OVERCOMING CHEMORESISTANCE IN OSTEOSARCOMA:
THE ROLE OF AUTOPHAGY IN CELL DEATH OR SURVIVAL FOLLOWING CHEMOTHERAPY

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Abstract

For the past 30 years, long-term survival rates in metastatic and recurrent osteosarcoma (OS) patients has remained unchanged. Drug resistance is thought to be the main cause and overcoming this phenomenon is a key step towards greater efficacy in OS therapy. Increasing evidence shows that autophagy, a 'self-degradation' pathway, can act as a protective mechanism to help cancer cells thrive under chemotherapeutic stress. The current study investigated the implication of autophagy in metastatic OS progression and further investigated the functional role of autophagy in two OS cell lines in response to two standard chemotherapy treatments, doxorubicin and cisplatin. The results from the tissue OS microarray showed that advanced grade and stage tumours express high levels of autophagy marker LC3. Bioinformatics showed how MAP1LC3B (LC3B gene) can be highly expressed in tumours associated with poor disease outcome. Drug-treated OS cell lines showed a remarkable increase in LC3 puncta (autophagy marker) detected by Immunofluorescence. Additionally, drug-treated OS cell lines showed increase autophagic flux (LC3-I/LC3-II protein conversion) that correlated with reduced p62/SQSTM1 expression, analysed by Western Blot. RT-PCR results confirmed this pattern. Combination of chloroquine (CQ) with chemotherapy had a significant effect on OS cell proliferation and cell death rate. However, the results of CQ combination could not be attributed to autophagy inhibition, due to the substantial cytotoxic effects that was associated with single CQ treatment. In conclusion, there is strong evidence to suggest advanced OS tumours highly express autophagy to aid in their progression and metastatic potential. Chemotherapy can significantly induce autophagy in OS cell lines, with more profound effect seen on the highly metastatic HOS-143B cells. Therefore, chemotherapy-induced autophagy is predominantly a survival strategy in metastatic OS, although further studies are required to understand the underlying mechanism.

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Table of contents

1. Introduction

1.1 What is osteosarcoma?

Osteosarcoma (OS) is the second most common type of primary bone tumour to affect children and adolescents (He, Ni and Huang, 2014). It is characterized by malignant osteoid (immature bone) that can be subdivided into five main types: osteoblastic, chondroblastic, fibroblastic, telangiectatic and small cell carcinoma (Klein and Siegal, 2006). These are the most aggressive forms of OS, accounting for 90% of all metastatic cases. The most common sites of metastasis seen in OS patients include the lungs and other long bones. The other 10% of cases refer to periosteal OS, a less aggressive form of this disease (Klein and Siegal, 2006).

OS predominantly affects people aged 10-25, with a greater prevalence seen in males (Longhi *et al*., 2006). Statistically, OS is a rare disorder amongst the population, with an incidence rate of 3 per every million people being affected (BRCT, 2017). In the UK, approximately 160 people are diagnosed with OS each year (BRCT, 2017). According to WHO (2014), OS accounts for about 5% of all paediatric malignancies worldwide. Although OS is a rare condition, there are considerable challenges in the management of a particular subset of OS patient tumours, mostly metastatic conditions, which do not respond well to current treatment strategies.

1.2 Current OS treatment strategy

Treatment consists of combining surgical resection and chemotherapy treatment (Ferguson and Goorin, 2001). Usually, patients will be administered a round of neo-adjuvant chemotherapy (before surgery) to reduce the overall mass of the tumour, facilitating its removal. Subsequently, adjuvant chemotherapy (after surgery) is also given to remove any remaining OS cancer cells in the patient's system, decreasing the chance of relapse and increasing prognostic outcome. Standard chemotherapy drugs used in OS patient's treatment include methotrexate, doxorubicin, cisplatin, ifosfamide and etoposide (Bacci *et al*., 2005). The use of neoadjuvant and adjuvant chemotherapy in OS treatment has significantly improved the 5-year survival rate of OS patients from 20% to almost 70% (He, Ni and Huang, 2014; Luetke *et al*., 2014; Bacci *et al*., 2005). However, this same improvement in not seen in metastatic patients, which is a major challenge in treatment of OS tumours.

1.3 The problem: Tumour Resistance to Chemotherapy

Survival rates of metastatic OS patients have plateaued over the past 30 years, and patients with recurrent tumours have less than 30% chance of long-term survival, as shown in figure 1 (Sakamoto and Iwamoto, 2008; Huang *et al*. 2012; Huang *et al*. 2012; Allison *et al*., 2012). A major reason for this phenomenon is the occurrence of chemotherapy resistance, which limits the effectiveness of current drugs (Sui *et al*., 2013; He, Ni and Huang, 201; Hu *et al*., 2016). To overcome this issue, a major focus in current research is centred on investigating the underlying molecular mechanism that govern OS chemoresistance. This can allow for new therapeutics to be developed that provide greater drug efficacy and ultimately lead to better survival rates in metastatic OS patient.

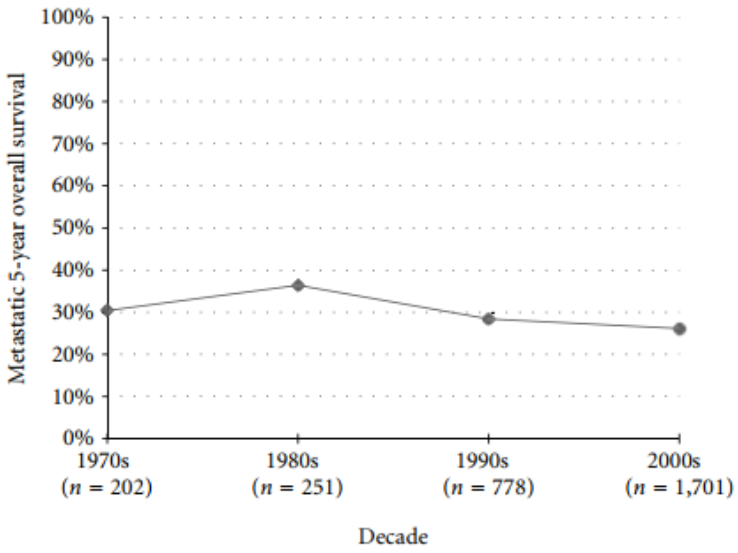


Figure 1. Meta-analysis data showing the 5-year survival rate of patients with metastatic OS (Allison *et al*., 2012).

1.4 *Mechanism of tumour resistance in OS*

Research has shown that many distinct mechanisms can be associated with chemoresistance in OS (He *et al*., 2014). These include decreased intracellular accumulation of drugs by reduced folate carrier (RFC) (Hattinger *et al*., 2003), increased P-glycoprotein (P-gp) expression (Pakos and Ioannidis, 2003) and human glutathione S-transferase P1 (GSTP1) mediated drug inactivation (Townsend and Tew, 2003). Notably, studies found that methotrexate resistance in OS correlated with decrease expression of RFC, a membrane bound transport protein that allows methotrexate to enter OS cells (Guo *et al*., 1999; Patino-Garcia *et al*., 2009; Ifergan *et al*., 2003, Flintoff, *et al*., 2004). Additionally, increased expression of P-gp, which is a

membrane-bound protein pump that removes drugs from the intracellular space (Weinstein *et al*., 1990), has been shown to play a role in doxorubicin resistance in OS cells (Bramwell, 2000; Park *et al*., 2001; Gomes *et al*., 2006). Furthermore, overexpression of GSTP1, a major phase II detoxification enzyme, has been shown to deactivate doxorubicin and cisplatin and contribute towards chemoresistance in HOS and SAOS-2 cells lines (Huang *et al*., 2007). However, chemoresistance in OS is still poorly defined, since the exact mechanism has not yet been established. Within these mechanisms, autophagy has been recently noted as a cellular pathway that can attribute chemoresistance in many cancers, including colorectal cancer (Li *et al*., 2010; Yang *et al*., 2010), leukaemia (Zhao *et al*., 2011), lung cancer (Han *et al*., 2011) and OS (O'Farrill and Gordon, 2014).

1.5 *What is autophagy?*

Macroautophagy (a major subtype of autophagy) is a tightly regulated and conserved catabolic pathway in eukaryotes that degrades intracellular macromolecules and whole organelles (Degenhardt *et al*., 2006). This system maintains intrinsic homeostasis by removing long-lived cytoplasmic components and recycling the resulting metabolic products for other basal functions such as energy production, growth and differentiation (Cecconi and Levine, 2008). Autophagy can be further subdivided into microautophagy and chaperone-mediated autophagy (CMA) [Boya and Codogno, 2012]. While macroautophagy consists of packaging cellular components in a double membrane vacuole (autophagosome) for lysosomal breakdown, microautophagy refers to a direct invagination of the cytoplasmic cargo by the lysosome (Li, Li and Bao, 2011; Boya and Codogno, 2012). Generally, the degraded cargo is non-specific, however, certain stimuli can selectively target specific cargo to be degraded, and the process is named based on this. For instance, mitophagy and ribophagy refer to specific elimination of mitochondria and ribosomes, respectively (Klionsky, 2005). Targeting cytoplasmic cargo can be done with ubiquitination, which allows binding of autophagy receptors, such as p62, for specific degradation (Weidberg, Shvets and Elazar, 2011). CMA refers to selective autophagy of single cytoplasmic proteins by means of recognition between the lysosomal receptor (Lamp-2A) with a chaperone complex (Hsc70 chaperone and KFERQ motif) that translocate the targeted protein across the lysosomal membrane (Boya and Codogno, 2012). Macroautophagy will be the main pathway of interest for this study and will hereafter be referred to as autophagy.

1.6 *Basal Autophagy and induced-autophagy*

Basal autophagy is essential to all cells and tissues, since it serves as a housekeeper to maintain quality control of altered proteins and organelles, reduce the accumulation of components that induce endoplasmic reticulum (ER) stress and reactive oxygen species, maintaining overall tissue homeostasis (Hara *et al*., 2006; Komatsu *et al*.,2006). Conversely, induced-autophagy is triggered under certain stress stimuli, such as hypoxia, nutrient starvation, growth factor deprivation, pathogens and chemotherapy (Klionsky, 2000; Mizushima 2005). When this occurs, intracellular components are recycled to generate ATP and nutrients that are important to sustain cell survival (Mizushima and Komatsu, 2011). Therefore, autophagy is a crucial survival mechanism that cells employ when encountering unfavourable environments.

1.7 *Molecular basis of autophagy pathway*

The process of autophagy can be divided into four main phases: initiation and nucleation, elongation, fusion and recycling (Figure 2). In recent years, extensive research has been conducted to elucidate the mechanism of autophagy and investigating the key mediators that act in different stages. Our understanding of this pathway took a breakthrough with studies of autophagy in yeast cells (*Saccharomyces cerevisiae*), that allowed the discovery of over 30 well-conserved autophagy-related genes (Atg) (Frake and Rubinsztein, 2016). Since autophagy is conserved through evolution, these findings could be reflected in human physiology by characterizing the respective orthologues in mammalian cells (Yang and Klionsky, 2010). Table 1 summarises the main Atg proteins that act in the autophagy pathway.

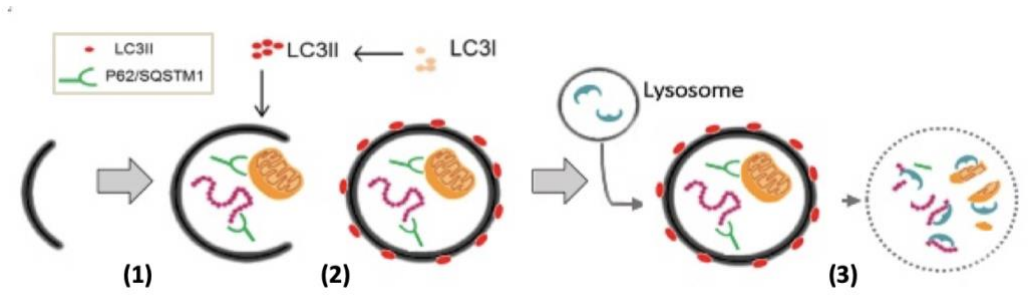


Figure 2. Overview of the process of induced-autophagy

Once a stress stimulus has been issued (e.g. nutrient deprivation), autophagy begins with the formation of an isolation membrane (also called a phagophore) from a preautophagosomal structure (PAS) (1). The PAS can originate from various organelles, including the endoplasmic reticulum (ER), mitochondria, mitochondria-associated membranes, Golgi, plasma membrane or recycling endosomes (Lamb, Yoshimori and Tooze, 2013). ER-mitochondria sites that contain DFCP1, known as omegasome, are the most common site of origin (Axe *et al*. 2008; Galluzzi *et al*. 2017). Subsequently, the isolation membrane expands around the cytoplasmic cargo to form an enclosed double membrane vacuole, known as a autophagosome (2). Then, the autophagosome fuses with a lysosome to form a complex, called autolysosome. Through hydrolase activity, the enclosed cytoplasmic components are degraded and recycled (3).

1.7.1 Initiation

Two main kinase complexes act to initiate the formation of the isolation membrane: ULK complex (1) and Class III phosphatidylinositol 3-kinase (PI3K) complex (2) [Figure 3]. The assembly of the ULK kinase complex, composed of ULK1 and ULK2, FIP200, Atg13 and Atg101 (Papinski et al, 2014; Stanley et al, 2014; Joachim et al, 2015; Karanasios et al, 2016), translocate to the site of PAS formation and recruits the activity of the Class III PI3K complex, composed of Beclin 1, Atg14, VPS34, p150 and AMBRA1 (Matsunaga et al, 2009; Zhong et al, 2009; Fan et al, 2011). In induced-autophagy, this initial step is controlled by two main regulators: the mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) (Nazio *et al*, 2013). Under normal conditions, the mTORC1 is bound to the ULK complex and renders it inactive (Kim *et al*., 2011, Jung

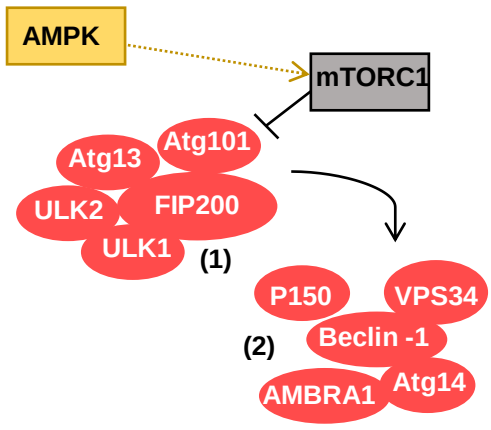


Figure 3. Regulation of ULK1 complex (1) and Class III PI3K complex (2) by mTORC1 and AMPK.

et al, 2009; Nicklin *et al*, 2009). Under conditions of cellular stress, mTORC1 is inactivated and dissociates from the ULK complex, allowing for increased ULK1 and ULK2 kinase activity and thus induction of autophagy (Lamb, Yoshimori and Tooze, 2013). Conversely, AMPK is a positive regulator that acts to promote autophagy by inactivating mTORC1 (Lee *et al*, 2010; Egan *et al*, 2011; Kim *et al*, 2011). The role of AMPK is particularly evident in changes of energy status, sensing the ratio of AMP to ATP ratio in the cell. In the case of decreased ATP level and consequent AMP accumulation, AMPK phosphorylates the TSC-TBC complex or RAPTOR subunit in mTORC1, resulting in its inactivation, and thus induction of autophagy (Lamb, Yoshimori and Tooze, 2013). However, not all stimuli activate autophagy through the mTORC1 pathway. For instance, hypoxic stimuli induce autophagy through hypoxia-inducible factor (HIF) mechanism, a mTORC1 independent-pathway (Russel *et al*., 2013; Lamb, Yoshimori and Tooze, 2013).

1.7.2 Nucleation

The dissociation of mTORC1 allows for increased activity of ULK1 and ULK2, which ultimately enhances the activity of the Class III PI3K complex by phosphorylating Beclin-1 and recruiting VPS34 (Russel, 2013). The Beclin-1/VPS34 domain in the Class III PI3K complex phosphorylate phosphatidylinositol phosphate (PIP2) contained in the membrane of the PAS to phosphatidylinositol-3 (PIP3) (Mizushima *et al*., 2011). During this process, the PAS structure detaches from the site (e.g. endoplasmic reticulum) to form the isolation membrane (Shimizu, Yoshida, Arakawa, 2014). The PIP3 contained in the isolation membrane is important for recruitment of other Atg proteins and PIP3 specific effectors (DFCP1 and WIPI) that drive the nucleation of the membrane (Proikas-Cezanne *et al*, 2015).

1.7.3 Elongation

Elongation of the isolation membrane into a mature autophagosome requires two important conjugation cascades: Atg12-Atg5-Atg16 conjugation system and microtubule-associated protein light chain 3 (LC3) processing (Figures 4 and 5). Atg5-Atg12-Atg16 complex is essential in LC3-II formation, since it determines the site of

Table 1 Comparison of main ATG proteins in yeast and mammalian cells

ATG protein (*S. cerevisiae*)	**Mammalian Homolog**	**Functional component**
Atg1	ULK1 and ULK2	ULK complex
Atg13	Atg13	
Atg17	FIP200	
Atg101	Atg101	
Atg 6	Beclin 1	PI3K complex
Vps34	VPS34	
Vps15	p150	
Atg14	Atg14L	
Atg18	WIPI1 and WIPI2	PIP3-binding protein
Atg3	Atg3	LC3 processing
Atg4	Atg4	
Atg8	LC3-A, LC3-B, LC3-C, GATE16, GABARAPL1, GABARAPL2 AND GABARAPL3	
Atg7	Atg7	LC3 processing and Atg12 conjugation
Atg5	Atg5	Atg5-Atg12 conjugation
Atg10	Atg10	
Atg12	Atg12	
Atg16	Atg16L1	
Atg9	Atg9A and Atg9B	Integral proteins
Atg2	Atg2A and Atg2B	Omegasome

LC3 lipidation (Fujita *et al*, 2008). LC3-II plays a vital role in fusion of the autophagosome with the lysosome and enables docking of specific cargo by binding to several autophagy receptors (Kabeya *et al*, 2000; Stolz *et al*, 2014; Wild *et al*, 2014), including p62/ sequestosome 1 (SQSTM1), which guide misfolded protein aggregates and cytoplasmic materials to the isolation membrane (Fujita *et al*., 2008).

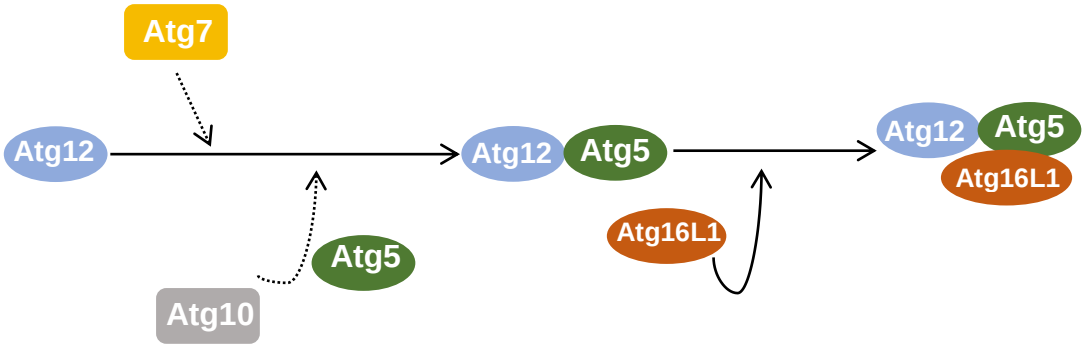


Figure 4. Atg12 conjugation. Atg 7 activates Atg12 in an ATP-dependent manner, which is then covalently linked to Atg 5 by Atg10. The conjugated Atg12-Atg5 is further cleaved with Atg16L1 to form a complex that associates with the isolation membrane (Glick, Barth and Macleod, 2010).

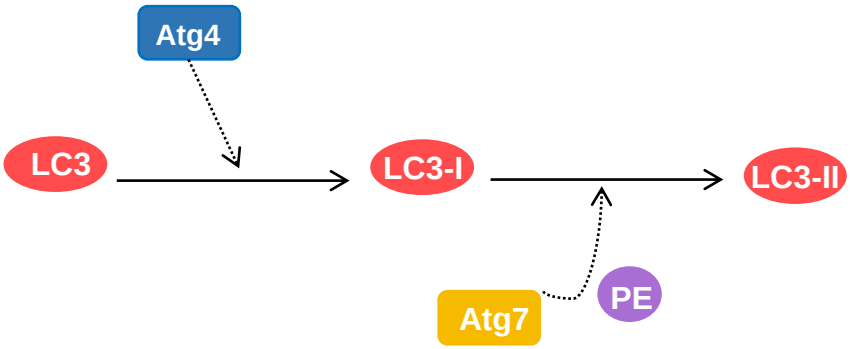


Figure 5. LC3 processing. In LC3 processing, LC3 found in the cytosol is proteolytically cleaved by Atg4 to make LC3-I. Atg7 then activates LC3-I and conjugates phosphatidylethanolamine (PE), found in the isolation membrane, to generate LC3-II (Glick, Barth and Macleod, 2010).

1.7.4 Fusion, degradation and recycling

When the autophagosome is fully formed, the next steps involve fusion of the mature autophagosome with the lysosome, through the action of receptors such as SNAREs (Fader *et al*., 2009; Furuta *et al*., 2010) and UVRAG (Liang *et al*., 2008), producing an autolysosome (Boya and Cogodno, 2012). Before fusion occurs, both structures move closer together and become tethered (Yu, Chen and Tooze, 2017). Evidence has suggested that prior to autolysosome formation, endosomes containing further cytoplasmic cargo can fuse with the autophagosome (Glick *et al*. 2010; Yang and Klionsky, 2010). Subsequently, the autophagic cargo contained in the autolysosome

is degraded by the action of hydrolases and the resulting metabolic products are recycled for other metabolic or cellular growth needs (Yang *et al*., 2006).

1.8 Autophagic Cell Death: Guardian or executioner?

In contrast to the pro-survival role of autophagy in cells, 'autophagic cell death' is a distinct cell death pathway from apoptosis and necrosis (Boya and Codogno, 2012; Kroemer and Levine, 2008). It is characterised by the absence of chromatin condensation and a large-scale accumulation of autophagosomes (occupy more than half of the cytosol) in dying cells, in which the high degree of self-degradation leads to cell death (Kroemer and Levine, 2008; Li, Li and Bao, 2011). This process is more commonly seen in cells with deficiency or a non-functional apoptotic mechanism. For instance, studies have shown that the absence of pro-apoptotic proteins Bax and Bak can induce autophagic cell death (Shimizu *et al*., 2004; Yu *et al*., 2004). However, many controversies are seen in research, as the exact connection between autophagy's ability to induce cell death is not well defined. Arguments hold that the presence of high numbers of autophagosomes in a dying cell does not necessarily imply a causal effect, since autophagy might simply be a pro-survival attempt in dying cells to prevent cellular demise (Liu and Levine, 2014). Recently, the Nomenclature Committee of Cell Death has proposed the use of the term autophagic cell death when the occurrence of cell death is supressed with autophagy inhibition (Choi, 2012). Yonekawa and Thorburn (2013) suggest that to define if cells are undergoing autophagic cell death the inhibition of autophagy or genetic ablation of this mechanism would result in the prevention of cellular death.

1.9 Autophagy and Cancer

Studies have noted that dysregulation of autophagy in different cell types is associated with the aetiology of many diseases, including cancer (White, 2015; Boya and Codogno, 2012; Mathew *et al*., 2007). However, research emphasises that the effects of autophagy in promoting tumorigenesis is highly context dependent, since autophagy can act as both a tumour suppressor and promoter (White, 2012; Marino and Lopez-Otin, 2004; Boya and Codogno, 2012).

1.9.1 Autophagy as a tumour suppressor

By preserving tissue homeostasis and preventing toxic build-up of intracellular waste, autophagy can reduce oxidative stress that would otherwise promote malignant transformation (Manic *et al*., 2014; Choi, 2012). Mathew *et al*. (2009) elaborates that the accumulation of p62/SQSTM1 aggregates, damaged mitochondria and misfolded proteins can cause the production of reactive oxygen species, which damage DNA and lead to chromatin instability. Therefore, defects in the autophagy pathway can predispose cells to oncogenic mutations. For instance, monoallelic loss of Beclin-1 expression has been frequently seen in breast, ovarian and prostate cancer (Choi *et al*., 2013) and mutations in Atg5 and Atg12 have been associated with colorectal neoplasms (Kang *et al*., 2009).

1.9.2 Autophagy as a tumour promoter

Conversely, cancer cells can upregulate autophagy as a pro-survival mechanism to cope with cytotoxic agents in their microenvironment (Chang *et al*., 2014). When this occurs, upregulation of autophagy can act as a protective mechanism by using aberrant cytoplasmic content to sustain energy yields and keep cancer cell integrity (Sui *et al*., 2013). Hence, autophagy can attribute a more chemoresistant phenotype in cancer cells against certain drug treatments (Kondo *et al*., 2005; Janku *et al*., 2011). On the contrary, autophagy overexpression can cause cancer cells to undergo autophagic cell death, particularly in tumours with non-functional apoptotic machinery (Yang *et al*., 2011). However, most research indicates that autophagy primarily aids in providing many different cancer cells with chemotherapy resistance (Levy and Thorburn, 2011; Maycotte and Thorburn, 2011; Xu *et al*., 2015). It is thought that cancer cells use autophagy to produce energy in the unfavourable metastatic environments (Kimura et al., 2012).

1.10 The role of autophagy in OS

To date, the role of autophagy in OS is still very complex and poorly understood. Whilst some studies suggest a pro-survival role (Huang *et al*., 2011; Shen *et al*., 2013; Li *et al*., 2016; Wang *et al*., 2018) others suggest a pro-death role (Voss *et al*., 2010; Salazar *et al*., 2009; Yamamoto *et al*. 2008). In the context of chemoresistance, autophagy acts as a pro-tumorigenic mechanism by enhancing tumour cells capacity to cope with

drug-induced cytotoxic stress (Li *et al*., 2016). A number of reports have shown how reducing the expression of autophagy related proteins could effectively obliterate the autophagy pathway in OS cell models and cause increased cytotoxic effects of chemotherapy (He *et al*., 2013; Chang *et al*., 2014; Wu *et al*., 2014; Mukherjee *et al*., 2017). A study by Wu *et al*. (2014) showed how reduced expression of autophagy regulator Beclin-1 in cisplatin resistant MG-63 cell lines resulted in a significant reduction of autophagy and increased drug sensitivity. Another study by Mukherjee *et al*. (2017) showed that cisplatin resistant HOS cell lines showed upregulation of autophagy upon cisplatin shock and downregulation of this pathway could increase cisplatin-induced cell death. He *et a*l, (2013) discovered that autophagy induced by HMGB1 is a dominant mechanism of resistance to standard OS chemotherapy, namely doxorubicin, cisplatin and methotrexate. A study by Chang *et al*. (2014) and Li *et a*l. (2014) showed that HMGB1 upregulation induced autophagy and contributed towards resistance to doxorubicin and cisplatin *in vitro*. Huang *et al*. (2012) explains that HMGB1 is a critical regulator in the formation of Beclin-1/Class III PI3K complex, which is important in elongation of the isolation membrane. Other studies have evidenced how induction of autophagy promotes OS cell survival and tumour resistance to most standard chemotherapy drugs (Meschini *et al*., 2007; Lambert *et al*., 2008; Zhang *et al*., 2009; Pan *et al*., 2010). Additionally, it has been shown in *in vivo* mouse models that Gemcitabine (GCB) treatment can induce autophagy, which was associated with GCB resistance in LM7 metastatic OS cells (Gordon *et al*., 2010).

In contrast to these findings, several agents have been shown to trigger increase of cell death in OS cells by mediating autophagic cell death (Meschini *et al*., 2008). A study by Meschini *et al*., (2008) showed how voacoamine (VOA) induced autophagic cell death in U2-OS cells and combining with doxorubicin could revert the multi-drug resistance seen in this cell line. They also showed how pharmacological or genetic inhibition of autophagy was associated with reduced ability of VOA to induce autophagic cell death and increase cytotoxicity mediated by doxorubicin treatment. Another study by Dhule *et al*. (2011) showed how DMSO-curcumin induced death by autophagy and was independent of apoptosis. Although these agents are not considered standard therapies in OS treatment, autophagy modulation with such agents could potentially serve an important step in therapy design. Additionally, it has been shown that chemotherapy agents' doxorubicin and cisplatin can induce autophagy mediated cell death in other cancer types (Zhang *et al*., 2016; Chen *et al*., 2018).

In summary, although current evidence indicates that chemotherapy mostly induces chemoresistance in OS, more precise research showing the connection between autophagy and resistance to standard therapies is required. The dual role of autophagy creates a confusing paradigm in modulation of autophagy for therapeutic use. It is not clear which method can attribute the most therapeutic benefit, whether to inhibit or enhance autophagy. Therefore, it is necessary to investigate the prime role of chemotherapy-induced autophagy in OS in order to develop a better treatment strategy for recurrent OS patients.

1. 11 Targeting Autophagy in OS Therapy

Targeting autophagy, whether to enhance or inhibit this pathway, can be an effective therapeutic approach in OS therapy. Many studies have proposed the use of a range of pharmacological inhibitors to promote chemosensitivity and enhance cytotoxic effects of chemotherapy in OS, including bafilomycin A1, 3-methyladenine and pepstain A (Kimura *et al*., 2013). Evidently, two antimalarial drugs, chloroquine (CQ) and hydrochloroquine (HCQ), can act as lysosomotropic agents that increase lysosomal pH and prevent autophagosome fusion (White and DiPaola, 2009). Ongoing phase I and II clinical trials are being conducted on CQ derivatives to be used alone and in combination with conventional chemotherapy in many different cancers (Cynthia and Amaravadi, 2017). In contrast, certain dietary phytochemicals, such as resveratrol, have shown to possess therapeutic effects in cancer cells by inducing autophagy (Park *et al*., 2015). Resveratrol is a natural phenol compound produced by several plants and can be found in dietary foods, such as wine (Angulo *et al*., 2017). A recent study has shown that resveratrol can inhibit mTOR activation by binding to the ATP-binding site of mTOR (Park *et al*., 2015). Additionally, a study by Zhang *et al*., 2015 revealed that the use of flavonoid luteolin, a polyphenolic compound that upregulates Beclin- 1 expression, caused increased autophagy in U2OS cell lines treated with doxorubicin, which ultimately lead to cell death. The inhibition or enhancement of autophagy provide a novel approach in therapy to OS patients.

1.12. Aim

This study aims to explore the role of autophagy in metastatic OS disease progression and investigate the functional role of autophagy in chemoresistance in two OS cell lines of varying metastatic capacity.

1.13 Translation into patient care

At present, the role of autophagy in chemoresistance OS has not been clarified. Therefore, the project envisions to enhance our current understanding of autophagy and the role it plays in chemoresistant OS. Additionally, these findings will contribute towards circumventing the issue of chemoresistance in OS and could lead to new potential treatment strategies and predictive markers to developed, conceptualizing the idea of personalised medicine.

2. Methods

2.1 Immunohistochemistry of OS tissue microarray

Human OS tissue microarrays (AMS Biotechnology, T261 and OS804c) were probed for LC3 expression. Sections were deparaffinised in xylene and rehydrated through graded ethanol. Then, antigens were retrieved with sodium citrate buffer in a microwave set between 95°-98°C for 10 min. Slides were washed three times with dH_2O and submerged in 3% hydrogen peroxide for 10min to quench endogenous peroxidase. Section were blocked in blocking buffer (5% Normal Goat Serum in 1X TBST) for 1h. Primary antibody LC3 A/B (D11) XP Rabbit mAb (#3868, Cell Signalling) diluted in SignalStain Antibody Diluent (#8112, Cell Signalling) was added and slides were left overnight in a humidified chamber at 4°C. Next day, slides were washed three times in 1X TBST and then incubated with SignalStain Boost Detection Reagent (#8114, Cell Signalling) for 45min in a humidified chamber at room temperature (RT). Slides were further washed three times and chromogenic reaction was performed using SignalStain DAB Substrate Kit (8059P, Cell Signalling). Slides were counterstained with haematoxylin. Images were obtained using Eclipse 50i (Nikon). Stain Intensity was quantified using Fiji software. For this study, an area fraction method was used to calculate LC3 expression in the OS histological samples. This calculates the % area of intense signal obtained from DAB chromogen and normalizes to the overall area of the biopsy tissue (quantification was performed at 40x magnification).

2.2 Cell lines, culture and drug reagents

HOS-143B (ATCC, CRL) and MG-63 (ATCC, CRL-1427) cell lines were grown in 1X Dulbecco modified Eagle medium (DMEM) + GlutaMAX (#61965026, Thermofisher) supplemented with 10% fetal bovine serum (#16000044, Thermofisher), 1% non-essential amino acids (#11140035, Thermofisher), 1% sodium pyruvate (#11360070, Thermofisher) and 1% penicillin-streptomycin (#15140122, Thermofisher,). HOS-143B medium was supplemented with additional 0.2% 5-bromo-2'-deoxyuridine (BrdU). Cells were grown in humidified chamber with 5% CO_2 at 37°C. Doxorubicin (#5927, Cell Signalling) was dissolved in DMSO (#D2650, Sigma) to make a 10mM Stock. Cisplatin (#C2210000, Sigma) was dissolved in Saline solution to make a 2mM stock. Chloroquine diphosphate salt (#C6628, Sigma) was dissolved in ddH_2O to make a 50mM stock. Rapamycin (#37094, Sigma) was dissolved in 100% ethanol to make a

55mM stock. BrdU (#5911439, BioGems) was dissolved in DMEM to make a 7.5mg/mL stock.

2.3 Cytotoxicity assay

1×10^4 cells were seeded in flat-bottomed 96 well plates and left overnight to attach. Cells were treated in triplicates with serial dilutions of both Dox and Cis to reach a final volume of 200μl/well. For assays containing CQ inhibitor, cells were pre-incubated with CQ for 1h before chemotherapy treatment. Plates were incubated at 48h, 72h, 96h and 120h and kept at 37°C with 5% CO_2. Cell viability was measured using acid phosphatase assay (Stordal *et al*., 2012). Plates were read on FLUOstar Omega microplate reader (BMG Labtech). Dose-response curve was calculated relative to drug free control and graphs were plotted using GraphPad (Prism) software.

2.4 Quantitative Real-time- PCR

20×10^4 cells were seeded on a 6 well plate and allowed to attach overnight. The next day, cells were drug treated and left to grow for an additional 48H. To purify RNA, cells were washed twice with PBS, scrapped and suspended in RNA lysis buffer containing 1% 2-mercaptoethanol. Then, RNA extraction was performed using PureLink RNA Mini Kit (Ambion) following the instructions set by the manufacture. Extracted RNA was quantified using the Nanodrop 2000 spectrophotometer. Samples were diluted to lowest RNA concentration. Total RNA was reverse transcribed to cDNA using a superscript IV Reserve transcriptase kit (Invitrogen) following the manufactures instructions. For quantitative RT-PCR, each reaction mix (10μL final volume) consisted of 2.5μL of 10ng cDNA, 5μLSYBR Green qPCR Master Mix (ThermoFisher), 1μL each of 200nM forward and reverse primer and 1.5μL or nuclease free water. Primer sequences were as follows: p62/ SQSTM1 forward, 5'-GACAATGGCCATGTCCTACG-3', reverse, 5'-GCACTTGTAGCGGGTTCCTA -3'; MAP1LC3B, forward 5'-TTGAGCTGTAAGCGCCTTCTA-3', reverse, 5'-GATGTCCGACTTATTCGAGAGC-3'. GAPDH acted as housekeeping gene: forward, 5'- CCTGCACCACCAACTGCTTA-3', reverse, 5'-GGCCATCCACAGTCTTCTGAG -3'. Samples were plated in triplicates on a 96 well-plate. No Template Control was also incorporated in each assay. Reactions were performed on Light Cycler 96 (Roche) thermocycler with a program set with 95°C for 10min initial denaturation, followed by 10s at 95°C (denaturation) and 60°C (annealing), and a final elongation step of 10sec

at 72°C, for 40 cycles. Relative gene expression was calculated by normalization to GAPDH using $2^{-\Delta\Delta CT}$ method (three biological replicates). Melting-curve data were then collected to verify PCR specificity, contamination and the absence of primer dimers.

2.5 Western Blotting

20×10^4 cells were seeded on a 6 well plate and allowed to attach overnight. The next day, cells were treated with single and combination of chemotherapy and autophagy modulators and left to grow for an additional 48H. Cells were washed twice with 1X PBS and lysed with 50µl RIPA (Thermo Scientific, PI89900) with protease inhibitor (complete midi, Roche Diagnostics) per $1x10^6$ cells and detached using cell scrapper. Lysates were sonicated at 50% amplitude three times for 2 seconds and centrifuged at 13,000rpm for 5min at 4°C. Supernatant was collected, and protein quantification was determined using a Bradford Protein Assay (Biorad). 40ug of protein was diluted in equal volume of 2x Laemmli loading dye and proteins were denatured at 95ºC for 5 min. Samples were loaded into 4-15% pre-cast polyacrylamide gel (#4561086dc, Biorad) and electrophoresed at 100V and 0.02A for 1h. Then, gels were electrotransfered to a 2µM nitrocellulose membrane using Trans-blot Turbo system (#1704156, Biorad) at 25V and 2.5A for 7 min. Membranes were washed three times with 1x TBST and blocked with 5% BSA (in TBST) for 1h at RT. Membrane was incubated overnight with primary antibody (#3868, LC3 A/B; p62, #5114S, Cell Signalling) diluted in 1:1000 ratio with blocking solution at 4°C. Next day, membrane was washed 3 times for 10 min and incubated for 2h with HRP-linked secondary antibody (7074S, NewEnglandBiolabs) diluted in 1:1000 blocking bluffer. Membranes were further washed, and signal was developed with ECL reagents and visualized on Odyssey® Fc Imaging system (LI-COR). Bands were quantified using ImageJ software. The intensity of band was quantified and normalized using b-actin (Biorad) as an internal control. Three biologically independent experiments were performed.

2.6 Immunofluorescence

1×10^4 cells were seeded in 8-well chamber slide (C7057, Sigma) and left to attach overnight. Cells were drug-treated the following day and incubated for an additional 48H. Then, growth medium was aspired, cells were washed twice with 1X PBS. Ice-cold 100% methanol was used to fix cells for 15 min at -20°C. Cells were further washed three times with 1X PBS for 5min each and blocked in blocking solution (5% Normal Goat Serum in 1 X PBS with 0.3% Triton X-100) for 1h at RT. Blocking solution

was removed and LC3 A/B (D11) XP Rabbit mAb (#3868, Cell Signalling) diluted in dilution buffer (1% BSA in 1X PBS with 0.3% Triton X-100) was added and slides were incubated overnight at 4°C. Then, cells were washed three times with 1X PBS for 5min each. Then, cells were incubated with Alexa Fluor 488 Conjugate secondary antibody (#4412, Cell Signalling) diluted in dilution buffer for 2 hours in the dark at RT. After incubation, cells were washed three times with 1X PBS for 5min each. Slides were then mounted with VECTASHIED Antifade Mouting Medium with DAPI (#H-1200, Vector Labs) stain and coverslip was applied and left to dry. Images were acquired with Confocal Microscopy Leica DMI4000 B (Leica Microsystems). The number of LC3 A/B puncta was quantified using ImageJ software.

2.7 Apoptosis using Annexin V/PI

4×10^4 cells were seeded on a 12 well plate and left to attach overnight. Cells were then drug-treated and incubated for an additional 48h. Apoptosis assay was carried out using Annexin V/Dead Cell Apoptosis Kit (#V13242, ThermoFisher) as described in the manufactures protocol. Briefly, cells were washed with 1X cold PBS and trypsinized. Supernatant was discarded, and cells were washed twice and then resuspended in 100µL binding buffer. Cells were stained with 5µL propidium iodide and Annexin V and left to incubate in the dark for 15 min before adding an extra 400 µL binding buffer. Samples were analysed using FACS Calibur (Biosciences) with CellQuest software. A minimum of 10,000 cells was acquired for each sample.

2.8 Bioinformatic analysis of genome-wide database

Database from R2 Genomics Analysis and Visualization platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) was used to perform bioinformatic analysis. Kaplein Meyer curves were calculated from 84 high-grade OS cohort (GSE42352). MAP1LC3B gene expression data analysis in OS tumour tissue was obtained from two genome-wide cohort studies (GSE14827 and GSE14359) of OS patient biopsies to evaluate metastatic development and tissue specific expression, respectively.

2.9 Statistical Analysis

Minitab software was used for descriptive and comparison statistics. For multiple comparison, ANOVA was performed, while two-sample t-test was used to analyse differences between two groups. $p<0.05$ was considered statically significant. GraphPad Prism 8 was used for graphical presentation of the data.

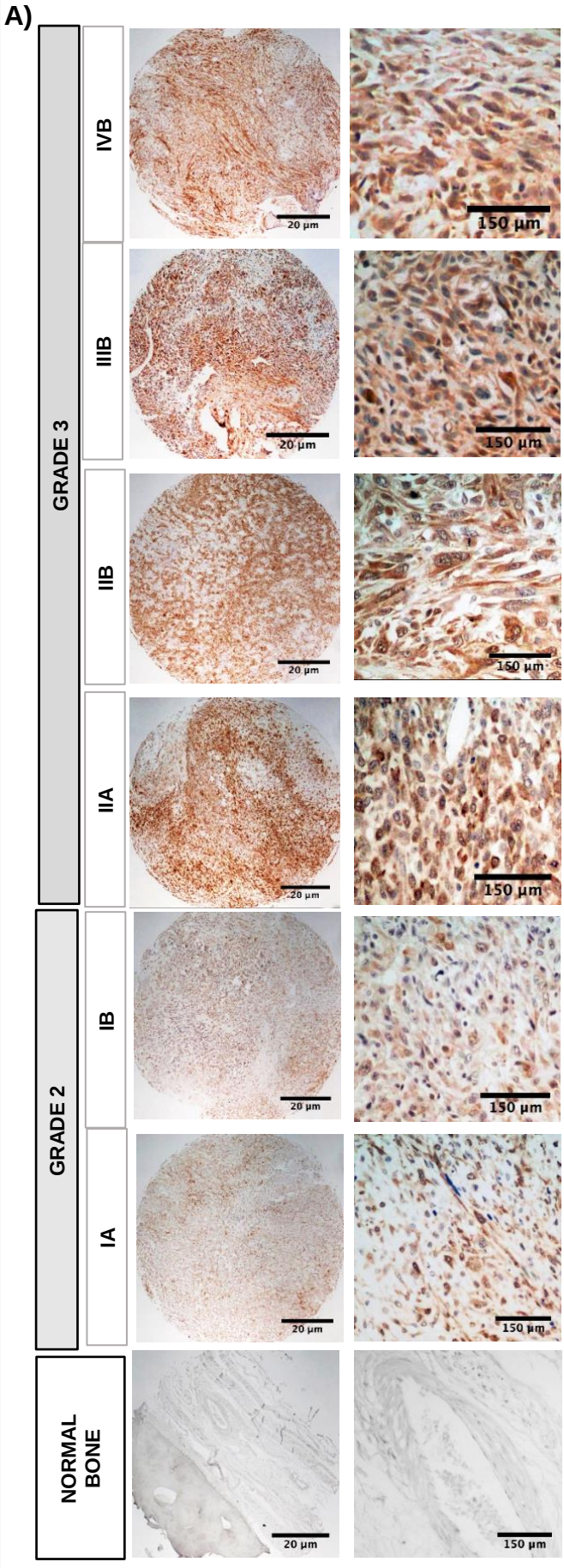
3. Results

3.1 Autophagy Expression in OS tissue

3.1.1 LC3 puncta expression in OS tissue by IHC

A total of 86 core biopsies comprising of OS tissue (41 cases) and normal bone tissue (2 cases) were immunohistochemically probed for LC3 protein expression. Autophagy was based on signal intensity of endogenous LC3-I and LC3-II expression (A/B subtypes). LC3-I is diffused in the cytoplasm and once converted to its lipid form (LC3-II) it integrates into the membrane of the autophagosome (Rosenfeldt *et al*., 2012). Therefore, LC3-II serves as a useful marker for autophagosome formation and reflects autophagy occurrence (Berezowska and Galvan *et al*., 2017). It is important to note that several reports mention how immunohistochemistry (IHC) detection of LC3 cannot distinguish between LC3-I and LC3-II (Guo *et al*., 2011; Shubtaku, 2011; Rosenfeldt *et al*., 2012). This inability to differentiate LC3 in current IHC methodologies limits interpretation of autophagy in human and mouse tissue (Diwan *et al*., 2012). Yet, Sato *et al*. (2007) states that IHC detection of LC3 can still serve as a useful indicator of autophagy activity since high signal detection of LC3 is physiologically abnormal and concomitant with overexpression of LC3-II.

The IHC results from OS biopsies revealed a clear pattern, where LC3 was highly expressed in grade 3 OS tumours compared to grade 2 ($p=0.0071$) and normal bone tissue ($p=0.0053$), as shown in figure 6. By further grouping the results based on stage, it was evident that LC3 was overexpressed in most advanced tumour subtypes (IVB, $p=0.0014$; IIIB, $p=0.0671$; IIB, $p=0.0001$; IIA, $p=0.0697$) compared to normal bone. Less aggressive subtypes (IB, $p=0.0496$; IA, $p=0.6089$) also showed high LC3 expression compared to normal bone, although not to the same extent. Control slides without the addition of primary antibody showed no detectable expression, indicating no false-positive results. Such OS biopsies reflected patients between the age of 13-64, with no significant difference in gender (male and female; $p>0.05$) and tumour location (femur, tibia, humerus, fibula, rib and jaw bone; $p>0.05$). Samples categorised as grade 3 and grade 2 were T2N0M0 and T1N0M0 classifications, indicating no record of nearby lymph node dissemination and metastasis (Bridge *et al*., 2013). The exception is an OS tumour from the lower jaw (Stage IVB), with a classification of T1N1M0 indicating cancer cell dissemination to regional lymph nodes. Collectively, these results suggest a potential link between autophagy expression and the aggressiveness of OS tumours.



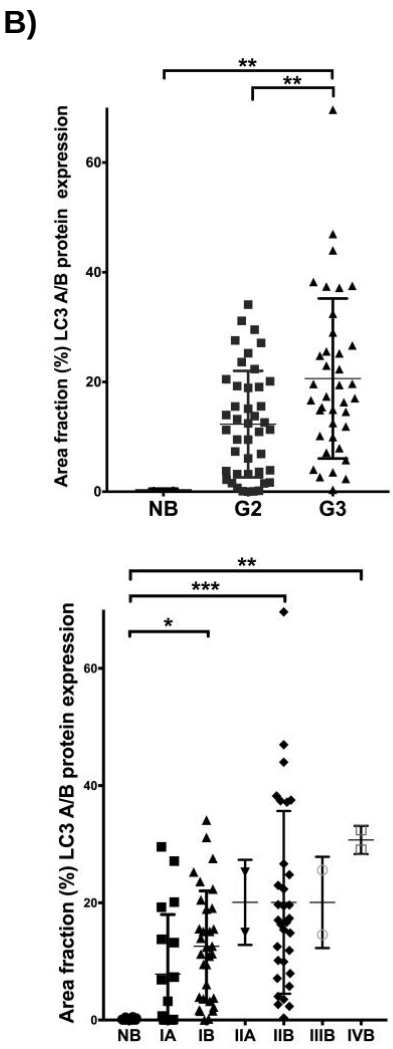


Figure 6. LC3 protein expression of varying grades of OS tissue (A) Immunohistochemical detection of LC3 A/B in primary osteosarcoma tumour tissue of varying grades and stages (n=37) and normal bone tissue (n=4). (B) Quantification analysis of positive LC3 A/B area coverage (%) based on tumour grade (G3, n=38; G2, n=42; NB, n=4) and tumour stage (IVB, n=2; IIIB, n=2; IIB, n=32; IIA, n=2; IB, n=32; IA, n=12; NB, n=4). Data is presented as mean ± SE. ***p<0.001; **p<0.01; *p<0.05. Abbreviation: G3, Grade 3; G2, Grade 2; NB, Normal Bone.

3.1.2 Genome-wide analysis of MAP1LC3B expression in OS

MAP1LC3B (LC3 coding gene) profile analysis from an integrative study of 84 high grade osteosarcoma tumours (GSE42352) revealed a strong link between MAP1LC3B expression and OS patient prognosis. From this cohort, only 53 samples were considered for analysis since the other 31 samples lacked survival data (Kuijjer *et al*., 2012). As shown in figure 7, the patient group with significantly higher MAP1LC3B expression (n=41) were associated with poor disease outcome compared to the low MAP1LC3B expressing group (n=12) [p=0.039]. Additionally, patients with high MAP1LC3B expression had less probability for metastasis free survival (p=0.0095), also shown in figure 7. Furthermore, analysis in another OS tissue database (GSE14827) revealed a strong connection between MAP1LC3B expression and metastatic development. In this group, OS patients who developed lung metastasis (n=7) were shown to have considerably higher expression of MAP1LC3B compared with OS patients' samples who did not develop metastasis (n=12) [p=0.06]. According to the investigators in this study, biopsies were retrieved after surgical resection and neoadjuvant chemotherapy, and profiling was performed before metastatic development (Kobayashi *et al*., 2010). Interestingly, analysis of another OS genome-wide database (GSE14359) shows how lung metastatic OS tissue express higher levels of MAP1LC3B compared to primary OS tissue counterpart (p=0.07).

Together, the results evidence a strong link between autophagy overexpression and the survival outcome and metastatic potential of OS tumours.

3.2 Chemotherapy-induced autophagy

The results from the IHC and bioinformatics suggests that autophagy overexpression can be strongly associated with aggressive OS tumours, including metastatic forms. Therefore, these prompt for further investigations to understand the role of autophagy in OS tumour development. In particular, to understand the functional implication of autophagy in metastatic OS patient chemoresistance, this study focused on investigating the influence of autophagy expression in OS tumour response to standard chemotherapy. To do this, *in vitro* studies using two OS cell models of varying metastatic potential (HOS-143B and MG-63) formed the baseline for such investigations. The purpose of using these aggressively different OS cell lines is to reflect the heterogeneous population in OS tumours, where HOS-143B represents more aggressive tumour types with potential for metastasis (Lauvrak *et al*., 2013). Two

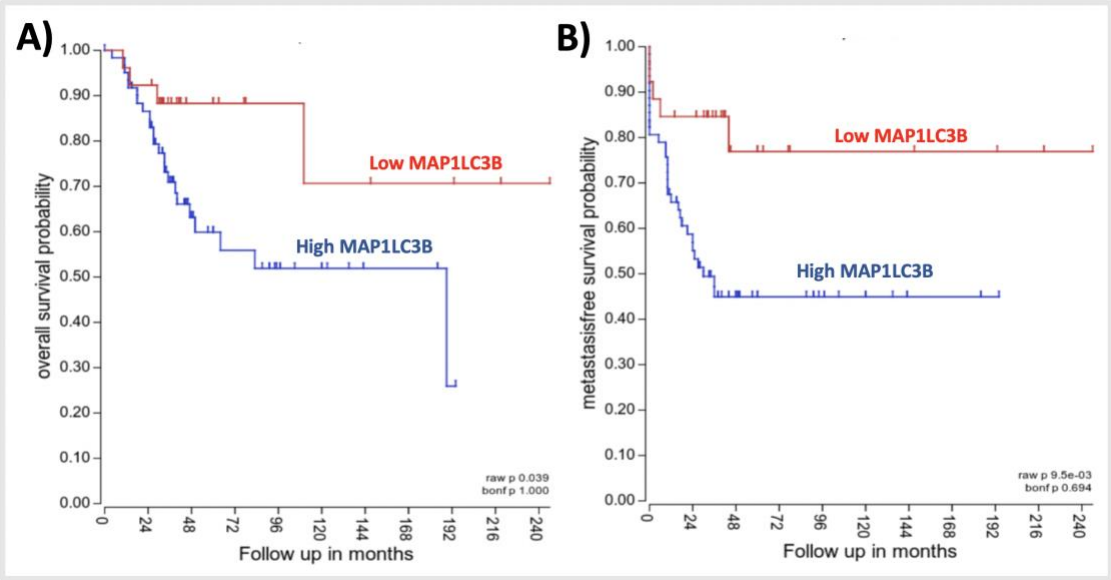


Figure 7. Kaplan-Meier curve of MAP1LC3B gene expression and OS patient survival. Comparison of overall survival (A) and metastasis free survival (B) probability between osteosarcoma patient group (GSE42352) with high (blue, n=62) and low (red, n=26) expressing MAP1LC3B tumours ($p<0.05$ and $p<0.001$, respectively).

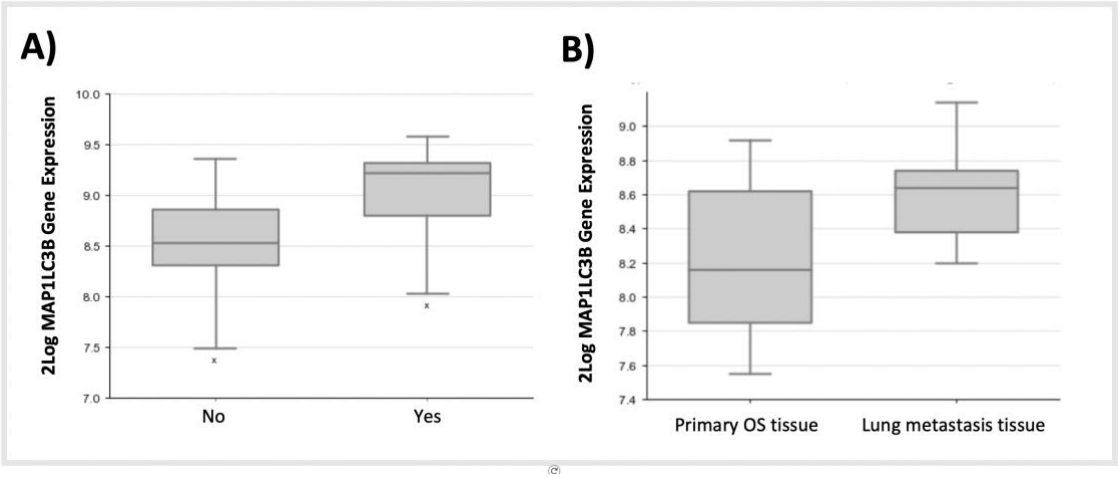


Figure 8. Relationship between MAP1LC3B gene expression and OS metastasis (A) MAP1LC3B gene expression in cohort of patients (GSE14827) that developed lung metastasis (Yes) and patients had no metastatic development (No) [p=0.06]. (B) Comparison of MAP1LC3B expression between primary OS and lung metastatic tumour tissue in OS patient group (GSE14359) [p=0.07].

standard chemotherapies were used in this study, doxorubicin (DOX) and cisplatin (CIS), which reflect current therapeutic drugs treatment used in clinics. Firstly, it was investigated whether chemotherapy treatment would lead to autophagy upregulation in OS cell lines. Further to this, a series of investigations were carried out to effectively delineate if autophagy serves as a mechanistic response to chemotherapeutic stress.

3.2.1 Chemotherapy effects on OS cells viability

Working Dox and Cis concentrations for both OS cell lines were established using cell proliferation assay to use for subsequent experiments. Also, the initial drug resistance profile between the two OS cell lines was established. A cytotoxicity assay was performed at two-time points (48h and 5 days) to illustrate the effect of prolonged drug exposure on cell viability. Figure 9 reveals the dose-response curves and the corresponding IC50 (concentration required to inhibit cellular growth by 50%). For Dox treatment, MG-63 showed a significantly higher IC50 at 48h (2.23 fold increase, $p<0.005$) and 5 days (11.3 fold increase, $p<0.005$) compared to HOS-143B. In Cis-treated cells, MG-63 also showed a significantly higher IC50 value at 48h (1.52 fold increase, $p<0.005$), however, the opposite pattern was seen at 5 days, with HOS-143B having a higher IC50 value (1.73 fold increase, $p<0.005$). In essence, MG-63 shows to have a higher tolerance to Dox and Cis treatment compared to HOS-143B at 48h.

Working concentration of Dox (1µM and 2 µM) and CIS (10µM and 20 µM) was established based on both the obtained cell proliferation results and concentration that have been reported to induce autophagy in other OS cell models (Huang *et al.*, 2011).

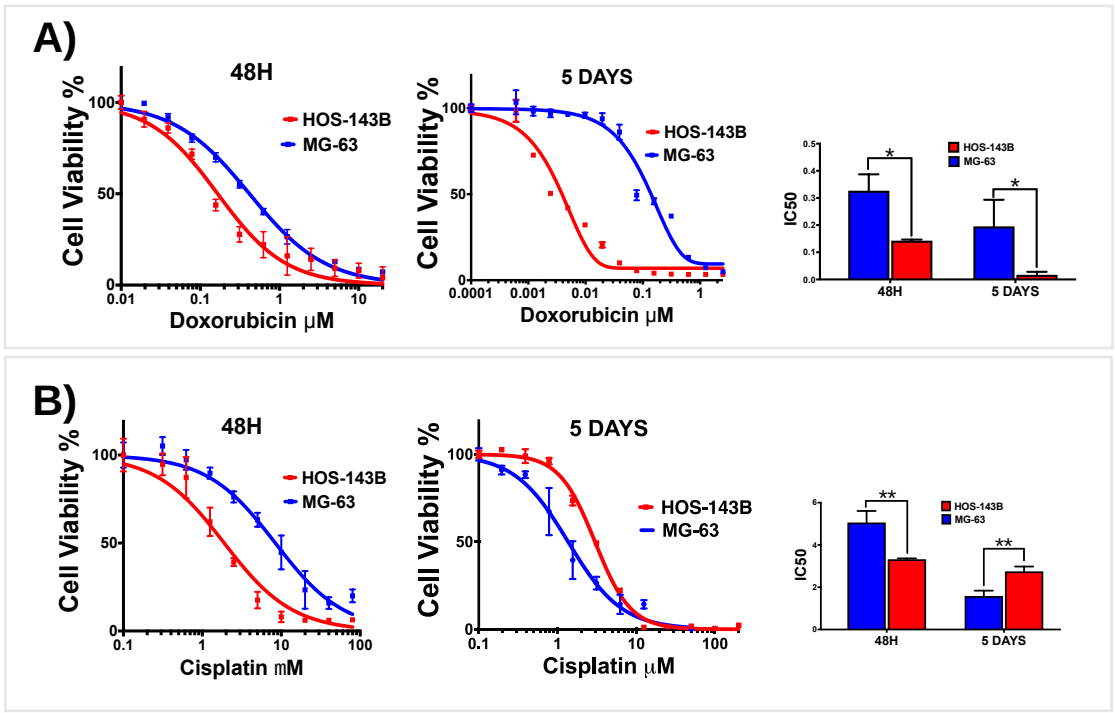


Figure 9. Effects of chemotherapy treatment on OS cell viability. Dose-response curve of HOS-143B and MG-63 treated with doxorubicin (A) and cisplatin (B) at 48H and 5 days. Quantitative comparison of IC50s between OS cell lines at each timepoint. Data is represented as mean ± SE (n=3). ***p<0.001; **p<0.01; *p<0.05. Doxorubicin IC50 mean values, at 48h: MG-63, 0.327µM; HOS-143B, 0.143 µM; at 5days: MG-63, 0.1957µM; HOS-143B, 0.01734 µM. Cisplatin IC50 mean values, at 48h: MG-63, 5.063µM; HOS-143B, 3.323µM; at 5days: MG-63, 1.586µM; HOS-143B, 2.751µM

3.2.2 Chemotherapy increases expression of LC3 puncta in OS cells detected by Immunofluorescence

To investigate the effects of chemotherapy treatment on autophagy induction, drug-treated OS cells were probed for LC3 puncta formation to detect changes in autophagic flux. Additionally, OS cells were treated with autophagy inducer rapamycin (RP) and inhibitor chloroquine (CQ) for comparison. As shown in figure 10, OS cells treated with two different concentrations of each chemotherapy drug led to significant increase in LC3 puncta, which is an index for the number of autophagosomes contained in the cytoplasm. 1μM and 2μM Dox treatment caused significant LC3 puncta formation in MG-63 cells compared to control (8.1 fold increase, $p<0.001$; 5.8 fold increase, $p<0.001$, respectively). However, 10μM and 20μM Cis treatment showed small increase in LC3 puncta compared to control. A similar pattern was seen in HOS-143B, with significant increase in LC3 puncta with 1μM Dox treatment (13.3 fold increase, $p<0.001$), 10μM and 20μM Cis (9.2 fold increase, $p<0.001$; 16.8 fold increase, $p<0.001$, respectively). In both MG-63 and HOS-143B, the autophagy-inducer rapamycin (mTOR inhibitor) caused a significant increase in autophagosome formation (6 fold increase, $p<0.05$; 5.5 fold increase, $p<0.001$, respectively). CQ treatment showed the most significant increase in LC3 puncta in both OS cell lines (13.4 fold increase, $p<0.05$; 22.6 fold increase, $p<0.001$, respectively), which is expected as this lysosome inhibitor causes accumulation of autophagic vacuoles (Mauthe *et al*., 2018). These results indicate that both chemotherapy drugs are able to induce autophagy upregulation, with greater significance seen in the highly metastatic HOS-143B cell line.

3.2.3 Increased expression of autophagy gene and proteins in chemotherapy-treated OS cells

To confirm expression patterns seen in immunofluorescence (IF), protein and gene expression of key autophagic markers was investigated in drug-treated OS cells. In protein analysis, autophagy induction was based on conversion of LC3-I to LC3-II (autophagic flux) and p62/SQSTM1 expression. p62/SQSTM1 is an autophagosome cargo binding protein that forms protein aggregates which can be directed to the autophagosome for degradation (Bjørkøy *et al*., 2005; Komatsu *et al*., 2007). This ubiquitin binding protein can interact with LC3-II (found in the autophagosomal

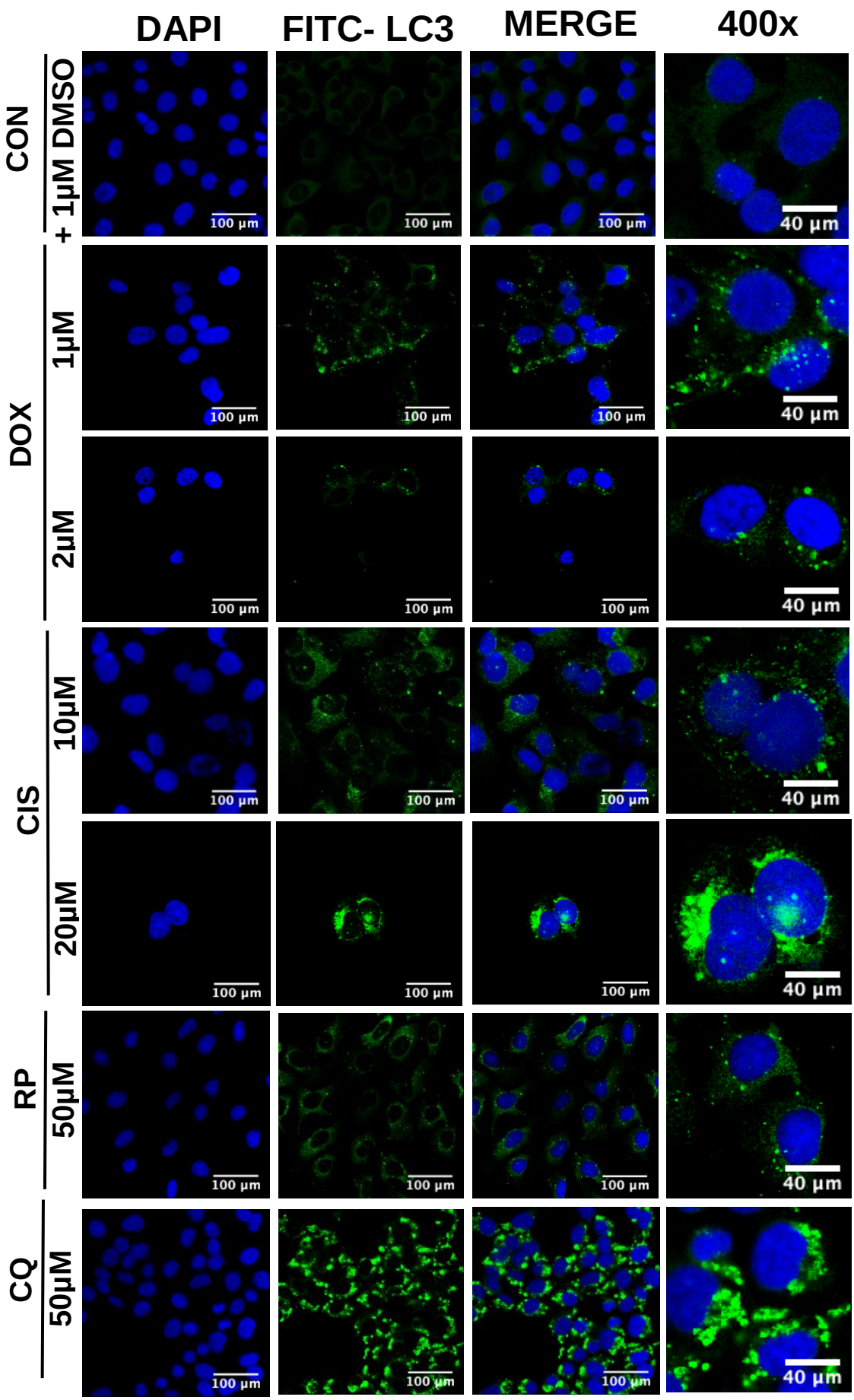


Figure 10A. Confocal imaging of LC3 A/B puncta protein expression in HOS-143B cells treated with chemotherapy and autophagy modulator for 48h. Samples: CON, 1μM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; RP, Rapamycin; CQ, Chloroquine.

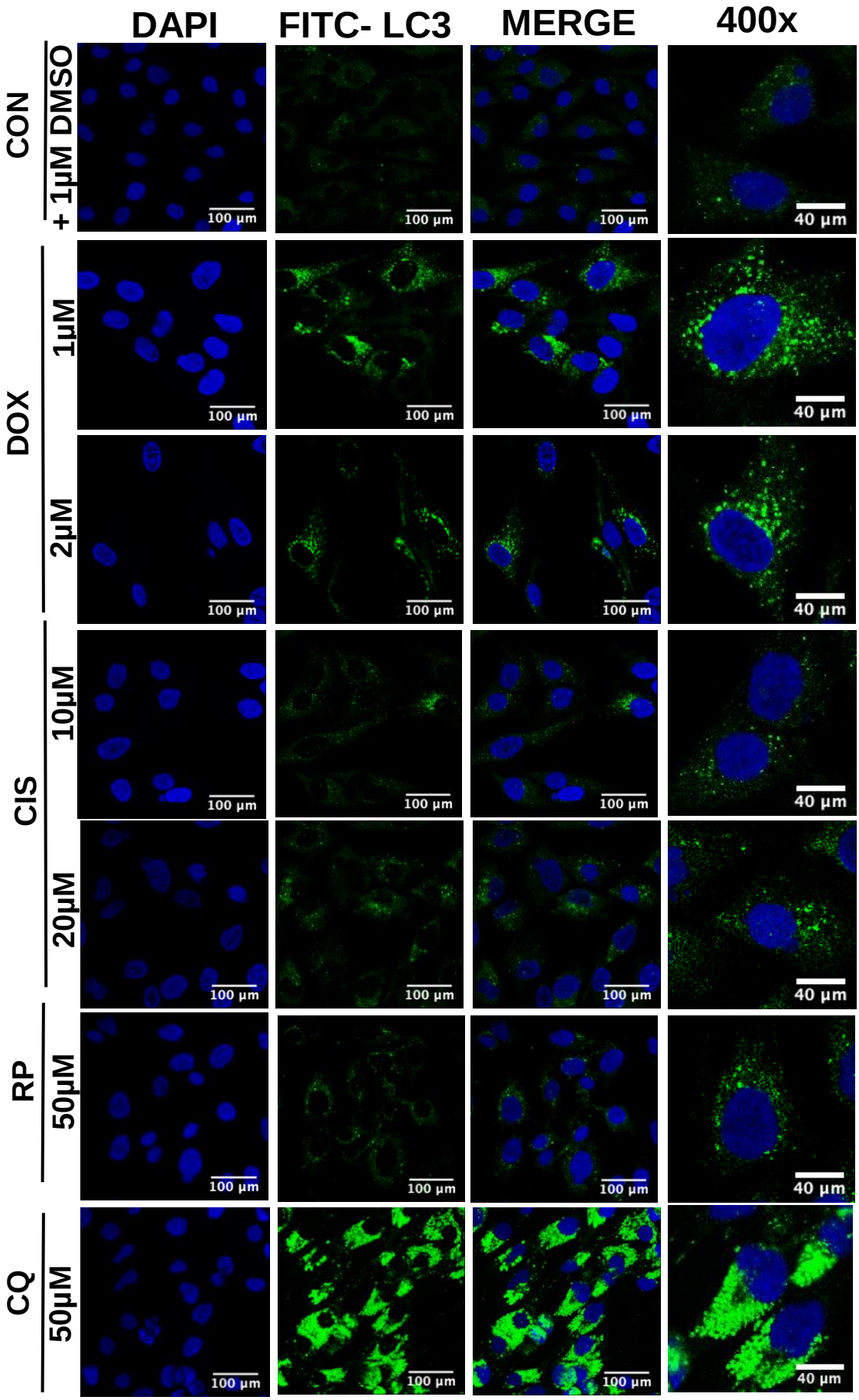


Figure 10B. Confocal imaging of LC3 A/B puncta protein expression in MG-63 cells treated with chemotherapy and autophagy modulator for 48h. Samples: CON, 1µM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; RP, Rapamycin; CQ, Chloroquine.

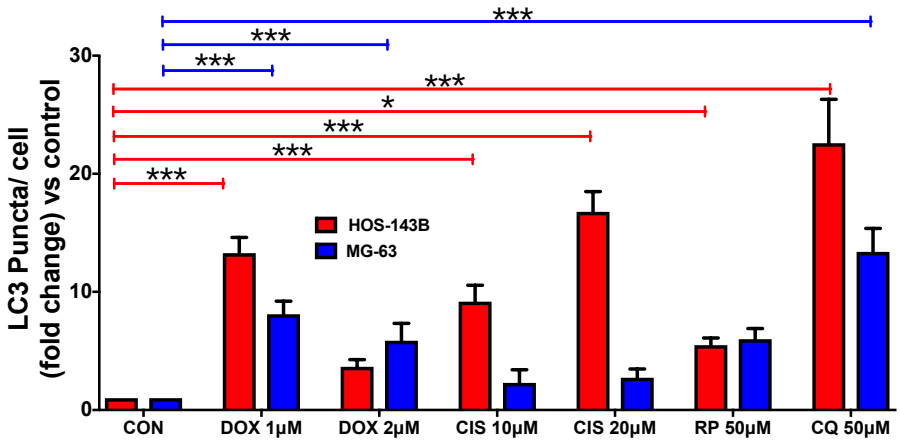


Figure 10C. Chemotherapy causes increase expression of LC3 puncta in OS cell lines
Quantitative analysis of LC3 puncta per cell in OS cell lines. Data is presented as mean ± SE (n = 3). ***p<0.001; **p<0.01; *p<0.05 vs Control. Samples: CON, 1μM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; RP, Rapamycin; CQ, Chloroquine.

membrane) and drag p62/SQSTM1-bounded cargo into the internal compartment of autophagosomes (Pankiv *et al*., 2007). Therefore, during autophagy induction the lysosomal degradation of autophagic cargo bound to p62/SQSTM1 effectively reduced its expression, and vice versa. Conversely, in the event that autophagy is being inhibited, for instance, using chloroquine (CQ), there is increased accumulation of autophagosomes, hence reduced degradation of autophagosomal cargo and accumulation of LC3-II and p62/SQSTM1. As shown in figure 11A, chemotherapy drugs had a significant impact on autophagic flux and p62/SQSTM1 levels in OS cell lines, with the highly metastatic HOS-143B revealing the most significant change. In HOS-143B, Dox caused 5.4 ($p<0.001$) and 3.7 ($p<0.001$) fold increase in autophagic flux with 1μM and 2μM treatment, respectively; Cis caused a 3.7 ($p<0.001$) and 4.5 ($p<0.001$) fold increase with 10μM and 20μM treatment, respectively. In parallel, Dox caused 2.5 ($p=0.002$) and 1.6 ($p=0.0136$) fold decrease in p62/SQSTM1 with 1μM and 2μM treatment, respectively; Cis caused 1.3 ($p=0.1426$) and 1.2 ($p=0.5024$) fold decrease with 10μM and 20μM treatment, respectively. In MG-63, although Dox and Cis treatment caused increase autophagic flux, only 1μM Dox treatment was associated with a significant fold increase of 3.4 ($p=0.093$). Interestingly, 1μM and 2μM Dox treatment were associated with decrease p62/SQSTM1 expression, which indicates autophagy upregulation, whilst 10μM and 20μM Cis treatment were associated with increase expression, implying decrease degradation of p62/SQSTM1

tagged cargo. The highest increase in both autophagic flux and p62/SQSTM1 was seen with CQ treatment in both cell lines, which was to be expected due to accumulation of these proteins caused by lysosomal dysfunction.

At the gene level, analysis was based on gene expression of p62/SQSTM1 and MAP1LC3B. The results achieved reflect very similar patterns seen with western blot analysis, as shown in figure 11B. Both chemotherapy drugs caused a significant increase in MAP1LC3B and p62/SQSTM1 expression in both OS cell lines, with HOS-143B having the most profound change in expression. In HOS-143B, Dox caused a 10.6 ($p=0.001$) and 6.2 ($p=0.001$) fold increase in MAP1LC3B expression with 1μM and 2μM treatment, respectively; Cis caused a 3.9 ($p=0.009$) and 5.2 ($p=0.001$) fold increase with 10μM and 20μM treatment, respectively. Concurrently, Dox caused a 32.2 ($p=0.001$) and 27.5 ($p<0.001$) significant fold increase in p62/SQSTM1 with 1μM and 2μM treatment, respectively; Cis caused 12.1 ($p=0.001$) and 17.5 ($p<0.001$) fold decrease with 10μM and 20μM treatment, respectively. In MG-63, Dox caused a 3.4 ($p=0.04$) and 3.6 ($p=0.03$) fold increase in MAP1LC3B expression with 1μM and 2μM treatment, respectively; Cis caused a 6.8 ($p<0.001$) significant fold increase with 10μM treatment and 3.3 ($p=0.06$) fold increase with 20μM treatment. Both doses of Dox and Cis caused a significant increase in p62/SQSTM1 gene expression; 9.1 ($p<0.001$) and 5.1 ($p=0.002$) fold increase with 1μM and 2μM Dox treatment, respectively; 4.8 ($p=0.003$) and 10.8 ($p<0.001$) fold increase with 10μM and 20μM Cis treatment, respectively. No significant change in both p62/SQSTM1 and MAP1L3B expression was seen with autophagy inhibitor CQ and inducer RP treatment.

These findings suggest that both chemotherapy drugs are able to effectively induce autophagy in OS cells, with Dox treatment having the most profound effect. This indicates a possible distinct mechanism of autophagy induction between the two chemotherapy drugs. Additionally, the highly metastatic cell line HOS-143B was associated more frequently with autophagy upregulation compared to the less metastatic MG-63, indicating a more critical role of autophagy in the survival of these fast dividing and aggressive cell type.

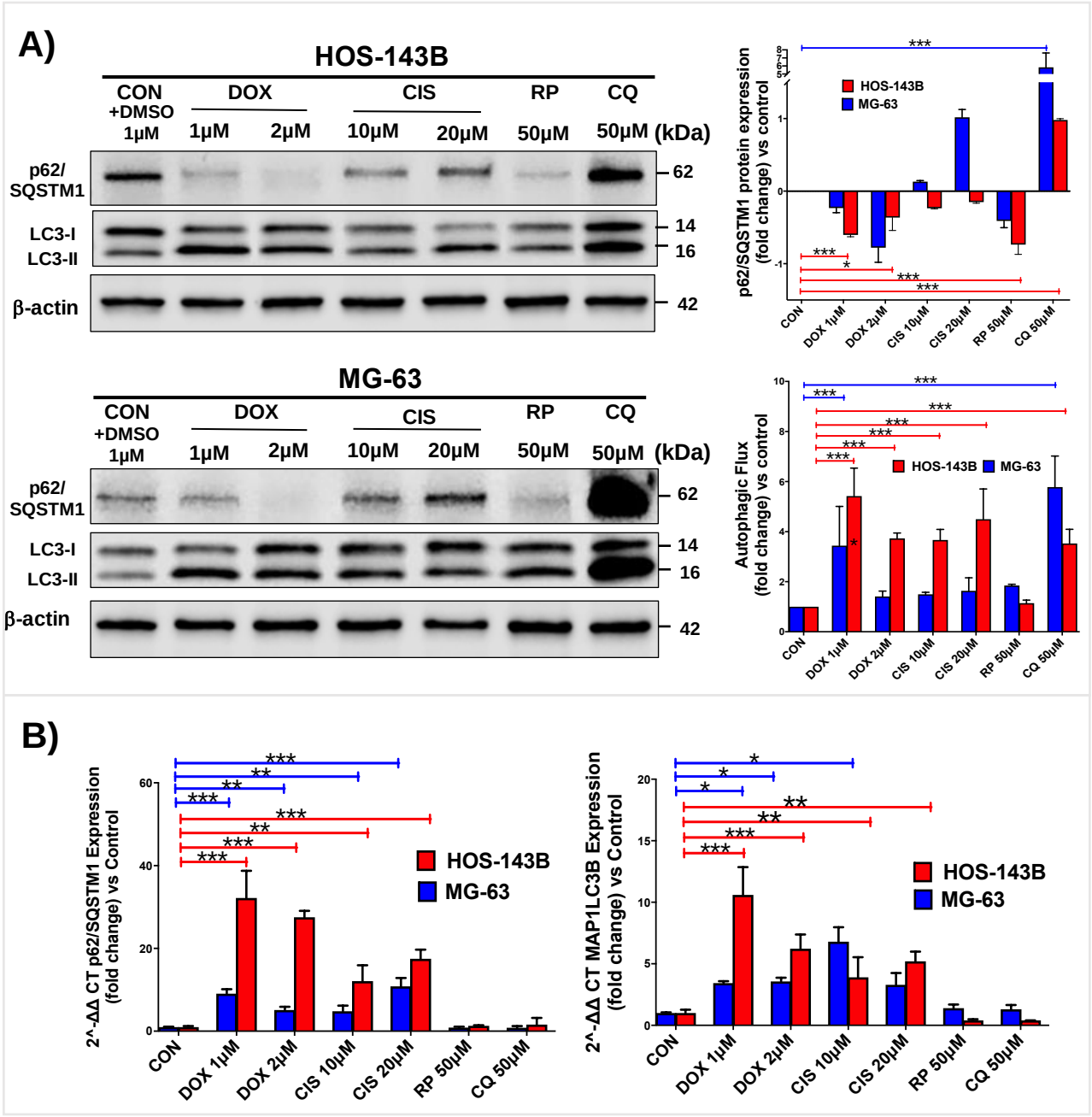


Figure 11. Chemotherapy treatment induces expression of autophagy markers in OS cell lines (A) Protein expression of p62/SQSTM1 and LC3-I and LC3-II in HOS-143B and MG-63 cells treated with two concentrations of both chemotherapy drugs and autophagy inducer rapamycin and inhibiter chloroquine at 48h. Bar charts showing quantitative analysis of p62 expression and autophagic flux (conversion of LC3-I to LC3-II); 40µg protein loaded in each sample. (B) Quantitative analysis of RT-PCR results showing p62/SQSTM1 and MAP1LC3B gene expression relative to GAPDH. Data is presented as mean ± SE (n = 3). ***p<0.001; **p<0.01; *p<0.05 vs Control. Samples: CON, 1µM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; RP, Rapamycin; CQ, Chloroquine.

3.3 Effects of autophagy modulation in OS cell response to chemotherapy

The previous results made evident how Dox and Cis treatment can significantly increase autophagy upregulation in OS cell lines. The next step consisted of investigating the actual role of such chemotherapy-induced autophagy, whether pro-survival or pro-death. To do this, autophagy was pharmacologically inhibited with CQ and the effects on increase or decrease chemotherapy ability to induce cell death in OS cells were evaluated with cell viability and apoptosis assays.

3.3.1 CQ pre-treatment causes decreased OS cell viability

To investigate the pro-survival or pro-death role of autophagy, the impact on cell viability was measured in OS cells pre-treated with CQ in combination with chemotherapy at 48h. As shown in figure 12, combination of CQ with chemotherapy had a profound effect on OS cell viability. However, these results could not directly reflect autophagy inhibition, as CQ treatment had severe effects on the ability of cells to proliferate. Upon further analysis of control samples treated with CQ, it was possible to devise a dose range (between 10μM and 90μM) of CQ which can significantly impact OS cell viability. Most studies use concentrations within these ranges to effectively inhibit autophagy, but do not mention the added cytotoxicity associated with CQ treatment (Wang et al., 2017, Kim *et al*., 2016, Zhou *et al*., 2015). Therefore, a degree of optimization was required to establish a causal link between autophagy inhibition and enhanced chemotherapeutic effect, without the added anti-proliferative effect of CQ on OS cell lines. CQ concentration required to be tailored to the specific cell type and drug treatment in order to achieve an effective working dose. As shown in figure 12, optimization was made possible with HOS-143B, in which a dose range between 20-30μM of CQ were suitable to cause autophagy inhibition without the added cytotoxic effect. In this case, Cis combination with CQ had a profound impact on HOS-143B viability as evidenced by a 2 fold decrease ($p<0.0001$) in IC50 compared to single Cis treatment. This result suggests that autophagy inhibition could sensitise HOS-143B to Cis treatment, indicating a possible protective role. Due to time constrains, it was not possible to optimize the other drug treatments and cell line.

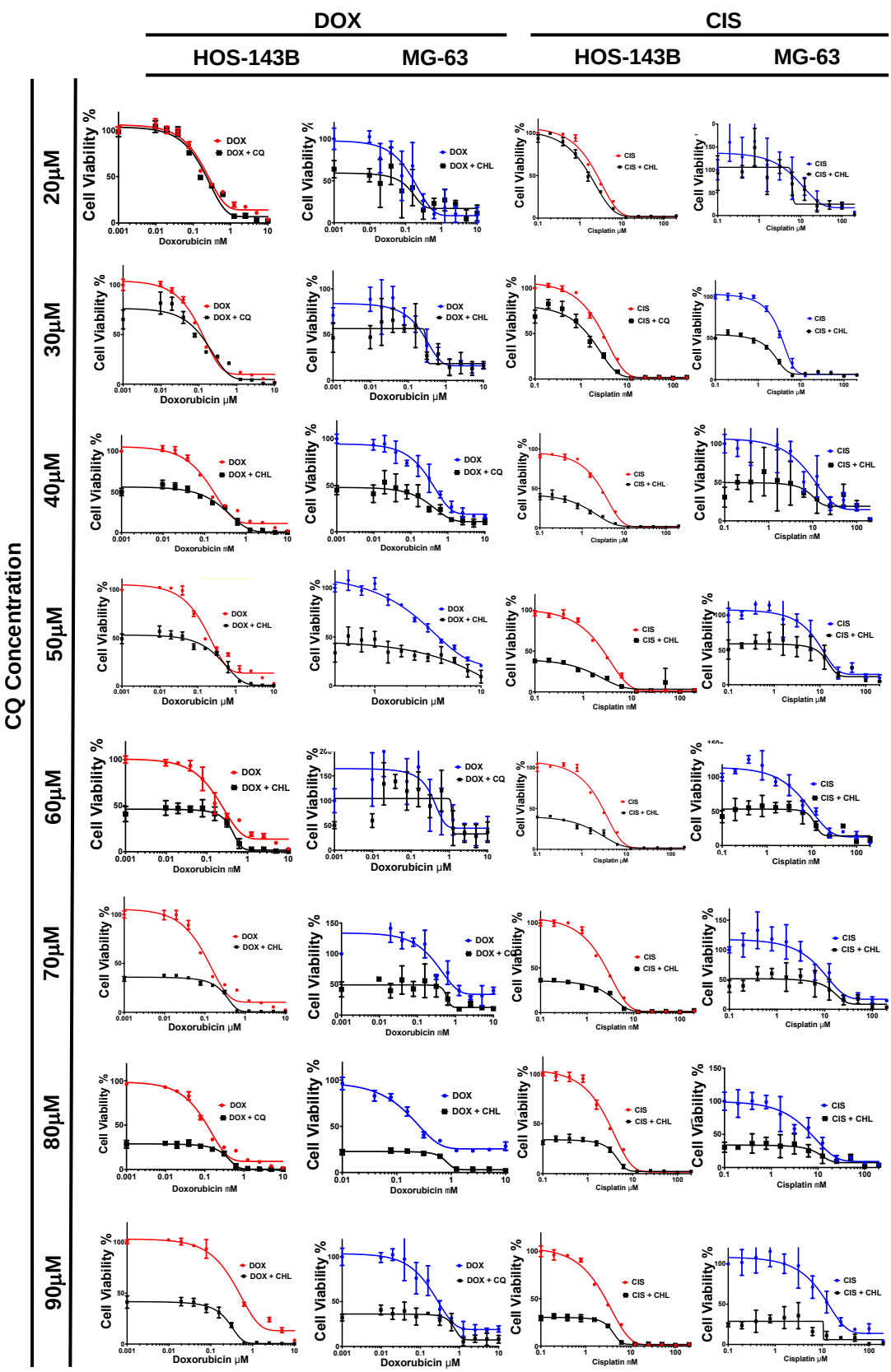


Figure 12a. Effects of CQ treatment on the sensitivity of OS cell lines to chemotherapy. Dose-response curve of CQ combination with chemotherapy in HOS-143B and MG-63 at 48h. Samples: DOX, Doxorubicin; CIS, Cisplatin; CQ, Chloroquine.

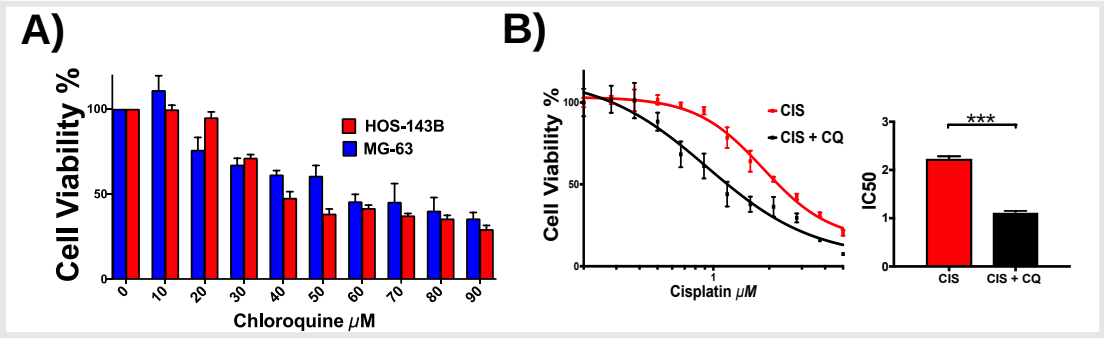


Figure 12b. Effects of CQ treatment on the sensitivity of OS cell lines to chemotherapy. (A) Cell viability of control samples treated with corresponding CQ dose (B) HOS-143B cell viability treated with combination of CIS and CQ (CIS + CQ) compared to single treatment CIS. Quantitative comparison of IC50s between each treatment. Data is represented as mean ± SE (n=3). ***p<0.001; **p<0.01; *p<0.05. Samples: CIS, Cisplatin; CQ, Chloroquine.

3.3.2 CQ pre-treatment causes increase cell death rate

Further investigations consisted of determining the effects of autophagy inhibition on the ability of chemotherapy to induce cell death. Such studies were carried using an Annexin V/PI apoptosis assay. As shown in figure 13, combination of CQ with chemotherapy lead to a substantial increase in the dead cell population compared to a single agent treatment. Both cell lines showed remark shift, suggesting that autophagy plays an important role in chemotherapy response. However, the same issue arose, where single CQ treatment alone lead to a significant increase in cell death. Because of this, the results obtained shadow the effects of autophagy inhibition, since the increase death rate could be a result of the anti-cancer effect of CQ single treatment, therefore, no conclusion can be drawn. Much like the cell viability, the optimization of CQ concentration is required, in order to use a dose range that can induce autophagy inhibition without the added cytotoxicity. Furthermore, the inhibition of autophagy in combination drug treatment using CQ was confirmed via IF and Western blot analysis of LC3 protein expression. As shown in figure 14, OS cell lines had a notable accumulation of LC3 puncta and increased autophagic flux with combination treatment. Therefore, this suggests that autophagy is effectively being blocked upon CQ treatment. However, significance could not be established due to time constrains in replicating such experiments. Although it was not possible to verify the role of autophagy in OS cells, these results indicate that autophagy can be a prime factor in cancer cell response to chemotherapy.

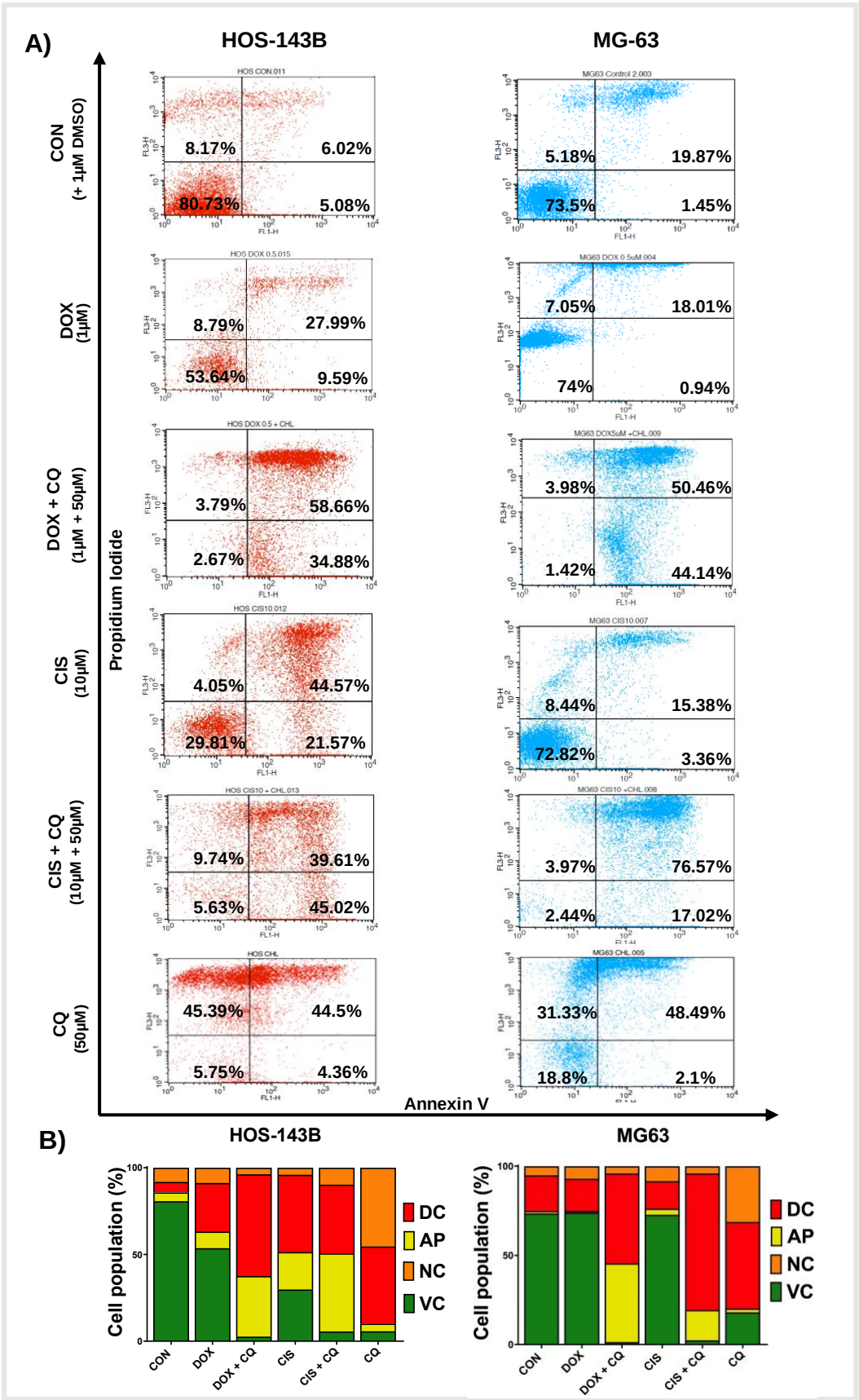


Figure 13. CQ combination with chemotherapy causes increase OS cell death (A) Annexin V/PI stain results of OS cell treated with single and combination therapies at 48h. (B) Quantitative comparison of IC50s between both cell lines at each timepoint. VC, viable cells; AP, apoptosis; DC, cell death; NC, necrosis. Quadrant guide: Lower left = viable cells; Lower right, early apoptosis; Upper left, necrosis; Upper right, dead cells. Samples: CON, 1µM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; CQ, Chloroquine.

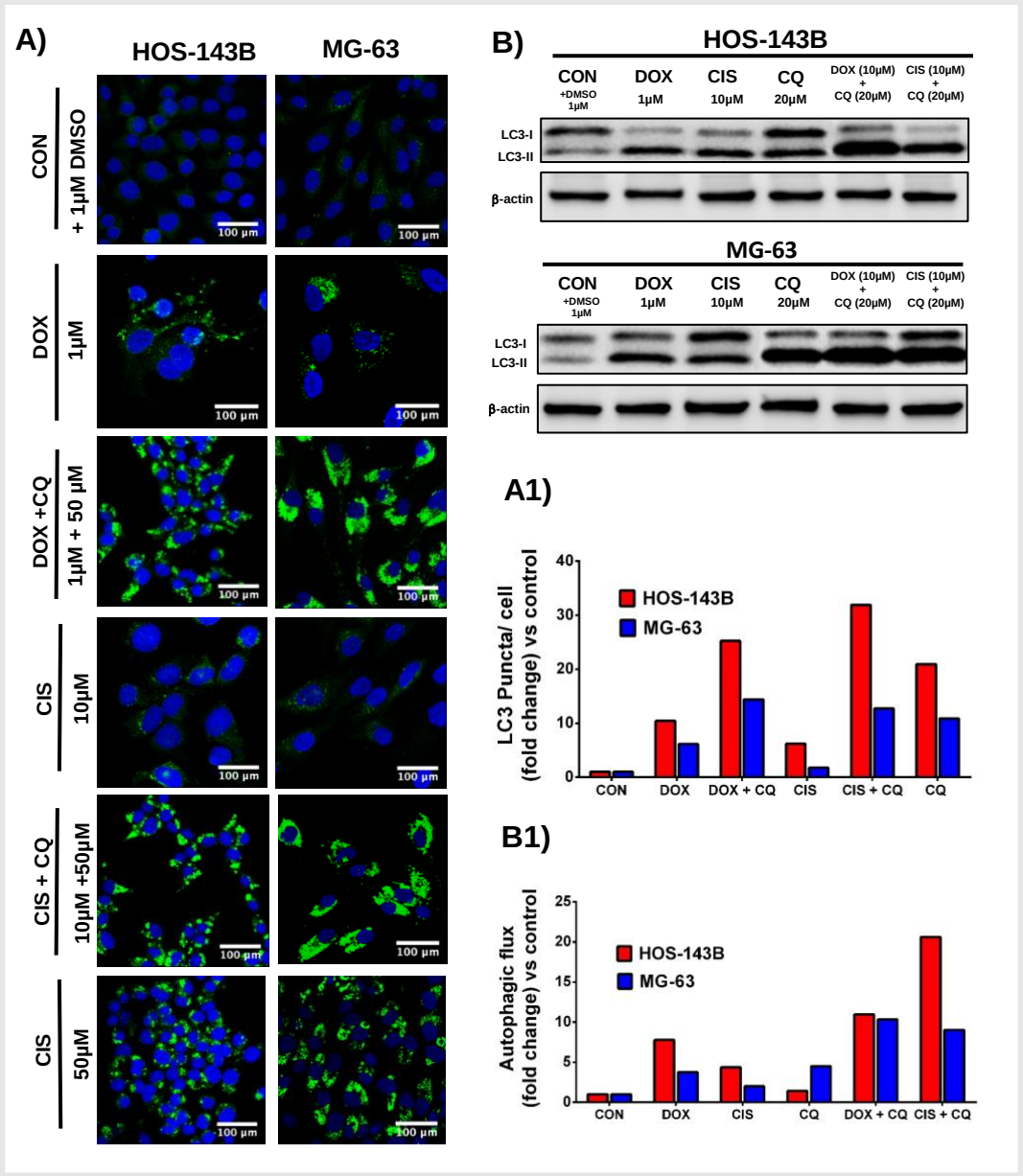


Figure 14. CQ combination with chemotherapy causes an increase LC3 protein expression. (A) Immunofluorescence images showing LC3 puncta accumulation in drug treated OS cells at 48h. (A1) Quantitative analysis of LC3 puncta per cell in OS cell lines. (B) Western blot protein expression of LC3-I and LC3-II in drug treated OS cells (B1) Quantitative analysis of autophagic flux (conversion of LC3-I to LC3-II); 40µg protein loaded in each sample. Samples: CON, 1µM DMSO Control; DOX, Doxorubicin; CIS, Cisplatin; CQ, Chloroquine.

4. Discussion

Poor prognosis in metastatic OS patients are frequently associated with resistance to conventional chemotherapy (Botter, Neri and Fuchs, 2014). Management and treatment of such resistant OS patients is a major challenge faced by clinicians, usually associated to poor disease outcome (Lindsey *et al*., 2017). To improve OS patient survival, current research is focused on depicting the underlying molecular mechanism implicated in tumour resistance. A better understanding of the mechanisms that drive OS resistance can allow novel targets to be developed that ultimately enhance therapeutic efficacy. Although many mechanisms have been suggested to drive OS chemoresistance (He *et al*., 2014), it is still not clear which has the most imperative effect. Within these, autophagy has been shown to promote tumour progression, metastasis and chemoresistance in a number of cancer types, including OS (Yang *et al*., 2011; Wei *et al*., 2013**;** Ojha *et al*., 2015). Autophagy is a conserved degradation process that maintains intracellular homeostasis by continuous removal and recycling of superfluous, ectopic or damaged cellular components (Glick, Barth and Macleod, 2010; Galluzzi *et al*., 2014; Kenific and Debnath, 2015). Physiologically, autophagy acts as a quality control system and a cell survival response to certain stimuli, including cytotoxic, metabolic and pathogenic cues (Boya, Reggiori and Codogno, 2013). In cancer, the functional role of autophagy is complex, since autophagy can act to either suppress oncogenesis or promote cancer cell survival in unfavourable microenvironments (Mathew, Karantza-Wadsworth and White, 2007). This dual-role can be context-dependent, for instance, autophagy may supress oncogenesis in early stages of tumour development, but in later stages can provide cancer cells with a selective advantage to cope with metabolic demand, hypoxia and therapeutic stress (Sehgal *et al*., 2015). Moreover, not only can autophagy act as a cell survival mechanism, but overexpression of autophagy can also be detrimental to cancer cells, since the excessive cellular breakdown can lead to activation of a death pathway known as ‘autophagic cell death’ (Chen *et al*., 2008). Factors including tissue type, tumour grade and concomitant drug therapy can influence autophagy to either function to protect or kill cancer cells (Eskelinen, 2011; Notte, Leclere and Michiels, 2011). This study explored the functional role of autophagy in OS tumour tissue, investigating autophagy expression in OS tumour progression and metastasis and particularly focusing on its function in OS cell models under chemotherapeutic stress. The results achieved from this investigation strongly links autophagy expression to metastatic OS patient tumours and chemotherapeutic response.

4.1 The role of autophagy in OS tumour progression

Increasing evidence reveals a strong connection between autophagy overexpression and the proliferative capacity of established tumours (White, 2012; Mathew, *et al*., 2007**;** Su *et al*., 2015). The common consensus is that an autophagic response supports tumour growth and progression by sustaining high energy yield in cancer cells, conferring better adaptability in adverse microenvironmental conditions, particularly in nutrient deficient and hypoxic situations (Mathew and White, 2011; Galluzzi *et al*., 2015). This is especially evident in poorly vascularized tumours, since the inadequate blood supply can lead to hypoxia and nutrient depletion, impairing ATP production and elevating metabolic stress (Vander Heiden, 2011; Eskelinen, 2011). Therefore, defects in angiogenesis are a predominant factor in hypoxia-mediated autophagy (Norman *et al*., 2011; Hu *et al*., 2012). Norman *et al*., (2011) not only showed how small cell lung carcinoma had substantial hypoxia-induced autophagy to promote their survival, but it was also linked to their increase resistance to cytotoxic T lymphocyte-mediated lysis. Another study by Hu *et al*. (2012) showed how hypoxia-induced autophagy caused by anti-angiogenic therapy can promote cell survival and bevacizumab resistance in glioblastoma cell lines. They found a strong link between autophagy mediated by BNIP3 and hypoxic growth of tumours, and how CQ inhibition could revert tumour growth in glioblastoma xenographs. Bellot *et al*. (2009) also saw similar patterns in TNF-resistant breast cancer, whereby BNIP3-induced autophagy conferred survival advantage in hypoxic environments. In OS, studies have shown how hypoxia-induction not only acts to support tumour growth, but also confer resistance to therapy in OS models (Feng *et al*., 2016; Zhao *et al*., 2016). Feng *et al*. (2016) disclosed how MG-63 OS cell line exposed to hypoxic conditions had marked overexpression of autophagy, which was associated with cell survival and increased resistance to radiation therapy. They also showed how overexpression of LC3 puncta in OS tumour tissue correlated with HIF-1α expression, a biological marker of hypoxia. Zhao *et al*. (2016) showed how HIF-1β could regulate hypoxia-induced autophagy and promote doxorubicin resistance in MG-63 and U2-OS cell lines.

Studies have also shown that autophagy can induce superior stress tolerance in nutrient depleted conditions (Degenhardt *et al*, 2006; Akin *et al*., 2014; Martin *et al*., 2018). Akin *et al*. (2014) indicate how OS cell lines can upregulate autophagy in adverse nutrient conditions (i.e. amino acid, serum, pyruvate, glucose) to aid in survival. In his study, starvation-induced autophagy allowed Saos-2 OS cells to grow in amino acid depleted conditions. Inhibition of autophagy by ATG4B knockdown

supressed starvation-induced autophagy, which had a negative impact on Saos-2 cell growth.

Advanced human tumours can also exhibit increased autophagy activity caused by high metabolic demand during invasiveness and metastatic transformation (Lazova *et al*., 2012; Mikhaylova *et al*., 2012). This fact can therefore indicate a link between autophagy upregulation and the relative tumour stage and grade, that is, overall progression, spread and speed of growth (Amin and Edge, 2018). The results from the OS tissue array reveals such a pattern. OS biopsies associated with advanced grade tumours (G3, abnormal cells with high proliferative capacity), have significantly higher LC3 protein expression compared to less aggressive tumours (G2). These results are consistent with the idea that as established tumours grow, they rely on autophagy activity in order to cope with the increased metabolic demand caused by the inadequate supply of nutrients and oxygen.

4.2 The role of autophagy in OS in metastasis

Metastasis is characterised by the ability of cancer cells to disseminate from the primary tumour and generate malignant growth in a distal site (Das *et al*. 2012). A sequence of steps occurs during this process, including the degradation of the extracellular matrix (ECM) and increased cell motility (invasion), migration of cancer cells from the primary site into the circulatory system (intravasation), circulation of cancer cells to reach capillary beds (dissemination), permeation of cancer cells from vessels to local tissue (extravasation) and metastatic cells colonization of secondary sites (Srivastava, 2008). Studies have shown that autophagy can promote metastatic cancer cell survival during dissemination and colonization events by conferring superior stress tolerance in inhospitable environments, such as the systemic circulation (Chambers, Groom and MacDonald, 2002; Kroemer *et al*, 2010; Guo *et al*., 2013). Malignant development is preceded with an exacerbated metabolic function that increases energy requirements to support dissemination (Galluzzi *et al*., 2015). Galluzzi *et al*., 2015 mention how metastatic tumours utilize autophagy as a means to sustain such energy yields during malignant transformation and maintenance. Additionally, it has suggested that upregulation of autophagy in metastatic cells can attribute higher resistance to apoptosis induction compared to their primary cell counterparts (Glinsky and Glinsky, 1996). For instance, a study by Han *et al*. (2008) showed how autophagy upregulation in metastatic cell models was strongly linked with resistance to TRAIL-induced apoptosis. Furthermore, autophagy can promote resistance to anoikis (i.e. apoptotic cell death that can occur during detachment ECM)

in metastatic cells (Simpson, Anyiwe and Schimmer, 2008; Fung *et al*., 2008; Galluzzi *et al*., 2015). A study by Fung *et al*. (2008) showed how autophagy upregulation was strongly linked with fibrosarcoma cell survival following ECM detachment. Furthermore, autophagy can promote the onset of senescence in cancer cells that fail to establish in secondary sites, aiding in their survival (Capparelli *et al*., 2012; Galluzzi *et al*., 2015). However, autophagy also has a tumour suppressor action that not only reduces the occurrence of malignant transformation, but also metastasis. Galluzzi *et al*. (2015) produced evidence showing that during the initial stages of metastasis, autophagy can restrict necrosis and inflammatory cell infiltration, which is required for ECM detachment**.** Galluzzi *et al*., (2015) explained that acquisition of malignant and metastatic features could involve a temporary loss of autophagic function, whereby a reduction in the suppressive function of autophagy facilitates cancer cells to acquire oncogenetic changes and how cancer cells reconstitute autophagy to promote stress tolerant and therapy insensitivity. Possible mechanisms that can restore autophagy function include genetic or epigenetic instability in advanced tumours, although these have not been tested.

In this study, OS biopsies associated with more aggressive stages (III and IV), characterised by cancer cells with metastatic potential (Amin and Edge, 2018), had the highest LC3 protein expression compared to other non-invasive stages. This suggests that overexpression of autophagy can be associated with the aggressive metastatic properties of OS. The genome-wide analysis of a metastatic OS patient database also showed similar patterns. As indicated in figure 8, in a cohort of 27 OS patients, those who developed metastasis were associated with higher expression of autophagy gene MAP1LC3B. Furthermore, another OS patient cohort showed how lung metastatic tissue showed higher expression of MAP1LC3B compared to primary OS tumours. These findings support the idea that autophagy serves an important function in metastatic cancer cell proliferation and dissemination in order to effectively colonize a secondary site. Survival curves showed a strong link between the expression of MAP1LC3B and poor disease outcome, whereby OS patients with high MAP1LC3B expressing tumours were associated with reduced survival probability. Interestingly, higher MAP1LC3B gene expression was also significantly associated with a lower probability of metastasis free survival.

Taken together, these results strongly suggest that autophagy expression can be associated with metastatic OS patient tumour growth and proliferation. Although not directly, the OS patient survival data suggests that autophagy upregulation can be involved in the unresponsiveness of aggressive OS tumours to therapy.

4.3 The role of chemotherapy-induced autophagy in OS

A growing body of evidence highlights the paradoxical role of autophagy in promoting cancer cell survival or death during chemotherapy treatment (Mainz and Rosenfeldt, 2017; Wilde *et al*., 2018; Das, Mandal and Kögel, 2018). This controversy in the current literature is seen in many cancer cell types, including OS. On one side, cancer cells upregulate autophagy as a survival strategy against anticancer agents, on the other, certain agents can induce autophagy to promote cell death (Levy *et al*., 2017). This variability in the role of autophagy makes it difficult to standardize therapeutic approaches that modulate autophagy to induce anti-cancer effects. In OS, most evidence shows that autophagy primarily acts as a pro-survival mechanism against chemotherapeutic stress (Huang *et al*., 2012; He *et al*., 2013; Wu *et al*., 2014, Mukherjee *et al*., 2017). However, there is still insufficient evidence to imply a strong causality. Therefore, it is vital to fully elucidate the mechanism of autophagy-mediated chemoresistance in OS to establish a uniform consensus in autophagy modulation, allowing the best therapeutic outcome in metastatic OS patients.

4.3.1 Autophagy promotes OS cell survival under chemotherapeutic stress

An overwhelming sum of *in vitro* and *in vivo* evidence shows how cancer cells can exploit autophagy to resist radiation and chemotherapy associated cell death (Chittaranjan *et al*., 2014; Filippi-Chiela *et al*., 2015; Liang *et al*., 2016; Piya *et al*., 2016). Studies in OS cell models have reported how standard chemotherapy drugs induce autophagy upregulation, and how inhibition of this pathway could augment the potency of chemotherapy drugs (Huang *et al*., 2011; Shen *et al*., 2013; Li *et al*., 2016; Wang *et al*., 2018). Not only has autophagy been implicated in resistance to chemotherapy drugs in cell lines, but also in cancer stem cells (Helgason *et al*., 2013).
The results from this study show how standard chemotherapy drugs doxorubicin and cisplatin significantly upregulated autophagy in the highly metastatic HOS-143B and poorly metastatic MG-63 cell lines. It was clearly evidenced by the increase in protein and gene expression of key autophagy markers (LC3-II and p62/SQSTM1), as shown in figure 10 and 11. The evidence also highlights an interesting occurrence, where the level of autophagy induction was dependent on chemotherapy dose. Increasing doxorubicin treatment from 1µM to 2µM was associated with decreased expression of autophagic markers. Conversely, this was the opposite for cisplatin treatment, where an increase in concentration from 10µM to 20µM caused increase expression of autophagic markers. This pattern was seen in all assays performed (Western blot, RT-

PCR and Immunofluorescence). Interestingly, it was observed that increasing the concentration of cisplatin caused accumulation of p62/SQSTM1 protein, as shown in figure 11. This was not expected since the observed increase in autophagic flux would indicate decrease p62/SQSTM1 expression. Cisplatin treatment did show very similar patterns in protein expression with chloroquine treatment, indicating that increasing such concentrations of cisplatin tended to block autophagy at the autophagosome level. This could reflect the cross talk between autophagy and apoptosis, where successful chemotherapy-induced cell death via apoptosis requires an effective mechanism to switch off autophagy (Sui *et al*., 2015; Das *et al.*, 2015). Additionally, a study by Yu *et al*. (2011) showed how an accumulation of p62/SQSTM1 was significantly associated with cisplatin resistance in human ovarian cancer cells, as it reduced the occurrence of ER stress induced apoptosis. Islam *et al*. (2018) further elaborated that p62 plays a critical role in apoptotic activity because of the interplay between apoptosis and autophagy. The significantly higher expression of p62 observed in MG-63 cells treated with 20µM cisplatin could reflect the resistance pattern seen in the viability assays (figure 9), where MG-63 was significantly more resistant to cisplatin compared to HOS-143B at 48h. Furthermore, doxorubicin treatment had a higher ability to induce autophagy in both OS cells compared to cisplatin. This fact could be due to the distinct mode of action of these two anti-cancer agents. Whilst both drugs effectively cause DNA damage, doxorubicin acts by blocking topo-isomerase 2, an enzyme that manages DNA tangles and supercoils, while cisplatin is an alkylating agent that intercalates with DNA, impeding effective replication (Thorn *et al*, 2012; Dasari amd Tchounwou, 2014). Although they exert similar modes of cellular damage, the type and amount of cellular stress caused by each drug can differ, and therefore, the autophagy induction could be distinct for each type of drug, although no study has been conducted to confirm this.

To further investigate the role of doxorubicin and cisplatin mediated autophagy, this pathway was pharmacologically inhibited with chloroquine to examine if cancer cells would become more resistant or sensitive to chemotherapy drug treatment. Although results from both toxicity and apoptosis assays showed that combination of CQ had significant impact on reducing cell viability and increasing cell death, no conclusions could be drawn in regard to possible acquired cancer cell chemosensitivity due to autophagy inhibition. This is primarily due to the anti-cancer effect of chloroquine single treatment on cancer cells. As shown in figure 13, a chloroquine dose range between 10µM- 90µM had a significant impact on cell viability in control samples. Therefore, it was paramount to optimize such drug doses to establish working concentrations that could effectively induce autophagy inhibition without the added cytotoxic effects.

However, it is important to note that using chloroquine in autophagy-based studies is arbitrary due to its lack of specificity in the autophagy pathway. Although studies use chloroquine as a mean to block autophagy, these results may not be representative and could relate to other pathways that are affected by lysosomal inhibition (Wang *et al*., 2014). Therefore, to imply a strong causal link between autophagy and chemotherapy resistance, the use of non-specific pharmacological inhibitors should be avoided and a more precise investigation, such as genetic targeting, should be employed.

4.3.2 Mechanism of autophagy-induced chemoresistance

To date, the precise mechanism by which autophagy promotes chemoresistance in cancer cells has not been well defined. It has been proposed that autophagy is able to attenuate a set of chemotherapy-induced cellular stresses to promote chemoresistance, including endoplasmic reticulum (ER) stress, genotoxic stress and oxidative stress (Huang *et al*., 2016).

Functionally, ER serves a biosynthetic and signalling process that is vital for protein synthesis, vesicular trafficking, protein modifications, lipid synthesis and intracellular calcium homeostasis (Cole and Kramer, 2016). Prolonged ER dysregulation (known as ER stress) can lead to accumulation of misfolded proteins and calcium imbalance, triggering a cascade of events that lead to cell death (Verfaillie *et al*., 2012; Han *et al*., 2013; Zheng *et al*. 2013). Tumorigenesis has been shown to cause increased ER stress in many solid cancers, however, cancer cells have evolved efficient mechanism to adapt to such ER stress (Yadav *et al*., 2014). Chemotherapy has been shown to interfere with ER stress balance in cancer cells by causing the accumulation of reactive oxygen species (Riha *et al*., 2017). In this scenario, the unfolded protein response (UPR) is one of the major adaptation mechanisms that are activated to overcome chemotherapy-induced ER stress (Avril *et al*., 2017). This highly conserved mechanism aims to attenuate ER stress by reducing the number of misfolded protein aggregates and re-establish ER homeostasis. However, sometimes the UPR system is unable to effectively manage ER stress (Avril *et al*., 2017). Studies indicate that when this occurs, UPR induces autophagy as an alternative protein degradation pathway (Qin *et al*., 2010; Hoyer-Hansen *et al*., 2007). In this way, autophagy can act as an antioxidant machinery to keep ROS levels under the death threshold and reduce the number of misfolded protein aggregates (Qin *et al*., 2010).

Furthermore, autophagy chemoresistance can be attributed to the DNA damage response caused by certain chemotherapy agents (Eliopoulos *et al*., 2016). The

process of DNA damage repair (DDR) is a major protective response in cells undergoing genotoxic stress (Chatterjee and Walker, 2017). The effectiveness of DNA damage-induced drugs, such as doxorubicin and cisplatin, largely depends on the balance between DNA damage and DDR (Roos, Thomas and Kaina, 2015). Evidence shows how DNA damage causes autophagy induction and is linked to genotoxic therapy resistance (Orlotti *et al*., 2012; Eapen and Haber, 2013). Studies have shown that DNA damage can activate autophagy via AMPK (Robert *et al*., 2011; Eapen and Haber, 2013; Orlotti *et al*., 2012; Park *et al*., 2015). It has been suggested that extensive induction of DDR causes increased ATP demand, implying that autophagy acts to upregulate ATP production to sustain effective repair (Park *et al*., 2015).

Another chemotherapeutic stress response that can induce autophagy is the production of reactive oxygen species (ROS) that cause random damage of intracellular components (Feinendegen, 2002). In cancer cells, there is large production of ROS species caused by high levels of metabolic waste (White, 2012). However, cancer cells have adapted antioxidant mechanisms against oxidative stress, maintaining normal ROS levels under the death threshold (Qin *et al*., 2010). Chemotherapeutic agents can disrupt this balance and elevate ROS, which imposes damaging risk to cancer cells (Feinendegen, 2002). ROS-induced damage of cellular components (particularly the mitochondria) can have lethal consequences to cancer cells (Filomeni, De Zio, Cecconi, 2015). Autophagy can act as a potent antioxidant system to effectively clear irreversibly oxidized biomolecules (Filomeni, De Zio, Cecconi, 2015). Therefore, autophagy plays an important role in redox homeostasis and reduces the induction of ROS mediated cell damage (Filomeni, Zio and Cecconi, 2014).

An additional avenue by which autophagy confers resistant phenotypes in cancer cells is related to the cross-talk between autophagy and apoptosis (Sui *et al*., 2015; Das *et al.*, 2015). Autophagy has been shown to precede and regulate caspase-dependent apoptosis via proteins that overlap these two pathways (Zhang *et al*. 2011; Abe *et al*., 2011; Yahiro *et al*., 2012; Franzetti *et al*., 2012; Francisco *et al*., 2012). The most well-established proteins are p62 and Beclin-1 (Sui *et al*., 2015). P62 has been shown to interact with several apoptotic proteins, including caspase-8, TRAF6 and ERK (Shubassi *et al*., 2012). The interaction between p62 and caspase-8 clearly demonstrates the engagement between these two pathways. Caspase-8 is crucial for the initiation of apoptotic signalling via the extrinsic pathway (Elmore, 2008). Not only is p62 important for activation of caspase-8 (Norman *et al*., 2010; Jin *et al*., 2009), but also caspase-8 can be actively degraded by autophagy, presumably via p62 tagging (Hou *et al*., 2010). These studies suggest an interesting paradigm where autophagy

can alter the kinetic works of apoptosis. Furthermore, it has been shown that autophagy regulator Beclin-1 has direct interactions with the anti-apoptotic protein Bcl-2 (Djavaheri-Mergny *et al*., 2010p; Marquez and Xu, 2012; Sinha and Levine, 2008). Marquez and Xu (2012) explain that when Bcl-2 binds to Beclin-1 it diminishes the activation of autophagy. The release of Beclin-1 from Bcl-2 by BH3 proteins, DAP kinase phosphorylation or Bcl-2 phosphorylation by JNK has been shown to revert autophagy induction (Wei *et al*., 2008; Pattingre *et al*., 2005). Additionally, it has been shown that overexpression of Bcl-2 can inhibit autophagy (Erlich *et al*., 2007; Levine *et al*., 2008). Moreover, apoptosis can inhibit autophagy through caspase-3 cleavage of Beclin-1, leading to a non-functional truncated protein (Luo and Rubinsztein, 2010). These examples show how apoptosis can mutually regulate autophagy and reveals an interesting pattern whereby efficient apoptosis induction requires the inactivation of autophagy.

Such mechanisms that attribute to autophagy-based chemoresistance are prime investigations that have not yet been published. Therefore, these should form the basis for future studies, since fully elucidating the molecular mechanism of autophagy mediated chemoresistance is crucial for our further understanding of the relevance of autophagy in tumour response to chemotherapy.

4.3.3 Chemotherapy promotes autophagic cell death in OS

In contrast to its protective role during chemotherapeutic stress, studies have shown that a specific set of chemotherapeutic agents can induce autophagy to evoke a distinct pro death mechanism, known as 'autophagic cell death' (Voss *et al*., 2010; Salazar *et al*., 2009; Yamamoto *et al*. 2008). For instance, the DNA-alkylating agent temozolomide (TMZ) has been shown to trigger autophagic cell death in U251 glioma cell line (Li et al., 2017). A study by Kanzawa *et al*. (2005) showed how arsenic trioxide upregulated BNIP3 to induce autophagic cell death induction in glioma cells. Based on current data, autophagic cell death is predominantly induced as an alternative cell death mechanism when apoptosis is defective (Xiong *et al*., 2010; Yuan *et al*., 2010; Shen and Codogno, 2011; Lee *et al*., 2012). For instance, Xiong *et al*. (2010) showed how human colon cancer cells with deficiency in apoptotic proteins PUMA or Bax induced autophagic cell death upon 5-FU treatment. Lee *et al*. (2012) showed how suberoylanilide hydroxamic acid (SAHA) induced autophagic cell death in tamoxifen-resistant MCF-7 breast cancer cells whilst detection of apoptotic markers was relatively low. Yamamoto *et al*., (2008) also showed how SAHA could induce autophagy associated cell death in chondrosarcoma cell lines and *in vivo* xenograft models.

Although no study has shown that standard chemotherapy drugs induce autophagic cell death in OS cell models, it has been shown in other cell models that doxorubicin and cisplatin can induce such autophagy-mediated cell death (Zhang *et al*., 2016; Chen *et al*., 2018). This switch between pro-survival and pro-death in autophagy could depend on a number of factors, such as the cellular context and the duration and extent of autophagy induction (Yamamoto *et al*. 2008), although these have still not been tested.

4.4 Autophagy modulation in cancer therapy – Potential Clinical Applications

The substantial progress in autophagy research has created considerable interest in the development of targeted therapies to modulate cancer progression and chemoresistance. However, its equivocal role makes it difficult to standardise such therapeutic approaches. On the one hand, autophagy inhibitors can be employed to decrease the cell survival function. On the other, cancer therapy can be designed to enhanced autophagic cell death (ACD). Many studies have employed such therapeutic concepts to evaluate the anti-cancer benefits of promoting or inhibiting autophagy.

4.4.1 Enhancing autophagy as a therapeutic in OS

The use of agents that stimulate autophagic cell death is a potential anti-cancer therapeutic approach. *In vitro* studies have reported a number of agents (alkylating agents, actinomycin D, arsenic trioxide) and natural compounds (resveratrol) that can induce autophagic cell death in a variety of cancer types (Chen and Karantza, 2011). Additionally, the therapeutic potential of autophagic cell death has also been shown *in vivo* (Lian *et al*., 2011). Lian *et al*., (2011) state how in a phase II and III clinical trial, BH3 mimetic treatment could effectively induced autophagic cell death in human prostate cancer, which correlated with a significant improvement in disease outcome. The main issue with the use of agents that promote autophagic cell death lies in the unwanted paradoxical effect where combination of chemotherapy agents could result in loss of drug potency caused by protective autophagy (Chen and Karantza, 2011). Apart from these, not many studies investigate the therapeutic benefit of autophagic cell death induction as an anti-cancer approach. Most studies focus on therapeutic inhibition of autophagy, since there is vast amount of preclinical evidence to suggest autophagy inhibition can provide higher therapeutic benefit.

4.4.2 Inhibiting autophagy in cancer therapy

In the context that autophagy promotes OS tumour cells progression and chemoresistance, targeting autophagy with inhibitors is a promising approach to increase cancer cell vulnerability to chemotherapy, and hence achieve greater treatment potency. A selection of inhibitory drugs have been devised and show to effectively inhibit autophagy. These include 3-Methyladenine (3-MA) and wortmannin, that inhibit autophagy by blocking class I and III PI3K activity (Wu *et al*., 2010). Studies indicate that the use of such inhibitors effectively impairs autophagy and cause increase sensitivity of OS cells to therapy (Feng *et al*., 2015). However, these inhibitors can also affect PI3K activity without blocking autophagy (Ito *et al*., 2007). Ito *et al*., (2007) show how 3-MA treatment could supress PI3K activity and reduce the invasion capacity of HT1080 fibrosarcoma cells without blocking autophagy. Inhibitors of key regulator ULK complex and Beclin-1 (important drivers of membrane nucleation) are also effective in obliterating the autophagy pathway (Adhikary *et al*., 2008; Rhee *et al*., 2004). These include the JNK inhibitor SP600125 that downregulates Beclin-1 expression or MAP Kinase Inhibitor U0126 that activates ERK expression and supresses ULK expression. Other more common inhibitors target later steps of autophagosome maturation, including Chloroquine and Bafilomycin A1, that act to block lysosome fusion with autophagosomes and consequently cause their accumulation (Mauvezin and Neufeld, 2015). Within many agents, CQ treatment is predominantly used method of autophagy in many preclinical studies since it is currently the only approved drug for clinical trials (Cook *et al*., 2014).

4.4.3 Clinical application of HCQ and CQ

CQ is an antimalarial drug with lysosomotropic properties that interfere with autophagosome-lysosome fusion, thereby supressing degradation of autophagic cargo (Wang *et al*., 2011). Mechanistically, CQ accumulates in lysosomes and mediates pronation reactions that effectively increase internal pH and causes their dysfunction (Kimura *et al*., 2012). The use of CQ and its derivative, HCQ, are currently the most widely used method for autophagy inhibition since these are clinically approved (Cook *et al*., 2014). HCQ is a preferred choice over CQ because of the reduced toxicity associated with higher concentrations (Warhurst *et al*., 2003; Golberg et al., 2012; Donohue et al., 2013; Manic *et al*., 2014). Preclinical investigations in both cell and animal models have evaluated the potential of HCQ to exert anti-cancer effects by inhibiting autophagy (Lee *et al*., 2015; Cook *et al*., 2014). The results

achieved from these studies have provided important insights that lay the foundation for clinical trials. The first trial (phase II) assessed the safety and anticancer activity of HCQ in 20 patients with metastatic pancreatic cancer with poor response to conventional therapy (Wolpin *et al*., 2014). Patients were administered 400 (n=10) and 600 (n=10) mg of HCQ twice daily as single-agent treatment. These doses were reasonably well tolerated, with only 2 patients experiencing treatment related grade 3 and 4 side effects. Additionally, only 2 patients showed no disease progression upon initiating HCQ regimen. This observation suggested that single-agent HCQ treatment did not provide significant anti-cancer therapeutic benefits. Subsequent investigations evaluated the efficacy of combining HCQ with various chemotherapy and targeted therapies, including bortezomib (Vogl, *et al*. 2014, temozolomide (Rangwala *et al*., 2014), temsirolimus (Rangwala *et al*., 2014a), radiotherapy and temsirolimus (Rosenfeld *et al*., 2014) and varinostart (Mahalingam *et al*., 2014). The results from these studies showed how HCQ combination could enhance chemotherapy potency in some patients, without exerting adverse side effects. A number of patients in these cohorts experienced good partial response and a period of stable disease, suggesting HCQ combination had significant antineoplastic activity and is effective to an extent in patients with melanoma, colorectal cancer, myeloma and renal cell carcinoma. For instance, Vogl *et al*., (2014) showed how some myeloma patients experienced a good response to a combination of HCQ treatment and bortezomib (proteasome inhibitor). Additionally, Vogl *et al*. (2014) and Rangwala *et al*., (2014) showed that combination of high dose HCQ (1200mg) and dose-intense bortezomib, temozolomide, temsirolimus, respectively, showed no dose-limiting toxicity and was well tolerated in patients with advanced solid malignancies. However, a clinical study by Rosenfled *et al*. (2014) showed that 800mg of HCQ daily administration with temozolomide combination therapy caused grade 3 and 4 associated neutropenia and thrombocytopenia in many subjects with malignant glioma and no significant improvement in patient survival. Mahalingam *et al*. (2014) also showed that combination of 600mg of HCQ with histone deacetylases inhibitor vorinostat caused subjects to experience fatigue and gastrointestinal side effects. It is stipulated that CQ treatment can sensitise both malignant and normal tissue to chemotherapy, so prolonged use of such agents can lead to undesirable effect and possibly further oncogenic predisposition. Additionally, studies have shown that concentration up to 1200mg of HCQ does not efficiently inhibit autophagy *in vivo* (Rangwala *et al*., 2014). Currently, there are 20 ongoing trials further to explore the therapeutic benefit of the anti-autophagic effect of chloroquine on cancer therapy (Clinicaltriasl.gov, 2018a). Within these, a phase I trial is being conducted in patients with bone metastasis,

combining HCQ with radiotherapy, for which results are yet to be published (Clinicaltrials.gov, 2018b). The outcome of this study could potentially reinforce the importance of autophagy modulation on osteosarcoma patient treatment.

One major downfall with use of CQ to imply causality in autophagy inhibition and OS patient outcome is the additional non-specific action that can be attributed by lysosomal dysfunction. For instance, Wang *et al*. (2014) eluded how increased cellular death can be caused by the actual lysis of lysosome due to its defective function. Therefore, the use of specific approaches that efficiently target autophagy in cancer therapy are more desirable. For instance, the use of ATG4B inhibitors such as NSC185058 and NSC377071 (Akin *et al*., 2015). Akin *et al*. (2015) proposed that NSC185058 could effectively supress OS tumour growth both *in vitro* and *in vivo* by targeting ATG4B.

Taken together, these results from these studies indicate that autophagy modulation in clinics is still at its infancy,

5. Conclusion

Tumour resistance to chemotherapy continues to be the main challenge in treatment of metastatic OS patients. By summarizing the most recent data, it was clear that autophagy has a complex interplay in OS, due to its dual role in promoting cell survival and cell death. The results from this study display how autophagy expression is associated with progression, metastatic potential and chemotherapy resistance in OS tumours. *In vitro* studies showed how standard chemotherapy drugs such as doxorubicin and cisplatin can induce autophagy upregulation in OS cell models. Furthermore, it was possible to show how a combination of the autophagy inhibitor CQ with cisplatin caused increased sensitivity of highly metastatic HOS-143B cells to chemotherapy, indicating a protective role of autophagy. The results from cell viability studies also indicate that the use of CQ as an autophagy inhibitor can have cytotoxic effects in OS cell viability at relatively low concentrations. For precise investigations focusing on autophagy modulation, specific pharmacological or genetic approaches should be used.

Advances in autophagy research have allowed the discovery of novel treatment strategies to target this pathway. However, the question still remains if anti-cancer approaches should enhance or inhibit autophagy. This requires careful assessment and further studies to understand the mechanism that are involved in autophagy-induced chemoresistance. Additionally, it is paramount to define the clinical context by which cancer cells exert autophagy-mediated chemoresistance to obtain the most therapeutic benefit. Equally important is to gain a greater understanding of the dynamic interplay in cellular response that causes a shift in the pro-survival role of autophagy to a cellular death pathway.

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