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Cysteine uptake capacity is a relevant mechanism of resistance to platinum salts, in ovarian cancer

Inês Pires dos Santos

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertação de Candidatura ao grau de Mestre em Oncologia, no ramo de Oncologia Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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DEDICATÓRIA

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A

AA - Antibiotic-Antimycotic

ABC - ATP-Binding Cassette transporters

Akt/PKB - serine/threonine-protein kinase B

AMPH - Amphetamine

AP-1 - Activator Protein-1

AP-2 - Activator Protein-2

ARE - Antioxidant-Responsive Element

***ARID1A* -** AT-Rich Interactive Domain-containing protein 1A

ASC - system Alanine-Serine-Cysteine

ASCT 1/2 - Alanine-Serine-Cysteine Transporter 1/2

ASP - Aspartate

ATCC - American Type Culture Collection

ATP - Adenosine Triphosphate

ATPase - Adenosine Triphosphatase

ATR - Ataxia Telangiectasia and Rad3-related protein

B

B.C. - Before Christ

BCRP - Breast Cancer Resistant Proteins

BER - Base Excision Repair

***BRAF* -** Murine Sarcoma Viral Oncogene Homolog B

***BRCA1* -** Breast Cancer gene 1

***BRCA2* -** Breast Cancer gene 2

BSA - Bovine Serum Albumin

BSO - Buthionine Sulfoximine

***B. subtilis* -** Bacillus subtilis

C

CAFS - Cancer Associated-Fibroblasts

CAT - Cysteine Aminotransferase

CBS - Cystathionine-β-synthase

cDNA - complementary DNA copy

CDO - Cysteine Deoxygenase

ChIP - Chromatin Immunoprecipitation

CNS - Central Nervous System

CSE - Cystathionine-ϒ-lyase

***CTNNB1* -** β-catenin

Cul1 - Cullin 1

Cul3 - Cullin 3

CYP 450 - Cytochromes P450

Cys - L-Cysteine

Cys-Gly - Cysteinyl-Glycine

D

DAPI - 4′-6-Diamidino-2-Phenylindole

DAT - Dopamine Transporter

ddH2O - Sterile ultra-pure water

DMEM - Dulbecco's Modified Essential Medium

DMES - Drug-Metabolizing Enzymes

DNA - Acid Deoxyribonucleic

dNTPs - Deoxynucleotides

DSB - Double Strand DNA Breaks

DTNB - 5,5'-dithiobis-2-nitrobenzoic acid

DTT - Dithiothreitol

E

EAATs - Excitatory Amino Acids Transporters

EAAT1/2/3/4/5 - Excitatory Amino Acid Transporter, member 1/2/3/4/5

EDTA - Ethylenediamine Tetra-acetic Acid

EGFR - Epidermal Growth Factor Receptor

EOC - Epithelial Ovarian Cancer

ERA - Erastin

***ERBB2/HER2* -** Receptor Tyrosine-Protein Kinase erbB-2

F

4F2hc - cell-surface antigen heavy chain

FA - Fatty Acids

FBS - Fetal Bovine Serum

FIGO - *Fédération Internationale de Gynécologie et d'Obstétrique*

FITC - Fluorescein Isothiocyanate

FOXM1 - Forkhead box protein M1

G

G2 - Gap 2 cell cycle phase

GCL - Glutamate Cysteine Ligase

GCLC - Glutamate-Cysteine Ligase Catalytic subunit

GCLM - Glutamate-Cysteine Ligase Modifier subunit

γ -Glu-Cys - gamma-L-Glutamyl-L-Cysteine

GPx - Glutathione Peroxidases

GR - GSH Reductase

GSH - Glutathione

GSS - GSH Synthetase

GSSG - GSH disulphide

GST - Glutathione S-Transferase

GSTP1 - Glutathione S-Transferase P1

GS-X pump - Glutathione S-conjugate export pump

GTPase - Guanosine Triphosphatase

H

h - hour

H_2O - Water

H_2O_2 - Hydrogen Peroxide

H_2S - Hydrogen Sulphide

HCys - Homo-L-cysteine

HGSC - High Grade Serous Carcinoma

HIP-A - 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d] isoxazole-4-carboxylic acid

HIP-B - 3-hydroxy-4,5,6, 6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid

HNF1β - Hepatocyte Nuclear Factor1 homeobox B

HO-1 - Hemoxygenase-1

HPLC - High-Performance Liquid Chromatography

HPLC-FD - HPLC with Fluorescence Detection

HPRT1 - Hypoxanthine Phosphoribosyl Transferase 1

HRP - Horse Raddish Peroxidase

I

IARC - International Agency for Research on Cancer

IC50 - half maximal Inhibitory Concentration

IF - Immunofluorescence

J

JNK - c-jun N-terminal kinase

K

Keap-1 - Kelch-like ECH-1

***KRAS* -** Kirsten Rat Sarcoma virus

L

LGSC - Low-Grade Serous Carcinoma

M

M - mitosis cell cycle phase

Maf - small Masculoaponeurotic fibrosarcoma

MAPK - Mitogen-Activated Protein Kinase

MDR - Multidrug Resistance

MDR1 - Multidrug Resistance protein (MRP) 1

miR - micro RNA

min - minutes

MMR - Mismatch DNA Repair

MODY - Maturity-Onset Diabetes of the Young

MpST - 3-Mercapto-pyruvate Sulphur Transferase

mPTP - mitochondrial Permeability Transition Pore

mTORC1 and 2 - mammalian Target of Rapamycin Complex 1 and 2

MVP - Major Vault Protein

N

NADH - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NaOH - Sodium Hydroxide

NER - Nucleotide Excision Repair

NFDM - Non-Fat Dry Milk

NFκB - Nuclear Factor Kappa B

NMDARs - N-methyl-D-aspartate receptors

NQO1 - NAD(P)H Quinone Oxidoreductase

NRF2 - Nuclear Factor Erythroid 2-P45-Related Factor2

O

OCCC - Ovarian Clear Cell Carcinoma

OEC - Ovarian Endometrioid Carcinoma

OMC - Ovarian Mucinous Carcinoma

OSC - Ovarian Serous Carcinoma

P

PARP - Poly (ADP-Ribose) Polymerase

PBS - Phosphate-Buffered Saline

PCR - Polymerase Chain Reaction

P-gp - P-glycoprotein

PI - Propidium Iodide

PICK1 - Protein Interaction with C-kinase 1

PI3K - Phosphoinositide 3-Kinase

***PIK3CA* -** Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP_2 - Phosphatidylinositol (4,5) - biphosphate

PIP_3 - Phosphatidylinositol (3,4,5) - triphosphate

PKC - Protein Kinase C

PKM2 - Pyruvate Kinase M2

PMA - Phorbol 12-Myristate 13-Acetate

PP2Ac - Protein Phosphatase 2A

PPP - Pentose Phosphate Pathway

PS - Phosphatidyl Serine

***PTEN* -** Phosphatase and Tensin homolog gene

R

RAC3 - Receptor Associated Co-activator 3

***RB* -** Retinoblastoma gene

RBX1 - Ring-Box protein 1

RNA - Ribonucleic Acid

RIPA - Radio-Immunoprecipitation Assay

ROS - Reactive Oxygen Species

RPA - Replication Protein A

RQ-PCR - Relative Quantifying Polymerase Chain Reaction

RT - room temperature

R.T. - Reverse Transcription

S

SAPK - Stress-Activated Protein Kinase

SBD-F - Ammonium 7-Fluoro-2,1,3-Benzoxadiazole-4-Sulfonate

SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Se - Selenium

sec - seconds

SeChry/SeC - Selenium containing Chrysin

SGLT2 - Sodium-Glucose cotransporter 2

***SLC1A1(EAAT3)* -** Solute Carrier Family 1 member 1

***SLC7A11(xCT)* -** Solute Carrier Family 7 member 11

SSZ - Sulfasalazine

SWI/SNF - SWItch/Sucrose Non-Fermentable complex

T

TBOA - dl-threo-β-Benzyloxyaspartic acid

TBS - Tris Buffered Saline

TCA - Trichloroacetic acid

TCA cycle - Tricarboxylic Acid cycle

TCEP - Tris (2-carboxyethyl) phosphine

***TCF-2* -** Transcription Factor 2 gene

TCGA - The Cancer Genome Atlas

TFIIH - Transcription Factor II Human

TGF-β - Transforming Growth Factor beta

TGS - TrisGlycine-SDS

TMZ - Temozolomide

TNBC - Triple Negative Breast Cancer

TNM - Tumour Node Metastasis

TrxR - Thioredoxin Reductase

U

UGT - UDP-glycosyltransferases

UGT1A1 - UDP-glycosyltransferase family 1, member 1

V

v - volume

VDACs - Voltage-Dependent Anion Channels

W

W - weight

WHO - World Health Organization

X

XP - Xeroderma Pigmentosum

XPA/B/D - Xeroderma Pigmentosum group A/B/D

XAR - Xenobiotic-Activated Receptor

XIAP - X-linked Inhibitor of Apoptosis Protein

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ABSTRACT

Ovarian cancer corresponds to the first leading cause of death from gynaecologic cancer in women, mainly due to molecular heterogeneity, diagnosis in advanced stages and chemotherapy resistance. Epithelial ovarian cancer corresponds to 90% of all ovarian cancers and includes two distinguishable histotypes in what concerns chemoresistance: ovarian serous carcinoma (OSC), presenting acquired resistance, and ovarian clear cell carcinoma (OCCC) characterised by intrinsic resistance. Chemoresistance can be associated with changes in detoxification activity where glutathione (GSH) has an important role. Cysteine constitutes a rate-limiting substrate for GSH synthesis and like GSH, has been implicated in platinum-based drugs resistance. Two different transporters were already reported to have a role in cysteine transport: xCT, part of system Xc- and excitatory amino-acid transporter 3 (EAAT3). Hence, we aimed to study the relevance of those transporters in cells response to cysteine and carboplatin exposure using two different ovarian cancer cells lines, ES2 cells (OCCC) and OVCAR3 cells (OSC). Moreover, since it was reported that xCT is also capable to uptake selenium and EAAT3 selenocysteine, we hypothesized that a selenated-compound, selenium-containing Chrysin (SeChry) was able to compete with cysteine transport, affecting ovarian cancer cells. Thus, we addressed the effect of SeChry in cells viability, capacity to uptake cysteine and in response to carboplatin.

The results have shown that cysteine was responsible for a reduction in cell death caused by platinum salts, and its transporters xCT and EAAT3 were influenced by cysteine supplementation and carboplatin exposure. Importantly, ES2 cell line showed increased levels of both transporters compared to OVCAR3, which could be related to the differences observed in chemoresistance mechanisms between OSC and OCCC histotypes. Our results also demonstrated that Nuclear Factor Erythroid 2-P45-Related Factor2 (NRF2) (involved in the regulation of xCT) and hepatocyte nuclear factor1 homeobox B (HNF1β) (which has a role in GSH dynamics) were modulated upon cysteine and carboplatin exposure.

Regarding SeChry effects, results shown that this selenium compound was able to induce cell death, not due to directly impaired cysteine uptake but probably due to oxidative stress, since GSH levels decreased intracellularly, concomitant with its increase and degradation extracellularly. Interestingly, its combination with carboplatin, another pro-oxidant drug, was unexpectedly favourable for ovarian cancer cells survival. Because there is evidence that selenium compounds are selective to cancer cells, this compound could present clinical benefits for ovarian cancer patients, but its combination with carboplatin should be approached with caution.

Key-Words: ovarian carcinoma, platinum drugs, GSH, cysteine, xCT, EAAT3, NRF2, HNF1β, SeChry.

RESUMO

Cancro é a designação atribuída a um conjunto de doenças que atualmente constitui a segunda maior causa de morte em todo o Mundo. É-lhe reconhecida uma grande complexidade que é despoletada por alterações genéticas e epigenéticas em células normais, conduzindo a alterações fisiológicas responsáveis por um crescimento descontrolado, proliferação e invasão. O cancro do ovário apresenta-se como o tumor maligno ginecológico com maior mortalidade no sexo feminino. Esta mortalidade deve-se essencialmente à sua elevada heterogeneidade molecular que compromete o recurso a um tratamento mais específico, à ausência de sintomatologia que inevitavelmente leva em muitos casos a um diagnóstico em estádios avançados e também ao fenómeno de quimioresistência.

Os carcinomas correspondem a 90% de todos os casos de cancro do ovário e englobam diversos tipos histológicos, incluindo os carcinomas serosos e os carcinomas de células claras. No que diz respeito às alterações genéticas, estes carcinomas são distintos, o que pode ser importante para justificar diferenças que ocorrem ao nível da quimioresistência. Neste contexto, o carcinoma seroso está associado a uma resistência que é adquirida e o carcinoma de células claras a uma resistência intrínseca. O tratamento comumente empregue no cancro do ovário alia a cirurgia à quimioterapia que é principalmente baseada na utilização da carboplatina em combinação com o paclitaxel. A carboplatina é responsável pela formação de ligações covalentes com o DNA (adutos) e pela formação de ROS que se associam a alterações no DNA, proteína e grupos -SH (presentes nos tióis como a glutationa (GSH)), desta forma a quimioresistência que lhe está associada pode basear-se em alterações nos mecanismos de destoxificação, onde a atividade do GSH se demonstra de grande importância. A cisteína ao constituir o substrato limitante na síntese de GSH, tem sido alvo de diversos estudos que acabam por suportar também o seu papel na resistência à carboplatina.

O xCT, que é parte integrante de um sistema designado Xc- , e o transportador de aminoácidos excitatórios, membro 3 (EAAT3) são dois conhecidos transportadores de cisteína, que geralmente se apresentam caracterizados no contexto cerebral/sistema nervoso central, por estarem concomitantemente associados ao transporte de glutamato. Assim sendo, o transportador xCT opera como um sistema de antiporte, importando cisteína na forma reduzida (cistina) e exportando glutamato, enquanto que o EAAT3 funciona em simporte, importando tanto cisteína como glutamato. O interesse nestes transportadores tem sido expandido a outros contextos, visto que ambos estão envolvidos na manutenção do equilíbrio do stress oxidativo a nível celular. Assim, o nosso objetivo primordial, foi estudar a relevância do xCT e do EAAT3 na capacidade de resposta das

células aquando da sua exposição à cisteína e à carboplatina. Para isto, duas linhas celulares de tipos histológicos distintos foram usadas, as ES2 (correspondentes a um carcinoma de células claras) e as OVCAR3 (representantes de um carcinoma seroso). Para além disto, uma vez que ambos os transportadores de cisteína conseguem também fazer a assimilação de compostos selenados, colocámos como hipótese o selénio funcionar como um competidor da cisteína, levando à diminuição da sua absorção, podendo ter como consequência efeitos deletérios nas células de cancro do ovário. Assim sendo, testámos o efeito do SeChry, um composto orgânico constituído por selénio e uma flavona (Crisina) – nas duas linhas celulares de cancro de ovário e analisámos os seus efeitos na viabilidade celular, na capacidade de assimilação de cisteína e na resposta à carboplatina.

Os nossos resultados mostraram que a cisteína tem um efeito protetor na viabilidade celular quando estas células estão sujeitas à ação tanto da cisplatina como da carboplatina. Tal como a cisteína, a carboplatina foi também responsável pela modulação destes dois transportadores, cuja expressão se revelou mais elevada na linha celular ES2, corroborando com o facto destas células estarem mais dependentes do metabolismo de cisteína. Como nas ES2, ao nível da proteína, se verificou uma redução do transportador EAAT3 perante a exposição à carboplatina, concomitante com o ligeiro aumento de xCT, considerámos que tal fenómeno se pudesse dever à acumulação de glutamato extracelular decorrente da atividade aumentada do xCT, criando assim instabilidade funcional no EAAT3.

O fator de transcrição NRF2 **(**Nuclear Factor Erythroid 2- P45 -Related Factor2) está envolvido na regulação do xCT e na manutenção da homeostase em condições de stress oxidativo, enquanto que o HNF1β (Hepatocyte Nuclear Factor-1β) constitui um marcador do carcinoma de células claras e está envolvido na regulação da subunidade catalítica glutamato-cisteína ligase (GCLC). Tomando tais factos em consideração, estes dois fatores de transcrição foram estudados de forma a compreender se a cisteína e a carboplatina têm efeitos na sua expressão e o seu papel na regulação do *xCT/SLC7A11* e do *EAAT3/SLC1A1*. Os nossos resultados mostraram que tanto a cisteína como a carboplatina induzem a expressão do NRF2 e do HNF1β, sugerindo um papel destes genes na resposta a este fármaco. É de salientar que a expressão do HNF1β só se verificou nas ES2. Demonstrou-se ainda, uma potencial regulação dos transportadores de cisteína por parte destes fatores de transcrição.

No que diz respeito aos efeitos do SeChry, verificámos que as ES2 são mais sensíveis a este composto em comparação com as OVCAR3, apresentando um IC50 mais baixo. A morte celular provocada por este composto foi muito elevada, revelando-se

altamente tóxico para as células de cancro do ovário testadas. No entanto, apurámos que a combinação deste composto selenado com a carboplatina, pode ser, contrariamente ao esperado, vantajosa para a sobrevivência das duas linhas celulares. Através de uma cromatografia líquida de alta eficiência (HPLC) foi possível verificar que o SeChry não inibiu a assimilação de cisteína nas células ES2 e OVCAR3, no entanto, levou a uma diminuição dos níveis intracelulares de GSH, concomitante com um aumento dos seus níveis extracelulares e aumento da sua degradação. Estes resultados sugerem assim que a indução da morte celular provocada por este composto se deve, não à inibição da assimilação de cisteína, mas à indução de stress oxidativo, podendo estar correlacionado com a disfunção de mecanismos de destoxificação e consequente aumento de espécies reativas de oxigénio, revelando-se como um composto pro-oxidativo.

São reconhecidos diversos tratamentos para o cancro, no entanto, a quimioresistência continua a ser um obstáculo no tratamento desta doença, pelo que se torna necessário desenvolver novas estratégias terapêuticas dirigidas a células malignas, tendo em conta as suas características e necessidades metabólicas. Denotadas evidências da seletividade e toxicidade de compostos orgânicos selenados para células cancerígenas, o SeChry poderá assim constituir um importante composto com futura aplicabilidade no contexto clínico do cancro do ovário, sendo, no entanto, necessária extrema precaução com a sua combinação com carboplatina.

Palavras-chave: Cancro do ovário, carboplatina/cisplatina, GSH, cisteína, xCT, EAAT3, NRF2, HNF1β e SeChry.

1. STATE OF THE ART

1.1. CANCER

Cancer is a generic concept that includes a group of complex diseases, corresponding to the world's second leading cause of death[1]. Nowadays, the expression "Cancer is the 21st century disease" is recurrently listened, this quote is true because, besides the increasing diagnosed number of cases, a revolutionary and intense research focused on this area of biology has been occurring, having a fundamental role for prevention, diagnostic improvement and treatment innovation. However, this is an "old" set of diseases whose first evidences remit for 1500 B.C. in the ancient Egypt[2].

According to World Health Organization (WHO), cancer was recognised as a misfortune of the developed countries. Since it has been identified as characteristic from elderly people and the average life expectancy increased, its incidence and prevalence has rapidly raised. However developing countries lodge nearly half of all cancers especially due to their social context and a high preponderance of infectious agents that make individuals more susceptible to cancer manifestation, where cervix cancer may be employed as an example[3,4]. Likewise the lack and deficiency of diagnostic methods and therapeutic approaches cannot be excluded[4–6].

As a very complex and interesting panel of diseases, cancer is triggered by diverse alterations in normal cells in charge of breaking with its normal behaviour and leading to modifications and adaptations in biological processes, such as uncontrolled growth and proliferation. In 2000, Hanahan and Weinberg considered six different hallmarks of cancer that lean on the capacity to **maintain the proliferative signalling**, the assortment of mechanisms necessary for the **growth suppressors disruption** that are responsible for **cell death resistance** and **cell immortality**, the local and distal cancer spread capacity leading to **cell invasion** and **metastasis**, respectively, and the **angiogenesis role**[7,8]. More recently, four new concepts started to be pointed. Two are contemplated as emerging hallmarks and rely on the capacity to **evade immune system** and the ability of **energy metabolism** reprogramming. On the other hand, two are enabling traits: **tumour turns into an inflammation promoter** and the **genome instability and mutation** are the core for these changes[8–12] .

As previously mentioned, transformation of normal cells into cancer cells occurs due to genetic and epigenetic alterations. Genetic modifications rely on both structural and numeric changes in chromosomes, a range of inactivating and activating point mutations and/or decreased expression of tumour suppressor genes and/or increased expression of protooncogenes[7,13]. In epigenetic modifications, DNA methylation and histone modifications are comprised. DNA hypomethylation was the first epigenetic change detected in malignant

tumours[14,15]. In addition to its presence in the early stages of carcinogenesis it is suggested to display functions in tumour progression[15–17]. Histone modifications, more specifically acetylation, can equally provide information regarding cancer prognosis and diagnosis[16,18,19]. Not only cell phenotype, but also all the cellular microenvironment are influenced by this set of mutations[11]. In figure 1.1. it is presented the complexity of carcinogenesis.

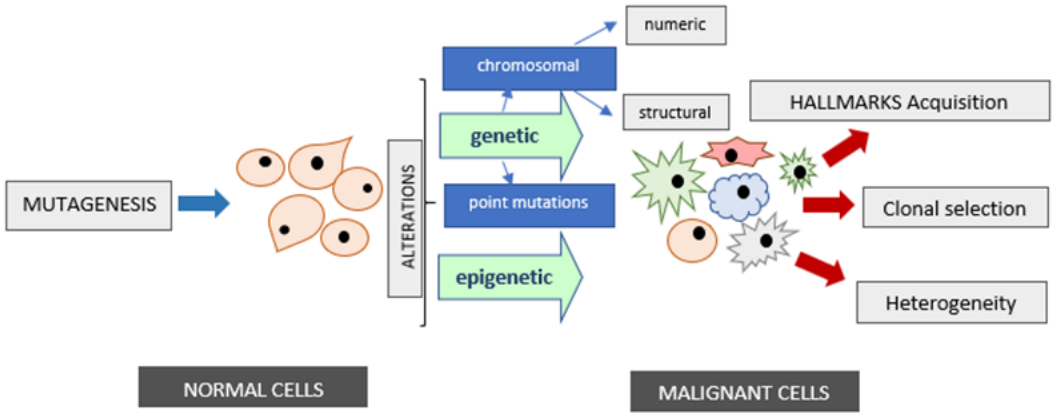


Figure 1.1. - Normal cell transformation into malignant. Normal cells undergo genetic and epigenetic alterations and are reprogrammed, acquiring cancer hallmarks. The tumour microenvironment is heterogenous and there is clonal selection favouring cells adaptation. Adapted from Fouad, Y.A. and Aanei, C., 2017.

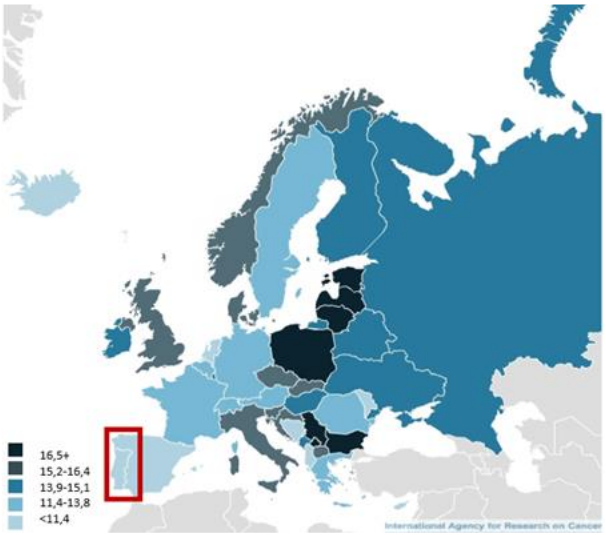
To explore better the tumour microenvironment, it is relevant to establish that the whole carcinogenic process is not linear: as the microenvironment is heterogenous, usually sub clonal tumour populations co-exist[20]. Heterotypism is the designation attributed to the interactions between cell lineages[8]. In a cross-talk between non-malignant and cancerous cells, is recognized the secretion ability of many proteins, enzymes, growth factors and adhesion molecules synthesis crucial to remodelling cell viability and proliferation[8,21,22]. Cell heterogeneity is thus characteristic from tumour microenvironment, being crucial to be explored and analysed in order to find the right treatment strategies.

Chemotherapy and surgery, or the combination of both, are still the main strategy treatments, however, hormonal therapy, radiotherapy, nuclear medicine, nanotechnology and more recently immunotherapy are other established ways to persuade a cure, or at least and more reasonably to minimise symptoms, decrease morbility and increase survival[2,23].

1.2. OVARIAN CANCER

1.2.1. EPIDEMIOLOGY

Ovarian cancer is characterised as a malignancy with high mortality levels, corresponding to the first leading cause of death from gynaecologic cancer in women and the 8th commonest female cancer death. Furthermore it is the 7th most common cancer diagnosed in female, accounting for about 240.000 cases every year[24–26]. Curiously, International Agency for Research on Cancer (IARC) pointed Portugal as one of the countries with the lower ovarian cancer prevalence (figure 1.2.)[27].



Statistical data revealed that the mean age at diagnosis is 63 years old and overall survival is about 40%[24,28,29]. Besides this, ovarian cancer is more common in caucasian women, mainly in the post-menopausal phase[30]. Moreover, in 70% of the cases, this disease is diagnosed in advanced stages[31].

Figure 1.2. - Ovarian cancer prevalence in the European Union. This figure was adapted from International Agency for Research on Cancer (IARC)/WHO, 2012.

The high mortality characteristic of this disease is mainly due to three factors: the **molecular heterogeneity -** consistent with difficulties to find out a specific treatment strategy, the **diagnosis in advanced stages -** based on the lack of symptoms and the **chemotherapy resistance** phenomenon, that will be exploited in this thesis[32–34].

1.2.2. ETIOLOGY

This tumour is classified by the WHO in 3 main groups depending on their cellular origin, corresponding to epithelial, germinal and sex cord-stromal type[25,34]. Whilst, epithelial ovarian cancer - the carcinomas - corresponds to 90% of all malignant ovarian tumours[25,31].

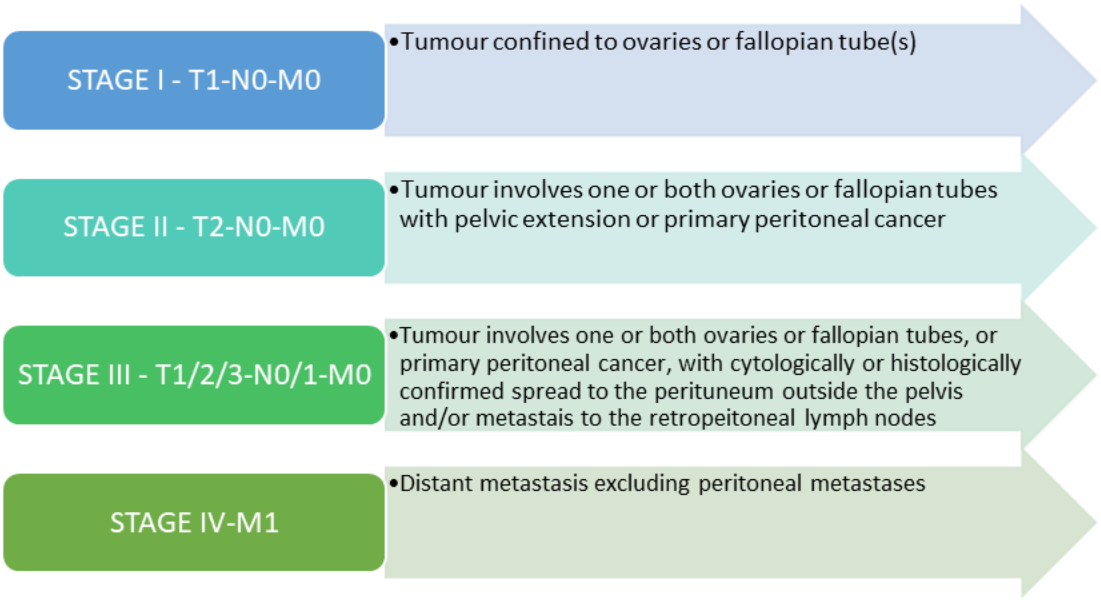
Ovarian cancer etiology is still little understood. In 1971 a model suggesting ovulation as being responsible for ovarian tumours due to the epithelium injury was proposed[35,36]. The most common hypothesis for their presentation is based on ovarian surface epithelium cell-lined inclusion cysts, that will be stimulated by stromal growth factors. There is evidence for this theory due to a transition period between non-malignant

and malignant ovarian surface epithelium, that was found in ovarian cancers in early stages[28,37]. However, cells from the fallopian tube, peritoneum and foci of endometriosis were also suggested as ovarian cancer precursors[32,34].

1.2.3. STAGING

As Prat, J. wrote we are "in the era of personalized cancer medicine", so in order to reach this, it is fundamental to set criteria for tumours staging. In 2014 by consensus agreement, the *Fédération Internationale de Gynécologie et d'Obstétrique* (FIGO) created the follow staging criteria for ovarian cancer (table 1.1.)[38].

Table 1.1. – Ovarian cancer staging criteria by FIGO. TNM is the system used for this cancer classification: T refers to the size and extent of the primary tumour, N to the lymph nodes metastases and M to the distal metastases. The classification can be divided in 4 main stages, ranging from stage I, which presents better prognosis, and stage IV associated with a worst outcome. Adapted from FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication. Prat. J, 2015.



1.2.4. RISK FACTORS, SCREENING and TREATMENT

The majority of ovarian carcinomas are sporadic, familial cases account just for about 5-10%[24,30]. Human papilloma virus, perineal talc and smoking increases the risk for this type of cancer, but risk can be reduced by anovulation, that happens with pregnancy or by oral contraception[29,37,39,40].

Ovarian cancer screening is mainly done through measurements of CA-125 and pelvic ultrasonography, this last method is also important in diagnostic context[24]. The

treatment keynote ways are based on surgical cytoreduction with platinum and taxan based chemotherapy[41–43].

1.3. EPHITELIAL OVARIAN CANCER

1.3.1. HISTOLOGICAL TYPES

Epithelial ovarian cancer (EOC) is divided in different histological types, being the most common the **serous (OSC)**, the **endometrioid (OEC)**, the **clear cell (OCCC)** and the **mucinous (OMC)** carcinomas. Ovarian serous carcinoma is subdivided in 2 groups, low grade or type I and high grade or type II[24,43].

High grade serous carcinoma (HGSC) is the most frequent subtype, corresponding to 70% of the all carcinomas. In contrast, **low-grade serous carcinoma (LGSC)** just accounts for about 5% or less of the cases, **OMC** is equivalent to approximately 3% and the **OEC** for 10% and it is usually diagnosed at early stages. Lastly, but not less important, **OCCC** appears as an enigmatic and uncommon subtype, which accounts for about 5%-10%. The unfamiliarity upon OCCC gives rise to an urgent need to undercover some aspects that are relevant for thesis theme[44].

In the present thesis the OSC and the OCCC histotypes will be addressed. The main reason to study them is the difference in chemoresistance mechanism. OSC are the most prevalent and develop chemoresistance in the course of therapy[45]. OCCC presents the worst prognosis as an extraovarian disease, mainly because it is intrinsically chemoresistant[45].

1.3.2. OVARIAN SEROUS CARCINOMA (OSC)

As previously presented, epithelial serous cancer is the most prevalent type. Type 1 and type 2 present different histological and genetic profiles.

1.3.2.1. HIGH-GRADE SEROUS CARCINOMA (HGSC)

Focusing on the histological features, HGSC, normally exhibit different patterns, enlarged nuclei, classified in grade 3, and abundant mitotic figures[31,43,46]. As an heterogenous tissue there is a mixture of low-cuboidal, columnar and hobnail cells that permit mimicking endometrioid carcinomas (i.e.) (figure 1.3.)[43].

In the majority of the patients that exhibit HGSC, somatic mutations as gene abnormalities in *TP53* are the main trigger. Nevertheless, this type of cancer is extensively known by breast cancer gene 1 (*BRCA1*) and breast cancer gene 2 (*BRCA2*) mutations at both somatic and germinal level and linked with defective double strand DNA breaks (DSB)

repair[24,47,48]. Malignancies based on these mutations present an adjusted therapy that relies on the use of Poly (ADP-ribose) polymerase (PARP) inhibitors (Olaparib) which induce DNA lesions selectively potent against cells with biallelic *BRCA1* or *BRCA2* deficiency[49–51].

Hereditary HGSC accounts for hereditary breast-ovarian cancer syndrome and Lynch syndrome. There is about 40-60% of increased ovarian carcinoma risk in women with *BRCA1* mutations *and* 11%–30% *BRCA2* mutations[52]. Germeline mutations of genes belonging to mismatch DNA repair (MMR) system, underlie Lynch and non-polyposis hereditary cancer[53]. Besides ovarian carcinomas other types of cancer are included in the spectra of these syndromes such as endometrial, urinary and colorectal cancer. Women with this familial susceptibility have higher risk (8%) of ovarian cancer comparing with general female population. When there is genetical predisposition women are normally diagnosed 10 years before the median age at diagnosis of sporadic cases[32,41,52].

Alterations based on somatic mutations, modifications in DNA copy-number, or even due to significant up- or down-regulation compared to expression in diploid tumours in signalling pathways as NOTCH and overexpression of transcription factor Forkhead box protein M1(FOXM1) and its proliferation-related target genes are also associated with high grade serous tumours pathophysiology and with its aggressive behaviour[24,54–56].

Patients with platinum-sensitive epithelial ovarian cancer harbours more frequently mutations in *BRCA1/2* compared with platinum – resistant ovarian cancers, a phenomenon that help highlighting the differences in chemoresistance between carcinomas[57].

1.3.2.2. LOW-GRADE SEROUS CARCINOMA (LGSC)

Regarding histological features, LGSCs present uniform/grade I nuclei and differentiated architecture. The nucleoli are more prominent, but do not let LGSC be distinguished from HGSC (figure 1.3.)[43]. These carcinomas do not present chromosome instability showing somatic mutations and lacking the complex genetic abnormalities observed in HGSCs[43].

Contrary to the previous carcinoma subtype, LGSCs have lower percentage of mutations in *TP53*. Somatic mutations in oncogenes *BRAF* (Murine Sarcoma Viral Oncogene Homolog B), *KRAS* (Kirsten Rat Sarcoma virus), *ERBB2/HER2* (Receptor tyrosine-protein kinase erbB-2), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha*)* and *CTNNB1* (β-catenin)*,* are quite frequent. Tumour suppressor genes *ARID1A* (encoding AT-rich interactive domain-containing protein 1A*)* and *PTEN* (encoding phosphatase and tensin homolog) are also commonly mutated[24,31,32,43,58]. The mutations that occur in *RAS*, *BRAF* and *ERBB2* affect the mitogen-activated protein kinase

(MAPK) pathway leading to its constitutive activity and consequently to uncontrolled proliferation[31]. *PIK3CA* and *PTEN* have been studied as being responsible for changes in the phosphatidylinositol 3′-kinase/AKT (PI3K/AKT) pathway. The amplification of *PIK3CA* is reported to have a role in invasion, metastasis and chemoresistance in ovarian cancer[24,31,59].

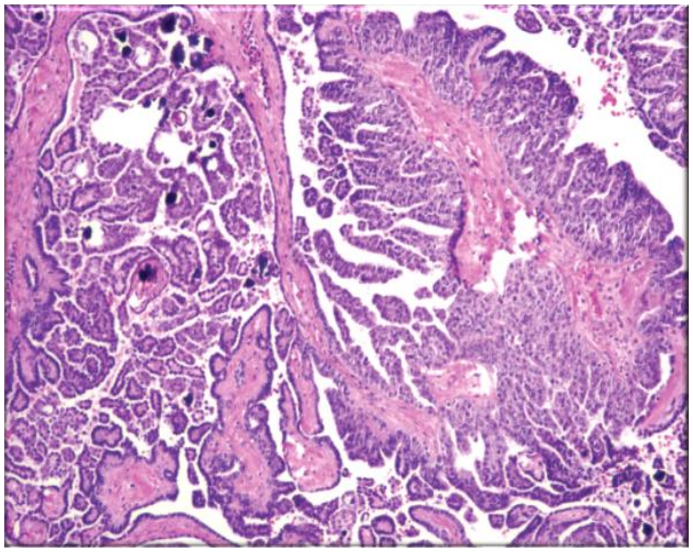


Figure 1.3. - Histological section representative of serous carcinoma.

1.3.3. OVARIAN CLEAR CELL CARCINOMA (OCCC)

OCCC was described by Schiller as mesonephroid carcinoma or mesonephroma in 1939, but only in 1973 this type of carcinoma was recognized by WHO[60–62]. One third of the cases are diagnosed at stage I (intraovarian), yet when presented at extraovarian disease the prognosis is poor[31,63,64].

OCCCs are characterised by a high heterogeneity[44], making difficult to classify the malignancy, due to other cell types that can mimic this ovarian tumour, such as endometrioid carcinomas (figure 1.4.)[44,46,65].

Endometriosis is a condition characterised by the presence of endometrial-type mucosa outside the uterine cavity that leads to endometrioid ovarian cysts formation, being OCCC and OEC associated with it[31,66]. Besides the huge heterogeneity and the similarity in the histological features between OCCC and OEC, there are differences at the genomic level that will be addressed. OCCC do not have a high mitotic rate, as it usually happens with other histological types, do not exhibit instability at the chromosomal level and karyotype complexity[65,67,68].

PTEN a tumour suppressor gene frequently mutated in this cancer type is responsible for the inhibition of phosphoinositide 3-kinase (PI3K) dependent activation of serine/threonine-protein kinase Akt/PKB[13,24,69]. Another molecular change that seems to be

important for the OCCC development, accounting for about 50% of the all cases, is the occurrence of truncating mutations in *ARID1A* tumour suppressor gene that leads to the loss of function of BAF250a, one of the noncatalytic subunit of the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex (chromatin remodelling complex) responsible for modulating the target specificity and activity of the ATPase of the complex[68,70,71]. Due to these genetic changes, there are diverse alterations in protein expression as the increase of Akt activity, mTORC1 and 2 (mammalian target of rapamycin complex 1 and 2) and the "de novo" expression of hepatocyte nuclear factor β (HNF1β)[65,69,72,73]. OCCC usually do not present mutations in *KRAS*, *BRAF* and *TP53*[31].

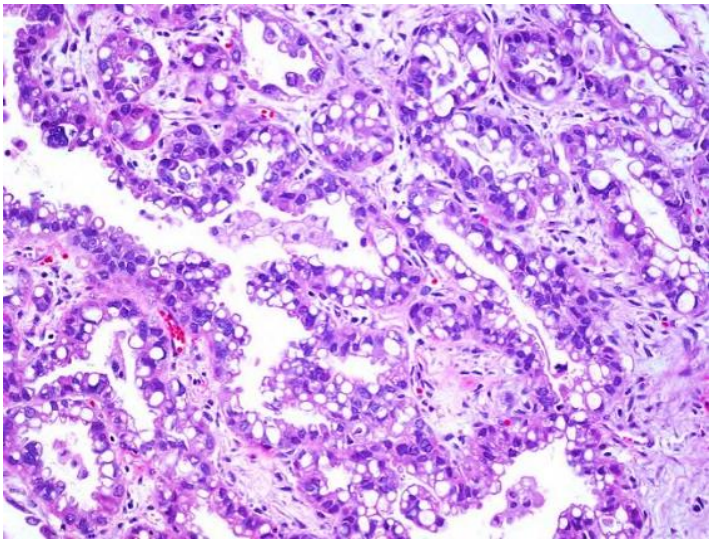


Figure 1.4. - Histological section representative of OCCC.

1.4. CHEMOTHERAPY

Ovarian cancer chemical treatment involves the use of two distinct type of drugs that can be applied in combination, resulting in more efficacy. Platinum drugs, as cisplatin, carboplatin or oxaliplatin and taxanes, such as paclitaxel or docetaxel are recurrently used. These drugs are applied intravenously in a 3 to 6 cycle treatment[24,74]. Drugs' mechanism of action is distinct: taxane chemotherapy is based on changes in β-tubulin structure – polymerization: so drug binds to the microtubules leading to its dynamics suppression and consequently to cell cycle arrest (G2/M phase), being also responsible for the formation of reactive oxygen species (ROS) and finishing with cell death by the activation of caspases 3 and 8[75,76]. Platinum-based chemotherapy is also recognized by ROS formation and adducts formation with DNA, that will be posteriorly explained.

1.4.1. PLATINUM-BASED CHEMOTHERAPY

Platinum-based chemotherapy is the elected treatment strategy, cisplatin (cis-diammine dichloroplatinum) was the gold-standard in first line chemotherapy in EOC, although it presents high nephrotoxicity, otoxicity and peripheral neuropathy[77–79]. Carboplatin (cis-diammine-cyclobutanedicarboxylato-platinum), a second-generation platinum-containing antineoplastic, just differs from cisplatin in cyclobutanedicarboxylate group, showing more stability and leading to less reactivity, nevertheless a higher concentration of this drug is needed to obtain the same effects of cisplatin[57,79,80]. This drug appeared as another and better option to neoadjuvant, adjuvant and palliative treatment, mainly due to the less side effects[24,57,77].

Platinum drugs' mechanism of action is based on intra/inter strand cross-links with DNA, being responsible for the activation of diverse signal transduction pathways, such as the ones that involve ataxia telangiectasia and Rad3-related protein (ATR), TP53, TP73, and MAPK[81]. The covalent adducts formation kinetics varies between the different cisplatin analogues, that are responsible for promoting cell cycle arrest and apoptosis, in which caspases 3, 8 and 9 are important[80].

Despite the function previously reported, other effects have been described in cell lines which include inhibition of sodium-potassium adenosine triphosphatase (ATPase), calcium channel function, and mitochondrial activity[79,82]. It was reported that platinum drugs exposure was responsible for intracellular ROS increase, suggesting their involvement in DNA attack, affecting severely its structure and function, leading to protein impairment[82,83]. ROS affect cell homeostasis that is maintained by the thiol group (–SH) containing molecules. Affected by ROS increase, GSH decreases inducing apoptosis (figure 1.5.)[82,84].

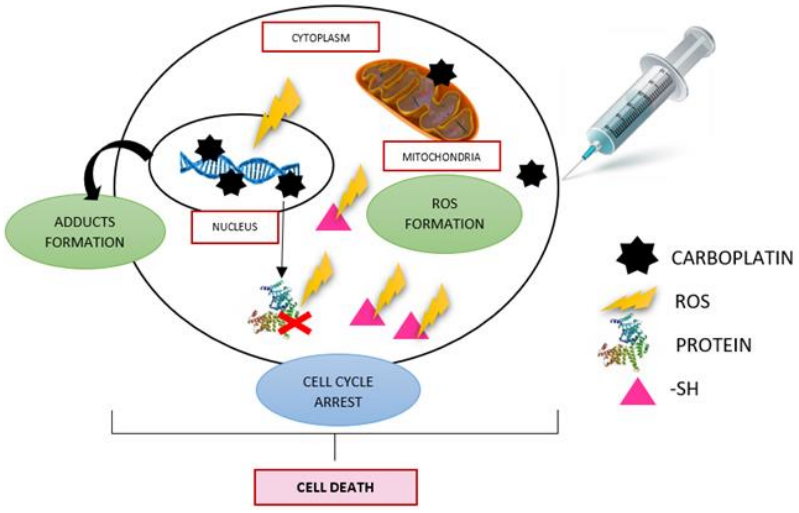


Figure 1.5. - Representation of platinum-based drugs' (carboplatin) mechanisms of action. These agents are responsible for adducts formation with DNA leading to cell cycle arrest and for an increase in ROS formation

that causes changes in DNA, protein and GSH. Both mechanisms culminate with cell death. Adapted from Nunes, S. and Serpa, J., 2018.

Reverting DNA-platinum adduct is possible by nucleotide excision repair (NER) pathway and dependent on xeroderma pigmentosum (XP) complementation groups. This reversible activity constitutes a mechanism of chemoresistance based on the augmented DNA repair activity, better explained in topic 1.4.2.[80,85].

1.4.2. MECHANISMS OF CHEMORESISTANCE

Chemoresistance (intrinsic and extrinsic) is the main hurdle for successful cancer therapy[86]. **Intrinsic chemoresistance** is also named *de novo* chemoresistance and occurs when cancer cells are resistant to chemotherapy since the beginning, being ovarian clear cell carcinoma one of the best examples[87]. **Extrinsic chemoresistance** designation is attributed when chemoresistance is acquired and genetic and epigenetic modifications are responsible for that, for instance mutations in *TP53*, retinoblastoma gene (*RB1)* and *KRAS*, are seen as a result of the repetitive treatment[85–87]. Ovarian cancer first line chemotherapy have response rates of approximately 80%, however in advanced disease many patients (about 85%) tend to relapse, and the re-treatment reveals itself less responsive[87–89]

Focusing on that is indispensable to make an approach on the diverse mechanisms of chemoresistance that seem to be highly correlated and “cross-talking”. The variety of mechanisms is based on **modifications in drug transporters**, **adjustments in drug-target interaction**, **changes in proteins related with the detoxification**, **augmented DNA repair activity, increased tolerance to drugs damage** and **defects in pathways involved in apoptosis**[90–92].

By recognising **transporter pumps** in this dynamic is pivotal to make reference to P-glycoprotein (P-gp), an ATP-binding cassette transporter (ABC), that functions as an energy-dependent efflux pump, decreasing drug accumulation in cells, for example, by alterations in intracellular pH[90,91]. It is associated with multidrug resistance (MDR) and its overexpression has been observed in many solid tumours as ovarian cancers[90]. In this context, there are other transporters that can be affected as breast cancer resistant proteins (BCRPs) and major vault protein (MVP)[90,91,93].

Changes involving proteins with detoxification activity as glutathione (GSH) and its associated enzymes, constitute other chemoresistance mechanism. GSH is very important in the detoxification process – high levels of GSH and overexpression of GST are intimately correlated with the phenomenon, where glutathione S-conjugate export pump (GS- X pump) also participates[81,94,95]. This chemoresistance mechanism is very important

in this thesis as we will focus on the role of cysteine - which is a component of this molecule - transporters in ovarian cancer. Besides GSH, metallothioneins – cysteine-rich intracellular proteins, that also function by protecting cells from oxidative stress, are very expressed in many types of cancer[81,90,92]. Cytochromes P450 (CYP 450) divided in Class I:CYP1A1, CYP1A2, CYP2E1 and CYP3A4 and Class II: CYP2B6, CYP2C9, CYP2C19 and CYP2D6 (characterized by being highly polymorphic), are mainly involved in cancer drug metabolism and drug resistance[96,97]. UDP-glycosyltransferases (UGT) superfamily, constitutes a group of phase II enzymes/transferases, responsible for glucuronidation catalysation by regulating the formation of inactive hydrophilic glucuronides with environmental carcinogens and cytotoxics[98,99].The expression of UDP-glycosyltransferase family 1, member 1 (*UGT1A1)* seems to be negatively regulated by DNA methylation, however the overexpression of *UGT1A1* also due to epigenetic changes is recurrent in cancerous states and leads to drug detoxification[96].

Conformational **alterations in drug targets** that lead to inhibitor drugs disruption is one of the most studied topics in cancer resistance. Paclitaxel, as a used drug in ovarian cancer, has been shown to have chemoresistance associated which relies on alterations in β-tubulin structure, that happen mainly due to differential β-tubulin isotype expression[75,100–102]. Mutations in topoisomerase genes also lead to a decrease sensivity for anti-tumour agents that target topoisomerases and are responsible for inhibition of DNA synthesis and its damage[90,92,96].

DNA repair mechanisms are very important to maintain genetic stability, however with alkylating agents and also topoisomerase inhibitors, DNA repair systems cooperate to maintain cell viability. There are different mechanisms with the aim of excising damaged bases as MMR, NER, base excision repair (BER) and DSB. After the damage recognition, there is splicing out the lesion, and then the insertion of new bases[92,93]. For instance, DNA lesions due to alkylant drugs and ROS can be repaired through NER, the process starts with the recognition of the damage by a complex constituted by zinc-finger protein xeroderma pigmentosum group A (XPA) and heterotrimeric replication protein A (RPA), that will recruit the transcription factor II Human (TFIIH), whose subunits XPB and XPD function as helicases allowing DNA incision[85,90,94].

Finally, **defective apoptosis** can happen due to aberrantly regulation of TP53 and PTEN that is an inhibitor of PI3K/AKT pathway, increasing chemotherapy tolerance to platinum drugs and leading to drug-induced apoptosis failure. It is the apoptotic inhibitor named X-linked Inhibitor of apoptosis protein (XIAP) that downregulates or inhibits PTEN activity, whose function is catalyse the phosphorylation of the 3’ phosphate of the inositol

ring in phosphatidylinositol (3,4,5) - triphosphate (PIP_3) leading to phosphatidylinositol (4,5) -biphosphate (PIP_2)[31,90,103,104]. In this situation, PTEN is inhibited, PIP_3 is not phosphorylated and Akt presenting a high affinity for it, is activated. TP53 inhibits *PI3K* expression by binding to PIK3C (encoding PI3K) promoters avoiding its transcription, so the increased transcription of Akt accounts for the degradation of TP53 by the activation of its inhibitor MDM2, abrogating the mitochondrial TP53-dependent apoptosis[81,90,93,103,104].

Drug Damage Tolerance is linked with decreased susceptibility to apoptosis. Genes that influence in the induction or inhibition of apoptosis are altered. Catalytic caspases, that correspond to a group of cysteine-dependent aspartate-directed proteases that have their activity dependent on the formation of a tetrahedral intermediate by promoting a cysteine residue to act as a nucleophile are highly correlated with apoptosis signalling and apoptotic activity itself[90,105,106]. Death receptor pathway fas-ligand was disclosed to be upregulated by cisplatin in ovarian cancers resulting in stimulation of caspase-8 and -3 triggering apoptotic induction, however in a chemoresistant variant, cisplatin is incapable of upregulating fas-ligand and induce apoptosis leading to cell survival[7,81,90,103].

1.5. CANCER METABOLISM

Reprogramming energy metabolism is considered one of the new hallmarks of cancer, as cells alter their metabolism in order to increase their capacity to grow, invade and metastasize[7,8,107]. In 1923, cancer and metabolism correlation was first done by Otto Warburg being the key basis of a new concept named the Warburg effect[108–111]. However, we have been assisting to diverse changes to this “theory”. Cancer cells manage different ways to support its viability, that are not based on mitochondrial function disruption neither on oxygen absence. Non-cancerous and cancerous cells stablished cooperation relationships in order to maintain tumours progression and cancer associated-fibroblasts (CAFS) are one of the best examples to support this affirmation[21,22]. In addition, cancer cells can perform the uptake of metabolic substrates as fatty acids (FA) towards the rapid growth support and in order to afford the energy necessary for biomass synthesis. FA are major contributors to the acetyl-CoA pool, important for tricarboxylic acid (TCA) cycle that consequently is important to the generation of nicotinamide adenine dinucleotide (NADH), essential for oxidative phosphorylation occurrence[22]. Serpa, J. *et a*l., in 2010, also recognised the ability of cancer cells remodelling metabolism, by being capable of resemble normal colonocytes, in the colon tumour microenvironment, this cells showed capacity to metabolize butyrate, whose carbons are also important for TCA[10,112]. ROS, are short-lived molecules, that result from the cellular metabolism and whose functions are very important

in diverse biological cell processes as proliferation, differentiation, immune system regulation and vascular remodeling, however, excessive ROS leads to oxidative stress[84]. This increase was shown to be responsible for the modification of a sulfhydryl group of pyruvate kinase M2 (PKM2) that becomes inactive and alters pentose phosphate pathway (PPP), that is responsible for nicotinamide adenine dinucleotide phosphate (NADPH) generation reducing glutathione (GSH) into an active antioxidant with a protective activity. This last mechanism is very important in cell redox homeostasis[113].

1.5.1. GLUTATHIONE (GSH) AND CYSTEINE

Glutathione (GSH) and cysteine are both thiols having a sulfhydryl group (-SH) or thiol group, that is composed by a sulphur and hydrogen atoms attached to a carbon[114]**.**

1.5.1.1. GLUTATHIONE (GSH)

GSH, the main cellular low molecular weight thiol, is constituted by glutamic acid, cysteine and glycine (tripeptide gamma-glutamyl-cysteinyl-glycine)[115,116]. It is synthesized intracellularly in cell cytosol and is mediated by two enzymes that require ATP that are glutamate cysteine ligase (GCL) and GSH synthetase (GSS)[115,117]. GCL is associated with a dipeptide bond between the glutamic acid and cysteine, presenting two catalytic subunits, one is named glutamate-cysteine ligase catalytic subunit (GCLC) and contains the active site important for the linkage between the amino group of cysteine and the γ-carboxyl group of glutamic acid. The other enzyme is glutamate-cysteine ligase modifier (GCLM) that interacts with GCLC, increasing its catalytic activity[116]. GSS catalyses the formation of a peptide bond between the gamma-L-Glutamyl-l-Cysteine (γ -Glu-Cys/ γGC) and glycine. Substrate availability, and GCL are rate-limiting in GSH synthesis. Cells capacity to transport GSH into the extracellular compartment is important in cysteine transfer and consequently in the de novo GSH production (figure 1.6.)[95,118,119].

The degradation of GSH comprises the catalysation done by Ɣ-Glutamyl transpeptidase and dipeptidase, Ɣ-Glutamyl transferase, Ɣ-Glutamylcyclotransferase and 5-Oxoprolinase. Ɣ-Glutamyl transpeptidase helps cleaving the gamma linkage between glutamate and cysteine in an ATP-dependent manner, being responsible for the generation of cysteinyl-glycine (Cys-Gly), that posteriorly is cleaved by dipeptidase giving raise to free cysteine and glycine [95,117,120] .

GSH is mainly present in the cytosol (about 90%), being the remaining specially present in the mitochondria and very few in the endoplasmic reticulum[115]. GSH is mostly in the reduced form (GSH) being oxidized (GSH disulphide - GSSG) through its direct interaction with ROS or, more commonly, acting as a cofactor for antioxidant enzymes as

GSH peroxidases (GPx) [95,118,121,122]. GPx are tetrameric enzymes members of the selenium-dependent family that can be distinguished from transferases mainly due to its activation with hydrogen peroxide (H_2O_2)[118]. Those enzymes use glutathione (GSH) as a ROS scavenger, to reduce H_2O_2 to water (H_2O) and lipid peroxides, which are consequently reduced to alcohols[121,123].

GSH is fundamental in the protection of cells against apoptosis, corroborating with this, GSH/GSSG is of extreme importance for the detoxification process, its disruption will activate diverse transcription factors that are redox sensitive as activator protein-1 (AP-1) responsible for the expression of transforming growth factor beta (TGF-β), activator protein-2 (AP-2), c-jun N-terminal kinase (JNK), stress-activated protein kinase (SAPK), protein kinase C (PKC) and also tyrosine kinases. Protein tyrosine phosphatases, serine/threonine kinases and tyrosine kinase receptors, as epidermal growth factor receptor (EGFR) constitute some of the main ROS targets that are consequently affected in this dynamic[95,123]. Besides the antioxidant function, GSH is also involved in immune activity enhancement, DNA synthesis and repair, protein and prostaglandin synthesis[124].

As previously reported, GSH has a role in resistance to platinum drugs through several mechanisms based mainly on its capacity to increase intracellular drug detoxification[77,80,81,94]. In ovarian cancer it has been reported an association between higher GSH levels and glutathione S-transferase P1 (GSTP1) activity with chemoresistance phenomenon[77,125–128]. The deletion of GSTP1 was verified to be responsible for an increase sensitivity to platinum drugs in A2780 ovarian cancer cells[127]. It was also verified the role of the microRNA miR-133b overexpression in the capacity to augment ovarian cancer cell sensitivity to the commonest drugs used (cisplatin and paclitaxel), this happened through glutathione S-transferase (GST) and multidrug resistance protein 1 (MDR1) decreased expression[129]. As explained, ovarian cancer histotypes present different chemoresistance behaviour being OCCC cells more resistant to carboplatin than OSC cells[45,130]. Beyond this GSH levels also vary between these two carcinomas, Lopes-Coelho, *et al.*, verified that GSH synthesis inhibition by buthionine sulfoximine (BSO) sensitized OCCC to carboplatin[45].

Moreover, one of the most significant functions of GSH is cysteine storage since this thiol is unstable extracellularly and can rapidly oxidize to cystine producing potentially toxic ROS[115]. The next section will address cysteine metabolism and functions in cells, focusing in ovarian cancer.

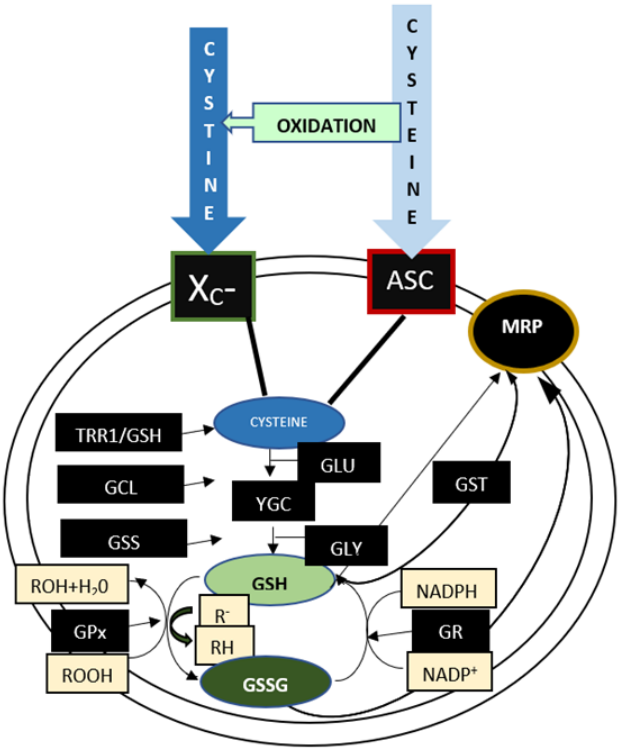


Figure 1.6. - Summary of GSH metabolism, showing the involvement of cysteine and glutamate/cysteine anti-porter system Xc - (xCT). xCT mediates the uptake of cystine that is reduced to cysteine through thioredoxin reductase 1 (TrxR1) or GSH. Glutamate cysteine ligase (GCL) and glutathione synthase (GSS) will catalyse the process responsible for the generation of GSH. GSH peroxidase (GPx) help in reducing radicals and GSH can be converted to GSH disulphide (GSSG). Than GSSG is recycled to GSH by another enzyme named GSH reductase (GR). NADPH is a co-factor of this reaction. GSH and GSSG are than exported by MRP. Adapted from The Cystine/Glutamate Antiporter System xc - in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities, Lewerenz, J. et al., 2013.

1.5.1.2. CYSTEINE (CYS)

Cysteine is a semi-essential amino acid, that is mainly provided through the diet or by trans-sulphuration pathway. So, its synthesis can happen through condensation of serine and methionine-derived homocysteine to form L-cystathionine, this reaction is catalysed by cystathionine-β-synthase (CBS), afterwards this pathway is mediated by cystathionine-ϒ-lyase (CSE) in the conversion of L-cystathionine to cysteine and α-ketobutyrate (trans-sulphuration pathway)[120,131,132]. Cysteine can also be obtained through L- cystine reduction and GSH degradation, previously described[117].

L-cysteine degradation mostly relies on a multi-enzyme pathway involved in sulfane sulphur generation from cysteine, homocysteine and their disulphides, through three enzymes that are CBS, CSE and by 3-mercapto-pyruvate sulphurtransferase (MpST) accompanied by cysteine aminotransferase (CAT). CSE is responsible for catalysing the conversion of L-cystine to thiocysteine, pyruvate, and ammonia. Then, thiocysteine, in the presence of thiols, forms hydrogen sulphide (H_2S) and cystine or the corresponding disulphide or even decomposes non-enzymatically to cysteine and inorganic sulphur[120,133]. The important enzyme MpST is responsible for catalysing cysteine degradation through the transfer of sulphur ion from 3-mercaptopyruvate to a thiol compound[132]. L-cysteine dessulphuration plays important roles in biological processes such as synthesis of iron sulphur, proteins and cyanide detoxification, but it also exerts anti-cancer and anti-oxidant effects. All these enzymes are also important for mitochondrial ATP production, while CAT and MpST are already in mitochondria, CBS and CSE are relocated there under cellular stress[134]. Other L-cysteine way of degradation is based on the activity of cysteine deoxygenase (CDO) that is responsible for the addition of an oxygen to the sulphur of cysteine, converting it to a sulphinic acid known as cysteinesulfinic acid (3-sulfinoalanine), being the final products taurine and sulfate[132,135].

It is recognized the importance of L-cysteine in the generation of GSH by being the rate-limiting substrate for GSH production, being the only thiol containing amino acid[114,136].Therefore, the maintenance of appropriate intracellular concentrations of cysteine is essential for redox homeostasis and for other mechanisms as protein synthesis[117,137].

Literature reports cysteine as a tumorigenesis promoter: in the context of ovarian cancer, it was revealed CBS importance in ovarian tumour growth, drug resistance and cellular bioenergetics[138]. Proteomic screen allowed to verify that CSE overexpression was common in OCCC, however the opposite pattern was observed in endometrioid and high grade serous ovarian cancer[139]. In addition, through metabolomics analysis ovarian cancer cells sensitive and resistant to platinum drugs presented differences in what concerns cysteine metabolism[140]. Corroborating with the previous data, Nunes, S. *et al*., supported the role of cysteine in hypoxia adaptation and its capacity to overcome carboplatin effects, recognizing an OCCC cell line (ES2) as more dependent on cysteine function than an OSC cell line (OVCAR3)[63]. In cancer context, cysteine role is also extended to other cancer types, in breast cancer cell lines, cysteine transport by SLC3A1/EAAT1 (excitatory amino acids transporters), decreased the stability and activity of catalytic subunit of protein phosphatase 2A (PP2Ac) through ROS level reduction and by the activation of AKT/GSK3β/β-catenin pathway[141].

1.5.2. CYSTEINE/GLUTAMATE TRANSPORTERS

There are three distinct pathways of cysteine transportation: the Na+ dependent transport mediated **by excitatory amino acids transporters (EAATs)** and the **system alanine-serine-cysteine (ASC)** that includes ASCT1 and 2, and the **Xc- system,** whose activity mainly relies on xCT transporter, that is dependent on proton electrochemical gradient[117]. EAAT3 and xCT transporters will be subject to a better characterization due to its role in ovarian cancer (cysteine transport) and chemoresistance that will be furthered explored.

1.5.2.1. SYSTEM Xc- (xCT)

The system Xc- was firstly described by Bannai and Kitamura in 1979 in cultured fibroblasts, being an antiporter system capable of transporting cystine and glutamate in both directions. As cystine, the oxidized cysteine form, is rapidly reduced and glutamate concentration is higher intracellularly, the procedure mainly relies on cystine import and glutamate export[117,136,142–145].

The xCT transporter is encoded by **solute carrier family 7 member 11 *(SLC7A11)***, which is localized in chromosome 4 (4q28.3). *xCT/SLC7A11* gene has an antioxidant-responsive element region (ARE) in its promoter that is under the regulation of nuclear factor erythroid 2-related factor 2 (NRF2), a major transcription factor involved in cellular defence against oxidative stress, posteriorly characterised. In a study led by Carpi-Santos, R. and Calaza, K.C., it was shown that *xCT/SLC7A11* expression is affected in the retina of diabetic rats by direct regulation of NRF2 activity[146]. *xCT/SLC7A11* gene knockdown resulted in higher endogenous ROS levels, leading to GSH impairment, and enhanced invasive properties[146]. *xCT/SLC7A11* regulation by NRF2 has many relevance in brain cancer context, by being responsible for glioma-induced neuronal cell death and tumour-associated epileptic events[147]. Experiments conducted by Qian, M. *et al.*, aimed to make an association between sepsis illness and protein interaction with C-kinase 1 (PICK1), so besides suggesting this, they found out that absence of PICK1 was responsible for xCT expression inhibition and impaired GSH synthesis, leading to severe oxidative stress[148]. In what concerns to microRNAs, miR-375 is recognized by being expressed in the pancreatic islet, brain and spinal cord and associated with many cancers, being reported to negatively regulate *xCT/SLC7A11* in oral squamous cell carcinoma (OSCC) cells[149]. So besides cancer context this gene is associated with other diseases as epilepsy, diabetes, sepsis and so on and its presence was also reported in kidney and duodenum[136].

Data proposes that transcriptional regulation of xCT, a 12-pass transmembrane protein xCT/the light chain element from system Xc-, is more important than the expression of the heavy chain named 4F2hc. Chains are linked by a disulphide bridge, however 4F2hc is capable to form heterodimers with other specific amino acid transporter light chains[110,117,150]. xCT is responsible for maintaining intracellular cysteine in normal physiological concentrations in order to achieve cell homeostasis. This role is better known in brain/central nervous system, because the neurotransmitter glutamate is dependent on xCT. Besides its contribution for oxidative protection, it is involved in the operation of the blood brain barrier, in neurotransmission, drug addiction, chemoresistance and brain tumour growth[117,151]. Studies performed in the light of this knowledge have been found that neuronal oxidative stress is related with CNS dysfunction, hypothesizing the occurrence of glutamate accumulation in the extracellular microenvironment that leads to the reduction of cysteine import to the cell through EAAT3 and the tendency to induce ROS formation which is a major contributor to mitochondrial dysfunction, thereby, increased ROS induce the formation of inflammatory factors that enhance excitotoxicity[117]. Equally the proper cancer cell can lead to ROS increase by augmenting the activity of this transporter. This process is observed in glioblastomas, a central nervous system tumour presentig high mortality rates. Corroborating with this, cells from glioblastoma, normally, show higher expression of xCT transcripts[117,144,152].

There is also xCT activity evidences in breast cancer, in which upregulation is negatively correlated with survival. These conclusions were derived from experiments in triple negative breast cancer (TNBC) that allowed to see that cystine availability through xCT inhibition resulted in decreased GSH levels and an increased in ROS levels, corroborating with the previous data[153,154]. In relation to ovarian cancer it was shown that oxidative stress was correlated with increased xCT expression, enhancing cysteine influx and resulting in a stimulation of GSH synthesis[155,156].

Besides the overall functions announced, a role of xCT in the immune system was also reported. There is evidence that system Xc – is important in the regulation of the innate immune response[117,157]. Macrophages activation is associated with ROS production, which are responsible for oxidative stress and directly upregulation of this system[117,157].

Giving the relevance of this transporter in cancer context and its association with increased GSH levels and chemoresistance, efforts in developing compounds capable of interfering with xCT expression and with its inhibition were done[150,158]. Sulfasalazine and erastin have been tested in this ambit[155,159].

Sulfasalazine (SSZ) is a drug with anti-inflammatory and immunomodulatory activity, that is used in rheumatic polyarthritis[160]. This drug is metabolized by bacteria into sulfapyridine and mesalazine and reaches the colon in 90% of the case[160]. However, more recently, it has been apointed as an inhibitor of xCT[161]. SSZ mechanism of action upon xCT still remains unclear, however a competition reaction has been appointed due to a reduction in drug efficacy by increasing cystine levels in cell medium[161]. There is evidence in the ocurrence of reduction in GSH and tumour proliferation in glioblastomas and small-cell lung cancer and also a decrease in cell metastasis in esophageal squamous cell carcinoma by SSZ that interfers with caveolin-1/β-catenin pathway and by GSH depletion[151]. Some new data, provided by in vivo assays, shows that sulphasalazine combined with chemotherapeutic drugs, as temozolomide (TMZ), an alkylating agent commonly used in glioblastomas treatment, resulted in more cell death,comparing with the use of SSZ and TMZ alone[162]. Other synersgitic effects with SSZ were reported even with radiotherapy[163]. So, SSZ is an inducer of cancer cell death through ferroptotic cell death/iron dependent. In prior studies, remitting to 1987, 0. H. Nielsen and colleagues showed that this drug constitutes an inhibitor of "lamina propria macrophage chemotaxis", limiting in their study case the intestinal inflammatory process, through a reduced recruitment of this cell type to the inflamed areas, thereby was found to be also responsible for leucocyte motility and nuclear factor kappa B (NFκB) inhibition[164,165].

Erastin (ERA) is also responsible for ferroptotic cell death through disruption of mitochondrial permeability transition pore (mPTP) interfering with cells mitochondrial energy supply and leading to cell homeostasis disruption[159,166]. Besides that, studies conducted by Yagoda, N., *et al*., 2007, allowed to see that ERA was responsible for tumour cells death harbouring mutations in RAS-RAF-MEK genes[167].The set of assays conducted allowed to conclude that ERA will act through mitochondrial voltage-dependent anion channels (VDACs), that are membrane-spanning channels responsible for ions and other metabolites transport. This was proven because RNA-interference-mediated knockdown of VDAC2 or VDAC3 conduct to ERA resistance. They found this drug as important for changes in outer mitochondrial membrane due to a decrease in the permeability to NADH and consequently to its oxidation[167]. Once more, xCT mechanism of inhibition by ERA still is under investigation[157].

Comparing both inhibitors, Dixon, S.J. *et al*., recognized that cystine inhibition by ERA through the system Xc- was more potent than with Sulphazalazine. ERA IC50 was approximately 2250 times lower than SSZ IC50, resulting in a great decrease in cysteine uptake, through irreversible xCT antiporter inhibition[157,168].

1.5.2.2. EXCITATORY AMINO ACID TRANSPORTERS, MEMBER3 (EAAT3)

Excitatory amino acid transporters (EAATs) are a class of membrane transporters which include five different types, EAAT1-5. These transporters are found in different cell types, but resembling xCT their function, as previously understood, is better known in central nervous system. EAAT1 and 2 are predominantly in the membrane of glial cells. EAAT3 and 4 are present in the neurons, more precisely in peri synaptic membrane, however EAAT3 and EAAT5 have been found in other cell types. In central nervous system their action is to export glutamate released during neurotransmission[169–173].

EAAT3 is encoded by **solute carrier family 1 member 1 (*SLC1A1*)**, it is located in chomosome 9 (9p24.2) and can be regulated at transcription and trafficking levels and by pharmacological modulation, as it will be exposed[173]. By being predominantly present in the brain reveals to have a role in diseases like epilepsy[173]. Studies by Davis, K.E. suggested that the trafficking of EAAT3 is regulated by PKC, demonstrating that when the cells are treated with a phorbol ester: phorbol 12-myristate 13-acetate (PMA), recognised as a PKC activator there was an increase in the plasma membrane localization of EAAT3[174].

hsa-mir-26a-5p (miR-26a), correlated with brain tumours and known for being a direct regulator of PTEN expression, was found in multiple sclerosis context to be responsible for *SLC1A1/EAAT3* regulation, by silencing it[175]. microRNAS regulation is not exclusive from miR-26a, miR-101 was found to be important in *SLC1A1/EAAT3* upregulation in depression states[175,176]. Still in depressive case, ketamine, as an antagonist of N-methyl-D-aspartate receptors (NMDARs), constitutes a good antidepressant which is associated with *EAAT3/SLC1A1* increased expression[172]. In rat retina, GluN2B-containing-NMDARs stimulated by caffeine was also responsible for the upregulation of this gene[177].

As previously revealed the glutamate transport mediated by EAATs is dependent on a sodium gradient[169]. There is the cotransport of three sodium ions plus one proton and the counter-transport of a potassium ion[170,178]. EAAT3 is ubiquitously expressed and is capable of transporting glutamate against a several thousand-fold concentration gradient[173]. Besides glutamate and cysteine it transports aspartate (ASP). Cysteine is transported in its deprotonated/reduced form (cysteine), interacting with a residue in the transmembrane helice 8[137,173].

As the uptake of cysteine in neurons was proven to be predominately mediated by the EAAT system, EAAT blockade induces a reduction in the synthesis of neuronal GSH[179].Experiments also showed that in EAAT3 deficient mice, neurons that were more

susceptible to ROS presented lower GSH levels and this was responsible for sustaining oxidative stress and decreased neuronal survival. In 2014, Watts *et al*. concluded that EAAT3 was important for cysteine uptake but not for its depletion, proving once again cysteine transport comprising intracellular redox potential homeostasis[137].

There are several no selective EAAT3 inhibitors, one of the best known is β-benzo-Asp analogs. As EAAT3 is also responsible for aspartate transport, **β-benzo-Asp analogs** have been studied, due to their competitive way of action: for example, dl-threo-β-Benzyloxyaspartic acid (TBOA) binds to the transporter like glutamate, being determinant for conformational changes during transport of the proper glutamate[180,181].The cotreatment with this drug is beneficial with irinotecan and oxaliplatin[182].

As non-competitive/allosteric inhibitors of glutamate uptake we can focus on the HIP-A (3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d] isoxazole-4-carboxylic acid) and the HIP-B (3-hydroxy-4,5,6, 6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid), both bind to EAATs, mainly to EAAT1, at a site different from the orthosteric substrate binding site, prompting a conformational change that affects the transport dynamic[180].

Amphetamine (AMPH), a central nervous system stimulant involved in glutamatergic signalling adaptations, was demonstrated to stimulate the endocytosis of EAAT3 through dopamine transporter (DAT) dependent on dynamin and AMPH-mediated activation of a Rho-GTPase[180,183].

Apart from these xCT and EAAT3 inhibitors, other types of inhibitors have also been reliable. Selenium (Se), is an essential nutrient required for mechanisms as the maintenance of cellular redox[184,185]. xCT has been demonstrated to uptake selenium compounds, as selenite and EAAT3 responsible for the uptake of selenocysteine, that is quite similar to cysteine, being the only difference based in the substitution of sulphur group by selenium that is less reactive and prefers higher oxidation states[117,137,184–186]. Their similarity in cell uptake give rise to a hypothesis that regards on the use of selenated-compounds leading to a competition for cysteine transport, abrogating chemoresistance pathways, that involve detoxification mechanisms[137,187]. In the context from the prior information, an organoselenium compound, constituted by a selenium group and chrysin, a flavone, with demonstrated anti-tumoral and anti-oxidant properties, named Selenium-containing chrysin (SeChry) showed promising properties for the chemoresistance abrogation in cisplatin resistant cell lines. Interestingly, in one hand, SeChry shows antioxidant properties by protecting cells from toxic compounds and on the other hand, can induce apoptosis[188–190].

1.5.3. ROLE OF NRF2 AND HNF1β IN THE REGULATION OF CYSTEINE UPTAKE

We choose to study these two transcription factors as putative regulators of *xCT/SLC7A11* and *SLC1A1/EAAT3* expression, because their binding sequence are present in the promoter regions of these genes and also because NRF2 and HNF1β have already been implicated in GSH synthesis and ROS control[45,117,191].

1.5.3.1. NUCLEAR FACTOR ERYTHROID 2- P45 -RELATED FACTOR2 (NRF2)

Nuclear Factor Erythroid 2- P45 -Related Factor2 (NRF2) is part of the basic region leucine zipper transcription factors, named the "cap 'n' collar" constituted by a family of six transcription factors: NRF2, NRF1, NRF3, Bach 1, and Bach 2[147,154]. NRF2 is a redox sensitive transcription factor that is involved in many cellular functions such as lipid synthesis, differentiation, proliferation, inflammation and apoptosis[107,192–194]. It has been reported in mammalian hematopoiesis by being responsible for the regulation of hemoxygenase-1 (HO-1) implicated in the management of iron[192,193,195].

NRF2 is a short-lived protein, which under basal conditions is maintained at low levels, in the cytosol and binding to its inhibitory protein: Kelch-like ECH-1 (Keap-1), that leads to NRF2 ubiquitination and subsequent degradation. When there are NRF2 stimulators or in stress conditions, NRF2 dissociates from Keap-1 , its degradation becomes difficult and the transcriptional factor is translocated to the nucleus[117,193,196]. The process of degradation is mediated by two regulatory mechanisms, the most recurrent has the involvement of Keap-1 the substrate adaptor protein for Cullin 3 (Cul3)/ Ring-box protein 1 (Rbx1) ubiquitin ligase. The other pathway is mediated by glycogen synthase kinase (GSK)3/β-TrCP-dependent (cullin 1) Cul1-based ubiquitin ligase[154,193,197].

In the nucleus NRF2 forms heterodimers with proteins that are called small masculoaponeurotic fibrosarcoma (Maf) that will help with the binding of this transcription factor to the antioxidant response element (ARE) that is present in the promoter regions of diverse genes[193,196,198]. So NRF2 is capable of recruiting transcriptional machinery, including receptor associated co-activator 3 (RAC3) to effectively transactivate the ARE-driven gene (figure 1.7.)[151,193,195,196,198].

NAD(P)H: quinone oxidoreductase (NQO1), GST, GPx, catalytic and regulatory subunits of GCL are examples of redox-balancing proteins that are directly regulated by NRF2[193] . These enzymes are commonly called drug-metabolizing enzymes (DMES). The induction of the DMEs is important for an increased detoxification. Thereby NRF2 acts as a

xenobiotic-activated receptor (XAR)[193]. Curiously, another transcriptional target of NRF2, already here contextualized, is the glutamate antiporter xCT from system Xc[-154,199].

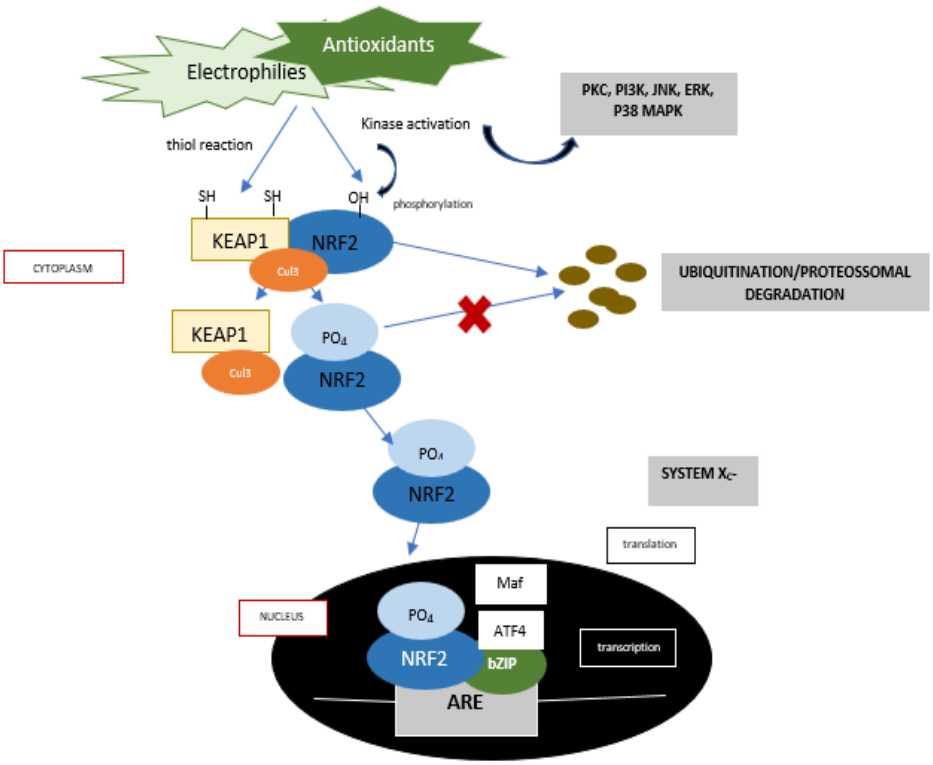


Figure 1.7. - Representation of the NRF2 regulation. NRF2 is activated by ROS and other agents and goes to the nucleus through Keap-1 pathway. Here it forms a heterodimer with Maf protein, interacting with ARE. This mechanism is essential for system Xc- activation and DMEs. Adapted from Bridges, R. J., Natale, N. R. and Patel, S. A., 2012.

The knockout of *NRF2* compared with the WT in mice increases the susceptibility to chemical toxicity and other diseases that are correlated with oxidative interactions. When working in aberrant conditions, NRF2 has been identified as a very important element in many pathologies, including cancer[87,154]. Cancer cells enhanced detoxification capacities. Proof of the prior data, *NRF2* increased expression leads to resistance of breast and ovarian cancer cells to doxorubicin and paclitaxel[87,154,200]. Doxorubicin, produced by *Streptomyces* is a drug commonly used to treat some cancers, by intercalating with DNA, inhibiting topoisomerase II and avoiding replication. In ovarian cancer it is mainly used in advanced and recurred disease[200,201].

Studies evidence that phosphorylation of NRF2 has a contribute to its nuclear exclusion, and this phenomenon is involved in some pathways as MAPK and PI3K/AKT[154].

1.5.3.2. HEPATOCYTE NUCLEAR FACTOR 1 HOMEOBOX B (HNF1β)

Hepatocyte nuclear factor 1 homeobox B (*HNF1B*) gene equally known by transcription factor 2 (*TCF-2*) gene, is a member of the homeodomain-containing superfamily of transcription factors (TFs) genes that encodes a protein (HNF1β or TCF2) of the homeobox-containing basic helix-turn-helix family[202]. HNF1β shares amino acid sequence homology in more than 80% with HNF1α, being capable of recognize the same binding sites of HNF1α and by forming heterodimers with it[203]. Therefore HNF1 consists in a dimeric protein composed by an amino-terminal, a DNA-binding and a carboxyl-terminal domains[204,205].

HNF1 is expressed in polarized epithelium of pancreatic, liver, digestive tract and kidney tissue[206,207]**.** By being present in these tissues, HNF1 exhibit involvement in the regulation of diverse genes such as maturity-onset diabetes of the young 1 and 3 (MODY), sodium-glucose cotransporter 2 (SGLT2), β-fibrinogen, α1-antitrypsin and albumin[204]. Consistently with this, phenylalanine catabolism, glucose metabolism/homeostasis and differences in the reabsorption of certain metabolites, as glucose, are severely affected by alterations, as heterozygous mutations, in these transcription factors[202,204,205,208]. Meanwhile HNF1β displays important activities in embryonal pancreas development by operating in pancreatic cells differentiation[205,207,209].

The occurred mutations in *HNF1B* can be *de novo* or inherited comprising both monoallelic changes or whole gene deletions. An analysis of The Cancer Genome Atlas (TCGA) data, conducted by Shen, H. *et al.,* led to prove that in OSC there is *HNF1B* downregulation that results due to epigenetic silencing in about 50% of the cases[202,210]. In contrast, CCC from different organs including ovary, shows “de novo” expression of HNF1β, being considered a CCC hallmark and a useful immunohistochemical marker[45,71,202,209,211]. The involvement in the maintenance of epithelial phenotype, links *HNF1*B downregulation to epithelial to mesenchymal transition (EMT) in OCCC[212]. Being demonstrated that is important for the maintenance of E-cadherin[71].

The study of HNF1β in cancer context has been increasing: OCCC presents osteopontin (OPN), a gene associated with tumour progression, as one of the most markedly upregulated genes. As OPN contains functional HNF1β binding sites in the promoter region it can constitute a direct target gene of it[213,214]. HNF1β is regulated at translational and post-translational level and experiments suggest that Notch2 pathway is responsible for the regulation of *HNF1B* gene expression[203,213,215].

Besides ovarian cancer its presence is recognized in prostate, kidney, endometrial and hepatocellular carcinomas[209,213,216,217]. Its characterization still remains under evaluation, some authors look at it as an oncogene mainly because its overexpression results in worse metastasis survival in HER2-positive breast cancer and due to its upregulation in cysts that remit for the initiation and survival of OCCC, being associated with chemotherapy resistance[71,208,210,218,220]. Controversially, it can be considered as a tumour suppressor gene in serous carcinomas, that loses its activity when silenced by promoter methylation (i.e.)[210,219].

Coelho, F. *et al*. showed the involvement of HNF1β in the regulation GCLC expression and consequently its importance in GSH production[45]. As higher GSH production is assumed to be an important key factor in OCCC chemoresistance, HNF1β is an important mediator in therapeutic resistance[122,208,220].

2. AIMS

The hypothesis of this thesis is: xCT and EAAT3 are crucial for cysteine uptake and chemoresistance. So, there are two main objectives, the first one is to consolidate the role of EAAT3 and xCT transporters in ovarian carcinoma chemoresistance and the second one is to evaluate the capacity of SeChry (selenium-containing chrysin) in the inhibition of cysteine uptake, and its effect in chemoresistance reversion.

Thus, the first objective will be accomplished by addressing the effect of cysteine and carboplatin in:

1 – the expression of transporters *encoded by xCT/SLC7A11* and *EAAT3/SLC1A1*, by accessing gene and protein expression; 2 – *NRF2* and *HNF1B* gene expression and the capacity of those transcription factors to differently modulate *xCT/SLC7A11* and *EAAT3/SLC1A1* expression upon different stimuli; 3 – cell viability.

The second objective will be accomplished by assessing the effects of SeChry exposure in:

1 – cell viability; 2 – thiols dynamics to ascertain its role in cysteine uptake, and 3 – carboplatin resistance reversion.

In order to fulfil our aims, two different ovarian cancer cell lines with different chemoresistance levels were used: ES2 (ATCC ® CRL-1978™) cells, as previously mentioned, are intrinsically resistant and OVCAR3 (ATCC ® HTB-161™) cells acquire resistance throughout therapy.

3. MATERIAL AND METHODS

3.1. CELL CULTURE

Two different ovarian cancer cells lines were used, one serous carcinoma (OSC) cell line (OVCAR3 - HTB-161™) and one clear cell carcinoma (OCCC) cell line (ES2 - CRL-1978™). Both were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Essential Medium 1X (DMEM) (41965-039, Gibco, Life Technologies) that contains 4,5 g/L of D-glucose and L-glutamine 0,58 g/L. Besides this, the medium was supplemented with 10% Fetal Bovine Serum (FBS) (S 0615, Merck), 1% Antibiotic-Antimycotic (AA) (P06-07300, PAN Biotech) and 1% Gentamicin (15750-060, Gibco, Life Technologies). Cells were maintained under a temperature of 37°C, 5% CO_2 and in a humidified environment. Cells were grown to approximately 75%-100% optical confluence before they were detached with 0,05% Trypsin- Ethylenediamine Tetra-acetic Acid (EDTA) (25300-054, Invitrogen, Thermo Fisher Scientific) at room temperature (RT) for approximately 5min. For each assay, the seeded cellular density and culture conditions are summarized in table 3.1. Cell number was determined with a Bürker counting chamber.

Table 3.1. - Cell plate/flask areas, cell number and cell culture conditions for each assay performed.

ASSAY	SEEDING	STARVATION	CONDITIONS	TIME POINT(S)
mRNA EXPRESSION	12 well-plates 2 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM) (102839, Merck); Carboplatin (0,025 mg/mL) (gently provided by IPO's pharmacy); L-Cysteine (0,402 mM) + Carboplatin (0,025 mg/mL) DMEM 1%FBS	16h
PULSE CHASE - mRNA EXPRESSION	6 well-plates 2,5 x 10^5 cells/mL	-	Control; L-Cysteine (0,402 mM) DMEM 1%FBS	30min, 1h, 2h, 4h, 6h, 8h
ChIP	tissue flask 175 cm^2 1 x 10^6 cells/mL	8h	Control; L-Cysteine (0,402 mM); Carboplatin (0,025 mg/mL); L-Cysteine (0,402 mM) + Carboplatin (0,025 mg/mL) DMEM 1%FBS	16h
	6 well-plates 2,5 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM); Carboplatin/Cisplatin (0,025 mg/mL); L-Cysteine (0,402 mM) + Carboplatin/Cisplatin (0,025 mg/mL) DMEM 1%FBS	30min, 2h
WESTERN BLOT - total content	6 well-plates 2,5 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM); Carboplatin (0,025 mg/mL); L-Cysteine (0,402 mM) + Carboplatin (0,025 mg/mL) DMEM 1%FBS	16h
WESTERN BLOT - mitochondrial content	tissue flask 150 cm^2 8,5 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM) DMEM 1%FBS	16h
IMMUNOFLUORESCENCE	24 well-plates 2 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM); Carboplatin (0,025 mg/mL); L-Cysteine (0,402 mM) + Carboplatin (0,025 mg/mL) DMEM 1%FBS	16h
CARBOPLATIN/CISPLATIN-CELL VIABILITY	24 well-plates 2 x 10^5 cells/mL	8h	Control; L-Cysteine (0,402 mM); Carboplatin/Cisplatin (0,025 mg/mL); L-Cysteine (0,402 mM) + Carboplatin/Cisplatin (0,025 mg/mL) DMEM 1%FBS	16h
SeChry IC50	24 well-plates 2 x 10^5 cells/mL	-	Control; SeChry: 0,5 μM, 1 μM, 5 μM, 10 μM, 20 μM and 40 μM DMEM 10%FBS	48h
SeChry – CELL VIABILITY CARBOPLATIN	24 well-plates 2 x 10^5 cells/mL	-	Control; SeChry: 40 μM and 100 μM; Carboplatin (0,025 mg/mL) (SeChry medium extracted before Carboplatin appliance in time-point 24h) DMEM 10%FBS	24h/48h (SeChry 24h (Carboplatin)
SeChry – CARBOPLATIN+CYSTEINE	24 well-plates 2 x 10^5 cells/mL	-	Control; SeChry: 19μM; L-Cysteine (0,402 mM); Carboplatin (0,025 mg/mL) DMEM 10%FBS	48h (SeChry) 24h (Carboplatin and/or L-Cysteine)
HPLC	6 well-plates 2,5 x 10^5 cells/mL	-	Control; L-Cysteine (0,402 mM); SeChry (19 μM); L-Cysteine (0,402 mM) + SeChry (19 μM) DMEM 10%FBS	24h (SeChry) 30min, 2h (L-Cysteine)

3.2. GENE EXPRESSION

To analyse the expression of *xCT/SLC7A11*, *EAAT3/SLC1A1*, *NRF2* and *HNF1B* genes, reverse transcription (R.T.) and relative quantifying polymerase chain reaction (RQ-PCR) were performed.

3.2.1. REVERSE TRANSCRIPTION (R.T.)

Cellular behaviour and response to microenvironment changes are accompanied by gene expression alterations, thus making transcription levels (messenger ribonucleic acid, mRNA) quantification, a valuable tool to assess gene function in biological samples[221]. Polymerase chain reaction (PCR) widely used in molecular biology to amplify DNA, gave rise to a variant technique named reverse transcription polymerase chain reaction (RT-PCR) that is employed in mRNA quantification. This technique is characterised by the reverse transcription based on the activity of the enzyme reverse transcriptase that catalyses the production of a single-stranded complementary DNA copy (cDNA) from an RNA template followed by its cDNA amplification by PCR[222].

To first study the influence of cysteine and carboplatin effect on mRNA expression of *xCT/SLC7A11*, *EAAT3/SLC1A1*, *NRF2 and HNF1B*, cells were seeded according to the conditions stablished in table 3.1. To further analyse the influence of cysteine in *xCT/SLC7A11* and *EAAT3/SLC1A1* expression, a pulse chase assay - a method extensively used to examine cellular activity over the time - was performed (table 3.1.).

Following cells collection, ribonucleic acid (RNA) was extracted by RNeasy Mini Extraction kit (74104, Qiagen), according to the manufacturer's protocol. RNA concentration was determined by measuring the absorbance at 260 nm in a Nanodrop 2000 (Thermo Scientific). cDNA was synthesized as follow in tables 3.2. and 3.3.

Table 3.2. – Reagents used for cDNA synthesis.

QUANTITY PER SAMPLE	REAGENT	REFERENCE
2 µL	DEOXYNUCLEOTIDES (dNTPs) mix (10 mM)	28-4065-22V, 28-4065-02V, 28-4065-12V and 28-4065-32V, GE Healthcare
2 µL	DITHIOTHREITOL (DTT) (0.1M)	Y00147, Invitrogen
1 µL	RNAse OUT™ Recombinant RNase Inhibitor (40 U/µL)	10777-019, Invitrogen
4 µL	FIRST STRAND BUFFER 5X	Y00146, Invitrogen
1 µL	Superscript II® (200 U/µL)	18064-022, Invitrogen
Volume necessary to complete a total volume of 12 µL	ddH_2O	

The table 3.3. present the detailed following steps performed after the addition of this mixture.

Table 3.3. – Thermocycler program used for cDNA synthesis.

STAGE	CYCLE NUMBER	TEMPERATURE (ºC)	TIME (min)
DENATURATION	1	70	10
COOLING	-	4	3
cDNA SYNTHESIS	1	42	90
Reverse transcriptase INACTIVATION	1	75	15
COOLING	-	4	∞

After R.T., RQ-PCR (detailed in 3.2.2.) was performed.

3.2.2. RELATIVE QUANTIFYING POLYMERASE CHAIN REACTION (RQ- PCR)

RQ-PCR is a technique distinguishable from other methods recurrently used to evaluate gene expression, mainly due to its sensitivity, accuracy and celerity to obtain results, making possible the absolute and relative quantification of cDNA related to a certain mRNA[223].

For RQ-PCR a reaction mixture was performed for all genes of interest for each sample. The reaction mixture required for mRNA expression evaluation of *xCT/SLC7A11*, *EAAT3/SLC1A1*, *NRF2* and *HNF1B* is detailed in tables 3.4. and 3.5.

Table 3.4. - RQ-PCR mixture used to analyse cDNA obtained from R.T.

QUANTITY PER SAMPLE	REAGENT	REFERENCE
1 µL	cDNA	-
4 µL	SYBR Green PCR Master Mix	4309155, Applied Biosystems
0,15 µL **0,15 µL**	Primer forward (10 µM) Primer reverse (10 µM)	Sigma Aldrich
3 µL	ddH_2O	

Table 3.5. - RQ-PCR mixture used to quantify mRNA obtained from pulse chase (3.2.1.) and to uncover NRF2 and HNF1β relative occupancy in *xCT/SLC7A11* and *EAAT3/SLC1A1* promoter, referred in 3.4.

QUANTITY PER SAMPLE	REAGENT	REFERENCE
1 µL	cDNA	-
3,5 µL	LightCycler 480 SYBR Green I master	04707516001, Roche
0.5 µL	PRIMERS: primer forward and reverse (5 µM)	Sigma Aldrich
2 µL	ddH_2O	

To all the experiments reported in 3.2.1., samples normalization was performed using the housekeeping gene (expressed constitutively) hypoxanthine phosphoribosyl transferase 1 (HPRT1). Besides this normalization, all cells were normalized to a control sample.

In the table 3.6 and 3.7. are present the amplification programs used in LightCycler 480 instrument (Roche), using Applied Biosystems and Roche reagents.

Table 3.6. - Amplification program for Applied Biosystems reagents presented in table 3.4.

STAGE	CYCLE NUMBER	TEMPERATURE (ºC)	TIME
HEAT ACTIVATION	1	50	2min
INCUBATION	1	95	10min
PCR DENATURATION	45	95	15sec
ANNEALING		60	1min
ELONGATION			
MELTING	1	95 60 95	15sec 15sec
COOLING	1	4	10sec

Table 3.7. - Amplification program for Roche reagents presented in table 3.5.

STAGE	CYCLE NUMBER	TEMPERATURE (ºC)	TIME
INCUBATION	1	95	10 min
PCR DENATURATION	70	95	15sec
ANNEALING		62	20sec
ELONGATION		72	15sec
MELTING	1	95 60 95	5sec 1min
COOLING	1	4	10sec

The primers used to assess *xCT/SLC7A11*, *EAAT3/SLC1A1, NRF2* and *HNF1B* mRNA expression are listed in table 3.8.

Table 3.8. - Primers used to assess the quantification of xCT/SLC7A11, EAAT3/SLC1A1, NRF2 and HNF1B genes expression. Gene encoding hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as the house keeping gene.

PRIMERS	FORWARD (5'-3')	REVERSE (5'-3')
SLC7A11/xCT	GGTCCTGTCACTATTTGGAGC	GAGGAGTTCCACCCAGACTC
EAAT3/SLC1A1	GTATCACGGCCACATCTGCC	GCAATGATCAGGGTGACATCC
NRF2	GCCAACTACTCCCAGGTTG	CGTAGCCGAAGAAACCTCATTG
HNF1B	CCGACAATTCAACCAGACAG	CAGAGCAGGCATCATCGGAC
HPRT1	TGACACTGGCAAAACAATG	GGTCGTTTTTCACCAGCAA

3.3. PROTEIN LEVELS

To investigate the effect of cysteine and carboplatin in xCT, EAAT3, NRF2 and HNF1β protein levels, western blot and immunofluorescence analysis were performed.

3.3.1 WESTERN BLOT (WB)

Western blot is a useful technique widely used to identify proteins. Gel electrophoresis, which is based on molecular weight, is used to separate a mixture of proteins in complex biological samples. After its separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transferring, the interaction with a specific antibody allow to obtain a band for each protein[224].

For the extraction of total protein content, cells were cultured as described in table 3.1. After tripsinization and centrifugation (150xg), cell extracts were obtained by lysing cells in Radio-Immunoprecipitation Assay (RIPA) buffer **(Appendix A)** and stored at -20ºC until use.

For the extraction of mitochondrial protein content cells were cultured as described in table 3.1. Then, cells were harvested, and mitochondria were isolated with Mitochondria Isolation Kit for profiling cultured cells (MITOISO2, Sigma). Briefly, cells were scraped, centrifuged for 5min at 600xg. and washed in ice cold phosphate buffered saline (PBS (1X)). Cell pellet was then resuspended in 650 µL of Lysis Buffer. A 5min period of incubation was performed on ice and samples were suspended at 1min intervals by pipetting up and down

once. Two volumes of Extraction Buffer were added and the homogenate was centrifuged at 600xg for 10min at 4°C. The supernatant was transferred to a fresh tube and centrifuged at 11000xg for 10min at 4°C, this new supernatant was removed and the pellet suspended in 75 µL of CelLytic M Cell Lysis Reagent.

The lysates were centrifuged at 20800xg for 5min at 4ºC. Protein concentration was determined based on Bradford method, using Bio-Rad protein assay reagent (500-0006, Bio-Rad) through spectrophotometric quantification at 595 nm in Bio-Rad, iMark, microplate reader. The same amount of total protein from each sample lysate was used. To perform electrophoresis, a mixture of loading buffer 5x **(Appendix A)** with 10% β-mercaptoethanol (M3148, Sigma Aldrich) was applied to each sample and boiled at 95-100ºC during 10min, to induce proteins denaturation. Samples were centrifuged at for 20800xg 2min at 4ºC, placed on ice and loaded in 12% polyacrylamide gel (Tris-glycine SDS-Polyacrylamide gel). Electrophoresis was performed in MINIPROTEAN Tetra Electrophoresis System (Bio-Rad) at 140 V during approximately 1h30min, buffered in 1XTGS buffer (TrisGlycine-SDS 10x (TGS)) (161-0772, Bio-Rad). The gel was transferred to an Immun-Blot® PVDF membrane with a Trans-Blot® TURBO (Bio-Rad), at 25 V, 1,3 A and during 10min **(Appendix A).** After the transfer, membrane blocking was done with 3% weight (w)/volume(v)) bovine serum albumin (BSA (A9647, Sigma)) in PBS (1X) 0,1% volume (v)/volume (v) Tween 20 (PBS(1X) - 0,1% Tween - 3%BSA), in the case of EAAT3 and xCT antibodies, or 5% w/v non-fat dry milk(NFDM (Molico, Nestlé) in Tris buffered saline (TBS) 1x 0,1%v/v Tween 20 (TBS (1X) - 0,1% Tween 20 - 5% NFDM), for NRF2 antibody, for about 1h at RT and in agitation **(Appendix A)**. This procedure is necessary to avoid non-specific bindings to the membrane. Membranes were incubated with primary specific antibodies (Mouse anti-human EAAT3, ab131429-Abcam; Rabbit anti-human xCT, ab175186- Abcam; Rabbit anti-human NRF2, ab62352- Abcam; diluted 1:1000 in PBS(1X)- 0,1% Tween - 3% BSA (EAAT3 and xCT) antibodies or TBS (1X) – 0,1% Tween 20 - 5% NFDM at 4ºC with agitation, overnight. To remove unbound primary antibody, the membrane was washed 3 times, for 5 min, with PBS (1X) 0.1% (v/v) Tween 20. Posteriorly, the membranes were incubated with the respective secondary antibody IgG-conjugated horse raddish peroxidase (HRP) (anti-rabbit, 31460, from Thermo Scientific or anti-mouse 31430 from Thermo Scientific; 1:5000 in PBS (1X)- 0,1% Tween - 3% BSA or TBS (1X) – 0,1% Tween 20 - 5% NFDM). To assess the peroxidase activity, it was used the ECL method and digital images were obtained through ChemiDoc XRS System (Bio-Rad) with Image Lab Software. The intensity of each band was quantified using ImageJ Software (http://imagej.nih.gov/ij/) and afterwards normalized to β-actin (A5441, Sigma Aldrich).

All the solutions used in this protocol are described in Appendix A.

3.3.2 IMMUNOFLUORESCENCE (IF)

Immunofluorescence (IF) is a technique that relies on the use of antibodies to label a specific target antigen combined with a fluorescent dye named fluorochromes, as fluorescein isothiocyanate (FITC). Using a fluorescent microscope it is possible to make visible the antigen presence in the cells and tissues. In this thesis context, it was performed an indirect immunofluorescence that relies on the use of a primary antibody (that binds to the antigen), followed be a secondary antibody (against the species specific isotype of the primary antibody) conjugated with a fluorochrome[225].

To analyse the effects of cysteine and carboplatin in xCT, EAAT3, NRF2 and HNF1β expression and cell localization, cells were seeded as described in table 3.1., but first lamellas were insert in wells, coated with 200 µL of 0,2% gelatine (G-1890, Sigma Aldrich). After 16h of experimental conditions, cells were fixed with 200 µL of 4% of paraformaldehyde (104003, Merck Millipore) **(Appendix A)** during 15min at 4°C. Posteriorly cells were rinsed with PBS (1X) and incubated with 50 mM of NH4Cl in PBS (1x). To permeabilize cells it was used PBS (1X) – 0,5% w/v BSA – 0,1% w/v Saponin (PBS (1X) - 0,5% BSA - 0,1% saponin) **(Appendix A)** for about 15min at RT. Cells were then incubated with primary antibody during 30min (Mouse anti-human EAAT3, ab131429-Abcam; Rabbit anti-human xCT, ab175186- Abcam; Rabbit anti-human NRF2, ab62352- Abcam; Anti-HNF1β, Rabbit anti-human antibody from Sigma Aldrich- HPA002083) (diluted in PBS(1X)-0,5% BSA -0,1% saponin) , 1:100). Cells were washed with PBS(1X) - 0,5% BSA - 0,1% saponin and incubated with secondary antibody for 2h at RT. Alexa Fluor® 488 anti-mouse (A-11001, Invitrogen) and Alexa Fluor® 488 anti-rabbit (A-11034, Invitrogen), were the secondary antibodies used. To ensure the specificity of the secondary antibody, controls without primary antibody (negative controls) were stained with the secondary antibody. Cells were then rinsed with PBS (1X) - 0,5% BSA - 0,1% saponin and the slides were mounted in VECTASHIELD media containing DAPI (4′-6-diamidino-2-phenylindole) (H-1200, Vector Labs). The analysis was performed by standard fluorescence microscopy using Zeiss Imajer.Z1 AX10 microscope. Images were acquired and processed with CytoVision software.

3.4. CHROMATIN IMMUNOPRECIPITATION (ChIP)

Chromatin is a structure composed by RNA, DNA and protein, providing crucial information regarding biological samples[226]. Chromatin immunoprecipitation (ChIP) assay allows to follow the interactions between DNA and proteins by determining if this interaction is present at a given location within the genome[226].

To examine if *xCT/SLC7A11* and *EAAT3/SLC1A1* expression is regulated by NRF2 and HNF1β, two different approaches were developed. In the first approach we examined the possible regulation of this transporters using a long time-point (16h), cells seeding was performed according to the conditions in table 3.1.

To a more detailed analysis of the effect of cysteine, carboplatin and cisplatin exposition on relative NRF2 occupancy in *EAAT3/SLC1A1* promoter, cells were exposed to shorter periods (30min and 2h) of the experimental conditions mentioned in table 3.1.

Cells were then treated with 37% formaldehyde, to trigger the cross link between proteins and DNA while keeping the chromatin structure. Glycine (0.125 M) was added to inhibit formaldehyde action. Cells were than scraped, collected and incubated with buffer and protease inhibitors. After this, samples were sonicated (Branson, digital sonifier). To evaluate the size of the chromatin fragments it was used an electrophoresis gel, in a 1.2% (w/v) agarose. The fragments obtained presented mainly sizes between 1000 bp and 500 bp that were considered suitable to go forward with the assay. The chromatin complexes were immunoprecipitated with specific antibodies (Rabbit anti-Human NRF2, ab31163 - Abcam; Anti-HNF1β, Rabbit anti-human antibody from Sigma Aldrich- HPA002083). ChIP assay was performed using OneDay ChIP kit (kch-onedIP-060, Diagenode) according to the manufacturer's protocol. Primers were designed to amplify a putative HNF1β and NRF2 binding sites at the *xCT/SLC7A11* and *EAAT3/SLC1A1* promoters (table 3.9.). The amplification of promoter regions sequenced from released DNA was performed by RQ-PCR as described in 3.2.2. (tables 3.5. and 3.7.). To find the relative occupancy of binding sites it was used the following formula: Relative occupancy = $2^{(CtNegCtl - CtTarget)}$

The primers herein used were designed to amplify a putative NRF2 and HNF1β binding sites at the *xCT* and *EAAT3* promoters.

Table 3.9. - Primers used in RQ-PCR to evaluate ChIP assay

	PRIMERS	FORWARD (5'-3')	REVERSE (5'-3')
SLC7A11/xCT promoter	NRF2 binding site	CCATTCTGTTTTCCTTTACAGGC	CACACAACTATAAGCCTTCCTC
	HNF1β binding site	GGGTCTTTGGCTCAACTTATG	GGAGCAGCTCTTCTTTCCCAG
EAAT3/SLC1A1 promoter	NRF2 binding site	GGTACTGTTGCTTGGCAGT	GCCGAGATCACGCTGCAG
	HNF1β binding site	CAGGTTCAAGCGATTCTCCTC	CTCTTGCCTGTAATCTCAGGAC

3.5. FLOW CYTOMETRY - CELL DEATH ANALYSIS

To evaluate the effect of cysteine, carboplatin, seleno-chrysin (SeChry) and erastin in ovarian cancer cells, cell death analysis through flow cytometry was performed.

Flow cytometry is a profitable and effective method that allows not only cell death analysis but also multiparameter measurements in a quite quick manner[227]. This technique makes possible, for example, to distinguish apoptosis from necrosis due to the differences in morphological and biochemical patterns. The key basis of flow cytometry in apoptosis is mainly related to a compound present in cell membrane facing the cytosolic side, that is named phosphatidyl serine (PS). When the apoptotic cascade initiates, this phospholipid is translocated from the inner to the external cell membrane surface. Annexin V allows the identification of apoptotic cells by binding to PS exposed on the outer leaflet. Besides annexin, red-fluorescent propidium iodide (PI) is used to stain necrotic/late apoptotic cells, whose cell membranes are disrupted, allowing the free entering of PI in the cell and binding to DNA[228,229].

To assess the effect of L-cysteine, carboplatin and cisplatin in cell death, and to evaluate the effect of SeChry, and SeChry combined with carboplatin and cysteine in cell death cells were cultured as described in table 3.1.

Besides the different experimental conditions, cells were collected equally. First, the supernatant from each well was collected to an eppendorf and cells were collected by harvesting with 100 µL of 0.05% trypsin-EDTA (25300-054, Invitrogen) and added to the same eppendorf. Cells were centrifuged at 150xg for 3 min, washed with PBS (1X) – 0,1% BSA and stained with 0,5 µL FITC-annexin V (640906, BioLegend) (100 µg/mL) in 100 µL annexin V binding buffer 1X and incubated, in dark for 15min at RT. After incubation, samples were rinsed with 100 µL of PBS (1X) - 0,1% BSA and centrifuged at 150xg for 3min. The supernatant was removed and cells were resuspended in 200 µL of annexin V binding buffer 1X (10 mM HEPES (pH 7.4), 0.14 M sodium chloride (NaCl), 2.5 mM calcium chloride ($CaCl_2$)) and 2,5 µL PI solution (50 µg/mL) **(Appendix A)**. Samples acquisition was performed with a FACScalibur (Becton Dickinson). To analyse flow cytometry data it was used FlowJo software (www.flowjo.com).

3.6. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Thiols are molecules that play important functions in cell homeostasis, metabolism and signalling[230]. Besides this, thiol groups (-SH) are critical intracellular and extracellular redox buffers being relevant for cell detoxification. Homo-L-cysteine (HCys), L-cysteine (Cys), cysteinylglycine (Cys-Gly) and glutathione (GSH), low molecular mass thiols, are

example of these molecules[231].

HPLC with fluorescence detection (HPLC–FD) is a useful method for determination and quantification of aminothiols mainly due to its sensitivity and simplicity. However, to perform chromatographic separation is necessary to prepare samples involving disulphide reduction, protein precipitation, and derivatization[231].To study the possible effect of SeChry in cysteine uptake impairment, HPLC was performed, according to Grilo, *et al*.[232].

ES2 and OVCAR3 cells were cultured as described in table 3.1. Cells were harvested with 0.05% trypsin, centrifuged at 150xg for 2min, rinsed twice in PBS (1X) and lysed with 120 µL PBS (1X) - 0.01% (v/v) Triton X - 100 and stored at -80°C until use. Cell lysates and supernatants were centrifuged at 10600xg for 2min prior to HPLC protocol.

Each sample was divided in two eppendorfs (50 µL each) in order to quantify total thiols levels (all thiols present in the sample) and free total thiols levels (thiols present in the free form in the sample, not bounded to proteins). For total free thiols samples, protein was first precipitated.

To total aminothiols quantification, cell supernatants and crude culture medium were reduced through the addiction of 5 µL of Tris (2-carboxyethyl) phosphine (TCEP) (100 g/L) in 50 µL of sample, during 30min incubation at RT. Then 45 µL Trichloroacetic acid (TCA) (100 g/L) with 1mM EDTA were added to stop the reduction reaction and induce protein precipitation and samples were centrifuged at 17900xg for 10min at 4ºC. The resultant supernatants were collected (25 µL) and derived due to the addiction of 5 µL 1.55 M Sodium hydroxide (NaOH), 62,5 µL 0.125 M Sodium tetraborate- 4 mM EDTA (Borate tampon) and 25 µL 1 g/L Ammonium 7-Fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). The samples were incubated at 60ºC for 1h, protected from light.

As previously referred, for free thiols the procedure is different by starting with protein precipitation with 45 µL of TCA (100 g/L) followed by the addition of 5 µL TCEP (100 g/L) in a new vial containing 50 µL of sample. Samples were frozen at -80ºC until its quantification.

Levels of Cys, GSH and Cys-Gly were quantified as described by Grilo *et al*.[232] by HPLC in Schimadzu (Kyoto, Japan) system, consisting of a LC 9-A solvent delivery pump, a 7725i injector, a RF-10AXL fluorescence detector and a CTO-10AS VP column oven. A LiChrospher 100 RP-18 (250 x 4 mm; 5 µm) column protected by a LiChrospher 100 RP-18e (4x 4mm; 5 µm) guardcolumn, both from Merck (New Jersey, USA), was used, at 25ºC. The detector was set at excitation and emission wavelengths of 385 nm and 515 nm and the mobile phase consisted of 100 mM acetate buffer (pH 4.5) and methanol [98:2 (v/v)].

The analytes were separated in an isocratic elution mode for 22 min, at a flow rate of 0.6 mL/min. Data acquisition and processing were performed in Shimadzu Class VP 7.X software. Calibration curves were constructed for each curve, the absolute peak-area ratios were calculated and plotted against the nominal aminothiol concentration.

3.7. STATISTICAL ANALYSIS

For all experiments, statistical analyses were performed in GraphPad Prism 6.0 software (www.graphpad.com). Data is presented as the mean ± SD. Assays were performed with, at least, 3 replicates per experimental condition, excepting the ChIP which just accounts for 2 replicates. For comparisons of two groups, two-tailed unpaired T-test was used. For comparison of more than two groups, One-way and Two-way analysis of variance (ANOVA) with Tukey's multiple-comparisons post hoc test was used. Statistical significance was established as $p<0.05$.

4. RESULTS

4.1. CYSTEINE TENDS TO PROTECT OVARIAN CANCER CELLS AGAINST PLATINUM-INDUCED TOXICITY

Since previous results of our lab have supported a role of cysteine in carboplatin response in ovarian cancer cells, we further aimed to address the effect of cysteine in ovarian cancer cells response to carboplatin and its analogue cisplatin when pre-reaction of drugs with cysteine was allowed (cysteinylated carboplatin), hypothesizing that this pre-reaction would be advantageous for cells as platinum-based drugs react with sulphur, allowing the abrogation of its toxicity.

Results have shown that in ES2 cells, with 16h and 24h of experimental conditions, no differences were found upon cysteine, cysteinylated carboplatin or carboplatin exposure (Figure 4.1.A.). However, with 48h of experimental conditions, cysteine presented a protective effect against carboplatin-induced cell death, both with (cysteinylated carboplatin) and without pre-reaction with it (cysteine plus carboplatin) ($p<0,05$ and $p<0,001$) (Figure 4.1.A.). Moreover, we also observed increased cell death levels with 48h of carboplatin exposure compared both with 16h and 24h ($p<0,05$ and $p<0,01$, respectively) (Figure 4.1.A.). Regarding cisplatin exposure, for 16h of experimental conditions, cysteinylated cisplatin presented lower cell death levels in relation to cisplatin alone and there was a tendency for lower cell death levels with cysteine (Figure 4.1.A.). At 24h, a tendency for decreased cell death with cysteine supplementation was also observed, both with and without pre-reaction with cisplatin (Figure 4.1.A.).

In OVCAR3 cells, there was also a tendency for a protective effect of cysteine upon carboplatin exposure at 16h and 24h of experimental conditions (Figure 4.1.B.). Interestingly, it was observed that cysteinylated carboplatin increased drug efficacy, inducing cell death at 48h of experimental conditions (Figure 4.1.B.). Regarding cisplatin, cysteine also presented a protective effect upon cisplatin exposure with 24h of experimental conditions ($p<0,01$) (Figure 4.1.B.). However, cysteinylated cisplatin also seemed to induce more cell death levels in this time-point when compared with non-cysteinylated cisplatin (Figure 4.1.B.).

Results also demonstrated that cisplatin induced more cell death compared to carboplatin in both cell lines ($p<0,001$) (Figure 4.1.).

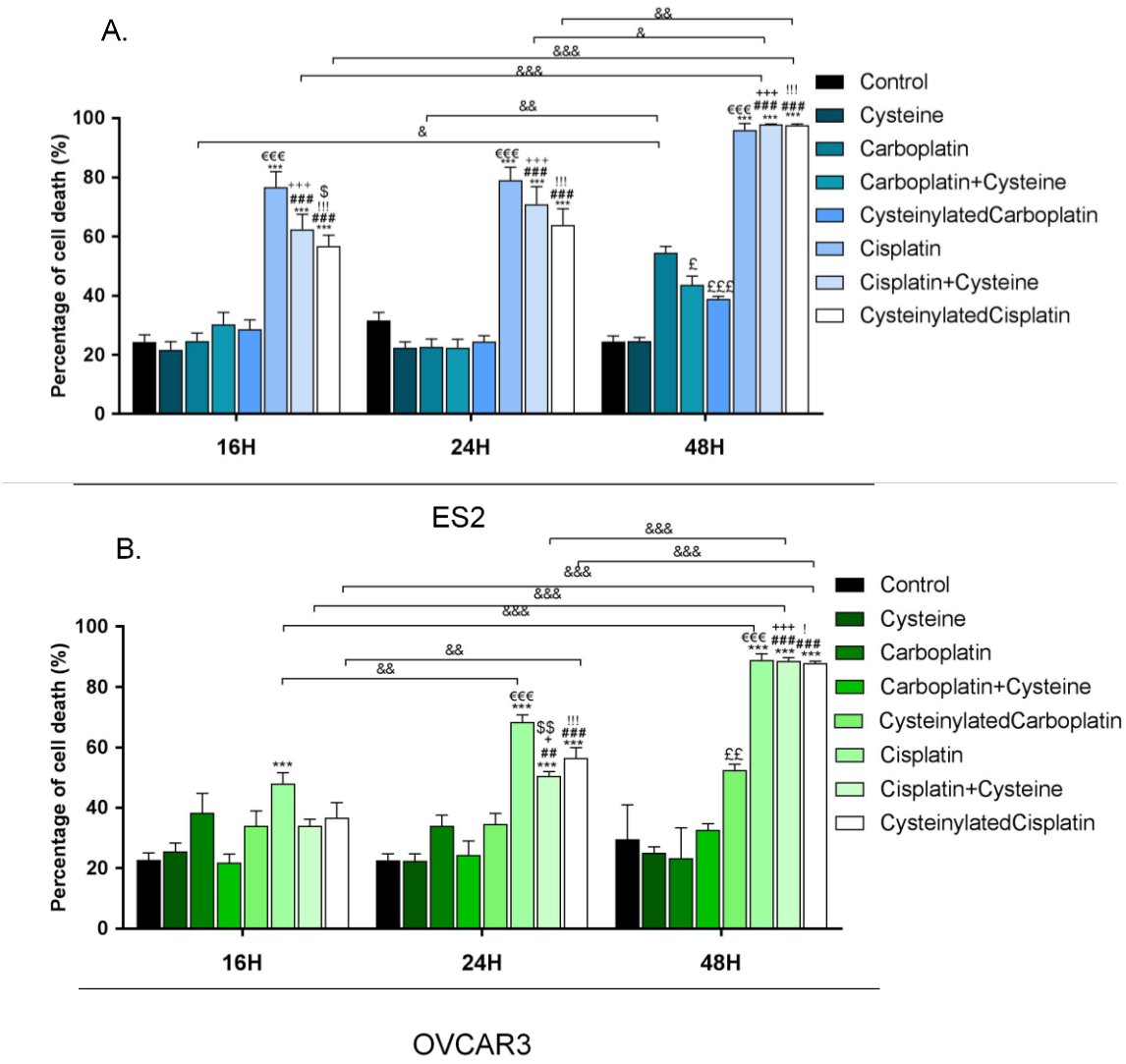


Figure 4.1. - The effect of cysteine, carboplatin, carboplatin plus cysteine, cysteinylated carboplatin, cisplatin, cisplatin plus cysteine and cysteinylated cisplatin, in ES2 and OVCAR3 cell lines. After 16, 24 and 48h, cells were collected, and the percentage of cell death was evaluated. **A.** Percentage of cell death in ES2. Cysteine has a protective effect upon drugs. **B.** Percentage of cell death in OVCAR3 cell line. Cysteinylated carboplatin and cysteinylated cisplatin increase drugs effect. Results are shown as mean SD *p<0,05, **p<0,01, ***p<0,001 (Two-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control; "#"-cysteine, "€"-carboplatin; "+" – carboplatin with cysteine; "!"- cysteinylated carboplatin; "$" –cisplatin; "£" – carboplatin (One-Way ANOVA, Tukey, post-test) and "&" reports the statistical significance between conditions in the different time-points

These results have shown that, in the presence of platinum-drugs, cysteine with and without pre-reaction with drugs led to different drug responses between cell lines. In ES2, cysteine both with and without its pre-reaction to both drugs leads to a protective effect, whereas in OVCAR3, cysteine is only advantageous when is not cysteinylated.

4.2. CYSTEINE AND CARBOPLATIN ROLE IN xCT AND EAAT3 EXPRESSION

4.2.1. CYSTEINE AND CARBOPLATIN ROLE IN *xCT/SLC7A11* AND *EAAT3/SLC1A1* mRNA LEVELS

Given the role of cysteine in protecting cells from platinum-induced toxicity, we aimed to investigate if this protection was related with xCT and EAAT3 expression, both known to have important roles in cysteine transport[117,137] . Thus, we started by investigating if cysteine supplementation was able to modulate *xCT/SLC7A11* and *EAAT3/SLC1A1* genes expression of these transporters in a short-time manner. For this, we accessed the mRNA expression by performing RQ-PCR with a pulse chase assay using 7 time-points: 0h, 30min, 2h, 4h, 6h, 8h, and results have shown that *xCT/SLC7A11* expression is independent of cysteine supplementation in both cell lines in a short-time exposure (figure 4.2.A. and B.). However, regarding EAAT3 expression, in OVCAR3 cell line, cysteine supplementation induced *EAAT3* mRNA expression within 30min, 1h, 2h and 4h of cysteine exposure ($p<0,05$; $p<0,05$; $p<0,01$; $p<0,01$, respectively) (figure 4.2.B.). We also addressed if there were differences among cell lines in basal expression levels of these transporters, and it was observed that relative *xCT/SLC7A11* and *EAAT3/SLC1A1* mRNA expression was significantly higher in ES2 cell line ($p<0,05$ and $p<0,01$, respectively) (figure 4.2.C. and D.)

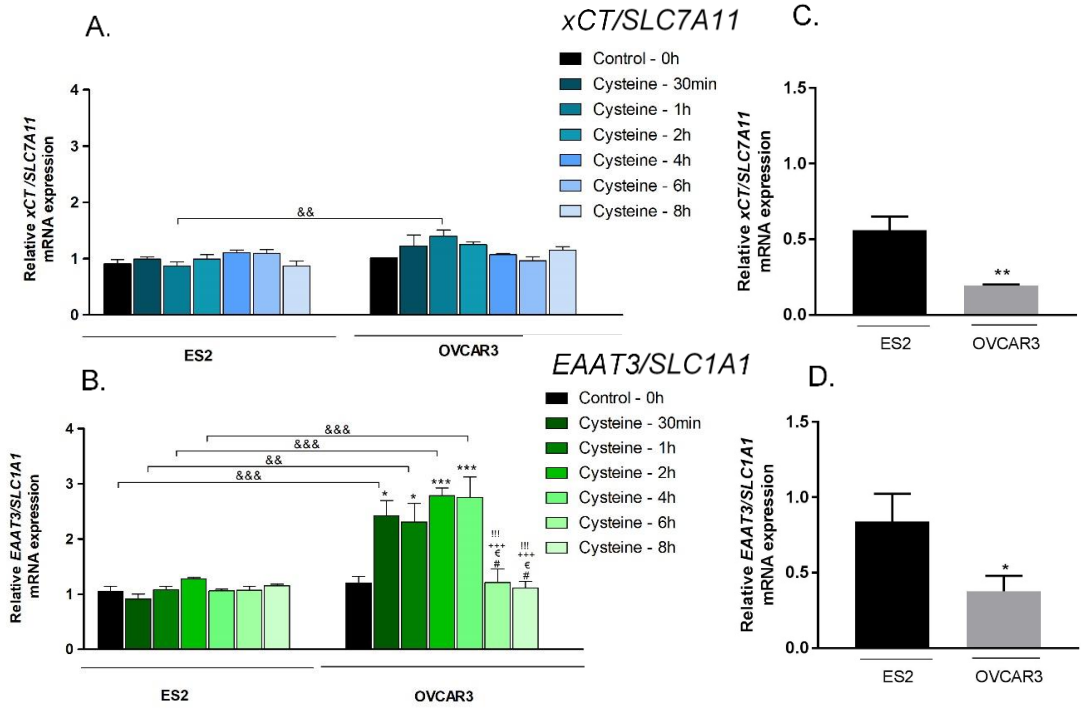


Figure 4.2. – The effect of cysteine supplementation over time in *xCT/SLC7A11* and *EAAT3/SLC1A1* mRNA expression, in ES2 and OVCAR3 cell lines. After 0h, 30min, 1h, 2h, 4h, 6h and 8h of cysteine supplementation the mRNA levels of *xCT/SLC7A11* and *EAAT3/SLC1A1* were analysed. **A.** Relative

xCT/SLC7A11 mRNA expression. *xCT/SLC7A11* is constitutively expressed. **B.** Relative *EAAT3/SLC1A1* mRNA expression. In OVCAR3, *EAAT3/SLC1A1* mRNA expression is higher at initial time-points. **C.** Basal relative *xCT/SLC7A11* mRNA expression. **D.** Basal *EAAT3/SLC1A1* mRNA expression. Basal relative *xCT/SLC7A11* and *EAAT3/SLC1A1* mRNA expression are higher in ES2 cell line. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (Two-Way ANOVA, Tukey post-tests (**A. and B.**), two-tailed unpaired t-tests (**C. and D.**)). "*" statistical significance in relation to control-0h (**A. and B.**) and in relation to ES2 (**C. and D.**); "#"-cysteine-30min; "€"- cysteine-1h; "+" - cysteine-2h; "!" - cysteine-4h and "&" reports the statistical significance between conditions among cell lines (**A. and B.**).

We then asked if an increase in cysteine exposure time was able to modulate the expression of those transporters, asking also if carboplatin and the combination of both, were able to modulate cysteine transporters expression. For *xCT/SLC7A11*, through a Two-Way ANOVA with Tukey post-tests, there were no significant differences among conditions, but when applying two-tailed unpaired t-tests, results shown that cysteine was responsible for a significant increase in relative *xCT/SLC7A11* mRNA expression in ES2 cell line, both with and without carboplatin exposure (p<0,05 and p<0,001) (figure 4.3.A). In OVCAR3 no significant changes between conditions were observed (figure 4.3.A). When comparing the experimental conditions between cell lines, it was possible to see that in OVCAR3 cells exposed to carboplatin, *xCT/SLC7A11* mRNA expression was significantly higher than in ES2 cells (p<0,01) (figure 4.3.A.).

Regarding *EAAT3/SLC1A1* mRNA levels, ES2 cells showed no differences between conditions, but in OVCAR3, carboplatin condition was responsible for a reduction in mRNA expression compared to the control (p<0.05) (figure 4.3.B.). This cell line also showed significant lower *EAAT3/SLC1A1* levels in relation to ES2 in all experimental conditions (p<0,001) (figure 4.3.B.).

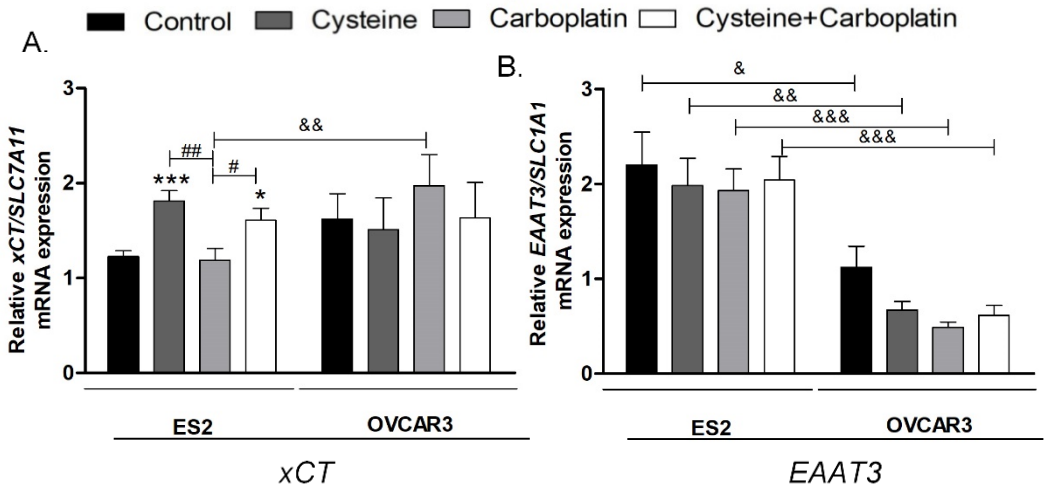


Figure 4.3. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in *xCT/SLC7A11* and *EAAT3/SLC1A1* mRNA expression, in ES2 and OVCAR3 cell lines. After 16h of conditions the mRNA levels of xCT and EAAT3 were analysed. **A.** Relative xCT mRNA expression. Cysteine increases the xCT levels in ES2 cell line. **B**. Relative EAAT3 mRNA expression. ES2 showed higher EAAT3 mRNA expression than

OVCAR3. Results are shown as mean SD ± *p<0,05, **p<0,01, ***p<0,001(two tailed unpaired t-tests). "*" statistical significance in relation to control within each cell line, "#" represent the statistical significance between conditions and "&" reports the statistical significance between conditions among cell lines.

Taken together, results support a role of cysteine in regulating *xCT/SLC7A11* mRNA levels in ES2 cells with a higher cysteine exposure time, but not in OVCAR3 cells.

Regarding EAAT3 transporter, the dynamics of *EAAT3/SLC1A1* mRNA levels differ in ES2 and OVCAR3 cells. OVCAR3 cells presented cysteine-induced *EAAT3/SLC1A1* mRNA levels in a short-time exposure, decreasing with 16h of experimental conditions, whereas ES2 cells showed no cysteine modulation both in a short-time exposure and at 16h.

4.2.2. CYSTEINE AND CARBOPLATIN ROLE IN xCT and EAAT3 PROTEIN LEVELS

We also addressed the effects of cysteine and carboplatin exposure in xCT and EAAT3 protein levels by western blot and immunofluorescence analysis.

Through western blot quantification, it was possible to observe a significant increase in xCT protein levels with the supplementation of cysteine combined with carboplatin exposure in ES2 cell line (p<0,05) (figure 4.4.). However, no significant differences were observed in OVCAR3 (figure 4.4.)

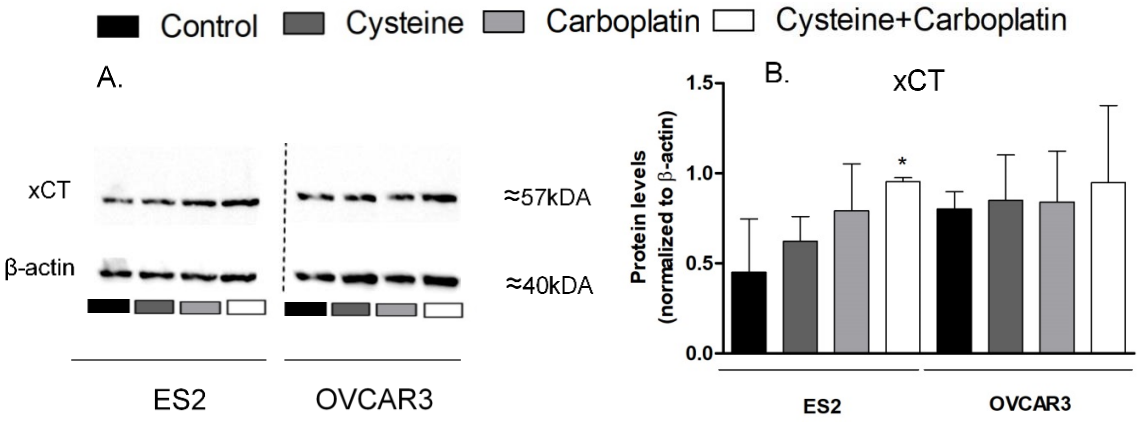
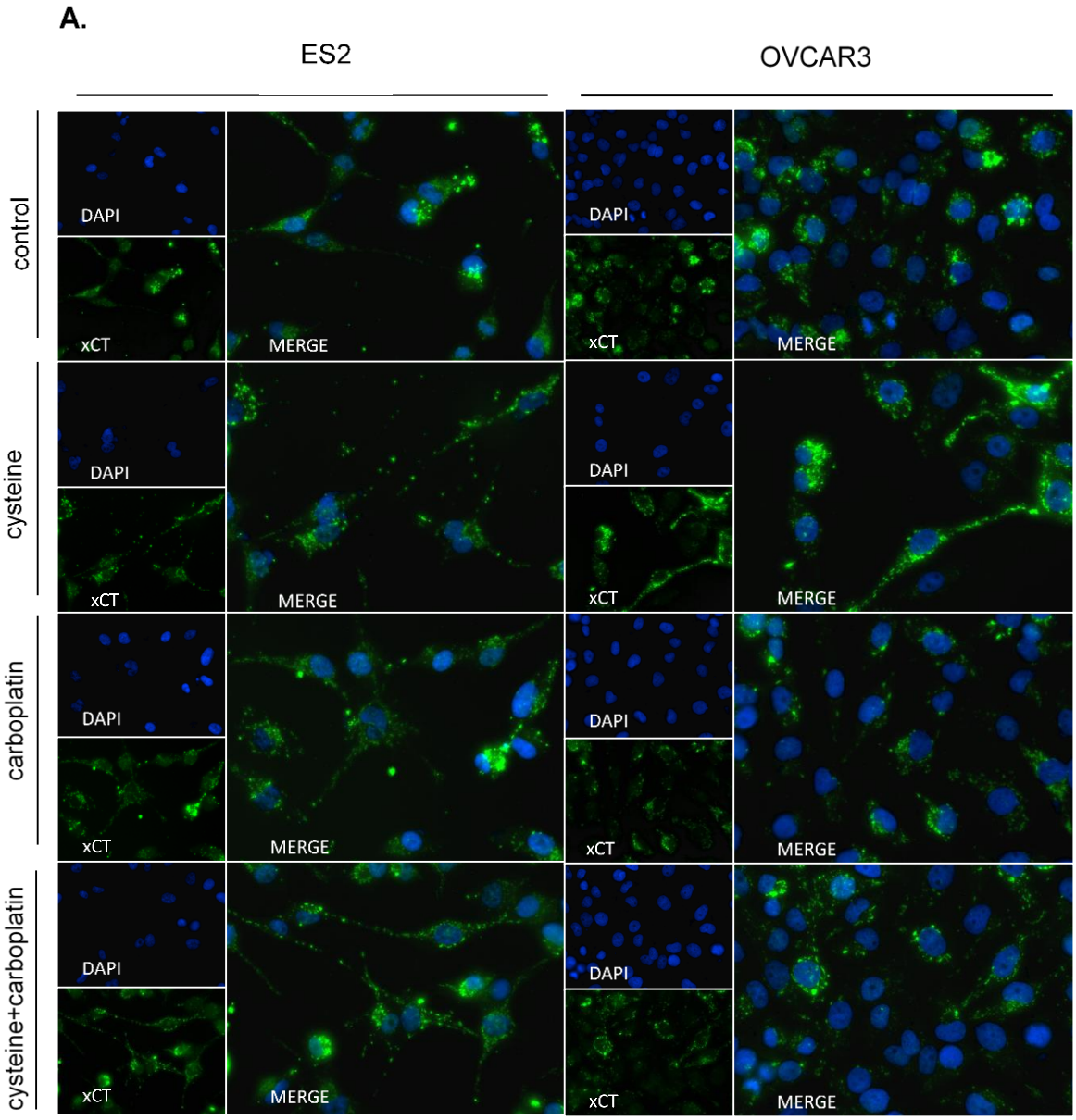


Figure 4.4. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in xCT protein levels, in ES2 and OVCAR3 cell lines. After 16h of conditions exposure the protein levels of xCT were analysed. **A.** Western blot membrane, β-actin was used for normalization **B.** Western blot quantification through Image J. Cysteine combined with carboplatin condition was responsible for an increase in the relative xCT mRNA expression in ES2 cell line. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (two tailed unpaired t-test). "*" statistical significance in relation to control.

In what concerns immunofluorescence analysis, results showed a slightly tendency (no significant) to an increase in xCT levels upon cysteine and carboplatin exposure in ES2 cells (figure 4.5.A. and B.). In OVCAR3 cells, cysteine induced higher xCT protein levels ($p<0,05$) (figure 4.5.A. and C.). Interestingly, immunofluorescence analysis suggested a mitochondrial localization of xCT transporter, thus we furthered confirmed this localization performing western blot analysis with isolated mitochondria (figure 4.5.D).



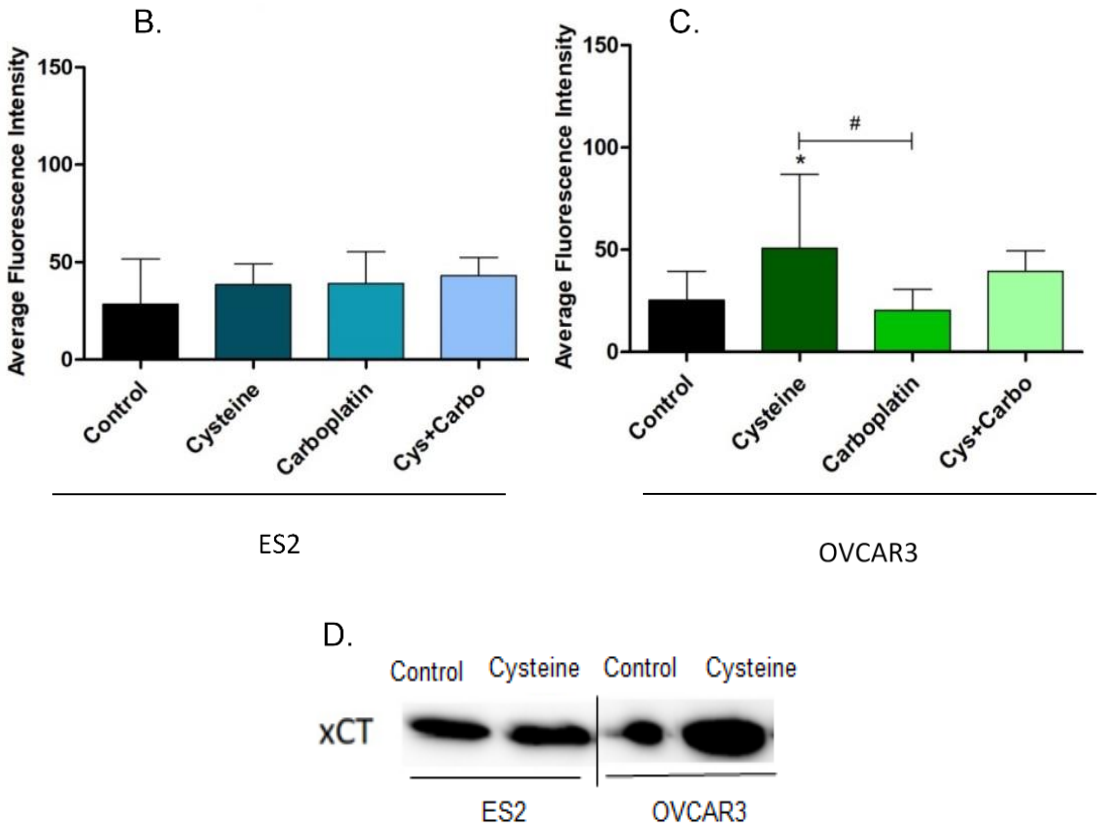


Figure 4.5. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in xCT protein levels, in ES2 and OVCAR3 cell lines. After 16h of conditions exposure the protein levels of xCT were analysed. **A.** Fluorescence microscopy (original magnification: 400 x). Nuclei were stained with DAPI (blue). **B.** Immunofluorescence quantification using Image J in ES2. **C.** Immunofluorescence quantification using Image J in OVCAR3. Cysteine is responsible for an increase of xCT protein levels in OVCAR3. **D. xCT localized in ES2 and OVCAR3 mitochondria**. Western blot membrane revealing xCT transporter presence in the mitochondria. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (One-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control and "#" statistical significance between conditions.

Regarding EAAT3 transporter, only results for immunofluorescence analysis are presented since we failed to achieve results with western blot analysis. Thus, fluorescence microscopy revealed lower EAAT3 levels in ES2 cells in the presence of cysteine with carboplatin in comparison with control and cysteine conditions ($p<0,01$ and $p<0,05$), indicating that carboplatin in the presence of cysteine leads to decreased EAAT3 protein expression in ES2 cell line (figure 4.6.A. and B.). However, OVCAR3 cell line did not show differences between conditions (figure 4.6.A. and C.).

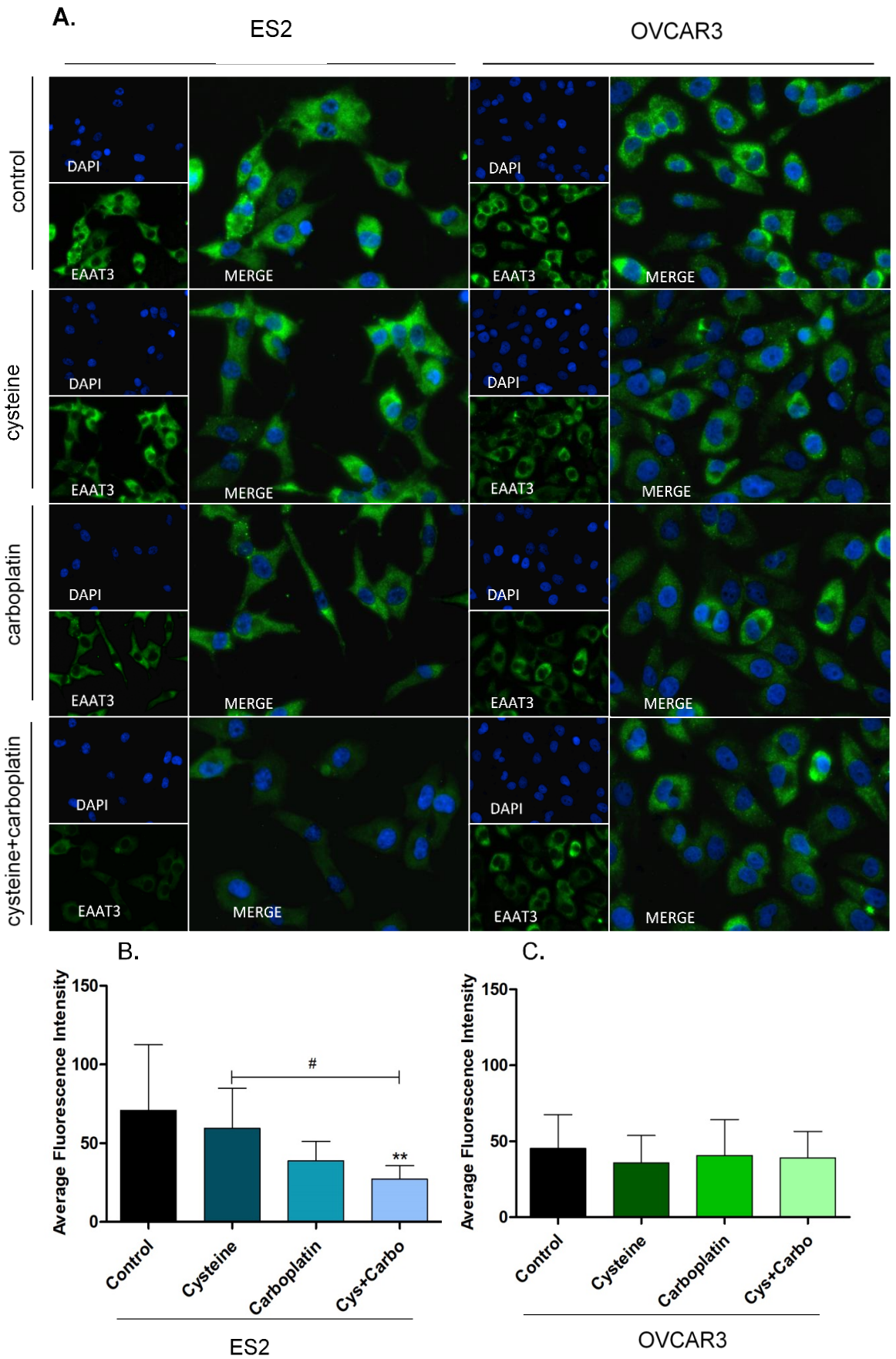


Figure 4.6. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in EAAT3 protein levels, in ES2 and OVCAR3 cell lines. After 16h of exposure, protein levels of EAAT3 were analysed. **A.**

Fluorescence microscopy (original magnification: 400 x). Nuclei were stained with DAPI (blue). **B.** Immunofluorescence quantification using Image J in ES2. Carboplatin is responsible for a decrease of EAAT3 protein levels in ES2. **C.** Immunofluorescence quantification using Image J in OVCAR3. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (One-Way ANOVA, Tukey post-test). "*" statistical significance in relation to control and "#" statistical significance between conditions.

Taken together, results indicate that cysteine and carboplatin affect differently mRNA and protein levels of xCT and EAAT3 in ES2 and OVCAR3 cells. Whereas in ES2 cells, results support a role of carboplatin and cysteine in the increase of both xCT mRNA and protein levels, *EAAT3/SLC1A1* mRNA levels were not affected by cysteine and carboplatin but protein levels were, in the opposite way of xCT. By looking at OVCAR3 cells, *EAAT3/SLC1A1* mRNA levels were not significantly affected by cysteine and carboplatin, but protein levels were and mainly by cysteine. Moreover, mRNA *EAAT3/SLC1A1* levels were modulated by cysteine only in a short-time manner, indicating expression modulation by cysteine.

4.3. NRF2 AND HNF1β MODULATION AND REGULATION OF xCT/SLC7A11 AND *EAAT3/SLC1A1*

4.3.1. NRF2 AND HNF1β ARE MODULATED BY CYSTEINE AND CARBOPLATIN

As NRF2 is involved in the regulation of *xCT/SLC7A11*[154] and HNF1β has a role in GSH dynamics[45], we decided to evaluate the effect of cysteine, carboplatin and the combination of both in *NRF2* and *HNF1B* mRNA expression.

Relative *NRF2* and *HNF1B* mRNA expression, showed no significant alterations under the different conditions and between the two cell lines (figure 4.7.), thus suggesting that those genes are not influenced by cysteine or carboplatin.

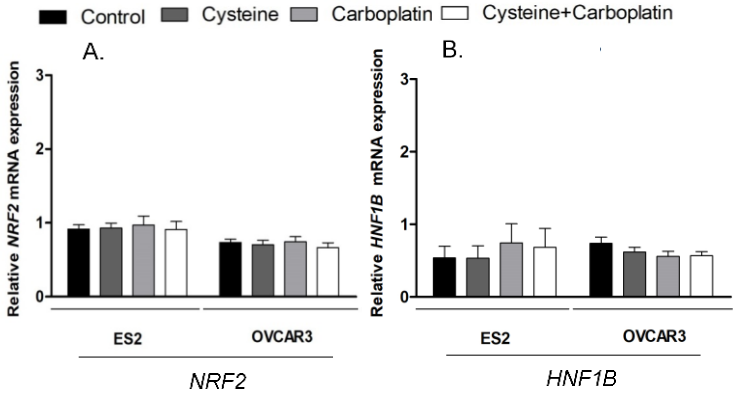
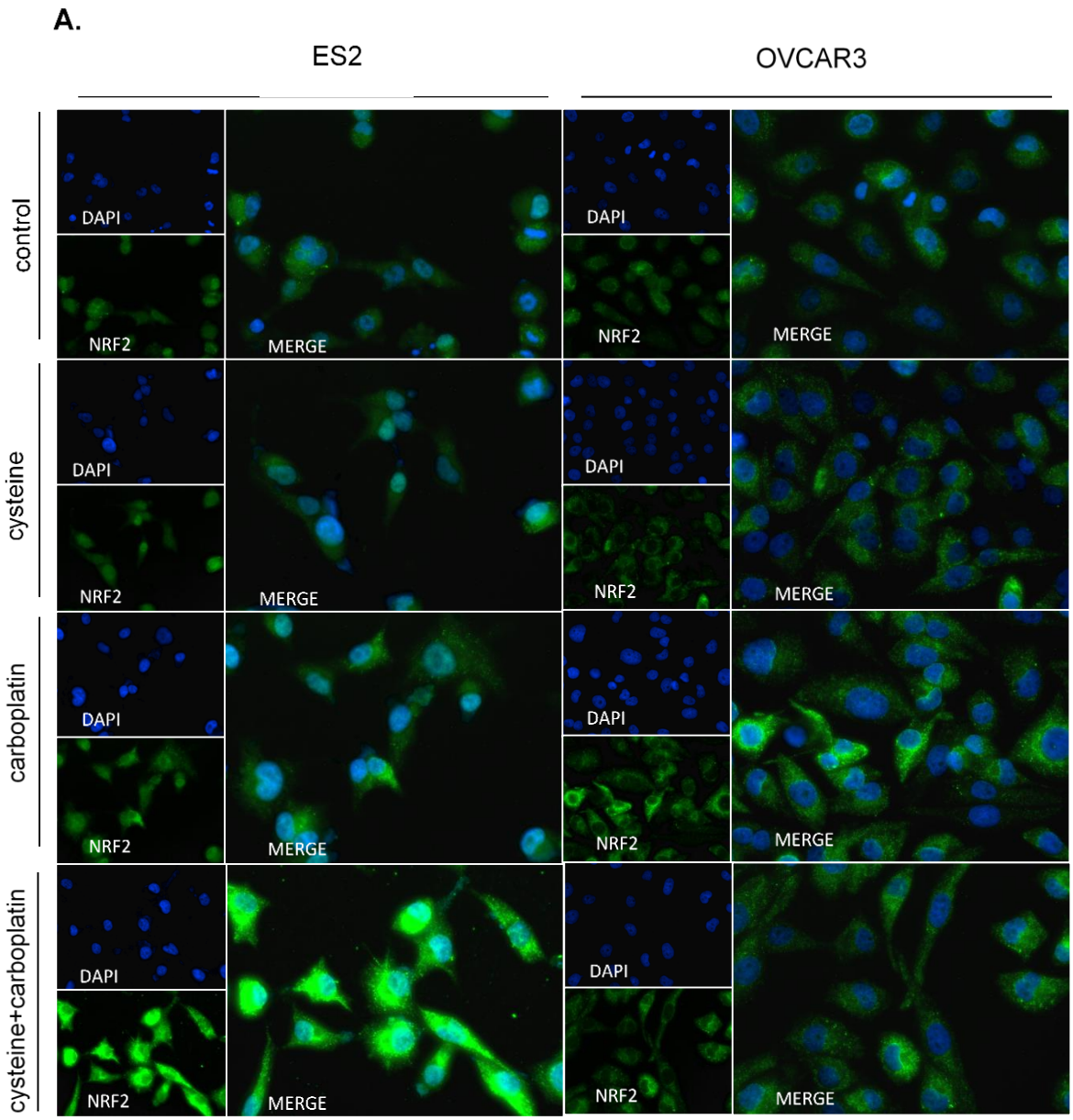
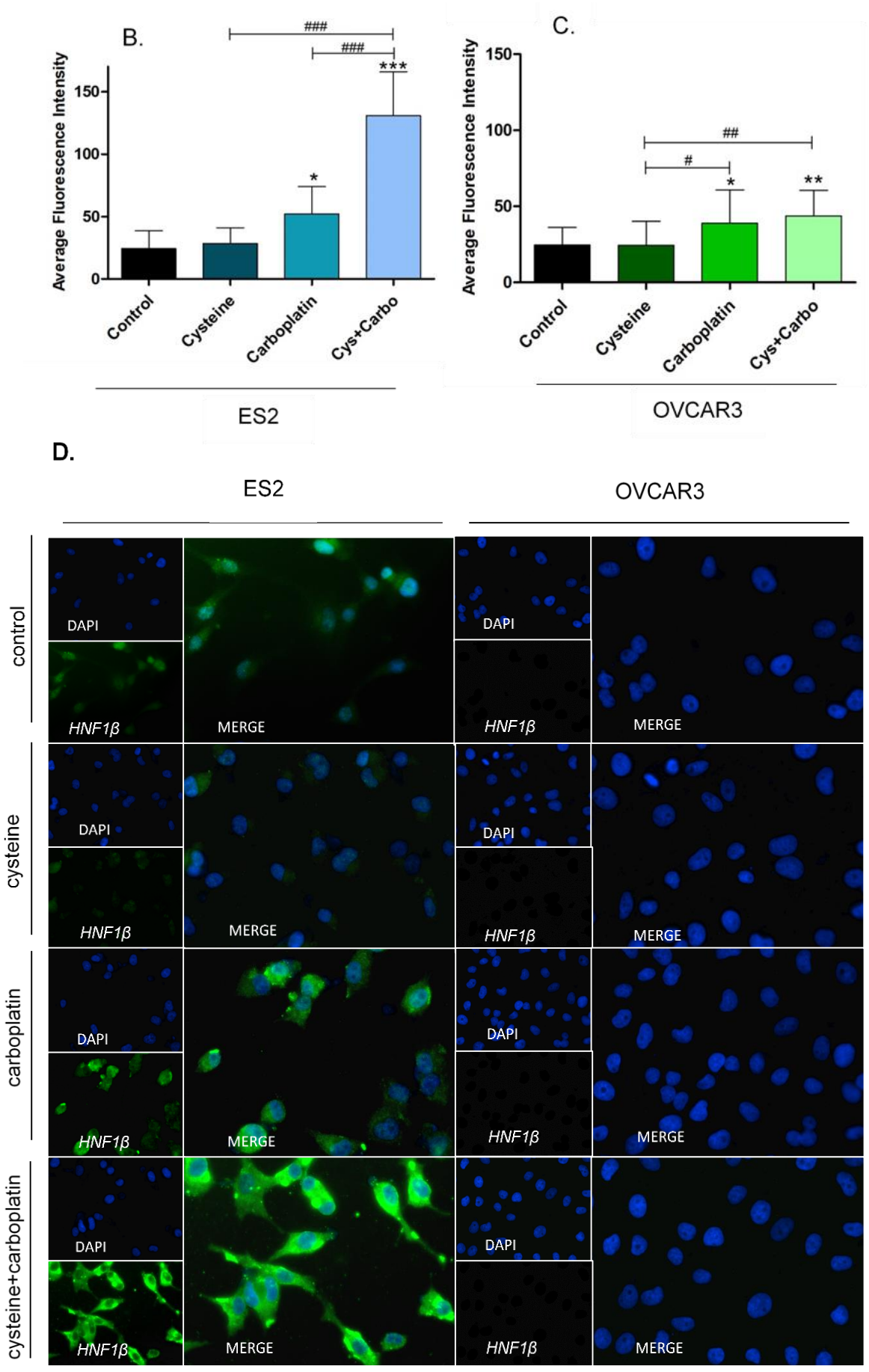


Figure 4.7. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in *NRF2* and *HNF1B* mRNA expression in ES2 and OVCAR3 cell lines. after 16h of cysteine and/or carboplatin exposure the mRNA levels of *NRF2* and *HNF1B* were analysed.**A.** relative *NRF2* mRNA expression. **B.** *HNF1B* mRNA expression. (Two tailed unpaired t-tests).

However, when analysing the protein levels through immunofluorescence, results demonstrated that NRF2 levels were significantly higher upon carboplatin exposure, alone ($p<0,05$) or in combination with cysteine ($p<0,001$) in both cell lines (figure 4.8.A.-C.). Regarding HNF1β, as expected, it was not observed in OVCAR3 cell line, but in ES2 a significant protein increase was observed with carboplatin alone or combined with cysteine ($p<0,05$ and $p<0,01$, respectively), suggesting that carboplatin is involved in the regulation of HNF1β, especially when combined with cysteine in this cell line (figure 4.8.D. and E.)




B.
Average Fluorescence Intensity
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Carboplatin
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DAPI
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MERGE

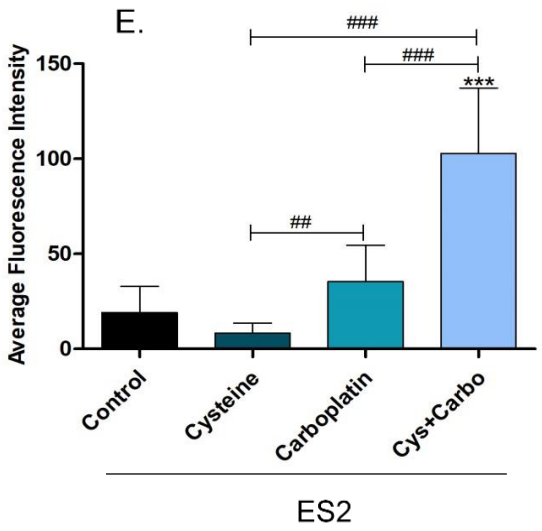


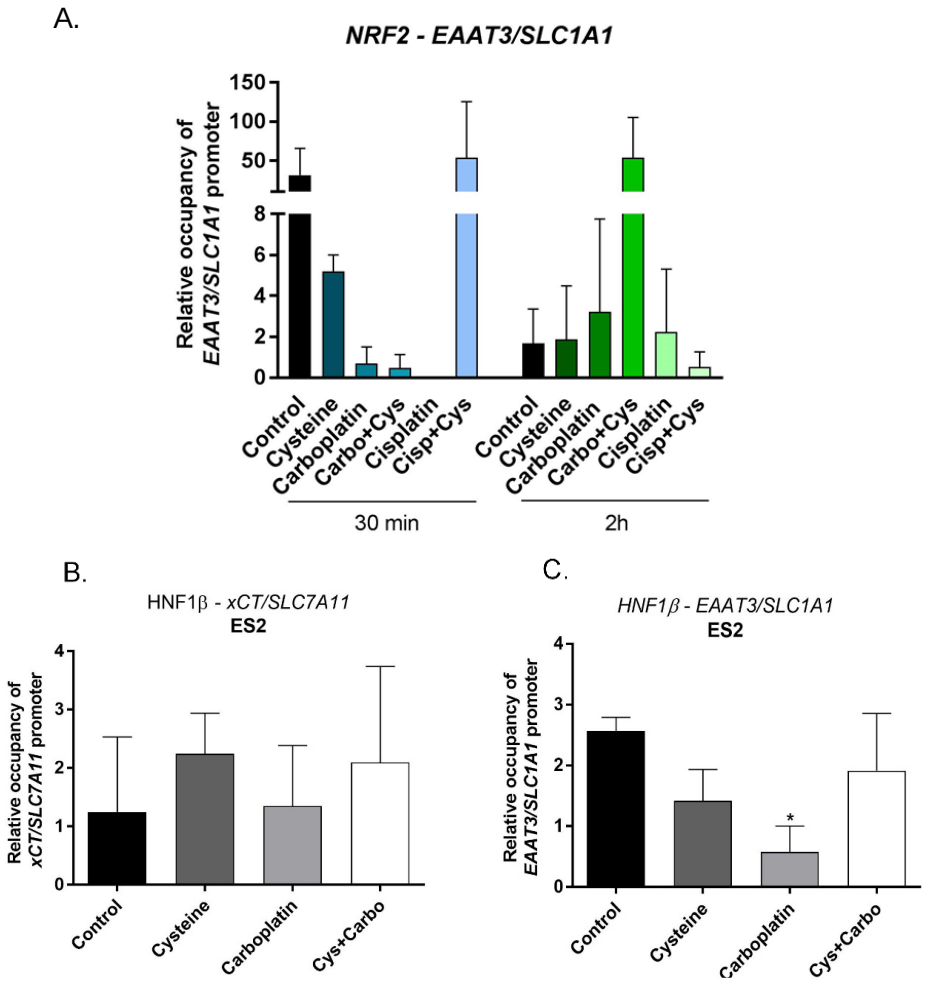
Figure 4.8. - The effect of cysteine, carboplatin and cysteine combined with carboplatin in NRF2 and HNF1β protein levels, in ES2 and OVCAR3 cell lines. After 16h of cysteine and/or carboplatin exposure the protein levels of NRF2 and HNF1β were analysed. **A.** Fluorescence microscopy for NRF2 (original magnification: 400 x). Nuclei were stained with DAPI (blue). **B.** Immunofluorescence quantification using Image J in ES2. **C.** Immunofluorescence quantification using Image J in OVCAR3. Carboplatin is responsible for an increase of NRF2 protein levels in ES2 and OVCAR3. **D.** Fluorescence microscopy for HNF1β (original magnification: 400 x). **E**. Immunofluorescence quantification using Image J in ES2. Carboplatin is responsible for an increase of HNF1β protein levels. Nuclei were stained with DAPI (blue). Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (One-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control and "#" statistical significance between conditions.

4.3.2. NRF2 AND HNF1β INVOLVMENT IN THE REGULATION OF xCT AND EAAT3 TRANSPORTERS EXPRESSION

To disclosure NRF2 and HNF1β role in the regulation of *EAAT3/SLC1A1* and *xCT/SLC7A11*, two different ChIP assays followed by RQ-PCR were performed. The relative NRF2 occupancy of *EAAT3/SLC1A1* promoter was analysed for a short-time period: 30min and 2 h for ES2 cells, under control, cysteine, carboplatin, carboplatin plus cysteine, cisplatin (a carboplatin analogue) and cisplatin with cysteine conditions. Results shown that after 30 min of exposure to cisplatin with cysteine there was a tendency to an increase in relative NRF2 occupancy of *EAAT3/SLC1A1* in ES2 cells. After 2 h and upon carboplatin and cysteine exposure, NRF2 occupancy of *EAAT3/SLC1A1* promoter also tends to increase, even in a not significant way (figure 4.9.A.).

We also analysed the regulation of *xCT/SLC7A11* and *EAAT3/SLC1A1* by NRF2 and HNF1β with 16h of experimental conditions for both ES2 and OVCAR3 cell lines when subjected to control, cysteine, carboplatin and cysteine with carboplatin conditions (figure

4.9.B.- G.). The results suggested that in ES2 cell line, cysteine tends to increase HNF1β binding to *xCT/SLC7A11* promoter (not significant) (figure 4.9.B.). However, in the same cell line, HNF1β relative occupancy of *EAAT3/SLC1A1* promoter is decreased when stimulated by carboplatin (p<0,05) (figure 4.9.C.). Relative HNF1β occupancy of both promoters in OVCAR3 was not analysed because we did not observed protein expression by immunofluorescence. In relative NRF2 occupancy of *xCT/SLC7A11* and *EAAT3/SLC1A1* promoters, we could not see any significant modulation, however we observed a tendency in the NRF2 increase regulation of *xCT/SLC7A11* in carboplatin condition (figure 4.9.D). Furthermore, cysteine led to an increased NRF2 binding to *xCT/SLC7A11* promoter in OVCAR3 cell line (p<0,01) (figure 4.9.F.). Still in OVCAR3, cysteine and carboplatin significantly reduced relative NRF2 occupancy in *EAAT3/SLC1A1* promoter (p<0,01) (figure 4.9.G.).



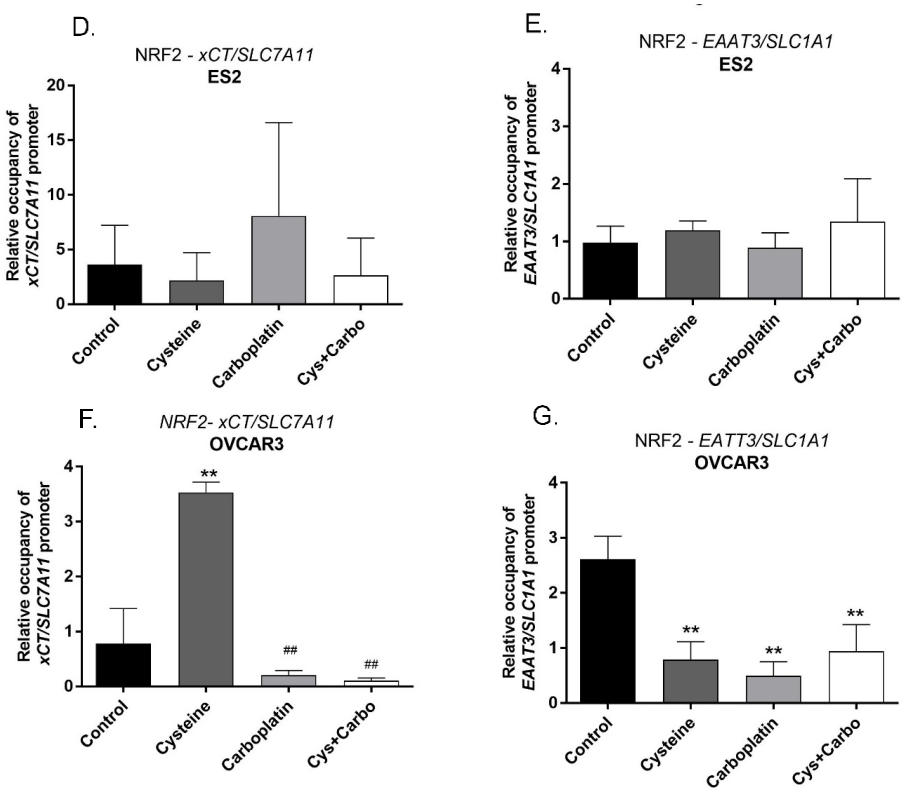


Figure 4.9. - The effect of cysteine, carboplatin, cysteine combined with carboplatin, cisplatin and cisplatin combined with cysteine in NRF2 and HNF1β relative occupancy of *xCT/SLC7A11* and *EAAT3/SLC1A1* promoters, in ES2 and OVCAR3 cell lines. After 30min, 2h and 16h of cysteine and/or Carboplatin/cisplatin exposure the ChIP assay was performed. **A.** Relative NRF2 occupancy of *EAAT3/SLC1A1* promoter**,** tested in 30min and 2h period**. B.** Relative HNF1β occupancy of *xCT/SLC7A11* promoter in ES2 cell line, tested for 16h. **C.** Relative HNF1β occupancy of *EAAT3/SLC1A1* promoter in ES2 cell line, tested for 16h. **D**. Relative NRF2 occupancy of *xCT/SLC7A11* promoter in ES2 cell line, tested for 16h. *xCT/SLC7A11* regulation tend to increase with carboplatin**. E.** Relative NRF2 occupancy of *EAAT3/SLC1A1* promoter in ES2 cell line, tested for 16h. **F.** Relative NRF2 occupancy of *xCT/SLC7A11* promoter in OVCAR3 cell line, tested for 16h. Cysteine augments the relative occupancy of *xCT/SLC7A11* promoter. **G.** Relative NRF2 occupancy of *EAAT3/SLC1A1* promoter in OVCAR3 cell line, tested for 16h. Results are shown as mean SD *p<0,05, **p<0,01, ***p<0,001 (One-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control and "#" statistical significance in relation to cysteine.

4.4. EFFECT OF Seleno-Chrysin (SeChry) IN OVARIAN CANCER CELLS

4.4.1. SeChry INDUCES CELL DEATH IN OVARIAN CANCER CELLS AND MIGHT POTENTIATE CARBOPLATIN EFFICACY AT LOW CONCENTRATIONS IN OVCAR3 CELLS

Selenium compounds can be uptaked by cysteine transporters EAAT3 and xCT [137,184]. Thus, we hypothesised that selenium compounds could compete with cysteine uptake, affecting ovarian cancer cells viability by impairing cysteine uptake. Hence, we

investigated the effect of SeChry, which is constituted by selenium, in ES2 and OVCAR3 cells viability by analysing the IC50 of this compound.

Results have shown that SeChry induces cell death in both ovarian cancer cell lines and that ES2 cells exhibit a lower IC50 (IC50=12,2µM) (figure 4.10.A.) than OVCAR3 cells (IC50=18,8µM) (figure 4.10.B.).

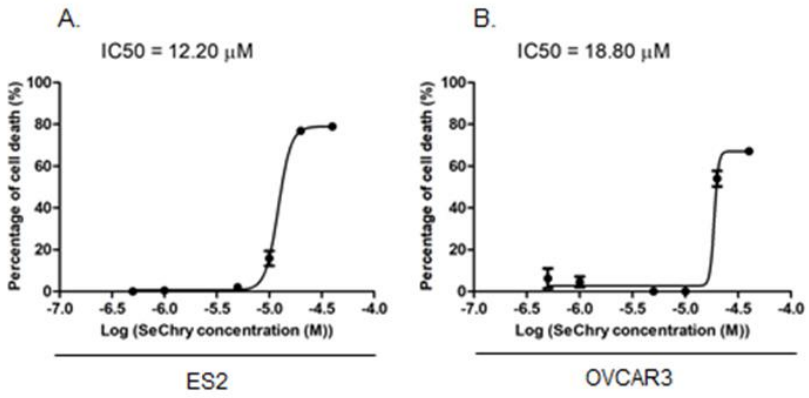
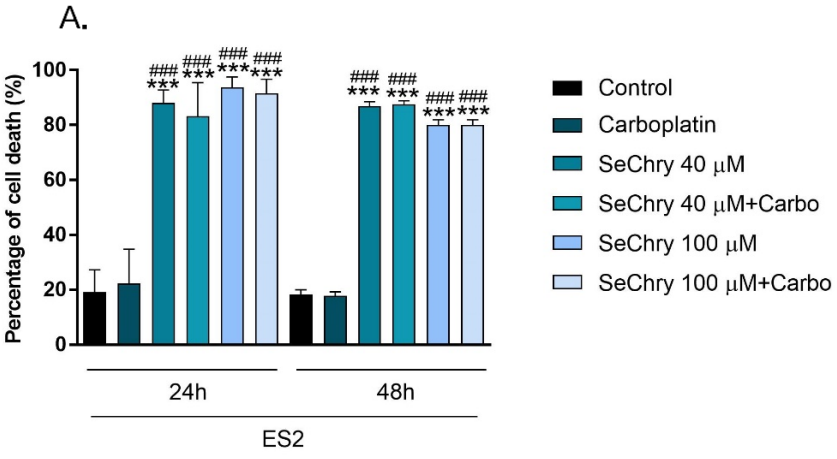


Figure 4.10. - SeChry IC50 determination for ES2 and OVCAR 3. After 48h of SeChry presence, cell death was determined by Flow cytometry. **A.** SeChry IC50 is 12,20 µM in ES2 cell line. **B.** SeChry IC50 is 18,80 µM in OVCAR3 cell line.

Furthermore, we also investigated if this selenium compound was able to increase carboplatin efficacy, thus acting synergistically with this drug in ovarian cancer cells. To test this hypothesis, cells were exposed to 2 different SeChry concentrations (40 and 100 µM) with and without carboplatin exposure.

Results demonstrated that SeChry leads to more cell death compared to carboplatin in both cell lines ($p<0,001$) (figure 4.11.). Moreover, whereas in ES2 cell line no differences between SeChry alone and SeChry with carboplatin were observed (figure 4.11.A.), in OVCAR3 at 24h, SeChry showed a synergistic effect with carboplatin at 40 µM ($p<0,05$) (figure 4.11.B.)



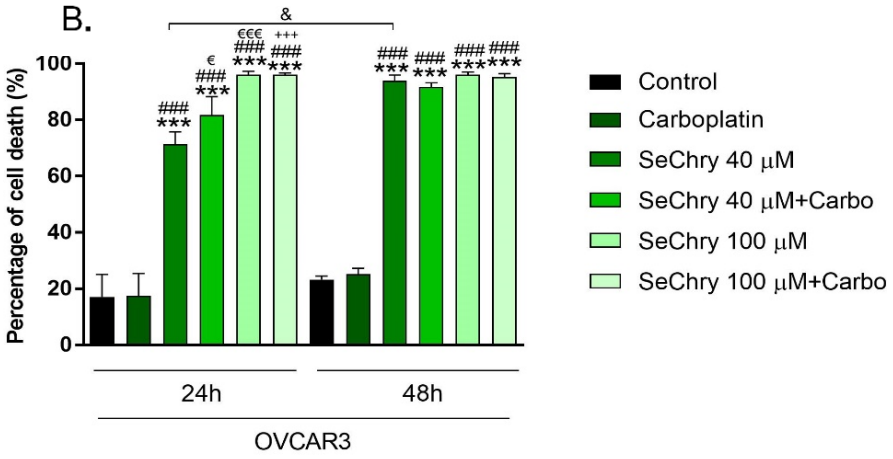


Figure 4.11. - Cell death effect of SeChry at 40 μM and 100 μM with and without carboplatin, in ES2 and OVCAR3. After 24 OR 48h of SeChry exposure followed or combined by 24h of carboplatin exposure, cells were collected to perform cell death analysis by Flow Cytometry. **A.** Percentage of cell death in ES2 cell line. **B.** Percentage of cell death in OVCAR3 cell line. No cell death differences were observed between SeChry and SeChry with carboplatin, excepting in OVCAR3, at the time-point of 24h, in which SeChry 40 μM with carboplatin is responsible for higher cell death than SeChry 40 μM. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (Two-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control, "#"- carboplatin, "€"- SeChry 40 μM, "+" – SeChry 40 μM with carboplatin and "&" statistical significance in relation between conditions in the different time-points.

4.4.2. THE COMBINATION OF CARBOPLATIN WITH CYSTEINE ABROGATE SeChry-INDUCED CELL DEATH IN OVARIAN CANCERS

After the observation that SeChry induces cell death and that this compound was able to potentiate carboplatin effect in OVCAR3 cells, we next aim to disclosure the impact of cysteine supplementation in SeChry effects, asking if the protective effect of cysteine against carboplatin toxicity was lost upon SeChry treatment. For this, 19 μM of SeChry (a proximate value to the IC50 for both cells lines) was used with and without cysteine and with and without carboplatin exposure.

In ES2 and OVCAR3 cells, SeChry acting alone showed increased cell death levels compared to the other conditions ($p<0,001$) (figure 4.12.). Interestingly, in ES2 cells, this effect was lost in the presence of cysteine, carboplatin and the combination of both ($p<0,001$) (figure 4.12.A.). In OVCAR3, cysteine potentiated SeChry-induced cell death ($p<0,001$), effect that was lost when SeChry was combined with carboplatin or with cysteine plus carboplatin ($p<0,001$) (figure 4.12.B.)

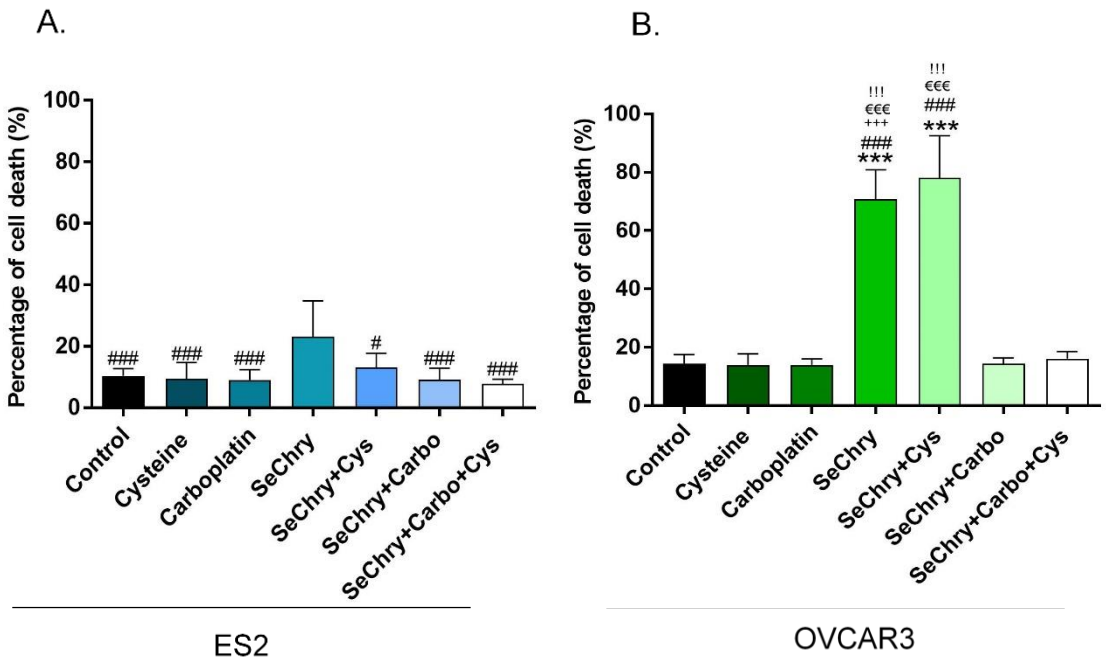


Figure 4.12. - The effect of cysteine, carboplatin, SeChry, SeChry plus cysteine, SeChry plus carboplatin and SeChry plus carboplatin and cysteine in cell death, in ES2 and OVCAR3. After 48h of SeChry exposure accompanied by 24h of cysteine and/or carboplatin exposure, cells were collected to perform cell death analysis by Flow Cytometry. **A.** Percentage of cell death in ES2 cell line **B.** Percentage of cell death in OVCAR3 cell line. SeChry itself is responsible for higher cell death, besides this, in OVCAR3, SeChry associated with cysteine also increased cell death. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (One-Way ANOVA, Tukey post-tests). "*" statistical significance in relation to control; "#"- SeChry (**A**.), cysteine (**B.**); "+" – carboplatin; "€"- SeChry with carboplatin and "!" – SeChry with cysteine and carboplatin.

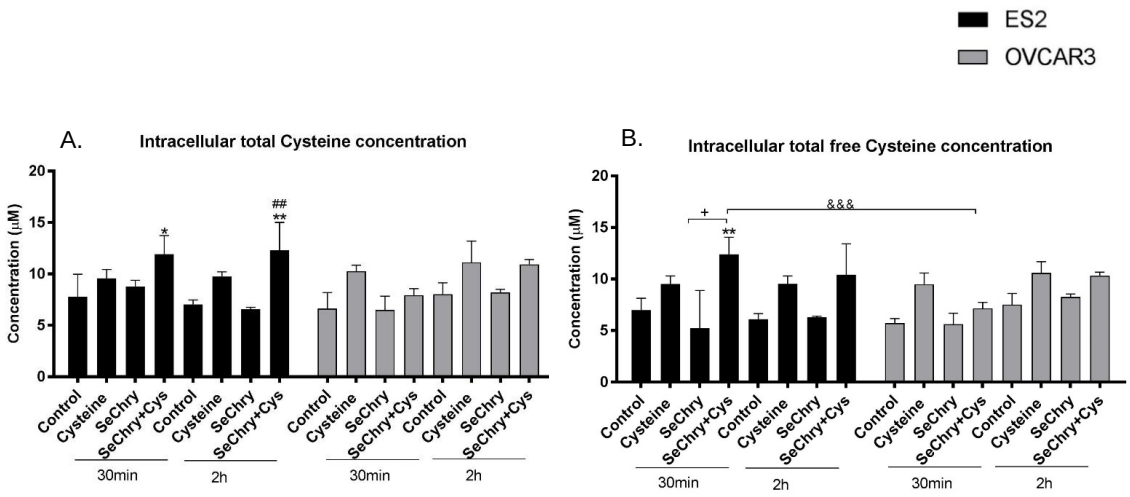
These results have demonstrated that SeChry-induced cell death and the synergistic effect with carboplatin is dose dependent.

4.4.3. SeChry INDUCES CELL DEATH BY PROMOTING OXIDATIVE STRESS

Given the results obtained concerning SeChry role in cell death induction, we next explored cells ability to uptake cysteine upon SeChry exposure, hypothesising that this selenium compound induced cell death could be due to cysteine uptake impairment. To test this hypothesis, 19 µM of SeChry (a proximate value to the IC50 for both cells lines) was used with and without cysteine and HPLC was conducted in cell lysates to determine intracellular thiols and in supernatants of cell cultures to determine extracellular thiols from cells that were exposed to cysteine, SeChry and cysteine with SeChry, during two different time-points: 30min and 2h. From each sample, total and free total thiols concentrations were determined.

Regarding intracellular cysteine levels, in ES2 cell line, intracellular total cysteine concentration was significantly higher under cysteine and SeChry exposure, for both time-points (30min $p<0,05$ and 2h $p<0,01$) compared to control and cysteine (just under 2h exposure) (figure 4.13.A.). However, OVCAR3 did not present significant differences among conditions (figure 4.13.A.). Intracellular total free cysteine concentration analysis revealed that this thiol was also augmented when ES2 cells were exposed to SeChry and cysteine for 30min ($p<0,01$) (figure 4.13.B.). By comparing this same condition between cell lines, higher cysteine concentrations were observed in ES2 cells than OVCAR3 ($p<0,001$) (figure 4.13.B.).

In ES2 cell line, GSH intracellular levels were lower in the cysteine plus SeChry condition at 30min ($p<0,01$). In OVCAR3, SeChry both with and without cysteine presented a significant GSH reduction in comparison with control and cysteine conditions, in both time-points ($p<0,001$ and $p<0,001$) (figure 4.13.C.). Moreover, at 2h cysteine combined with SeChry lead to a significant GSH concentration increase in comparison with SeChry alone ($p<0,05$) (figure 4.13.C.). In control and cysteine conditions, OVCAR3 showed higher GSH concentration than ES2 ($p<0,001$) (figure 4.13.C.). In what concerns the intracellular total free GSH concentration, at the 30min time-point, SeChry in combination with cysteine led to decreased GSH concentration in relation to cysteine alone in ES2 ($p<0,05$) (figure 4.13.D.). OVCAR3 cells presented the same dynamic as in intracellular GSH concentration, SeChry and SeChry with cysteine presented lower GSH levels in comparison with their respective controls ($p<0,001$ and $p<0,05$) (figure 4.13.D.). The concentration of protein thiolation was also analysed allowing to observe that in ES2 GSSP, GSH-protein thiolation concentration tended to increase with SeChry plus cysteine exposure at 2h but this was not significant (figure 4.13.E.).



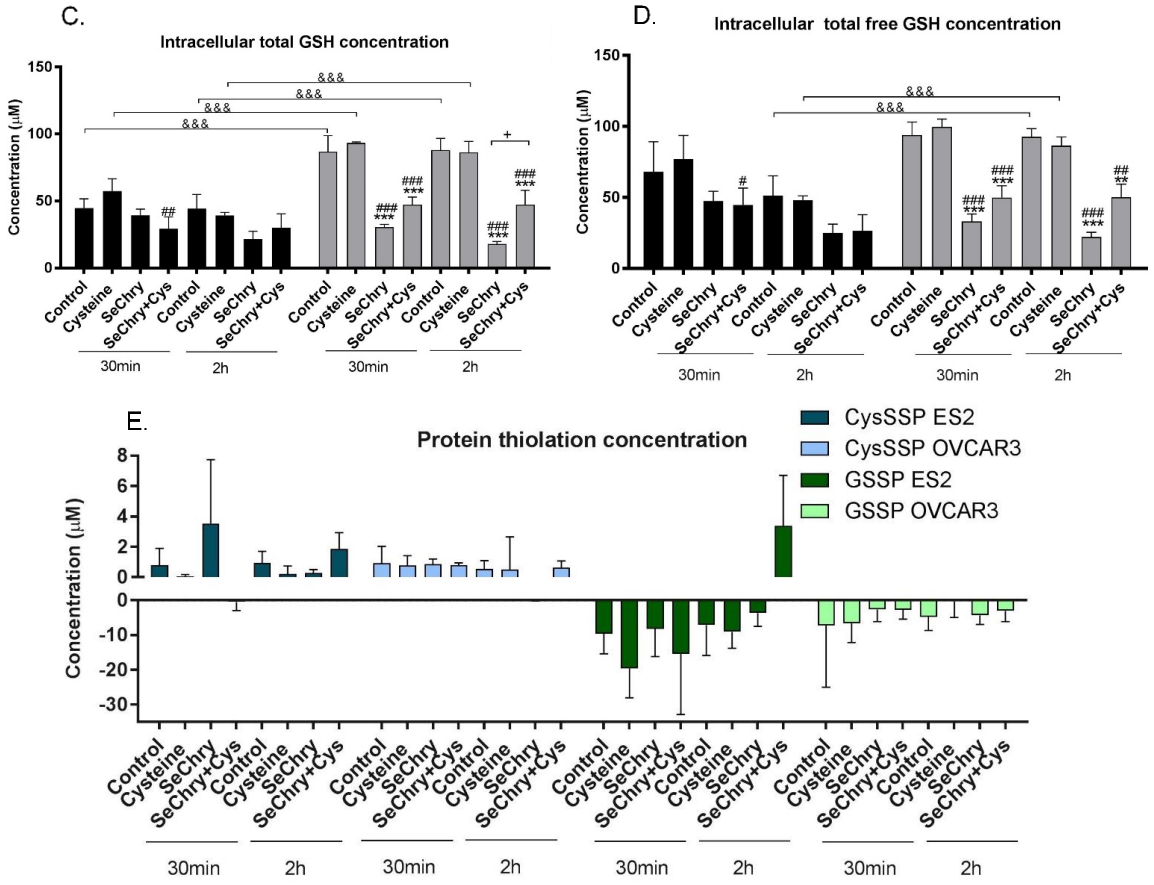


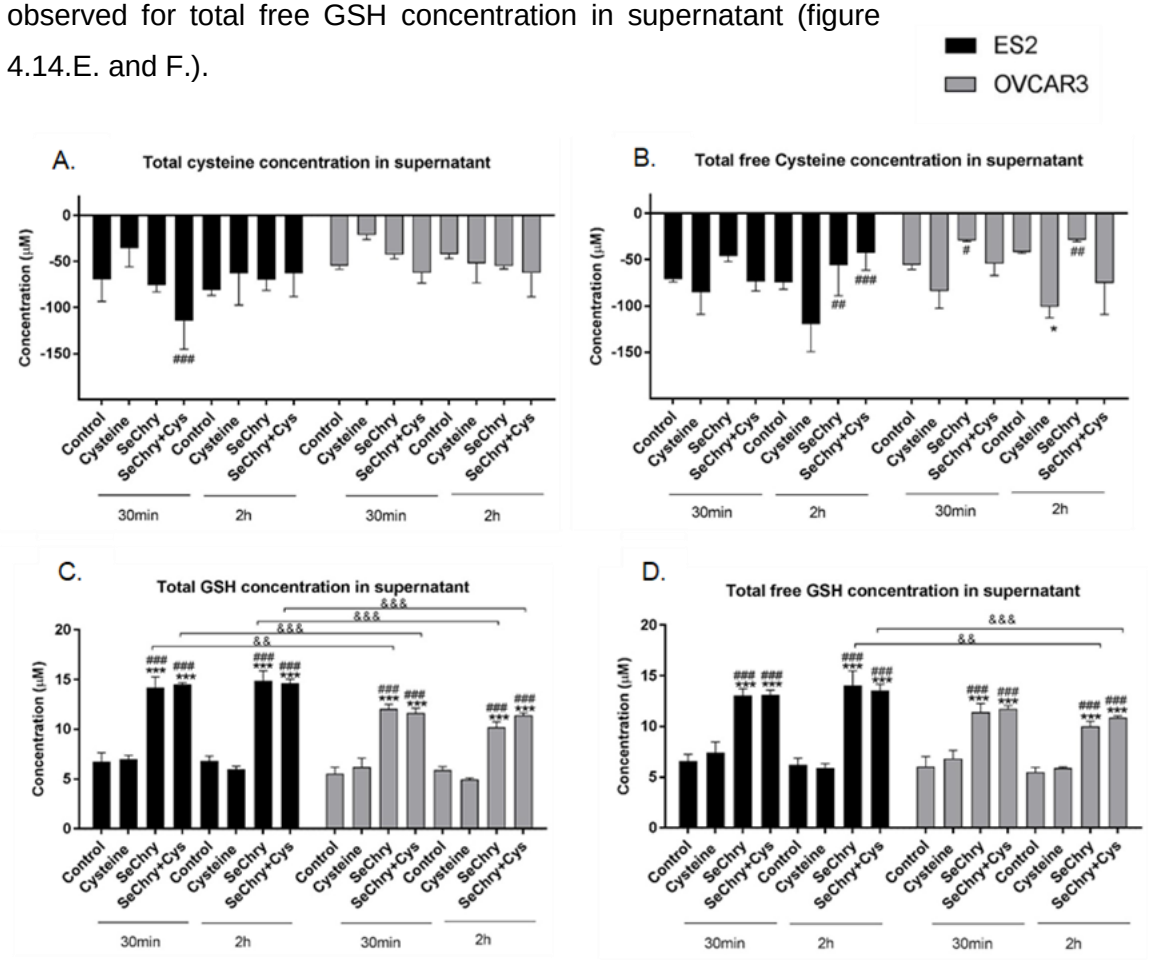
Figure 4.13. - The effect of SeChry in intracellular total and total free cysteine and GSH concentration, in ES2 and OVCAR3. After 24h of SeChry exposure by 30min and 2h with or without cysteine supplementation, cells were collected to perform HPLC, according to Grilo, N.M., *et al.*[232]. **A.** Intracellular total and free total cysteine concentration in ES2 cell line. **B.** Intracellular total and free total cysteine concentration in OVCAR3 cell line. In ES2, SeChry combined with cysteine is responsible for more cysteine concentration**. C.** Intracellular total and free total GSH concentration in ES2 cell line. **D**. Intracellular total and free total GSH concentration in OVCAR3 cell line. In OVCAR3, SeChry and SeChry combined with cysteine is responsible for less cysteine concentration**.** Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (Two-Way ANOVA, Tukey post-tests). "*" is the statistical significance in relation to control; "#"- cysteine; "+" – between conditions in the same time-point and "&" reports the statistical significance between conditions among cell lines.

After pellet cells evaluation, cysteine, GSH and Cys-Gly concentration in cells supernatant were also analysed. Regarding cysteine extracellular levels, the negative values observed are due to cysteine uptake, where lowest values correspond to highest cellular uptake (figure 4.14.A. and B.). In ES2 cell line, with 30min of experimental conditions, total cysteine concentration in supernatant was lower in SeChry with cysteine condition compared to cysteine (p<0,01) (figure 4.14.A.). There were no differences in OVCAR3 total cysteine extracellular levels (figure 4.14.A.). Regarding total free concentration, in ES2 cells under 2h exposure, SeChry with and without cysteine led to

higher free cysteine levels compared to cysteine (p<0,01 and p<0,001) (figure 4.14.B.). In OVCAR3, SeChry exposure at both time-points, conducted to higher total free cysteine concentration in relation to cysteine (30min p<0,05; 2hmin p<0,01) (figure 4.14.B.).

Regarding GSH levels in supernatants, SeChry with and without cysteine supplementation led to higher total extracellular GSH concentration in the two time-points and for both cell lines (p<0,001) (figure 4.14.C.). Besides this, in ES2 supernatants, GSH concentration was higher in SeChry and SeChry combined with cysteine conditions compared to OVCAR3 (p<0,001) (figure 4.14.C.). Total and total free GSH concentration showed the same differences pattern, with the exception that at 30min, SeChry and SeChry with cysteine condition did not lead to differences between cell lines (figure 4.14.D.).

The total and total free Cys-Gly (a product of GSH degradation) concentration in cells supernatant were also determined. In ES2 cells, SeChry with and without cysteine exposure during 30min lead to a Cys-Gly increase in relation to its control conditions (p<0,01) (figure 4.14.E. and F.). The same effect was observed with 2h of exposure, however the significance level was higher (p<0,001) (figure 4.14.C.) (figure 4.14.E. and F.). In OVCAR3 cells there were no differences between conditions, however this cell line showed lower extracellular GSH concentration in relation to ES2. Similar results were observed for total free GSH concentration in supernatant (figure 4.14.E. and F.).



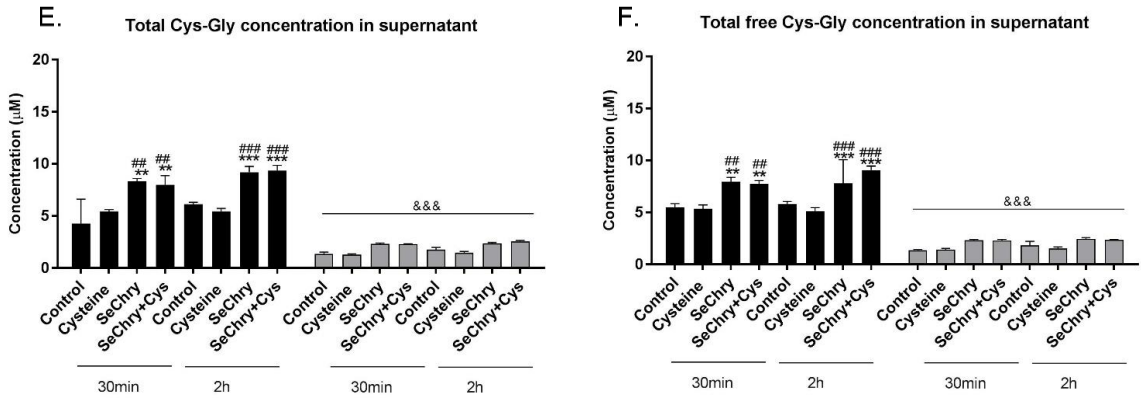


Figure 4.14. - The effect of SeChry in total and total free cysteine, GSH and Cys-Gly concentration, , in ES2 and OVCAR3. After 24h of SeChry exposure by 30min and 2h with or without cysteine supplementation, cells were collected to perform HPLC, according to Grilo, N.M., *et al*[232]. **A.** Total cysteine concentration in the supernatant in ES2 and OVCAR3 cell lines. **B.** Free total cysteine concentration in the supernatant in ES2 and OVCAR3 cell lines. **C.** Total GSH concentration in the supernatant in ES2 and OVCAR3 cell lines. **D**. Free total GSH concentration in the supernatant in ES2 and OVCAR3 cell lines. SeChry and SeChry combined with cysteine increased GSH concentration in both cell lines and time-points. **D.** Total Cys-Gly concentration in the supernatant in ES2 and OVCAR3 cell lines. **E.** Free total Cys-Gly concentration in the supernatant in ES2 and OVCAR3 cell lines. Results are shown as mean ± SD *p<0,05, **p<0,01, ***p<0,001 (Two-Way ANOVA, Tukey post-tests). "*" is the statistical significance in relation to control; "#"- cysteine; "+" – between conditions in the same time point and "&" reports the statistical significance between conditions among cell lines.

Taken together, results indicated that SeChry is not inhibiting cysteine uptake in ovarian cancer cells. Instead, results have demonstrated that this selenium compound is inducing cysteine uptake. Moreover, data also supports that SeChry induced GSH export to the extracellular environment, resulting in increased degradation of this thiol. Those results strongly support that SeChry-induced cell death is due to oxidative stress induction in ovarian cancer cells but not due to cysteine uptake impairment, nevertheless SeChry combination with carboplatin, another pro-oxidant drug, can be unexpectedly favourable for ovarian cancer cells.

5. DISCUSSION

Regarding the maintenance of redox equilibrium, GSH is recognized as a pivotal player in the cell homeostasis. This thiol interacts directly with platinum drugs as alkylant agents and it is also a direct target of ROS[95,118,121,122]. However, its role in ROS scavenging can constitute a mechanism of resistance, in which cysteine seems to have a pivotal role by being a rate limiting substrate and the thiolic component of GSH[80,140,120,130,136].

5.1. CYSTEINE HAS A PROTECTIVE EFFECT AGAINST PLATINUM-INDUCED TOXICITY

In ovarian cancer, it is recognized a variety of mechanisms implicated in chemoresistance as alterations in drug targets, that happen mainly with paclitaxel, DNA repair mechanisms, recognizing nucleotide excision repair (NER) as very relevant upon the activity of platinum salts, defective apoptosis and drugs tolerance, highlighting the one corresponding to drugs inactivation through thiol-containing molecules as GSH[77,80,81,90]. The identification of chemoresistance mechanisms in ovarian carcinomas must be the first step to uncover its behaviour under therapeutical conditions[77,80,81,90].

To access chemoresistance, we evaluated cell death under cisplatin and carboplatin as the platinum drugs mostly used in ovarian carcinoma treatment[80,233]. Cisplatin leads to more cell death than carboplatin, predicting the necessity to use a high carboplatin concentration to achieve the same results as cisplatin[57]. This is in accordance with what happens in the clinical context where cisplatin is characterised by higher toxicity[77]. For both cell lines we recognized the time-dependent efficiency, significantly noticed with cisplatin (figure 4.1.).

Our first aim was to recognize cysteine as a cell protector or as capable of overcoming platinum drugs effect. For that we used cysteine in combination with both drugs and in their majority, results confirmed our hypothesis (figure 4.1.). Extended exposure time (48h), led cysteine effect upon cisplatin to be nullified proposing the rapid consume of cysteine in an attempt to protect cells from the potent cisplatin effect at the initial time-points (figure 4.1.). Through direct chemical interaction as oxidative/alkylant agents and through ROS formation, platinum drugs are responsible for GSH decrease, which requires cysteine to supply its synthesis[80–82] .

As reported by Nunes, S.C., *et al.*, OCCC (ES2) are more dependent on cysteine metabolism than OSC (particularly OVCAR3), recognizing its metabolism as a major contributor to a poor disease outcome[63]. Differences in cysteine role between cell lines were just observed in cell death due to carboplatin and cisplatin cysteinylation. Implementing these conditions was relevant to undercover drugs thiolation role, by considering the two

follow hypothesis: by drugs cysteinylation, drug uptake is accompanied by more cysteine cell entrance, and cells can better accomplish their detoxifying activity, or on the other hand, this strongest linkage between thiol and drug could potentially lead to more cell death, due to the more cysteine request that necessarily comes along with carboplatin or cisplatin. In fact, regarding our results, we can suggest the attribution of the first hypothesis to ES2 cells behaviour (figure 4.1.A.), and the second one to OVCAR3 cell line (figure 4.1.B.).

Taking a look at other biological models and thiolation function, in *B. subtilis* this mechanism was shown to be crucial in the prevention of the irreversible oxidation of cysteine residues during oxidative stress leading consequently to redox regulation and homeostasis[234]

In what concerns platinum drugs cysteinylation applicability, there are evidences that cisplatin reacts spontaneously in solution with GSH or cysteine to form its respective conjugates, being shown that GSH high doses combination with cisplatin was responsible for protecting cells against cisplatin-induced nephrotoxicity[235] . In figure 5.1. we summarize the three mechanisms here exposed.

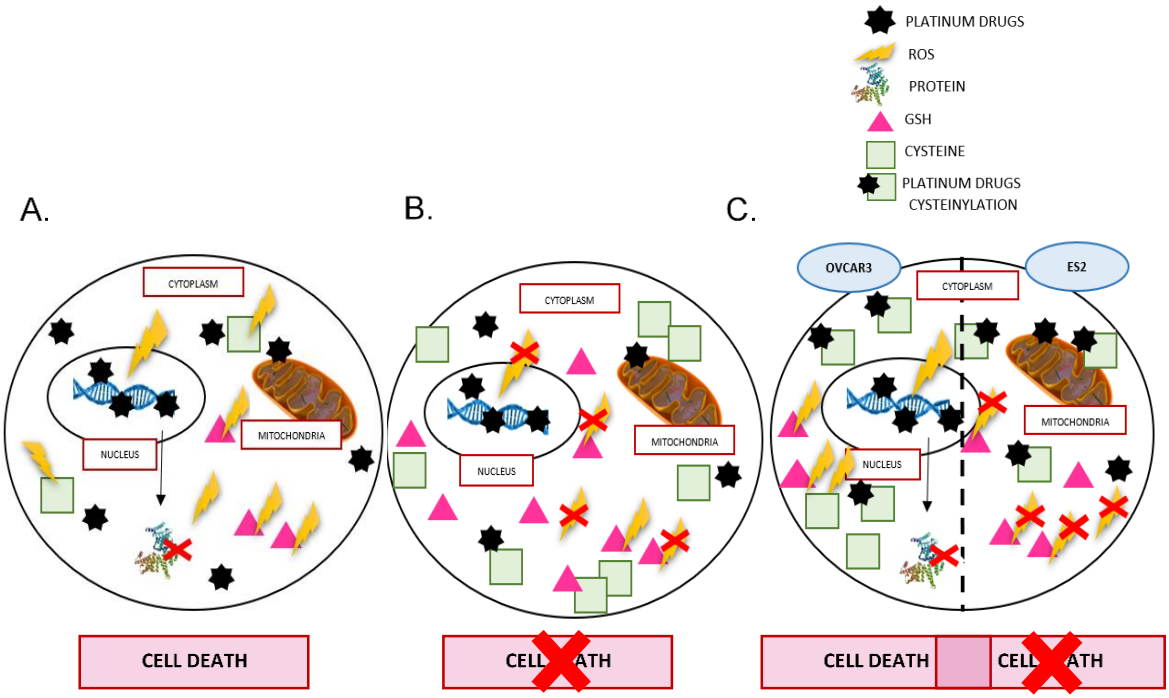


Figure 5.1. - Representative scheme from the three hypothesis of platinum salts activity with and without cysteine. A. Platinum salts lead to adducts formation with DNA and increase ROS formation that consequently leads to decreased GSH. **B.** Cysteine supplementation is responsible for less cell death, probably because it is a rate-limiting substrate in GSH synthesis, which consequently leads to efficient ROS scavenging, and due to its sulphur group capable of binding to platinum salts. **C.** Carboplatin and cisplatin cysteinylation have different effects within cell lines. In OVCAR3 this process leads to more cell death, in the opposite, in ES2 is responsible for a reduction in cell death.

5.2. xCT AND EAAT3 ARE DIFFERENTLY MODULATED BY CYSTEINE AND CARBOPLATIN

As we were capable of identifying cysteine as an important molecule involved in cell homeostasis by having a protective effect against platinum salts, it became fundamental to understand how this thiol modulates its transporters.

xCT and EAAT3, also glutamate transporters, are recognized in a variety of contexts and diseases, including cancer[117,179,137]. Both transporters functions are intimately correlated with chemoresistance phenomenon's, that relies on their increased expression with subsequent cysteine increased uptake, that is why we sought to be important in ovarian cancer chemoresistance[117,182,236,237]. Firstly, xCT and EAAT3 transporters stimulation by cysteine allowed to confirm their presence in ovarian cancer, even knowing that its characterization is commonly done and more complete in the central nervous system (CNS) context (figure 4.2.)[125,136,238,239]. xCT showed constant mRNA levels in short-time periods for both cell lines (figure 4.2.A.). However, in OVCAR3, we recognized initial time-points, upon cysteine stimulation, important for *EAAT3/SLC1A1* mRNA expression (figure 4.2.B.). ES2 presented higher basal mRNA expression of *xCT/SLC7A11* and *EAAT3/SLC1A1* in comparison with OVCAR3, corroborating with previous data indicating that ES2 is more dependent on cysteine (figure 4.2.C. and D.)[45,63].

xCT/SLC7A11 mRNA expression tended to be unaltered under short-time periods, however in 16h is still possible to observe a significant increase in *xCT/SLC7A11* expression upon cysteine exposure (figure 4.3.A.). In this experience, as we were also testing carboplatin effect, we equally verified that cysteine accompanied with carboplatin showed higher *xCT/SLC7A11* mRNA expression, which was not verified under carboplatin (figure 4.3.A.). With these results we claimed the importance of cysteine in the modulation of xCT transporter and particularly in ES2 cell line, as in OVCAR3 no obvious differences were observed (just a slightly increase with carboplatin) (figure 4.3.A.).

EAAT3/SLC1A1 mRNA expression did not present any modulation by drugs in ES2, but interestingly, in OVCAR3, *EAAT3/SLC1A1* mRNA expression was decreased under carboplatin exposure and quite lower in comparison with *xCT/SLC7A11* suggesting that these cells upon carboplatin exposure may be in a compensatory mechanism using xCT transporter, instead of EAAT3, to support the detoxification process (figure 4.3.B.).

To reveal if the same modulation was present in protein levels we used two distinct widely used techniques: western blotting and Immunofluorescence.

For xCT transporter, by western blot analysis, we observed in ES2 cell line an increase in its protein levels under carboplatin plus cysteine exposure that was in accordance with mRNA results (figure 4.4.B.). However, cysteine alone was not responsible for an increase in protein levels. This phenomenon could be explained by post-translational modifications that are responsible for modulating molecular interactions, protein localization and also its stability giving rise to a large heterogeneity of the protein population[240]. This aspect explains the difficulties to achieve complete characterization and accurate quantitation of proteins[240]. Besides this, it is known that many mRNAs are short-lived/unstable, an important feature for limiting the synthesis of encoded proteins that in this case can be involved in these differences[241].

The immunofluorescence assay performed in ES2 just showed a not significant increase in xCT transporter when stimulated by carboplatin and cysteine (figure 4.5.A and B.). In western blotting analysis, in OVCAR3, there were no differences in xCT protein levels upon experimental conditions, however by immunofluorescence cysteine seem to induce xCT expression (figure 4.5.A. and C.).

The differences observed between the results obtained through western blotting analysis and immunofluorescence can be explained: western blotting technique is a semi-quantitative method, since it is a relative and not an absolute quantification , yet is more accurate in which concerns to specificity than immunofluorescence, because this last technique has more applicability in what concerns protein localization, being very difficult to evaluate the specificity of antibodies[224,225].

Interestingly, by looking at the immunofluorescence results, xCT seemed to co-localize with mitochondria (figure 4.5.A.). By western blotting with isolated mitochondria, xCT was detected, reinforcing this observation (figure 4.5.D.). The importance of xCT in the mitochondria could be associated with ATP synthesis. Cysteine uptake that can be done by xCT transporter, can be metabolized in the mitochondria by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercapto-pyruvate sulphurtransferase (MpST) conjugated with cysteine aminotransferase (CAT) activity, these last two enzymes are already in the mitochondria, however CBS and CSE are dependent on its translocation from the cytosol that is described under stress conditions[242,243]. They are all associated with mitochondrial hydrogen sulphide (H_2S) generation and ATP production and evidences for this are in cysteine degradation in H_2S and pyruvate[242,243]. H_2S may function as an energy substrate to sustain ATP production in these cases of oxidative stress. Pyruvate is pivotal in supplying the Krebs cycle and supporting the mitochondrial electron transport chain and ATP production[120,242,244].

Towards EAAT3 transporter we also performed western blotting analysis that ended up failing, we believe that the antibodies tested were just not suitable.

Based exclusively on immunofluorescence, EAAT3 protein levels, in OVCAR3, were not altered between conditions (figure 4.6.A. and C.). Nevertheless, unexpected effects conducted by cysteine and carboplatin were verified in ES2 cell line (figure 4.6.A. and B.). EAAT3 protein levels decreased when cells underwent cysteine and carboplatin combination exposure, that can be explained by a compensatory mechanism, in which cells tend to use xCT. Hypothesized by Lewerenz, J. *et al*., in brain disease, xCT (glutamate/cystine antiporter) activity increase to augment cysteine uptake and minimize cell injury, consequently glutamate at the extracellular level augments, and EAAT3 transporter (glutamate/cysteine symporter), that is important in Xc- system balance regulation, is decreased being consequently responsible for diminishing glutamate in the cell, this toxicity is translated by an intracellular ROS accumulation that can continue stimulating xCT expression[117]. The aforementioned hypothesis can be corroborated through the fact that glioblastomas exhibit higher system Xc- activity, but in contrast, lower Na+ dependent glutamate transport activity[152]. In what concerns to metabolism, it is known that up regulation of *xCT/SLC7A11* by NRF2 is a mechanism in which glutamine-dependent processes, that drive mitochondrial respiration, presents reduced efficiency, that is explained as being result of the high glutamate export[245].

We can refer ES2 as more dependent on cysteine than OVCAR3, this cell line tends to present higher mRNA expression of *xCT/SLC7A11* and *EAAT3/SLC1A1* whose regulation is modulated by cysteine and carboplatin. Nevertheless, in both cell lines we suspected from the higher xCT activity in relation to EAAT3 due to a compensatory mechanism or an imbalance of xCT activity in disease context, that decreases EAAT3 (figure 5.2.).

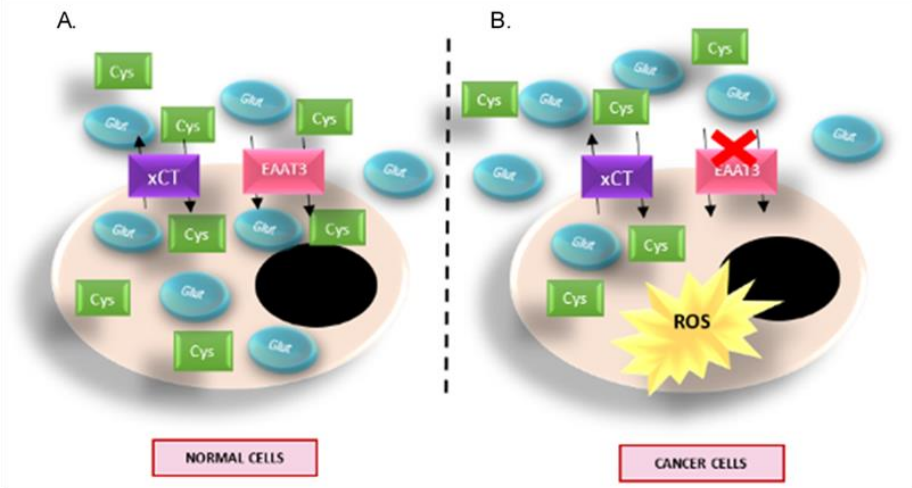


Figure 5.2. - Hypothesis for the imbalance of xCT activity leading to decreased of EAAT3 activity. A. In normal cells, xCT and EAAT3 present a normal activity, being responsible for the uptake of cysteine and

glutamate (EAAT3) or the glutamate export (xCT). **B.** In cancer cells, xCT transporter is upregulated leading to the increase of glutamate export, that consequently diminishes the EAAT3 transporter activity. This is responsible for ROS increase that at the same time can stimulate xCT. Glut means glutamate and Cys cysteine.

5.3. NRF2 AND HNF1β ARE AUGMENTED IN STRESSFUL CONDITIONS

NRF2 as a transcription factor involved in xCT regulation was considered to be studied in this context also due to its recognized role in detoxification process[117,154,193]. There is a variety of mechanisms involved in the constitutive activation of this gene in cancer cells, as the gain-of-function mutations in *NRF2* and as the loss-of-function mutations in the Kelch-like ECH (*Keap-1)* leading to an impairment of the binding to Keap-1. Keap-1 is the substrate adaptor for CUL3, and both lead to NRF2 ubiquitination[154,195]. Moreover, somatic mutations and promoter hypermethylation in *CUL3* gene are also responsible for *NRF2* activation in ovarian cancers[195].

So, *NRF2* mRNA expression was evaluated to verify if cysteine and carboplatin were modulating its expression. No differences were observed in both cell lines, seeming that this gene is not affected by these two compounds (figure 4.7.A.). Contrarily, protein levels revealed to be increased under carboplatin and carboplatin combined with cysteine exposure (figure 4.8.A. – C.). This clearly showed, that carboplatin is responsible for increasing NRF2, in both cell lines, so the oxidative stress caused by this drug, triggered NRF2 activity. Consistent with this, other researchers have been showing that GSH depletion achieved by drugs is responsible for increasing the regulation of antioxidant genes in which NRF2 has an important role. NRF2 dissociation from Keap-1 and posteriorly binding to ARE is responsible for triggering a cytoprotective adaptative response, characterised by upregulation of several cytoprotective and detoxification genes, including ferritin, NQOI, GSH-S-reductase (GSR), GST, GCLM, and GCLC, multidrug resistance protein (MRP), and *xCT/SLC7A11*[115,117,195,246].

Likely the other cytoprotective genes, *NRF2* is upregulated and it is associated with chemoresistance. Studies performed, allowed to verify that *NRF2* overexpressed in A549 cells (non-small cell lung carcinoma cell line) presented higher resistance to cisplatin compared to NCI-H292 and LC-AI cells[195].

Even though, these facts cannot distinguish cell lines in their response to the oxidative stress stimulation.

NRF2 regulates *xCT/SLC7A11,* it is involved in oxidative stress protection, so we thought that it could also be important in the regulation of *EAAT3/SCL1A1*. Concomitantly, we analysed the possible regulation of this transcription factor upon *xCT/SLC7A11* and

EAAT3/SCL1A1 and the modulation by cysteine and carboplatin (figure 4.9.). NRF2 relative occupancy in *xCT/SLC7A11* promoter is modulated by cysteine in OVCAR3, corroborating the differences observed in xCT protein levels in this cell line (figure 4.9.F.). Regarding *EAAT3/SCL1A1*, in ES2 any differences were observed in *EAAT3/SCL1A1* modulation by cysteine and carboplatin but in OVCAR3, this NRF2 relative occupancy was reduced with cysteine and carboplatin (figure 4.9.G.). These experiments must be validated, in order to confirm the results.

In accordance with discussion in 5.2., ES2 cell line, is more dependent on cysteine metabolism in comparison with OVCAR3, and it belongs to an histological type (OCCC) that exhibits intrinsic chemoresistance and expresses the transcription factor HNF1β[72]. A study conducted by Lopes-Coelho *et al*., had as main objective to find out if HNF1β was underlying the intrinsic chemoresistance of OCCC (ES2) to platinum-based chemotherapy. The authors demonstrated through knockdown ShRNA-HNF1β assays, that HNF1β (a hallmark of OCCC) was regulating the expression of the gene encoding the catalytic subunit of glutamate cysteine ligase (GCL), the limitant enzyme of GSH synthesis, accounting for the higher levels of GSH in ES2 comparing to OVCAR3 (negative for HNF1β)[45].

Hence, we first wanted to understand how *HNF1B* gene was modulated by cysteine or carboplatin. *HNF1B* mRNA showed no differences between ES2 and OVCAR3 cell lines or cell culture conditions (figure 4.7.B). However, immunofluorescence granted differences between cell lines, as OVCAR3 did not present any protein expression (figure 4.8.D.).

In ES2 HNF1β protein levels were increased by carboplatin plus cysteine but neither cysteine nor carboplatin alone were able to induce significant changes in HNF1β expression (figure 4.8.D. and E.). So, cysteine requisition due to the oxidative stress caused by carboplatin and the supplementation of cysteine in the medium are responsible for an augment in GSH levels, a mechanism in which HNF1β is a contributor[45,208,220].

Testing the interaction of this HNF1β with *xCT/SLC7A11* and *EAAT3/SCL1A1* promoters in ES2, we can suggest a possible role of HNF1β as an activator of *xCT/SLC7A11* transcription induced by cysteine (figure 4.9.B. and C.). Regarding *EAAT3/SCL1A1*, the role of HNF1β seems to be as a transcriptional repressor.

Nevertheless, it is known that ChIP is a valuable tool to find out protein interactions with DNA, but, from all the assays conducted, this one was the more difficult to analyse due to the variability of the results obtained and also because the biological replicates were low[247–249]. To assure the regulation of these genes by these transcription factors we should

recognize the importance on using other techniques, such as a luciferase assay, in which through light emission it is possible to access gene promoters activity[250].

5.4. Seleno-CHRYSIN (SeChry), A PUTATIVE CYTOTOXIC DRUG

Selenium (Se), a cofactor of mammalian enzymes such as glutathione peroxidase (GPx) has been a target of cancer research due to its chemopreventive effects, functioning as a great antioxidant[184,251]. This fact led to a variety of studies demonstrating that Se pharmacological targeting exerts an anti-tumoral effect[184,251]. As EAAT3 and xCT are intimately correlated with drugs resistance in cancer, in an attempt to decrease both transporters detoxifying activity, by decreasing cysteine uptake, selenated-compounds were taking in consideration as a putative EAAT3 and/or xCT inhibitor[117,137]. Based on the capacity of both transporters being capable of performing selenium uptake, we thought that probably this could lead to a competitive reaction with cysteine. We focused on the use of a selenated-compound named Selenium-containing chrysin (SeChry), which has been described as having an anti-tumour effect[188,189,251]. ES2, through IC50 determination, showed to be more sensible to SeChry than OVCAR3 (figure 4.10.). As ES2 are more dependent on cysteine, this hypothesis based on a competitive reaction could be suggested. This observation is consistent with what was observed in two small cell lung cancer cell lines, H157 and U2020, in which a higher xCT activity was associated with higher selenium salt (selenite) sensivity[252].

Regarding these observations, we tried to find out if this competitive reaction would synergistically act within the appliance of platinum drugs. The results promptly showed that SeChry was responsible for higher levels of cell death, that were quite similar to the levels of cell death induced by carboplatin (figure 4.11.).

This cell death conducted by SeChry could be explained by the cysteine impairment or by the newly recognized pro-oxidant role of selenated-compounds, that is based on ROS generation, oxidation of protein, thiols and direct or indirect DNA binding, leading to impaired protein function and apoptotic cell death[188,251]. Presenting antioxidant or pro-oxidant properties dependent on selenated-compounds concentrations[253,254].

To attest if the prior results were consequence of a higher SeChry concentration, and also to explore cell ability to uptake cysteine upon SeChry exposure, the assay was performed by using less SeChry concentration (a proximate value to the IC50 for both cells lines) with and without cysteine (figure 4.12.A.). In ES2, cell death was significantly higher with SeChry, in comparison with all the other conditions (figure.4.12.B.). In OVCAR3, cell death was also augmented with SeChry plus cysteine (figure 4.12.A.) which could be an evidence for the necessity of a reduced microenvironment to the improve of selenium-

compounds activation, previously described[252] . These results were conclusive in what concerns to SeChry cytotoxicity previously reported by Martins, I.L., *et al.,* based on changes in mitochondrial membrane potential. however some doubts remain in which concerns to cysteine uptake[188]. Regarding SeChry with carboplatin (SeChry medium was not taken before carboplatin exposure), if we assume that both compounds are chemically reacting with each other, they would become less toxic and it would justify the cell death decrease mainly in relation to SeChry condition (figure 4.12.).

The SeChry IC50 was lower in ES2 cell line (12,2 µM) in comparison with OVCAR3 (18.8 µM), however, in cell death assay, percentage of cell death was minor than the expected. There is a wide variety of factors that compromise cell culture, and cell confluency is one of the most important as it can affect cell response to pro-apoptotic drugs[255].

High-performance liquid chromatography (HPLC) technique comes with the evaluation in what concerns cysteine transport impairment by SeChry (figures 4.13. and 4.14.). This assay helped to verify thiols dynamic and intracellular total and total free cysteine concentrations seemed to be higher in cells exposed to cysteine and SeChry with cysteine, suggesting that SeChry was not inhibiting cysteine uptake. Total and free total cysteine concentrations in the supernatant of cell culture were lower in accordance with the intracellular results. Revealing once more that cysteine uptake is not affected by SeChry (figures 4.13.A. and B./4.14.A. and B.).

Regarding intracellular GSH total and free total thiols, their concentrations were lower in the presence of SeChry, so, SeChry was responsible for GSH decrease, which is characteristic of oxidative stress cell states (figure 4.13.C. and D.)[256]. This effect was in accordance with Martins, I.L., *et al.* observations, obtained through thiols absorbance spectrometric measurements based on Ellman's reagent (5,5'-dithiobis –(2-nitrobenzoic acid) (DTNB) assay[188]. In its turn, total and free total GSH concentration in supernatants of cell culture was higher in SeChry and SeChry plus cysteine (figure 4.14.C. and D.), the opposite from what was observed at intracellular level, appointing for GSH degradation, which was confirmed by high levels of extracellular Cys-Gly (resulting from GSH degradation). Collectively, data strongly supported the pro-oxidative role of SeChry (figure 4.14.E. and F.)[95,120]. According with these results, it was reported that GSH is released by cells that enter in the apoptotic process[257]. Moreover, those GSH levels could explain the decreased cell death observed upon the combined exposure of SeChry and carboplatin, that may lead to decreased cell death due to its extracellularly abrogation by GSH.

As previously debated, protein thiolation is a common modification and important component of cellular housekeeping, being also relevant in key signalling cascades. This phenomenon can be considered a defensive complex induced by oxidative stress through radical and protein sulphenic acids mediated reactions and by exchange reactions between thiols and disulphide[258,259]. If cells are under a reducing cell environment, protein thiolation is maintained at relatively low levels due to the recycling of GSH[258]. In this case, no significant changes in protein thiolation were observed, however after 2h under SeChry and cysteine exposure, ES2 cells tended to augment their protein thiolation concentration, maybe in an attempt to counteract the oxidative stress led by this compound (figure 4.13.E.). Figure 5.3. summarizes the possible role of SeChry in cancer cells.

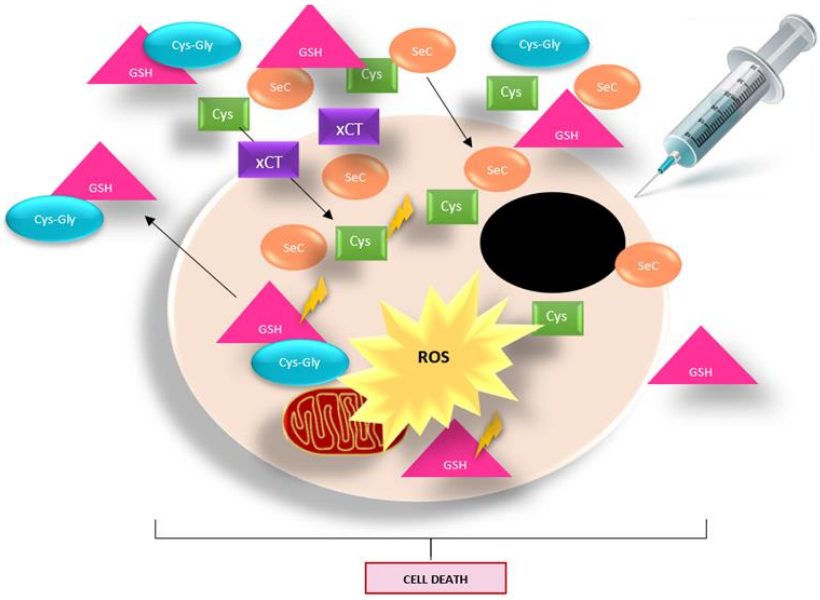


Figure 5.3. - Possible SeChry's mechanism of action in ovarian cancer cells. SeChry exposure leads to oxidative stress that consequently leads to the reduction of GSH intracellularly and its increase extracellularly accompanied by its degradation, not affecting cysteine uptake, through xCT transporter, that can be even more required to GSH synthesis. SeC corresponds to SeChry.

Research in this ambit have been demonstrating differences between selenium compounds: inorganic are suggested to present higher genotoxic stress leading to higher systemic toxicity and organic have anti-tumour activity, preventing metastasis and related to a more directed target activity[184,251]. As SeChry is an organo-selenium compound capable of inducing ovarian cancer cell death, it's our main interest to study its function and possible selectivity.

6. CONCLUSIONS / FUTURE PERSPECTIVES

Chemoresistance is undoubtedly one of the major problems in what concerns overcoming cancer. Nowadays, there is recognized an extensive range of therapies that are quite efficient, nevertheless, in many cases patients tend to relapse. So, to undercover what is beyond this phenomenon can be the trigger to new target therapies.

In the case of ovarian cancer, a highly heterogeneous disease, this cell resistance behaviour is based in a set of mechanisms, in which ROS scavenging through the increased of GSH is relevant. Exploring the pathways that contribute for this phenomenon is pivotal to understand the consequent dynamic. By recognizing cysteine implication in cancer cells resistance, research works have been exploring the role of cysteine transporters, showing their critical importance.

Our work allowed to undercover cysteine role in ES2 (OCCC) and OVCAR3 (OSC) cell death resistance, showing a protective effect upon carboplatin and cisplatin stimuli and also to unveil the pivotal role of xCT transporter in cysteine management. These cell lines presented different response patterns in what concerns transporters activity, helping highlight the differences between chemoresistance pathways. Besides xCT, EAAT3 also presented to be involved in this dynamic, and to be deregulated/downregulated when xCT activity is increased.

This work was important to undercover the importance of NRF2 and HNF1β in oxidative stress situations, as they showed to be overexpressed under carboplatin exposure and because in certain cases they seemed to be involved in *xCT/SLC7A11* and *EAAT3/SLC1A1* regulation. As protein levels of HNF1β were not detected in OVCAR3 cell lines, these results helped to emphasize differences between OCCC and OSC,

SeChry failed to inhibit cysteine uptake but this pathway, instead, led us to recognize its pro-oxidative function, leading to ES2 and OVCAR3 cell death. There is evidences that cancer cells are more sensitive to cytotoxicity induced by selenium than non-cancer cells, so this can be selective in cancer cell death and a future strategy to overcome ovarian cancer. Importantly to highlight that its conjugation with other cytotoxics as carboplatin can be detrimental, as cells tend to survive.

So as future perspectives, it would be interesting:

- To verify xCT and EAAT3 transporters modulation upon SeChry and carboplatin exposure.

- To test the effect of SeChry in a non-malignant cell line, in order to characterize its cytotoxic capacity.

- To evaluate the effects of sulfasalazine and erastin, xCT inhibitors, in cell viability and in response to carboplatin in ES2 and OVCAR3 cell lines.

- To evaluate the repercussions in the use of an EAAT3 inhibitor upon ES2 and OVCAR3 cell viability.

- To verify the amount of mitochondrial xCT in the presence of cysteine and carboplatin.

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8. APPENDICES

Appendix A. Solutions used in the experimental work:

-10X PBS (pH 7.4-7.6)

1 L= 80 g NaCl (1,37 M) (106404, Merck Millipore)

2 g KH2PO4 (14,7 mM) (104873, Merck Millipore)

11,1 g Na2HPO4 (78,1 mM) (S-0876, Sigma Aldrich)

2 g KCl (26,8 mM) (104936, Merck Millipore)

ddH2O to 1 L

-PBS(1X) – 0,1% (v/v) Tween 20

1 L= 1X PBS 1 L

1 mL Tween 20 (20605, USB)

-PBS(1X) - 0,1% (v/v) Tween 20 - 3% (w/v) BSA

100 mL= PBS 1X – 0,1% (v/v) Tween 100 mL

3 g BSA (A9647, Sigma Aldrich))

-PBS(1X) – 0,5% (w/v) BSA – 0,1% (w/v) saponin

100 mL= PBS 1X 100 mL

0,5 g BSA (A9647, Sigma Aldrich)

0,1 g saponin

-PBS(1X) – 0,1% (w/v) BSA

100 mL= PBS 1X 100 mL

0,1 g BSA (A9647, Sigma Aldrich)

-PBS(1X) – 0,01% (v/v) Triton X-100

5 mL= PBS 1X 5 mL

50 µL 10% Triton X-100 diluted in ddH_2O (100% Triton X-100, T8787, Sigma)

-10X TBS (pH 7.4-7.6)

1 L= 8 g NaCl (106404, Merck Millipore)

0.63 g Tris (15504-038, Gibco BRL)

4,4 mL hydrochloric acid (1 M)

ddH_2O to 1 L

-TBS (1X) - 0,1% (v/v) Tween 20 - 5% (w/v) NFDM

100 mL= TBS 1X – 0,1% (v/v) Tween 100 mL

5 g NFDM (Molico, Nestlé)

- **5X SDS gel loading buffer**

250 mM Tris HCl (pH 6,8) (0,5M 161-0799, Bio-Rad)

10% SDS (V6551, Promega)

0,5% bromophenol blue

50% glycerol (1.04094.1000, Merck Millipore)

- **Transfer buffer**

500 mL= 7,5 g glycine (US16407, USB)

1,5 g Trizma-base (T-8524, Sigma Aldrich)

100 mL Metanol (107018, Merck Millipore)

0,5mL 10% SDS (V6551, Promega)

ddH_2O to 400 mL

- **RIPA buffer**

For 10 mL= 20 mM Tris-HCl pH 7,5

150 mM NaCl (106404, Merck Millipore)

5 mM KCl (104936, Merck Millipore)

5 mM MgCl2 (M-8266, Sigma Aldrich)

1% Triton X-100 (T8787, Sigma Aldrich)

1 Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablet (11836170001, Roche)

1 mM Orthovanadate (Na3VO4)

1 mM Sodium fluoride (NaF) (201154, Sigma Aldrich)

ddH_2O to 10 mL

-PFA 4%

30 mL= 27 mL de ddH_2O (60ºC)

1,2 g (37%) paraphormaldeyde (104003, Merck Millipore)

60 µL (1M) NaOH

3 mL de PBS (10X)

- **50 µg/mL Propidium Iodide (PI) solution – Cell Death assay**

For 50 mL: 1 mL of 2,5 mg/mL PI solution (P4170, Sigma Aldrich) (prepared in PBS(1X)) 49 mL PBS(1X)

-TCA 100 g/L

10 mL= 1g TCA

2, 922 mg (1mM) EDTA

ddH_2O to 10 mL

-NaOH

10 mL= 0,62 g (1,55 M) NaOH

ddH_2O to 10 mL

-Borate tampon

50 mL= 2,384 g (0,125 M) sodium tretaborate

58,45 mg (4mM) EDTA

ddH_2O to 50 mL