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The neurotoxicity of doxorubicin and mitoxantrone: chemobrain studies using infant, adult, and old mice

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The neurotoxicity of doxorubicin and mitoxantrone: chemobrain studies using infant, adult, and old mice

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Abstract

The number of cancer survivors has increased gradually over the last decades and the number will continue to grow due to earlier diagnosis and better treatments. These increases raise concerns about the post-treatment life quality of patients. Cancer patients are submitted to extremely aggressive treatments, being chemotherapy still the most used against a wide range of cancers and with high success rates. However, it has several side effects. In the past decades, cognitive deficits have been acknowledged as a serious side effect on chemotherapy-treated patients, being 'chemobrain' the designation given to the long-term cognitive dysfunction caused by systemic chemotherapy. Doxorubicin (DOX) and mitoxantrone (MTX) are topoisomerase II inhibitors used to treat a wide range of tumors. Despite their effectiveness in cancer treatment, their brain toxicity is poorly understood.

This work aimed to evaluate the underlying neurotoxicity mechanisms of clinically relevant doses of DOX or MTX in the brain of male CD-1 mice of different ages [infant (4 weeks), adult (3 months) and old (18-20 months)]. All animals received biweekly intraperitoneal administrations of each drug, for 3 weeks, as to mimic human clinical administrations of the drugs done in cycles. All groups received a total cumulative dose of 6 mg/kg MTX, while adult and infant mice had a total cumulative dose of 18 mg/kg DOX and old mice received a cumulative of 9 mg/kg DOX. In a subsequent experiment, adult mice were subjected to a total cumulative dose of 9 mg/kg DOX or 6 mg/kg MTX. During the experimental period, the mice well-being was monitored, as well as food and water consumption. Mice were euthanized one week (adults and old animals) or seventeen days (infant) after the last injection. To assess the brain's redox and energetic status, total glutathione (tGSH), reduced glutathione (GSH), oxidized glutathione (GSSG), the GSH/GSSG ratio, and ATP levels were measured. In the adult mice, coronal sections of fixed brains were used for immunofluorescent detection of Bax, phosphorylated Tau (pTau), and p53 in the hippocampal formation and prefrontal cortex (PFC) formations. The volume and the total number of glial fibrillary acid protein (GFAP)-immunoreactive (ir) astrocytes of the hippocampal formation were determined through stereological methods.

DOX provoked considerably less food and water intake in infants and adults, being that in the later that effects were more pronounced in the highest cumulative dose tested. Those changes reflected on less weight gain or weight loss in those populations. In 18mg/kg DOX animals, brain tGSH and GSH decreased in the adults, while ATP increased in infant mice. On the other hand, MTX administration did not cause significant alterations neither in the glutathione parameters nor in the ATP levels in any of the ages tested.

Considering the stereological measures, the hippocampal volume increased in mice administrated with a total cumulative dose of 18 mg/kg DOX and decreased in MTX-treated mice. The total estimated GFAP-ir astrocytes increased in hippocampal formation regions of MTX mice, whereas both DOX doses caused dissimilar effects on GFAP-ir astrocytes throughout the hippocampal formation. Regarding the apoptotic markers, Bax increased in the PFC of 9 mg/kg DOX and MTX (6 mg/kg) exposed mice, whereas the highest dose of DOX (18 mg/kg) decreased Bax expression in the PFC. Meanwhile, p53 expression increased in hippocampal CA3 region of the 9mg/kg DOX-treated mice, though, in MTX mice, a decline in p53 expression was observed in all analysed areas. In PFC MTX administration increased pTau, but the highest DOX dose administered to adults decreased pTau in the DG and CA3 regions.

These results show that although DOX and MTX share pharmacological anticancer proprieties and both DOX and MTX cause significant alterations in the mice brain, they have dissimilar neurotoxic mechanisms that need to be further elucidated as to contribute to the understanding and mitigation of chemobrain on cancer-treated patients. Moreover, dosing and aging of administration impact greatly on the results obtained and need careful attention in the future studies.

Keywords: doxorubicin, mitoxantrone, chemobrain, chemotherapy, age, neurotoxicity.

Resumo

O número de pessoas que sobrevivem ao cancro tem vindo a aumentar gradualmente ao longo das últimas décadas e esse aumento será maior com a implementação de técnicas de diagnóstico mais precoces e também com os tratamentos mais eficazes existentes na terapêutica. O aumento da taxa de sobrevivência dos doentes oncológicos tem feito com que a qualidade de vida no pós-tratamento seja cada vez mais um assunto relevante, visto que os doentes são submetidos a tratamentos extremamente agressivos, como a quimioterapia. A quimioterapia apresenta uma elevada taxa de sucesso e um largo espectro de ação. Não obstante, também apresenta vários efeitos adversos medicamentosos. Nas últimas décadas, a disfunção cognitiva tem sido reconhecida como um dos efeitos adversos mais graves da quimioterapia e o déficit cognitivo causado pela quimioterapia é muitas vezes referido como “chemobrain”. A doxorubicina (DOX) e a mitoxantrona (MTX) são dois fármacos amplamente usados na clínica, uma vez que apresentam uma elevada taxa de eficácia em diferentes tipos de cancro. Apesar da sua eficácia no tratamento do cancro, os seus mecanismos de neurotoxicidade ainda estão pouco investigados.

Os estudos incluídos nesta dissertação têm como principal objetivo avaliar a neurotoxicidade decorrente da administração de doses clinicamente relevantes de DOX ou MTX a ratinhos CD-1 de diferentes idades (infantil [4 semanas], adultos [3 meses] e idosos [18-20 meses]). Durante 3 semanas, os ratinhos receberam injeções via intraperitoneal 2 vezes por semana dos fármacos: uma dose total cumulativa de 6 mg/kg MTX em todas as idades ou uma dose total cumulativa de 18 mg/kg DOX nos ratinhos infantis e adultos e, ainda, uma dose total cumulativa de 9 mg/kg DOX nos ratinhos idosos. Numa experiência subsequente, animais adultos receberam 9 mg/kg DOX ou 6 mg/kg MTX. Durante o período experimental, o bem-estar animal, assim como o consumo de ração e água, foi monitorizado. Os animais foram sacrificados uma semana (adultos e idosos) ou 17 dias (infantis) após a última administração. Para avaliar o stress oxidativo no cérebro avaliou-se os níveis de glutathiona total (tGSH), glutathiona reduzida (GSH), glutathiona oxidada (GSSG) e foi ainda calculado o rácio GSH/GSSG. O estado energético do cérebro foi avaliado através da medição dos níveis de ATP. Secções cerebrais foram usadas para deteção por imunofluorescência, na área do córtex pré-frontal (PFC) e na formação do hipocampo, da proteína Bax, da proteína Tau fosforilada (pTau) e da proteína p53. Na formação do hipocampo foi ainda determinado o seu volume e o número total de astrócitos reativos à proteína glial acídica fibrilar (GFAP-ir) através de métodos estereológicos.

Os grupos infantis e adultos administrados com DOX ingeriram significativamente menos ração e água. Nos adultos a maior dose causou um maior impacto nesses parâmetros. Essas alterações levaram a menor ganho ou a uma perda de peso nessas populações. Nos adultos (18 mg/kg DOX), os níveis cerebrais de tGSH e GSH diminuíram significativamente, em comparação com os controles, não existindo alterações significativas nos restantes grupos. A DOX (18mg/kg) causou um aumento dos níveis cerebrais de ATP na população infantil. Por outro lado, o tratamento com MTX não levou a alterações significativas nem nos níveis de glutathione nem de ATP em nenhum dos grupos estudados. O volume da formação do hipocampo aumentou com a dose mais elevada de DOX e diminuiu nos animais tratados com MTX. O número total de GFAP-ir aumentou na formação do hipocampo dos adultos tratados com MTX e, por outro lado, as duas doses cumulativas de DOX causaram variações dependentes da dose. Relativamente aos marcadores de apoptose avaliados no cérebro adulto, a expressão da proteína Bax aumentou no PFC dos animais tratados com uma dose total cumulativa de 9 mg/kg DOX e nos adultos tratados com MTX (6 mg/kg). Por outro lado, a sua expressão nesta área diminuiu com o tratamento de 18 mg/kg DOX. Enquanto isso, a expressão da proteína p53 aumentou na região CA3 do hipocampo nos animais tratados com 9mg/kg, mas a MTX causou uma diminuição da expressão de p53 em todas as áreas cerebrais analisadas. Relativamente à expressão da pTau no cérebro de ratinhos no PFC, o tratamento com MTX aumentou os níveis de pTau, ao passo que a dose cumulativa de 18 mg/kg DOX diminuiu a sua expressão nas regiões DG e CA3 do hipocampo.

Estes resultados mostram que, embora a DOX e a MTX compartilhem propriedades farmacológicas e ambas causem alterações significativas no cérebro dos ratinhos, os dois fármacos têm mecanismos neurotóxicos diferentes que precisam ser mais bem elucidados a fim de contribuir para a compreensão e mitigação do 'chemobrain' em doentes tratados com estes agentes quimioterapêuticos. Além disso, a dose cumulativa administrada, assim como a idade a quando da administração têm um grande impacto na neurotoxicidade obtida e precisam de maior pesquisa científica no futuro.

Palavras-chave: doxorubicina, mitoxantrona, chemobrain, quimioterapia, idade, neurotoxicidade.

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Abbreviations

•NO - Nitric oxide

ANOVA - One-way parametric analysis of variance

ApoA1 - Apolipoprotein A1

ApoE - Apolipoprotein E

ATP - Adenosine 5'-triphosphate

BBB - Blood-brain barrier

Bcl-xL - B-cell lymphoma-extra-large

BDNF - Brain derived neurotrophic factor

CNS - Central nervous system

COMT - Catechol-O-methyltransferase

DG - Dentate gyrus

DNA - Deoxyribonucleic acid

DNM - Default network mode

DOX - Doxorubicin

DTI - Diffusion-Tensor imaging

EEG - Electroencephalogram

EORTC QLQ-C30 - European organisation for research and treatment of cancer quality of life questionnaire c30

ERP - Event related potentials

FDA - American Food and Drug Administration

fMRI - Functional magnetic resonance imaging

GCEE - γ -Glutamyl cysteine ethyl ester

GFAP - Glial fibrillary acid protein

GSH - Reduced glutathione

GSSG - Oxidized glutathione

HSCS - High sensitivity cognitive screen

i.p. - Intraperitoneal

iNOS - Cytokine-inducible form of nitric oxide synthase

ir - Immunoreactive

MMSE - Mini mental state examination

MnSOD - Manganese-containing superoxide dismutase

MRI - Magnetic resonance imaging
MTX - Mitoxantrone
PET - Positron emission tomography
PFC - Prefrontal cortex
pNH-F - high-molecular-weight neurofilament subunit
pTau - Phosphorylated Tau
RONS - Reactive oxygen and nitrogen species
ROS - Reactive oxygen species
SD - Standard deviation
tGSH - Total glutathione
TNF- α - Tumor necrosis factor alpha
VBM - Voxel-based morphometry

1. Introduction

Cancer affects several organs and has a heterogeneous nature, being mostly related with uncontrolled and massive cell growth and division (1). It is the second leading cause of death worldwide, after cardiovascular diseases (2). In 2018, 18.1 million new cases of cancer were diagnosed, and 9.6 million deaths occurred due to cancer. Over the years, cancer incidence has been increasing, mainly due to the growing and ageing population (3). Risk lifestyle factors, such as exposure to carcinogenic substances (e.g. smoking) or sedentary behavior [high fat diets and lack of physical activity (4)] also contribute to the increase in cancer incidence, although epidemiological profiles of cancer burden show worldwide heterogeneities in distribution and other underlying contributing factors (2). Nevertheless, survival rates in cancer patients have steadily increased over the years mainly because of new methods for diagnosis in earlier stages, new and improved therapies, and combined modality implementations [surgery, chemotherapy, and radiotherapy (5)]. In fact, the American Cancer Society verified that in 2019 in the United States of America alone, there were 16.9 million cancer survivors, and this number is expected to rise to more than 22.1 million by 2030 based on the 5-year survival rate (6). Nevertheless, cancer survivors are at risk for long-term physical and psychosocial side-effects, either because of cancer or due to the anticancer treatment put in place (7). Hence, the life-quality of cancer survivors has gained a higher priority, and the goal nowadays is not only to treat cancer but also to insure the minimal long-term effects and easier adjustment to the life after treatment (8).

Currently, common approaches to treat cancer are radiotherapy, immunotherapy, surgery, hormonal therapy and chemotherapy (9). Despite the numerous therapies available, chemotherapy is still the most used, due to its high rate of success against a wide range of cancers and proven efficacy over the years (10). Chemotherapy comprises drugs that have cytotoxic effects and mainly target the dividing cells like the neoplastic cells, being often used in combination (11). The chemotherapeutic agents can be grouped according both to their chemical structure and also their mechanism of action. The principal chemotherapeutic groups are: the alkylating agents (e.g. cyclophosphamide), intercalating and anti-topoisomerase II agents [anthracyclines, such as doxorubicin (DOX), daunorubicin, idarubicin and epirubicin or the anthracenedione mitoxantrone (MTX)], enzyme inhibitors (e.g. etoposide and irinotecan), antimetabolites (e.g. 5-fluorouracil and methotrexate) and mitotic spindle inhibitors [e.g. docetaxel (12)]. They can target deoxyribonucleic acid (DNA) synthesis, DNA transcription, DNA duplication, and mitosis. Nevertheless, the chemotherapeutic agents can interfere with cell

cycle by more than one way and the classification being used is found to be very simplistic in several aspects (11).

Focusing on the chemotherapeutic agents used in this dissertation, DOX and MTX, they are two widely used chemotherapeutic agents that not only are topoisomerase II inhibitors but also bind and intercalate into the DNA (13). DOX is an anthracycline (Figure 1) used in a wide range of solid tumors and hematological malignancies, including breast, prostate, uterus, ovary, esophagus, liver, bile ducts and stomach tumors, child solid tumors, soft tissue sarcomas, multiple myeloma, and Wilms' tumor (14). Usually, the intravenous administration schedule is 60-75 mg/m² every 3 weeks, depending on the tumor, but should not surpass the total lifetime cumulative dose of 400-550 mg/m² (15). DOX metabolism is mainly hepatic (16). According to a study on DOX comparing its pharmacokinetics in women and men, the elimination rate of DOX is higher in men, even though, the half-life of DOX is superior in men [54 h in men and 34 h in women (17)]. Elimination rate of DOX is higher in children over 2 years when compared to adults. For children under 2 years, the elimination rate decreases when compared to older children and it is similar to elimination rates in adults (18).

MTX is an anthracenedione, closely related to the anthracyclines group, being the main chemical difference the replacement of the amino sugar on the anthracyclines by an aminoalkylalcohol chain (Figure 1). MTX is used to treat breast cancer, prostate cancer, acute leukemia, and lymphomas (19) and was approved by the American Food and Drug Administration (FDA) for the treatment of the autoimmune-mediated neurologic disease multiple sclerosis in adults (20). MTX has immunomodulatory and immune suppressive effects, which abrogates T helper activity, increases T suppressor function and inhibits B lymphocyte function and antibody secretion (21). The treatment dose of MTX is lower than DOX, being the ratio approximately 4.5 mg of DOX to 1 mg of MTX, but clinically they are equally effective (16). MTX is intravenously administered, in various doses that should not exceed the total lifetime cumulative dose of 140 mg/m² (20). In multiple sclerosis, the recommended dosage schedule is 12 mg/m² every 3 months, while in prostate cancer patients' the given dosage is 12 mg/m² every 3 weeks. MTX and the metabolites are eliminated in urine or feces but, several aspects of the metabolism of MTX are still not fully understood (16). MTX-treatment causes hematological toxicity, nausea, fatigue and significant cardiac toxicity, affecting 2.6 - 13% of the treated patients (16, 22).

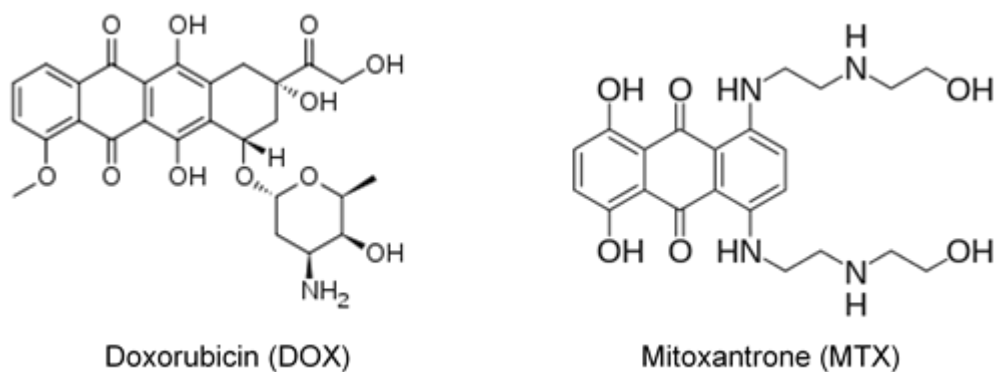


Figure 1: Chemical structure of DOX and MTX.

Despite the high efficacy of cancer treatment, chemotherapy is not specific for tumor cells and can affect healthy cells. It is a very aggressive treatment with an extended list of short-term and long-term side effects. The most common immediate side effects of chemotherapy are nausea and vomiting (23), alopecia, anemia, myelosuppression (24) and haematotoxicity (25). Chemotherapy can also cause cardiac toxicity, secondary leukemia (13), chronic fatigue, and sleep disorders (26). Of relevance, in the last decades, more attention has been given to the fact that chemotherapy can cause neurotoxicity (27). Chemotherapy-inflicted side effects lead to high morbidity, and as the number of cancer survivors increases, a wider concern about the long-term consequences of chemotherapy exposure is happening. Actually, the impact of cancer treatment side effects on the long-term life quality of patients, and also the burden that it may cause on the social and economic levels have gain more relevance in the last years (28). Forty to 60% of the cancer survivors are younger than 65 years old and potentially still a part of the labor active force (29). When the treatments end, the patients seek to return to their routine and the ability to go back to work represents a sign of recovery, being an important contributor to the patients' quality of life. However, many cancer survivors struggle to return to work due to the long-term effects of cancer and cancer treatment (30) and reported cognitive limitations (31) take an important aspect of that post-cancer life and will be addressed next.

1.1. Neurotoxicity of anticancer therapies

Chemotherapy-induced cognitive impairment has been established as a long-term secondary effect of chemotherapy treatment that may persist for years following the treatment and that significantly diminishes the life quality of cancer survivors and has a reported prevalence of cognitive impairment between 28% (32) and 75% (33). Nowadays,

neurological disorders are being more acknowledged as major causes of death and disability in the world (34). In a growing ageing population, the prevalence of the non-communicable neurological disorders has increased (34). The brain is a highly specialized and complex organ that integrates information and generates a response. It is the responsible for creativity, memory, thought process, emotion, among other aspects that make us humans (35). The central nervous system (CNS) integrates the brain and spinal cord. Neurons are excitable cells, specialized for stimulus reception and nerve impulse that have a key role in the brains' function. The neurons are supported by non-excitable cells that account for half of the brain's volume. There are three major types of neuroglial cells in the brain: astrocytes, oligodendrocytes, and microglia. Neuroglial cells retain the capacity of undergoing cell division in the adult brain, whereas mature neurons do not divide and replicate (36). Still, in the adult brain, neurogenesis, which is the generation of new neurons, occurs in two distinct areas, the lateral subventricular zone and in the dentate gyrus (DG) of the hippocampal formation (37). Adult neurogenesis is thought to have an important role in memory and learning, as well as in the protection of the brain against stress-induced attrition (38). However, it is a slow process forming only a limited number of neurons, being therefore an unsuccessful route to cope with neurotoxic insults.

The microenvironment in the brain is tightly regulated and protected from foreign compounds, mostly due to the presence of the blood-brain barrier (BBB). The BBB is both a physical and a functional barrier. The endothelial cells are linked by tight junctions, they lack fenestrations and have low pinocytotic capacity, which creates an almost impermeable barrier. Since transport across the membrane is limited, the requirements of the brain are assured by specialized membrane transporters. Solute-linked carriers, ion pumps and receptors control the entrance of substances from the blood to the brain, even xenobiotics, while adenosine 5'-triphosphate (ATP)-dependent efflux transports (such as P-glycoprotein) extrude substances from the brain to the blood. Most xenobiotics, and in particular anticancer drugs, are usually not believed to cross the BBB. Therefore, in cases of brain cancer, local chemotherapy needs to be given instead of systemic administration (39). Nevertheless, the BBB protects almost all brain areas, except the circumventricular organs that are located along the midline of the brain ventricular system. The circumventricular organs include the subfornical organ, vascular organ of the lamina terminalis, median eminence, area postrema and choroid plexus (40). That absence of BBB allows the passage of circulating peptides and other small molecules on that space, which cannot cross the BBB elsewhere. This discontinuity also allows the hormones produced in the hypothalamus to enter the blood stream and maintain the human body homeostasis (41). Studies performed in mice showed that blood-born substances (e.g.

macromolecules from the immune and complement system) can enter the brain parenchyma through the endothelial cells of the subfornical organ and reach the white matter of the corpus callosum (42) and the hippocampus (43). Therefore, the areas not protected by the BBB are potential places for the entry of xenobiotics, including anticancer drugs.

Both cancer and neurological disorders represent a burden that drastically affects patients' life quality. As stated, anticancer therapy usually compromises the combination of several drugs or strategies. The CNS effects of radiotherapy, hormonal therapy and immunotherapy have been thoroughly studied over the years (44-52). Radiotherapy can induce acute encephalopathy, BBB impairment, lesions in the white matter, cognitive dysfunction and, in some cases, severe dementia (46). Hormone therapy is usually prescribed for women with hormone receptor-positive breast cancer and it can cause memory and spatial cognitive deficits, since estrogens play an important role in cognition and neuroprotection (47, 52). In men, androgen-deprivation therapy can cause visuomotor deficits (48). Regarding immunotherapy, the cytokines have both antineoplastic and immunomodulatory effects. They can induce changes in mental state, cognitive deficits associated with frontal-cortical dysfunction and, in some cases, white matter abnormalities (49). Moreover, the direct administration of chemotherapy to the brain (usually intrathecal or intraventricular administration) for the treatment of brain tumors or metastasis has been documented to induce leukoencephalopathy, cognitive dysfunction and, in more severe cases, dementia (50, 51).

Cognitive function is the most complex attribute of the human being. It involves memory, orientation, awareness, focus, comprehension, perception, and rational thinking (53). In cases of cognitive dysfunction, the brains' ability to integrate information is impaired, which can demonstrate abnormalities in the brains' physiology and structure (54). The link between anticancer drug and cognitive dysfunction is of paramount importance to the patients' life quality. The first papers revealing the putative link between cognitive impairment and neurotoxicity induced by chemotherapeutic agents were published in 1974 by Weiss and co-workers (55, 56). The articles reviewed the neurotoxicity of commonly used antineoplastic agents (L - asparaginase, 5 - fluorouracil, methotrexate, nitrogen mustard, procarbazine, vincristine and vinblastine) and highlighted the correlation between the increase of neurologic problems in cancer patients and chemotherapy usage (55, 56). Following these pioneer works, in 1978 Levine *et al.*, reviewed 100 cases of cancer patients referred for psychiatric consultation, 56% of whom were diagnosed with depression and 40% diagnosed with organic brain syndrome (the authors did not clarify further this concept). Twenty-six of these patients were

misdiagnosed as having depression, but in reality, they were suffering from organic brain syndrome. The authors accentuated the need for assessment of the mental status in cancer patients throughout the treatment, especially the cognitive function since they considered it to be the most neglected part of the patient care at that time (57).

Despite the recurrent complaints of memory and attention/concentration problems of the chemotherapy-treated patients since earlier reports, the systemic research on the cognitive side effects of chemotherapy only started in the 1980s (58). The first clinical study dedicated to the evaluation of putative cognitive deficits on cancer patients after chemotherapy was performed by Oxman and Silberfarb. The study compared nine women and one man that did cognitive testing before chemotherapy, 24 hours and 1 month after chemotherapy. Patients had been treated with several cytostatic agents that included DOX and cyclophosphamide. The study found no differences between the three time points analyzed but authors acknowledged that the small sample size and the practice effect due to the patients performing the same tests in a minor time frame, may have influenced results. The patients also received different regimens that could have led to different results and higher dispersion on the results halting accurate statistical significance. However, the authors also recognized the possibility that chemotherapy could induce cognitive dysfunction and the need for further studies to evaluate cognitive function (58).

Ever since that, numerous studies were done to assess the effects of chemotherapy on cognition using standardized neuropsychological testing. However, at first, the terminology used to describe the possible cognitive dysfunction on treated cancer patients was very broad, such as “dementia” “delirium” (59, 60) or unspecific like “organic brain disorder” (61). This terminology did not clarify what domains of cognition were affected. Furthermore, only one or two tests were used in patients, usually for general cognition and not for particular domains (62, 63). Moreover, the tests first applied were not very sensitive and most of the times simple observation of the patients was used to detect cognitive changes (54), thus were of subjective nature. As mentioned above, the ‘practice effect’ that results from the patients knowing and repeating the same tests in different time-points can cause apparent improvements overtime that do not correspond to the reality (64, 65). Actually, all the pitfalls of the first clinical study (58) led to some degree of uncertainty about the real existence of chemotherapy-induced cognitive dysfunction. Even when the clinical data clearly evidenced a decline on cognition after cancer treatment, the results were often attributed to stress, anxiety, and fatigue due to cancer diagnosis (66). Self-reported cognitive problems by chemotherapy-treated patients included difficulties in memory (e.g. remembering names of everyday things) and concentration (67). They also described a feeling of “fogginess” in their mind after receiving chemotherapy, calling it

“chemofog” or “chemobrain” (68). These terms started to be used among patients and in the lay press, being after adopted by the scientific community (27). The term “chemobrain” appeared in the scientific literature for the first time in 2004 in an article by Wefel *et al* (27). Alongside with the term chemobrain, the literature also uses the term “chemofog” (although less used) to acknowledge the long-term cognitive deficits induced by chemotherapy (28). In particular, the effects of systemic chemotherapy in children cancer survivors have been more thoroughly studied, since the developing brain is considered to be at higher risk for long-term sequels, and because it was easier to perform longer longitudinal studies because children may be potentially monitored during many years (69). Despite the presence of a functional BBB in early stages of embryotic development, this barrier is still not fully mature until 6 months of age (70), therefore exogenous compounds can more easily modulate the BBB permeability and harm the developing brain (71). On the other hand, in the adult brain, the effects of chemotherapy were, in the past, less studied and understood, because it was assumed that most chemotherapeutic agents could not cross the mature BBB and even those that can cross the barrier, were assumed harmless to the brain since the neurons are post-mitotic cells and chemotherapy acts on cell division (72, 73). Furthermore, despite the neuroglial cells being as abundant as neurons and can undergo cell division in the adulthood, little attention was given to the effects that cytostatic agents could have on these cells (74). Finally, the long-term neurotoxicity of chemotherapeutic agents was not seen as a concern until recently since the life expectancy of cancer patients was short. The improvements in cancer treatment that lead to an increase in patient’s survival and extra knowledge of the neurotoxicity of the chemotherapeutic drugs have shifted that paradigm and life quality of cancer survivors is now pivotal (75).

1.2. Neuropsychological testing

Chemotherapy-induced cognitive dysfunction can be defined as changes in cognitive domains such as memory, attention, processing speed, and executive function that happen during the chemotherapeutic treatment and last longer after the cessation (76). A large bulk of literature emerged trying to evaluate the prevalence of chemotherapy-induced cognitive dysfunction on cancer patients. To evaluate the changes in cognition, physicians used mainly standardized neuropsychological tests, electrophysiological and neuroimaging techniques. These studies are predominantly longitudinal and have cross-sectional designs.

Actually, a multitude of neuropsychological tests have been developed and validated throughout the years to assess the various domains of cognitive function, such as attention and concentration, executive function, information and processing speed, language, motor function, visuospatial skill, learning and memory (77). Nevertheless, the use of objective and standardized tests requires trained professionals, usually either a neuropsychologist or a psychometrician, and depending on the battery of tests used, they could take between 4 to 7 hours per patient (64). Moreover, the tests chosen to evaluate chemotherapy-induced cognitive dysfunction need to be validated, sensitive and specific to the domain being studied, and also replicable and feasible (78). Only objective evaluation can in fact confirm or dismiss the effect of chemotherapy on the patients' cognitive function.

Before chemotherapy-induced long-term cognitive impairment was proven, only a few clinical trials done in cancer patients incorporated neuropsychological assessment as part of the routine evaluation. One of the first studies was performed by Van Dam and colleagues (79). They compared the effects of high-dose chemotherapy versus standard-dose chemotherapy on cognition. Thirty-six breast cancer patients that received four cycles of cyclophosphamide, epirubicin and 5-fluorouracil followed by tamoxifen and 35 patients that besides the 4 cycles also received a fifth course of cyclophosphamide, thiotepa and carboplatin with autologous hematopoietic progenitor cell support (high dose chemotherapy) were evaluated. The study also included 34 aged-matched control patients with stage I breast cancer that only had surgery. The patients performed a battery of neuropsychological tests, at least 6 months after the hormonal therapy that covered a broad spectrum of cognitive functions. The authors found that chemotherapy-treated patients had more subjective complaints of memory and concentration loss. In the neuropsychological testing, patients treated with high dose chemotherapy had higher cognitive impairment, 8.2 higher fold comparing to the control group and 3.5 higher fold than the standard dose chemotherapy patients. In fact, the high-dose group had significantly impaired attention/concentration, speed of information processing, visual memory, visuospatial and motor function. Moreover, the authors found that the fatigue, stress, and depression tests scores did not correlate with the cognitive deficits observed (79). This kind of matching, with cancer-diagnosed patients with no treatment other than surgery, eliminates the question of stress.

The longitudinal studies that started to emerge mainly in the last decade tried to bridge some limitations of the early studies and provided an insight into the cognitive domains more affected and how long after treatment, the patients were still affected. The first longitudinal trial was performed by Wefel and colleagues. They conducted

neuropsychological evaluation on 18 breast cancer women, before they initiated the systemic chemotherapy consisting of 5-fluorouracil, DOX and cyclophosphamide, 3 weeks after the last cycle of chemotherapy and 1-year post-chemotherapy. The authors compared the results obtained by the patients with population normative data, adjusted for age and education, for each test. Before chemotherapy, 33% of the breast cancer patients were classified as having cognitive impairment, with the domains of verbal learning and memory being the most frequently impaired. Three weeks' post-chemotherapy, 61% of the patients exhibited a decline from the baseline evaluation. Furthermore, nonverbal memory, psychomotor processing speed and attention, confrontational naming, visuoconstruction, and upper-extremity fine motor dexterity were impaired more commonly than expected after chemotherapy. One-year after the end of chemotherapy, only 50% of the patients presented an improvement on their cognitive function (80). This longitudinal study compares the patients' scores to published normative values in healthy populations (80). Other authors also used the same method of comparison for patients scores (81-83). The normative values of healthy populations represent a demographic sample with age and education that differ from the patient sample (84). Even if the populations can be matched on age and education, cultural and socio-economic status can also influence cognitive scoring. Moreover, how the tests are undertaken differs among the groups producing different results that may not be comparable (85). Moreover, and ideally the testing should be applied by the same professionals to reduce confounding factors and pre-treatment evaluation should be set.

Summary data and results extracted from studies that evaluated cognitive function through neuropsychological testing on chemotherapy-treated patients are presented in Table 1, highlighting the disparities of testing, of results and of groups matching. Data referring to anthracyclines were more focused, as DOX is the drug of study. Importantly, no clinical studies were identified so far with MTX.

Table 1: Clinical studies with neuropsychological testing and major findings.

Number and conditions of patients	Anticancer treatment	Clinical and neuropsychological evaluations	Major findings	Study
10 patients admitted for chemotherapy treatment.	Patients received different regimens that included DOX with varying combinations of cyclophosphamide, cis-platinum, and vincristine.	Patients were evaluated before the start of chemotherapy (T1) with vocabulary, reading and information subtests of the Wechsler Adult Intelligence Scale, the Halstead-Reitan Battery, 24 hours (T2) after chemotherapy and a month after (T3), the patients did only the Halstead-Reitan Battery tests.	No differences were found between the 3 time points.	(58)
Breast cancer patients treated with high-dose chemotherapy plus tamoxifen (n = 34) or treated with standard-dose chemotherapy (n = 36). 34 control patients with stage I cancer and no chemotherapy (all patients admitted to the study were off hormonal treatment for at least 6 months).	Standard dose consisted of 4 cycles of 5-fluorouracil, epidoxorubicin and cyclophosphamide and the high-dose regimen included a fifth course of cyclophosphamide, thiotepa and carboplatin.	Battery of 19 neuropsychologic tests.	Patients treated with high dose chemotherapy had higher cognitive impairment (8.2-fold higher comparing to the control group and 3.5-fold higher than the standard dose chemotherapy patients).	(79)
Group A: 39 breast carcinoma patients treated with adjuvant chemotherapy (mean time since last treatment was 2 years).	Group A treatment: 6 cycles of cyclophosphamide, methotrexate 5-	Battery of 14 neurologic tests screening for a wide range of functions	Group A patients presented significantly more concentration and memory problems than group B. Cognitive impairment was found in 28% patients of group A versus 12% in group B.	(86)

Group B: 34 control patients with axillary lymph node negative breast carcinoma not treated with adjuvant chemotherapy: patients only underwent surgery. Women with breast cancer receiving adjuvant chemotherapy (n = 31, group A) and women that had completed the treatment at least 1 year before the study (n = 40; Group B) and 36 healthy control women (Group C).	fluorouracil followed by 20 mg of tamoxifen daily for 3 years (20 patients) or not (19 patients). Group A received every 4 weeks cycles of cyclophosphamide, epirubicin and 5-fluorouracil for at least 8 weeks. Group B completed a full course of adjuvant chemotherapy at least a year before.	mainly focusing on the cognitive function. High Sensitivity Cognitive Screen (HSCS) and Profile of mood states.	No differences in performance were found in cancer patients treated with chemotherapy alone or chemotherapy plus tamoxifen. Group A presented cognitive impairment when compared to group C. Group B had intermediated scores but with no substantial difference when compared with group A and C.	(87)
5 years post-systemic chemotherapy cancer survivors (breast cancer n = 35; lymphoma n = 36) and patients who received local therapy only (breast cancer n = 35; lymphoma n = 22)	Systemic chemotherapy was indicated as 'standard dose chemotherapy', while no specific regimens were mentioned. Local therapy includes radiation and surgery.	Battery of neuropsychologic tests and self-reported measures of cognitive and psychologic function.	Patients treated with systemic chemotherapy presented dramatically lower scores on the neuropsychologic tests than the patients with local treatment, mainly in the domains of verbal memory and psychomotor function.	(88)
109 patients on 3 different cancer regimens and 27 control patients with stage I breast cancer.	5 cycles of chemotherapy (5-fluorouracil, cyclophosphamide and epirubicin), radiotherapy and tamoxifen (n = 36) or high-dose chemotherapy where the fifth course of chemotherapy was replaced by cyclophosphamide, thiotepa and carboplatin with peripheral blood	Series of neuropsychological tests 4 years post-therapy (T1) and 2 years after the last evaluation (T2).	At T1, 28% of the patients had cognitive dysfunction comparing to 12% in the control group. However, at T2 there were no differences among the set groups.	(32)

progenitor cell transplantation (n = 34).
6 cycles of cyclophosphamide, methotrexate and 5-fluorouracil (n = 39).
The control group, after surgery, was only treated with radiotherapy.

Long-term survivors of breast cancer with the allele ε4 of the Apolipoprotein E (ApoE) (n= 17) or absence (n=63)	Standard-dose systemic chemotherapy	Series of neuropsychological tests. ApoE genotyping.	Patients with at least one allele ε4 had lower scores on the domains of visual memory and spatial ability when compared to no-carriers.	(89)
100 women treated with adjuvant chemotherapy for breast cancer, each one matched by age and education with a healthy control person.	Several chemotherapy regimens including: 6 monthly cycles of cyclophosphamide, methotrexate and fluorouracil or 6 cycles of cyclophosphamide, epirubicin and 5-fluorouracil or 4 cycles of DOX and cyclophosphamide sometimes followed by paclitaxel.	Several neuropsychological tests including the Mini mental state examination (MMSE)	Cancer patients presented higher levels of cognitive dysfunction when compared with the matched control. In addition, the patients experienced more fatigue, menopausal symptoms and poorer quality of life.	(90)
53 breast cancer survivors (2 to 5 years post-diagnosis): 36 received adjuvant chemotherapy either alone (n = 18) or plus tamoxifen (n = 18) and 17 did not received chemotherapy. 19 healthy controls were used as comparison.	Chemotherapy regimens included several combinations: cyclophosphamide, methotrexate and 5-fluorouracil; a DOX-containing regimen along with cyclophosphamide alone or with	Neurocognitive performance tasks, self-reported cognitive function and assessment of depression, anxiety and fatigue.	Among the cancer patients, those who received adjuvant chemotherapy scored significantly lower in the domains of visual memory, visuospatial and verbal learning compared to cancer patients not treated with chemotherapy. Cancer patients that received chemotherapy plus tamoxifen had the worse scores among all groups.	(91)

	cyclophosphamide, methotrexate and 5-fluorouracil; DOX, cyclophosphamide and taxane.			
84 patients with breast cancer	Patients received 5-fluorouracil, DOX and cyclophosphamide.	Battery of neuropsychologic tests before chemotherapy (baseline), short-term post-chemotherapy (3 weeks) and long-term post-chemotherapy (1 year).	Before chemotherapy, 33% of the patients presented cognitive impairment, when comparing to published normative values. Short-term post-chemotherapy assessment revealed cognitive impairment in the domains of verbal learning (18%) and memory function (25%) when compared to the baseline evaluation. Furthermore, nonverbal memory (17%), psychomotor processing speed and attention (13%), confrontational naming (13%), visuoconstruction (13%), and upper-extremity fine motor dexterity (12%) were significantly impaired. At the long-term evaluation, 50% of the patients that were previously cognitive impaired demonstrated improvement.	(80)
104 cancer patients did a first evaluation at the end of 3 cycles of chemotherapy. One-year post-chemotherapy, 91 of these patients were evaluated. Two-years post-chemotherapy, 81 of the patients were evaluated again. Cancer patients were matched with an equivalent healthy control.	6 cycles of cyclophosphamide, epirubicin, and fluorouracil or 4 cycles of DOX and cyclophosphamide.	Battery of tests to evaluate cognitive function, levels of depression and fatigue.	At the initial assessment, 16% of cancer patients presented moderate-severe cognitive dysfunction comparing to 5% in the control group. One year after, the patients with cognitive dysfunction decreased to 4.4% and at the 2-year follow up, decreased to 3.8%, versus 3.6% and 0% respectively in the control group.	(92)
702 cancer survivors over the age of 65 years were compared with their cancer-free twins.	The authors did not have access to the cancer treatments received by the patients.	Cognitive screening assessing orientation, short-term memory, general knowledge, verbal recall and verbal abstract reasoning. Dementia screening using Diagnostic and Statistical Manual-IV criteria.	Long-term cancer survivors presented higher rates of cognitive dysfunction then their respective twin.	(93)

Group 1: 19 women with hormone receptor negative breast cancer received only chemotherapy Group 2: 19 women with hormone receptor positive breast cancer received chemotherapy plus tamoxifen Group 3: 19 control women with ductal carcinoma <i>in situ</i> that did not received chemotherapy nor tamoxifen.	In group 1, 20% received cyclophosphamide, methotrexate and 5-fluorouracil and 80% received cyclophosphamide and DOX. In group 2, 75% received cyclophosphamide and DOX with (56%) or without (44%) a taxane. Three women in group 2 received DOX plus docetaxel.	Cognitive function measures at baseline, prior to chemotherapy (T1), 6 months after chemotherapy (T2) and 18 months (T3).	There were no significant differences between groups in the baseline evaluation. Group 3 improved scores from T2 to T3. At T3, groups 1 and 2 performed poorly when compared to group 3.	(94)
45 patients $\geq$ age 65 with stage I to III breast cancer	All patients received adjuvant chemotherapy. 31% received an anthracycline-based regimen and the others received cyclophosphamide, methotrexate, and 5-fluorouracil.	The Squire Memory Self-Rating Questionnaire (pre-chemotherapy baseline evaluation and 6 months post-chemotherapy evaluation).	51% of the patients perceived a decline in memory when comparing to the baseline evaluation.	(95)
28 patients $\geq$ age 65 with stage I to III breast cancer	71% received cyclophosphamide, methotrexate, 5-fluorouracil every 3 weeks for 8 cycles; 7% received DOX and cyclophosphamide every 2 or 3 weeks for 4 cycles; 18% received DOX and cyclophosphamide followed by paclitaxel	All patients completed age-adequate cognitive assessments before chemotherapy (T1), at the end of chemotherapy (T2), and 6 months after chemotherapy (T3).	Before chemotherapy, 11% of the patients scored 2 standard deviation (SD) below the standard values on two or more neuropsychological tests. Six-months after chemotherapy (T3), 29% of the patients had 2 SDs below the standard values on two or more neuropsychological tests.	(96)

	and 4% DOX, cyclophosphamide and paclitaxel followed by trastuzumab.			
85 women scheduled to received chemotherapy. 43 women received either hormonal therapy or/and radiotherapy. 49 healthy controls	Most of the treated patients received 6 cycles of 5-fluorouracil, epirubicin and cyclophosphamide.	Cognitive assessments were made at baseline (T1), 4 weeks after the final chemotherapy session (T2) and 12 months after the last session of chemotherapy (T3).	After normalizing the groups for age and intelligence, repeated measures analysis found no relevant differences between the groups. On an individual level, there was a decline in more than 2 measures, from T1 to T2, in 20% of the chemotherapy-treated group, versus 25.65 and 18.4% in the non-chemotherapy and control group, respectively. In T3, the chemotherapy group had a decline on cognitive function of 18.1% versus 14.6% and 10.6% in the non-chemotherapy and control group, respectively.	(97)
35 patients with metastatic gastric cancer admitted for second-line chemotherapy.	Most patients treated received epirubicin, cisplatin, 5-fluorouracil or capecitabine. The first line of treatment, in most cases, consisted of cisplatin-based chemotherapy and surgery.	EORTC QLQ-C30 questionnaire before the start of the treatment and at the start of each cycle.	There was an improvement in the cognitive parameters from the start of the chemotherapy until the last cycle of treatment.	(98)
71 patients with gastric adenocarcinoma randomized to receive one of two regimens.	Regimen A (n = 35): epirubicin, cisplatin and 5-fluorouracil. Regimen B (n = 36): docetaxel, cisplatin and 5-fluorouracil.	EORTC QLQ-C30 questionnaire before the start of the treatment and after the third treatment.	Before the start of the treatment, there were no differences among the groups. After treatment, there was improvement, in both groups, in all domains except the cognitive domain that deteriorated. Both treatments affected the patients in a similar way.	(99)
39 cancer patients treated with regimen A. 28 cancer patients treated with regimen B. 57 cancer patients that did not received chemotherapy. 60 healthy controls.	Regimen A: 5 cycles of 5-fluorouracil, epirubicin and cyclophosphamide. Regimen B: 4 cycles of regimen A	Neuropsychologic examination before the start of the treatment and 6 months after the end of treatment.	No differences between groups were observed in the first assessment. Patients in regimen B (high-dose chemotherapy) presented a decline in cognitive performance when compared to the healthy control group 6 months after treatment. This decline was not observed in any other group.	(100)

	followed by cyclophosphamide, thiotepa and carboplatin.			
30 women with breast cancer	Regimen with cyclophosphamide and DOX alone or followed by taxane.	Repeatable Battery of Adult Neuropsychological Status prior to the treatment (T1), 1 week after the fourth cycle of treatment (T2) and 6 months after the completion of chemotherapy (T3).	At T1, 4 women had cognitive impairment namely in memory, language and visuospatial skill. Overall, in T2, visuospatial skill and total cognition scores decrease over-time. On contrary, executive function improved over-time. On an individual level, 10 women (33%) decreased one or more SDs on two or more tests at T3.	(101)
57 cancer patients scheduled to received chemotherapy.	Patients treated with chemotherapy were randomized to receive methylphenidate (n = 29) or placebo (n = 27).	MMSE HSCS Revised Hopkins Verbal Learning Test The tests were performed before the treatment (T1), at the end of the treatment (T2) and 6 months after the treatments' end (T3).	There were no significant differences in cognition in the 3 time points evaluated between the methylphenidate group and the placebo group.	(102)
Cancer patients aged more than 65 years divided into 3 groups: no-chemotherapy (control group; n = 32); chemotherapy group (n = 16) and hormonal therapy group (n = 13).	Chemotherapy treatments differed among patients but included 5-fluorouracil, cyclophosphamide, methotrexate, DOX, paclitaxel and epirubicin. The hormonal therapy was anastrozole or tamoxifen.	Cambridge Cognitive Examination performed before chemotherapy or endocrine therapy, (T0) and after 3 (T1) and 6 months (T2).	Overall, there was no deterioration in cognitive function in the 3 time points tested for the 3 groups. However, in the chemotherapy group, more patients displayed reduction of scores in the domains of learning memory and total memory, from T1 to T2.	(103)

Patients with testicular cancer divided into 3 groups: group 1 (n = 70) received chemotherapy after surgery; group 2 (n = 57) were only treated with radiotherapy after surgery; group 3 (n = 55) patients received surgery only.	4 courses of bleomycin, etoposide, and cisplatin.	Battery of neuropsychological tests approximately 3 years after the finalization of treatment.	Both in group 1 and group 2, 32% of the patients reported cognitive problems. However, in the neuropsychological tests done no differences between groups were seen. In individual scores, patients in group 1 presented more cognitive dysfunction when compared to group 3, but no differences were observed when compared to group 2.	(104)
61 breast cancer patients scheduled to receive chemotherapy and 51 control women receiving hormonal therapy.	Most women received anthracycline-based chemotherapy.	Neuropsychological testing after surgery and prior to initiation of any chemotherapy (T1) and after the last chemotherapeutic treatment (T2).	Mean scores for both groups were similar and within the norms for cognitive tests; however, on individual scores in T2, chemotherapy-treated patients were 3.3 times more likely to had cognitive decline than the hormonal therapy group.	(105)
38 women who received chemotherapy 5 to 39 months prior to the study (39 to 71 years old) and 55 control healthy women aged matched.	Most women received regimens that included cyclophosphamide plus methotrexate and 5-fluorouracil, or anthracycline-based regimens.	Useful Field of View assessment program.	Older (more than 54 years old) chemotherapy-treated women performed worse on speed of processing than controls and younger chemotherapy-treated women.	(106)
87 patients 12 to 30 months post-chemotherapy of whom 45 received epoetin-alfa and 42 received standard care.	Most patients received an anthracycline-based regimen or received cyclophosphamide, 5-fluorouracil, methotrexate and taxane. Besides the chemotherapy treatment, the patients were randomized to receive epoetin-alfa.	MMSE HSCS Revised Hopkins Verbal Learning Test. Functional Assessment of Cancer Therapy questionnaires assessing fatigue and general life quality. Hospital Anxiety and Depression Scale.	Although there were no significant differences in cognition with or without epoetin-alfa, those whom received epoetin-alfa reported better quality of life.	(107)
187 breast cancer survivors of whom 97 patients were treated with chemotherapy only or chemotherapy plus radiotherapy and 90 patients received radiotherapy only.	Most chemotherapy regimens included DOX and cyclophosphamide.	Battery of neuropsychological tests 6 months after completion of treatment.	Small differences in cognition were found in women treated with chemotherapy when compared to the age-matched controls. No differences were observed among the other groups.	(108)

187 aged-matched healthy controls.

36 female breast cancer patients receiving chemotherapy.
14 cardiac patients (cancer-free).
17 healthy controls.

Chemotherapeutic regimen consisted of cyclophosphamide, epirubicin and 5-fluorouracil.

Battery of neuropsychological tests performed before the beginning of chemotherapy, 4 to 6 weeks after the last treatment and 25 weeks after the first test. Cardiac patients were tested 4 days after hospitalization and at a 3-month follow up. Healthy controls were tested within an interval of 12 to 16 weeks.

No differences were found between groups tested. Cancer patients did not differ from healthy controls neither before nor after chemotherapy.

(109)

Breast cancer patients divided into 2 groups: 41 patients received chemotherapy followed by radiotherapy in 38 of these patients. The other group of 40 patients received radiotherapy without chemotherapy. The study also included a control group of 45 healthy women.

23 patients received DOX and cyclophosphamide; 12 patients received taxotere, DOX and cyclophosphamide; 6 patients received 5-fluorouracil, epirubicin and cyclophosphamide.

Battery of neuropsychological tests prior to the initiation of the treatment (T1), instantly after the first treatment (T2) and following the end of the last treatment (T3).

Both groups had difficulties for recruiting information in verbal memory, compared to T1. However, only patients treated with chemotherapy presented impaired verbal fluency.

(110)

46 women receiving chemotherapy for stage II-III breast cancer.

Patients received DOX and cyclophosphamide with or without paclitaxel or taxotere.

Neuropsychological tests to evaluate skills in five cognitive domains: immediate memory, delayed memory, attention, executive function and verbal fluency. Tests

Patients had cognitive deficits in executive functioning and verbal fluency when comparing to calculated expected values.

(111)

were performed 1 month following the end of chemotherapy.

136 women with breast cancer scheduled to receive chemotherapy and 21 women also with breast cancer but not receiving chemotherapy.	Several chemotherapeutic regimens that included 5-fluorouracil, epirubicin, cyclophosphamide, DOX, methotrexate.	Neuropsychological tests and self-report measurements at baseline (before chemotherapy and after surgery) and 4 weeks after the last dose of chemotherapy.	16.9% of the women treated with chemotherapy presented decline in verbal learning and memory abstract reasoning, and motor coordination. Moreover, the study also found that a decline in hemoglobin and increase in anxiety, over the course of chemotherapy significantly predict impairment in multiple cognitive measures.	(112)
Cancer patients scheduled to be treated with chemotherapy (n = 60) and cancer patients not receiving chemotherapy (n = 72) and 45 female healthy controls.	Several chemotherapeutic regimens that included combinations of DOX, cyclophosphamide, paclitaxel, taxotere, fluorouracil, epirubicin and methotrexate.	Standardized neuropsychological tests. For chemotherapy-treated patients, the tests occurred after surgery but before the initiation of the treatment and 1, 6 and 18 months after treatment end. The other patients and healthy controls had the same testing scheduled.	Older patients (60 to 70 years old) had lower cognitive reserves in the pre-treatment and performed worse after the treatment compared to non-chemotherapy patients and healthy controls (of the same age range).	(113)
124 breast cancer women aged <60 years and 224 healthy controls aged-matched to the patients.	Among the breast cancer patients, 75 received cyclophosphamide 600 mg/m ² intravenously, epirubicin 60 mg/m ² and 5-fluorouracil 600 mg/m ² every 3 weeks for seven cycles, 26 patients received tamoxifen and	Neuropsychological testing was performed before any adjuvant treatment (T1) and approximately 6 months after the first test (T2).	No differences were found between groups in both time-points.	(114)

	19 received no adjuvant treatment.			
101 cancer patients.	Patients were randomized to receive one of two treatments: Treatment A (48 patients): epirubicin, cyclophosphamide and paclitaxel. Treatment B (53 patients): epirubicin and paclitaxel combined with cyclophosphamide, methotrexate and 5-fluorouracil. In each group, about half of the patients also received erythropoietin.	The neuropsychological evaluation and self-perceived cognitive function took place before the start of preoperative chemotherapy (T1), before the last cycle of chemotherapy (T2, about 5 months later), and about 1 year after the first evaluation(T3).	No significant changes in self-reported cognitive changes nor in the neuropsychological tests were found.	(115)
61 women aged between 45 to 70 years old with breast cancer and post-menopausal. 30 of these women received chemotherapy and the remaining did not and were taken as controls.	14 women received DOX, cyclophosphamide and docetaxel/paclitaxel; 7 women received DOX, cyclophosphamide and the other 9 received cyclophosphamide, methotrexate and 5-fluorouracil.	Battery of standardized neuropsychological tests and self-rated surveys at 3 times: before treatment (T1), 6 months after the first test (T2) and then 12 months after (T3).	The motor domain was the only one significantly worse in women treated with chemotherapy. In post-menopausal women, chemotherapy does not seem to induce cognitive deficits.	(116)
42 patients with breast cancer.	Patients were treated with 5-fluorouracil, DOX, cyclophosphamide with or without paclitaxel.	Battery of cognitive tests, mood measures and quality of life questionnaire. Tests were conducted before treatment, and then on average 2.9 months and 7.0	21% of the patients met the criteria of cognitive impairment before any treatment and the domains more affected were learning and memory, executive function, and processing speed. In the acute interval, the number increased to 65% being the same domains the most affected. In the late interval evaluation, 61% of the patients had cognitive decline, of whom, 30% presented new domains affected that were not affected before, mainly learning and memory impairments.	(117)

		months (acute interval) and 13.1 months after the first assessment (late interval).		
30 women aged over 65 years and long-term survivors of breast cancer. They were at least 50 years old and at least 10 years post-chemotherapy. The patients did not have any cancer recurrence. Each survivor was matched to a healthy control person.	Patients were administrated with different combinations of anti-cancer drugs that included cyclophosphamide, methotrexate and 5-fluorouracil or DOX.	Battery of neurocognitive tests selected to evaluate a broad spectrum of cognitive domains.	The long-term survivor's group had impaired attention, working memory, psychomotor speed, and aspects of executive functioning, performing 0.75 to 2.0 standard deviation below their matched healthy control. However, no impairment was detected in the domains of language, visuospatial and memory functioning.	(118)
42 patients with breast cancer.	Patients did 4 cycles of paclitaxel or 4 cycles of 5-fluorouracil, DOX, and cyclophosphamide	Neuropsychological testing that included measurement of cognition, mood and quality of life performed before treatment, and on average 3.7 and 13 months after the first evaluation.	Before treatment, 21% of the patients had evidence of cognitive dysfunction, with the most common domains affected being: learning and memory, executive function, and processing speed. Acute decline in cognitive function occurred in 65% of the patients. Late cognitive decline happened in 61% of the patients, of whom, 30% presented new domains affected that were not affected in the acute phase.	(119)
71 women with breast cancer received chemotherapy.	Group A: 22 patients had four cycles of DOX and cyclophosphamide. Group B: 49 patients had four cycles of DOX and cyclophosphamide plus taxane.	Neuropsychological testing was performed before the start of chemotherapy, 1 week after 4 cycles of treatment, 1 week after taxane treatment and 6 months after finishing chemotherapy.	At baseline, 23% of the women had scores consistent with cognitive dysfunction when comparing with pre-established scoring criteria. The main functions affected were language, visuospatial skill and motor function. Throughout the study, in both groups, 52% of the women had a decreased of 1 or more SD for 2 or more tests, after chemotherapy, being the most impaired: visuospatial skills followed by motor function, attention, language, immediate memory and delayed memory. At the 6-months evaluation, 20% of the women still presented a decreased of 1 or more SD from baseline scores.	(120)
40 women with breast cancer that finished the treatment at least 18 months prior to evaluation.	20 women did taxane and/or DOX-based chemotherapy and the	Self-reported cognitive problems questionnaire and neuropsychological	Overall, the patients performed worse when comparing to the standard scores. When comparing the tamoxifen group versus chemotherapy group, there were no differences. In both	(121)

	other 20 women only took tamoxifen.	tests for assessment of long-term memory.	groups, 40% of the women had scores consistent with mild cognitive impairment when compared to comparative values.	
40 women undergoing chemotherapy for breast cancer.	Patients received cycles of an anthracycline-based therapy.	Cognitive testing and self-perception of cognition before (T1) and 6 months after chemotherapy (T2).	Comparing the pre-evaluation to the tests 6 months after treatment, there was a worsening in the overall cognition and in the visual selective attention. Still, the patients did not had perception of cognitive impairment as the self-perception results did not correlate with objective evaluation.	(122)
34 women with breast cancer and premenopausal treated with chemotherapy. 16 women with breast cancer and not treated. 19 healthy controls age matched.	Patients did 6 cycles of 5-fluorouracil, epirubicin, and cyclophosphamide or 3 cycles of 5-fluorouracil, epirubicin, and cyclophosphamide plus 3 cycles of paclitaxel.	Neuropsychological assessment before any treatment (T1) and 3 to 4 months after the last treatment (T2).	At T1, there were no differences among the 3 groups. In the T2 evaluation, the chemotherapy-treated group had significantly lower scores on attention and concentration tests, psychomotor speed and memory when comparing to the first assessment. Both groups not treated and controls improved their scores over-time due to the learning ('practice') effect.	(123)
40 breast cancer patients treated with chemotherapy and 40 age-matched healthy controls.	Chemotherapy treatment with DOX, paclitaxel, cyclophosphamide, and 5-fluorouracil.	Neuropsychological tests that include event-based prospective memory task and time-based prospective memory task, 4 weeks after the end of the treatment.	The chemotherapy group had lower scores in the MMSE, verbal fluency and digit span. This group also presented significantly lower scores in the event and time-based prospective memory task.	(124)
60 women with early breast cancer. 60 healthy control women.	47 women received 5-fluorouracil, epirubicin, cyclophosphamide with or without taxotere. 7 women received cyclophosphamide plus taxotere. 5 women received DOX and cyclophosphamide with or without paclitaxel.	Battery of neuropsychological assessment after surgery but before chemotherapy and after each cycle of chemotherapy (7 time-points evaluated).	In the first assessment, both groups scored within the normal average scores and there were no differences among groups. In the chemotherapy group, the cognition scores constantly changed from T1 to T7, reaching statistically difference in T5 (fourth cycle of chemotherapy). The domain that declined the most was working memory on the chemotherapy group. There was no difference in the scores of different anticancer treatments.	(125)

18 patients with breast cancer and 20 healthy controls.	Cycles of DOX or epirubicin and cyclophosphamide followed by paclitaxel/docetaxel.	Battery of neuropsychological tests, before the treatment, 4 weeks after the last treatment and 6 months after the last treatment.	Neither differences were found in cognition between the 3 time-points, nor between breast cancer patients and the healthy controls.	(126)
Breast cancer patients treated with chemotherapy (n = 28) or not (n = 37) and a control group (n = 32).	Chemotherapy regimen included several cycles of DOX and cyclophosphamide with or without docetaxel or paclitaxel.	Neuropsychological testing before chemotherapy and 1 month after the last treatment.	No significant baseline differences were found regarding cognition among the groups. However, after treatment, the chemotherapy group had worse cognition scores than the other two groups.	(127)
60 breast cancer patients that received chemotherapy. 60 individually matched (age and education) controls.	Most of the patients received cycles of 5-fluorouracil, epirubicin and cyclophosphamide.	Standardized neuropsychological testing before the start of chemotherapy, at the end of each cycle (short-term evaluation) and 1 year after the last treatment (long-term evaluation).	At an individual level, there was a decline in score of 48% of the patients versus 9% of the control group, in the short-term evaluation. From the baseline evaluation to the long-term evaluation, the patients group presented a decline in 22% of the patients versus 6% in the control group.	(128)
32 women treated for breast cancer. 32 healthy controls.	41% of the patients received cycles of cyclophosphamide, epirubicin and 5-fluorouracil. The remaining patients received DOX in combination cyclophosphamide or docetaxel or paclitaxel.	Standard cognitive testing done approximately 2 months after chemotherapy ended.	Women treated with chemotherapy had higher percentage of cognitive impairment, being the domains of attention and working memory the most affected.	(129)
1300 breast cancer survivors.	93% of the patients received chemotherapy.	Neuropsychological testing was done 18 months after diagnosis, while 1059 of these patients were	Despite the treatment, the women improved their cognitive function scores from the 18 th month to the 36 th month.	(130)

		evaluated again 36 months post-diagnosis.		
99 women with breast cancer that received chemotherapy.	Women received an anthracycline-based regimen consisting of DOX 60 mg/m ² and cyclophosphamide 600 mg/m ² or a taxane-based regimen consisting of docetaxel 75mg/m ² and cyclophosphamide 600 mg/m ² .	Computerized neuropsychological testing and measurement of plasma cytokines levels before the start of the treatment (T1), after the 3 rd cycle of chemotherapy (T2) and after the end of treatment (T3).	At T3, patients had dysfunction in memory (13.2%) and attention (7.3%) when comparing to T1. Considering the cytokines levels, only the IL-6 was significantly altered and increased from T1 to T3.	(131)
76 cancer patients of whom 5 had not started chemotherapy, 20 had completed 1 cycle, 20 had completed 3 cycles, 19 had completed 7 cycles and 12 had completed the treatment at least 24 months prior to the study.	Patients received cycles of 5-fluorouracil, epirubicin and cyclophosphamide or cyclophosphamide and DOX.	Standard neuropsychological testing and measurement of blood levels of high-molecular-weight neurofilament subunit (pNH-F), a potential biomarker of neuronal or axonal damage.	28.8% of all patients that received chemotherapy had increased levels of pNH-F, but not the patients still waiting to receive treatment. There was a positive correlation between the number of cycles and higher elevated levels of pNH-F. No differences were observed in the neuropsychological tests among the groups of patients.	(132)
Early-stage breast cancer patients, over 65 years old, treated with chemotherapy (n = 58) or radiotherapy (n = 61). Sixty-two aged matched healthy controls were used.	Chemotherapy treatment consisted of 5-fluorouracil, epirubicin and cyclophosphamide with or without docetaxel.	Neuropsychological testing after radiotherapy or before chemotherapy (T1) and after the end of the treatment (T2).	At T1, 46% of the patients that received chemotherapy had cognitive impairment. At T2, 64% of all patients had cognitive impairment. No differences in cognition were found between patients that received chemotherapy or radiotherapy.	(133)
75 women with early-stage breast cancer.	Most women received cycles of DOX, cyclophosphamide and docetaxel.	Cognitive testing and plasma concentration of several cytokines measured before the start of chemotherapy (T1), halfway through the treatment (T2), 6	At T1, the granulocyte colony stimulating factor concentration was positively correlated with cognitive flexibility, executive functioning and psychomotor speed. On the other hand, IL-17 was negatively correlated with psychomotor speed. At T2, various cytokines levels were correlated with psychomotor speed, complex attentions, executive function, verbal memory and visual memory. At T3 and T4, there were also multiple	(134)

		months after the beginning of the treatment (T3), 1-year after the beginning (T4) and 2 years after the beginning (T5).	relations between the levels of cytokines and cognition. At T5, less relations between levels of cytokines and cognition deficits were set when compared to earlier time-points.	
Breast cancer patients received chemotherapy, with or without endocrine therapy, (n = 31) or did not receive systemic chemotherapy (n = 24). The study also included 33 age-matched healthy controls.	Anthracycline-based therapy.	Standardized neuropsychological testing was performed before the start of the treatment (T1) and six months after chemotherapy (T2).	At T2, 16% of the patients treated with chemotherapy had impaired cognition, compared to 4% of the patients that did not received chemotherapy and 6% in the healthy controls.	(135)
106 patients with non-Hodgkin or Hodgkin lymphoma and 53 healthy matched controls.	Anthracycline-based chemotherapy.	Neuropsychological testing on average 4 years after chemotherapy.	A subgroup of 16% of the patients had abnormal neuropsychological performance (1.5 SD below the norm in 3 of the 14 tests).	(136)
Women diagnosed with breast cancer scheduled to received chemotherapy with taxane (n = 25) or without (n = 26).	Patients received cycles of 5-fluorouracil, epirubicin and cyclophosphamide followed or not by taxane.	Standard neuropsychological testing was performed before the start of chemotherapy (T1), after the end of the treatment (T2) and 75 weeks after T2 (T3).	At baseline, none of the patients had cognitive impairment. At T2, the taxane group performed worse than the group without taxane when compared to baseline. At T3, both groups still had cognitive impairment when compared to baseline.	(137)
480 breast cancer patients that received chemotherapy.	Patients received several cycles of chemotherapeutic agents that included DOX, cyclophosphamide, 5-fluorouracil, epirubicin or docetaxel.	Montreal cognitive assessment and hospital anxiety and depression scale tests were performed before the start of the treatment and at the 1-year follow-up.	At the 1-year follow-up, 81% of the patients had cognitive impairment. Moreover, there was an increasing risk of cognitive impairment in patients that did not presented anxiety in the first assessment.	(138)
403 cancer survivors, at least 4 years post diagnosis and treatment.	Treatment data was available for 235 patients: 46.8% received exclusively chemotherapy, 32.3%	Standard neuropsychological testing.	The study found no association between cognitive function decline and chemotherapy treatment.	(139)

received only surgery
and 20.8% received only
radiotherapy.

Twenty-two female breast patients that received chemotherapy.	Anthracycline or non-anthracycline-based chemotherapy.	Neuropsychological testing and serum levels of inflammatory cytokines during chemotherapy treatment.	Patients that had higher levels of soluble tumor necrosis factor receptor 1 had lower cognitive scores.	(140)
142 patients with breast cancer (51.4%) or colorectal cancer (48.6%) submitted to chemotherapy.	Breast cancer patients received combination of 5-fluorouracil, epirubicin, cyclophosphamide, DOX with or without docetaxel/paclitaxel. Colorectal cancer patients received cycles of 5-fluorouracil, leucovorin, oxaliplatin, irinotecan and capecitabine.	Psychomotor vigilance test and the trail-making test were performed prior to the start of the treatment (T1) and after the end of treatment (T2).	After chemotherapy, patients had decreased reaction time in the psychomotor vigilance test, indicating impaired orientation and attention. However, in the trail-making test, the patients did the test faster (6.7 s) and there were no significant alterations from T1 to T2.	(141)

EORTC QLQ-C30 - European organization for research and treatment of cancer quality of life questionnaire C30; HSCS - High sensitivity cognitive screen; pNH-F - High-molecular-weight neurofilament subunit; MMSE - Mini mental state examination; SD - Standard deviation.

The data of Table 1 highlights several aspects: 1) the number of patients per study is very diverse and clearly influences the conclusions of the study, being larger studies more prone to detect chemobrain; 2) women (with breast cancer) are the large majority of patients included in the studies; 3) the studies that undergo baseline evaluations (before treatment) are more prone to detect cognitive dysfunctions after treatment; 4) it is not clear if age of diagnosis or age of treatment impacts on cognitive dysfunction outcome; 5) although hormonal therapy has been described (142) as causing cognitive dysfunction, when placed with chemotherapy, the effects of the latter seem to cause more impact; 6) the effect of practice (or education on neurophysiological tests) is commonly seen between baseline evaluation and longer periods of evaluation, which can mask underlying and persisting cognitive dysfunction and chemobrain; 7) whether chemobrain is transient or not is still not clear and other studies are required. Despite some methodological issues regarding the chemotherapy-induced cognitive dysfunction, it is clear, at this point, that chemotherapy affects patients' cognition.

In summary, chemobrain is now a well-recognized secondary effect of chemotherapy (68), but there is still a lot of uncertainty to what extent it affects treated patients as well as the underlying mechanisms involved. Hence, the research on the neurotoxicity of chemotherapy is crucial to: 1) access the real incidence of cognitive dysfunction on chemotherapy-treated patients and individual susceptibility factors; 2) clarify the putative mechanisms of chemobrain; and 3) establish standard care and management options to ameliorate the quality of life of cancer survivors (143). In addition, what are the mechanisms and the possible measures to be undertaken to ameliorate its impact on cancer survivors daily living are still largely unknown.

1.3. Electrophysiological and neuroimaging studies

The brain optimal function is highly dependent on the maintenance of its integrity and structure. The grey matter comprises the regions of the CNS enriched in cell bodies, unmyelinated axons, dendrites and glial cells (36). Beneath the grey matter cortex, lies the white matter, which is composed by oligodendrocytes that involve the axons in lipidic myelin sheets. Myelin is essential for the fast transmission of electrical impulses and the loss of integrity and axonal demyelination of the white matter occur in some neurodegenerative diseases, such as Alzheimer's and multiple sclerosis (144). The performance of cognitive tasks depends on the highly organized and synchronized brain networks and the integrity of brain white matter pathways to transmit the neural signals

effectively (76). Therefore, changes in structure and brain-behavior could translate into cognitive dysfunction (145) and are probably less subjective measures of damage.

Multiple structural and neuroimaging techniques have been used to understand the changes in the neural substrate of chemotherapy-treated patients that may lead to poor cognition. Voxel-based morphometry (VBM) and Diffusion-Tensor imaging (DTI) are structural neuroimaging techniques that can detect alterations in the grey matter and white matter tissue, respectively (146). Functional Magnetic Resonance Imaging (fMRI), Positron Emission Tomography (PET) and electroencephalogram (EEG) can find changes in brain function even when structural changes are not evident (146). Scans that use magnetic resonance imaging (MRI) such as VBM, DTI and fMRI are not invasive and provide the best image quality both in 2 or 3 dimensions without exposing the patients to ionizing radiation or radioactive probes (147). These techniques provide an insight on brain of chemotherapy-treated patients attempting to explain why they have declines in cognitive functions. Summary data and results of studies using electrophysiological and neuroimaging techniques are presented in Table 2, thus demonstrating the impact of chemotherapy on brain structures.

Table 2: Clinical studies with neuroimaging and neurophysiological testing

Number and conditions of patients	Anticancer treatment	Clinical neuroimaging and neurophysiological evaluation	Major findings	Study
6 patients with breast cancer treated with 5-fluorouracil, whom developed leukoencephalopathy.	4 patients received 5-fluorouracil, cyclophosphamide, epirubicin and carmofur, one received the same regime except the 5-fluorouracil and the last patient only received carmofur.	MMSE. Routine laboratory tests, brain biopsies and electroencephalography.	5-Fluorouracil and carmofur seem to be the cause of leukoencephalopathy in these patients and could be a possible cause of chemotherapy-induced neurotoxicity.	(148)
33 post-surgery high-risk cancer patients that received chemotherapy. 14 stage I breast cancer control patients.	16 patients received 5 cycles of chemotherapy (5-fluorouracil, cyclophosphamide and epirubicin) radiotherapy and tamoxifen. 17 patients received high-dose chemotherapy where the fifth course of chemotherapy was replaced by cyclophosphamide, thiotepa, carboplatin and peripheral blood progenitor cell transplantation. The control group, after surgery was treated with radiotherapy only.	Event-related potential quantitative electroencephalography performed 2 years post-treatment.	Cognitive dysfunction was established in 32% of the patients treated with high-dose chemotherapy, in 17% of the standard-dose patients and in 9% of the stage I breast cancer control group. Regarding the electroencephalograph results, 7 patients of the high-dose regimen and 2 patients of standard dose regimen had asymmetric alpha rhythms versus no alteration in the control group.	(149)

12 patients with breast cancer treated with high-dose chemotherapy. 17 patients with breast cancer treated with standard-dose chemotherapy. 23 control patients with breast cancer that did not received chemotherapy.	The standard-dose group received 5 courses of 5-fluorouracil, epirubicin, and cyclophosphamide every 3 weeks. In the high-dose group, the fifth course was replaced by a course of cyclophosphamide, thiotepa and carboplatin with autologous peripheral blood hematopoietic progenitor cell transplantation. All patients received locoregional radiation therapy after the end of chemotherapy and were treated with tamoxifen for 2-5 years if they had hormone receptor-positive cancer.	EEG and neuropsychological testing. All patients were evaluated approximately 4 years after treatment.	Comparing the evaluated parameters between the 3 groups, only event-related Potential (P3 peak amplitude) was reduced. The group treated with high-dose chemotherapy had significantly lower P3 component compared with control patients. However, there were no significant differences between the standard-dose group and the control group.	(150)
16 women who had breast cancer and received chemotherapy 5 to 10 years prior. 8 control women, who did not had chemotherapy and had breast cancer (n = 5) or not (n = 3).	Not described.	PET scan.	Chemotherapy-treated women had significantly altered blood-flow in specific regions of frontal cortex and cerebellum while performing short-term recall tasks. Moreover, these patients' cerebral activation differed from untreated patients in inferior frontal gyrus. Metabolism of the basal glia was considerably decreased in chemotherapy patients that also received tamoxifen when compared to the other groups, even when compared with other women that underwent chemotherapy.	(151)
17 post-chemotherapy women with breast cancer. 18 healthy controls. 10 non-chemotherapy treated women (radiotherapy or hormonal therapy).	Chemotherapy cycles included 5-fluorouracil, epirubicin, and cyclophosphamide.	DTI. Neuropsychological testing.	White matter integrity was diminished in chemotherapy treated patients when comparing to healthy controls and non-chemotherapy treated patients. Moreover, there were significant correlations between white matter integrity and neuropsychological tests covering the domains of attention and processing/psychomotor speed in the temporal and parietal areas.	(152)

32 patients with Hodgkin's lymphoma or non-Hodgkin's lymphoma treated with chemotherapy. 18 healthy controls.	Cancer patients were treated according to the ABVD (Bleomycin-Dacarbazine-DOX-Vinblastine) protocol and the CHOP (Cyclophosphamide-DOX-Vincristine-Prednisolone) protocol.	Neuropsychological testing and PET scanning in average 7 months after the last treatment.	The chemotherapy-treated group had lower glucose metabolism in multiple regions of the brain being the prefrontal cortex (PFC) the main area affected.	(153)
34 women with history of breast cancer and treated with chemotherapy. 27 healthy controls matched for age, education and overall intelligence.	Most women received cycles of DOX, cyclophosphamide and taxol.	Resting state fMRI.	Breast cancer patients presented altered global brain network when comparing to the healthy controls.	(154)
Chemotherapy group: 17 breast cancer patients treated almost 10 years prior to evaluation. No chemotherapy group: 15 patients that had stage I breast cancer and where currently disease-free.	4 cycles of 5-fluorouracil, 500 mg/m ² , epirubicin, 90 mg/m ² , cyclophosphamide, 500 mg/m ² , followed by one cycle of high-dose cyclophosphamide, 6 g/m ² , thiotepa, 480 mg/m ² , carboplatin, 1600 mg/m ² , and autologous peripheral blood hematopoietic progenitor-cell transplantation. Patients also took 40 mg of tamoxifen daily on average for 3.8 years.	Neuropsychological testing. MRI scanning.	Almost 10 years after, the chemotherapy group had significant hyporesponsiveness of dorsolateral PFC when comparing to the non-chemotherapy group. The chemotherapy-treated group also performed worse on the neuropsychological testing.	(155)
37 female breast cancer survivors (in average 4 years after chemotherapy). 38 age-matched healthy controls.	Most women did several cycles of DOX and cyclophosphamide.	MRI scanning.	Women treated with chemotherapy for breast cancer had alterations in the properties of grey matter, particularly in the frontotemporal regions when comparing to the control group.	(156)
184 invasive breast cancer survivors on average 21 years after chemotherapy. 368 age-matched healthy controls.	6 cycles of cyclophosphamide, methotrexate, and 5-fluorouracil.	MRI and VBM for analysis of different brain regions and measurement of total	The chemotherapy-treated group had smaller total brain volume, and smaller grey matter volume than the control group. No differences were found in the volume of the	(157)

		brain volume and hippocampal volume.	hippocampal region, white matter and cerebral-spine fluid volume compared to the control group.	
Female breast cancer patients treated with chemotherapy (n = 16) or without (n = 12). Healthy controls (n = 15).	Most patients that received chemotherapy did cycles of DOX, cyclophosphamide and paclitaxel or docetaxel, DOX and cyclophosphamide or DOX and cyclophosphamide.	MRI scan performed before any treatment, 1 month after chemotherapy and 1 year later.	At baseline, both group of patients had increased bifrontal and decreased left parietal activation and at the 1-month follow up, those groups had reduced frontal hyperactivation with partial return to the baseline patterns after one year. Comparing chemotherapy-treated patients with non-treated, they differ in the pattern of activation in the frontal lobes.	(158)
9 women with breast cancer	Not described.	Functional MRI session before the start of the chemotherapy (T1), 1 month after (T2) and 1 year after the last treatment (T3).	At T2 exam, there was decreased functional connectivity in the dorsal attention network that partially returned to baseline values after 1-year. Regarding the default mode network, there was decreased connectivity that did not return to normal after 1-year.	(159)
44 women survivors of breast cancer, on average 4.8 years post-chemotherapy. 38 healthy female controls.	36 women received DOX, cyclophosphamide or paclitaxel. 6 women received cyclophosphamide, 5-fluorouracil and paclitaxel or methotrexate.	MRI exam, hippocampal volume measurement and plasma cytokines measurement.	The left hippocampal volume was significantly reduced in the chemotherapy when comparing with the control group. The right hippocampal volume was slightly reduced ($p=0.07$) compared to the controls. Concerning the cytokines levels only tumor necrosis factor alpha (TNF- α) and IL-6 levels were significantly higher in the chemotherapy group, comparing to the control group.	(160)
21 early breast cancer patients. 21 matching controls.	15 women received 5-fluorouracil, epirubicin, cyclophosphamide and docetaxel or with epirubicin. 6 women received cyclophosphamide with docetaxel or DOX.	Neuropsychological assessment and self-report mood state, MRI scanning and verbal recall task before and after chemotherapy.	Before chemotherapy, cancer patients activated the anterior cingulate fewer times than controls during memory recall. In the patients' group, there were also changes in brain activation after the treatment. On contrary, the control group had no changes in activation in either time-points assessed.	(161)

Female with breast cancer treated with chemotherapy (n = 27) or without (n = 28). 24 healthy controls.	Standard dose chemotherapy that included taxane.	ApoE genotyping and MRI scanning before any treatment and a month after the end of chemotherapy.	No significant changes were observed among groups in the first evaluation. Chemotherapy-treated patients, in the second evaluation, had reduced grey matter density, in the left middle frontal gyrus, when compared to the controls. This change was not observed in the patients that did not received chemotherapy. No correlation was found between changes in the grey matter size and the ApoE gene.	(162)
Cancer patients scheduled to received chemotherapy (n = 18) or not (n = 16). Age matched healthy controls (n = 17).	Patients received cycles of 5-fluorouracil, epirubicin, cyclophosphamide.	fMRI scanning pre-treatment and also 4 to 6 months after the end of treatment.	Before chemotherapy, there were no significant differences among the groups. At the 6 months follow-up, the chemotherapy group had decreased brain activity in the multitasking network.	(163)
19 female breast cancer patients. 19 individually matched controls.	Most patients received 5-fluorouracil, epirubicin and cyclophosphamide plus docetaxel.	Neuropsychological testing and MRI scanning before chemotherapy (T1), one month after the last treatment (T2) and one year (T3) after the last treatment.	In the treated group, at the one-year evaluation (T3), the MRI scan showed a decrease in the grey matter volume in the frontal, temporal, parietal and occipital regions, comparing to the last evaluation. The control had no significant alterations. Regarding the neuropsychological testing, scores decreased over time in the chemotherapy-treated group.	(164)
8 breast cancer patients. 8 matched controls.	Not described.	EEG after physical and cognitive testing, performed before chemotherapy (T1), after 3/4 cycles of chemotherapy (T2) and one year (T3) after completion of the treatment.	Before treatment, there were no significant differences between patients and controls. However, during the treatment (T2), the EEG power value was higher in patients compared with the controls. At the one-year assessment (T3) there were no differences among the two groups.	(165)
Breast cancer patients that received chemotherapy (n = 27) or not (n = 26). Healthy controls (n = 26).	Standard dose chemotherapy that included DOX and cyclophosphamide.	MRI scanning before treatment and one month after the last cycle of chemotherapy.	At baseline, there were no differences among groups. After treatment, the chemotherapy group had an increased perfusion comparing to baseline assessment and significant differences in the precentral gyrus when compared to the control group.	(166)

21 patients with non-Hodgkin lymphoma.	Not described.	PET imaging before and after chemotherapy.	After the treatment, the patients had overall reduced cerebral cortical metabolism, reduced partial volume-corrected average glucose uptake. However, the overall brain volume was not altered.	(167)
Breast cancer patients treated with chemotherapy within 2 years of the study and that had cognitive complaints (n = 15) or not (n = 13).	Not described.	MRI scanning.	Women who reported cognitive complaints had disrupted functional connectivity between brain networks implicated in cognitive control.	(168)
Cancer patients more than 2 years post-treatment, that received an anthracycline based chemotherapy (n = 20) or non-anthracycline based chemotherapy (n = 19) or did not receive chemotherapy at all (n = 23).	Anthracycline based therapy consisted of DOX, cyclophosphamide and paclitaxel. Non-anthracycline based therapy consisted of cyclophosphamide, methotrexate and 5-fluorouracil.	Neuropsychological testing. MRI scanning.	The anthracycline group had considerably lower verbal memory and left precuneus connectivity than those who received non-anthracycline regimens and the non-chemotherapy group.	(169)
22 female breast cancer patients. 22 healthy controls.	Patients received cyclophosphamide, DOX and taxotere.	Neuropsychological testing. MRI scanning.	Breast cancer patients had lower connectivity in the medial temporal lobe and dorsal medial PFC when compared to the healthy controls.	(170)
15 cancer patients receiving chemotherapy. 14 age-matched healthy controls.	Not described.	MRI scanning after 3 cycles have been completed.	Patients had lower PFC activation when compared to the control group.	(171)
Breast cancer patients treated with chemotherapy (n = 28) or without (n = 34). Healthy controls (n = 30).	Most patients received a combination of DOX, cyclophosphamide, and paclitaxel.	fMRI scanning and verbal working memory task were performed before the start of the treatment (T1), 5 months after (T2) and approximately 1 year after the first test (T3).	The chemotherapy-treated group showed impaired verbal working memory task aggravated from T1 to T3 and the fMRI showed changes in the network connectivity during the task when comparing to the non-chemotherapy group and healthy controls.	(172)

Breast cancer patients received chemotherapy (n = 28) or not (n = 24). Healthy controls (n = 31).	Patients that received chemotherapy had an anthracycline-based therapy.	fMRI scanning while performing executive functioning task and episodic memory task. The scans were done before and 6 months after the end of the treatments.	No changes were seen in the execution of the tasks among the groups at baseline and after the treatment. However, the chemotherapy-group had increased parietal activation compared to baseline results.	(173)
19 patients received chemotherapy. 11 healthy controls.	Patients received combinations of 5-fluorouracil, cyclophosphamide, epirubicin and paclitaxel.	Resting-State fMRI, diffusion tensor imaging and neuropsychological testing before the treatment (T1) and after the treatment (T2).	Chemotherapy-treated group performed poorly in the memory test when comparing T1 time-point with controls. Regarding the neuroimaging scans, at T1 there were no differences between the two groups. After treatment, the patients had significant alterations in some brain areas when comparing to T1 but after normalizing for anxiety and depression scores the changes were no longer meaningful.	(174)
33 breast cancer patients. 31 healthy controls.	Patients received cycles of DOX, paclitaxel, and cyclophosphamide without any hormonal treatment.	Resting-state fMRI. Neuropsychological testing.	The chemotherapy-group had lower functional connectivity.	(175)
19 women that had breast cancer and received chemotherapy less than 6 months before assessment started. 20 healthy control women.	Patients received cycles of taxotere and epirubicin.	Diffusion MRI scanning. Neuropsychological testing.	The chemotherapy group had lower white matter integrity. Moreover, the brain network integration also deteriorated in the patients group.	(176)
28 breast cancer patients. 29 matched healthy controls.	Patients received cycles of DOX and paclitaxel.	MRI scanning was performed after each cycle of chemotherapy.	The grey matter density decreased in the chemotherapy group comparing with the control group.	(177)
Breast cancer patients received chemotherapy (n = 26) or not (n = 23). Healthy controls (n = 30).	Patients received combinations of DOX, cyclophosphamide, epirubicin, docetaxel, paclitaxel, and 5-fluorouracil.	MRI scanning and neuropsychological testing before the start of chemotherapy (T1) and at 6 months follow-up (T2).	At T2, the chemotherapy group had subtle but significant deterioration in white matter integrity when comparing to the patients that did not receive chemotherapy and to healthy controls.	(178)

122 women with early stage breast cancer that received chemotherapy (n = 72) or not (n = 49). 59 healthy controls.	Women received cycles of 5-fluorouracil, epirubicin and cyclophosphamide with or without docetaxel.	Neuropsychological testing and assessment of volume changes through VBM before the start of the treatment (T1) and after the end of the treatment (T2).	At T1 assessment, there were no differences among groups. In T2, chemotherapy-treated patients performed worse on attention/concentration, memory and processing speed. In opposition, the other groups improved their scores in the testing done. In the younger patients (<52 years old) treated with chemotherapy, the VBM results showed widespread volume alterations when comparing to the other groups.	(179)
19 women with breast cancer and over the age of 60. 14 age-matched healthy controls.	Most chemotherapy-treated patients received docetaxel in combination with DOX or cyclophosphamide.	Neuropsychological testing and DTI before treatment (T1) and one month after chemotherapy (T2).	In T1, both groups performed similarly in the neuropsychological testing. At T2, the chemotherapy group performed worst only in the oral recognition test. However, from T1 to T2, the chemotherapy group had significant white matter alterations when compared to the control group.	(180)
13 breast cancer patients received chemotherapy. 13 matched healthy controls.	Patients received cycles of DOX, cyclophosphamide and taxol or cyclophosphamide, methotrexate, and 5-fluorouracil.	fMRI scanning during memory encoding and retrieval before and after chemotherapy.	After chemotherapy, patients had diminished activity in posterior brain regions, especially those involved in visual processing when compared to the control group.	(181)
19 breast cancer patients that received chemotherapy within 6 months of the study. 20 matched healthy controls.	Patients received docetaxel and epirubicin.	MRI scanning and neuropsychological testing.	Patients had alterations in the frontoparietal lobe and occipital lobe when compared to the healthy controls.	(182)
28 female patients that received chemotherapy for breast cancer. 22 healthy women.	Not described.	Neuroimaging techniques to evaluate the striatal dopamine transporter binding ratio.	Chemotherapy-treated patients had significantly dopaminergic decrease in all the dorsal striatum when comparing with the healthy controls.	(183)
20 patients with gynecological cancer. 20 healthy controls.	Chemotherapy regimens included paclitaxel, carboplatin, cisplatin, DOX, bleomycin, and methotrexate.	Neuropsychological testing and MRI scanning before the start of the treatment (T1) and after treatment end (T2).	In T1, besides psychomotor speed, the groups did not present significant differences. However, at T2, the chemotherapy group performed significantly worst in the domains of verbal memory, immediate and delayed recall and information processing speed.	(184)

ApoE - Apolipoprotein E; DTI - Diffusion tensor imaging; EEG - Electroencephalogram; ERP - Event related potentials; fMRI - Functional magnetic resonance imaging; MMSE - Mini mental state examination; MRI - Magnetic resonance imaging; PET - Positron emission tomography; PFC - Prefrontal cortex; TNF- α - Tumor necrosis factor alpha; VBM - Voxel-based morphometry.

Electrophysiology studies or brain function assays can be diverse and expensive; however, they are an objective way, without interferences of practice (or education factor) or evaluator experience, to assess neurotoxicity. Nevertheless, earlier studies on chemobrain using these techniques suffered the same problems of grouping, namely controls, or even low number of subjects when compared to cognitive testing. In 1992, Siddiqui and colleagues (185) made the first EEG study in cancer treated-patients to assess the baseline cognition before the treatment. They evaluated patients that did not have brain metastasis nor presented signs of dementia or other neurologic problems and used an electroencephalographer to measure the ERP P300. The P300 is a positive component of the event-related potential that peaks at 300 ms or more after a stimulus and integrates cognitive domains such as attention and working memory. A change on these cognitive domains leads to a latency in the potential registered (186). The study aimed to detect subclinical cognitive defects in cancer patients and in the 16 patients analyzed, 9 presented abnormalities in the P300, while the 28 healthy controls had no abnormalities (185). The authors acknowledged the P300 test as a feasible method to measure subclinical cognitive impairments in cancer patients.

The need to use other techniques able to identify specific neural changes, namely brain imaging techniques, as to understand the basis of chemotherapy-induced cognitive dysfunction is crucial. Five to 10 years' post-treatment were analyzed using PET scans. The chemotherapy-treated group had hypometabolism in the inferior frontal cortex during the cognitive test. On the other hand, the non-chemotherapy group had higher cortical activation in the parietal cortex and occipital cortex while performing the same test. Thus, chemotherapy exposure could alter cortical activation while performing a short-term memory recall (151). In another study and after chemotherapy (on average 4 months after), the treated patients had significantly reduced global cortical glycolysis (167).

To further assess metabolic differences due to chemotherapy exposure later in life, Ponto *et al.* compared the PET scans of 10 breast cancer survivors (more than 10 years after treatment) with 10 age-matched healthy controls. The survivors were older than 65 years at the time of the evaluation and had breast cancer in their mid-life. They had been treated with standard multi-agent therapy that included cyclophosphamide, methotrexate, 5-fluorouracil, and DOX. When comparing with healthy controls, the cancer survivors treated with chemotherapy had hypometabolism in the bilateral orbital frontal gyri, in the basal ganglia (right substantia nigra), brainstem, left postcentral gyrus of the parietal lobe and corpus callosum (187).

Moreover, the large portion of clinical neuroimaging studies on chemobrain has used MRI-and fMRI-based techniques. De Ruiter and colleagues used fMRI and investigated the effects of chemotherapy on the brain of breast cancer survivors more than 10 years. The results showed that chemotherapy, even more than 10 years after drug treatment, had hyporesponsiveness of the dorsolateral prefrontal cortex (PFC) and parahippocampal gyrus while performing the tower of London test and the test of episodic memory, respectively, when comparing with the non-chemotherapy group (188).

In order to determine white matter integrity after chemotherapy, Deprez *et al.* performed DTI (an MRI-based neuroimaging technique) in 17 breast cancer patients, approximately 3 months' after treatment (3 to 6 cycles of 5-fluorouracil, epirubicin and cyclophosphamide with or without taxol). They also examined 18 age-matched healthy controls and 10 breast cancer patients only treated with radiotherapy or hormonal therapy. The DTI revealed that chemotherapy-treated patients had decreased frontal and temporal white matter integrity when comparing with healthy controls and non-chemotherapy treated patients (152). Furthermore, the authors verified that the loss white matter integrity in the temporal and parietal areas correlated with poor outcomes in neuropsychological tests done in these patients to assess the domains of attention and processing/ psychomotor speed (152).

The white matter microstructure alterations were also detected in older women (more than 60 years old) that underwent chemotherapy. Nineteen women with breast cancer got MRI scanning with DTI before the start of chemotherapy and one month after chemotherapy and were compared with 14 age-matched healthy controls. The chemotherapy regimen for most women consisted of docetaxel and cyclophosphamide. At the baseline, the groups had no differences regarding the DTI parameters, but, after chemotherapy, the cancer patients had significant alterations in the genu of corpus callosum and left superior longitudinal fasciculus (180).

Chemotherapy damages to the white matter structure and its connectivity might also cause grey matter dysfunction in critical brain areas and thus leading to cognitive dysfunction (189). Decrease grey matter volume in chemotherapy treated patients was reported by Lepage *et al.* when evaluating 19 female breast cancer survivors compared to 19 individually age-matched healthy controls, after 5 or 6 cycles of 5-fluorouracil, epirubicin and cyclophosphamide. At one-year examination, partial recovery happened in some areas, but the frontal and temporal regions were still affected when compared to baseline (164). The grey matter volume and proper function of the hippocampus have also been evaluated in some studies. This area is of particular importance, since, as previously mentioned,

neurogenesis occurs here in the adult brain, which is reported to have an important role in memory and learning (160). The MRI analysis showed that breast cancer patients (most patients received DOX and cyclophosphamide) had significantly reduced left hippocampal volume when comparing to the healthy controls and that the right hippocampal volume was slightly decreased ($p = 0.07$) when comparing to healthy controls (160). Apple *et al* using MRI in 16 breast cancer patients, 18 months' post-chemotherapy (anthracycline, taxane and cyclophosphamide) but still taking hormonal therapy (tamoxifen) found average hippocampal volume reduction in the breast cancer group when comparing to the healthy controls. Moreover, they also measured hippocampal deformation and the right hippocampus of breast cancer survivors was significantly inwardly deformed when comparing to the control group (190).

On a structural level, chemotherapy affects both the white and grey matter of the brain (164, 191). These changes overall will affect core processes of the brain and the default network mode (DNM) connectivity has been reported to be decreased in chemotherapy-treated patients. In a sample of nine women with breast cancer (treated with cyclophosphamide being that eight also received taxane, while another four received an anthracycline and cyclophosphamide), fMRI scanning was performed before the start of the chemotherapy, a month, and a year after the completion of chemotherapy treatments. From the baseline evaluation to the 1-year following chemotherapy time-point a decrease connectivity in the DMN was observed in chemotherapy treated women (159). In addition, Miao *et al.* examined 22 breast cancer patients (cyclophosphamide, DOX and taxotere), 1 month prior to the fMRI scan found that breast cancer patients had lower connectivity in the medial temporal lobe and dorsal medial PFC when comparing to the healthy controls, thus, indicating inefficient functional integration of the DMN, 1 month after treatment (170).

In summary, electrophysiological and neuroimaging studies provided evidence for the functional and structural damages evoked by chemotherapy, being even more sensitive and by far more objective than cognitive studies and done very often after longer periods post-treatment. Moreover, more distinctive chemotherapy regimens were done with these methods comprising drugs other than anthracyclines. Nevertheless, fewer patients per study were evaluated, and breast cancer and women were more represented in the studies. In summary, chemotherapy causes brain abnormalities in the values of P300, reduced metabolism in certain areas, alterations in the white matter microstructure and integrity, reduction of the grey matter volume, especially in the frontal region, and alters the brain networks. Furthermore, in several studies, those changes are reported to remain years after chemotherapy. These data substantiate the real and significant effect of chemotherapy on the brain and/or its function.

1.4. Animal studies

Even though the number of clinical studies correlating the use of systemic therapy with cognitive dysfunction in cancer patients has steadily increased in recent years, most of them fail to provide biological mechanisms by which chemotherapy affects the brain. Some inherent methodological issues such as small sample sizes, discrepancies in patient characteristics (age, education, stress and anxiety), type of tumor and location, and the combination of anticancer treatments (e.g. hormonal and radiotherapy) can interfere with an objective assessment of the effects of chemotherapy on the brain. Moreover, invasive techniques on patients without clinical purpose are obviously not ethical. To overcome some of these issues, animal studies arose as a valid means to study chemobrain in a controlled environment and assess the underlying physiological mechanisms involved in cognitive decline due to systemic chemotherapy (192), even if species differences could obscure some results.

As it happens in cancer patients, the rodents display cognitive impairment following systematic chemotherapy treatment (193, 194). Moreover, there are wide varieties of behavioral tests that can evaluate the effects of chemotherapy on rodents and determine what cognitive domains are impaired. Other than behavioral tests, in these studies the underlying cell-biological and molecular mechanisms affected by chemotherapy are researched (195). The use of laboratory animals also allows evaluating the effects of commonly used drugs either as chemotherapeutic combination regimens or as single chemotherapeutic agents and to understand potential differential effects in male and female brain. All of these aspects are difficult or even impossible to assess in a cohort of cancer patients because of lower number of enrolled patients (196) or even gender specific or gender prone cancers or obvious ethic reasons.

It is irrefutable that the most widely studied chemotherapeutic agent is DOX in human reports and it has been also the most addressed on animal studies. Furthermore, as it is one of the drugs of study in this dissertation, it will be more deeply addressed. Male adult rats (2 to 3 months old) received intraperitoneal (i.p.) single doses of 0.5, 2 or 8 mg/kg of DOX and underwent behavioral tasks 180 minutes, 24 hours, and 7 days after the drug administration. The results were compared to the control group that only received a saline solution, to which the same procedure was given. At the 180 minutes time-point, there were no differences between groups, while at 24 hours and 7 days, only the two higher doses caused impaired memory retention. These results suggest a dose-dependent neurotoxic response caused by DOX (193). For systematic reasons, a table with dosage, animal species and strain, and also main results regarding putative neurotoxicity of DOX drug

treatment will be presented next (Table 3), similar to what was observed in clinical studies. At the moment that this thesis is being written and using the best knowledge available, no animal studies evaluating the neurotoxicity of systemic use of MTX have been identified.

Table 3: Animal studies revealing DOX-induced neurotoxicity. Cytostatic drug used, animal species and strain, and major results regarding neurotoxicity are highlighted.

Experimental setting	Animals	Major results found in the drug-treated animals	Reference
0.5, 2 and 8 mg/kg i.p. Animals were tested 3 hours, or 1 or 7 days after administration.	Adult male Wistar rats	Impaired learning capacity and long-term memory and sparing short-term memory.	(193)
1.5 and 3 mg/kg i.p. Animals were tested 3 or 24 hours after administration.	Adult male Wistar rats	Inhibition of the dopamine uptake system in the frontal cortex, hippocampus, and striatum.	(197)
25 mg/kg i.p. Animals were sacrificed within 24 hours after treatment.	Male B6C3F1J mice	Slower uptake of glutamate in the frontal cortex and DG.	(198)
25 mg/kg i.p. Animals were sacrificed 72 hours after treatment.	Male B6C3 mice	Higher levels of protein carbonyls and protein-bound 4-hydroxynonenal in the brain and plasma; decreased locomotor activity; decreased neuronal integrity in the hippocampus.	(199)

DG - dentate gyrus i.p. - intraperitoneal

Other studies were done and are summarized next. DOX is a well-characterized inducer of oxidative stress. Moreover, the putative involvement of oxidative stress on DOX toxicity has been extensively studied in other organs, in particular in the heart and that has been the starting point for the studies evaluating DOX-induced neurotoxicity (15, 200). To investigate if DOX caused oxidative stress in the brain, Joshi *et al.* divided male mice (2 to 3 months old) into 4 groups. Two groups received saline and the 2 other groups received 150 mg/kg of γ -glutamyl cysteine ethyl ester (GCEE) and one mouse from each group received a single i.p. dose of 20 mg/kg of DOX, 4 hours after saline or GCEE injection. GCEE is a precursor for glutathione (GSH) synthesis, which is an important nonprotein thiol antioxidant. Seventy-two hours after DOX administration, the levels of GSH, protein carbonyls, 3-nitrotyrosine (biomarker of protein oxidation), and protein-bound 4-hydroxynonenal (a product of lipid peroxidation) were determined on the brain. The mice that only received DOX had lower levels of GSH when compared to the saline controls or the mice that only received GCEE, meanwhile co-administration of GCEE partially restored

the levels of GSH on the DOX + GCEE group. The administration of DOX *per se* caused elevation of protein carbonyl, 3-nitrotyrosine and protein-bound 4-hydroxynonenal when comparing to the saline control and GCEE-administrated mice. Importantly, GCEE partially avoided the effects of DOX, on the brain levels of GSH, of protein carbonyl, 3-nitrotyrosine and protein-bound 4-hydroxynonenal (201). Therefore, the authors found that DOX induces oxidative stress on the brain, dependent on GSH. These claims were also supported by the study of Keeney *et al.*, where they searched on brain's and plasma of male mice (2 to 3 months old) biomarkers of oxidative stress. The mice were injected with a single i.p. dose of 25 mg/kg of DOX and equivalent volume of saline in control mice. Seventy-two hours after administration, DOX-treated mice had increased protein-carbonyl and protein-bound 4-hydroxynonenal levels in the brain and in the plasma (199).

Nonetheless, the question regarding DOX induced oxidative stress is placed: how can DOX induce stress oxidative on the brain? The possible entrance of DOX into the brain was considered. In fact, *post-mortem* studies in cancer patients treated *ante-mortem* with DOX and in 2 out of 5 five patients, DOX and its metabolite doxorubicinol were present in their brain (202). Moreover, in a study where male adult rats were pre-treated with morphine (10 mg/kg, three times over 24 hours) and received DOX (12 mg/kg single i.p.) brain accumulation of DOX occurred, while in rats that only received DOX, it was not detectable on the brain. In fact, morphine causes BBB loosening and DOX would access the brain in those conditions (203). Nevertheless, the current data advocates that DOX in an intact BBB is not able to reach the brain in significant amount, (204, 205), therefore its evoked neurotoxicity could be indirect.

Still some studies were conducted on redox pathways. Pal and colleagues administrated mice with a single i.p. injection of 25 mg/kg DOX and 7 days after the administration the mice were euthanized. DOX administration increased brain lipid and protein oxidation, as well as reactive oxygen species (ROS) levels when comparing to the saline control group. Moreover, the brain activities of antioxidant enzymes such as SOD, glutathione S-transferase (GST), glutathione reductase, and glutathione peroxidase (GPx) were decreased when comparing to the control group, further contributing for the increase in the levels of oxidative stress (206). In male rats administrated with a total cumulative dose of 2.5 mg/kg given through 10 i.p. injections, every 5 days, it was observed increased levels of malondialdehyde (marker of lipid peroxidation) and nitrite in the frontal cortex and hippocampus in comparison to the vehicle control. The DOX-treated group also presented more than 50% of increase in myeloperoxidase in both hippocampus and frontal cortex when comparing to the control group (207).

DOX redox cycle generates reactive oxygen and nitrogen species (RONS) as stated. Tangpong and co-workers studied the role of nitric oxide radical ($\bullet\text{NO}$). $\bullet\text{NO}$ is a reactive specie, which plays an important role in some physiological and pathological conditions. NO is synthesized from L-arginine by two different enzymatic pathways: the tightly regulated nitric oxide synthase (NOS) pathway that is mainly involved in blood flow and pressure; the other pathway is by the cytokine-inducible form of nitric oxide synthase (iNOS). In the CNS, microglia and astrocytes can generate $\bullet\text{NO}$ from iNOS activation. The radical $\bullet\text{NO}$ can rapidly react with superoxide radicals and form peroxynitrite, a potent oxidant that causes tissue injury and pro-inflammatory responses. Moreover, this downstream of events can culminate in the inhibition of mitochondrial electron transport and inactivation of iron-sulfur containing enzymes (208). In the mitochondria, the manganese-containing superoxide dismutase (MnSOD), a crucial primary antioxidant defense, can be nitrated by peroxynitrite and lose its enzymatic activity. Eight-week-old male mice received a single i.p. dose of 20 mg/kg of DOX and the authors observed that DOX administration caused elevation of circulating tumor necrosis factor alpha (TNF- α) levels in both the wild type and iNOS knockout model, when comparing with the respective saline controls. The brain expression of iNOS was more elevated in the wild type DOX-treated mice when comparing with the wild type saline control. The brain mitochondrial function was measured through the respiration control ratio, and in the wild type mice treated with DOX, the value was significantly lower when comparing to the wild type control, whereas in the drug-treated knockout mice no differences were seen when comparing to both control groups. Moreover, the brain levels of nitrated/ inactive MnSOD were considerably high in the wild type DOX-treated mice when comparing with both the saline controls and the drug-treated knockout mice (209).

To further research DOX-induced neurotoxicity and its link to TNF- α levels, Ren and colleagues used male TNF- α knockout and wild type mice (both 2 to 3 months old). The mice received a single i.p. injection of 25 mg/kg DOX or a saline solution and 72 hours after the administration, the animals were sacrificed and biomarkers of oxidative stress and proper mitochondrial function were evaluated (210). Regarding the results, protein carbonyls and protein-bound 4-hydroxynonenal levels were significantly increased in the plasma of both the TNF- α knockout and wild type mice administrated with DOX when comparing with their respective saline control. However, when analyzing the brain protein carbonyls and protein-bound 4-hydroxynonenal levels, the DOX-treated TNF- α knockout mice had similar values as TNF- α knockout saline controls while the DOX-treated wild type had increased brain levels of both biomarkers when compared to the wild type saline controls, confirming that TNF- α is a mediator for brain protein and lipid oxidation. To access

mitochondrial function, authors measured oxygen consumption rate in mitochondria isolated from the total brain and observed that DOX-treated wild type mice had even reduced rate of oxygen consumption when compared to the wild type saline control. In DOX-treated TNF- α knockout mice, no significant differences were seen when comparing to both controls [wild type and the knockout (210)].

In another study, rats administrated with a total cumulative dose of 100 mg/kg DOX i.p. given every day for 7 days and then sacrificed 4 weeks later displayed cognitive impairment as seen by the Morris water maze and step-down avoidance tests. Moreover, the animals also presented decreased in hippocampal neurogenesis and increase neuronal apoptosis. It was also observed decreased levels of brain derived neurotrophic factor (BDNF) that is responsible the maintenance of mature neurons, decreased levels of tropomyosin-related kinase B (TrkB) to which BDNF binds to exert anti-apoptotic effects. Furthermore, the hippocampus also had diminished Ca^{2+} retention, increase mitochondria permeability transition pore and hydrogen peroxide (H_2O_2) emission. DOX administration impaired cognitive function, neuroplasticity, and mitochondrial function (211).

One study that addresses fairly well all these issues was performed by Tangpong and colleagues that investigated the role of the pro-inflammatory cytokine TNF- α in DOX-induced neurotoxicity. Moreover, the authors also determined if DOX was found in the brain areas protected by the BBB through fluorescence. Eight-week-old male mice received a single i.p. dose of 20 mg/kg of DOX. DOX was not detected in areas protected by the BBB, such as the cortex and hippocampus, but the drug was detected in the choroid plexus that is protected by the more permeable blood-cerebrospinal fluid barrier (205). Concerning TNF- α levels, both the circulating and brain levels were significantly higher in the drug-treated group when compared to the saline control group. In addition, in DOX-treated mice, TNF- α was particularly concentrated in the cortex and hippocampus when comparing with the saline controls. The authors also observed that DOX administration caused brain mitochondrial dysfunction, mainly decreased complex I activity and mitochondrial release of cytochrome c, increased caspase-3 activation, and cell death. Moreover, results showed that p53 and Bax expression increased significantly 3 hours after administration, whereas B-cell lymphoma-extra-large (Bcl-xL) increased significantly 6 hours after treatment, when comparing to the saline controls. Mitochondrial membrane permeabilization (via p53 pathways either by forming complexes with Bcl-xL or by via regulation of Bax) and cytochrome c release triggered increasing in apoptotic cell death in cortical and hippocampal regions of the brain. Nonetheless, when circulating TNF- α was neutralized through the administration of anti-mouse TNF- α antibodies, the levels of brain TNF- α

decreased and the mitochondrial injury was prevented. This indicates that TNF- α plays an important role mediating the DOX neurotoxicity (205).

Taking this into account, Aluise and colleagues investigated the possible link between DOX-induced peripheral oxidative stress and peripheral elevation of TNF- α . For this study, male mice received a single i.p. dose of 25 mg/kg DOX, and the results showed that DOX-administration increased plasmatic protein carbonyls, protein-bound 4-hydroxy-2-nonenal levels and peripheral levels of TNF- α when comparing to the saline control group. Using redox proteomics, the authors identified an increase in the specific protein carbonyl level of plasma apolipoprotein A1 (ApoA1) in DOX-treated mice. Furthermore, the authors also demonstrated that DOX-induced oxidation of ApoA1 compromises its function and when oxidized, this protein loses the function of suppressing TNF- α increase in the periphery, which can pass the BBB and activate centrally that cytokine formation (212), which, on the other hand favors oxidative stress. Actually, on chemotherapy-treated patients circulating pro-inflammatory cytokines increase was registered also (213) and they can reach the brain and activate the microglia to produce other pro-inflammatory cytokines and other inflammatory mediators harmful to the brain (214). Based on the studies presented previously, the possible mechanisms of DOX-induced neurotoxicity are presented in Figure 2.

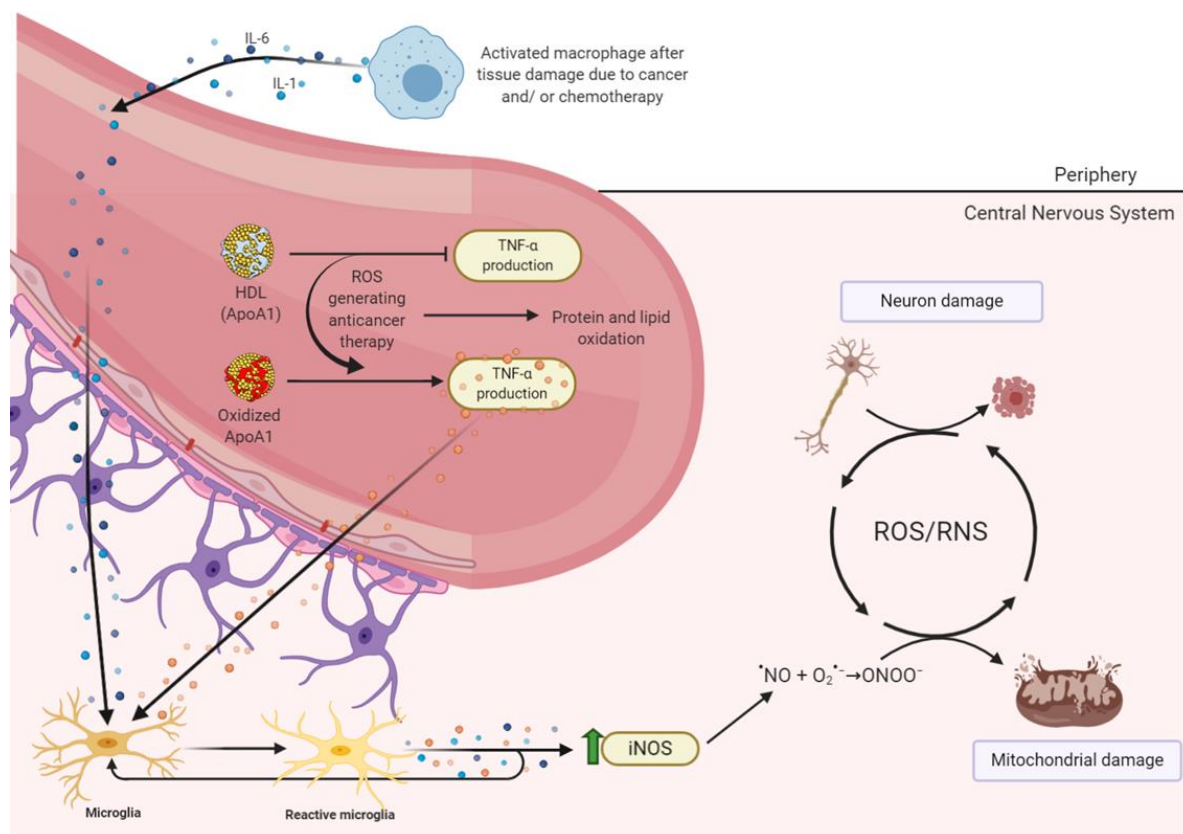


Figure 2: Schematic illustration of the sequence of events in the mice brain after DOX administration. The semi-quinones generate a redox cycle that leads to oxidation of the high-density lipoprotein (HDL) ApoA1 and lipids that consequently causes an increase in the levels of plasmatic TNF- α . Moreover, DOX can also activate macrophages due to tissue damage. The produced cytokines can readily cross the BBB and induce damage by itself or it can activate the microglia to produce other cytokines. Moreover, TNF- α leads to $^*NO$ production by the iNOS activation in the microglia that increases the oxidative stress in the brain and mitochondrial dysfunction. The interplay between them causes neuron damage/death.

Oxidative stress and neuronal damage have been the most studied aspects on *in vivo* settings, being that neuro-inflammation can be the common link between several anticancer drugs. In fact, several clinical studies have (161, 215) shown increased cytokine levels, thus showing the clinical relevance and clinical translation of animal studies. Nevertheless, the anticancer therapies usually use combination regimens and those are still under evaluated and the combination of several cytostatic agents might exacerbate the neurotoxic actions of a single drug.

1.5. *In vitro* studies

In vitro neuronal models have been extensively used in the field of neurotoxicology since they allow a better understanding of the molecular and cellular mechanisms by which xenobiotics affect the different cell types that compose the brain. There are several models available with different complexities, ranging from subcellular systems, single cell types to systems that try to maintain the tissue structure and function, (e.g. brain layers). The most *in vitro* suited model depends on the hypothesis being tested and the previous knowledge of the pharmacokinetics and pharmacodynamics of the compound being studied (216). Moreover, the use of *in vitro* models allows for a reduction in the number of animals used and elimination of influence of several factors, namely hormonal or other.

Almeida and co-workers compared the neurotoxicity of DOX and MTX (0.13, 0.2, and 0.5 μ M) in the SH-SY5Y human neuronal cell model. The authors observed that, despite both chemotherapeutics agents causing significant cytotoxicity, MTX was more neurotoxic than DOX in differentiated SH-SY5Y cells, while in undifferentiated (and therefore more proliferative) SH-SY5Y cells, DOX was more neurotoxic than MTX, in the same experimental conditions. However, both DOX and MTX caused a decrease in the transmembrane mitochondrial potential following a 48-hour exposure in the lowest concentration. The authors also sought to evaluate the neuroprotective effect of some compounds and observed that tiron (100 μ M), an antioxidant was the only drug among those tested capable of partially avoid the toxicity of MTX. On the other hand, cycloheximide (10 nM), a protein synthesis inhibitor, and Ac-DEVD-CHO (100 μ M), an inhibitor of caspase-3/7, were able to partially protect against DOX-induced neurotoxicity and unexpectedly, the antioxidant tiron and the GSH precursor N-acetylcysteine did not provide any neuroprotection. The higher neurotoxicity of MTX in differentiated SH-SY5Y could be due to its greater lipophilicity, as it most easily accumulates inside the cells (217). Its higher neurotoxicity when compared to DOX is of concern as its neurotoxic effects have been poorly studied over the years and the BBB is impaired in multiple sclerosis patients (218).

In fact, due to the extensive use of MTX in multiple sclerosis patients, Li *et al.* evaluated the immunomodulatory effects of MTX in murine BV2 activated microglial cells (219). The results demonstrated that cell viability decreased in doses exceeding 2ng/ml in a dose-dependent manner and cell death occurred by late apoptosis or even necrosis. Moreover, MTX altered the cytokine network by increasing the production of IL-10 (anti-inflammatory) cytokine and decreased the secretion of IL-23p19 (proinflammatory cytokine) in activated microglial cells (219). Another study used primary mice astrocytes cultures and observed that MTX decreased lipopolysaccharide-induced production of $\bullet$ NO, TNF- α , IL-1 β

and monocyte chemoattractant protein-1 (MCP-1) in astrocytes. Furthermore, MTX also inhibit the IL-12 and IL-13 production by astrocytes and decrease expression of C-reactive protein, while inducing nuclear factor kappa B (NF- κ B) DNA binding activity (220). Nevertheless, the data presented reports to models of multiple sclerosis, where the BBB integrity might be compromised (218), and so far, data regarding MTX neurotoxicity in other models is scarce and is crucial for other conclusions.

Regarding DOX, a study by Lopes and colleagues evaluated its neurotoxicity in rat cortical neurons exposed to concentrations ranging from 0.01 to 20 μ M, for 24 or 48 hours. The obtained results showed that DOX induced neuronal cell death in differential ways, depending on the concentration, being that concentrations up to 0.5 μ M of DOX, neuronal death is due to apoptosis while at higher concentrations, necrosis becomes prevalent (221). The exposure of rat cortical primary cell cultures to DOX resulted in a significant generation of ROS/RONS, activation of NF- κ B, depletion of GSH and ATP, and cell death, in a concentration biphasic manner. Death in the lower concentration was prevented using a NOS pharmacological inhibitor. DOX also significantly increased protein-bound quinone products in neurons in a concentration-dependent manner (222), demonstrating local oxidative stress.

The emerging high-throughput technologies have also been used to study the phenomena of chemotherapy-induced cognitive dysfunction. Seo and colleagues evaluated the changes on transcriptome of human T98G neuroglial cells exposed to the combination of 5-fluorouracil (50 μ g/ml), epirubicin (0.5 μ g/ml), and cyclophosphamide (2 μ g/ml), commonly used in the treatment of breast cancer (223). The results obtained showed that this anticancer combination deregulated 67 genes involved in neuronal development, 37 genes involved in development of the sensory system, 12 genes in extension of axons and 3 genes in migration of neurons. Moreover, this combination downregulated the expression of integrins subunits β 1 and α V and biosynthesis cell adhesion molecule 1, which are essential for development and maintenance of synaptic structure plasticity and synaptic long-term potential (224).

In vitro methods can be a more simplistic approach when comparing to animal models, but they allow a better understanding of the intracellular pathways impaired due to chemotherapy exposure.

1.6. Possible mechanisms of chemobrain

1.6.1. Direct passage of chemotherapeutic agents through the blood-brain barrier

As stated before, the BBB represents a first line of defense of the brain. The endothelial cells linked by tight junctions, the lack of fenestrations and low pinocytic capacity do not allow for intercellular passage of molecules (192). The transport of compounds and nutrients essential for proper brain function is assured by selective uptake and efflux transport mechanisms. Nevertheless, compounds without endogenous selective transporter can only enter the brain by passive diffusion, therefore, the ability of a compound to cross the BBB is established by their lipid solubility, molecular weight (less than 400-500 daltons), degree of ionization, plasma protein and tissue binding, and local cerebral flow (225). Regardless, many compounds are unable to enter the CNS because they are substrates of the efflux ATP-binding cassette transporters of the multidrug resistance protein family in the BBB, in which the P-glycoprotein and the breast cancer resistance protein (BCRP) are included and most prevalent (226).

DOX is a water-soluble molecule with the molecular weight of 580 g/mol (227). Moreover, DOX is a substrate of the efflux pump P-glycoprotein, that significantly restricts its entrance (228). It is assumed that DOX could not cross the BBB, as it was not detected after systemic administration (205). However, as referred previously, post-mortem analysis of human brains detected the presence of DOX (202). *Postmortem* distribution has not been described from the deep tissues where DOX accumulates or even studies on cancer patients to assess the BBB integrity on treatment. Despite this, DOX neurotoxicity caused by direct entrance of the molecule seems highly unlikely due to its physical properties and because it is a substrate of P-glycoprotein.

MTX has a molecular weight of 444.5 g/mol, is sparingly soluble in water and it is a substrate of the efflux pump BCRP, that considerably limits the tissue penetration of this molecule (229). Therefore, with an intact BBB it is not expected that MTX gains access to the brain (230). To ascertain the ability of MTX to enter cerebral tissue, Green and colleagues administrated in a cohort of 10 patients 5 to 6 mg/m² MTX (i.v.) before surgical resection of intracerebral tumor and brain tumor tissue was collected. Despite the difficulty in accessing the integrity of the BBB in brain tumor cases, which might be damaged related to the disease, the authors detected MTX in all tumor samples in concentrations ranging from 4 to 322 ng/g, being higher than the plasmatic levels at the same time-point (231). In another study, autopsy tissue of 11 patients who received a total cumulative life-time dose varying

from 6 to 100 mg/m² MTX (i.v.) 10 to 272 days *antemortem* was analyzed. The results also demonstrated higher levels of MTX in brain tumor, but MTX was also detected in the cerebrum and cerebellum (232). Due to high use of MTX in multiple sclerosis patients, where one of the hallmarks of this disease is the loss of BBB integrity (218), it is important to access how MTX can reach the brain. Nevertheless, when the BBB integrity is maintained, it seems unlikely that MTX can enter the brain (233).

Nonetheless, the BBB integrity can be compromised after chemotherapy. A study in rat brain endothelial cell line to access integrity of the BBB after treatment with a ROS-generating agent (oxaliplatin), observed dysregulation of the expression of tight junction proteins zonula occludens-1 and F-actin, causing the disruption of the monolayer and pore-formation, making that barrier no longer impermeable to the entrance of the compound. The ROS formation in tight junction proteins caused significant increase of intracellular ROS, which increased endoplasmic reticulum stress and activation of caspase-3 (234). The ROS formation leads to the loosening of BBB and could cause further damage on the brain of chemotherapy-treated patients, increasing its permeability. As stated before, morphine can increase BBB permeability and allow the entrance of xenobiotics that would be otherwise halted (203). The use of combination chemotherapy or auxiliary pain treatments can in fact change the BBB role on the entrance of both MTX and DOX,

1.6.2. Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced mostly by activated macrophages but can also be produced by monocytes or by nonimmune cells such as fibroblasts and endothelial cells (235) and prompt the organism inflammatory response in complex manners that are interlinked. In fact, pro-inflammatory cytokines control the induction, production, stimulation, and inhibition of other cytokines. Some pro-inflammatory cytokines are IL-1 β , IL-6 and TNF- α (236). These molecules can easily cross the BBB by simple diffusion (TNF- α), saturable influx transport or retrograde axonal transport system (IL-1 α , IL-6 and TNF- α) or gain access to the brain through the circumventricular organs, where the barrier is more permeable (237). In the brain, the pro-inflammatory cytokines can activate the microglia that will exacerbate the production of pro-inflammatory cytokines *in loco*. The microglia functions as brain macrophages, being the primary source of pro-inflammatory cytokines and pivotal in the mediation of neuro-inflammation (238). Nevertheless, to some extent astrocytes and neurons can also produce some pro-inflammatory cytokines (239). Moreover, even without entering the brain, the pro-inflammatory cytokines can damage the BBB by activating and

destroying tight junction of microvascular endothelial cells of the BBB and increasing its permeability (237).

Cytokines also have significant impact on brain function. Interferon- α can increase the levels of IL-1 α , IL-6 and TNF- α , and high levels of these pro-inflammatory cytokines are associated with impairments of memory, fine motor skills, executive functions, and mood that indicate frontal-subcortical defects (240). In addition, microglia activation and pro-inflammatory cytokines have been linked to several neurodegenerative diseases (241). Considering this, the increase in pro-inflammatory cytokines due to chemotherapeutic agents has been pointed out as possible mechanism for the cognitive changes seen in chemotherapy-treated patients (Table 1 and 2). Actually, in 42 chemotherapy-treated (DOX, cyclophosphamide, and paclitaxel) breast cancer patients, on average 4 years after the end of chemotherapy, and 35 age- and education-matched healthy controls, the serum levels of pro-inflammatory cytokines and hippocampal volume were measured, and cognitive tests were performed. The results revealed that chemotherapy-treated patients had higher serum levels of IL-6 and TNF- α when comparing to the control group. Moreover, in the chemotherapy group, higher levels of TNF- α were associated with reduced left hippocampal volume and the levels of IL-6 and TNF- α correlated with reduced performance on verbal memory (160). Pomykala and co-workers also sought to evaluate the relationship between chemotherapy-induced cognitive dysfunction and pro-inflammatory cytokines. In 33 breast cancer patients (23 received chemotherapy and 10 only had surgery and radiotherapy) the levels of serum pro-inflammatory cytokines were measured, and PET scans were performed to evaluate regional cerebral metabolism. The evaluations were performed immediately after treatment and one-year later. At baseline, in the chemotherapy group, positive correlation between left medial frontal and right inferior lateral anterior temporal cortices metabolism and the pro-inflammatory cytokines IL-1 α , IL-6, TNF- α and C-reactive protein (whose synthesis is induced by IL-6 and TNF- α) were seen. At the one-year assessment, the correlations between cytokines and metabolism continued in the medial frontal cortex and temporal cortex in the chemotherapy group. The placed hypothesis states that the initial inflammatory response causes a downstream of events that has long-term impact on the brain metabolism (242). In summary, cell death and injury caused by chemotherapeutic agents in tumor cells and healthy tissues trigger an inflammatory response where the activated peripheral macrophages release several pro-inflammatory cytokines (243). These molecules can reach the brain and activate the microglia to produce local pro-inflammatory cytokines that further enhance neuro-inflammation. Neuro-inflammation causes synaptic impairment, decrease in myelination and axon thickness (244), inhibition of neurogenesis and neuronal death (245). The postulated mechanism for

pro-inflammatory cytokines neurotoxicity due to systemic chemotherapy is schematized in Figure 3.

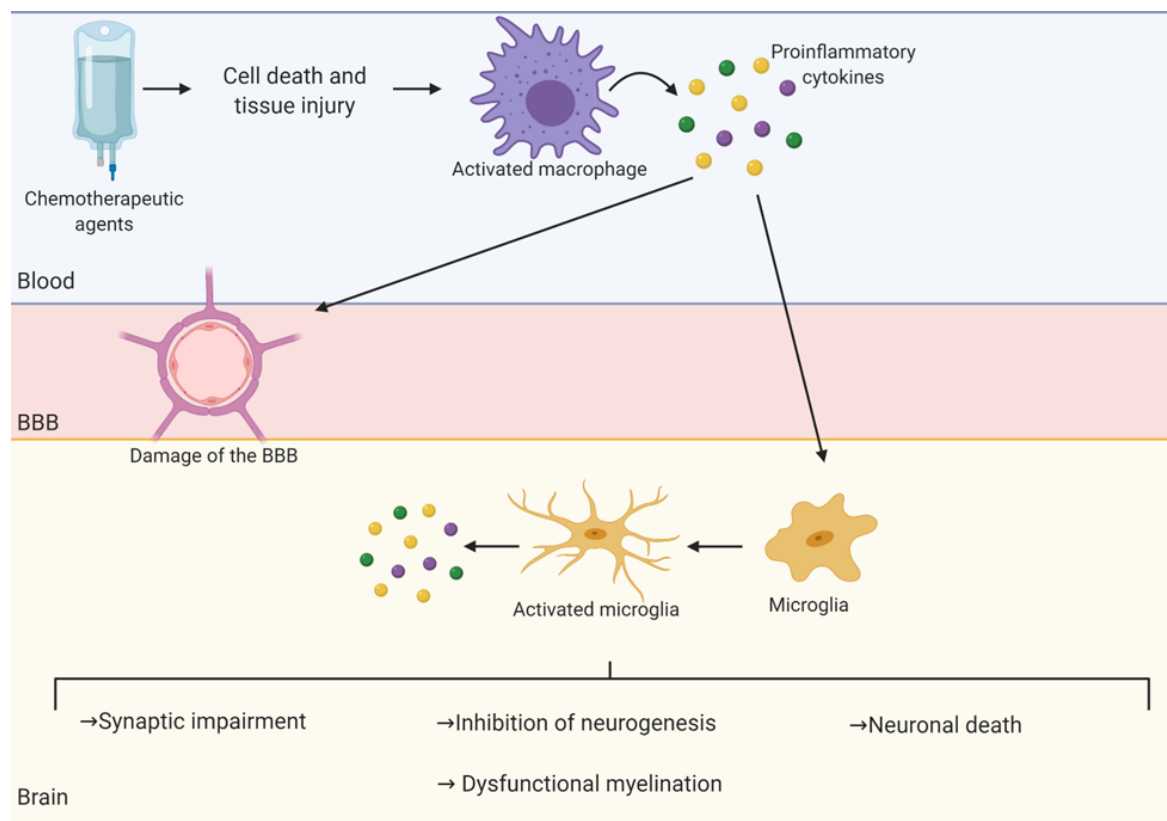


Figure 3: Schematic illustration of the mechanism of pro-inflammatory cytokines neurotoxic effects due to chemotherapy. The systemic administration of chemotherapeutic agents causes damage in non-target tissues and in response to the damage caused, the macrophages are activated and produce pro-inflammatory cytokines. The pro-inflammatory cytokines can cross the BBB passably, by active transporters of cytokines or by the leaky area of the circumventricular organs. These small molecules can also damage the BBB and make it more permeable. Once inside the brain, the pro-inflammatory cytokines can activate the microglia prompting the inflammatory response. The neuro-inflammation caused can lead to synaptic impairment, inhibition of neurogenesis, decreased myelination and axon thickness and neuronal death.

Considering MTX, since it has immunomodulatory and immunosuppressive effects, the impact on cytokines will probably differ from DOX, however these effects have not been studied on MTX-treated cancer patients or representative animal model. The data regarding MTX effects on cytokines has been collected in multiple sclerosis patients. A study performed by Angelucci and colleagues evaluated pro- and anti-inflammatory cytokines production by peripheral blood mononuclear cells of 18 secondary progressive multiple sclerosis patients treated with a dose of 12 mg/m² every 3 months over a year. The proinflammatory cytokines IL-6 and IL-12p40 and anti-inflammatory cytokines IL-10 and transforming growth factor- β (TGF- β) levels were evaluated on blood samples collected before treatment, and 1 month and 12 months after treatment. No changes were seen in

any of the time-points evaluated (246). Nonetheless, enlightenment of the effects of MTX in cancer is difficult without proper patient selection, as for instance the population of the previous study had different characteristics from cancer patients.

1.6.3. Oxidative stress

The brain is highly susceptible to oxidative stress due to its high demand for oxygen, high content in polyunsaturated fatty acids, and relatively low antioxidant capacity (201). An imbalance in the production and elimination of reactive species leads to cell damage since RONS can cause protein, DNA, RNA oxidation or lipid peroxidation and compromise essential cellular functions (247). Although it is known that ROS formation in cancer cells by some chemotherapeutic agents is responsible for tumor regression, more than half of the approved chemotherapeutic agents by FDA have been reported to induce oxidative stress in non-targeted tissues (248). Among those, anthracyclines such as DOX or epirubicin, platinum-based compounds, alkylating agents produce the highest levels of ROS. Chemotherapeutic agents can cause oxidative stress by undergoing redox cycle and disrupt essential cellular functions or by depleting antioxidants that inhibit cellular detoxification, or even causing mitochondria dysfunction, for instances (249). The generation of oxidative stress in the brain causes a downstream of events that can cause mitochondrial dysfunction, glial cell activation, programmed cell death triggering and proteasomal malfunction (250). Moreover, DOX increase of plasma TNF- α is due to its oxidation of plasma ApoA1, an endogenous inhibitor of TNF- α production by macrophages (212). That peripherally generated TNF- α crosses the BBB and promotes neuro-inflammation in the brain, as referred in the *in vivo* studies section.

Regarding MTX, the main goal of this synthetic molecule was to avoid the induction of a redox cycle, which was believed to be the source of DOX-induced cardiotoxicity (251). However, in a cohort of 60 multiple sclerosis patients, a higher level of oxidative modification of serum proteins was found in patients treated with MTX when comparing with patients treated with interferon β 1a and β 1b (252).

Oxidative stress seems a common link in chemobrain, either by direct action of reactive species or resulting of neuroinflammation.

1.6.4. Other factors

Other factors can also contribute to chemobrain. Neuronal apoptosis or dysfunction on neurotransmitters pathways or glial activation seem to be involved on chemobrain. The alteration of the neurochemical profile was observed in a study performed by Pal and colleagues (206). They also administrated mice with a single i.p. injection of 25 mg/kg DOX and 7 days after the administration the mice were euthanized. DOX-treated mice had significantly lower levels of the brain biogenic amine neurotransmitters dopamine, noradrenaline and 5-hydroxytryptamine (serotonin) when comparing to the saline control group. The activity of the monoamine oxidase was also increased in DOX-treated mice when comparing to the control group. The activity of acetylcholinesterase was significantly increased in the hippocampus and moderately increased (but no statistical difference) in the cerebral cortex of the DOX-treated rats (207). Moreover, DOX administration resulted in decreased activity of both brain phospholipase C and D (210). Similarly, when analyzing the neurochemical profile in the hippocampus, mice treated with DOX had a significant decrease in the levels of choline-containing biomolecules when comparing to saline control (210). As stated before, the hippocampal neurogenesis has been impaired after chemotherapy (211). But also, glial activation has been seen after chemotherapy (253), that could influence or contribute to the chemobrain seen in patients. The levels of neurotransmitters have been also changed after anticancer drugs administration (206). These factors, nevertheless, have been poorly explored so far and need further attention in the future.

Moreover, many researchers have focused their attention on genetic variations that play important roles in cognitive function. The screening for potential genetic risk factors could be important to identify patients more susceptible to chemotherapy-induced cognitive dysfunction and allow a closer monitoring. ApoE is a plasmatic protein involved in lipid and lipoprotein metabolism, but it also plays a role in stress-response depending on the isoform involved. The allele ϵ A is associated with an increased risk for Alzheimer's disease and mild cognitive impairment (254). Ahles and colleagues compared the cognitive outcome of cancer survivors (on average 4 years after chemotherapy) who carried the ϵ A allele with those absent for at least one ϵ A allele. The patients that underwent chemotherapy and that had both ϵ A alleles presented significantly impaired visual memory and spatial ability and tended to score lower in executive functioning when compared to patients who did not carried and ϵ A allele. Therefore, patients homozygous for the ϵ A alleles could be at higher risk for cognitive dysfunction after receiving chemotherapeutic treatment and this could be due to differential capacity to repair the microvascular and neuronal damage caused by chemotherapy (89).

Catechol-O-methyltransferase (COMT) is an enzyme involved in the metabolism of catecholamines such as dopamine, adrenaline, and noradrenaline. The activity of this enzyme is particularly important in the breakdown of dopamine in the PFC, since in this brain area the dopamine transporter has low expression within synapses, thus, COMT is essential to continue the dopamine flux and degradation (255). Proper dopamine signaling in the PFC is essential for cognitive functions such as executive cognition and working memory (256). The homozygous for the valine isoform has a higher rate of dopamine metabolism leading to less availability of this neurotransmitter. Small and co-workers performed cognitive and genotype testing in breast cancer survivors treated with chemotherapy or/and radiotherapy and compared their scores healthy controls. The chemotherapy regimen consisted of an average of 5 anthracycline-based cycles. The COMT homozygous for valine had significantly impaired attention, verbal fluency, and motor speed when comparing to COMT methionine homozygous. Moreover, the chemotherapy-treated group COMT homozygous for valine performed worst on tests of attention than COMT homozygous for valine healthy controls (257), placing the hypothesis that cancer patients COMT homozygous for valine could be at higher risk for chemotherapy-induced cognitive impairment.

BDNF is a neurotrophic factor that it is highly expressed in the brain, in particular in the PFC and hippocampus, and it is essential for the survival and maintenance of mature neurons. Moreover, BDNF impairment has been associated with some neurodegenerative diseases (258). The single nucleotide substitution at nucleotide 196 results in an amino acid exchange (methionine to a valine) and the protein formed has decreased activity on BDNF secretion. In a prospective cohort study in 145 breast cancer patients scheduled to received anthracycline or taxane-based chemotherapy, BDNF genotyping was performed. Furthermore, neuropsychological testing was done prior to treatment, 6 weeks after the start of the treatment and at the end of the treatment. The homozygous patients for methionine had significantly lower chances of developing cognitive impairment. Moreover, the valine homozygotes were more likely to have impaired verbal fluency and multitasking ability when comparing to the methionine homozygotes (259).

2. Aims

Despite all the available data, several questions remain unanswered mainly referring to intracellular mechanisms involved and how age affects the brain susceptibility towards anticancer drugs. Therefore, the aims of this dissertation were:

- to evaluate the brain redox and energetic status after administration of clinically relevant doses of DOX or MTX to mice;
- to determine the impact of age of mice on the aforementioned parameters;
- to study different biomarkers of brain damage after DOX or MTX in the adult population, by histological analysis and immunohistochemistry.

3. Materials and methods

3.1. Chemicals

Sodium hydroxide (NaOH) was purchased from VWR chemicals (Leuven, Belgium), sodium carbonate (Na₂CO₃) from Panreac (Barcelona, Spain) and monosodium phosphate (NaH₂PO₄) from J.T. Baker (Deventer, Holland). Potassium sodium tartrate (KNaC₄H₄O₆), perchloric acid (HClO₄), potassium hydrogen carbonate (KHCO₃), 4',6-diamidino-2-phenylindole (DAPI), NeuroTrace™ 530/615 red fluorescent Nissl stain and NeuroTrace™ 500/525 Green Fluorescent Nissl stain were purchased from Fisher scientific (Loughborough, United Kingdom). Copper (II) sulphate (CuSO₄), Folin-Ciocalteu reagent, ethylenediaminetetraacetic acid (EDTA), triton X-100, paraformaldehyde, immersion oil and FluorSave™ were purchased from Merck (Darmstadt, Germany). Isoflurane was obtained from Abbott Animal Health (North Chicago, IL, USA). The primary antibodies polyclonal rabbit anti-Bax (sc-493) and polyclonal rabbit anti-p53 (sc-6243) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The primary antibody polyclonal rabbit anti-glial fibrillary acidic protein (GFAP; Z0334) was purchased from Agilent Dako (CA, USA) and the primary polyclonal rabbit anti-pTau (pSER³⁹⁶; SAB4504557, phosphorylation site at serine 396) was obtained from Sigma-Aldrich. Histomount was obtained from National Diagnostics (Atlanta, GA, USA). All the other reagents used were obtained from Sigma-Aldrich at the highest purity available (St. Louis, MO, USA).

3.2. Animals

In this study, mice with different ages were used. Infant, adult, and old male CD-1 mice were purchased from Charles River Laboratories (L'Arbresle, France) or born in the Institute for Biomedical Sciences Abel Salazar University of Porto (ICBAS-UP) vivarium (namely the old and infant) and maintained in this facility until the day of the sacrifice. The experiments were held with the supervision of trained veterinarians. Before the start of the experimental protocol, the mice were divided into groups and allowed to adjust. The animals were housed in IVC Sealsafe plus mouse Green Mouse 500 cages, in a temperature (22 ± 2°C) and humidity-controlled environment, in a 12-hour light-dark cycle. Standard rodent 4RF21 certificate diet (Mucedola, Settimo Milanese, Italy) and water were provided *ad libitum*. Housing and experimental treatment of the animals were in accordance with the guidelines defined by the European Council Directive (2010/63/EU) transposed into Portuguese law (Decreto-Lei no. 113/2013). Each cage held a maximum of 3 animals to allow socialization and maintain the animals' welfare. Mice were also weighted every day in

the week before the first injection, to allow them to be accustomed to the handler, minimize stress, and improve the animal's welfare. The experiments were performed with the approval of the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (process no. 0421/000/000/2016) and the local Committee Responsible for Animal welfare (ORBEA) of ICBAS-UP (process no. 140/2015).

The mice corresponding to the infant group were 4 weeks old, the adult group were 3 months old, and finally, the old group were 18-20 months old. According to the literature it is widely accepted that at these animal ages, infants did not enter puberty, while the adult group reached sexual maturity, and the old group reached reproductive senescence, therefore being considered a good model to perform experiments and mimic the effects of MTX and DOX therapy in three different life-stages (260, 261).

3.3. Experimental protocol

As mentioned, three groups of differently aged mice were used, and two different experiments were performed. In the first experiment, 16 infant mice, 16 adult mice and 15 old mice were used. The animals in each group were equally divided into three groups: control, MTX and DOX. Based on the results obtained in the first experiment, a second experiment with only adult mice was performed and included 36 animals equally divided into four groups: control, MTX and two different DOX dosages. The administration schedule of MTX and DOX was designed to mimic the human cancer therapy, since these drugs are given in cycles and in multiple administrations (262, 263). In both experiments, the mice received i.p. injections twice a week, for three weeks and therefore receiving six injections, as schematized in Figure 4.

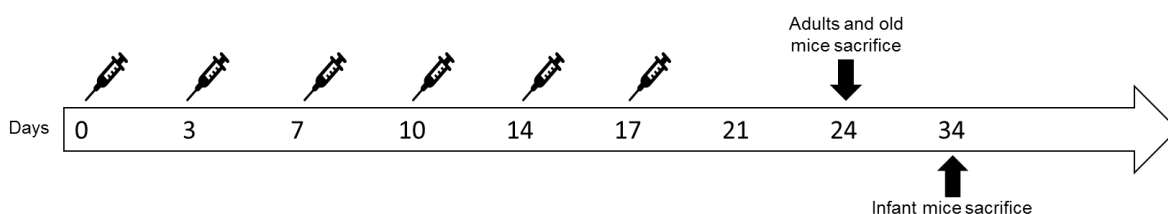


Figure 4: Schematic representation of the administration schedule and time of sacrifice of the mice.

In the first experiment, mice of all ages received a total cumulative dose of 6 mg/kg of MTX (1 mg/kg in each administration). The infant and adult mice received a total cumulative dose of 18 mg/kg of DOX (3 mg/kg in each administration) and the old mice received a total cumulative dose of 9 mg/kg of DOX (1.5 mg/kg in each administration).

Regarding the second experiment made only with adult mice, the MTX group received a total cumulative dose of 6 mg/kg of MTX, the first DOX group received a total cumulative dose of 9 mg/kg of DOX and the second DOX group received a total cumulative dose of 18 mg/kg of DOX. Regarding control groups, mice received injections of saline (NaCl 0.9%) in the same volume of the drug-treated mice and at the same time-points. All the administrated solutions were prepared in a laminar flow chamber to guarantee sterility.

To determine the clinical relevance of the doses used, a conversion of animal doses to human doses was performed using an allometric scaling equation (Figure 5).

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

Figure 5: Equation to convert animal doses into the equivalent human doses. Adapted from Hayes, 2007 (264).

Nevertheless, in human cancer therapy the doses are usually calculated in mg per body surface area (265), consequently the value obtained in the previous equation needed to be multiplied by 37, which is the value of the conversion factor between mice and humans used by the FDA. The final value obtained is roughly the equivalent dose in human in mg/m² (266). Table 4 has the conversion into the equivalent cumulative dose in humans calculated as previously explained, assuming average weights.

Table 4: Allometric conversion of animal doses (mg/kg) into equivalent in human doses (mg/kg) and application of the body surface area conversion factor from mice to human to obtain the equivalent dose in mg/m².

Animal dose	Ages	Animal body weight (kg)	Human body weight (kg)	Human dose (mg/kg)	Human dose equivalent (mg/m²)
6 mg/kg MTX	Infants	0.02	50	0.85	31.4
	Adults /old	0.04	70	0.93	34.3
18 mg/kg DOX	Infants	0.02	50	2.55	94.2
	Adults	0.04	70	2.78	102.9
9 mg/ kg DOX	Adults/Old	0.04	70	1.39	51.5

Food and water consumption, as well as mice weight were registered at least twice a week. Throughout the experimental period, mice well-being was monitored according to a pain scoring system approved in the project evaluation regarding animal welfare. 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 sum values were between 9 and 12 points, an urgent intervention was recommended in order to avoid further suffering (Table 5). Water and food consumption were also assessed, but since the animals were in groups, the consumption of food and water were determined for the entire group, and then calculated per animal based in the individual body weight of each animal.

Table 5: General distress scoring sheet implemented for the evaluation of the general welfare of mice during the experimental protocol. Adapted from Lloyd and Wolfensohn, 1999 (267).

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 (infants)/ ↓ 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 membrane (M), respiratory frequency (RF)/ cardiac frequency (CF)	0
	T; M; RF or CF with little alterations	1
	Irregular T; M; RF or CF with little alterations	2
	T > or < 2°C; pale/ cyanosis M; irregular RF or CF (↓ or ↑)	3
	T > or < 4°C; very altered M; RF or CF very altered (↓ or ↑, abdominal or noisy respiration, orthopneic position, etc.)	4
Intraperitoneal administration		
Response	Without vocalized or general discomfort (GD)/ without abdominal pain (AP)	0
	Do not vocalize; GD (transitory immobilization, local irritation; AP < 5 minutes)	1

	Vocalizes; evident Gd; complete/ reversed with analgesia AP < 10 minutes	2
	Vocalizes; evident GD; partial/ reversed with analgesia Ap > 10 minutes	3

3.4. Euthanasia and sample collection

After the last administration, the animals were maintained in a drug-free period to assess the cumulative toxicity of the administrated drugs. The adult and old population were sacrificed a week after the last administration, whereas the infant mice sacrifice occurred 17 days after the last dose, since a prior study showed that the infant mice are more resilient to the MTX-induced toxicity (268). The animals were anesthetized through the inhalation of isoflurane and once the animals had no reflex to stimulus, they were sacrificed by exsanguination, with blood removal in the inferior vena cava. Mice were decapitated and after uncovering the cranium, left and right anterolateral cuts from the interparietal bone to the frontal bone were done to remove the frontal, parietal, and interparietal bones and expose the brain that was afterwards carefully removed and weighed (Figure 6). In the first experiment, the whole brain was collected to tubes containing 1.5 ml of 5% (w/v) HClO_4 . The samples were homogenised and centrifuged at 16,060 g for 10 minutes (4°C). The pellet was frozen at -20°C for protein quantification and the supernatant was kept at -80°C for total glutathione (tGSH), reduced glutathione (GSH), oxidized glutathione (GSSG), and ATP levels determination. In the second experiment, after brain removal, the right and left hemisphere were separated, and the left hemisphere of each group was collected and fixated in 4% paraformaldehyde for histological analysis.

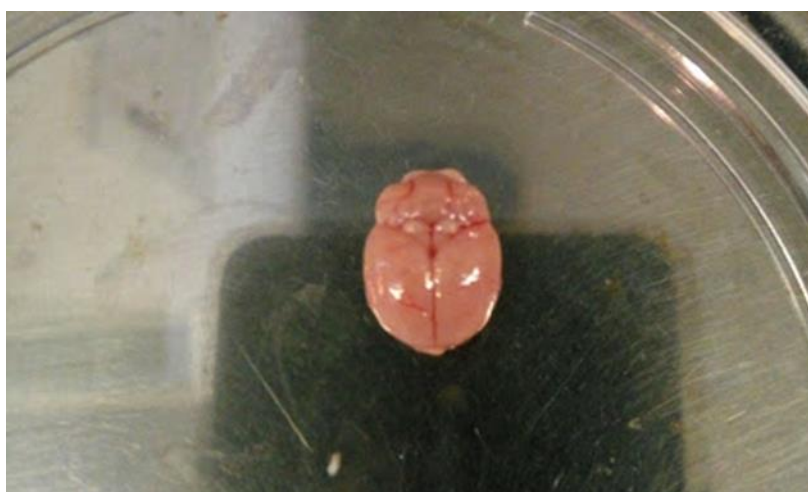


Figure 6: Mice brain.

3.5. Determination of tGSH, GSH and GSSG

The tGSH content of the sample was quantified through the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG recycling assay (268). Standard solutions of glutathione were prepared in 5% HClO₄ in a range concentration of 0.5 to 15 µM. The samples, standards and blank were neutralized using 0.73 M KHCO₃ and then centrifuged for 2 minutes at 16,060 g at 4°C. To a 96-well microplate, 100 µl of the neutralized solutions were added in triplicates and then 65 µl of reagent solution [1.3 mM of DTNB and 0.24 mM of nicotinamide adenine dinucleotide phosphate (NADPH) in phosphate buffer (71.5 mM Na₂HPO₄, 71.5 mM NaH₂PO₄ and 0.63 mM EDTA, pH 7)] prepared beforehand and kept protected from the light, were added. The microplate was incubated for 15 minutes at 30°C and after this period of incubation, 40 µl of glutathione reductase solution (10 U/ml in the phosphate buffer earlier described) prepared extemporaneously were added to each well. The absorbance was measured in kinetic mode for 3 minutes in 10 seconds intervals at 415 nm to follow the formation of the coloured compound 5-thio-2-nitrobenzoic acid (TNB). In the GSSG quantification, before the neutralization of samples, as well as to the standards or blank, 10 µl of 2-vinylpyridine were added. Then they were incubated for 1 hour on ice to block GSH. The standard solutions of GSSG were prepared also in 5% HClO₄ (w/v) but using GSSG in concentrations ranging from 0.25 to 8 µM. After the period of incubation with 2-vinylpyridine, the levels of GSSG were determined as described for tGSH. The levels of GSH were calculated using the formula: $GSH = tGSH - 2 \times GSSG$. The results of tGSH, GSH, and GSSG were normalized to the total protein content and expressed as % control. The ratio of GSH/GSSG was also calculated.

3.6. Determination of cellular ATP levels

The levels of brain ATP levels was determined through the firefly luciferin-luciferase bioluminescent assay (269). D-luciferin 90.9 mg/L stock reagent and luciferase from *Photinus pyralis* (firefly: 3.000.000 U/ml final concentration) were prepared in luciferin-luciferase buffer [50 mM glycine, 10 mM MgSO₄, 1 mM Tris, 0.55 mM EDTA, 0.1% (w/v) bovine serum albumin (BSA); pH = 7.6] and the light-protected aliquots were stored at -80°C until use. Standard solutions of ATP were prepared in 5% HClO₄ in a range concentration from 0.625 to 10 µM. Prior to the determination, the luciferin-luciferase reaction system was formed by mixing the two solutions and then kept at room temperature for optimal enzymatic reaction. A volume of 150 µl of samples, standards or blank were neutralized with 150 µl of 0.76 M of KHCO₃ and then centrifuged for 2 minutes at 16.060 g at 4°C. In a 96-well white microplate, 100 µl of the neutralized samples, standards or blank were added in duplicate, followed by 100 µl/well of the luciferin-luciferase reaction mixture.

The reaction was read immediately in the Biotek Synergy HT (Winooski, VT, USA) plate reader. The results obtained were normalized to the total protein content and expressed as % control.

3.7. Determination of protein levels

Protein content of collected samples was measured through the method previously described by Lowry (270). Standard solutions of BSA were prepared in concentrations ranging from 25 to 250 µg/ml in 0.3 M NaOH. Samples were placed on a 96-well microplate. Fifty µl triplicates of blank, standards or samples were placed, followed by 100 µl of a recently prepared mixture reagent [reagent solution: 9.8 ml of 2% (w/v) Na_2CO_3 , 100 µl of 2% (w/v) $\text{KNaC}_4\text{H}_4\text{O}_6$ and 100 µl of 1% (w/v) CuSO_4]. A 10-minute period incubation followed, protected from the light. After that, 100 µl of freshly prepared Folin-Ciocalteu reagent (1 ml of Folin-Ciocalteu diluted in 14 ml H_2O) were added, and the plate was kept protected from light. After a 20-minute period wait, absorbance was measured in a 96-well plate reader (Biotek PowerWaveX plate reader, VT, USA) at 750 nm.

3.8. Processing of the brain tissue for histologic analysis

The histological processing of the left hemisphere was performed in the Unit of Anatomy of the Department of Biomedicine, Faculty of Medicine of the University of Porto. The histological evaluation was performed only in the adult mice included in the second experiment. After brains' removal from the cranium, they were weighed and bisected sagittally through the midline, separating the left from the right hemisphere. Then, they were coded for blind processing and analysis. The left hemispheres were fixated in 4% paraformaldehyde in 0.01 mM phosphate buffer solution (PBS; pH = 7.4) for 24 hours, in low agitation at 4°C. After the 24-hour fixation, the hemispheres were immersed for 1 hour in the same fixative solution, in low agitation at room temperature, and afterwards were transposed for a 10% sucrose solution diluted in PBS and kept overnight at 4°C. After trimming away the cerebellum through the coronal plane, the block of tissue obtained was mounted on a Leica Vibratome VT1000S (Nuchloss, Germany) with the rostral surface up and serially sectioned in the coronal plane at 40 µm from the olfactory bulb to the caudal limit of the ventral hippocampus (Figure 7). The 40 µm sections were collected in PBS and

then stored at -20°C in the cryoprotectant Olmos solution [30% sucrose, 30% ethylene glycol and 1% polyvinylpyrrolidone-40 in PBS (271)] until further processing.

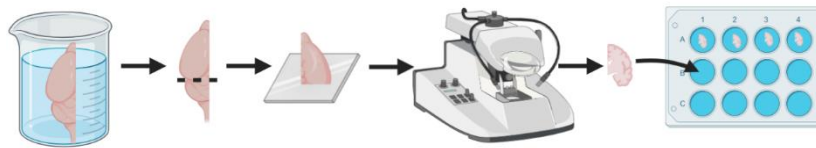


Figure 7: Fixated left brain hemispheres after removing the cerebellum were mounted in a vibratome and serially sectioned through the coronal plane at 40 μ m. The obtained sections were sequentially collected in PBS.

3.9. Giemsa-staining

For identification of neuronal perikarya, the brain sections placed in gelatine-coated slides were stained with Giemsa as followed: lipid extraction by washing 2 times with distilled water for 5 minutes, 1 minutes in 70% ethanol, 1 minutes in 96% ethanol, 2 times washing in absolute ethanol for 1 minutes, washing 2 times for 5 minutes in xylol, 1 minutes in absolute ethanol, 1 minutes in 70% ethanol, and 2 times washing in distilled water for 1 minutes. For the actual staining, the brain sections were immersed for 20 minutes in a Giemsa solution (20% Giemsa stock mixture in distilled water), followed by a 5 minutes wash in running water, 15 seconds in 0.25% acetic acid and another 5 minutes wash in running water, 2 minutes in 0.05% lithium carbonate, another 5 minutes wash in running water, 30 seconds in 70% ethanol, 30 seconds in 96% ethanol, 2 times washing in absolute ethanol for 15 seconds, and finally washing 2 times for 1 minutes in xylol. Afterwards, the slices were coverslipped with Histomount.

3.10. Stereological analysis of left hippocampal formation volume

The volume of the left hippocampal formation was estimated by using the Cavalieri's Principle, which states that if, in two solids of equal altitude, the sections made by planes parallel to and equally distanced from their respective bases are always equals, therefore the volumes of the two solids are equal (272, 273). This principle was adapted for stereology as a method to relate three-dimensional structure to two-dimensional measurements obtained on sections of the structure (274). The Giemsa-stained sections were analysed using a modified Olympus BH-2 microscope interfaced with a colour video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized part, and an object rotator (Olympus, Albertslund, Denmark). A computer equipped with a framegrabber (Screen Machine II, FAST Multimedia, Germany) was connected to the

monitor. The hippocampal formation profile was delineated at a total 100x magnification based on the Paxinos and Franklin's mouse brain atlas (275) and divided into the subregions CA3, CA1, DG and Hilus. Because of the difficulty to delineate the boundary between CA1 and CA2 with accuracy, the combined volumes of these subregions were determined. Estimations were carried out using C.A.S.T – Grid system software (Olympus Albertslund, Denmark) and a mean of 6 systematically sampled sections containing dorsal hippocampal formation area were used per animal. In each section, the cross-sectional area of each subregion was estimated by point counting (276), using an appropriated grid of test points. The area of the counting frame in the DG and Hilus was 15979.95 μm^2 and in CA1 and CA3 was 78913.35 μm^2 (Figure 8). The volume of each subregion was calculated from the total number of points that fell on each subregion and the thickness of the sections [the distance between the first and last focused plan measured with microcator at a total 1000x magnification using immersion oil (277)]. The coefficient of error (CE) of the individual estimates was calculated according to Cruz-Orive (273) and the mean value was 0.10.

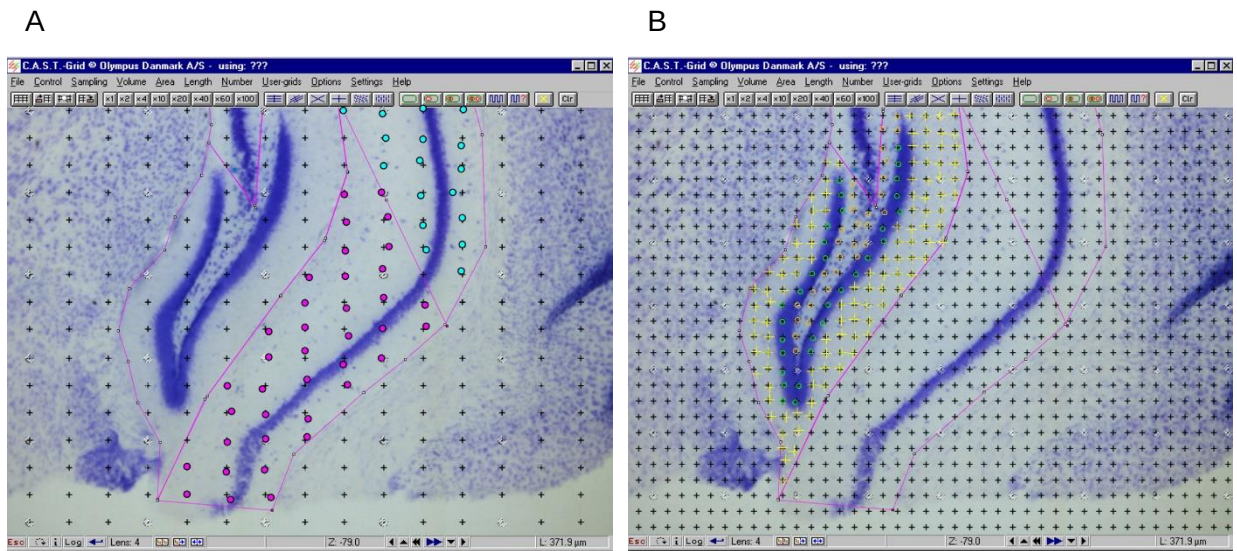


Figure 8: Giemsa-stained sections of the hippocampal formation delineated in pink full line at a 100x magnification in the C.A.S.T. Grid system. (A) Counting frame showing point-counting estimation in the CA1 (pink dots) and CA3 (cyan dots) with 78913.35 μm^2 . (B) Counting frame showing point-counting estimation on the Hilus (orange circles), molecular DG (green circles) and cellular DG (yellow crosses) with 15979.95 μm^2 .

3.11. Immunohistochemistry

GFAP-immunoreactive (GFAP-ir) astrocytes total number estimation was made in free-floating sections showing hippocampal regions and sampled at regular intervals of 480 μm (one out of 12). The previously sliced sections were washed with PBS (15 minutes x 4) to remove the Olmos solution and treated with 10% H_2O_2 in PBS for 10 minutes to inactivate endogenous peroxidase and then blocked with 5% normal goat serum (NGS) in PBS for 1

hour, at room temperature. Following this, the sections were incubated with the primary antibody polyclonal rabbit anti-GFAP at a 1:1000 dilution, for 72 hours at 4°C in PBS with 0.5% triton X-100 and low agitation. The secondary antibody, biotinylated goat IgG anti-rabbit antibody, was used at a 1:400 dilution for 1 hour, followed by the incubation with avidin-biotin peroxidase complex at a 1:800 dilution for 1 hour. Afterwards, the sections were incubated for 1 minute with 0.05% 3,3'-diaminobenzidine (DAB) to which 0.01% H₂O₂ was added and rinsed with PBS. The solutions were prepared in PBS with 0.5% triton X-100, and the incubation was carried out at room temperature in constant agitation. Between the different incubations, the sections were washed 3 x 10 minutes in PBS with 0.5% triton X-100. The stained sections obtained were mounted in gelatine-coated slides and were let to air-dry, overnight at room temperature and then were dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%), cleaned in xylol and sealed permanently with Histomount. The numerical density (Nv) of GFAP-ir astrocytes was estimated by applying the physical dissector method, which consists of two parallel sections generated at a known distance apart from the same tissue block (278), in an average of 6 systematically sampled sections (1 out 6) per animal containing the dorsal hippocampal formation. Six to 8 photographs per section were taken at a total 200x magnification in a Zeiss scope A1 Axio microscope coupled with a coloured camera and a computer with the software Carl Zeiss AxioVision Rel. 4.8, making an average of 40 dissectors. The images were upload to the software ImageJ 1.52a and the area of interest was delimited and measured using the tool "polygon selection" then, the number of GFAP-ir astrocytes presented in one focal plane and not in the other were counted. The thickness of the sections was also measured, using a microcator applied to the microscope, and the volume was determined using the Cavalieri's Principle. The total number of GFAP-ir astrocytes was calculated by multiplying Nv by the volume of the hippocampal formation.

Immunofluorescence detection of p53, pTau and Bax was performed in sections containing PFC or hippocampal regions, which were sampled at regular intervals of 480 µm (one out of 12) and used for immunofluorescence. The previously sliced sections were washed with PBS (15 minutes x 4) to remove the Olmos solution and then were incubated in 5% normal horse serum in PBS with 0.25% triton X-100, for 1 hour at room temperature to block nonspecific binding sites, and then incubated with the primary antibodies diluted in PBS with 0.25% triton X-100, for 72 hours at 4°C, in low agitation. The dilution ratio of primary antibodies was as follows: rabbit polyclonal anti-p53 at a dilution of 1:250, anti-Bax at a dilution of 1:1000 and anti-pTau at a dilution of 1:1500. After the incubation period, sections were washed in PBS (10 minutes x 3) and mounted in gelatine-coated slides and let to adhere to the slide, without letting the sections dry. Once the sections were clinging

to the slides, a PAP pen was used to draw a hydrophobic barrier around the sections to keep the reagents on small droplets on top of the tissue. The fluorescent secondary antibodies polyclonal anti-rabbit Alexa Fluor 488 (green; A32731) or polyclonal anti-rabbit Alexa Fluor 546 (red; A11035) were added to the samples at a dilution of 1:1000 in PBS with 0.25% triton X-100 and incubated for 1 hour, at room temperature, protected from light and after washed in PBS (10 minutes x 3). In order to identify neuronal perikarya, the slides were counterstained with NeuroTrace™ 530/615 red fluorescent Nissl stain or NeuroTrace™ 500/525 Green Fluorescent Nissl Stain, in a dilution of 1:750 in PBS with 0.25% triton X-100, for 40 minutes, at room temperature and kept from the light. The PBS-rinsed sections (10 minutes x 3) were coverslipped with Fluorsafe™ mixed with DAPI at a 1:100 dilution. Photographs of p53, Bax and pTau immune stained sections were taken with identical exposure times, gain and offsets for image acquisition in a Carl Zeiss Axio Imager 2.0 microscope coupled with a coloured camera and a computer with the software Carl Zeiss AxioVision Rel. 4.8 (New York, USA) for image capture. The appropriated exposure time was determined by analysing multiple areas. Images were obtained by manually scanning the PFC and hippocampal formation regions at a 20x magnification and at the following excitation/emission wavelengths: 358/461 (for DAPI), 500/525 (for Alexa Fluor 488 or NeuroTrace™ 525) and 555/565 (for Alexa Fluor 546 or NeuroTrace™ 530/615). A z-stack procedure was applied, with a total of 12 photos at different focal distances per fluorescent marker being evaluated. For measurement of intensity per area, the software ImageJ 1.52a was used. Images of the three different fluorescent markers in each optical plane were combined in stacks and, using the tool “polygon selection”, the area of interest was delimited in the DAPI picture, since it allows a better identification of the regions of interest. Next, using the same selected area, the image was changed to the Alexa fluor dye (that corresponds to antibody labelled) and the total intensity of labelling was measured using the “measure” tool (Figure 9). The results obtained were determined in areal intensities (in arbitrary units per unit area) and expressed as % of control.

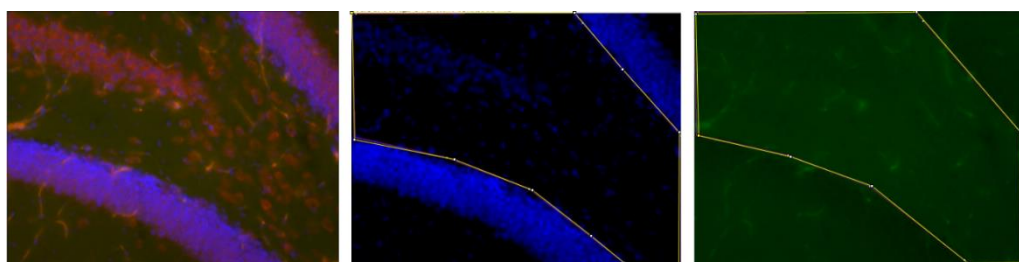


Figure 9: Microphotographs of immune stained sections to illustrate the quantification of the intensity per area in the ImageJ software of p53, Bax and pTau at a total 200x magnification. The images of the three different fluorescent markers merged (A). To accurately outline the area of interest the “polygon selection” tool (yellow full line) was used in the DAPI (B) and the selection was applied to the Alexa Fluor (C).

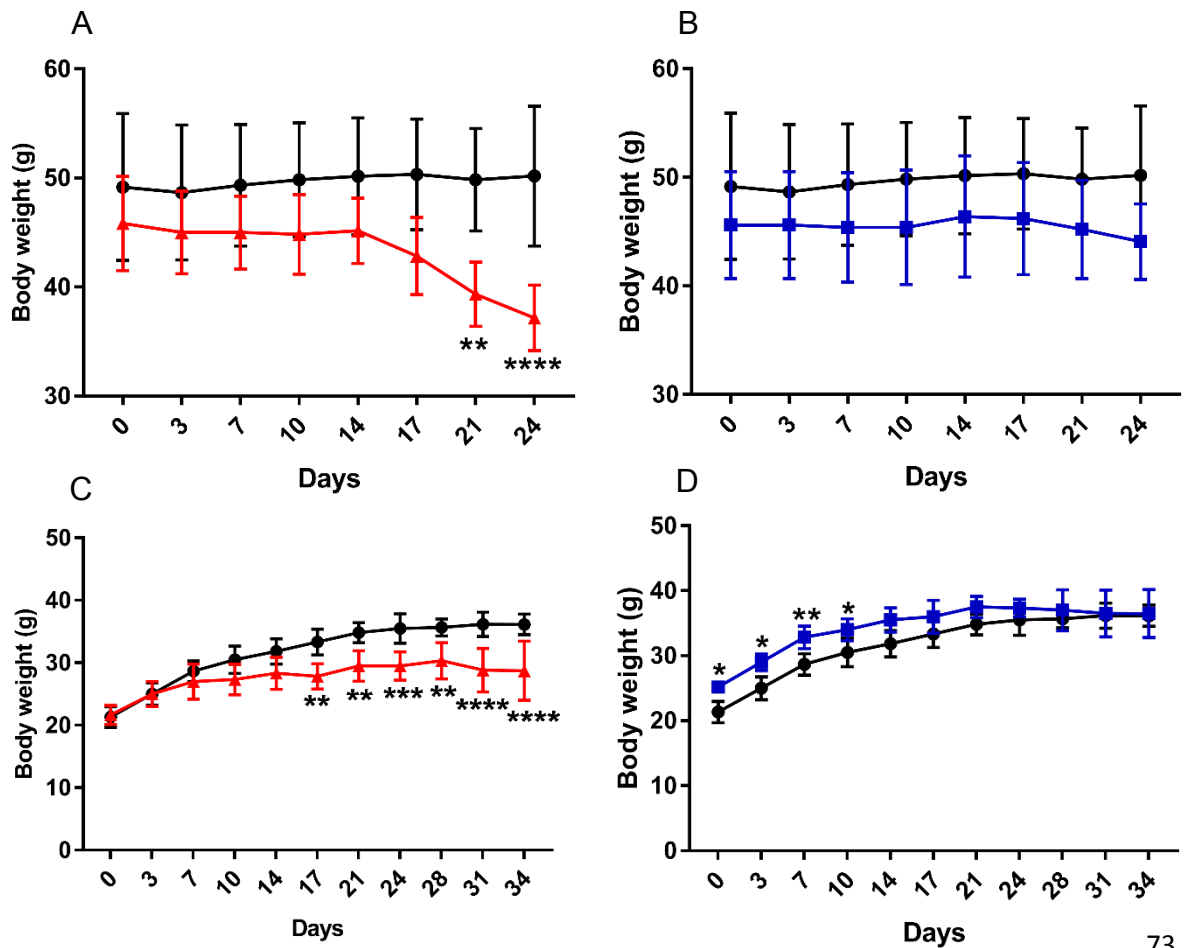
3.12. Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad Prism version 7 (GraphPad Software, CA, USA). Outliers were identified by the ROUT test ($Q = 1\%$) and removed before further statistical analyses. When 3 groups were compared, a one-way parametric analysis of variance (ANOVA) was performed, followed by the Tukey's *post-hoc* test, once a significant p was achieved. In 2 groups comparisons, a t-test analysis with Welch's correction was performed. Regarding the statistical analysis of the temperature, as well as animal weight, food, and water intake of experiments 1 and 2, the two-way repeated measurements analysis was used, followed by Bonferroni *post-hoc* test once a significant p was achieved. Details of statistical analysis are described in each figure legend. Statistical significance was accepted at p values lower than 0.05.

4. Results

4.1. DOX administration caused weight loss in adult mice

In the first experiment, adults and old animals were sacrificed a week later after the last administration and infants were sacrificed 17 days later. Adults administered with DOX at a total cumulative dose of 18 mg/kg, presented significant weight loss when compared to the controls (Figure 10A) after day 21. The average body weight in DOX infants also decreased significantly from day 17 onwards, when compared with respective controls (Figure 10C). In the old mice treated with a total cumulative dose of 9 mg/kg DOX, no significant changes were seen in average body weight in comparison to their respective control group (Figure 10E). The adult MTX-treated mice presented a tendency for a lower weight at the end of experience, however not statistically meaningful, when comparing to respective control group (Figure 10B). Initial significant differences in average body weight were observed in the MTX-treated infant population (Figure 10D) due to the random distribution of mice from different litters for each cage, but throughout the experimental protocol those differences were no longer seen from day 14 onwards. The MTX-old mice did not present any significant variations in average body weight comparing to the saline group (Figure 10F).



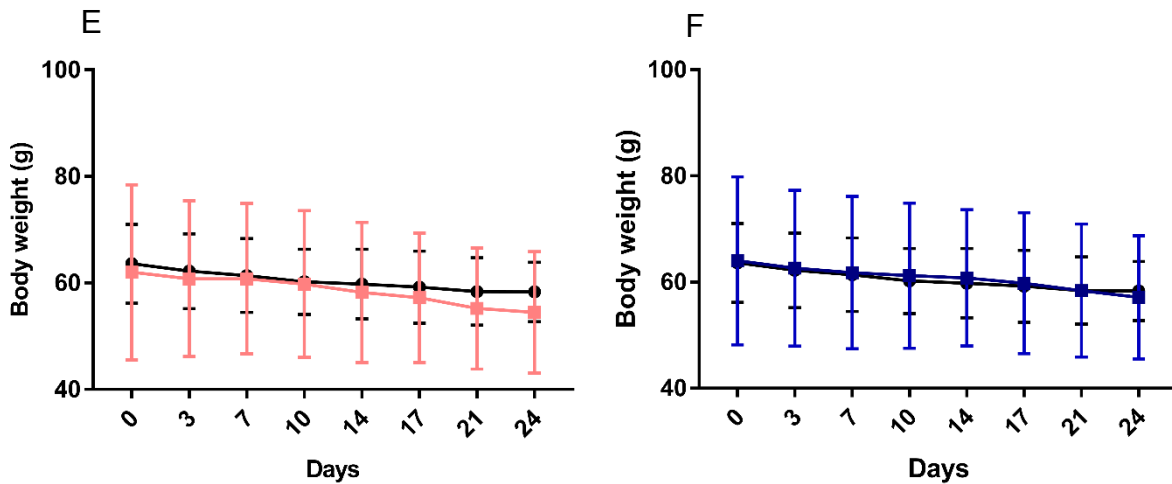


Figure 10: Average body weight in the first experiment of DOX-treated groups of adults (A) and infants (C) (total cumulative dose of 18 mg/kg) and old (E) (total cumulative dose of 9 mg/kg) and MTX-treated groups of adults (B), infants (D) and old (F) (total cumulative dose of 6 mg/kg). Results in grams (g), presented as mean $\pm$ SD from 6 animals in each group, except in the adult MTX-treated group and the old population that have 5 animals each. Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX in adults and infants, pink squares (■) represent total cumulative dose of 9 mg/kg DOX in old mice, and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. controls at the same time-points).

In the second experiment only with adult mice, the average body weight of 18 mg/kg DOX-treated animals decreased significantly on day 14 and in the following days when comparing to the control group or to the lower dose of 9 mg/kg DOX group. The animals treated with 9 mg/kg DOX presented considerable weight loss on day 21 when comparing to the saline control (Figure 11A). In the MTX-treated adult mice, the only significant difference showing weight loss to controls was registered in the day 24, the day of sacrifice (Figure 11B).

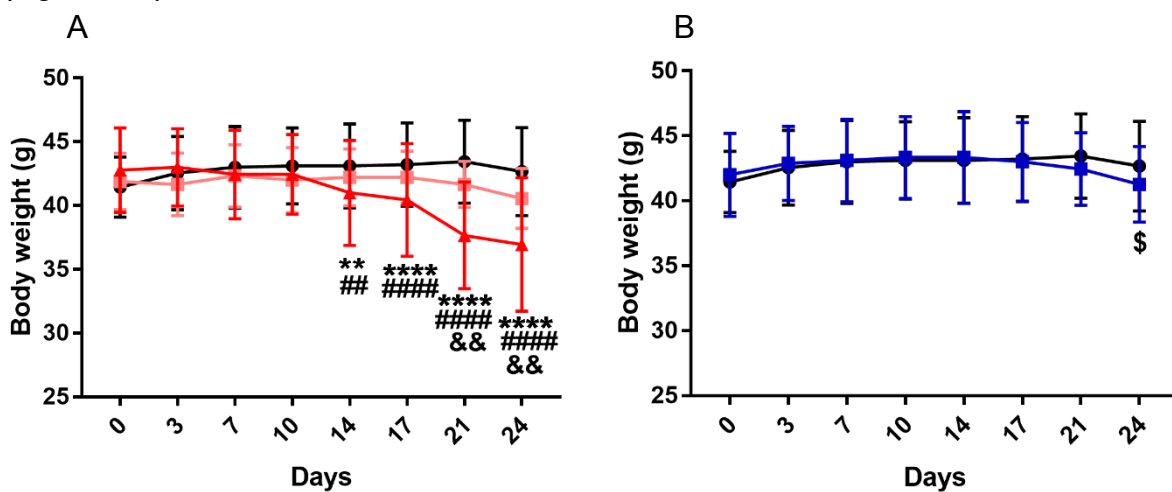
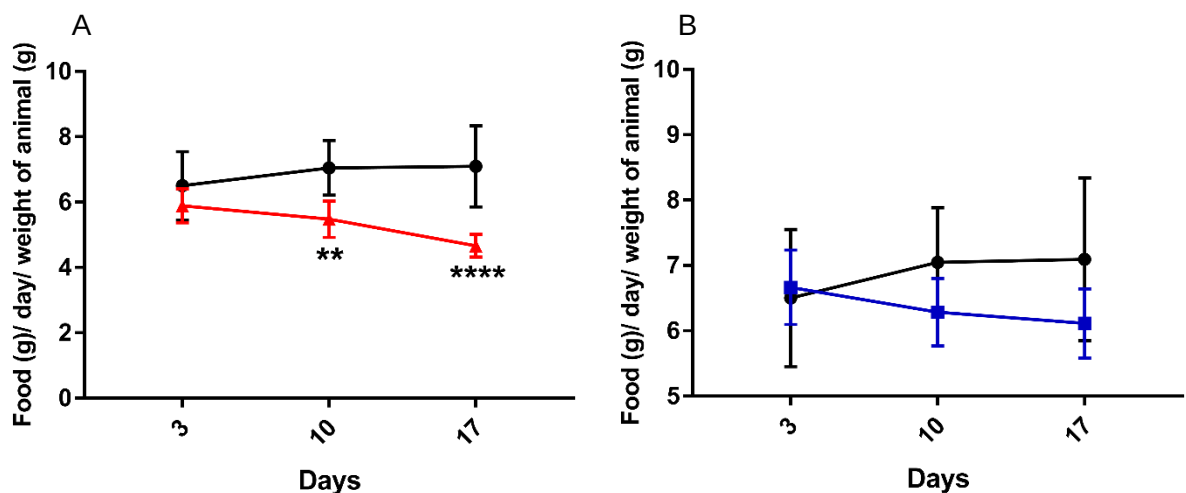


Figure 11: Average body weight in the second experiment of DOX-treated groups of adults (A) and MTX-treated groups of adults (B). Results in grams (g), presented as mean $\pm$ SD from 9 animals in each group. Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX, pink squares (■) represent total cumulative dose of 9 mg/kg DOX and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (** $p < 0.01$ and **** $p < 0.0001$ control vs 18 mg/kg DOX, && $p < 0.01$ control vs 9 mg/kg DOX, ### $p < 0.01$, #### $p < 0.0001$ 9 mg/kg DOX vs 18 mg/kg DOX and \$ $p < 0.05$ control vs MTX 6mg/kg, when assessing the same time-points).

The daily food and water intake were determined for each cage and then assessed for each mouse based on their weight. The precise consumption rates of each animal were not possible to determine, as they were housed grouped to keep social interaction as a standard of animal welfare. Therefore, it was assumed that the food consumption of each animal was proportional to mice weight and that a weight loss or gain was due to lower/higher consumption.

Regarding food intake in the first experiment, the 18 mg/kg DOX-treated adults presented significantly reduced food intake on day 10 and 17 when comparing to the saline-control (Figure 12A). Infant DOX-treated mice also consumed substantially less food on day 10 and forward when comparing to the saline group of the same age (Figure 12C). The old group that received 9 mg/kg DOX had no significant alterations in the food consumption pattern (Figure 12E). The MTX-administrated adult mice did not display any significant alterations in food intake throughout the experiment (Figure 12B). In the beginning of the protocol, infant MTX mice exhibit a significant different food consumption when comparing to the control group, possibly due to the initial difference in average body weight among controls and treated animals (Figure 12D). However, during the remaining protocol no significant differences were seen. The older MTX mice had no considerable alterations in comparison to the respective control group (Figure 12F).



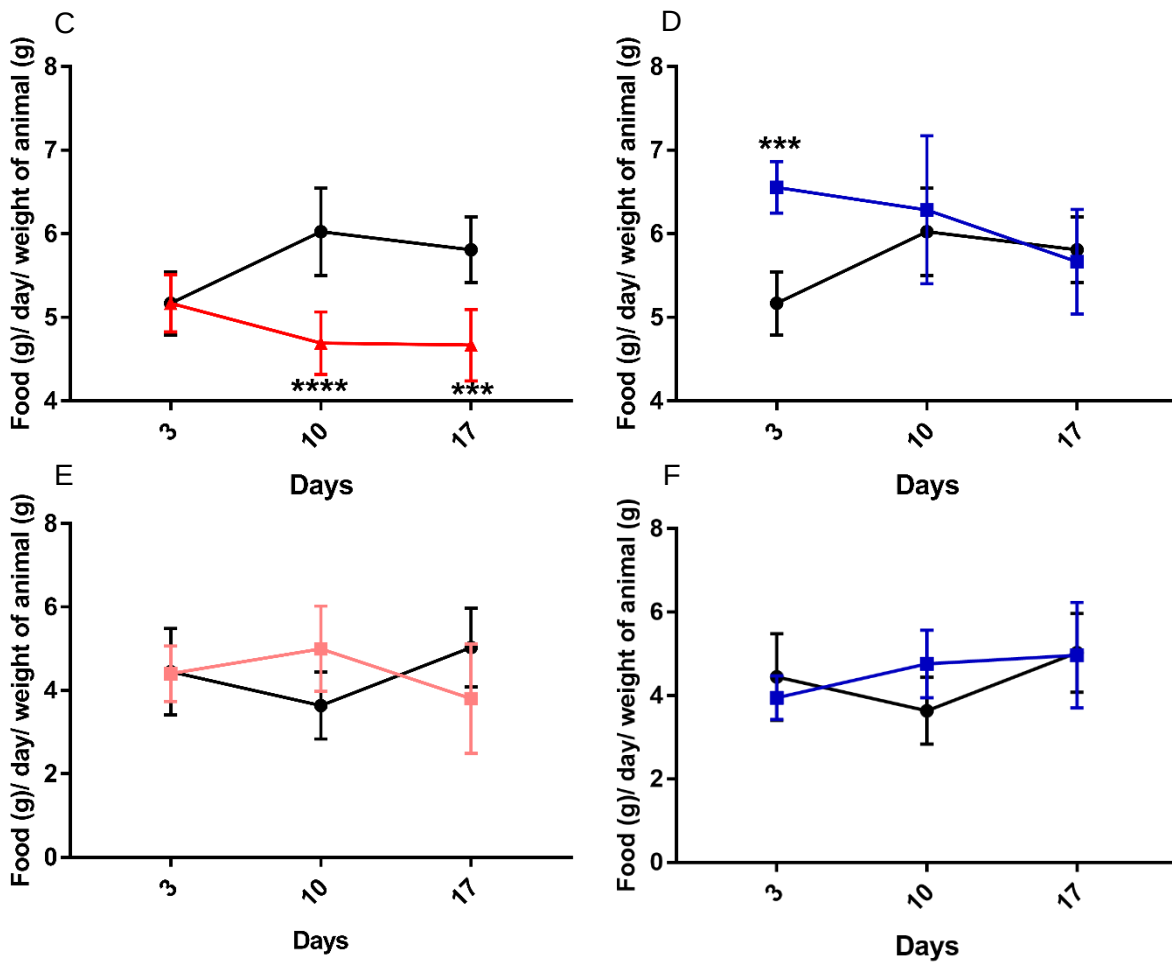


Figure 12: Food consumption in the first experiment of DOX-treated groups of adults (A) and infants (C) (total cumulative dose of 18 mg/kg) and old (E) (total cumulative dose of 9 mg/kg) and MTX-treated groups of adults (B), infants (D) and old (F) (total cumulative dose of 6 mg/kg). Results in food intake (g)/day/animal weight (g), presented as mean $\pm$ SD from six animals (except in the adult MTX-treated group and the old groups that have an N of five animals). Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX in adults and infants, pink squares (■) represent total cumulative dose of 9 mg/kg DOX in old mice and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (**p<0.01, ***p<0.001 and ****p<0.0001 vs controls at the same time-points).

In the second experiment, the food and water consumption were assessed in several time-points. The 9 mg/kg DOX-treated mice started the protocol consuming less food than the control group and this behaviour was maintained on day 3, 14, 17 and more accentuated on day 21. The 18 mg/kg DOX group began eating less than the control group on day 3, and that decrease also occurred from day 10 on until day 21. In this last evaluated day, the 18 mg/kg DOX treated animals consumed less also when comparing with the 9 mg/kg DOX mice (Figure 13A). The MTX group had no considerable changes in food intake patterns in comparison to the control group (Figure 13B).

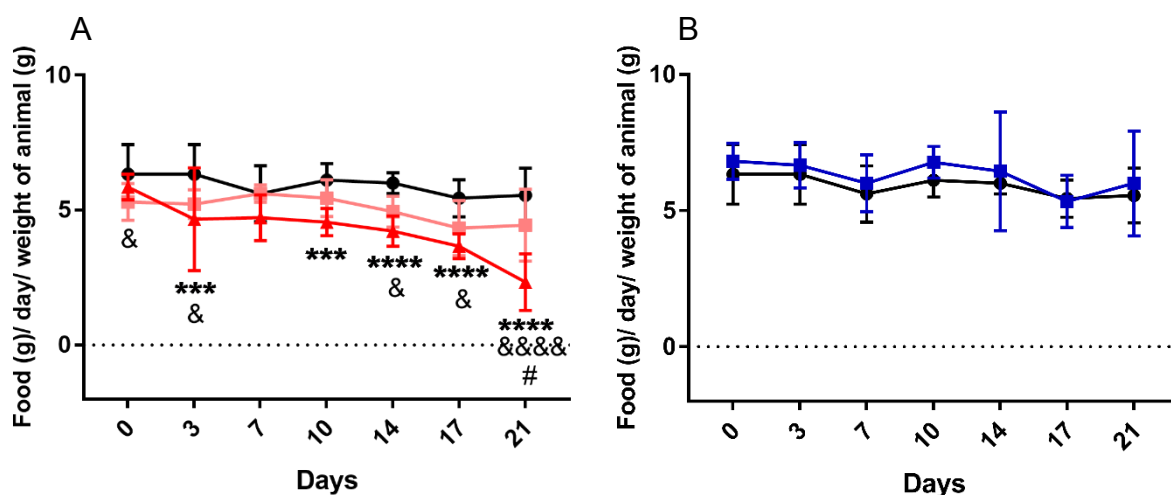


Figure 13: Food consumption in the second experiment of DOX-treated adults (A) and food consumption of MTX-treated groups of adults (B). Results in food intake (g)/day/animal weight (g), presented as mean $\pm$ SD from 9 animals in each group. Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX, pink squares (◻) represent total cumulative dose of 9 mg/kg DOX and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (** $p < 0.001$ and **** $p < 0.0001$ controls vs 18 mg/kg DOX, & $p < 0.05$ control vs 9 mg/kg DOX, # $p < 0.05$ 9 mg/kg DOX vs 18 mg/kg DOX, at the same time-points).

Regarding water consumption in the first experiment, adult mice treated with a total cumulative dose of 18 mg/kg of DOX had significantly decreased water intake on day 14, and this tendency was maintained on day 17 in comparison to the saline group (Figure 14A). The DOX-treated infant population had increased water consumption at the beginning of the experiment, but throughout the protocol consume decreased, reaching statistical significance on day 17 (Figure 14C). The older mice administrated with a total cumulative dose of 9 mg/kg DOX did not present any significant changes in water intake (Figure 14E). The MTX-administrated adult mice had no considerable changes in water intake patterns when comparing to the control group (Figure 14B). Similar to the food intake, in the MTX-infants there was also an initial increase in water consumption, but later on the protocol that was no longer meaningful. Older MTX mice did not show any changes in water consumption throughout the protocol (Figure 14E). Of note, on day 14 there is not data available of water consumption for the infant population in both DOX and MTX groups since the water bottle opened up (figure 14C and D).

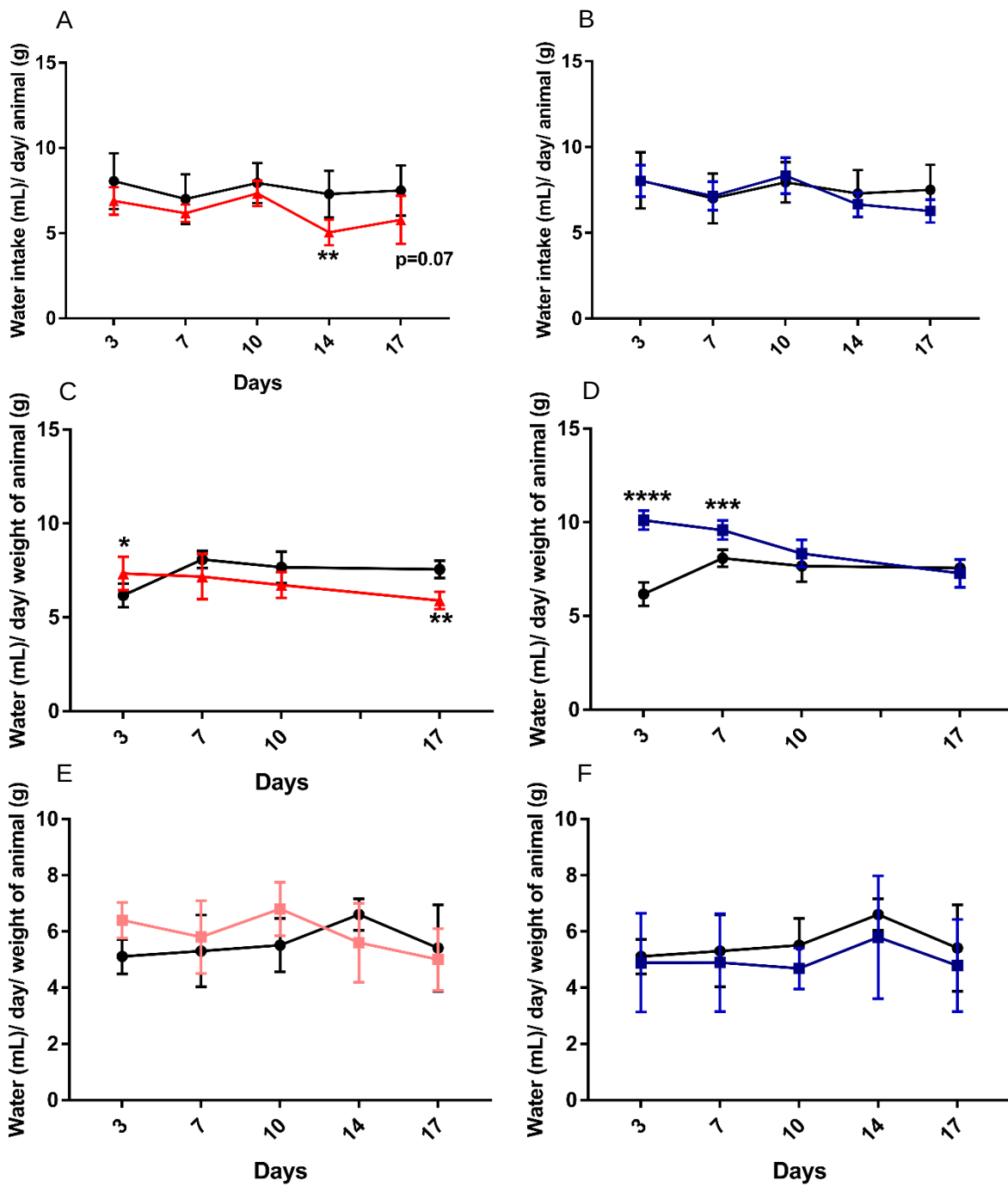


Figure 14: Water consumption in the first experiment of DOX-treated groups of adults (A) and infants (C) (total cumulative dose of 18 mg/kg) and old (E) (total cumulative dose of 9 mg/kg) and MTX-treated groups of adults (B), infants (D) and old (F) (total cumulative dose of 6 mg/kg). Results in water intake (mL)/day/weight of animal (g), presented as mean $\pm$ SD from six animals, except in the adult MTX-treated group and the old groups that have five animals each. Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX in adults and infants, pink squares (■) represent total cumulative dose of 9 mg/kg DOX in old mice and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (** p <0.01, *** p <0.001 and **** p <0.0001 vs controls at the same time-points).

The DOX-treated adult animals of the second experiment had decreased water intake. In fact, the 9 mg/kg DOX mice had lower water consumption from day 0 to day 14, with no further differences in the rest of the protocol, while the 18 mg/kg DOX mice had lower water ingestion since day 3 until the end of the protocol when comparing to the control group (Figure 15A). Regarding the MTX-treated adult mice, no statistical differences were observed among control and treated animals (Figure 15B).

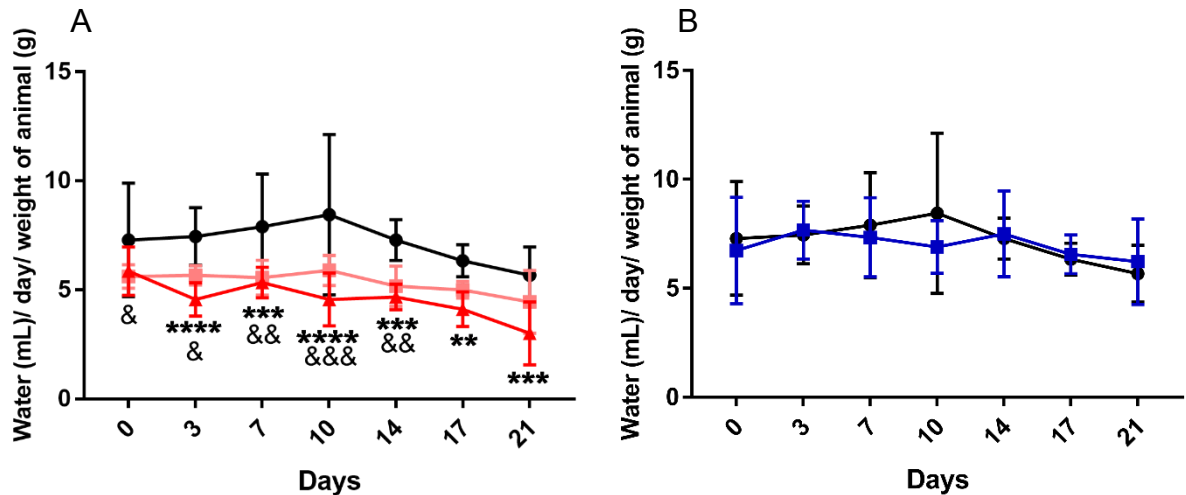


Figure 15: Water consumption in the second experiment of DOX-treated adults (A) and MTX-treated groups of adults (B). Results in water intake (mL)/day/weight of animal (g), presented as mean $\pm$ SD from 9 animals in each group. Black full dots (●) represent saline-control, bright red triangles (▲) represent a total cumulative dose of 18 mg/kg DOX, pink squares (■) represent total cumulative dose of 9 mg/kg DOX and dark-blue squares (■) represent the cumulative dose of 6 mg/kg MTX. Statistical comparisons were made using two-way ANOVA repeated measures followed by Bonferroni *post-hoc* test (** p <0.01, *** p <0.001 and **** p <0.0001 controls vs 18 mg/kg DOX, & p <0.05, && p <0.01, and &&& p <0.001 controls vs 9 mg/kg DOX at the same time-points).

4.2. High DOX dose promoted a decrease in brain relative weight

The adult mice treated with 18 mg/kg DOX had a reduction in brain weight when comparing with the mice administrated with the lower dose of 9 mg/kg DOX (Figure 16A). No significant changes were seen in the infants (Figure 16B) and old mice (Figure 16C).

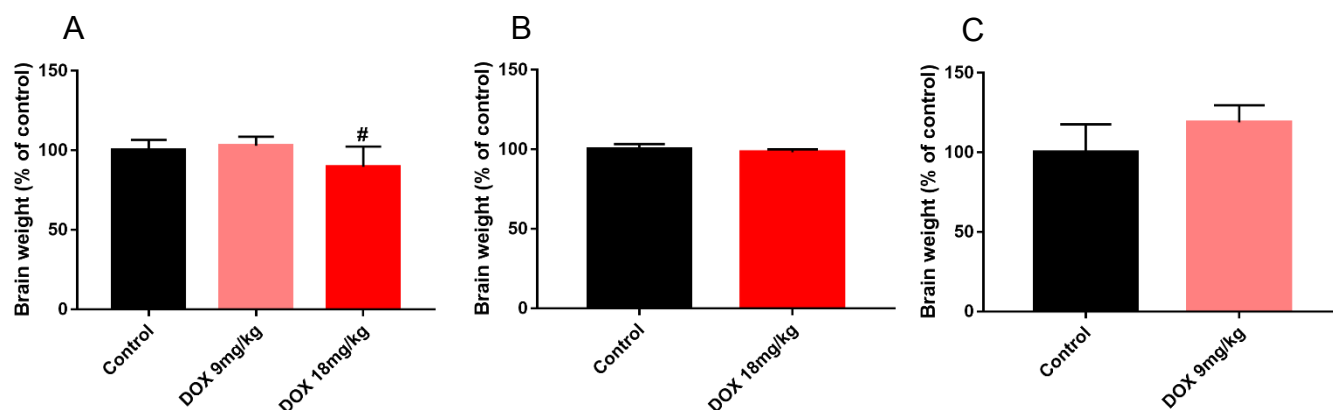


Figure 16: Brain relative weight of DOX-treated groups of adults (A), infants (B) and old (C). Results, in % of mean controls, are presented as mean $\pm$ SD from 6 animals in each group (except in the adult DOX-treated group and the old population that has 5 animals each and the adult control group with 12 animals). Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test for data in graph A, meanwhile a t-test was conducted in graphs B and C data ([#] $p < 0.05$ vs 9 mg/kg DOX).

4.3. The brain weight of old mice treated with MTX slightly increased when compared to control

No changes in brain weight were seen in the adult (Figure 17A) and infant mice (Figure 17B). The MTX-treated older mice presented a statistical tendency ($p = 0.07$) to have a higher brain weight than the saline-controls (Figure 17C).

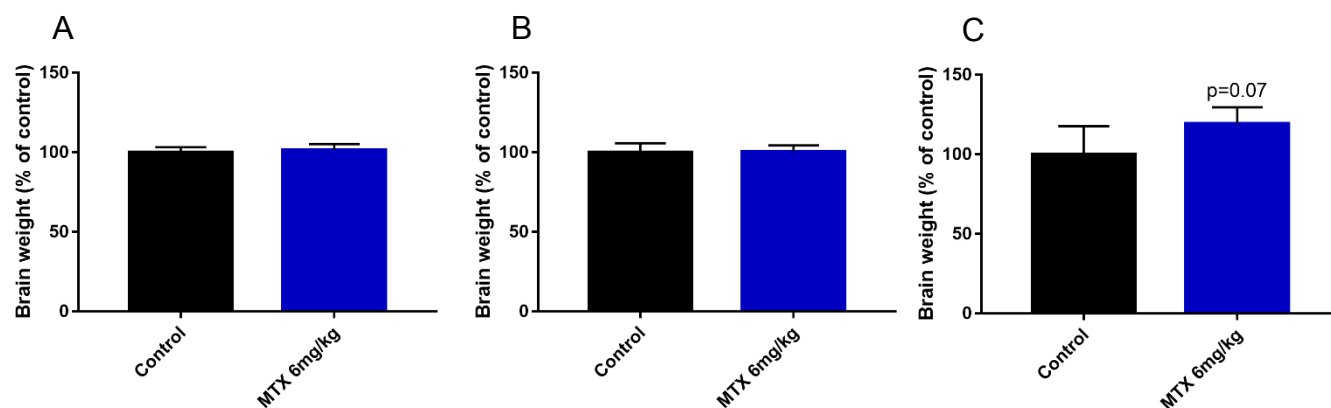


Figure 17: Brain weight of the MTX-treated groups of adults (A), infants (B), and old (C). Results, in % of mean controls, are presented as mean $\pm$ SD from 6 animals in each group except in the adult MTX-treated, and old population that has 5 animals each. Statistical comparisons were made using the t-test.

4.4. Glutathione levels decreased in adult animals treated with the highest DOX dose

Adult mice treated with a total cumulative dose of 18 mg/kg DOX had significantly lower levels of tGSH when comparing to the control group and the group that received the 9 mg/kg DOX dose (Figure 18A). No significant alterations in GSSG levels were seen in either DOX doses (Figure 18B). Moreover, the higher dose of DOX caused a decrease in the GSH levels when comparing to the controls (Figure 18C). No significant changes in the GSH/GSSG ratio were observed in any of the DOX cumulative doses administrated (Figure 18D).

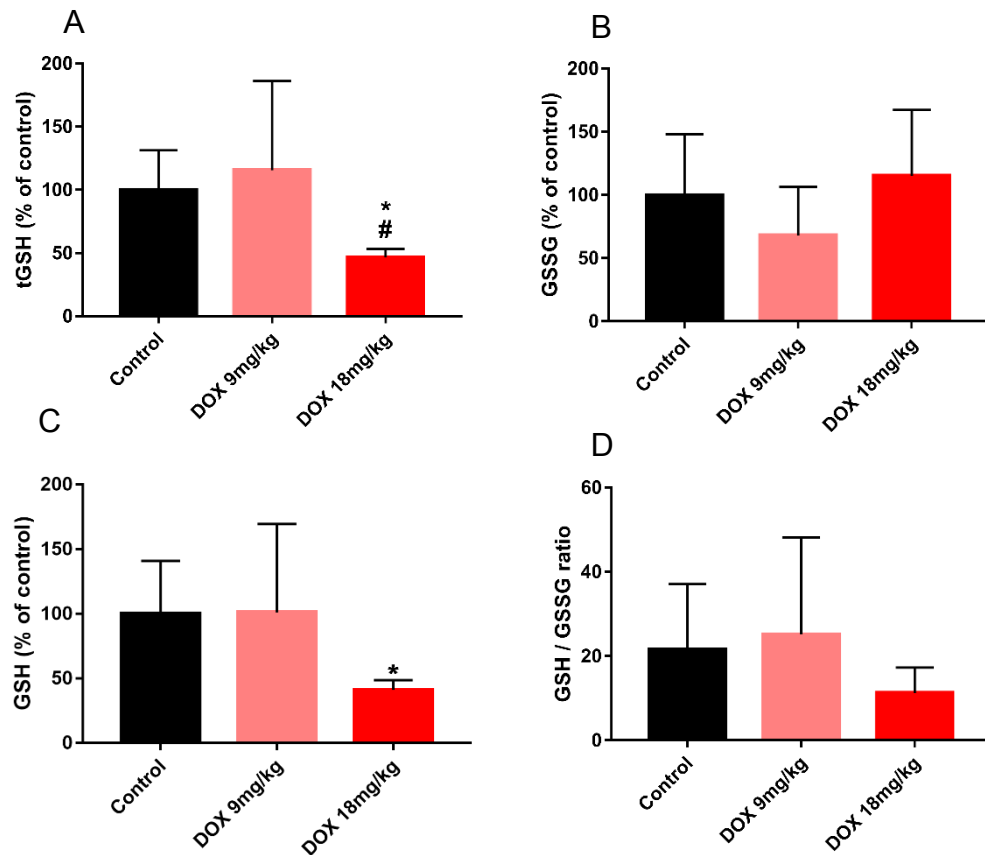


Figure 18: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control adults, were determined after DOX and the GSH/GSSG ratio calculated (D). Results, as % of mean control, are mean $\pm$ SD from 6 animals in each group, except in the control group that had 12 animals. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* $p < 0.05$ vs control; # $p < 0.05$ vs 9 mg/kg DOX).

The infant mice that received a total cumulative dose of 18 mg/kg DOX did not present any significant differences in the glutathione parameters evaluated (Figure 19).

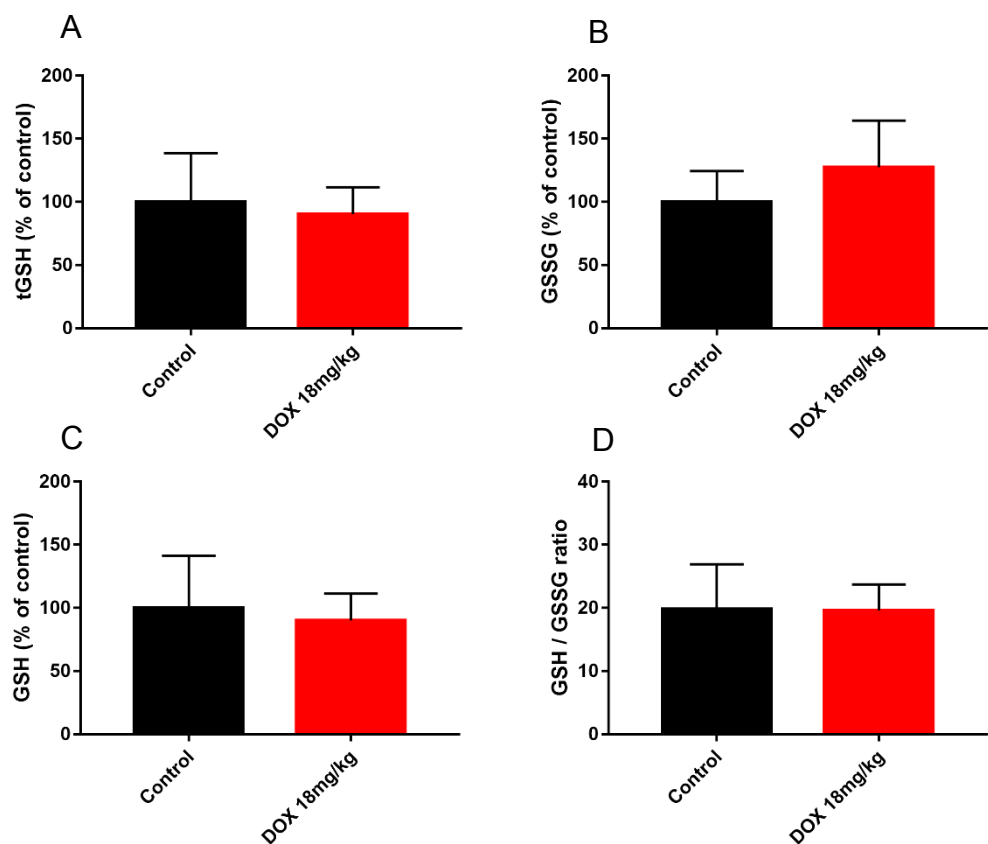
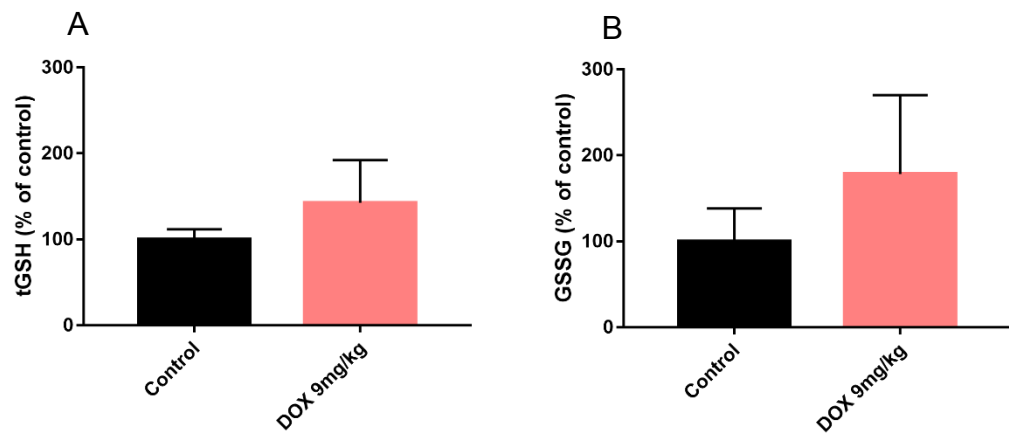


Figure 19: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control infants, were determined after DOX and the GSH/GSSG ratio calculated (D). Results are mean $\pm$ SD from 6 animals in each group. Statistical comparisons were made using the t-test.

Regarding the older DOX-treated mice that received a total cumulative dose of 9 mg/kg DOX, no significant statistical changes in the tGSH, GSSG and GSH levels nor in the GSH/GSSG were seen when comparing to the respective saline-controls (Figure 20).



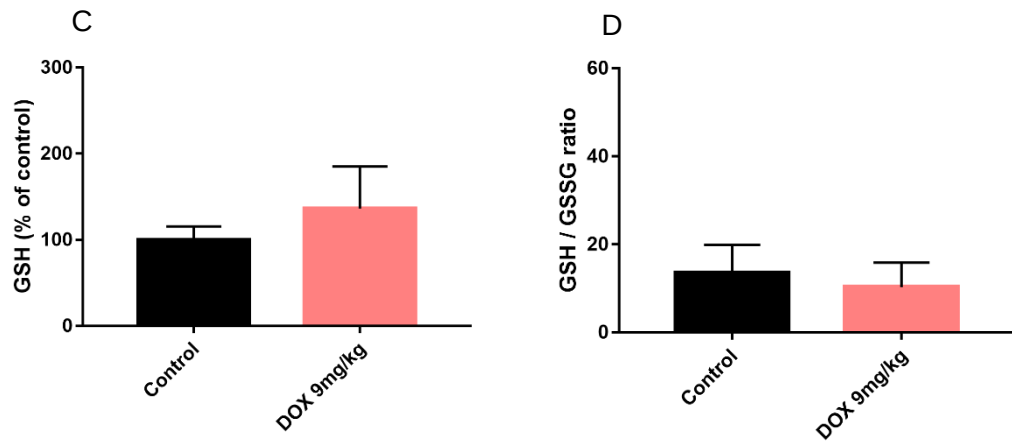


Figure 20: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control old, were determined after DOX and the GSH/GSSG ratio calculated (D). Results are mean $\pm$ SD from 5 animals in each group. Statistical comparisons were made using the t-test.

4.5. MTX caused no significant impact on the brain redox status of differently aged mice

The administration of 6 mg/kg did not cause significant alterations in the adult brain levels of tGSH, GSSG, GSH, nor altered the ratio GSH/GSSG (Figure 21).

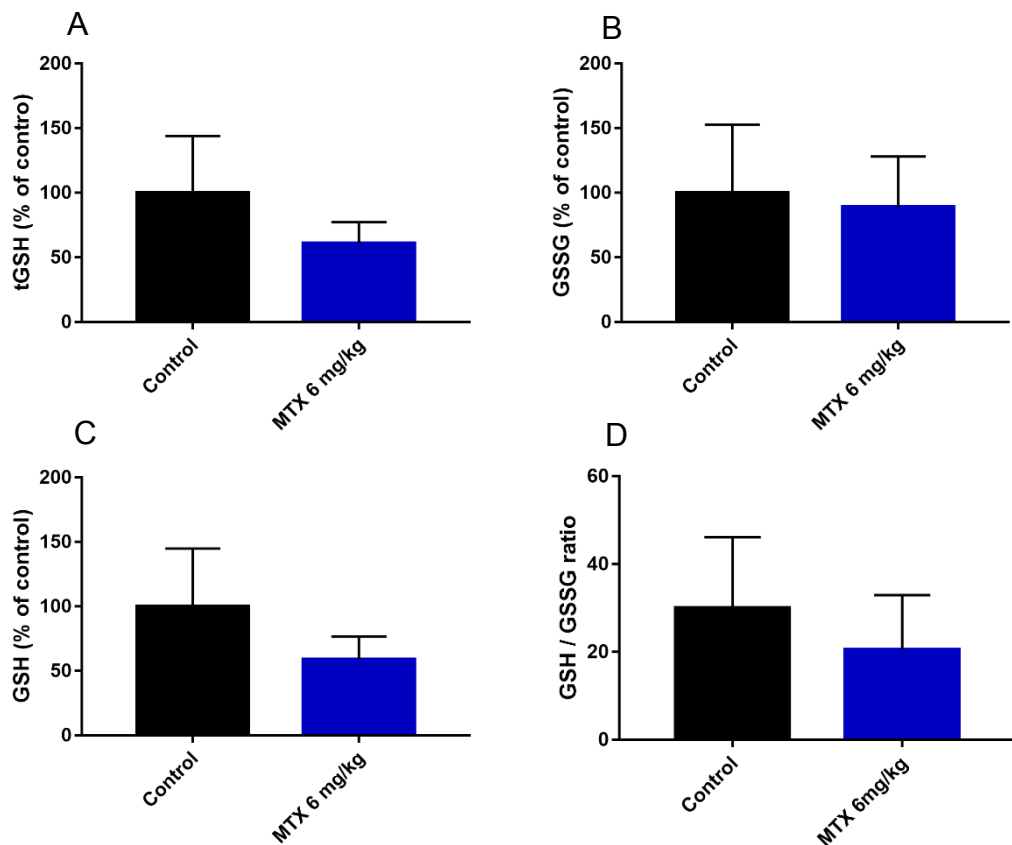


Figure 21: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control adults, were determined after MTX and the GSH/GSSG ratio calculated (D). Results are mean $\pm$ SD from 6 animals in each group. Statistical comparisons were made using the t-test.

The infant population administrated with a total cumulative dose of 6 mg/kg MTX did not present considerable alterations in the glutathione parameters evaluated (Figure 22).

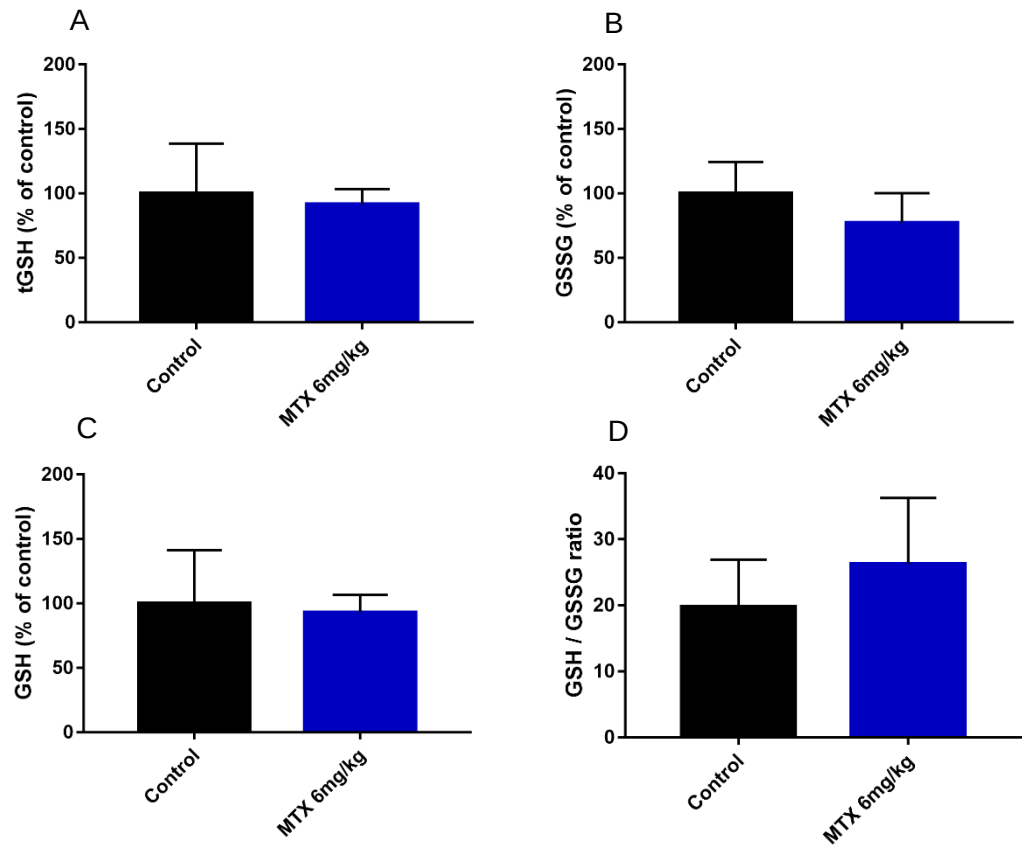
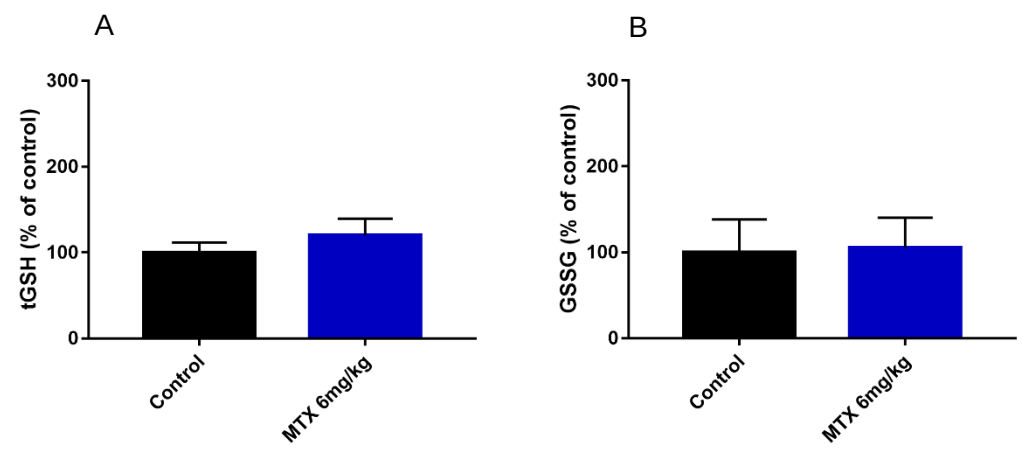


Figure 22: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control infants, were determined after MTX and the GSH/GSSG ratio calculated (D). Results are mean $\pm$ SD from 6 animals in the control group and 5 animals in the MTX group. Statistical comparisons were made using the t-test.

Considering the older MTX-treated mice, no significant statistical changes in the tGSH, GSSG and GSH levels nor in the GSH/GSSG ratio were observed in comparison to the saline-control mice (Figure 23).



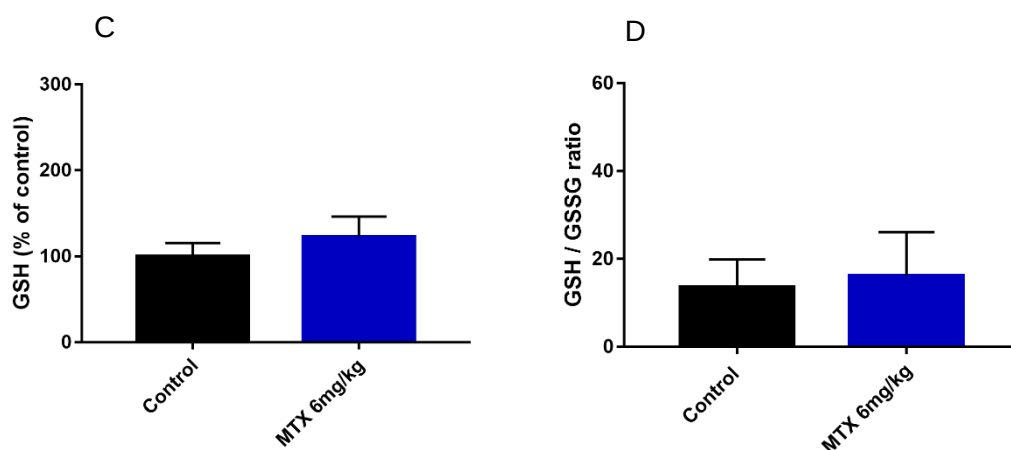


Figure 23: Total glutathione (tGSH) (A), oxidized glutathione (GSSG), (B), reduced glutathione (GSH) (C), as % of control old, were determined after MTX and the GSH/GSSG ratio calculated (D). Results are mean $\pm$ SD from 5 animals in each group. Statistical comparisons were made using the t-test.

4.6. Infant DOX-treated animals had an increase in brain ATP levels

The administration of the total cumulative dose of 9 mg/kg DOX did not alter the ATP levels in the adult (Figure 24A) nor in the older mice (Figure 24C). However, the higher total cumulative dose of 18 mg/kg DOX increased significantly the ATP levels in the infant population (Figure 24B), while not causing changes in the 18 mg/kg DOX-treated adults.

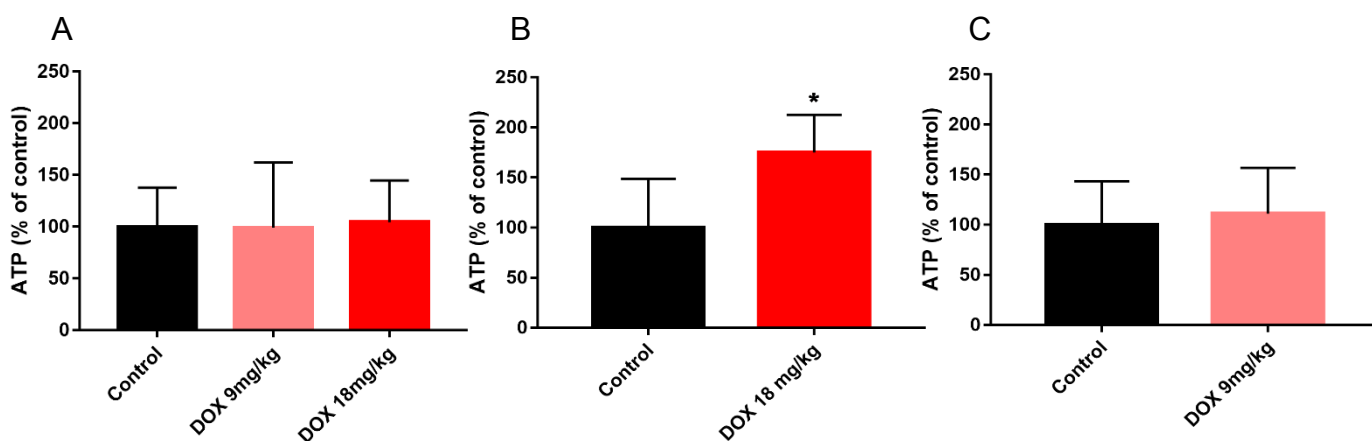


Figure 24: Brain ATP levels after DOX treatment. Data, as % of mean controls, in DOX-treated adult (A), infant (B) and old (C) groups. Results are mean $\pm$ SD from 6 animals in each group, except the adult control group that has 12 animals and the old groups that has 5 animals each. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test for data in graph A, meanwhile a t-test was conducted in graphs B and C data (* $p < 0.05$ vs control).

4.7. MTX administration caused no changes in brain ATP levels in mice of different ages

The MTX administration in the total cumulative dose of 6 mg/kg did not led to significant alterations in the whole brain ATP levels in none of the different ages tested (Figure 25).

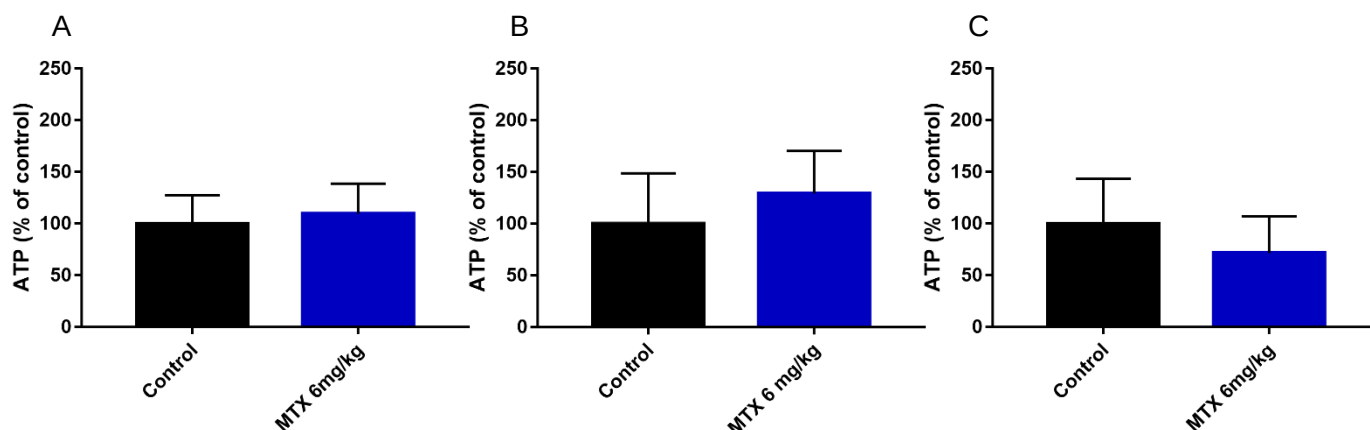
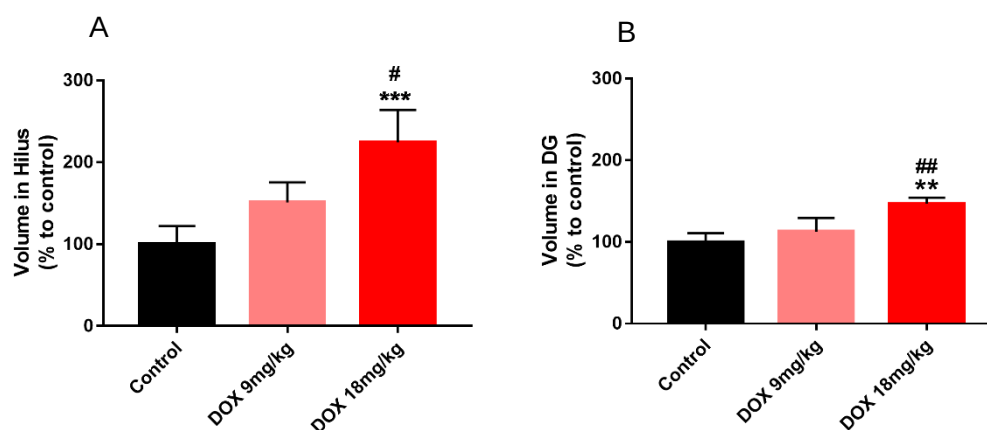


Figure 25: Brain ATP levels after MTX treatment. Data, as % of mean control, in MTX-treated adult (A), infant (B) and old (C) groups. Results are mean $\pm$ SD from 6 animals in each group, except the infant MTX group and the old groups that have 5 animals each. Statistical comparisons were made using one-way ANOVA.

4.8. The highest dose of DOX increased the hippocampal formation volume in mice

Considering the hippocampal formation volume, the highest total cumulative dose of 18 mg/kg DOX caused a significant increase in hippocampal volume in all regions evaluated (Figure 26), when compared to the control group and the lowest DOX dose group. That increase was more noticeable in the Hilus region (Figure 26A). The lowest DOX dose did not induce alterations in any of the evaluated hippocampal brain regions.



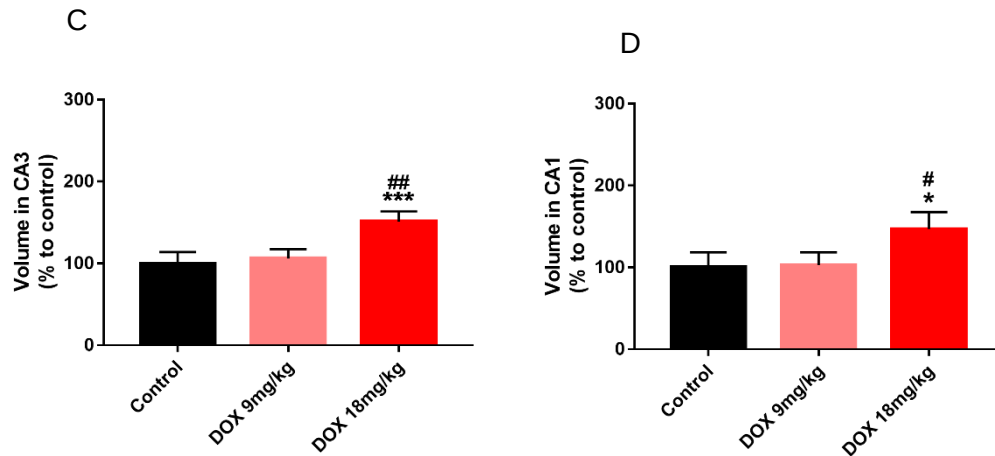
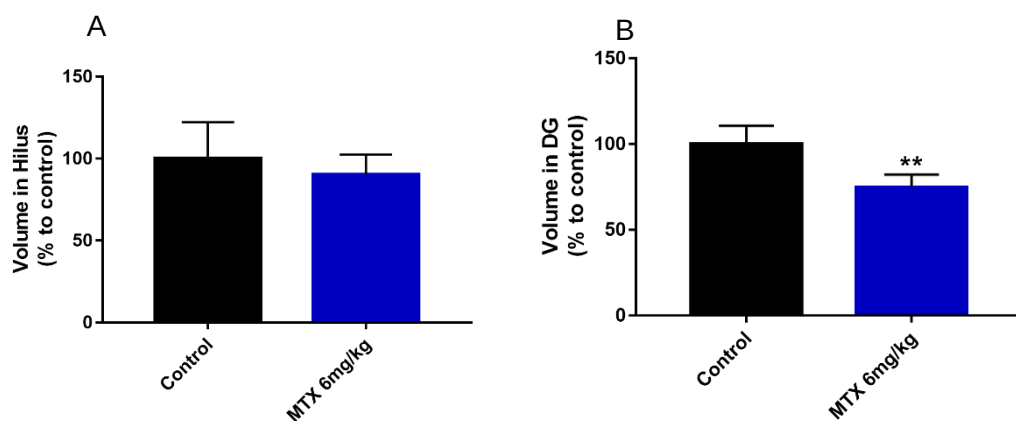


Figure 26: Volume in brain hippocampal regions Hilus (A), DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 9 mg/kg DOX or a total cumulative dose of 18 mg/kg DOX. Data, as % of mean control, are mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using a t-test analysis with Welch's correction (* p <0.05, ** p <0.01 vs control).

4.9. MTX-treated mice presented a decreased hippocampal volume in DG and CA1 regions

The hippocampal volume in the MTX-treated mice decreased in the DG and CA1 regions (Figure 27 B and D), whereas in the Hilus and CA3 no significant alterations were seen (Figure 27A and C).



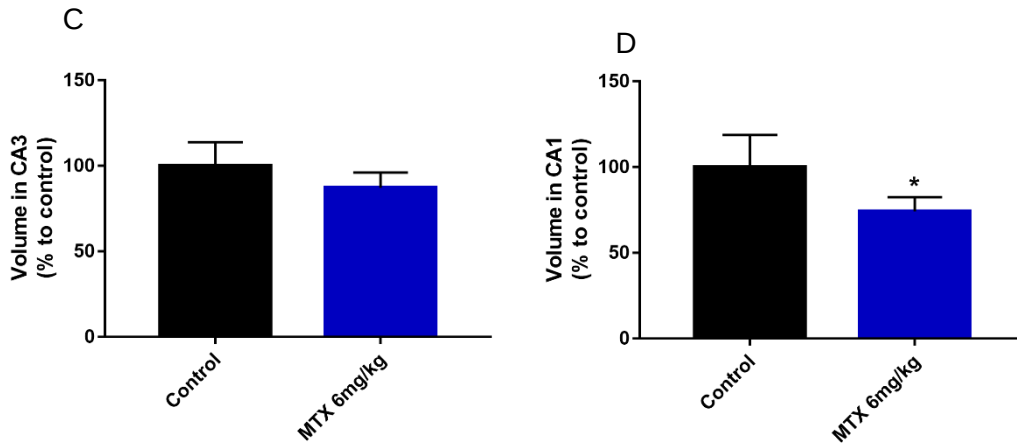
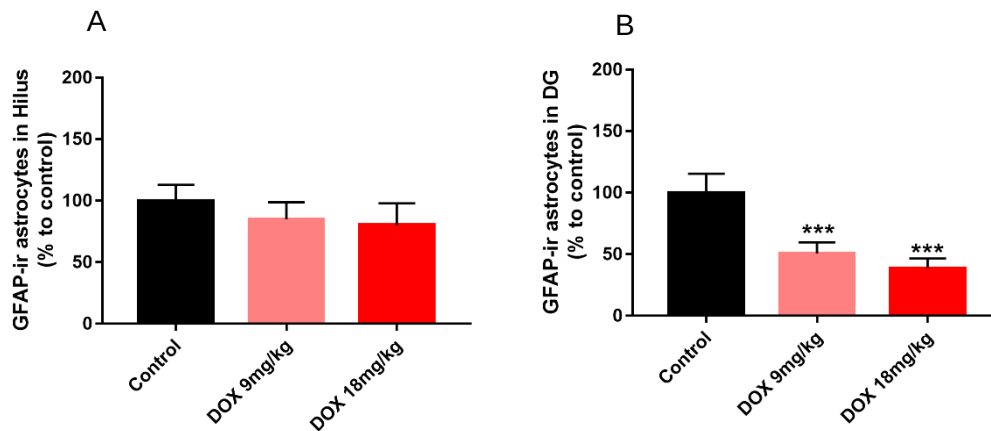


Figure 27: Volume in brain hippocampal regions Hilus (A), DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 6 mg/kg of MTX. Data, as % of mean control, are mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* $p < 0.05$, ** $p < 0.01$ vs control).

4.10. DOX increased GFAP-ir astrocytes in the CA3 region, but decreased in the DG hippocampal region

None of the DOX administrated doses altered the estimated number of total GFAP-ir astrocytes in the Hilus (Figure 28A) or the CA1 brain regions of mice (Figure 28D). However, both the lower (9 mg/kg) and higher (18 mg/kg) DOX doses decreased the GFAP-ir astrocytes in the DG (Figure 28B). Importantly, there was a substantial increase in the CA3 region of GFAP-ir astrocytes evoked by both DOX doses (Figure 28C).



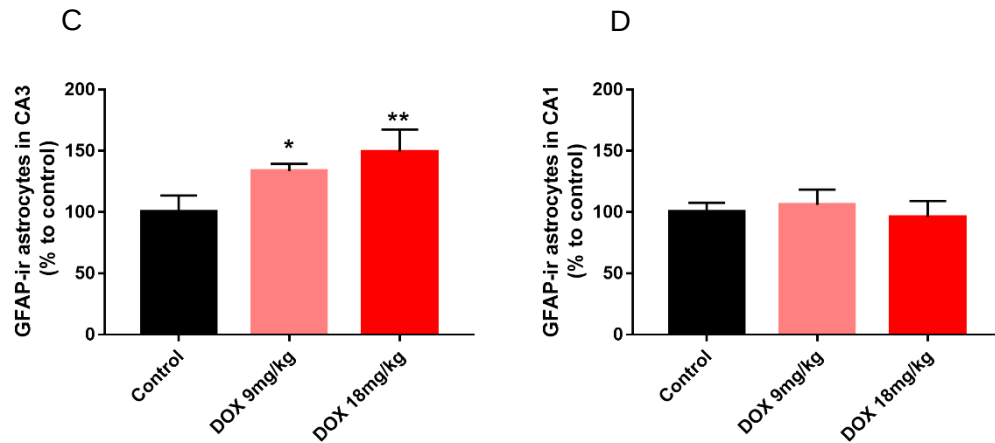
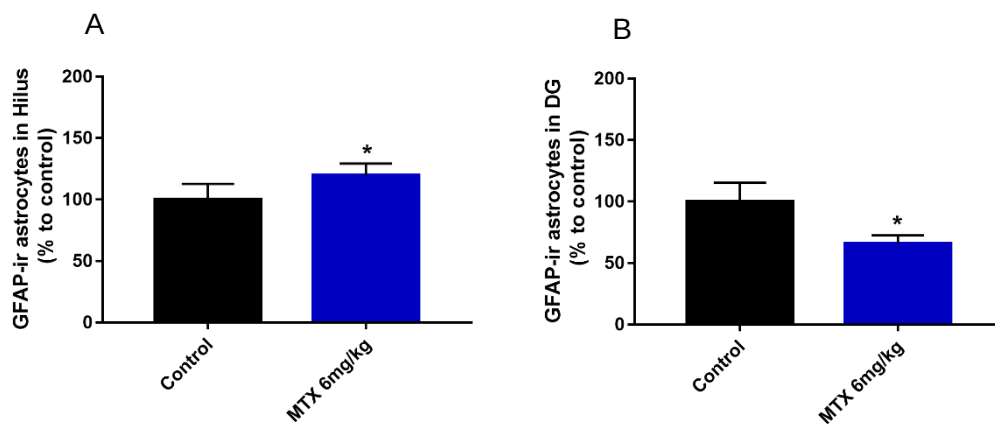


Figure 28: GFAP-immunoreactive astrocytes (GFAP-ir astrocytes) in the hippocampal formation regions Hilus (A), DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 9 mg/kg DOX or a total cumulative dose of 18 mg/kg DOX. Data, as % of mean control, are mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* p <0.05, ** p <0.01, *** p <0.001 vs control).

4.11. MTX-treated animals showed an increase in GFAP-ir astrocytes in three hippocampal regions

A total cumulative dose of 6 mg/kg MTX significantly increased the total number of GFAP-ir astrocytes in the Hilus, the CA3 and CA1 regions (Figures 29A, C, and D respectively). Whereas in the DG, a significant decrease towards controls was seen (Figure 29B).



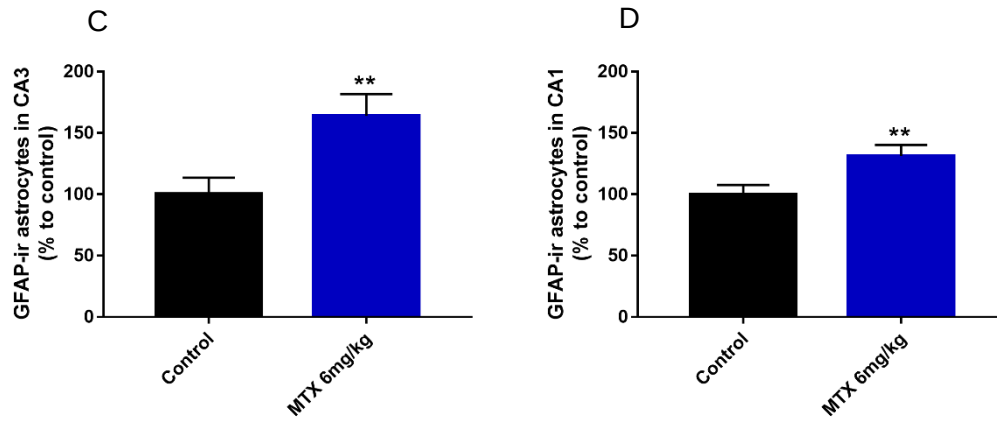
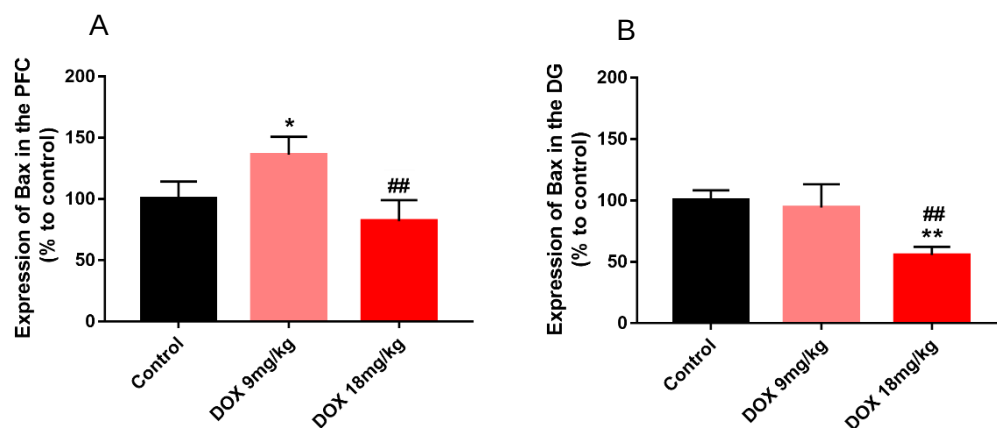


Figure 29: GFAP-immunoreactive astrocytes (GFAP-ir astrocytes) in the hippocampal formation regions Hilus (A), DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 6 mg/kg MTX. Data, as % of mean control, are represented as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using a t-test analysis with Welch's correction (* $p < 0.05$, ** $p < 0.01$ vs control).

4.12. DOX caused dissimilar changes in Bax expression depending on dosing and brain area

Regarding the markers of cell death, the administration of a total cumulative dose of 9 mg/kg DOX lead to an increase in Bax expression in the PFC when comparing to the saline control, whereas the higher dose of DOX (18 mg/kg) caused a decrease in Bax expression in comparison to the 9 mg/kg DOX-treated animals (Figure 30A). Regarding the hippocampal formation, the total cumulative dose of 18 mg/kg DOX diminished the expression of this pro-apoptotic protein when comparing to both the saline control and the lower dose of DOX (Figure 30B). Nevertheless, in CA3 region, the administration of 9 mg/kg DOX lead to an increase in Bax expression in relation to the control group expression (Figure 30C). The CA1 region did not present any statistically significant alterations in Bax expression in any of the DOX doses administrated (Figure 30D).



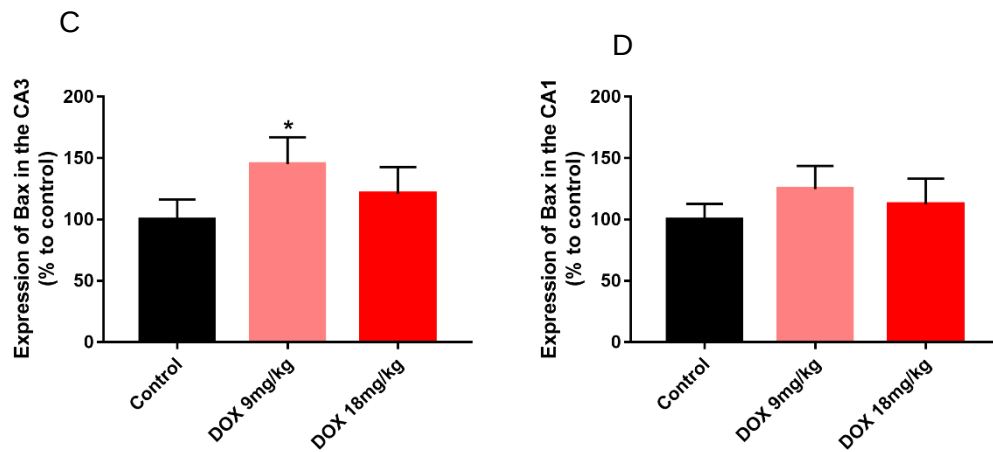
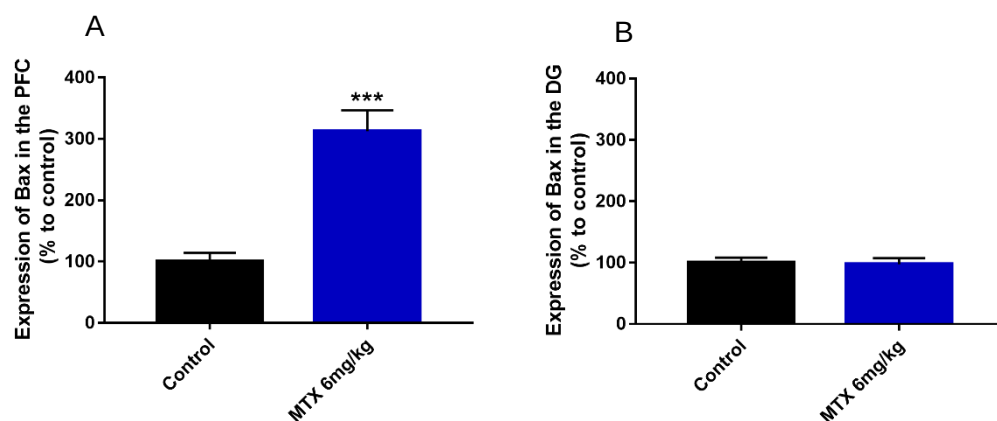


Figure 30: Bax expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 9 mg/kg DOX or a total cumulative dose of 18 mg/kg DOX. Results, as % of mean control, are mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* p <0.05 and ** p <0.01 vs control, ### p <0.01 vs 9 mg/kg DOX).

4.13. MTX increased Bax expression in both PFC and CA3 region of the hippocampus

The total cumulative dose of 6 mg/kg MTX caused approximately a 3-fold significant increase in PFC Bax expression in comparison to the saline control group (Figure 31A). The hippocampal CA3 region displayed an increase in Bax expression in MTX-treated animals, (Figure 31C), when compared to control animals. Meanwhile the DG and CA1 (Figure 31B and D) MTX promoted no significant alterations when comparing to the control group.



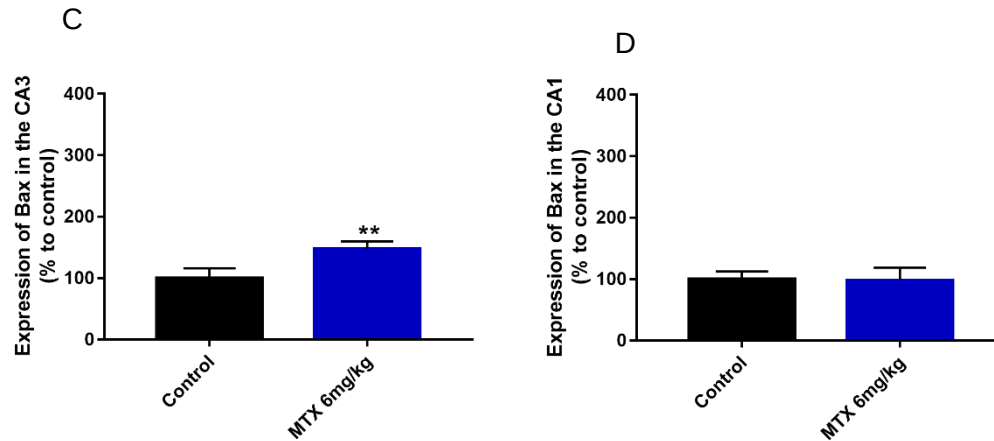
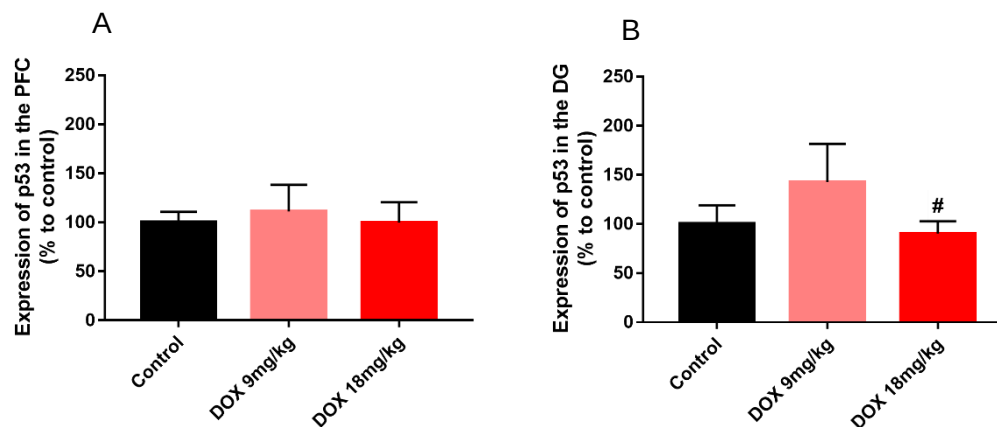


Figure 31: Bax expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 6 mg/kg of MTX. Data, as % of mean control, are represented as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using a t-test analysis with Welch's correction (* p <0.05 and ** p <0.01 vs control).

4.14. The lowest dose of DOX increased p53 expression in the CA3 region

Neither the lower (9 mg/kg) nor the higher (18 mg/kg) total cumulative dose of DOX affected the expression of p53 in the PFC area (Figure 32A). The treatment of 18 mg/kg DOX evoked a decrease expression of p53 when comparing to the lower DOX dose in the DG and CA3 of the hippocampal formation (Figure 32B and C), but not in comparison to control animals. In contrast, the total cumulative dose of 9 mg/kg increased p53 expression in the CA3 region when comparing to the control expression (Figure 32C). Meanwhile, the CA1 region was unaffected by any of the DOX doses administrated (Figure 32D).



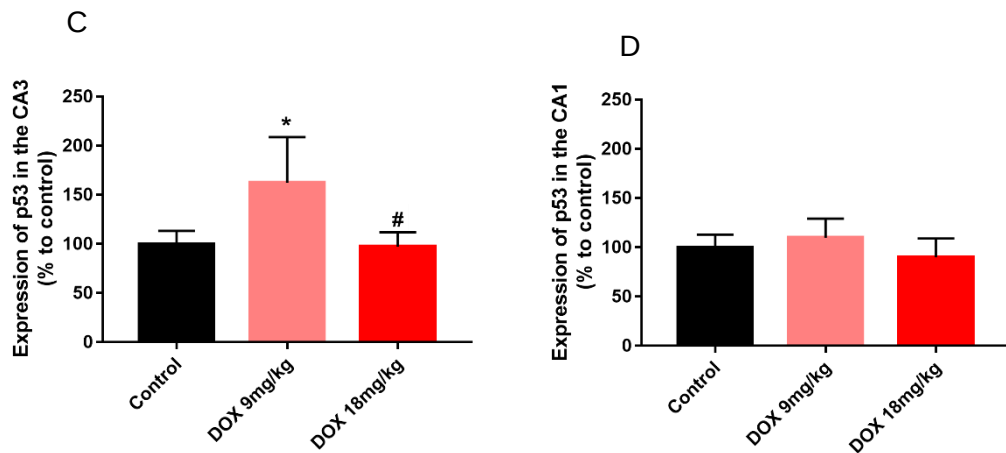
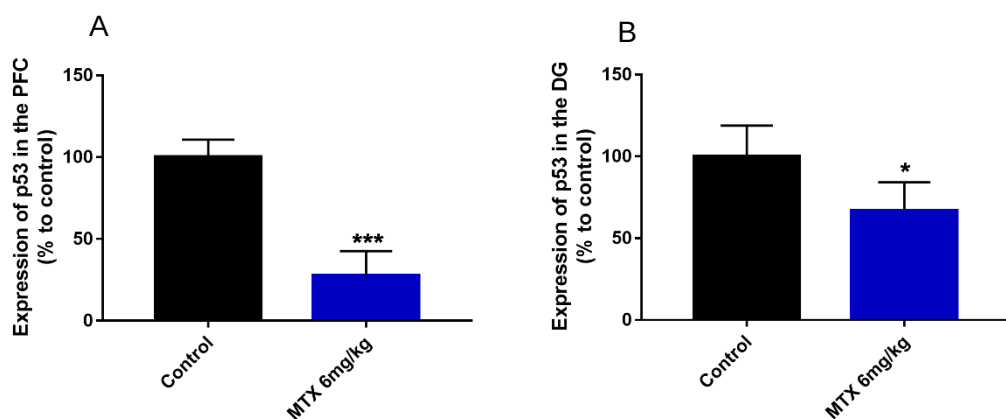


Figure 32: p53 expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 9 mg/kg DOX or to a total cumulative dose of 18 mg/kg DOX. Data, as % of mean control, are represented as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* p <0.05 vs control, # p <0.05 vs 9 mg/kg DOX).

4.15. MTX-treated mice presented decreased expression of p53 in all brain areas evaluated

The administration of a total cumulative dose of 6 mg/kg MTX decreased significantly the p53 expression in all the areas evaluated, being the higher reduction in the PFC area in comparison to the control groups (Figure 33).



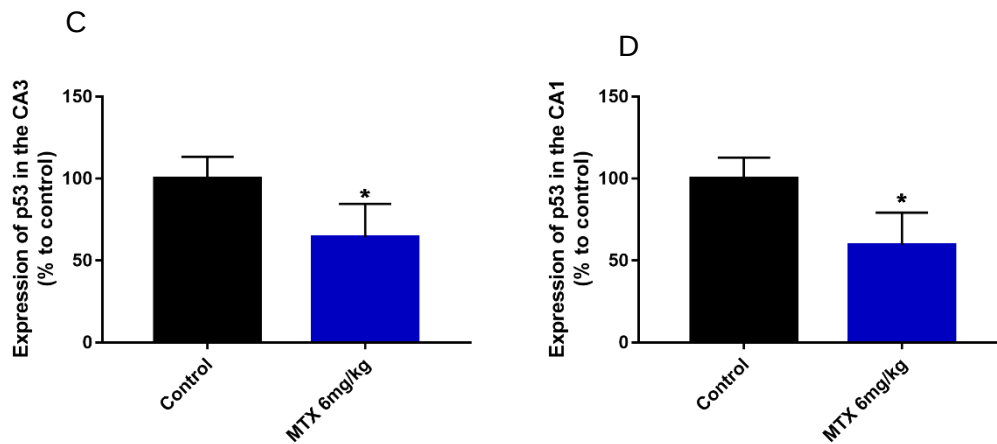
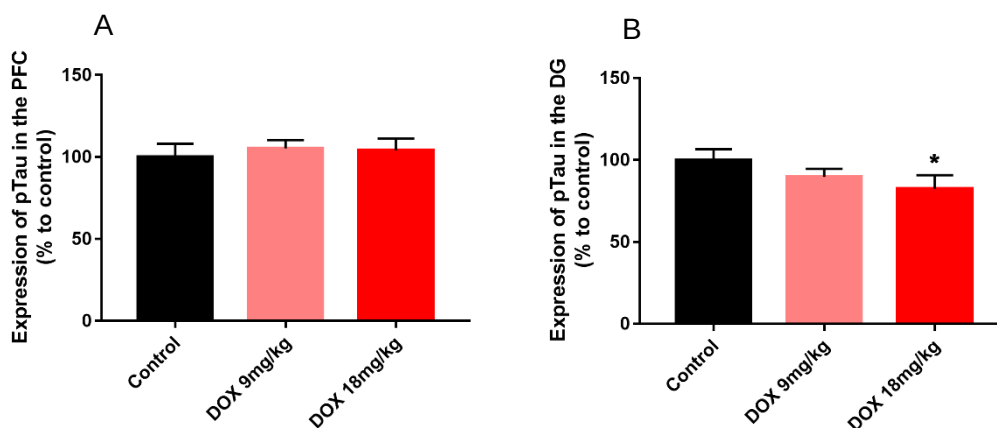


Figure 33: p53 expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 6 mg/kg MTX. Data are presented in % of mean control and as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using a t-test analysis with Welch's correction (* p <0.05 and *** p <0.001 vs control).

4.16. Mice given the dose of 18 mg/kg DOX had less pTau in the DG and CA3 regions

None of the administrated doses of DOX altered the levels of phosphorylated Tau protein in the PFC (Figure 34A). In the hippocampal formation, the administration of a total cumulative dose of 18 mg/kg caused a decrease in pTau levels in the DG and CA3 regions in comparison to the saline controls (Figures 34B and C), whereas the CA1 region did not evoke changes (Figure 34D).



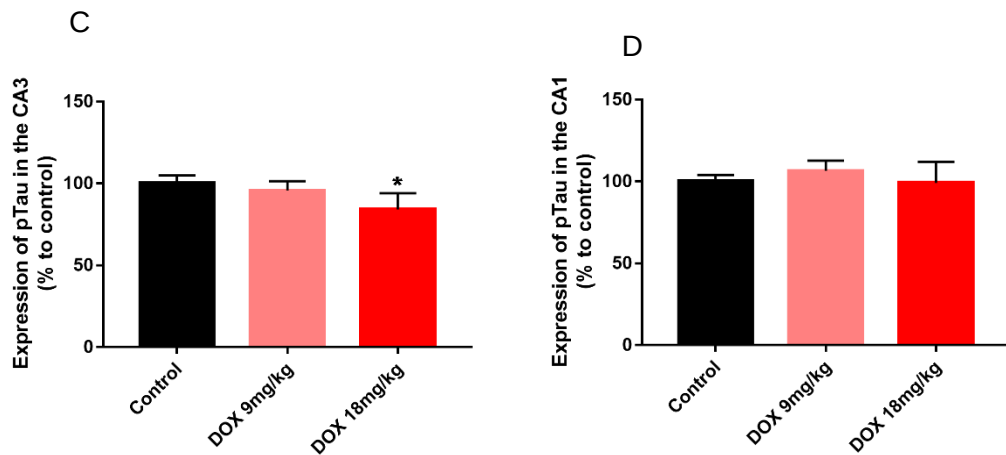
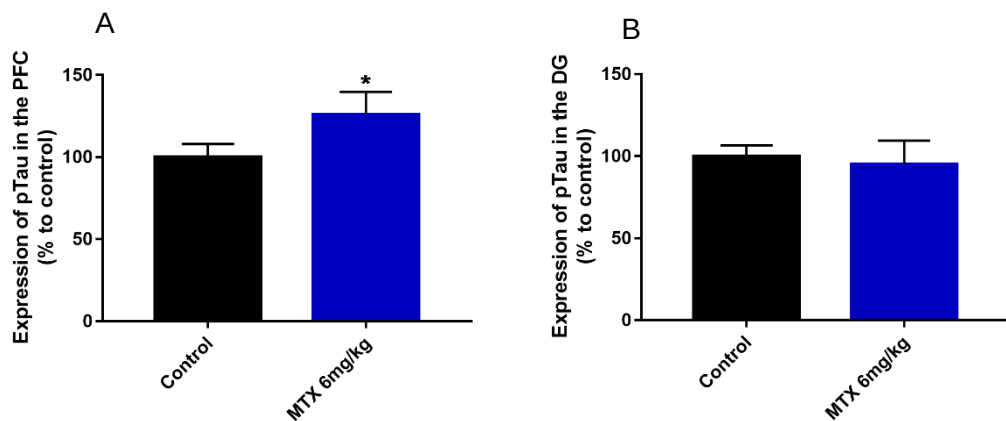


Figure 34: pTau expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 9 mg/kg DOX or a total cumulative dose of 18 mg/kg DOX. Data, as % of mean control, are presented as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test (* $p < 0.05$, vs control).

4.17. MTX increased pTau in the PFC area

MTX-treated mice had an increased in Tau phosphorylation in the PFC when comparing to the saline-control group (Figure 35A). The regions of the hippocampal formation evaluated in this work did not present significant alterations in pTau expression when compared to controls (Figure 35B to D).



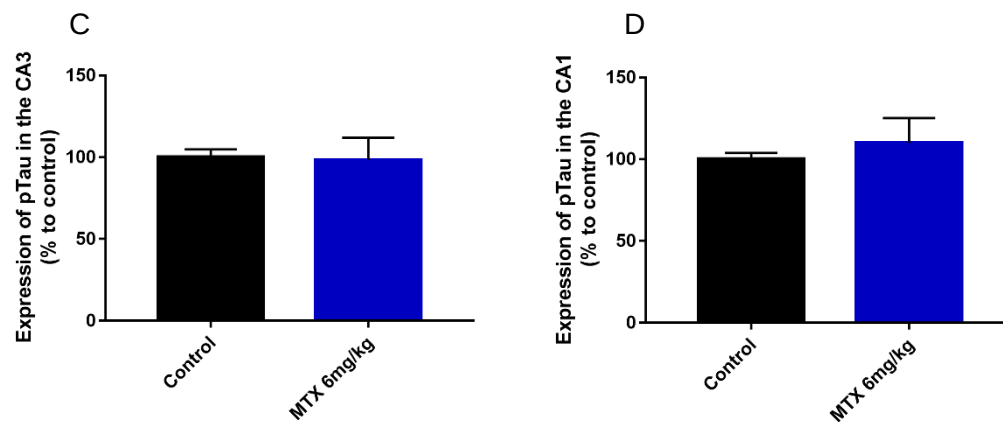


Figure 35: pTau expression in the PFC (A), and hippocampal formation regions DG (B), CA3 (C) and CA1 (D) in adult mice brain exposed to a total cumulative dose of 6 mg/kg MTX. Data, as % of mean control, are presented as mean $\pm$ SD from 4 animals in each group. Statistical comparisons were made using a t-test analysis with Welch's correction (* $p < 0.05$ vs control).

5. Discussion

The major findings of this dissertation were: 1) DOX, especially in the highest cumulative dose used, caused a significant weight loss in the adult and infant population; 2) adult and infant DOX-treated mice presented considerable less food and water intake than control mice; 3) DOX treatment did not elicit changes towards controls in brain weight, meanwhile MTX-treated old mice had a slight increase; 4) only the highest dose of DOX (18 mg/kg) caused redox homeostasis changes, with decreased tGSH and GSH levels; 5) only the total cumulative dose of 18 mg/kg DOX caused meaningful alterations in ATP levels, that was increased the infant-treated population; 6) in adults the hippocampal volume increased in the all the regions of the mice administrated with a total cumulative dose of 18 mg/kg DOX; 7) MTX-treated mice had decreased hippocampal volume in the DG and CA1 regions; 8) DOX increased GFAP-ir astrocytes in the CA3 region, but decreased in the DG hippocampal regions in adult mice; 9) MTX caused an increase in the total estimated GFAP-ir astrocytes in the Hilus, CA3 and CA1 regions, while a significant decrease was seen in the DG region, when compared to control adult animals; 10) Bax expression in adult mice brain was increased by the lowest dose of DOX (9 mg/kg) in the PFC and CA3, whereas the highest dose of DOX (18 mg/kg) decreased Bax expression in the PFC and DG; 11) MTX-treated adult mice expressed significant higher levels of Bax in the PFC and CA3; 12) the p53 expression increased in the CA3 regions of 9 mg/kg DOX-treated adult mice; 13) MTX-administrated adult mice presented a decline in p53 expression in the PFC and all of the hippocampal formation areas evaluated; 14) and the highest DOX dose (18 mg/kg) triggered a decrease in pTau in the DG and CA3, while 6 mg/kg MTX increased pTau in the PFC.

Chemotherapy has high rates of success in the treatment of cancer. However, the use of chemotherapeutics, such as DOX and MTX, has several adverse short and long-term effects, diminishing the patients' quality of life (16). After receiving chemotherapy, cancer survivors present cognitive deficits. Initially, that cognitive dysfunction was attributed to anxiety or depression, but longitudinal studies, mostly on women with breast cancer concluded that chemotherapy in fact can cause long-term cognitive impairment also known as chemobrain (279-281). While the BBB has several mechanisms that protect the brain from DOX and MTX access, it seems that theses chemotherapeutic agents can damage the brain through still not fully disclosed mechanisms.

The animal model used in this study allowed to study several underlying molecular mechanisms of chemotherapy-induced neurotoxicity, including the long-term effects on 3 different aging stages. The administration schedule of the CD-1 male mice was designed

to mimic the human anticancer therapy with MTX or DOX, since these drugs are given in cycles and multiple administrations in the anticancer therapy (and also in multiple sclerosis in the case of MTX) (262, 263). This procedure allowed the translation to clinic setting and to access the gradual development of neurotoxicity, as it occurs in real human scenario, instead of evoking acute neurotoxicity, which does not correlate with the clinical reality of anticancer therapy nor to the clinical manifestations of chemobrain. The clinical relevancy of the total cumulative doses used in this dissertation was calculated through allometric scaling to achieve clinically relevant doses and, importantly, far below the total lifetime cumulative dose recommended for DOX and MTX in humans (13). The injections were administrated in the afternoon period since mice are described to be less susceptible to the toxicity of MTX due to the circadian changes in plasma pharmacokinetics of MTX (282) and alternating between the left and right side of the abdomen to decrease discomfort. Despite the intravenous route being more used in human anticancer therapy (283), in mice there is the high risk of extravasation of the drug which could cause necrosis. Therefore, the i.p. administration route was chosen since it is more accessible, provides a large absorption area and causes less discomfort to the animal. Furthermore, a previous study demonstrated that MTX when administrated by the i.p. injections, it causes similar toxic effects to those observed in humans (268). Moreover, the fast growth rate of the animal model used herein allowed to observe the cumulative neurotoxic effects after chemotherapy in the drug-free period (a week in adult and old mice, and seventeen days in infant mice after the last administration). When comparing development in mice to humans (284, 285), at the beginning of the experiment to the day of sacrifice the 'human age' of the infant, adult and old mice was approximately 180 days, 20 years and 56 to 62 years respectively, whereas at the end of the experiment the corresponding 'human age' was 9 years, 25 years, and 64 to 71, respectively. Therefore, the three aging stages used in this experiment encompass different age groups affected by cancer diagnosis (2) and most of all address neurotoxicity aspects that may subside few years after remission.

5.1. Mice weight, food, and water intake

Throughout the experiment, the mice well-being was monitored, as well as food and water consumption. In the first experiment, the total cumulative dose of 18 mg/kg DOX significantly decreased the average body weight of adult and infant mice. The significant weight loss in adult mice started soon after they received the last i.p. injection, whereas the infant population did not gained weight as control animals after they received the 5th i.p. injection. In opposition, older mice treated with the lowest total cumulative dose of 9 mg/kg

DOX did not suffer significant weight variations. In contrast to DOX-treated animals, MTX treated did not show any weight differences to controls. Of note, infant mice had significant weight differences in the beginning of the experiment due to poor random distributions, but weight gain was comparable to controls, ruling for a non-interference of MTX in this parameter. In the second experiment only done with adult mice, the highest DOX dose caused significant weight loss after the 4th administration. While in the first experiment, the total cumulative dose of 9 mg/kg DOX did not affect the older population, in this cohort of adults that same dose caused significant weight loss after the last injection. Moreover, in opposition to what was observed in the first experiment, the MTX-treated adults of the second experiment presented significant weight loss at the day of sacrifice. The higher number of animals per condition in the second experiment allowed to observe more subtle differences. Weight loss has been associated with DOX treatment in infant (286, 287) and adult (287, 288) mice. Regarding to older mice, so far, no data was found. Likewise, weight loss has been reported as a common side-effect of MTX in infant (268) and adult (268, 282) mice, with no data so far available on older mice.

To ascertain if differences in average body weight were related to food/ water consumption, these parameters were registered at least twice a week. The food and water were provided *ad libitum* for each cage and the amount consumed was calculated per animal based on individual animal body weight, assuming that every animal consumed in a proportional manner to the respective body weight. In the first experiment, comparing to the respective control-saline group, adult mice treated with a total cumulative dose of 18 mg/kg DOX decreased progressively the food intake after the 2nd administration and drunk less water after the 4th DOX-injection. The infant mice treated with the same DOX cumulative dose also consumed less food after the 2nd administration and drunk less water after the 5th administration. The older DOX-treated mice suffer no major changes in consumption patterns. Meanwhile, MTX-treatment caused no significant alterations in all ages. Of note, MTX-treated infant mice ate more in the beginning of the experiment in comparison to the saline-control group, a result that is line with the higher weight of this animal group. However, as previously mentioned, this difference was possibly due to the random assorting of the litters. Concerning the adult mice of the second experiment, the consumptions were registered more frequently allowing a more precise monitorization. Both DOX doses caused a steady decrease in food and water intake; however, the higher DOX dose caused the most abrupt decreases in food and water consumption. MTX administrations also did not affect this cohort of adult mice. Clearly, the weight loss of DOX-treated adult and infant animals is correlated with less food intake, ruling for a DOX promoted effect. Taken together, these DOX results and the weight loss in infant and adult mice population, it is

evident that DOX cycles of administration cause higher morbidity in these ages and affect more the mice well-being than MTX administration in the doses under study. One could assume that the difference is due to the higher dose of DOX, however approximately 4.5 mg DOX are clinically equivalent to 1 mg MTX (13). Moreover, despite the concern of the putative more fragile state of the older mice, where only a lower cumulative DOX dose was used, their weight and food intake was not affected either by DOX or MTX administration. Loss of appetite, nausea, vomiting, diarrhoea, and other gastrointestinal problems are common side effects due to the cytostatic properties of chemotherapeutic agents in the non-targeted healthy cells of the gastric tract (289-291) and consequent result in weight loss. Nevertheless, MTX in clinical settings is reported to cause less nausea, vomiting and overall, it is more well-tolerated (292, 293), which can explain the different results in mice weight and food intake that are here reported for the two drugs. In agreement, in 90 breast cancer patients randomized to either receive MTX (total cumulative doses ranging from 12 to 177 mg/m²) or DOX (total cumulative dose ranging from 182 to 491 mg/m²) as a second line treatment in breast cancer, both with comparable clinical efficacy, MTX caused less nausea and vomiting, diarrhoea, anorexia and stomatitis (294).

At the day of sacrifice, the brain of adults was removed and weighted. DOX animals revealed no changes towards controls, but MTX-treated old mice had a slight increase in brain weight. Changes in brain weight may indicate either edema or that changes at the structural level might be happening, albeit it is merely indicative (295) and more detailed studies need to be done, as proceed here.

5.2. Brain redox status

As mentioned, the brain is highly susceptible to oxidative stress mainly due to its high oxygen consumption and lipid-rich content (296). To further enhance this susceptibility, it has poorer antioxidant defences in comparison to other organs (297). When the antioxidant defences ability is impaired and they cannot detoxify at the rate that the reactive species are generated, a redox imbalance happens leading to oxidative stress (296). Oxidative stress has been pointed as one of the main causes of chemotherapy-induced neurotoxicity (210, 298), and both DOX and MTX are known to induce oxidative stress in non-target organs, either directly or indirectly (268, 299). Considering this, the total brain levels of tGSH, GSH, GSSH were measured as possible indicators of the redox balance in the brain of DOX- and MTX-treated mice of different ages. The antioxidant GSH is vital for the redox balance. It is a tripeptide (γ -L-glutamyl-L-cysteinylglycine) and the most abundant non-protein thiol (300). It acts as an antioxidant by directly binding with electrophiles (with

100

or without catalysis of glutathione S-transferases) or by forming the dimeric oxidized form GSSG and eliminating peroxides (301). In this dissertation, the highest total cumulative dose of DOX caused brain oxidative stress as seen by a decrease in brain levels tGSH and GSH only in the adult mice. These changes were not accompanied by changes in GSSG turnover and consequently decrease in GSH/GSSG. Moreover, the total cumulative dose of 9 mg/kg DOX did not cause any significant alteration in the glutathione parameters of the adult population, suggesting a dose-dependent action of DOX. Moreover, in oxidizing conditions GSH can form adducts with electrophilic reactive molecules, which can explain DOX-induced oxidative stress with no concomitant increase in GSSG levels (302), but rather a decrease in GSH. Moreover, high intracellular levels of GSSG are toxic and to avoid major change in the redox balance when oxidative stress overcomes the capacity of the cell to reduce GSSG to GSH, GSSG can be actively exported out of the cell or it could react with a protein thiol and originate protein disulphides that are not detectable by the used method (303). Studies in male mice (2 to 3 months old) administrated with a single i.p. dose of 20 mg/kg DOX and sacrificed 72 hours after, reported increase in whole brain GSH levels (201) and a significant decrease in the GSH/GSSG ratio (304). A single dose of 25 mg/kg DOX also significantly raised brain levels of GSH and increased GSSG levels (206). This clearly shows that a lower cumulative dose done in cycles, like the 18mg/kg use in this work, can cause decrease on GSH levels with great impact on brain redox status, when compared to higher doses seen in the literature. Therefore, it seems important to mimic real clinical scenarios using cycles of chemotherapy administration instead of single drug administrations.

Even though the infant mice received the same total cumulative dose of 18 mg/kg as the adult mice, no significative changes in the redox status were observed, demonstrating the better resilience to the DOX-induced oxidative insult than adults. Antioxidant defences decrease with age (305) and oxidative stress occurs as part of natural aging (306), so one could assume that, in comparison to adult mice, infant mice can revoke the oxidative stress more efficiently. Notwithstanding the fact that the total cumulative dose of 9 mg/kg DOX did not affect the glutathione parameters in adult mice, the controls of the older mice that have lower basal levels of GSH should in theory be more susceptible. Surprisingly, the total cumulative dose of 9 mg/kg DOX did not cause considerable alterations in the brains' redox balance of older mice. A study using young (2 months), adult (10 months) and old (18 months) rats administrated with a single i.p dose of 10 mg/kg DOX evaluated the basal and DOX-induced antioxidant enzyme activities on the brain. The brain enzymatic antioxidant defence was evaluated 12 hours after administration. The basal activity (untreated rats) of catalase, GPx and GR did not differ significantly among age

groups, but the SOD activity was significantly lower in elderly when comparing to the other ages. Following the DOX treatment, only the young animals had significant induction of the enzymatic antioxidant defence by increasing the activity of the enzymes evaluated in comparison to the basal levels. When a biomarker of lipid peroxidation (thiobarbiturate acid reactive species) was evaluated in the different age-grouped animals 24 hours after the i.p. treatment with the same DOX dose, the older animals had the greatest increase over the basal level, followed by the adult rats, while no significant changes were seen in young rats (307). Even so, with the present protocol and administration regimen we did not find significative redox changes in DOX-treated elderly mice.

Regarding the administration of a total cumulative dose of 6 mg/kg MTX, none of the age-groups studied presented significant alterations in the brain redox balance. Nonetheless, MTX is reported to induce peripheral oxidative stress in multiple sclerosis patients by increasing oxidative modification of blood-serum proteins (252), but so far there are no data available on brain redox status. Of relevance, in the SH-SY5Y human neuronal cell model, MTX caused significant neurotoxicity and the induced neurotoxicity was partially prevented by using tiron, an antioxidant (217). Moreover, MTX causes peripheral oxidative stress but related with mitochondrial damage and electron leakage (308). Finally, although DOX and MTX share the same pharmacological anticancer mechanism of action, it has been observed that in regards to their toxicity in other organs, DOX is more prone to redox imbalance, whereas MTX induces energy imbalance (309).

5.3. Brain energetic status

ATP is a high-energy phosphate compound and supplies the energy needs of several cellular activities. The brain high demand for energy is assured in the most part by ATP that is formed through mitochondrial oxidative phosphorylation of adenosine diphosphate (ADP) catalysed by the ATP synthase (310). Due to the core role of ATP in cerebral bioenergetics, it was measured in the brain of differently aged mice submitted to DOX or MTX. In the present study, only the total cumulative dose of 18 mg/kg DOX caused meaningful alterations by increasing the ATP levels of the infant population. An increase in ATP levels was found to occur in cerebellar granule cells when apoptosis was occurring (311). In rat cortical neurons exposed to DOX, significant depletion of ATP was observed in a biphasic manner, being that at 0.1 μM and 0.5 μM DOX, ATP levels decreased, then recover at 5 μM DOX to approximately control levels and decreased again at 20 μM DOX (222). In the infant mice in the protocol set in this dissertation, an adaptive phenomenon might have happened to cope with DOX-induced injury. Considering MTX, although it is

known to deplete hepatic (312), and cardiac (308) ATP, the data on this thesis does not corroborate that and the total cumulative dose of 6 mg/kg did not interfere with the brain ATP levels in any of the differently aged mice tested. Furthermore, there is no data available on the effect of MTX in brain or other brain in vitro model ATP levels to the best of existing knowledge.

5.4. Hippocampal formation volume

The hippocampal formation is a brain structure that has been extensively studied for its major role in memory and cognition (313). It has been often found impaired in chemotherapy-treated patients, especially in those treated with anthracyclines such as DOX (91, 131). Starting from edge of the “C-curve” of this structure is the DG, where neuronal stem cells give rise to neurons in the adult brain (38). It presents 3 distinct layers, an outer molecular layer, a middle granule cell layer and an inner polymorphic layer also known as the hilus, followed by the cornu ammonis subfields: CA3, CA2, CA1 or also known as the hippocampus proper (313). Shrinkage of this structure has been seen as a result of toxic insults and with consequent cognitive decline (314, 315). Hippocampal formation volume of DOX and MTX treated adult mice was measured through the Cavalieri’s Principle. Additionally, only the dorsal part of the hippocampal formation was evaluated as it is the part correlated primarily to cognitive functioning, which is found to be impaired after chemotherapy (65), whereas the ventral is more involved in stress, emotion and affect (316).

In the present dissertation, the highest total cumulative dose of DOX increased hippocampal formation volume in all of the regions evaluated with the most pronounced increase seen in the hilus, which is the inner polymorphic layer of the DG (317). Based on the literature available, there are no reports of chemotherapy-treated patients or animals studied displaying an increase in hippocampal volume. Nevertheless, hippocampal volumetric enlargement can happen due to cytotoxic edema, which is swelling of brain cells that precedes the onset of cell death (318). Oxidative stress has been reported to be involved in increased vascular permeability and edema development (319). As previously mentioned, DOX is a well characterized inducer of oxidative stress (320) and in this dissertation the total cumulative dose of 18 mg/kg DOX induced oxidative stress in the adult mice brain.

On the other hand, the total cumulative dose of 6 mg/kg MTX decreased the volume of DG and CA1. Despite the lack of data regarding MTX effects on the brain, this result

corresponds to what has been observed for other chemotherapeutic agents. Chemotherapy decreases hippocampal cell proliferation, neuroplasticity, blood vessel density (321-323) and increases neuronal apoptosis (323), resulting in loss of hippocampal integrity and volume with consequent cognitive dysfunction (324).

5.5. GFAP-ir astrocytes

Astrocytes are the largest and most abundant glial cell, outnumbering neurons by five times. They support the neuronal function in a variety of functions, such as homeostatic regulation, increasing synaptic plasticity and are involved in neuroinflammatory process as astrocytes have both pro- and anti-inflammatory functions (325). During brain insult, mature astrocytes become more reactive by releasing extracellular molecules such as neurotrophic factors, inflammatory factors and cytotoxins. Those changes are accompanied with morphological changes such as increased and longer processes and more thick and larger cellular bodies (326, 327). Furthermore, it is also observed increased astrocyte proliferation and increased GFAP expression in reactive astrogliosis (328). Therefore, one of the hallmarks of astrogliosis is the increase in GFAP expression (329). GFAP is the principle intermediate filament protein in mature and reactive astrocytes but it also belongs to part of the cytoskeleton in developing astrocytes (330), so it is also present in non-reactive astrocytes but less immunohistochemically detectable (329). Given that neuroinflammatory processes have been associated with chemotherapy-induced cognitive dysfunction (244), the total number estimation of GFAP-ir astrocytes was made in the hippocampal formation.

The lowest and highest total cumulative dose of DOX caused the same alteration brain patterns: total number of GFAP-ir astrocytes decreased in the DG whereas in the CA3 region an increased number was seen. The pronounced decrease in the DG is most likely due to both increased astrocyte death and decreased proliferation as a consequence of the DOX insult. While there is not data reporting GFAP-ir decreases in the DG due to DOX administration, reduced number of GFAP-ir in the DG was found in individuals with major depressive disorder (331). The effects of DOX administration are further seen in the CA3 region with the increase in the reactive astrocytes. Similar results were obtained in the hippocampus of adult (3 months) rats treated i.p. with a total cumulative dose of 10 mg/kg DOX over 4 weeks (332) and in rats treated with a total cumulative dose of 8 mg/kg DOX also in the time-span of 4 weeks (332). All animals of the mentioned studies had considerable impaired cognitive function in domains dependent on proper hippocampal function, similar to what is observed in cancer patients treated with regimens that include DOX (333).

MTX administration also caused decreased GFAP-ir astrocytes in the DG, possibly due to the same reason that was pointed in the DOX treatment, meaning astrocyte death and decreased proliferation. In other three hippocampal regions evaluated, the number of GFAP-ir astrocytes increased considerably, a clear indication of astroglyosis and a possibly linked to the cognitive dysfunction described in patients (180). Exposure to MTX in concentrations of 10 and 50 µg/L MTX enhanced mRNA levels of GFAP in Zebrafish embryos (334). Apart from this study, so far, there are no studies to corroborate the obtained results, but in studies with other cytostatic agents described as to not cross the intact BBB, the total number of GFAP-ir astrocytes also increased (332, 335).

5.6. Bax expression

Bax is an apoptosis-promoting factor of the Bcl-2 family (336) usually located in the cytosol. Upon an apoptotic stimulus, it promotes cell death by suffering conformational changes and consequent translocation to the mitochondria and insertion in the outer mitochondrial membrane. This translocation results in a subsequent pore formation and cytochrome c leakage to the cytosol culminating in irreversible apoptosis (337, 338). This cell-programmed mitochondrial pathway is regulated mainly by the Bcl-2 protein family (339). Considering that DOX and MTX induce cell apoptosis in other non-target tissues (340, 341), the effects on Bax expression in the PFC and hippocampal formation of the systemic administration of these two cytostatic agents was evaluated.

DOX administration showed dissimilar effects depending on the dose administrated, being that the total cumulative dose of 9 mg/kg DOX increased Bax expression in the PFC and CA3 region, while the total cumulative dose of 18 mg/kg DOX decreased Bax expression in the PFC and DG region. Bax increased expression was observed in mice administered with a total cumulative dose of 8 mg/kg DOX for 4 weeks (323), however the effects of higher DOX doses were not evaluated by the authors. It is possible that the highest dose of DOX caused a similar Bax increase expression earlier in the experiment described in this dissertation or most likely that, at the highest cumulative DOX caused other mechanism of cellular death, namely necrosis. Dual-way cell death depending on the DOX dose was reported in an *in vitro* study where cortical neurons exposed to lower doses of DOX (up to 0.5 µM) elicited apoptotic death whereas for higher concentrations apoptosis was inhibited and necrosis became dominant (221).

In regard to MTX-treated mice, Bax expression largely increased in the PFC and CA3 region, suggesting apoptotic pathway activation. Unfortunately, until this moment,

there are no studies evaluating the effects of Bax expression on the brain upon MTX use. More studies with other brain biomarkers of cell death and apoptosis are needed to clarify whether neuronal death is induced following exposure to chemotherapeutic drugs.

5.7. p53 expression

The tumour suppressor p53 plays a key role in the response to cellular stress by acting as a transcription factor that regulates the expression of genes responsible for cell proliferation and death, and DNA repair (342). Among the many cellular processes controlled by this factor, p53 mediates cellular apoptosis by activating the proapoptotic proteins of the Bcl-2 family, such as Bax to permeabilize the mitochondrial membrane (343). In this dissertation, the expression of p53 was determined in the PFC and hippocampal formation of DOX and MTX administrated mice.

The total cumulative dose of 9 mg/kg DOX increased considerably p53 expression in the CA3 region in comparison to control animals; meanwhile the highest dose did not cause any significant changes. Considering the influence of p53 in Bax activation, it is important to note that Bax expression also increased in 9 mg/kg DOX-treated mice. The lowest DOX dose induced increase of both p53 and Bax expression in the CA3 region, indicating that apoptotic death might be occurring in this region; however other biomarkers are needed to evaluate this. On the contrary, in the DG and CA3 of 18 mg/kg DOX treated mice, Bax decreased and p53 only decreased towards the lower dose of DOX, which indicates that apoptotic death might have happened in another time-point before the mice sacrifice or most likely necrosis became the major cell death pathway. Supporting this notion is the study of Tangpong where a single i.p. dose of 20 mg/kg DOX increased p53 expression 3 hours after DOX administration accompanied with Bax increase, initiating apoptotic cell death. Twenty-four hours after administration the levels returned to normal, due to compensating increase in anti-apoptotic factors such as Bcl-xL that promote cell survival (205).

The MTX treatment decreased p53 in all brain areas evaluated, being the most robust decrease seen in the PFC. These results do not match the increase of Bax expression seen in two hippocampal regions, and further studies would be needed to understand this mechanism. The p53 is key on regulating the cell cycle by inducing cells cycle arrest, senescence or apoptosis when there is cell damage. Nevertheless, its deletion causes several mitochondrial pathway disruptions (344) and has been described as involved in neurodegenerative diseases, (345) however other p53 roles are still under investigation.

5.8. Tau expression

Tau is a microtubule-associated protein primarily expressed in the axons of neurons and its main functions are stabilization of microtubules packs and control microtubule assembly, dynamic behaviour, and spatial organization (346). Tau function is mainly regulated through post-translational modifications, being the phosphorylation at many sites the highly prominent (347). Increasing Tau phosphorylation reduces its interaction/ affinity with microtubules and consequently the neuronal cytoskeleton becomes unstable (348). The detachment of hyperphosphorylated Tau results on its abnormal aggregation and deposition, forming paired helical filaments and progressing to intracellular neurofibrillary tangles (349). Phosphorylation of Tau is a hallmark of aging brain but also of many neurodegenerative diseases with concomitant cognitive impairment (350). Therefore, impact of DOX and MTX administration in phosphorylation of Tau was evaluated. Tau phosphorylation expression was evaluated by the levels of Tau protein phosphorylated at site S396 as it is reported to cause weaken interaction with the microtubules (351).

The highest dose of DOX induced a decrease in the pTau expression in the DG and CA3 regions. One possible explanation is that the Tau expressing cells such as neurons are less present due to necrotic death, or other internal mechanisms of cellular regulation are occurring that rendered a decrease in pTau expression.

The PFC of the MTX treated adult mice was the only area that displayed significant increases in pTau levels. Once again, due to the poorly studied neurotoxic effects of MTX, there are no comparative studies. Nevertheless, increasing in Tau aggregates and premature brain aging accompanied with cognitive impairments occurred in the hippocampus of cisplatin-treated adult (7-8 months) mice (352). Herein it is demonstrated that MTX might accelerate brain aging in the PFC, as it is evident by the increase in pTau. Also, this event could contribute to the cognitive dysfunction seen in patients following MTX treatment. One of the proposed causes for increasing pTau may be linked to increased levels of brain mitochondrial oxidative stress (353) and MTX is known to induce mitochondrial damage in non-targeted organs (308).

6. Conclusions

The main goal of this dissertation was to evaluate the effects of clinically relevant doses of DOX or MTX in brain redox and energetic status of differently aged mice (infants, adults and old) and evaluate markers of brain damage in the adult mice population. The monitorization of the mice weight and food intake showed that adults are more susceptible to DOX evoked changes, since considerably less food and water intake with consequential weight loss/less weight gain, whereas MTX administration did not cause significant alterations in any of these parameters. Regarding the brain redox status, only the adult population treated with the total cumulative dose of 18 mg/kg had decreased tGSH and GSH ruling for redox imbalance. Additionally, the highest DOX dose also increased ATP levels in infant mice. On contrary, the MTX administration did not cause brain redox or energetic alterations in any of the tested ages. This clearly highlights the dissimilar brain actions of these drugs.

Considering the stereological measures, mice administrated with a total cumulative dose of 18 mg/kg DOX had increased hippocampal formation volume, whereas mice treated with a total cumulative dose of 6 mg/kg MTX presented a significant reduction. The total estimated GFAP-ir astrocytes increased in hippocampal formation regions of MTX-treated mice. Meanwhile, both DOX doses caused dissimilar effects on GFAP-ir astrocytes, that is, in the DG region the count decreased, while in the CA3 region increased being the most pronounced effect the increment obtained with the highest DOX dose.

Regarding the immunofluorescent detection of the apoptotic markers, while in the 9 mg/kg DOX-treated mice Bax expression increased in the PFC and CA3 region, the 18 mg/kg DOX administration reduced Bax expression in the PFC and DG region. MTX administration increased Bax expression considerably in the PFC and CA3. Meanwhile, p53 also presented dissimilar expression depending on the DOX dose, being that the highest DOX dose decreased its expression in the DG and CA3, and the lowest DOX dose increased p53 expression in CA3 region. Concerning MTX-treated animals, p53 expression decreased in all brain areas evaluated. The neurodegenerative marker pTau decreased only in the DG and CA3 of the 18 mg/kg DOX mice, whereas in the MTX-treated mice pTau increased in the PFC.

DOX and MTX have similar pharmacological anticancer properties, by intercalating with the DNA and inhibiting topoisomerase II, however, the data gathered in this dissertation shows that they induce neurotoxicity by dissimilar mechanisms. Considering the studies available so far, the results here presented are the first showing that systemic administration

of MTX causes neurotoxicity in the adult mice brain, as it has been a drug poorly studied regarding 'chemobrain'. Furthermore, dose and age seem to have an important role in the neurotoxic events seen herein. Nevertheless, further studies are needed to elucidate on the underlying mechanisms of chemotherapy-induced neurotoxicity of DOX and MTX to promote actions (pharmacological or not) that improve the quality of life of chemotherapy treated cancer patients.

7. References

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