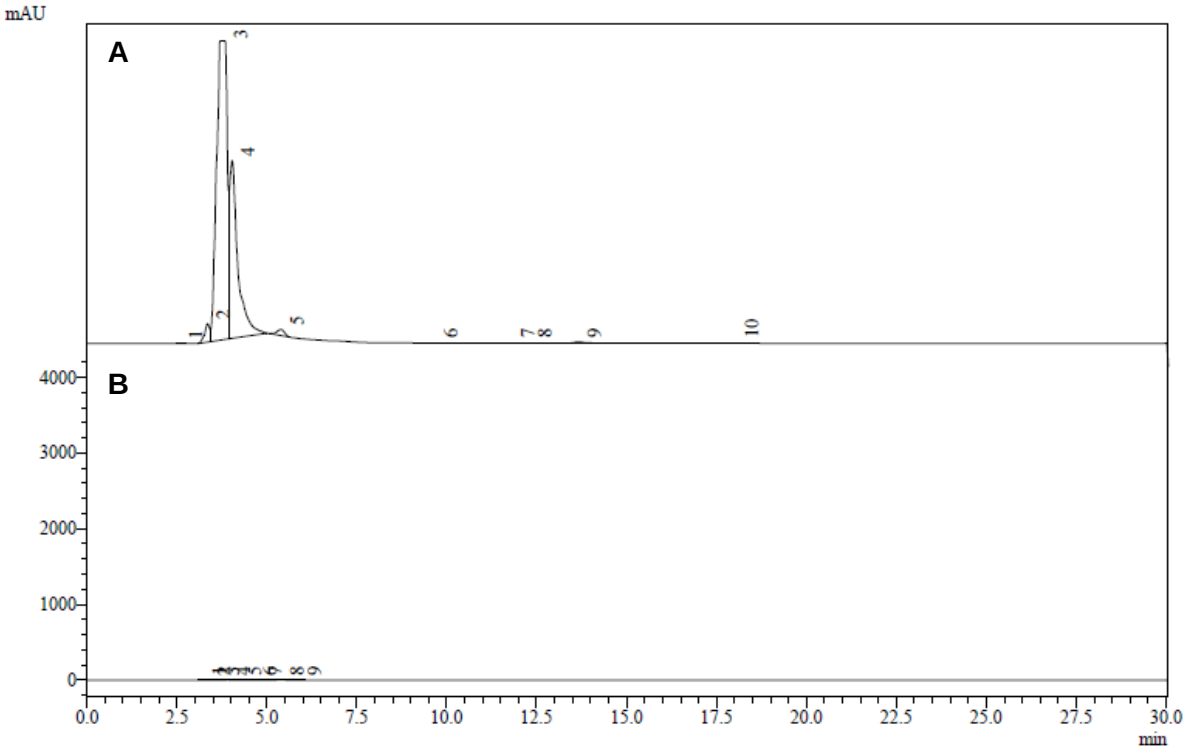


Figure 42. Chromatogram of a plasma sample from a **MTX-treated adult** mouse (assay 2), representative of the ten analyzed treated-mice plasma samples. An HPLC-DAD system was used, with a mediterranea sea_{18} column. The mobile phase was composed by acetonitrile: 0.5% formic acid (30:70, v/v) pH 3.0, and was kept at a flow rate of 0.6 mL/min. Wavelengths were set to 254 nm (**A**) and 658 nm (**B**) and the running time to 30 minutes. The band 5 and the band 8 of the chromatograms **A** and **B**, respectively, correspond to AMT, which had a retention time of 5.40 minutes ($\kappa = 0.86$). NAPHT was not detected.

CHAPTER 5.

Discussion

5. DISCUSSION

The major findings of this dissertation were: MTX (cumulative dose of 6 mg/kg) caused a significant weight loss in the adult mice; MTX caused a significant reduction in food and water consumptions in the adult and infant mice, when compared with the respective controls; MTX treatment significantly decreased all the organ weight/ brain weight ratios in the adult population and the spleen weight/ brain weight ratio on the elderly mice, in comparison with the respective controls; MTX caused a significant increase in the ALT levels and, subsequently, a decrease in the AST/ ALT ratios of the infant and adult (assay 1) populations; MTX caused morphologic and structural changes in the heart of all the treated populations; MTX and NAPHT could not be detected in any of the mice plasma samples analyzed by HPLC-DAD, at the conditions set by us.

Chemotherapeutic treatment with MTX has been widely used; however, despite its benefits, it can be responsible for several negative side effects, such as cardiotoxicity. It is known that this anticancer drug has some similarities with anthracyclines (Alberts et al., 1985). Nevertheless, their mechanisms of toxicity differ and the ones regarding MTX remain not properly clarified at this point. In human therapy, MTX is commonly administered at doses ranging from 12 to 14 mg/m^2, monthly or every three weeks, depending on the type of cancer. The toxic effects induced by this chemotherapeutic drug have been sometimes observed years after treatment ended, being a late event (BC_Cancer, 2017; NIH, 2015b). With this in mind, the administration schedule adopted in the present work consisted of multiple administrations of the chemotherapeutic drug, in separated time-points: the animals were subjected to a total of six intraperitoneal injections, administered twice a week, in order to mimic the human anticancer therapy. This allowed to evaluate the toxicity induced by a similar cumulative dose, over time, instead of an acute response to the drug. This way, the mice were afterwards submitted to a drug-free period to allow the development of the toxic effects as a response to the MTX-treatment. Additionally, it is known that several risk factors could aggravate the incidence of MTX toxicity, such as the age at diagnosis. So, three different age groups (infants, adults, elderly) were analyzed, in order to assess age as risk factor for the MTX-induced toxicity and the subsequent influence on the metabolic profile. The administrations were given in the beginning of the afternoon because MTX has proven to be better tolerated at this point due to the circadian rhythm differences in the clinical toxicity of this drug (Lévi et al., 1994). This way, MTX can maintain its anticancer activity with an associated lower toxicity. Since the criteria used for sacrifice was based on a pain scoring system, the adult and elderly mice were sacrificed 7 days after the last administration, while infants were euthanized 17 days after the last administration. In a

previous study, the infant population was proven to be more resistant than the adult one (Dores-Sousa et al., 2015).

5.1. General welfare

The obtained results demonstrated that treatment with MTX at a cumulative dose of 6 mg/kg caused a significant weight loss only in the adult mice: in assay 1, a tendency for a significant decrease in the body weight gain of the MTX group was observed, in comparison with the controls, which was achieved in the assay 2, where the cohort was higher. The infant population only showed initial significant differences in the body weight gain between the MTX-treated and the control groups due to the poor random distribution of the mice belonging to different litters. In fact, a more stable growth between the two groups was observed with the course of the experiment, which was already expectable since, at the moment of sacrifice, these mice had already reached puberty, where that rapid initial body weight gain started to stabilize. In the elderly population, the body weight of the animals was constant through the all experiment, even when MTX was administered.

The last administration day corresponded to the 17th day of the protocol. The significant differences observed in the adult population occurred from this day on, which could mean that these effects on the body weight of the animals may have resulted from the total cumulative dose of MTX. Other studies have already reported body weight losses in MTX therapy. For example, in a study performed by Lévi *et al.*, male B6D2F$_1$ received a single dose of 13, 14.5 or 16 mg/kg of MTX (Lévi et al., 1994). The authors recorded the body weight of the animals 3 times a week, during 60 days and they observed significant body weight losses in these animals, in comparison with controls (higher with increasing doses) (Lévi et al., 1994). In another study, Rossato *et al.* evaluated the mitochondrial effects induced by MTX treatment in male Wistar rats, who received 2.5 mg/kg of MTX, at days 0, 10 and 20, for 3 cycles (cumulative dose of 7.5 mg/kg) (Rossato et al., 2014). To evaluate the early and late cardiotoxicity of MTX, the rats were sacrificed in two different moments: 22 or 48 days after the beginning of the experiment. A reduction in the relative body weight gain of both groups was observed, reaching statistical significance after the 3rd cycle, that is, when a cumulative dose of 7.5 mg/kg was reached, meaning that this effect was due to the MTX cumulative dose (Rossato et al., 2014). In another study, the MTX-induced toxicity was assessed in infant and adult male CD-1 mice with a similar administration schedule as the one described in the present work, however with a cumulative dose of 9.0 mg/kg (Dores-Sousa et al., 2015). The sacrifices occurred in two time-points: 24 hours or 20 days after

the last administration, in order to evaluate the early and late cardiotoxicity, respectively. Analyzing the results of the sacrifices that occurred 20 days after the last injection, since that was a similar period to the drug-free period employed in this dissertation, body weight changes were statistically significant in the adult population, corroborating with the observed results in the present dissertation (Dores-Sousa et al., 2015).

The hypothesis if these significant differences in the body weight of the animals may be due to differences in the food and water consumptions was analyzed. In the present work, it was verified that, with a cumulative dose of 6 mg/kg of MTX, infant and adult mice ate and drank significantly less, when compared to the respective control groups. The increase in the number of adult mice (assay 1 to assay 2) allowed to ascertain those differences. No significant differences were observed in the elderly population. Once again, the differences observed between the MTX-treated and the control groups acquired statistical significance after the last administration day, where the total cumulative dose was reached. In a previous study performed by Niang *et al.*, similar results as the ones in the present dissertation were observed (Niang et al., 2011). Female NMRI mice were administered with 6, 9 or 12 mg/kg of MTX (single injection). The sacrifice occurred on the 14th day and the authors observed a significant reduction in the body weight of these animals (higher with increasing doses), accompanied with a significant decrease in the food consumption (Niang et al., 2011). In another study, infant and adult male CD-1 mice received a cumulative dose of 9.0 mg/kg of MTX by a similar administration schedule as the one described herein and, alongside with the body weight changes observed in the adult population (sacrifice 20 days after the last administration), food and water consumptions of the MTX-treated adults also decreased, in comparison to controls (Dores-Sousa et al., 2015). The infant MTX-treated mice also presented reduced food and water intakes; however, the differences were more significant in the adults. In general, this study also demonstrated a higher susceptible of the adult mice to the cardiotoxicity induced by MTX treatment, as observed herein (Dores-Sousa et al., 2015).

With the obtained results, one could assume that the body weight losses may result from these differences in the food and water consumptions, since the MTX-treated adult mice presented significant differences in both body weight and food and water consumptions, when compared to controls, while the elderly population did not present significant changes in any of these parameters. Nevertheless, infant mice presented reduced food and water intakes, when compared to controls, but no significant differences in the body weight gain were observed. So, it could be assumed that the observed differences in the body weight of the animals were not necessarily entirely related to

decreased food and water consumptions. In fact, in a previous study where male Wistar rats received MTX in a cumulative dose of 7.5 mg/kg, the authors did not observe significant differences between the food and water consumptions of the MTX-treated and control groups, despite the significant body weight changes in these animals (Rossato et al., 2014). Likewise, Dores-Sousa *et al.* observed that when infant male CD-1 mice received a cumulative dose of 9 mg/kg of MTX and were sacrificed 20 days after the last administration, they presented significant decreases in their food and water consumptions but without significant body weight losses, in comparison with controls (Dores-Sousa et al., 2015). These studies support the hypothesis that these parameters (body weight loss and decreased food and water consumptions) are not necessarily correlated.

With this in mind, it is necessary to assess other possible factors that could lead to body weight losses. In humans, it is already known that MTX could induce gastrointestinal toxicity, such as nausea, vomiting, diarrhea and mucositis (NIH, 2015b; Seiter, 2005). The chemotherapeutic drugs are not selective and, therefore, cannot make the distinction between cancer and normal cells, which is a major problem, since some normal cells can also grow and divide quickly, such as the ones from the gastrointestinal tract (NIH, 2015a). This way, the treatment with MTX at a cumulative dose of 6 mg/kg could have led to gastrointestinal disturbances that were reflected in the differences observed in the body weight of the animals and in their food and water consumptions. Gathered the results, the adult population seemed to be more susceptible to the treatment, exhibiting signs of severe gastrointestinal toxicity. In infants, since no differences in the body weight of the treated mice were observed, the gastrointestinal disturbances may have been less significant than in adults, since they started to consume less after receiving the total cumulative dose of MTX, but without body weight changes, when compared to controls. This way, it can be assumed that the infants are more resistant to a MTX cumulative dose of 6 mg/kg than adults. In fact, at sacrifice it was observed that some MTX-treated infant mice presented ascites (accumulation of fluid in the peritoneal cavity), which causes abdominal swelling. Additionally, one of them presented a swollen stomach and with much air, possibly aerophagia, and its intestines were dark and swollen. This latter event was observed in another mouse. In the MTX-treated adult population, some of them also presented ascites and one mouse presented a very swollen stomach, as well, and he was clearly in pain, since was not very active, in comparison with the remaining treated adults. With these observations, one could conclude that MTX caused similar effects in both age groups, regarding abdominal symptoms; nevertheless, together with the gathered data from the body weight gain and food and water consumptions, the idea that the younger mice were more resistant to a cumulative dose of 6 mg/kg of MTX than adults is supported. On the

other hand, it is known that a younger age may favor weight gain, which was observed throughout the experiment and that could explain the obtained results (Gadéa et al., 2012). Regarding the elderly, the observed results suggest that they were more resistant to the effects of the treatment than the other two age groups, regarding the gastrointestinal toxicity, since no significant differences were observed in the body weight and food and water intakes of the treated animals, in comparison with controls. In fact, it is known that the aging process induces a decline in the function of the gastrointestinal system by its own (Drozdowski and Thomson, 2006). This way, this occurrence could turn this population less susceptible to gastrointestinal toxicity induced by MTX, explaining the obtained results.

As already mentioned, some organs were collected at sacrifice: brain, heart, kidneys, spleen and liver. The brain weight was used to assess the changes in the weight of the remaining organs collected at sacrifice instead of the body weight, since the weight of the animals decreased in some groups. With the obtained results, it was possible to verify that, with a cumulative dose of 6 mg/kg of MTX, the weight of the organs of the adult and elderly mice were affected by the administration of this anticancer drug; however, in a greater extent in the adult population. No significant differences were observed between the organ weight/ brain weight ratios of the MTX-treated and control infant mice, suggesting, once again, that this population was more resistant to the treatment. Regarding the adult population, significant changes were observed in the kidneys and liver of both trials, in the mice administered with MTX. In the heart, a tendency for decreased heart weight/ brain weight ratio was observed in the MTX-treated group from assay 1, comparing with the controls, achieving statistical significance in assay 2, where the cohort was higher. The second group of MTX-treated adults also presented a significantly decreased spleen weight/ brain weight ratio, in comparison with controls. With these results, it is clear that an increase in the number of analyzed animals exacerbated the differences between MTX-treated and control mice, demonstrating the potential toxicity of MTX, at a cumulative dose of 6 mg/kg, for all the organs in question. At sacrifice, besides the gastrointestinal changes already mentioned, also some changes in the liver of some MTX-treated mice, such as reduced size, were observed. These results support the previously mentioned hypotheses that the adult mice have a higher propension to the toxicity induced by MTX treatment. It is known that MTX and its metabolites are excreted through the hepatobiliary and renal systems (BC_Cancer, 2017). So liver and renal impairment can result in delayed excretion and metabolism of the drug, leading to increased system toxicity (Merchan and Jhaveri, 2018). Hepatotoxicity had already been reported in mice treated with MTX (Llesuy and Arnaiz, 1990). In a study reporting the possible hepatotoxicity in female Swiss mice, MTX was given in a single dose of 15 mg/kg. The animals were sacrificed 3, 4 or 5 days after injection, in

order to evaluate the steady state concentration of singlet molecular oxygen (through *in situ* liver chemiluminescence), to measure the rate of *in vivo* lipid peroxidation (through malonaldehyde measurements) and to evaluate the amount of chain-breaker antioxidants in tissue and the existence of oxidative stress (through hydroperoxide-initiated chemiluminescence). Four days after injection, significant increases of 15%, 73% and 52% in liver spontaneous emission, malonaldehyde levels and hydroperoxide-initiated chemiluminescence of liver homogenates, respectively, were observed in the MTX treated mice. In the other two evaluated days, no significant changes were observed. When measuring the hepatic antioxidant enzyme levels (superoxide dismutase, catalase and glutathione peroxidase) 4 days after injection, the authors observed a diminished activity of these antioxidant enzymes in the liver of the MTX-treated mice. Additionally, the assessment of the secondary antioxidant defenses showed a decrease of 39% in glutathione levels, also 4 days after injection. Then, when analyzing the histopathology of the livers, damaged hepatocytes were observed in the mice treated with MTX (Llesuy and Arnaiz, 1990). In the present dissertation, the hepatotoxic potential of MTX was observed through decreased liver weight/ brain weight ratio of the MTX-treated adult population. Similar results had already been previously observed. For example, Niang *et al.* administered MTX to female NMRI mice, through a single injection, in doses of 6, 9 or 12 mg/kg (Niang et al., 2011). When the animals were sacrificed 14 days after the beginning of the experiment, the authors observed significant differences in the relative liver weight of the mice injected with the chemotherapeutic drug, in comparison with controls (Niang et al., 2011). On the other hand, studies reporting the renal toxicity induced by MTX-treatment are scarce. Nevertheless, it is known that MTX is extensively bounded to this tissue, meaning that is slowly eliminated from the kidneys, which contributes to the MTX potential of inducing toxicity (PDR, 2018e). Regarding the possible negative effects to the spleen, few studies had been made so far. Nevertheless, when assessing the circadian changes in MTX toxicity in male $B6D2F_1$ mice, Lévi *et al.* were able to observe splenic lesions induced by the treatment (Lévi et al., 1994). The animals received 14.5 mg/kg of MTX as a single dose at one of 6 or 4 circadian changes differing by 4 or 6 hours. A decrease in the spleen weight of approximately 60% was observed in the MTX-treated mice, 4 and 8 days after injection (Lévi et al., 1994). In another study, Levine *et al.* conducted an experiment using Lewis rats of both genders, which were administered with a cumulative dose of 3 mg/kg of MTX (Levine and Gherson, 1986). Likewise, the authors observed a reduction in the spleen weight of these animals only 1 day after the last administration, demonstrating the toxic potential of this chemotherapeutic drug (Levine and Gherson, 1986). This way, damages to the spleen cannot be rule out when administering MTX as part of the chemotherapeutic regimen. Concerning the cardiotoxicity caused by MTX therapy, this remains one of the main side

effects of this treatment and, therefore, has been extensively study in humans and experimental models (Cornbleet et al., 1984; Fleischer et al., 2014; Rossato et al., 2014; Viglione et al., 1993). In the present dissertation, the cardiotoxic potential of MTX was observed through decreased heart weight/ brain weight ratio of the MTX-treated adult population. Similar results had already been found in a study conducted by Zbinden and Beilstein, for example (Zbinden and Beilstein, 1982). Female Sprague-Dawley rats received a cumulative dose of 2 mg/kg of MTX. Two weeks after the last administration, the animals were sacrificed and the authors observed a decrease in the heart weight of the animals treated with the chemotherapeutic drug, in comparison with controls (Zbinden and Beilstein, 1982). This way, the observed results in the present work regarding the organ weight/ brain weight ratios of the treated-adult mice suggested, once again, that this population was particularly at risk when receiving MTX at a cumulative dose of 6 mg/kg.

Concerning the elderly population, there was a tendency for reduced liver weight/ brain weight ratio of the MTX-treated mice, in comparison with controls. In fact, at sacrifice, some liver changes were observed in two of them: whitish liver and an abnormal structure. Nevertheless, significant differences between the two groups were only achieved in the spleen weight/ brain weight ratio. It is known that the spleen is related with the production of lymphocytes, that are responsible for the formation of antibodies, and with the removal of old and damaged erythrocytes. It also acts as a reservoir of blood and cells from the immunologic system (monocytes) (Hoffman, 2017). This way, spleen has an important immunologic function, helping to protect against infections. However, some studies already reported age-related changes in the spleen, namely with increasing age. In fact, Cheung and Nadakavukaren observed a decrease in the cellularity of the spleen in older rats: a lower number of lymphocytes and increased structural disturbance were found in the elderly, demonstrating a significant age-related decline of spleen lymphocyte functions (Cheung and Nadakavukaren, 1983). In another study, spleen structural changes were also observed in aged mice, affecting the immune cells work, which results in a less effective or a decrease in immune responses (Turner and Mabbott, 2017). These studies demonstrated that older age, by itself, induces significant changes in the spleen, which could explain the observed results in the present dissertation. Nevertheless, MTX could have worsened the aging effects on the spleen, since Lévi *et al.* observed splenic lesions induced by MTX-treatment (14.5 mg/kg as a single dose) in male $B6D2F_1$ mice (Lévi et al., 1994).

5.2. Plasma biomarkers levels

Blood samples were collected at sacrifice for posterior biochemical determinations: ALT, AST, total-CK and CK-MB plasma levels. Regarding ALT and AST determinations, only significant changes in the plasma biomarkers levels (ALT) in the infants and in the adults from assay 1 were found. No significant differences were observed between the plasma biomarkers levels of the elderly MTX-treated mice and the control group.

ALT is an aminotransferase that is mainly present in the hepatocytes (Varaldo, 2001; Vroon and Israili, 1990). This enzyme is not exclusive produced by the liver, but it has a high amount there. When the liver cells are damaged, ALT is released to the blood stream, which allows the measurement of its plasma levels. In a much lower extent, it can be found in the kidneys, heart, skeletal muscle, pancreas, spleen and lungs, in this order. However, in these tissues its activity is significantly lower; thus, increased plasma ALT levels from non-hepatic disorders are much less common. This way, the measurement of ALT plasma levels is commonly used to assess the hepatic function. Regarding AST, it can be found in the high amount in the liver, and also in other tissues with high metabolic activity. Tissue activity for AST is, in a decreased concentration order, heart, liver, skeletal muscles, kidneys, pancreas, spleen and lungs. So, AST can also be released to the blood stream when any one of these organs is damaged, meaning that this enzyme is not a specific indicator of liver damage, as ALT (Varaldo, 2001; Vroon and Israili, 1990). Instead, AST has been more used to assess myocardial damage since myocardium is the highest source of this enzyme (Vroon and Israili, 1990). Nevertheless, it cannot be considered a specific marker for this condition, and an increased AST/ ALT ratio has been proposed as a marker of heart damage (Nyblom et al., 2004; Nyblom et al., 2006; Vroon and Israili, 1990).

In the infant population, ALT levels were significantly elevated and, consequently, the AST/ ALT ratio was found significantly decreased, when compared with the control group. Since AST levels from the MTX-treated mice were not significantly different from the ones of the control group, regarding all age groups, one can assume that the obtained results were due to hepatic damage. Although the liver weight/ brain weight ratio remained similar to the one found in the control group in infants, ALT increased after MTX treatment, meaning that the infant started to demonstrate signs of liver malfunction. This way, it can be concluded that a cumulative dose of 6 mg/kg of MTX caused hepatic damage in the infant population. Regarding the adult population, the changes were only observed in the animals from assay 1. The ALT levels were found significantly increased, while the AST/ ALT ratio was significantly reduced, when compared with the levels from the control group. In the assay 2, where there was a higher number of mice, those changes in the ALT plasma levels

(and AST/ ALT ratio) were expected; however, it was not the case, possible due to a higher resistance of the litter from assay 2 or to an earlier damage. Despite not being possible to take accurate conclusions regarding this aspect, the gathered data suggest that it is highly likely that MTX caused hepatotoxicity in the adults. In fact, significant differences in the liver weight/ brain weight ratio between MTX-treated and control mice were found in adults, as previously reported. One study, which also used infant and adult CD-1 mice in order to analyze age as a risk factor for MTX's toxicity, also determined the plasma biomarkers levels discussed herein (Dores-Sousa et al., 2015). MTX was administered at a cumulative dose of 9.0 mg/kg and the mice were sacrificed in two time-points. At 20 days after the last administration, only two MTX-treated infant mice survived until the end of the experiment (all the adults died), which presented significantly increased AST plasma levels, and subsequent an increased AST/ ALT ratio, in comparison with controls, suggesting cardiac damage for higher MTX cumulative dose or due to a more extensive period of time till sacrifice (Dores-Sousa et al., 2015). In the work developed in this dissertation, the administered cumulative dose of MTX was lower, as so for the free-drug period, when compared to the data obtained in the study described above, which could explained the higher rates of toxicity induced by MTX treatment in the study conducted by Dores-Sousa *et al.*. Still, although using lower doses, but given 17 days after the last administration, the MTX-treated infants showed signs of liver damage. In another study, similar results were found (Niang et al., 2011). Female NMRI mice received a single dose of 6, 9 or 12 mg/kg of MTX. The sacrifice occurred 14 days after the beginning of the experiment and the authors observed significantly increased AST and ALT plasma levels, especially at the higher dose (Niang et al., 2011). This way, the study performed by Niang *et al.* also demonstrates the cardiotoxic and hepatotoxic potential of MTX.

Regarding total-CK and CK-MB, their plasma measurements were only possible to be performed in the adults from assay 2 and in the elderly mice, due to insufficient amount of the gathered plasma in the remaining populations. CK is an enzyme that catalyzes the reversible conversion of adenosine diphosphate (ADP) in ATP, when the muscle contracts, using creatine phosphate as the phosphorylation reservoir (MayoClinic, 2018a). This reaction occurs when a muscle contracts, meaning that is found in tissues with high energy demands. CK activity is higher in the striated muscle, heart tissue and brain (MayoClinic, 2018a). CK-MB is an isoenzyme of CK (MayoClinic, 2018b). The myocardium contains approximately 30% of CK-MB, what makes it a cardiospecific marker. In fact, its values are increased when myocardial infarction occurs (MayoClinic, 2018b). When analyzing the obtained results, it was found that the total-CK plasma levels from the MTX-treated mice did not significantly differ from control groups, for both adult (assay 2) and elderly mice.

Since CK is mainly found in the skeletal muscle, it is possible to conclude that MTX treatment does not interfere with the overall muscle activity. In fact, one study where MTX was administered in a higher cumulative dose (9 mg/kg), the results were similar (Dores-Sousa et al., 2015). Regarding CK-MB, no significant differences were found between the MTX-treated and control mice of the analyzed populations, as well. These results suggest that no severe cardiac damage was induced in the elderly and in the second group of adults when receiving a cumulative dose 6 mg/kg of MTX. In fact, no significant changes were reported in the AST plasma levels of these two age groups, as previously mentioned. In one similar study using a higher cumulative dose of MTX (9 mg/kg), CK-MB decreased levels in infant mice were found (Dores-Sousa et al., 2015). Likewise, Rossato *et al.* observed that the CK-MB levels of rats that received 3 cycles of 2.5 mg/kg of MTX (at day 0, 10 and 20) and where sacrificed 2 days after the last administration, also decreased, when compared to controls (Rossato et al., 2014). The results observed in these two studies may occur as an adaptive process to an injured heart, which allows the preservation of ATP, diminishing the contractile activity of the heart, and its posterior use in other metabolic processes (Rossato et al., 2014; Ventura-Clapier et al., 2004).

Regarding the elderly population, no significant changes were observed in the plasma biomarkers levels, which corroborates with the results found in the analysis of the organ weight/ brain weight ratios. So far, the adults seemed to be the age group more susceptible to the toxicity induced by MTX treatment at a cumulative dose of 6 mg/kg, as already evaluated in similar experimental conditions (Dores-Sousa et al., 2015).

5.3. Histological cardiac evaluation

Through light microscopy, the cardiac morphology from control and MTX-treated mice of the different age groups was analyzed. Cardiac lesions were observed in the three age groups treated with MTX. In both infant and adult MTX-treated mice, cellular edema, necrotic zones, vacuolization, and large and uncondensed nucleus were observed, all signs of cellular degeneration. Furthermore, inflammatory infiltrations in the interstitial spaces of the cardiomyocytes were also observed in these two age populations treated with MTX. When myocardial injury occurs, several inflammatory mediators are released in the cardiomyocytes and an infiltration of inflammatory cells, such as macrophages, to the site of injury is observed (González et al., 2011). In the MTX-treated adult mice, vascular congestion was also observed. Other studies had already reported these observed morphological changes in the heart as potential toxic effects induced by MTX treatment,

such as the one conducted by Shipp *et al.* (Shipp et al., 1993). In that study, cultured neonatal rat cardiac cells were incubated with MTX and exposed to different concentrations (0.05 to 5 µg/ mL) drug for 3 hours. Then, the myocyte cultures were washed out and incubated in a drug-free medium for 72 hours. At 72 hours, through microscopically analyzes of the cardiac MTX-treated cells, significant alterations were verified, in comparison to controls, such as extensive cytoplasmatic vacuolization and nuclear changes (Shipp et al., 1993). Likewise, Rossato *et al.* also observed signs of cardiac damage induced by MTX in male Wistar rats administered with a cumulative dose of 7.5 mg/kg (Rossato et al., 2014). Either 22 or 48 days after the beginning of the experiment, the authors found, through histologic evaluations of the heart tissue of these animals, significant cardiac lesions similar to the ones found in the present dissertation, such as cellular edema and inflammatory infiltrations (Rossato et al., 2014).

Despite similar toxic effects being observed in infant and adult MTX-treated mice, these histological changes were more prominent in the adults, corroborating the previous results found in this dissertation, where this population seems to be more susceptible to the toxicity induced by a cumulative dose of 6 mg/kg of MTX. In fact, similar results to the ones described herein were observed in another study, where the infant mice also demonstrated to be more resilient to the MTX therapy (Dores-Sousa et al., 2015). Through light microscopy, the authors also observed interstitial inflammatory cell infiltration, cellular edema, signs of vacuolization and necrotic zones in the MTX-treated infant mice, but in a much lower extent than in the adults, when receiving a cumulative dose of 9 mg/kg of MTX (Dores-Sousa et al., 2015).

Regarding the elderly population, as far as we know, no other study was done with MTX in this population. Microscopical cardiac lesions were seen in the controls, such as necrotic zones, large and uncondensed nucleus, inflammatory infiltrations, vacuolization and a higher amount of loose connective tissue around the vessel walls and macrophages, than the other two age populations. In the MTX-treated elderly mice, the same structural changes as the controls were observed, with the addition of cellular edema and vascular congestion. The loose connective tissue is constituted by fibers and fibroblasts, which are widely dispersed in this tissue (González et al., 2011). The macrophages can stimulate fibroblasts, leading to fibrosis, which can induce an excessive formation of fibrous connective tissue (González et al., 2011). So, the high presence of macrophages in the heart of the elderly mice can suggest that the aging process induces some lesions to the heart, and an immunologic response of the organism is induced, resulting in an infiltration of inflammatory cells. This way, one can assume through the microscopic observations in the heart of the

elderly mice that an increased age would lead to a higher susceptibility for cardiac damage, by its own. In fact, it is already known that the elderly (> 65 years) account for the higher number of patients with cardiovascular diseases, among all population (Dai and Rabinovitch, 2009). The vulnerability of these patients is verified by an age-dependent progressive degeneration and decline in the function of the heart (Dai and Rabinovitch, 2009). The same observations had been made in another study with elderly mice, where intrinsic cardiac alterations included fibrosis, vacuolization, hypertrophy of the cardiomyocytes, among others (Treuting et al., 2008). As far as we know, no studies had been made regarding the effects of the MTX-treatment in the morphology and structure of the heart regarding an elderly mice population. Nevertheless, it could be assumed that the MTX-treatment exacerbated the cardiac changes observed in the elderly population, since other types of lesions were observed in the MTX-treated mice, when compared with the controls (cellular edema and vascular congestion).

In comparison with the previous results found, no correlations can be made, since CK-MB levels were unaltered in the analyzed populations, as so in AST. When myocardial injury occurs, in humans, AST plasma levels can be detected at its highest within 24 hours after lesion, returning to its normal levels after 3 to 6 days (Nigam, 2007). Also, CK-MB plasma levels peak occurs between 16 to 20 hours after onset and it presents a high sensitivity for detecting myocardial injury 6 hours after the cardiac damage (Jaffe et al., 2006; Nigam, 2007). These pharmacokinetic values may not be the same for mice. Nevertheless, the time-points selected in the present work may have not detected those changes, since the damage could have occurred in an earlier point, meaning that at the moment of the plasma biomarkers' determinations their values could have already returned to normal. Other possible explanation is if the histological lesions found in the MTX groups were too small to be detected by these assays.

5.4. HPLC-DAD analysis

The mice plasma samples were analyzed by HPLC-DAD to determine if MTX or NAPHT could be detected in the time of the sacrifice and to assess the possible metabolic differences between the different age groups. In order to do so, the most adequate chromatographic conditions had to be found.

All the solutions and samples were prepared in polypropylene tubes since MTX is known to adsorb in glass and other types of plastic (Priston and Sewell, 1994). As mentioned

before, the mice plasma samples were stored in tubes containing ascorbic acid in order to prevent the oxidative degradation of MTX. It is known that MTX is unstable in plasma and the addition of antioxidants has been proposed in order to overcome this aspect (Reynolds et al., 1981). Ascorbic acid, either in the presence of a citrate buffer or not, has been the most frequently used antioxidant (Priston and Sewell, 1994). In fact, a study where MTX was incubated with various antioxidants demonstrated that ascorbic acid alone was capable of stabilizing it, without any loss of the compound after the incubation, contrary to what happened with the other antioxidants tested (Priston and Sewell, 1994). In the absence of ascorbic acid, the authors observed a rapid degradation of the chemotherapeutic drug, at 24ºC and 37ºC (faster at the higher temperature) (Priston and Sewell, 1994). Hu *et al.* also studied the stability of MTX in plasma with or without the addition of ascorbic acid and several storage temperatures were also tested: 4ºC, -20ºC and -60ºC (Hu et al., 1990). At 4ºC, the half-life of MTX in plasma without the addition of ascorbic acid was 13.6 days and more than 10% of the initial concentration of the drug was degraded within the first day of storage. However, when the storing temperature was lowered, the observed loss in the initial MTX concentration was reduced. At -20ºC, MTX presented a half-time of 31.0 days, while at -60ºC this value was increased to 62.0 days (a decomposition of 10% of the initial MTX concentration was observed after 2.8 and 5.0 days, respectively). With the addition of ascorbic acid, these values significantly improved: a 10% loss of MTX was observed after 15, 112 and 175 days, at 4ºC, -20ºC and -60ºC, respectively. What happens is that, at 4ºC, the ascorbic acid in plasma possibly starts to gradually oxidize, leading to an increased degradation of MTX, which is reflected in the lower values of the time necessary to 10% of the initial MTX concentration degrade. Therefore, the addition of ascorbic acid to the plasma samples not only showed that it was capable of stabilize the MTX, but also lower storage temperatures are preferable to maintain the integrity of this chemotherapeutic drug without further losses (Hu et al., 1990). Further precautions were taken in the moment of the blood collection, where EDTA containing tubes were used in order to minimize the MTX loss during blood centrifugation (Priston and Sewell, 1994). In the work developed in this dissertation, the blood from mice was kept under agitation and rapidly centrifuged. Then, as previously mentioned, plasma samples were stored at -20 ºC until analysis and in the presence of ascorbic acid, to ensure that MTX was not degraded.

In the beginning, an HPLC-UV system with a Spherisorb RP-18 ODS2 column (250 x 4.6 mm, 5 µm; reversed-phase) was available. With reversed-phase conditions, meaning that the stationary phase is non-polar and the mobile phase is polar, the more polar substances are less retained and first detected that the non-polar ones. This way, it was necessary to find an appropriate mobile phase that worked well in this octadecylsilica

stationary phase and that detected properly the chemotherapeutic drug. Based on the literature of MTX analysis, some mobile phases were investigated. The differences found in the detection of a standard solution of MTX with different mobile phases may be related to the fact that MTX has two ionizable amines with pKa values of 8.3 – 8.6, meaning that MTX could be presented in solution in several forms. In fact, one study conducted at pH 7.4 and pH 10 stated that at pH 7.4 MTX has two positive charges (protonated nitrogen) on its lateral chains, while at pH 10 the deprotonation of the amines in the lateral chains occurs and the chemotherapeutic drug remains uncharged (and more hydrophobic) (Enache and Toader, 2018). This way, one aspect to take into account in the appropriate mobile phase selection is the pH, since pH variations could induce the protonation of the amines, which leads to chromatographic complications, such as broadening the chromatographic peak or not being detected at all (Enache and Toader, 2018; Raghunand et al., 2003). Among the tested isocratic mobile phases, only the one composed by acetonitrile: ammonium acetate 0.2 M (25:75, v/v), adjusted to pH 4.0 with acetic acid, previously used in a study conducted by Peng *et al.*, seemed to be suitable for MTX detection on the chromatographic system firstly used in this dissertation (Peng et al., 1982). Afterwards, in order to obtain a better peak resolution from the solvent front and to not overcome the recommended pressure of this equipment, the flow rate was set to 0.6 mL/min. Lower flow rates led to higher retention time of MTX.

For the analysis of the mice plasma samples, a different HPLC-DAD system was employed, allowing to perform the analyses using two wavelengths: 254 nm and 658 nm. These two wavelengths had already demonstrated to properly detect MTX in plasma and tissues; nevertheless, at 254 nm usually a large number of interfering peaks from biological fluids is present (Hu et al., 1990; Rentsch et al., 1996). Several columns were again investigated in order to find the most adequate for MTX analysis (similar retention factor and resolution as previously established), and a mediterranea sea_{18} (250 x 4.6 mm, 5 µm; reversed-phase) was chosen. The reason why the detection of MTX was not accomplished with the other previously investigated columns may be due to their efficiency, its prior uses or its age.

Then, with the possibility of analyzing the mice plasma samples with an LC-MS system in mind, the concentration of ammonium acetate in the mobile phase was reduced to 0.1 M. However, due to a malfunction in the LC-MS system, the analysis of the mice plasma samples in this equipment with these optimized conditions was rule out. The determination with an LC-MS combines the advantages of chromatography (high sensitivity and efficient separation) with the advantages of mass spectrometry (structural information, higher

selectivity and detectability), meaning that it would have allowed to acquire results with a higher precision and to detect lower concentrations of the anticancer drug, in comparison with the HPLC-DAD system (Oshita and Jardim, 2015). Regarding the metabolic profile of MTX, it is known that other metabolites besides NAPHT had already been detected (Hrynchak et al., 2017; Rossato et al., 2013a). This way, the use of an LC-MS system would have facilitated the detection of other possible MTX metabolites, present in trace amounts in biological samples.

Later in the work, a mobile phase composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0 was also tested. It was done in order to decrease the amount of salts from the mobile phase, which is preferred for HPLC-DAD determinations, since they can form complexes with the existent metal compounds naturally present in plasma samples and interfere with the analyzes. Since resolution of the MTX peak was accomplished (retention time of 16.57 minutes), this mobile phase was preferred and chosen as the most appropriate to analyze the mice plasma samples, since it was already investigated in a gradient profile (10% of acetonitrile for 1 minute and then ramped linearly to 80% over 20 minutes), to investigate MTX concomitantly with other metabolites (Rossato et al., 2013a).

Then, in order analyze the influence of age on the metabolic profile of MTX, NAPHT was chosen as the metabolite to be detected in the mice plasma samples, since a synthetic standard was available from a previous work in the laboratory and due to the fact that it is one of the MTX metabolites more prevalent in mice (Reis-Mendes et al., 2017). As mentioned in the previous chapter, the chromatographic conditions chosen for MTX detection did not worked for NAPHT. Due to technical problems, the determinations could only be performed in an isocratic mode, so it was necessary to separately investigate the ideal conditions for the resolution/ detection of these two compounds. This way, different proportions of acetonitrile: 0.5% formic acid were tested and it was found that a proportion of acetonitrile: 0.5% formic acid (30:70, v/v) was the best one to detect the MTX metabolite (retention time of 16.26 minutes). With these new proportions, MTX was detected together with the solvent front, which was not adequate to properly detect this drug. Therefore, in order to detect the metabolite and its parental drug, each mice plasma sample was injected two times. With the change of the acetonitrile: 0.5% formic acid proportion to (30:70, v/v), the amount of water in the mobile phase was reduced. It was verified that AMT and NAPHT were the most and less polar compounds, respectively. This way, with a 20:80 (v/v) proportion of acetonitrile: 0.5% formic acid, NAPHT demonstrated to be highly retained, being difficult to be detected. When the amount of water in the mobile phase was reduced,

its detection was possible for an appropriate running time and the retention times for AMT and MTX decreased.

Afterwards, MTX calibration curves were drawn using human plasma and PBS as matrixes, in order to determine the concentration range in which MTX presented linearity and the minimal concentration in which it was possible to detect the chemotherapeutic drug. In biological samples, MTX was expected to be found in small amounts (Catalin et al., 1994; Lin et al., 1989; Peng et al., 1982; Slørdal et al., 1993; Zhang et al., 2010). As referred before, AMT was used as the internal standard. Besides the similar structure with MTX, AMT also exhibits comparable retention characteristics and a similar absorption spectrum to its analog, MTX, proving to have a good potential for being its internal standard (Priston and Sewell, 1994). Since no calibration curves were done for NAPHT, AMT was not assessed if it was also a good internal standard for this compound.

Summing up, mice plasma samples were analyzed in an HPLC-DAD system, using a mediterranea sea_{18} (250 x 4.6 mm, 5 µm; reversed-phase) column and a mobile phase composed by acetonitrile: 0.5% formic acid (20:80, v/v) pH 3.0. The flow rate was kept at 0.6 mL/min, the running time to 30 minutes and the wavelengths to 254 nm and 658 nm. Additionally, in order to detect NAPHT, the same chromatographic conditions were used, except for the acetonitrile: 0.5% formic acid proportion, which was set to 30:70 (v/v). AMT, MTX and NAPHT standards presented retentions times of 10.21 minutes, 16.57 minutes and 18.05 minutes, respectively, in these chromatographic conditions.

The main purpose of the HPLC-DAD analysis was to detect MTX and NAPHT in the mice plasma samples. After some preliminary samples being analyzed, it was observed that MTX was not detected. Therefore, using the calibration curve with a plasma matrix, the LOD and LOQ were determined, for the chromatographic conditions reported in the presented dissertation. At 254 nm and 658 nm the obtained LOD's were 260 nM and 370 nM, respectively. Other studies have already demonstrated to properly detect MTX in plasma samples through HPLC-DAD. Among the ones that also determined the LOD, the values varied from 1.12 nM to 169 nM, which are lower than the LOD's determined for the method employed herein (Catalin et al., 1994; Lin et al., 1989; Peng et al., 1982; Slørdal et al., 1993; Zhang et al., 2010). The fact that our method had much higher LOD's than the ones found in the literature could be one possible reason that enabled the analysis of the mice plasma samples. Furthermore, it is known that MTX presents a fast elimination rate from plasma, which could also explain the observed results. Regarding the attempts to detect NAPHT, it was not possible to verify its presence in the plasma samples from any of the age groups, as well. Therefore, at the moment of the chromatographic analyses, the

compounds may not be present in plasma or else the employed chromatographic method could not detect the chemotherapeutic drug and its metabolite, in these chromatographic settings. This way, the possibility of their presence in the plasma of the treated mice should not be ruled out.

Nevertheless, as already stated, plasma samples were stored at -20 ºC until analysis and in the presence of ascorbic acid. The plasma samples were kept frozen for a maximum period of 9 months. Regarding the MTX pharmacokinetic features, it presents a slow elimination phase from the organism with a half-life of 5 - 18 days, in humans (Reis-Mendes et al., 2015). All these data together substantiate the hypotheses that MTX may be still present in the organism of the animals in considerable amounts, at the moment of sacrifice (7 or 17 days after the last administration). Its presence would be possible mostly seen in the form of its metabolites, namely NAPHT (Rossato et al., 2013a). So, acknowledging the pharmacokinetic characteristics of this chemotherapeutic drug and that MTX largely distributes and accumulates in tissues, a future possibility would be to analyze some tissues, as the heart and liver, by an equal or similar method as the one described in this work and compare with the obtained results. This way, more conclusions would be possible to taken and perhaps assess the real influence of age in the metabolic profile of MTX.

CHAPTER 6.
Conclusions

6. CONCLUSIONS

MTX therapy is an asset; however, it can induce several toxic effects. Alongside, there are some known risk factors that can influence the toxicity induced by the chemotherapeutic drug, such as administered dose, age, gender and pre-existent comorbidities. This way, several studies have been conducted in order to better understand how the mechanisms of toxicity work, in order to try to contradict the incidence of these side effects in cancer patients, improving their quality of life. Nevertheless, these mechanisms are not well understood. With all this in mind, one of the main objectives of this work was to assess the influence of age on the toxicity induced by MTX and, possibly, on its metabolic profile, in CD-1 male mice. This way, three age groups were studied: infants, adults (divided in two trials) and elderly.

The obtained results concerning the general welfare of the animals suggested that the adult mice are particularly susceptible to the toxicity induced by MTX at a cumulative dose of 6 mg/kg: a significant body weight loss was observed, as so for a decreased food and water consumptions, when compared to controls. These body weight changes are not necessarily entirely correlated with decreased intakes. Therefore, body weight losses could be an indicative of gastrointestinal toxicity. Moreover, all the organ weight/ brain weight ratios were significantly changed, in comparison with the control group. This way, at a cumulative dose of 6 mg/kg, the adult mice demonstrated to be susceptible to several types of toxicity induced by MTX, such as hepatotoxicity, renal toxicity and cardiotoxicity. The incidence of this later event due to MTX-treatment was also assessed through histologic cardiac evaluations, where intense cardiac lesions were seen in the MTX-treated adult mice. With these histological observations the cardiotoxic potential of MTX was, once again, proved to be one of the major problems related with this chemotherapeutic drug. Regarding the plasma biomarkers levels, only ALT showed to be significantly increased (assay 1) and, subsequently, decreased AST/ ALT ratio, when compared to controls, meaning that MTX treatment induced hepatic damage to these animals. Due to the histologic observations in the heart and the remaining analyzed parameters, it would be expected that plasma cardiac markers of toxicity would be increased. However, it was not the case. The time-points selected for the sacrifice may have not detected changes in the biochemical markers of cardiac damage, since at the moment of sacrifice they may have already returned to normal values. Other possibility is if the cardiac lesions were too small to be detected by these assays. Moreover, either infants as elderly mice presented toxic effects when administered with MTX, but at a lower extent than adults, as seen in the microscopic analyzes.

Concerning the analyses of the mice plasma samples through HPLC-DAD analysis, the detection of MTX and NAPHT was not possible, regarding all age groups. The pharmacokinetic characteristics of MTX in plasma, such as its rapid elimination, suggest the possibility that this drug could not be still present in the blood from the animals at the moment of sacrifice. Nevertheless, these known pharmacokinetic features for humans could be different in mice. Therefore, the presence of MTX (and/ or NAPHT) should not be ruled out and, in this case, one possible explanation for the obtained results lies in the fact that the concentrations of these compounds could be under the LOD of the method used herein.

Gathered the data, it was possible to determine a higher susceptibility of the adult population for MTX. Regarding the possible influence of age in the metabolic profile of MTX, no accurate conclusions were possible to be taken. Nevertheless, the development of this work demonstrated the difficulty to evaluate the toxic profile of this chemotherapeutic drug in an *in vivo* study. More studies are needed in order to better assess the influence of these three age groups in the pharmacokinetic features of MTX. It is known that both MTX and NAPHT largely distribute and accumulate in tissues so, a future work should analyze these samples, employing a similar HPLC method to the one performed in the present dissertation. This would allow to give a more holistic view of the results found in this dissertation and probably demonstrate the influence of age on the metabolic profile of MTX.

CHAPTER 7.
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7. REFERENCES

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