EVALUATING THE ROLE OF METFORMIN ON DNA DAMAGE AND DNA DAMAGE RESPONSE IN OVARIAN CANCER

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ABSTRACT

Ovarian cancer is one of the most common and lethal types of cancer among women. The increased mortality rate reflects the need for discovery of new therapeutic venues. The anti-diabetic drug Metformin has started to show anti-cancer potential as epidemiological studies have associated its long-term use with reduced risk of ovarian cancer and increased survival for ovarian cancer patients. However, the underlying mechanisms of Metformin's effects are not well understood, especially its association with DNA damage which remains controversial. In this study, we evaluated the role of Metformin in DNA damage and DNA damage response in two ovarian cancer cell lines. Immunofluorescence experiments and colony formation assays suggest that Metformin potentiated the DNA-damaging effects of irradiation as it delayed the resolution of protein foci associated with DNA double-strand breaks and reduced cell survival respectively. Furthermore, Metformin had a greater effect in replicating cells and increased the cytotoxic effects of the nucleoside analog Gemcitabine. Thus, our data suggest that Metformin may delay or impair the DNA damage response and may have a stronger effect during replication. However, further studies are essential for verification and discovery of the underlying mechanisms of Metformin in the DNA damage response in ovarian cancer.

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LIST OF ABBREVIATIONS

53BP1: p53 binding protein 1

AMPK: AMP-activated protein kinase

ATM: Ataxia-Telangiectasia Mutated

BRCA1: Breast Cancer 1 early onset gene

BRCA2: Breast Cancer 2 early onset gene

DDR: DNA Damage Response

DSB's: Double Strand Breaks

EOC: Epithelial Ovarian Cancer

H2DCF-DA: 2',7'-dihydro-dichlorofluorescein diacetate

HNPCC: Hereditary Non-Polyposis Colorectal Cancer

IF: Immunofluorescence

IR: Irradiation

IRS-1: Insulin Receptor Substrate-1

LKB1: Liver Kinase B1

MDC1: Mediator of DNA damage Checkpoint 1

MRN: Mre11/Rad50/Nbs1

mTOR: Mammalian/Mechanistic Target of Rapamycin

OC: Ovarian Cancer

OCT: Organic Cation Transporter

PARP1: (Poly (ADP-ribose) polymerase 1)

PEITC: Phenethyl Isothiocyanate

RHEB: Ras Homolog Enriched in Brain

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RPA: Replication Protein A

S6K : S6 (ribosomal protein) kinase

ssDNA breaks: single strand DNA breaks

TSC2: Tuberous Sclerosis 2

γH2AX: phosphorylated histone H2AX

1. INTRODUCTION

1.1 Ovarian Cancer

Ovarian Cancer (OC) is the 5th most common type of cancer in the UK and is considered a "silent killer" [1,2]. In 2011, 4300 women died of the disease, making it the 4th most common cause of cancer death in the UK. Furthermore, worldwide statistics show that 140,000 women died of ovarian cancer in 2008 [1]. The term "silent killer" arises from the fact that 80% of women suffering from OC are diagnosed in stages post-metastasis (stages II, III and IV); resulting in dramatically poorer outcomes [2].

Stage I OC is confined to the ovaries, while in stages II, III and IV the cancer has metastasized to organs of the pelvic cavity, in the abdomen or further than the peritoneal cavity, respectively [2]. OC can arise from germ, granulosa-theca or epithelial cells; however, more than 60% of ovarian cancers have epithelial origin and can develop into 4 different histotypes [1,2]. Serous epithelial ovarian cancer (EOC) lines the fallopian tubes, while endometrioid and mucinous EOC line the endometrium and endocervix, respectively, and clear-cell EOC forms vaginal cysts [2]. It is noteworthy that these histotypes have different gene expression patterns and tumour markers and respond differently to alternative therapies [2].

Treatment strategies for OC mostly involve surgery and chemotherapy using platinum based- or taxane- based drugs. Platinum drugs e.g. Cisplatin and Carboplatin, are DNA damaging agents (i.e. cause DNA-DNA crosslinks or DNA-protein crosslinks), thus impairing DNA replication and repair, eventually leading to apoptosis [3]. On the other hand, taxane drugs e.g. Paclitaxel, are mitotic spindle poisons as they inhibit the de-polymerisation of microtubules, thus inhibiting mitosis [4]. The treatment for OC depends on the stage of cancer as well as whether the cancer has relapsed (Table 1) [1,5]. Notably, serous and endometriod EOC are

more responsive to platinum and taxane-based therapies compared to mucinous and clear-cell EOC [2]. For women with increased risk of developing OC, prophylactic surgery is also recommended [1,5] (Table 1). Women are at increased risk of OC when they have mutations on breast cancer 1/2, early onset genes (BRCA1/ BRCA2 respectively) or have Hereditary Non-Polyposis Colorectal Cancer (HNPCC) [2,5]. BRCA1 and BRCA2 mutations were associated with an increased risk of 30-60% and 15-30% respectively, while HNPCC increases the risk to 10-12% for OC development [2,17].

TABLE 1: Ovarian Cancer Treatment Strategies Depending on Stage of Disease

Stage of Disease	Treatment Strategy	
Increased risk for developing OC • BRCA1/BRCA2 mutation carriers • HNPCC patients (Hereditary Non Polyposis Colorectal Cancer)	Prophylactic surgery	Depending on menopausal status and family history: • Oophorectomy (removal of ovaries) • Salpingo-oophorectomy (removal of ovaries and fallopian tubes) • Hysterectomy (removal of uterus)
Early OC (stages I and II)*	Surgery and Adjuvant Chemotherapy	Depending on menopausal status : • Oophorectomy • Salpingo-oophorectomy • Hysterectomy Platinum-based chemotherapeutic drugs e.g. Cisplatin, Carboplatin or Taxane-based chemotherapeutic drugs e.g. Paclitaxel
Advanced OC (stages III,IV)	Maximal surgical cytoreduction (diminishes tumour burden and increases effectiveness of chemotherapy**) and Adjuvant Chemotherapy	• Total abdominal hysterectomy • Bilateral salpingo-oophorectomy • Pelvic and para-aortic Lymphadenectomy • Omentectomy (removal of omentum fat) Platinum-based combined with Taxane-based chemotherapy (e.g. Paclitaxel)**
Recurrent OC	Chemotherapy	Platinum sensitivity:Platinum-based chemotherapy combined with taxane-based or novel drugs (e.g. Carboplatin and Paclitaxel or Carboplatin and Pegylated Liposomal Doxorubicin (PLD)) Platinum resistance: Single-agent therapy (Topotecan, Docetaxel, oral Stoposide, PLD, Gemcitabine, Ifosfamide) Novel strategies : Olaparib (PARP inhibitor) for BRCA1/BRCA2 mutation carriers (Phase I clinical trials)

Note 1*: Clear Cell EOC (Early stage OC) is mostly resistant to Carboplatin and Paclitaxel

Note 2**: Disagreements in literature on the benefits of this treatment strategy

Even though treatment in early stages of the disease (stage I or II) results in 90% 5-year survival rate, only 25% of women are diagnosed in these stages (due to ineffective screening procedures) [5]. Thus, the majority of OC patients are in advanced stages (III or IV) and the 5-year survival rate drops dramatically to 10-30% [1,5]. This may be attributed to the effectiveness of treatment; the maximal cytological reduction surgery is thought to increase the efficacy of adjuvant chemotherapy (by relieving as much tumour burden as possible) but there is insufficient evidence to fully support this [5]. Furthermore, in advanced OC, the adjuvant chemotherapy mostly involves a combination of platinum and taxane drugs because they are thought to improve survival [1]. Whereas one study supported that platinum drugs combined with Paclitaxel (taxane drug) increases survival [7], another study showed no substantial benefit in outcome [1]. In the case of relapsed OC, it was shown that the residual disease could not be detected in the first 5 months after treatment in 50% of patients with OC [2]. Unfortunately, in recurrent OC, the survival rates are even lower than advanced OC, especially since some patients become refractory to platinum-based therapy [5]. These patients generally don't respond well in other single-agent treatments that they are offered (Table 1); thus there is great need to develop new therapies which will increase their response and survival.

The overall late diagnosis of OC (and recurrent OC) due to ineffective screening programs (lack of imaging studies and appropriate serum biomarkers), the debate on treatment strategies, as well as the general inefficiency in current therapies, may be major causes of high mortality rates in OC [2]. Consequently, further investigation to design screening procedures and to find new therapeutic options is essential to improve survival of patients suffering from OC. A drug that started to emerge in the OC setting and started showing promising results in OC patients is Metformin.

1.2 Association of Metformin and Ovarian Cancer

1.2.1 Epidemiological Studies

Metformin (1-(diaminomethylidene)-3,3-dimethylguanidine) is derived from *Galega officinalis* (French lilac) [6,10] and is commonly used for the management of type II diabetes [8]. Currently around 120 million people are taking Metformin as a treatment for diabetes [8,10]. Metformin is an anti- hyperglycaemic drug which inhibits glucose absorption in the intestine and gluconeogenesis in the liver; thus, it results in reduced levels of circulating insulin and glucose, as well as increased glucose uptake in muscles and adipose tissue [6,8,9].

Various epidemiologic studies have shown that Metformin is associated with reduced risk of cancer in diabetic patients [11,12]. Other studies have shown that the use of Metformin significantly reduces the risk of cancers like breast [13], pancreatic [14] and prostate [15]. Moreover, a recent meta-analysis involving a larger cohort of patients, demonstrated that Metformin reduces the risk of cancer incidence and reduces mortality by 31% when is used by diabetic cancer patients suffering from colorectal, pancreatic, hepatocellular, breast or lung cancer [16]. Further studies focused on OC, validated that the long-term use of Metformin is associated with a decreased risk of OC [18] and increased survival for OC patients [19].

However, the above-mentioned epidemiologic studies have some drawbacks. Most of them were retrospective and their cases were obtained from hospitals or clinics (suggesting selection bias) [6,9]. Furthermore, these studies were only based on diabetic patients; thus, it is unknown whether the difference in physiology between a diabetic and non-diabetic will translate into different effects or efficacy of the drug [9]. Nonetheless, a recent pre-operative “window of opportunity” trial in non-diabetic women with breast cancer, has shown that Metformin treatment reduced tumour proliferation and altered expression of genes involved in inflammation, metabolism and growth [20]. Moreover, the observations of the above-mentioned studies were in agreement with following *in vitro* and *in vivo* studies, proposing that Metformin has an anti-

proliferative effect across various cancer cell lines and animal models [8,9]. It's noteworthy to mention that Metformin is a relatively inexpensive drug that is already approved and used since the 1950's [8]. This drug is generally well tolerated with a very low risk for harmful side-effects (lactic acidosis in 3 out of 100,000 patients) [6]. Consequently, Metformin use has several advantages as a novel therapy; thus, it can be considered a good candidate for further investigation in order to identify its potential therapeutic role in ovarian cancer.

1.2.2 Proposed Mechanisms of Metformin action in ovarian cancer

The role of Metformin has been investigated in numerous studies across various cell lines and animal models [6,8,9]. For the purpose of this study we will mostly focus on literature investigating the roles and proposed mechanisms of Metformin in OC. Briefly, Metformin regulates insulin and glucose levels, induces growth inhibition and cell death, potentiates the cytotoxic effects of drugs commonly used in chemotherapy, and potentially has a role in DNA damage and DNA Damage Response (DDR) [6,8,9] (Fig.1). Metformin is transported into cells through organic cation transporters (OCT1, -2 and -3) [9,21]. These transporters are essential for Metformin transportation, as previous studies using OCT1-deficient mice or the potent OCT1 inhibitor KU-55933, showed that Metformin cellular uptake and subsequent effects were significantly reduced or abolished, respectively [21,22].

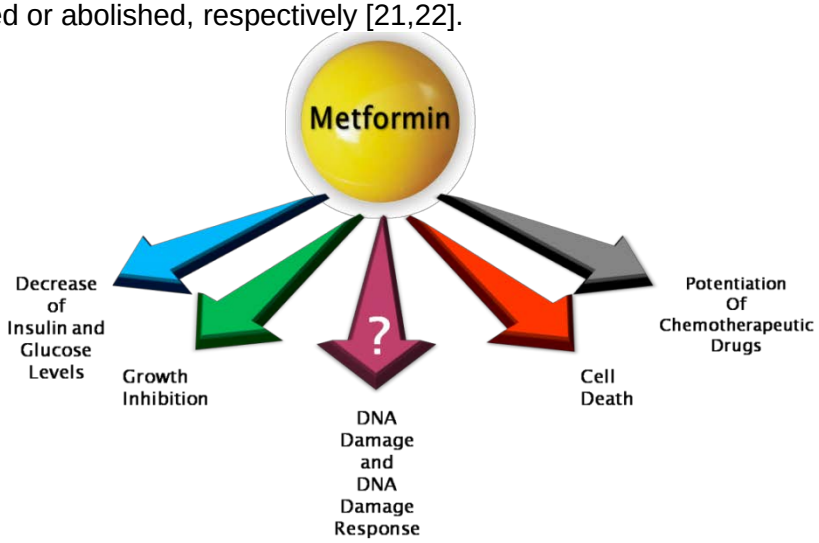


Figure 1. Proposed mechanisms of action of Metformin

1.2.2.1 **Metformin induces growth inhibition**

When Metformin is transported into the cells, it inhibits mitochondrial complex I (though the precise mechanism is unknown, to date) [23] (Fig.2). Metformin's ability to inhibit complex I compromises the mitochondrial respiratory chain, eventually leading to decreased ATP synthesis [8]. As a result, the AMP: ATP ratio in the cell is increased, leading to energy stress and activation of AMPK (AMP-activated protein kinase) [24]. AMPK is activated through conformational changes by AMP, which uncover the α-catalytic subunit of AMPK, leading to phosphorylation of residue Thr172 by LKB1 kinase (liver kinase B1) [25]. AMPK is considered a regulator of energy homeostasis; thus, in cases of energy stress it becomes activated resulting in down-regulation of energy-consuming pathways (e.g. protein synthesis) and up-regulation of energy-producing pathways (e.g. fatty acid oxidation) [6,8]. Activation of AMPK leads to a cascade of downstream events resulting in mTOR pathway down-regulation, which eventually induces protein synthesis arrest and growth inhibition [26,27]. Consequently, as observed in other cancer types, Metformin is thought to cause growth inhibition in OC by indirect activation of AMPK, leading to mTOR pathway downregulation and eventually protein synthesis and proliferation arrest [27,28]. Both *in vitro* and *in vivo* studies have shown the growth-inhibitory effects of Metformin in OC [26-32].

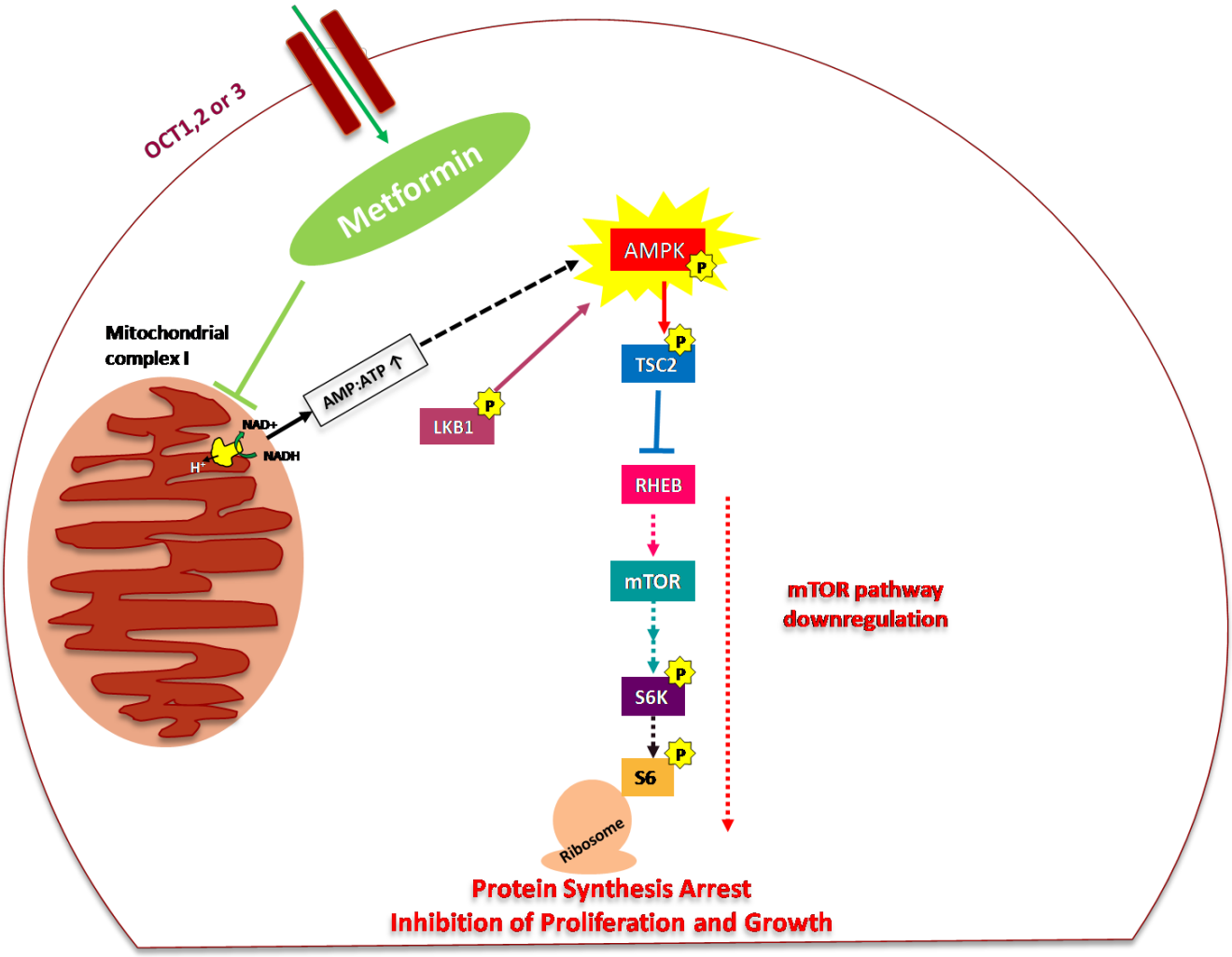


Figure 2. Metformin induces growth inhibition though AMPK activation and mTOR pathway inhibition. Metformin is transported into the cell through organic cation transporters (OCT1, 2, or 3). Metformin inhibits complex I of the mitochondrial respiratory chain leading to reduced ATP synthesis. This increases the AMP: ATP ratio so the increased AMP and LKB1 (Liver Kinase B1) activate AMPK (AMP-activated protein kinase) through allosteric interactions and phosphorylation on Thr172. Activated AMPK phosphorylates TSC2 (Tuberous Sclerosis 2, GTPase Activating Protein), which function inhibits RHEB (GTP-binding protein, Ras Homolog Enriched in Brain). RHEB inhibition results in reduced activation of its target, mTOR (mammalian/mechanistic target of Rapamycin-Ser/Thr kinase). This leads to inhibition of mTOR signalling resulting to down-regulation of proteins involved in translation like S6K (S6 kinase) and S6 (ribosomal protein); thus, mTOR pathway downregulation by Metformin eventually leads to inhibition of protein synthesis, growth and proliferation.

1.2.2.2 Metformin regulates insulin and glucose levels

Besides growth inhibition, Metformin regulates insulin and glucose levels in a similar way as in Metformin-treated diabetic patients [9]. As mentioned before, Metformin activates AMPK which results in inhibition of gluconeogenesis (energy-consuming process) in the liver, eventually reducing insulin and glucose levels and increasing glucose uptake in skeletal muscle [6,8,9]. By reducing circulating insulin, Metformin is thought to reduce ligand binding to insulin receptors; thus, Metformin can indirectly down-regulate the insulin signaling pathway in tumours [9,33]. Recently, Metformin was shown to exert a more direct effect on insulin signaling; by down-regulating a downstream target of the insulin receptors called IRS-1 (Insulin Receptor Substrate-1) [33]. IRS-1 is a widely expressed protein that, in the presence of insulin, becomes phosphorylated by insulin receptors (or by Insulin Growth Factor-1 Receptors, IGF1-R) resulting in activation of downstream insulin-associated signalling pathways like PI3K-AKT/Protein Kinase B(PKB) and Ras-MAPK [34]. IRS-1 is regulated by phosphorylation and has various phosphorylation sites (for tyrosine and serine) [35]. Depending on the phosphorylation status, IRS-1 may be activated or inhibited. In general, tyrosine phosphorylation activates IRS-1, while serine phosphorylation inhibits IRS-1 function [34,35]. It was proposed that activation of AMPK by Metformin, lead to phosphorylation of IRS-1 in Ser794, resulting to inhibition of IRS-1 and decreased activation of PI3K pathway, suggesting that Metformin down-regulates insulin-associated signalling pathways [33].

1.2.2.3 Metformin induces cell death

Metformin was also shown to promote cell death across multiple cell lines through both caspase-dependent and caspase-independent mechanisms [36-40]. A recent study in OC showed that Metformin induces apoptosis through caspases [37]. It is known that the BCL-2 protein family is a major regulator of apoptosis through caspases [41]. Anti-apoptotic BCL-2 proteins, such as BCL-2, BCL-w, BCL-xL and MCL1 are incorporated into the outer membrane

of mitochondria and inhibit pro-apoptotic BCL-2 family proteins *via* direct binding [42]. The pro-apoptotic BCL-2 proteins are either signal mediators or effectors; for example, BAD and BIK mediate stress signals when they interact with other BCL-2 proteins, while the effectors BAX and BAK1 form proteolipid pores on the outer membrane of mitochondria [37,42]. In cases where the ratio of anti-apoptotic to pro-apoptotic proteins is decreased, the pro-apoptotic effector proteins permeabilise the mitochondrial membrane, leading to release of apoptotic mitochondrial proteins that activate caspases resulting in apoptosis [41]. In the OC study, it was shown that Metformin decreases the expression of anti-apoptotic proteins BCL-2, Bcl-xL and Mcl-1, resulting to induction of the pro-apoptotic proteins, BAX and BAD which lead to activation of caspases and apoptosis [37]. In breast cancer, apoptosis by Metformin is also conducted by a caspase-independent mechanism involving the activation of PARP (poly (ADP-ribose) polymerase) which results in nuclear translocation of AIF (apoptosis-inducing factor) that leads to apoptosis [36]. It is unknown, however, if this Metformin-induced mechanism of apoptosis also occurs in OC.

1.2.2.4 Metformin potentiates the cytotoxicity of chemotherapeutic drugs

Due to epidemiological data suggesting that Metformin reduces cancer risk and increases survival, many studies have combined Metformin with other drugs to assess its potential use in chemotherapy. In OC, Metformin was shown to potentiate the cytotoxic effects of Cisplatin *in vitro* [26,29] and *in vivo* (Metformin and Cisplatin synergistically reduced size, proliferation and mitotic count of OC tumours in mice) [29]. Furthermore, another study using OC cell lines and primary cultures from OC patients in advanced stages (III-IV) showed that Metformin potentiates the cytotoxic effects of Carboplatin but not Paclitaxel or Doxorubicin [30]. Moreover, a study showed that the combination of Metformin with PEITC (phenethyl isothiocyanate) increases growth inhibition and cytotoxicity in OC cell lines in a synergistic manner [31]. However, in other cell lines there are contradicting studies. For example, *in vitro* studies showed that Metformin

acts synergistically with Paclitaxel and potentiates its growth inhibitory effects in endometrial cancer [43], while in ovarian cancer no such effects were observed [30]. Another interesting example is that Metformin, in contrast to its effect on OC cell lines, is reducing the cytotoxicity of Cisplatin in other human cell lines (U251 glioma, SHSY5Y neuroblastoma and HL-60 leukemia cell lines [44]). These observations suggest that depending on the cell line of interest and possibly the dose of Metformin, the combination of drugs exerts different effects; thus, further investigation on the adjuvant potential of Metformin is essential.

1.2.3 Controversy surrounding Metformin's role in DNA Damage and the DDR

Besides the established roles of Metformin detailed above, recent studies have proposed that the drug is involved in the DNA damage and DDR [6,8,9].

1.2.3.1 DNA damage and DDR

Integrity of the cellular genome is constantly threatened by DNA damage, which can be induced endogenously or exogenously, and can cause tens of thousands of DNA lesions per day [45,46]. These lesions can impair DNA synthesis and replication and can even lead to genomic abnormalities, mutagenesis and neoplastic transformation if left unrepaired or incorrectly repaired [45,47]. Single strand DNA breaks (ssDNA breaks) and double strand breaks (DSBs; the most damaging lesions) are two of the possible lesions that can occur after exposure to DNA damaging agents [47]. These breaks can occur either endogenously (e.g. by oxidative stress through reactive oxygen or nitrogen species, ROS and RNS) or exogenously (e.g. through ionizing radiation (IR) or ultraviolet light (UV)) [45]. Free radicals derived from ROS (e.g. hydroxyl radicals) and RNS (e.g. nitrogen oxides) can be produced endogenously, for example from mitochondria. At high concentrations, these radicals can cause oxidative stress, inducing damage to proteins, lipids and DNA. DNA damage lesions include oxidation of bases (e.g. formation 8-Hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine), modifications of

purines, pyrimidines or deoxyribose, DNA-protein crosslinks, ssDNA breaks or induction of DSBs [71].

In order for the cell to counteract these DNA damage lesions, a network of pathways has developed that detects lesions, amplifies the signal for detection, and induces appropriate cellular responses e.g. DNA repair; this network of pathways is termed the DNA Damage Response (DDR) [45].

Briefly, upon DNA damage and formation of DSBs, the Mre11/Rad50/NBS1 (MRN) complex recognizes DSBs and activates a member of the phosphatidylinositol-3 (PI3) kinases family, namely Ataxia-Telangiectasia Mutated (ATM) protein kinase [45,47] (Fig.3). ATM can then phosphorylate the Ser139 residue of the histone H2AX (forming phospho-H2AX (γH2AX)). Then, MDC1 (Mediator of DNA damage Checkpoint 1) is recruited to γH2AX and stabilizes the MRN complex leading to further ATM-activation and expansion of γH2AX along chromatin [47,48]. Furthermore, RNF8, RNF168 and HERC2, are recruited to adjacent chromatin leading to histone H2A and H2AX ubiquitination [48]. This ubiquitination event is thought to relax chromatin in order to enable 53BP1 (p53-binding protein 1) binding to histone methyl groups surrounding DSBs [48]. 53BP1 binding to DSBs sites can further activate ATM leading to ATM-dependent phosphorylation of 53BP1, DNA repair proteins or proteins involved in the DNA damage cell cycle checkpoints [47]. More specifically, ATM together with its target (CHK2) co-operate to downregulate the function of Cyclin-dependent kinases (CDK's) in order to induce delay or cell cycle arrest in G1–S, intra-S and G2–M cell cycle points (termed "DNA damage cell cycle checkpoints"). The delay on cell cycle progression enables more time for completion of DNA repair before the cell enters replication or mitosis [45].

Evidently, the cell has evolved multiple DNA repair mechanisms designated for different types of DNA lesions [45]. For DSB repair, two main mechanisms can take place: Non-

Homologous End Joining (NHEJ) and Homologous Recombination (HR). NHEJ is an error-prone repair mechanism that can occur at any phase of the cell cycle and it generally involves joining of the damaged ends [45]. Conversely, HR occurs only in S or G2 phase, as sister chromatids are used as templates for this error-free DNA repair [49]. Briefly, during HR at the DSBs site, the MRN complex creates ssDNA breaks by resection [45]. These strand ends are then coated by Replication Protein A (RPA) and Rad51 and Rad52, which can lead the damaged strand to hybridise with the sister-chromatid homologous template strand, in order for the repair to take place [49]. Besides the importance of Rad51 in HR, another important protein for DNA repair is PARP1 (Poly (ADP-ribose) polymerase 1) which is an ADP-ribosylating enzyme [50]. PARP1 recognizes ssDNA breaks and DSBs, it becomes activated and can further mediate the assembly of multi- protein complexes involved in DDR; for example it can recruit ATM in order to activate downstream signalling for DNA damage and DNA damage cell cycle checkpoint arrest [50].

A useful experimental tool to study DNA damage and DSBs detection is through Immunofluorescence (IF), where several proteins involved in DDR such as γH2AX and 53BP1, can be visualized by their recruitment to large protein complexes, as distinct structures named “foci” [47]. Thus, γH2AX and 53BP1 foci are considered valuable DNA damage signaling markers used regularly in the literature [46,51,52]. Furthermore, several studies measure the levels of Ser1981 phosphorylated-ATM (activated ATM) as indicative of active DNA damage signaling [53,54].

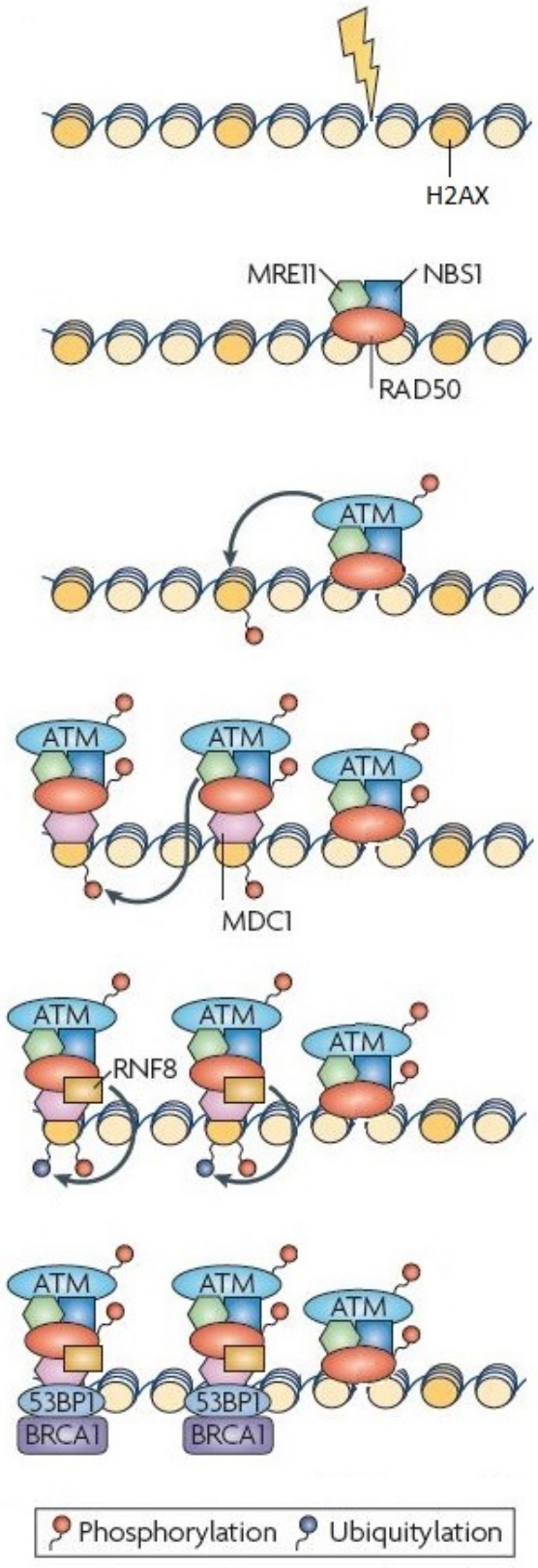


Figure 3. Protein complexes formation in the DNA Damage Response. Upon DNA damage causing DSBs, Mre11/Rad50/NBS1 (MRN) complex recognizes DSBs and activates ATM protein kinase via phosphorylation (Ser1981). ATM then phosphorylates adjacent H2AX (Ser139, γH2AX). MDC1 is recruited to γH2AX and leads to retention of MRN resulting in further ATM phosphorylation and expansion of γH2AX. RNF8 (with RNF168 and HERC2-not shown for simplicity) is recruited to adjacent chromatin leading to histone H2A and H2AX ubiquitination. Ubiquitination opens chromatin and enables 53BP1 binding.53BP1 binding further activates ATM and can recruit DNA repair proteins like BRCA1.DNA repair events and DNA damage cell cycle checkpoint arrest then follow.

Adapted from Misteli and Soutoglou (2009)

1.2.3.2 Metformin's association with oxidative stress, DNA damage and DDR

Several studies have associated Metformin's role with oxidative stress, DNA damage and the DDR; however, there are disagreements in the literature concerning the actual role of the drug. It's noteworthy that there are no current studies on OC investigating this potential role of Metformin.

Some studies have indicated that Metformin exerts a protective effect from DNA damage as indicated by reduced DNA damaged signaling (reduced γH2AX expression [53], reduced γH2AX foci formation [55] or reduced ATM activation [53]). More specifically, Halicka *et al.* (2011) observed that A549 lung adenocarcinoma, TK6 human lymphoblastoid and A431 epidermoid carcinoma cells treated with Metformin, had reduced levels of expression of γH2AX and activated ATM (Ser1981 phospho-ATM) as an indication of attenuating the DDR [53]. Furthermore, they demonstrated that Metformin effectively reduced ROS levels as evidenced by their reduced ability to oxidize 2',7'-dihydro-dichlorofluorescein diacetate (H2DCF-DA) [53]. ROS can oxidize H2DCF-DA (non-fluorescent) to a fluorescent compound called DCF, measured by flow cytometry to indicate levels of ROS [56]. Similarly, Algire *et al*. (2012) observed that Metformin and combination of Metformin with paraquat (stimulator of endogenous source of ROS) resulted in reduced γH2AX foci formation and reduced levels of DCF, suggesting that Metformin reduces ROS levels in human mammary epithelial cells [55]. Collectively, these studies suggested that Metformin reduces DNA damage signaling, and attributed this effect on Metformin-induced reduction of oxidative stress (reduction of ROS levels) [53,55]. The proposed mechanism is that Metformin reduces ROS levels by inhibiting mitochondrial complex I of the respiratory chain [53,55]. Inhibition of complex I compromises the electron flow in the electron transport chain, leading to reduced production of ROS by complexes I and III (mitochondrial ROS producers) [8,57].

Conversely, other studies propose that Metformin induces DNA damage as indicated by increased DNA damage signaling (increased γH2AX expression [58] or increased γH2AX foci formation [59]). Liu *et al.* (2012) supported that Metformin treatment induces DNA damage evident from comet assays and increased DNA damage signaling (increased γH2AX expression) in hepatocellular carcinoma (HepG2 and Bel-7402 cell lines [58]). Furthermore, they hypothesized that Metformin impairs DNA repair as evidenced from persistently increased DNA damage signaling (increased γH2AX expression long after Metformin treatment) [58]. Fasih *et al.* (2014) observed increased γH2AX foci formation after Metformin treatment in pancreatic cancer cell lines (MiaPaCa-2 and Panc1) [59]. Whereas Liu *et al.* (2012) supported that Metformin impairs DNA repair [58], Fasih *et al.* (2014) supported that Metformin does not affect DNA repair since the formation of γH2AX foci did not persist over time [59]. Furthermore, Liu *et al.* (2012), Fasih *et al.* (2014) and Song *et al.* (2012) observed increased DNA-damaging effects of Metformin when combined with IR [58,59,60]. They suggested that Metformin may be a promising radiosensitising agent of hepatocellular, pancreatic or breast carcinoma respectively [58,59,60]. This was evident from increased DNA damage signaling (increased γH2AX expression [58] or increased γH2AX foci formation [59], respectively) or from decreased survival of cells treated with Metformin and IR (compared to Metformin or IR treatment alone) [58-60].

Collectively, the above-mentioned studies hypothesised that the DNA-damaging effects of Metformin may be attributed to impaired DNA repair from ATP deprivation (Metformin-induced inhibition of mitochondrial complex I that reduces ATP synthesis [8]) [58]; or to Metformin-induced AMPK-dependent activation and consequent mTOR pathway downregulation [59,60].

Interestingly, another study supported that Metformin protects from DNA damage by activating the DDR as evidenced from increased ATM activation and increased γH2AX foci formation in A431 epidermoid carcinoma cells [54]. They hypothesised that Metformin's role was to protect from DNA damage by activating a "pseudo-DDR" in the absence of actual damage (supported

by absence of phosphorylated-ATM and γH2AX foci co-localisation) [54]. However, Halicka *et al.* (2011) has repeated the experiments of this study and provides opposing observations, directly contradicting the original study [53,54].

In conclusion, the numerous disagreements in the literature have raised a controversy surrounding the role of Metformin in DNA damage, oxidative stress and DDR. Combined with the absence of studies on the potential role of Metformin in OC, this highlights the importance of further investigation of the link between Metformin, DNA damage and OC.

1.3 Ovarian cancer cell lines representative of ovarian tumours

It is known that genomic differences exist between cell lines and tumours [61]. Hence, it was important for this study to acquire cell lines that are appropriate ovarian tumour models. A recent publication compared genetic profiles of OC cell lines (e.g. gene mutation and protein expression profiles) with ovarian tumours, defining which cell lines have the highest genetic similarity with clinical tumour samples; thus indicating which OC cell lines are deemed more suitable and representative of ovarian tumours [61]. This study reported that the most commonly used cell lines for ovarian cancer research e.g. SK-OV-3, A2780, CAOV3 and IGROV1 are not representative of tumour samples [61]. On the other hand, they indicated that OVCAR3 is a “moderately good” non-specified epithelial OC model, while COV318 (rare OC cell line) was classified as a “good” serous epithelial OC model [61]. Thus, OVCAR3 and COV318 cancer cell lines were chosen for this study for their classification as representative OC cell lines.

2. AIMS OF THE PROJECT

This project is focused on evaluating the role of Metformin in the DNA damage and the DDR in the ovarian cancer cell lines OVCAR3 and COV318 (high-grade epithelial OC cell lines). DDR will be determined by monitoring the expression of DNA repair proteins (Rad51 and PARP1) and the recruitment of DNA damage markers (γH2AX and 53BP1 foci) post-Metformin treatment. Furthermore, the potential effect of Metformin in the proliferation and survival of ovarian cancer cells will be determined in conditions when Metformin is combined with IR or Gemcitabine treatment (chemotherapeutic drug commonly used in ovarian cancer treatment).

3. MATERIALS AND METHODS

3.1 Cell lines and chemical reagents

The OC cell line COV318 was sourced from Sigma-Aldrich Company and the OC cell line OVCAR3 was a kind gift from Dr. Sudha Sundar (University of Birmingham). The breast cancer cell line (MCF-7), the osteosarcoma cell line (U2OS) and the cervical cancer cell line (HeLa) are routinely cultured in the Morris Laboratory. COV318, MCF-7, HeLa and U2OS cells were cultured at 37˚C and 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) high glucose media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. OVCAR3 cells were cultured at 37˚C and 5% CO_2 in DMEM high glucose media supplemented with 20% FBS, 1% Penicillin/Streptomycin, 0.01mg/ml insulin and 1% MEM Non-essential Amino Acid Solution. 1x PBS (Phosphate Buffered Saline) solution was made from PBS tablets and 1X Trypsin EDTA solution was made from Trypsin-EDTA Solution 10X. PBS and Trypsin-EDTA were also used for cell culture. Metformin Hydrochloride and Gemcitabine Hydrochloride were dissolved in water, as recommended by the supplier. The above mentioned reagents and cell lines are distributed by Sigma-Aldrich Co.

3.2 Western Blot analysis

Western Blot analysis was performed to determine the expression levels of Rad51, PARP1 (DNA repair proteins) and IRS-1 after Metformin treatment. MCF7, COV318 and OVCAR3 cell lines were seeded in 6-well plates one day prior to treatment (seeding confluency 1 x 10^6 cells per well). The next day, the cells were treated with various doses of Metformin (0, 50 μM, 500 μM, 1 mM, 2 mM, 5 mM) for 24 hours (MCF7 cells were left untreated). After treatment, the cells were washed with PBS (to remove Metformin) and trypsinised (with Trypsin-EDTA solution) followed by the addition of the appropriate cell culture media to inactivate trypsin. The cells were transferred in falcon tubes and were centrifuged at 1800 rpm for 5 minutes. The media was then

removed (supernatant) and the pellet (concentrated cells) was washed with PBS followed by another centrifugation (1800 rpm for 5 minutes). The supernatant was removed, the cells were lysed in 8M Urea Lysis Buffer (8M Urea, 0.1M Na_2PO_4/NaH_2PO_4, 0.01M Tris-HCl (pH 8) and 10mM β-mercaptoethanol) and the cell lysates were sonicated to further disrupt cellular membranes. A Bradford Assay was conducted to quantify protein concentration (using Bradford Dye Reagent (AlfaAesar Co.)) using known protein concentrations of BSA (Bovine Serum Albumin: Fisher BioReagents™) as protein standards. Cell lysates (50 μg total protein) were separated by SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis) (150V for 60 minutes). SDS-polyacrylamide gel preparation involved preparation of a 10% separating gel (ddH2O, 20% SDS, 1.5M Tris-HCl (pH 8.8), 30% acrylamide, TEMED (Tetramethylethylenediamine) and APS (Ammonium Persulfate) (10%)) and preparation of a 5% stacking gel (ddH2O, 1M Tris (pH 6.8), 30% acrylamide, TEMED, and APS (10%)). Acrylamide 30% Solution is supplied by Protogel-Gene Flow Co. TEMED, SDS and APS were sourced from Sigma-Aldrich Co. For SDS-PAGE, 2 protein ladders were used: BLUeye Prestained Protein Ladder (GeneFlow) and HiMark™ Pre-stained Protein Standard (Invitrogen). After SDS-PAGE, the proteins were transferred to PVDF membranes (Immobilon®-P Polyvinylidene difluoride membranes: Sigma-Aldrich Co.) and were blocked for 45 minutes in block solution (5% Marvel in PBS-T (PBS, 0.1% Tween™ 20 (sourced from Fisher BioReagents™))). After blocking, the membranes were probed with primary antibodies overnight at 4^oC. The membranes were then washed with PBS-T and incubated with HRP-conjugated secondary antibodies for 90 minutes in room temperature (RT). After washes with PBS-T the proteins were detected using the EZ-ECL Chemiluminescence detection kit for HRP (BI Co).

The primary antibodies used for Western Blot analysis were Rad51 (rabbit 1:500), α-tubulin (mouse 1:10.000), PARP1 (mouse 1:1000) and IRS-1 (rabbit 1:500) which were diluted in block

solution. Rad51 and α-tubulin were purchased by Santa Cruz Biotechnology, Inc., PARP1 was a kind gift from Dr. Grant Stewart and IRS-1 was purchased from Millipore™. Secondary antibodies were the HRP-conjugated rabbit-anti mouse (1:10.000 for PARP1 and 1:30.000 for α-tubulin) and the HRP- conjugated swine-anti rabbit (1:20.000 for Rad51 and 1:10.000 for IRS-1) which were also diluted in block solution (Dako Co.).

3.3 Immunofluorescence (IF) Experiments

3.3.1 IF of OVCAR3, COV318, U2OS and HeLa cells stained for 53BP1 and γH2AX to determine the most appropriate DNA damage marker

OVCAR3, COV318, U2OS and HeLa cells were seeded on circular cover slips (1 x 10^5 cells) in 24-well plates and left overnight to adhere. The following day the cells were irradiated with various doses of irradiation (IR: 0, 2 and 8 Gy) and were fixed at several different time points post-IR (0.5, 1, 4 and 24 hours). For fixation, the media was firstly removed followed by addition of 4% Paraformaldehyde (PFA: Fisher-Scientific) for 10 minutes. The cells were then washed with PBS, followed by addition of cold absolute methanol for 10 minutes (chilled to -20^{o}C) for permeabilisation. The cells were then washed with PBS prior to incubation in block solution (10% FBS in PBS) for 5 minutes.

After blocking, the cells were stained with the primary antibodies 53BP1 (rabbit, 1:2000: Abcam) and γH2AX (rabbit, 1:2000: Millipore), which were incubated for 1 hour RT. After one wash with PBS, the secondary antibody was added for 1 hour RT (AlexaFluor® 488 anti-rabbit (1:2000)). Next, the cells underwent an additional fixation step with 4% PFA for 10 minutes, washed with PBS, and briefly incubated with Hoechst dye 33258 (1:25 000, purchased from Sigma-Aldrich Co.) to stain the cell nucleus followed by one PBS wash. The cover slips were then mounted onto slides (using IMMU-MOUNT® mounting medium: Thermo Scientific) and imaged using the Fluorescence Microscope Nikon E600. Images were processed with the ImageJ software.

3.3.2 IF of OVCAR3, COV318 and HeLa cells co-stained with 53BP1 (DNA damage marker) and Cyclin A (S/G2 cell cycle phase marker) to evaluate the effects of Metformin on DNA damage and DDR and cell cycle.

OVCAR3, COV318 and HeLa cells were seeded on circular cover slips (1 x 10^4 cells) in 24-well plates and left overnight to adhere. The following day, the cells were treated with Metformin (0 or 5 mM) for 24 hours and then treated with various doses of IR (0, 2 and 8 Gy).

The cells were then fixed at several different time-points post-IR (0.5, 1, 4, 24, 48 and 72 hours). The cells irradiated with 2 Gy IR were fixed 0.5, 1 or 4 hours post-IR, while cells irradiated with 8 Gy IR were fixed 24, 48 or 72 hours post-IR. Fixation, permeabilisation and blocking of the cells were done similarly as above. After blocking, the cells were stained overnight with the primary antibody Cyclin A (mouse, 1:1000, purchased from Abcam) at 4^{o}C. The next day, Cyclin A antibody was removed and the cells were stained with the primary antibody 53BP1 (rabbit, 1:2000) for 1 hour RT. After one PBS wash, the cells were incubated with secondary antibodies for 1 hour RT (AlexaFluor®533 anti-mouse (1:2000) and AlexaFluor®488 anti-rabbit (1:2000)). Next, the cells were washed, post-fixed, stained with Hoechst dye 33258, washed once with PBS and the cover slips were mounted onto slides, as above. The images were taken with the Fluorescence Microscope Nikon E600 and processed with the ImageJ software (including 53BP1 foci counting). 53BP1 foci were counted in 50 cells per condition per treatment (total of 3600 cells) and were quantified.

3.4 Colony Formation Assays

3.4.1 Colony Formation Assay to determine if Metformin potentiates the effects of IR by assessing clonogenic survival

COV318 and OVCAR3 cells were treated with Metformin (0 or 5mM) for 24 hours (acute treatment). The cells were then washed (PBS), trypsinised (Trypsin EDTA solution) and irradiated with various doses of IR (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 Gy). Next, after cell counting (using Fast-Read 102® counting slides) the cells were seeded in 6-well plates (in sextuplicates), in different seeding confluencies (1.000, 1.300, 1.600, 2.000, 4.500, 7.000, 11.000, 16.000, 20.000 or 40.000 cells/well depending on the IR dose). The cells were left to form colonies in 2ml of culture media (as appropriate for each cell line) at 37 ˚C and 5% CO_2 for 7 days. After 7 days, the media was removed and the colonies were fixed and stained using a dye of 0.5% Crystal Violet (Sigma-Aldrich Co.) diluted in 50% methanol and 50% ddH2O. After washes with water to remove the excess dye, the colonies were counted for each condition. Colonies containing >50 cells were counted and the survival curve was plotted. We designated non-irradiated (-/+ Metformin) cells as 100% colony survival to assess relative survival percentages of irradiated samples. This was done to exclude the growth-inhibitory or apoptotic effects induced by Metformin alone, so that the impact of Metformin on DNA damage/repair pathways alone could be assessed in response to IR.

3.4.2 Colony Formation Assay to determine if Metformin potentiates the effects of Gemcitabine by assessing clonogenic survival

OVCAR3 cells were washed (PBS), trypsinised (IX Trypsin EDTA solution) counted (using Fast-Read 102® counting slides) and were seeded in 6-well plates (2000 cells/well in sextuplicates) one day prior to treatment. The next day, the cells were treated with Metformin (0 or 5mM) for 22 hours followed by addition of Gemcitabine in various doses (0, 0.1, 0.5, 0.75, 1 and 2 μM) for 2 hours. Next, the cells were washed with PBS to remove the drugs, and then they were left to form colonies in 2ml of culture media (the appropriate media for OVCAR3) at 37˚C and 5% CO_2 for 7 days. After 7 days, the media was removed and the colonies were fixed and stained using the 0.5% Crystal Violet dye. After washes with water to remove the excess dye, the colonies were counted for each condition. Colonies containing >50 cells were counted and the survival curve was plotted. We designated as 100% colony survival the percentage of colonies that were untreated, as well as the percentage of colonies treated with 5mM Metformin only. This was done to exclude the growth-inhibitory or apoptotic effects induced by Metformin alone, so that the impact of Metformin on DNA damage/repair pathways alone could be assessed in response to Gemcitabine.

3.5 Statistical Analysis

The Western Blot analysis and the colony formation assay experiments were repeated 3 times (see Results for further detail). The graphs in IF and Colony Formation Assay experiments represented mean values, error bars denoted standard error. P-values were calculated using the Student-T test and the p-values $p<0.05$ or $p<0.01$ were considered statistically significant.

4. RESULTS

The role of Metformin in the DNA damage and DNA damage response (DDR) has been the centre of controversy in the literature [26,27,55,57]. Besides the disagreements between studies, there is currently no literature in OC focusing on these potential functions of Metformin. Consequently, it is of great interest to attempt to elucidate the role of Metformin in the DNA damage and DNA damage response in ovarian cancer. To achieve this, we selected two ovarian cancer cell lines OVCAR3 and COV318.

4.1 Evidence that OVCAR3 and COV318 ovarian cancer cell lines are able to uptake Metformin

A current disadvantage found in several studies focusing on Metformin, is the lack of evidence proving that the drug is effectively transported into the cells [9]. This raises the concern that some observed effects of Metformin may not be attributed to the actual function of the drug, but to adverse side-effects like cytotoxicity or necrosis [9]. Furthermore, it was observed that some cell lines are insensitive to the drug, for example, the breast cancer cell line MDA-MB231 [36]. For these reasons, our first goal was to verify that Metformin can be successfully transported into our OC cell lines OVCAR3 and COV318. One way to test if Metformin is transported intracellularly is by observing its effect on the insulin pathway.

As already mentioned in the Introduction, Metformin can reduce insulin levels leading to reduced ligand binding to Insulin Receptors [9]. Hence, it is proposed that this effect induces a down-regulation of the downstream pathway, including a negative regulation of IRS-1 (Insulin Receptor Substrate-1) [9]. The down-regulation of IRS-1 by Metformin can either be achieved by reducing protein expression or, as other studies have suggested, by affecting the

phosphorylation status of IRS-1. It was proposed that Metformin-induced AMPK phosphorylation of IRS-1 in Ser794 leads to reduced IRS-1 activation [33] (serine phosphorylation generally exerts negative regulatory role on IRS-1 [34]). Thus, we hypothesised that if Metformin is successfully transported into OVCAR3 and COV318 cells, it would lead to negative regulation of IRS-1 either by inducing down-regulation of IRS-1 expression or by alteration of its phosphorylation levels.

To address this issue, Western Blot analysis was carried out to detect IRS-1 in OVCAR3 and COV318 cells after a 24-hour Metformin treatment in various doses (5mM, 500μM and 50μM). This experiment was repeated 3 times. Note that the IRS-1 antibody used in this experiment recognises both unphosphorylated and phosphorylated forms of IRS-1 (it is not phospho-Ser794 IRS-1 specific). Observations in OVCAR3 cells revealed that various doses of Metformin induced reduced expression of both unmodified and phosphorylated IRS-1 compared to untreated cells (Fig. 4A). Consequently, Metformin treatment in OVCAR3 cell lines appears to result in general down-regulation of IRS-1 protein expression. In untreated COV318 cells, we observed increased presence of the phosphorylated forms of IRS-1 relative to unphosphorylated IRS-1. However, in Metformin-treated cells (independently of dose) the levels of phosphorylated IRS-1 appear to be reduced, relative to unmodified IRS-1 (Fig. 4B). These observations suggest that Metformin is indeed transported into OVCAR3 and COV318 cells as it causes a decrease in IRS-1 expression or modified phosphorylation status of IRS-1, respectively.

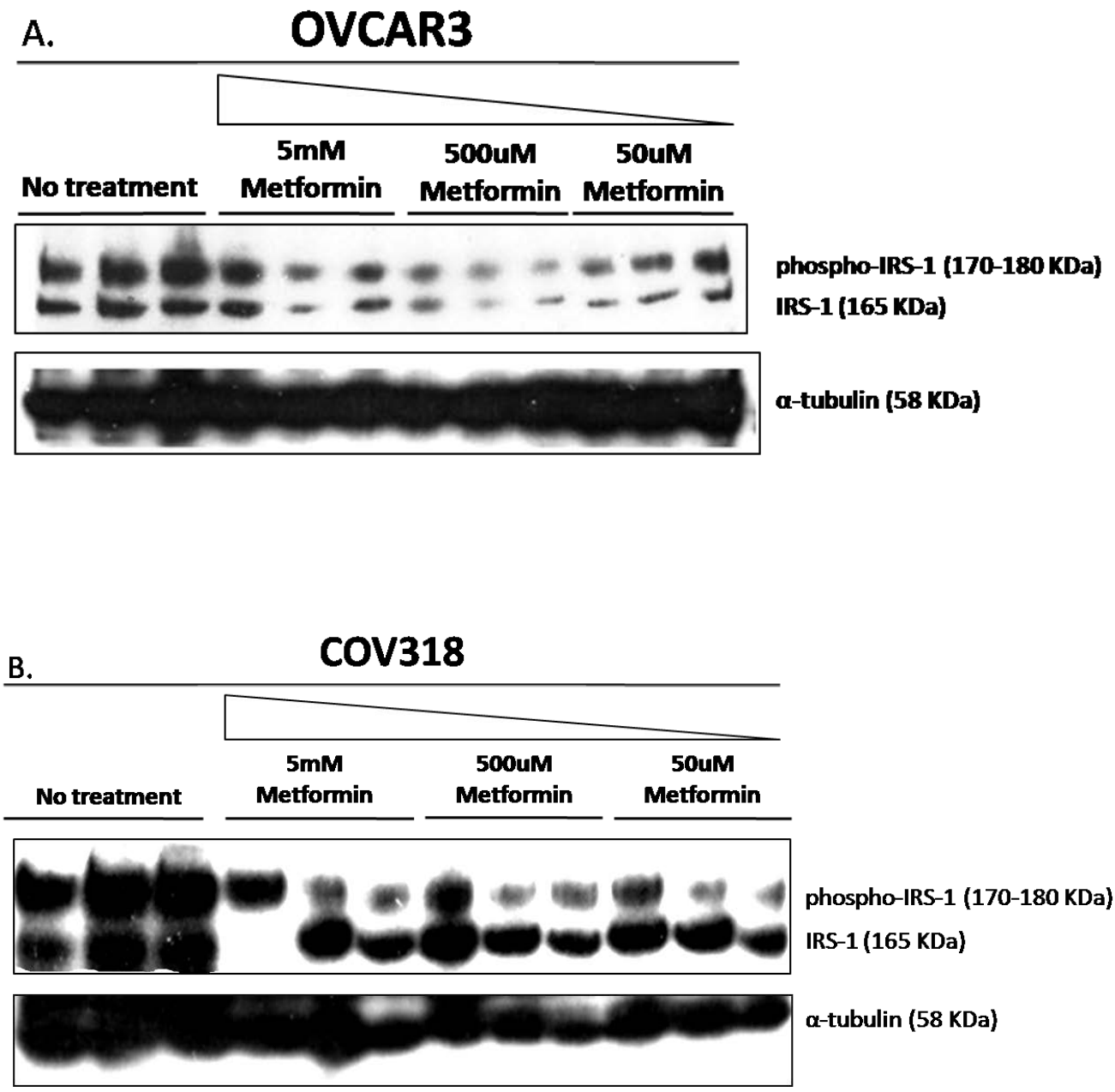


Figure 4. Western Blot analysis on OVCAR3 and COV318 cell lysates showing that Metformin is transported into the cells as evidenced by the alteration of IRS-1 expression

A) OVCAR3 cells were lysed after treated with Metformin (5 mM, 500 μM or 50 μM for 24 hours). Western Blot analysis on OVCAR3 cell lysates (probed for IRS-1 and a-tubulin) showed reduced expression of IRS-1 (both phosphorylated and unphosphorylated forms) compared to untreated cells. **B)** COV318 cells were lysed after treated with Metformin (5 mM, 500 μM or 50 μM for 24 hours). Western Blot analysis on COV318 cell lysates (probed for IRS-1 and α-tubulin) showed different expression levels of unphosphorylated and phosphorylated IRS-1 (compared to untreated cells) suggesting a reduction in IRS-1 phosphorylation. α-tubulin levels provided a loading control.

4.2 Metformin does not affect protein expression of Rad51 or PARP1 DNA repair proteins

Next, we evaluated whether Metformin has a role in the DNA damage and DNA damage response, firstly by assessing the effect of the drug on DNA-damage associated proteins. Previous unpublished data supported that Metformin treatment lead to down-regulation of Rad51 and PARP1 mRNA levels (genes implicated in DNA repair) in OVCAR3 cells (personal communication with Dr.Sudha Sundar). Hence, to determine whether Metformin treatment also down-regulates the protein expression of Rad51 and PARP1, Western Blot analysis was carried out in OVCAR3 and COV318 cells in order to examine the protein levels of these proteins after Metformin treatment. To achieve this, OVCAR3 and COV318 cells were treated with various doses of Metformin (0, 1, 2 and 5mM) for 24 hours followed by Western Blot analysis. The breast cancer cell line MCF7 was used as a positive control for Rad51 and PARP1 (non-treated controls; previously observed to have detectable levels of Rad51 and PARP1). This experiment was repeated 3 times. Metformin treatment was observed to have no effect on protein levels of Rad51 or PARP1 (independent of dose) relative to untreated cells in either OVCAR3 (Fig. 5A) or COV318 cells (Fig. 5B). In summary, Metformin does not have an effect on the protein expression of the DNA repair proteins Rad51 or PARP1.

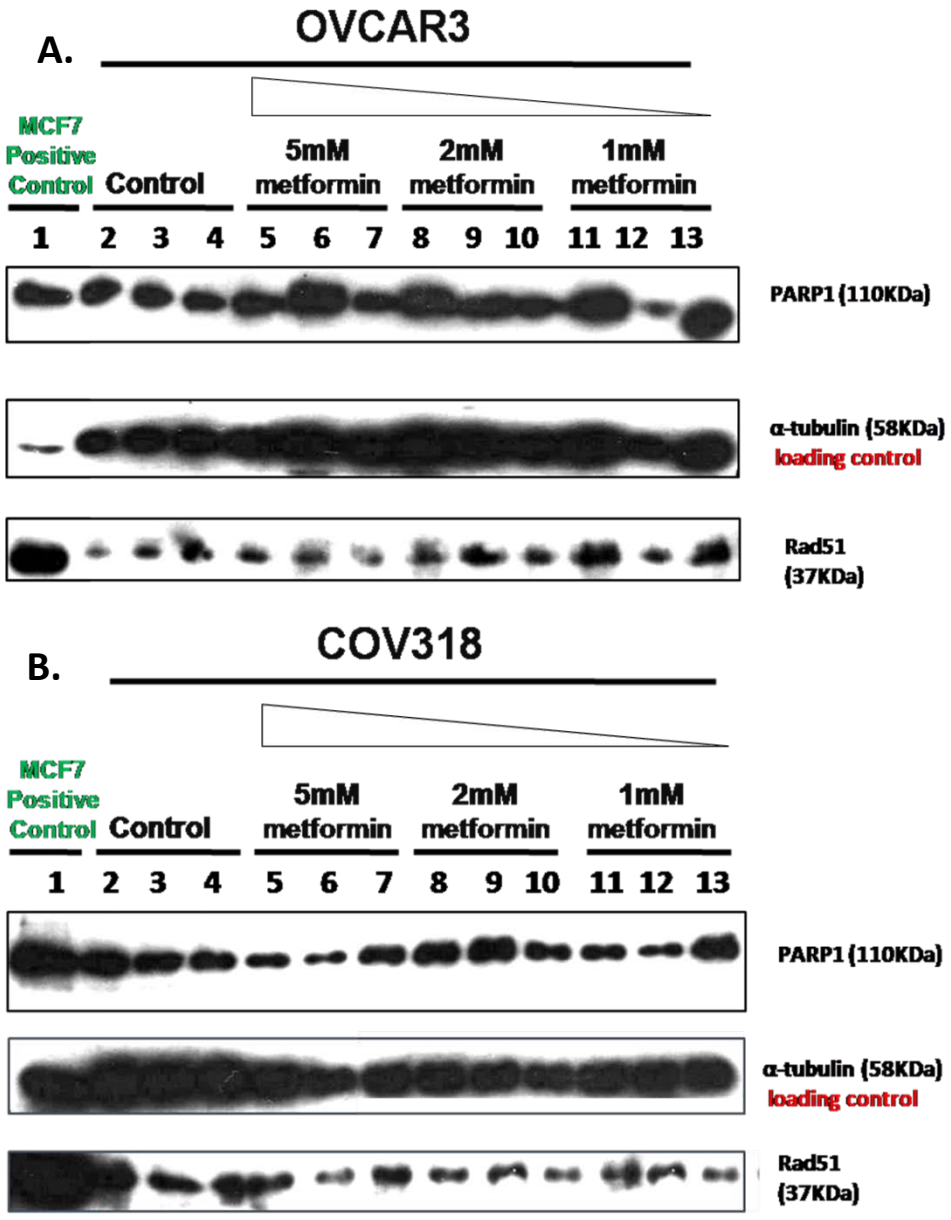


Figure 5. Western Blot analysis on OVCAR3 and COV318 cell lysates showing that Metformin does not down-regulate the protein expression of Rad51 or PARP1.

A) OVCAR3 cells were lysed after treated with Metformin (5 mM, 2 mM or 1 mM for 24 hours). Western Blot analysis on OVCAR3 cell lysates (probed for PARP1, Rad51 and α-tubulin) showed similar expression levels of Rad51 and PARP1 between Metformin-treated and control (untreated) cells. **B)** COV318 cells were lysed after treated with Metformin (5 mM, 2 mM or 1 mM for 24 hours). Western Blot analysis on COV318 cell lysates (probed for Rad51, PARP1 and α-tubulin) treated with Metformin showed similar expression levels of Rad51 and PARP1 compared with the control (untreated) cells. MCF7 cells were lysed and used as a positive untreated control (probed for Rad51, PARP1 and a-tubulin) in the Western Blot analysis. α-tubulin levels provided a loading control.

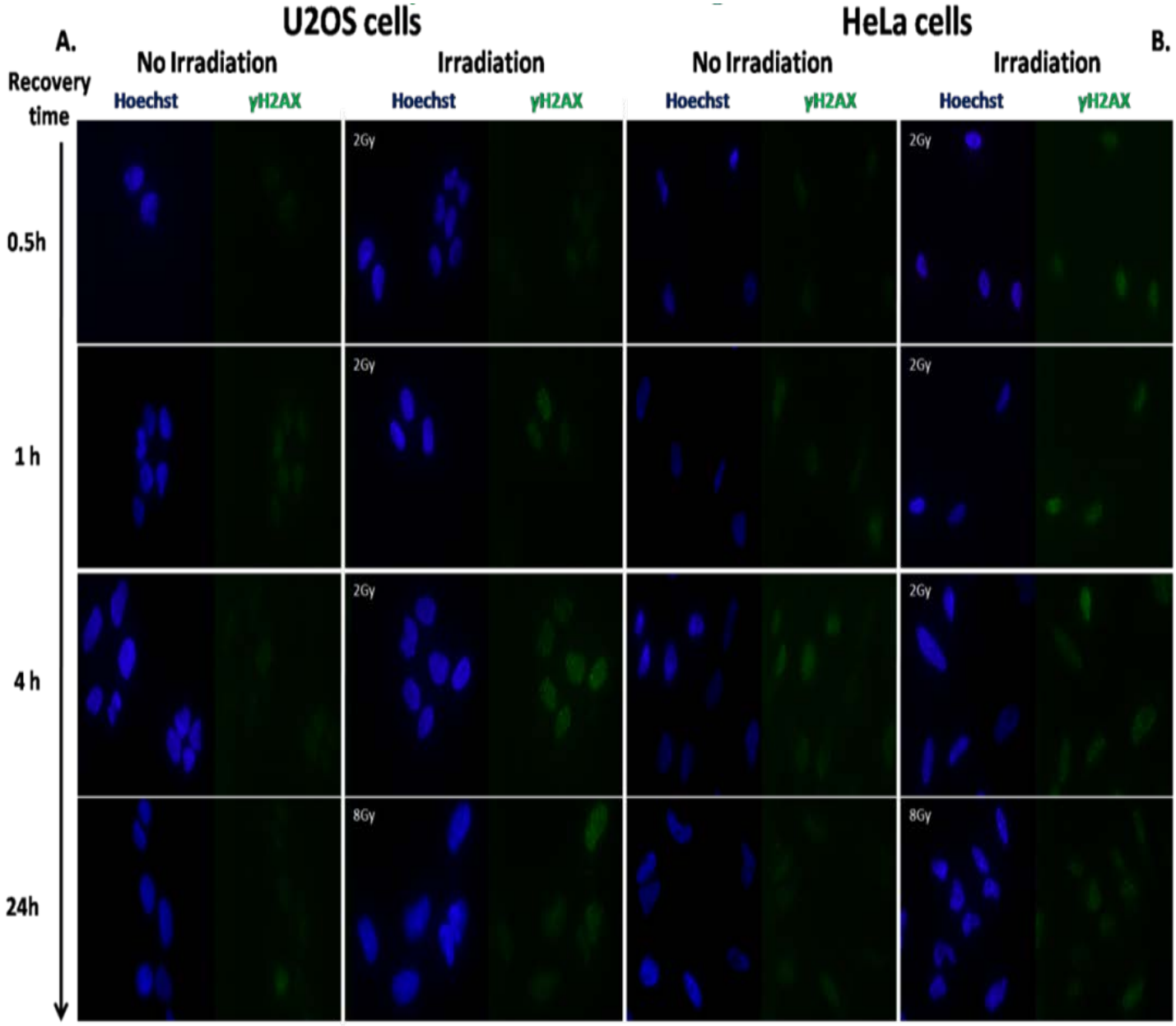
4.3 53BP1 is a more suitable DNA damage marker (associated with DNA double-strand breaks) than γH2AX in OVCAR3 and COV318 cells.

The failure of Metformin to down-regulate Rad51 or PARP1 protein expression prompted us to examine whether the drug has an alternative role in DNA damage or exerts a different effect on the DDR. However, due to the fact that COV318 is a rare cell line with limited literature available concerning DNA damage, it was essential to first find a suitable DNA damage marker. In subsequent experiments, the chosen DNA damage marker could be then used to determine the effect of Metformin on DNA damage in OVCAR3 and COV318 cells. To address this issue, we evaluated DNA damage by assessing the formation of γH2AX foci (DNA damage marker) and 53BP1 foci (DSBs marker) through IF in OVCAR3 and COV318 cells and compared them with HeLa and U2OS cells. HeLa and U2OS cell lines were selected as controls, as they are commonly used in DNA damage-associated studies (especially involving IF experiments). COV318, OVCAR3, HeLa and U2OS cells were subjected to IR at various doses (0, 2 and 8 Gy) in order to induce DNA damage, and then were allowed to recover for various time intervals (0.5, 1, 4 and 24 hours post IR). At these timepoints, the cells were stained for γH2AX and 53BP1 (following the IF experimental protocol) and imaged using a Fluorescence Microscope. The formation of 53BP1 and γH2AX foci at the above-mentioned timepoints was used to observe the DNA damage and repair over time in COV318, OVCAR3, HeLa and U2OS cells for the purpose of comparison and selection of the most suitable marker for following experiments.

U2OS and HeLa cells have increased numbers of γH2AX foci after IR (Fig. 6A and 6B) as expected, since this is an indication of IR-induced DNA damage. Furthermore, at later timepoints these γH2AX foci appear to be reduced as a result of DNA damage repair. In contrast to HeLa and U2OS cells, COV318 cells have increased numbers of γH2AX foci in both irradiated and non-irradiated conditions (Fig. 6C). In addition, this high abundance of γH2AX foci does not

reduce significantly over time as indicated from increased foci at 24 hours (in irradiated and non-irradiated cells). Similarly, OVCAR3 cells have high numbers of γH2AX foci even when they are not subjected to IR (Fig. 6D). Furthermore, like COV318 cells, OVCAR3 cells have high γH2AX foci at later timepoints (Fig. 6D). Collectively, these data suggest that γH2AX is an unsuitable DNA damage marker as increased numbers of γH2AX are problematic for processing and analysis. This high abundance of γH2AX foci also suggests that COV318 and OVCAR3 cells may have constitutive DNA damage.

Concerning 53BP1 foci (DSBs marker), similarly to γH2AX, HeLa and U2OS cells have increased formation of 53BP1 foci after IR-induced DNA damage (compared with non-irradiated cells) (Fig. 7A and 7B). Furthermore, 53BP1 foci were reduced at later timepoints as an indication of undergoing DNA repair. In contrast to previous observations using γH2AX foci as DNA damage markers, the numbers of 53BP1 foci and the kinetics in COV318 and OVCAR3 cells were relatively similar to HeLa and U2OS cells (Fig.7C and 7D). Hence, 53BP1 is a more suitable DNA damage marker for OVCAR3 and COV318 cells; 53BP1 foci can be easily analysed and quantified in order to provide insight into DNA damage and repair mechanisms. As a result, 53BP1 was used as a DNA damage marker in following experiments.


A.
U2OS cells
HeLa cells
B.
Recovery time
No Irradiation
Irradiation
No Irradiation
Irradiation
Hoechst
γH2AX
Hoechst
γH2AX
Hoechst
γH2AX
Hoechst
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4 h
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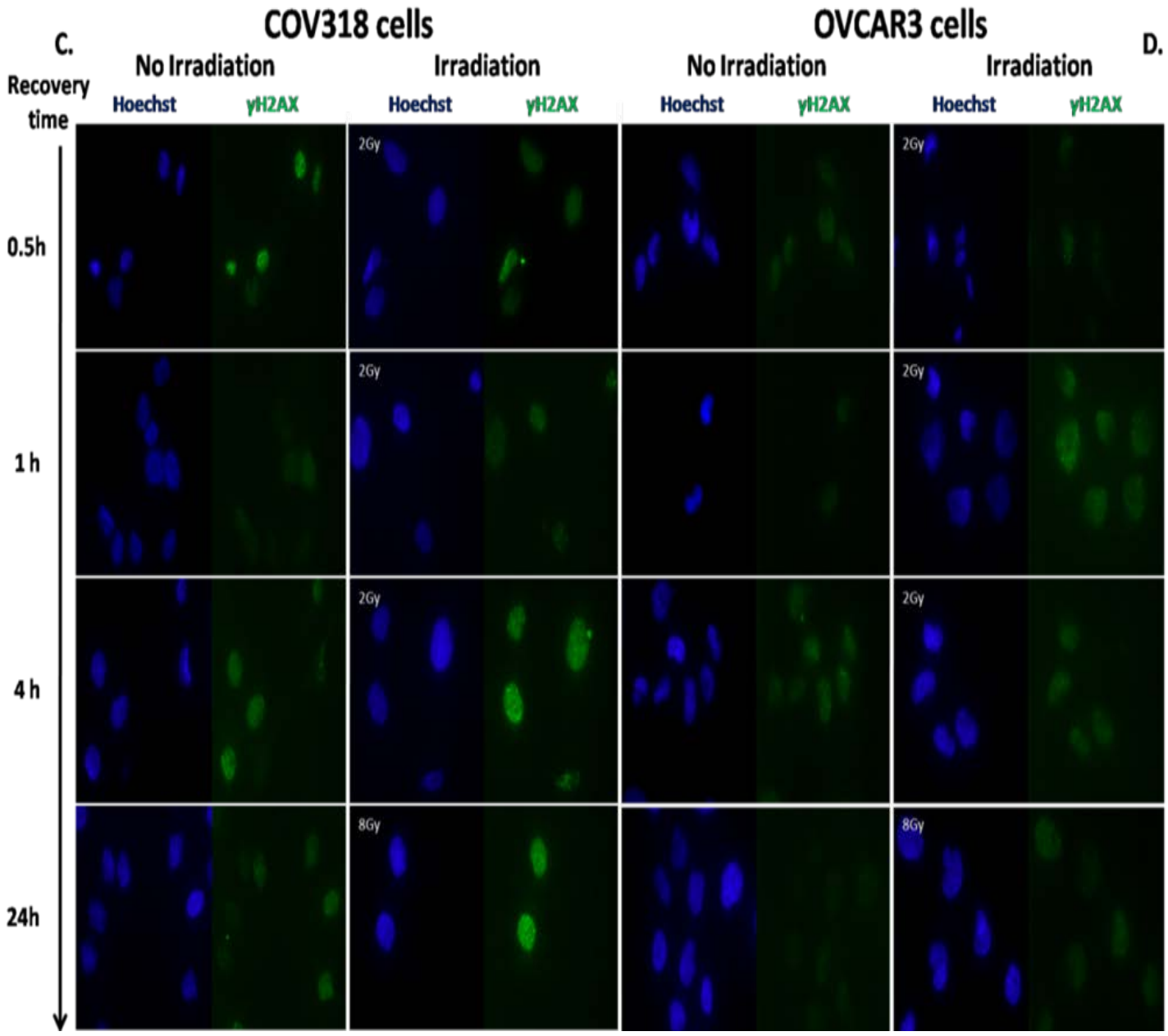
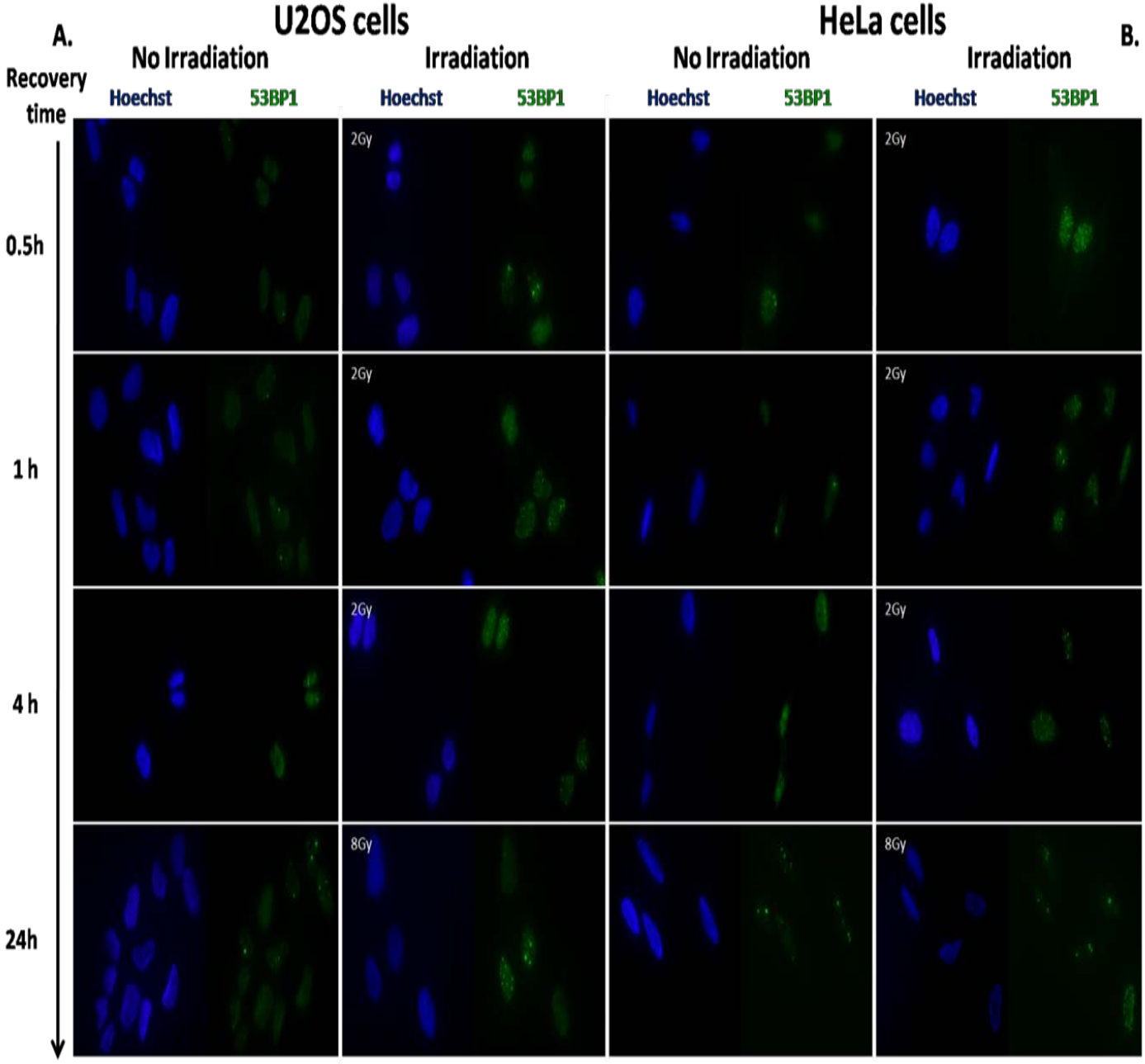


Figure 6. IF experiment in COV318, OVCAR3, U2OS and HeLa cells showing that γH2AX is not an appropriate DNA damage marker for COV318 and OVCAR3 cells. U2OS, HeLa COV318 and OVCAR3 cells received 0, 2 or 8 Gy IR and were fixed at different timepoints post IR (0.5h,1h, 4h, 24h). After fixation they were stained for γH2AX and with Hoescht dye (γH2AX is a DNA damage marker and Hoescht dye stains the cell nucleus) and imaged under a Fluorescence Microscope **A)** Non-irradiated U2OS cells had low levels of γH2AX foci (limited DNA damage). Irradiated U2OS cells (2 or 8 Gy) had increased γH2AX foci as a result of DNA damage (0.5 to 4 hours post IR) which gradually disappeared as DNA repair took place (24h post IR) **B)** HeLa cells formed γH2AX foci similarly to U2OS cells (with and without IR) **C)** and **D)** COV318 and OVCAR3 cells respectively, had high amounts of γH2AX foci in both IR and non-IR conditions. The γH2AX foci remained for even 24h post IR in both COV318 and OVCAR3 cells.


A.
U2OS cells
HeLa cells
B.
No Irradiation
Irradiation
No Irradiation
Irradiation
Recovery time
Hoechst
53BP1
Hoechst
53BP1
Hoechst
53BP1
Hoechst
53BP1
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1 h
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8Gy
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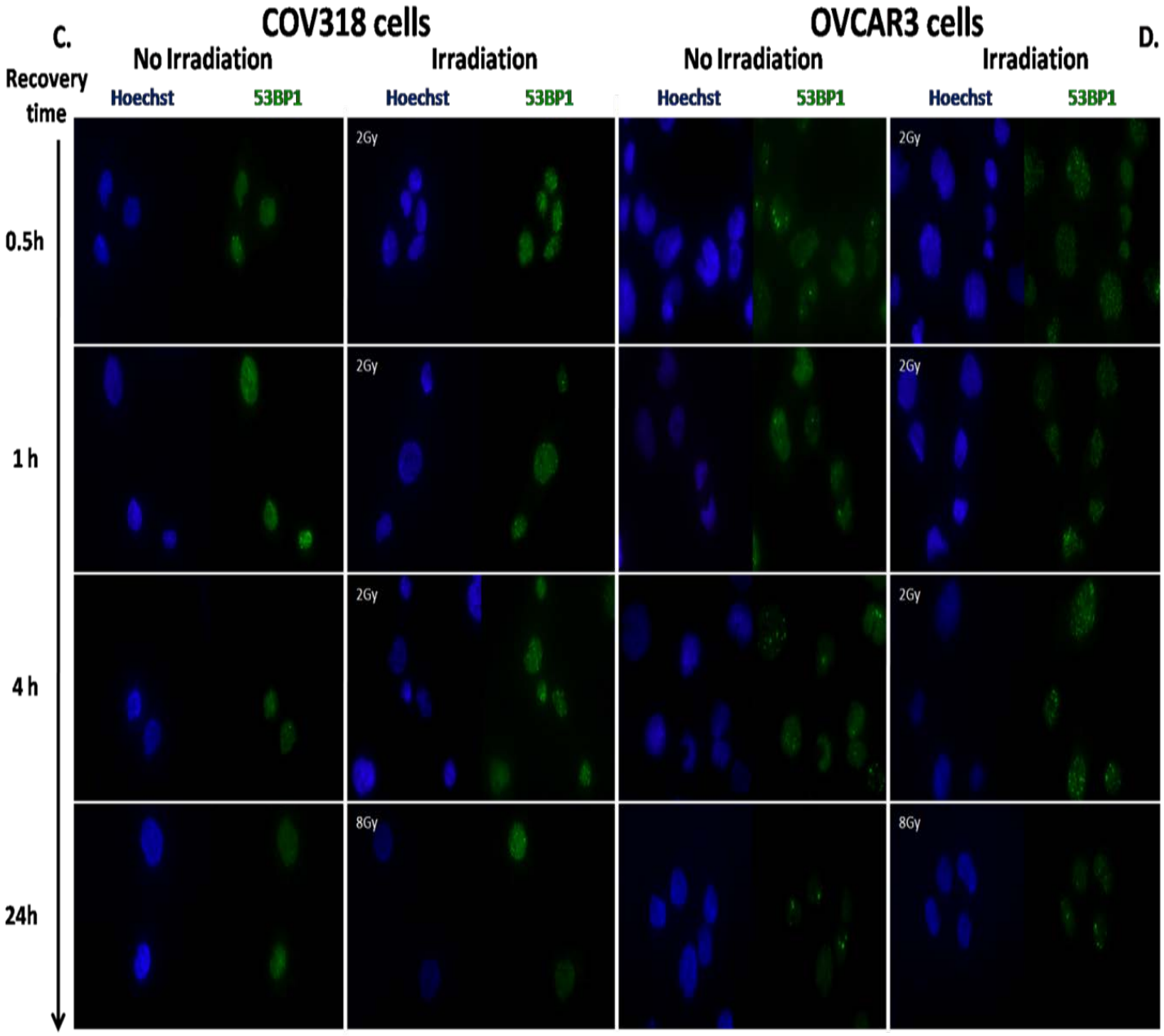

Figure 7. IF experiment in COV318, OVCAR3, U2OS and HeLa cells showing that 53BP1 is a suitable DNA damage marker for COV318 and OVCAR3 cells. U2OS, HeLa COV318 and OVCAR3 cells received 0, 2 or 8 Gy IR and were fixed at different timepoints post IR (0.5h,1h, 4h, 24h). After fixation they were stained for 53BP1 and with Hoescht dye (53BP1 is a DNA DSB damage marker and Hoescht dye stains the cell nucleus) and imaged under a Fluorescence Microscope **A)** Non-irradiated U2OS cells have low 53BP1 foci (limited DNA damage). U2OS cells receiving IR (2 or 8 Gy) have increased 53BP1 foci as a result of DNA damage (0.5 to 4h post IR) which gradually disappeared as DNA repair took place (t=24h post IR). **B) C)** and **D)** HeLa, COV318 and OVCAR3 cells respectively, had a similar pattern of 53BP1 foci formation and disappearance as U2OS cells.

4.4 Metformin causes delayed disappearance of DNA damage markers (associated with DNA double-strand breaks) when combined with irradiation

After we established that 53BP1 foci is an appropriate DNA damage marker for OVCAR3 and COV318 cells, we aimed to determine the effect of Metformin in the DNA damage and DDR in these ovarian cancer cell lines over time. To achieve this, we conducted another IF experiment in which COV318 ,OVCAR3 and HeLa cells were treated with Metformin (0 and 5mM) for 24 hours, then were irradiated at various doses (0,2,8 Gy) and finally were allowed to recover for different time intervals (0.5, 1, 4, 24, 48,72 hours post IR). At these time-points, the cells were fixed and stained for 53BP1, followed by imaging in a Fluorescence Microscope. To compare the DNA damage and repair between different conditions, 53BP1 foci were measured in 50 cells (per condition) and were quantified. Graphs were plotted for each cell line depicting the percentage of cells having more than 10 53BP1 foci/ per cell. In our experiments, we designated “more than 10 53BP1 foci per cell” as a cell with DNA damage markers (i.e. marked with probable DNA damage).

In OVCAR3 cells, Fig. 8A depicts the percentage of cells with more than 10 53BP1 foci in control (untreated) and 5mM Metformin-treated cells which have not received IR. We observed that the Metformin-treated cell population has a slightly higher percentage of cells with DNA damage compared to the control population. We hypothesized that Metformin treatment leads to more cells having an increased number of 53BP1 foci suggesting that this drug is implicated in DNA damage/repair pathways. Fig. 8B depicts the percentage of cells that have a high number of 53BP1 foci in untreated (IR only) and Metformin –treated cells (IR +5mM Metformin). At 30 to 60 minutes post-IR, we can observe that untreated cells (IR only) have an increased percentage of cells with probable DNA damage compared with the Metformin-treated cells (IR+5mM Metformin). This observation is more obvious from Fig. 8C which depicts the same graph as

Fig.8B with the timeline lasting until 4 hours post-IR. At 30 to 60 minutes post IR the untreated cell population (IR only) has 30% more cells with probable DNA damage, compared with the Metformin-treated cell population (IR+5mM Metformin). However, after 1 hour post-IR, the percentage of cells with probable DNA damage seems to increase for the Metformin-treated cell population (IR+5mM Metformin) and remains stable for 24 hours (Fig.8B). Similarly, the percentage of probable damaged cells is also increasing for the untreated cell population (IR only) for the first hour post IR; however, in contrast with Metformin-treated cells, this percentage gradually decreases over time. The gradual decrease of cells having high numbers of 53BP1 foci is expected as DNA repair takes place. However, the interesting fact concerning Metformin-treated cells (IR+5mM Metformin) is that, even though the percentage of cells with high numbers of 53BP1 foci is decreasing over time, the rate of this decrease is significantly lower than the untreated cells. This is more evident at 48 hours to 72 hours post-IR, when the Metformin-treated cell population has 50% more cells with probable DNA damage compared with the untreated cell population (Fig. 8B 48 to 72 hours post-IR). To sum up, the Metformin-treated cell population has originally lower percentage of cells with probable DNA damage (30 to 60 minutes post-IR); however, at later time-points (48 to 72 hours post-IR) it has persistently much higher percentage of cells with probable DNA damage compared to untreated cells. These data suggest that Metformin may exert a negative effect on the DDR and/or DNA repair in OVCAR3 cells. The original delay of 53BP1 foci formation (early after IR) may indicate a delayed or impaired DDR, while long after their exposure to IR the delay of their disappearance may suggest a delayed or impaired DNA repair.

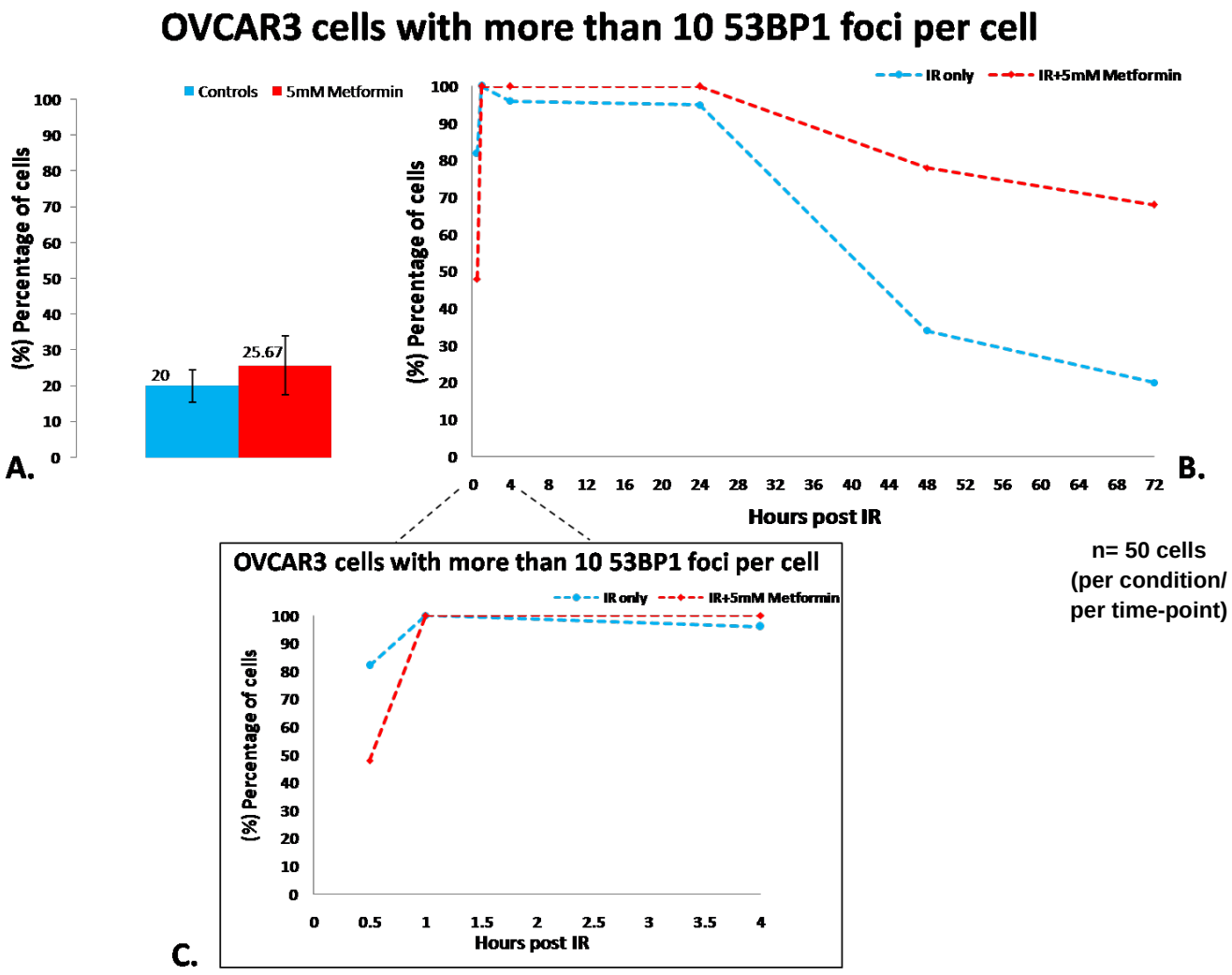

Figure 8. IF experiment quantifying the percentage of OVCAR3 cells with more than 10 53BP1 foci, suggests that Metformin has a negative impact on DDR or DNA repair pathways. OVCAR3 cells were treated with Metformin (0 or 5 mM) for 24 hours, followed by irradiation (0, 2 ,8 Gy) and fixation at different timepoints after IR (0.5, 1, 4, 24 ,48, 72 hours). The cells were stained for 53BP1 and imaged under a Fluorescence Microscope where 53BP1 foci were measured in each cell. 50 cells were analysed for each condition and time-point, and the percentage of cells with more than 10 53BP1 foci was quantified. **A) Without IR:** the OVCAR3 cell population treated with Metformin (5mM for 24 hours) has increased percentage of cells with more than 10 53BP1 foci/cell (DNA damage markers) compared with the Control population (untreated cells) **B) With IR:** OVCAR3 cell population treated with Metformin exhibits originally less percentage of cells with DNA damage markers (0.5 to 1 hour post-IR) compared with untreated cells. After 1 hour post-IR, the Metformin-treated cell population has a higher percentage and a slower rate of decrease of cells with DNA damage markers compared with untreated cells.**C) With IR:** Focus on the first 4 hours post-IR, where at 0.5 to 1 hour post-IR, the OVCAR3 cell population treated with Metformin has lower percentage of cells with DNA damage markers compared to untreated cell population.

In COV318 cells, Fig. 9A depicts the percentage of cells with more than 10 53BP1 foci in control (untreated) and 5mM Metformin-treated cells which have not received IR. In contrast to OVCAR3 cells, we did not observe a similar difference in the percentage of damaged cells between the Control and Metformin-treated cell population. Fig. 9B compares untreated and Metformin-treated cells that have received IR. Similarly with OVCAR3 cells, at 30 to 60 minutes post IR we observed that untreated cells (IR only) have an increased percentage of cells with probable DNA damage (high number of 53BP1 foci) compared with the Metformin-treated cells (IR+5mM Metformin). This is more evident from Fig. 9C which focuses on the first 4 hours post-IR when the untreated cell (IR only) population has 10% more damaged cells compared with the Metformin-treated cell population (IR+5mM Metformin). However, this difference was not of the same magnitude as observed in OVCAR3 cells (Fig. 8B). Similarly with OVCAR3 cells, the percentage of cells with DNA damage markers gradually decreases over time in untreated (IR only) cells as indicative of DNA repair (72 hours post-IR Fig. 9B). Whereas, the percentage of cells with DNA damage markers in the Metformin-treated (IR+5mM Metformin) cell population is decreasing at a slower rate over time. This is more evident 72 hours post-IR (Fig. 9B) where the Metformin-treated cell population has 40% more cells with DNA damage markers compared with the untreated cell population. This observation corresponds to what we have previously observed in OVCAR3 cells.

In Hela cells, Fig. 10A shows that the 5mM Metformin-treated cell population has a slight increase in the percentage of cells with probable DNA damage compared to the controls (these two populations did not receive IR) . This increase, however, is less than the one observed in OVCAR3 cells. In contrast to OVCAR3 and COV318 cells, HeLa cells treated with Metformin (IR+5mM Metformin) have originally higher percentage of cells with DNA damage markers (Fig. 10B and Fig. 10C 0.5 to 1 hour post-IR) compared with the untreated cells (IR only). Moreover, the Metformin-treated cell population (IR+5mM Metformin) has less percentage of cells with

DNA damage markers compared with the untreated cells (IR only) and this percentage gradually decreases over time with a similar rate as the untreated cells (IR only). Consequently, in contrast to OVCAR3 and COV318 cells, the untreated and Metformin-treated HeLa cell populations do not have major differences in the decrease rate of the percentage of cells with probable DNA damage (overlap from 48 to 72 hours Fig. 10C).These findings were surprising because previous studies showed that HeLa cells were insensitive to the growth inhibitory effects of Metformin [63]. Hence, we did not expect to see an effect of Metformin in HeLa cells. Because the differences between untreated and Metformin-treated HeLa cells were not of the same magnitude as observed in OVCAR3 or COV318 cells and the overlap between metformin-treated and untreated cells (at 72 hours post-IR) indicate that these findings may just reflect experimental variation. Alternatively, there might by an actual effect of Metformin in the DNA damage and /or DNA repair in HeLa cells, which is different than the one observed in COV318 or OVCAR3 cells. However, further studies must take place to determine if this is actually the case for HeLa cells.

Collectively, this IF experiment showed that Metformin probably has a negative impact in the DDR and/or DNA repair pathways of OVCAR3 and COV318 cells. Metformin treatment resulted in lower percentage of cells with DNA damage early after IR. Whereas, Metformin treatment in later timepoints after IR resulted in increased populations of cells with DNA damage that did not decrease significantly over time. This was evident from the slower disappearance of cells having more than 10 53BP1 foci (markers of probable DNA damage), which was indicative of persistent DNA damage. All in all, the delayed appearance of cells having DNA damage markers and their delayed disappearance long after IR exposure, suggests that Metformin may exert a negative role by delaying or impairing the DDR and/or DNA repair.

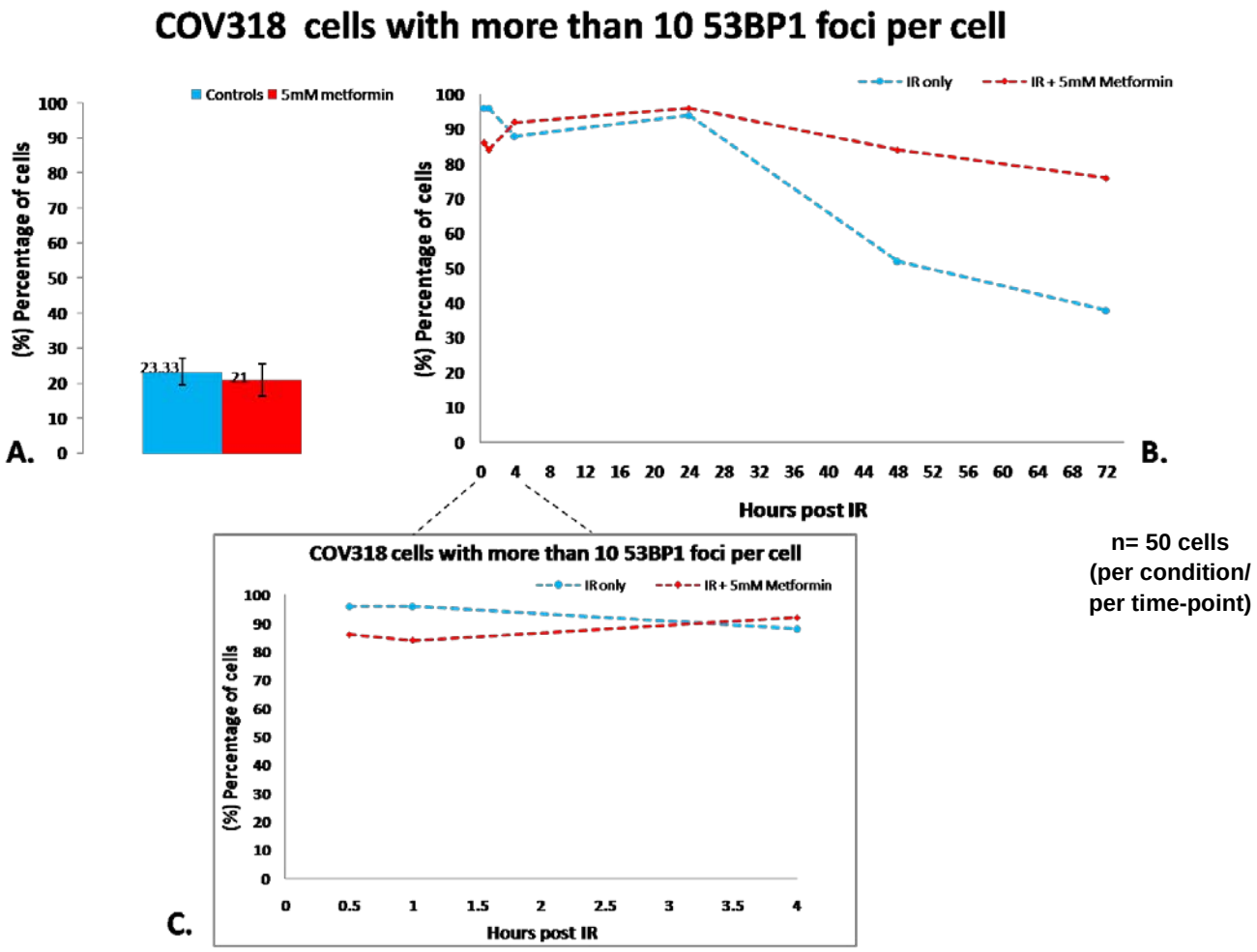

Figure 9. IF experiment quantifying the percentage of COV318 cells with more than 10 53BP1 foci, suggests that Metformin has a negative impact on DDR or DNA repair pathways. COV318 cells were treated with Metformin (0 or 5mM) for 24 hours, followed by irradiation (0, 2 ,8 Gy) and fixation at different timepoints after IR (0.5,1,4,24,48,72 hours). The cells were stained for 53BP1 and imaged under a Fluorescence Microscope where 53BP1 foci were measured in each cell. 50 cells were analysed for each condition and time-point, and the percentage of cells with more than 10 53BP1 foci was quantified. **A) Without IR:** the COV318 cell population treated with Metformin has decreased percentage of cells with more than 10 53BP1 foci/cell (DNA damage markers) compared with the Control population (untreated cells) **B) With IR:** COV318 cell population treated with Metformin exhibits originally less percentage of cells with DNA damage markers (0.5 to 1 hour post-IR) compared with untreated cells. After 4 hours post-IR, the Metformin-treated cell population has a higher percentage and a slower rate of decrease of cells with DNA damage markers compared with untreated cells. **C) With IR:** Focus on the first 4 hours post IR, where at 0.5 to 1 hour post-IR, the COV318 cell population treated with Metformin has lower percentage of cells with DNA damage markers compared to untreated cell population.

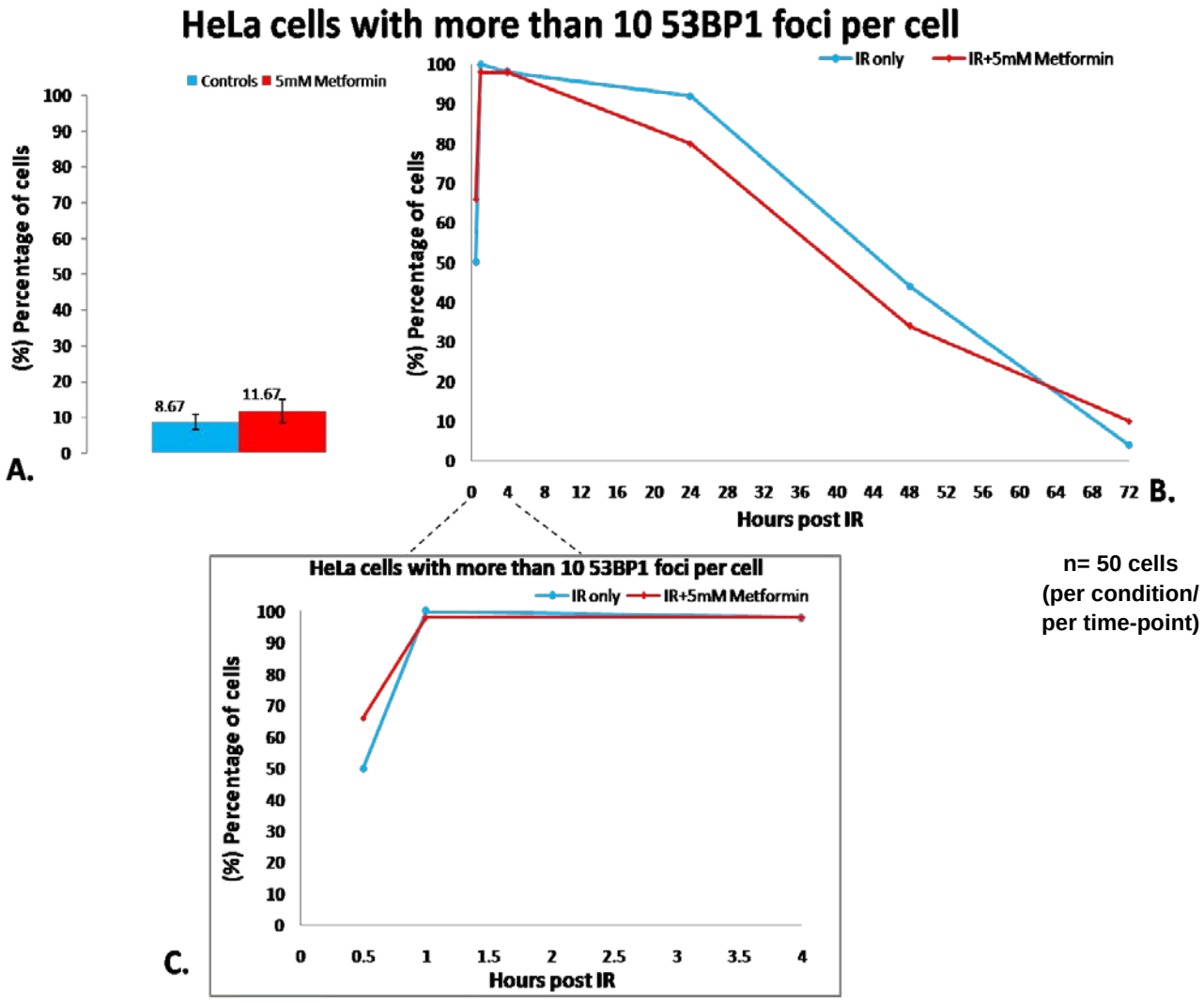

Figure 10. IF experiment quantifying the percentage of HeLa cells with more than 10 53BP1 foci, suggests a role of Metformin on DDR or DNA repair pathways.

HeLa cells were treated with Metformin (0 or 5mM) for 24 hours, followed by irradiation (0, 2 ,8 Gy) and fixation at different timepoints after IR (0.5, 1, 4, 24, 48, 72 hours). The cells were stained for 53BP1 and imaged under a Fluorescence Microscope where 53BP1 foci were measured in each cell. 50 cells were analysed for each condition and time-point, and the percentage of cells with more than 10 53BP1 foci was quantified. **A) Without IR:** the HeLA cell population treated with Metformin has increased percentage of cells with more than 10 53BP1 foci/cell (DNA damage markers) compared with the Control population (untreated cells) **B) With IR:** HeLa cell population treated with Metformin exhibits originally higher percentage of cells with DNA damage markers (0.5 to 1 hour post-IR) compared with untreated cells. After 4 hours post-IR, the Metformin-treated cell population has a lower percentage but similar rate of decrease of cells with DNA damage markers compared with untreated cells. **C) With IR:** Focus on the first 4 hours post-IR, where at 0.5 to 1 hour post-IR, the HeLa cell population treated with Metformin has higher percentage of cells with DNA damage markers compared to untreated cell population.

4.5 Metformin does not induce DNA damage in OVCAR3 and COV318 cells

Because of our previous findings in OVCAR3 and COV318 cells, further analysis of the IF data were done to attempt to determine whether Metformin primarily causes more DNA damage or hinders the DNA damage response and repair. Previous studies suggest that Metformin induces DNA damage [64], others that Metformin prevents or reduces DNA damage [53,55] while others propose that it may impair the DNA repair [58].

To address this issue, we calculated the average number of 53BP1 foci per cell for OVCAR3, COV318 and HeLa cells. We hypothesised that if Metformin causes more damage, then the average of number of foci per cell would be greater than the untreated cells (in both IR and non-IR conditions).Alternatively, we hypothesised that if Metformin delays or impairs the DNA damage response and repair, the average number of foci per cell will not have substantial differences in Metformin-treated compared to untreated cells. Fig. 11Ai and 11Bi, show that the average number of foci per cell (OVCAR3 and COV318 respectively) is similar between control (untreated) cells and 5mM Metformin treated cells (non-irradiated conditions). Fig. 11Aii and 11Bii (OVCAR3 and COV318 respectively), show that untreated cells have a higher number of 53BP1 foci per cell compared with Metformin-treated cells during the first hour post-IR. This is in agreement with Fig. 8C and 9C, where at the first hour post-IR, the untreated cell population has higher numbers of cells with probable DNA damage compared to Metformin-treated cells. Furthermore, in the same figures, we observe that at 1 to 4 hours post-IR both untreated and Metformin-treated cells have similar average of foci per cell. At 48 to 72 hours post-IR, Metformin-treated cells have slightly more 53BP1 foci per cell compared with the untreated cells. Comparing these with Fig. 8B and 9B, we can assume that, even if at 48 to 72 hours post-IR, the Metformin-treated cell population has an increased amount of cells with DNA damage (Fig.8B

and 9B), the magnitude of DNA damage is similar with the one observed in untreated cells (indicated from similar average of 53BP1 foci per cell- Fig. 11Aii and 11Bii).

Collectively, there is a similar trend in OVCAR3 and COV318 cells and one can hypothesise that Metformin is more likely to delay the DNA damage response and/or DNA repair, as both Metformin-treated and untreated cells have similar average of 53BP1 foci per cell (both in IR and non-IR conditions) indicating similar magnitude of DNA damage.

Concerning HeLa cells (Fig. 11Ci), like OVCAR3 and COV318, the average number of foci per cell is similar between control (untreated) cells and 5mM Metformin treated cells (non-irradiated conditions). However, in IR conditions (Fig. 11Cii) we observe variability in the data, as in some time-points (0.5, 4 and 72 hours post-IR) Metformin-treated cells have higher average of 53BP1 foci per cell, while in others (1h and 48 hours post-IR) they have a lower average compared with the untreated cells. Interestingly, this trend correlates with Fig. 10B; however, it may still be a product of experimental variation and requires further investigation.

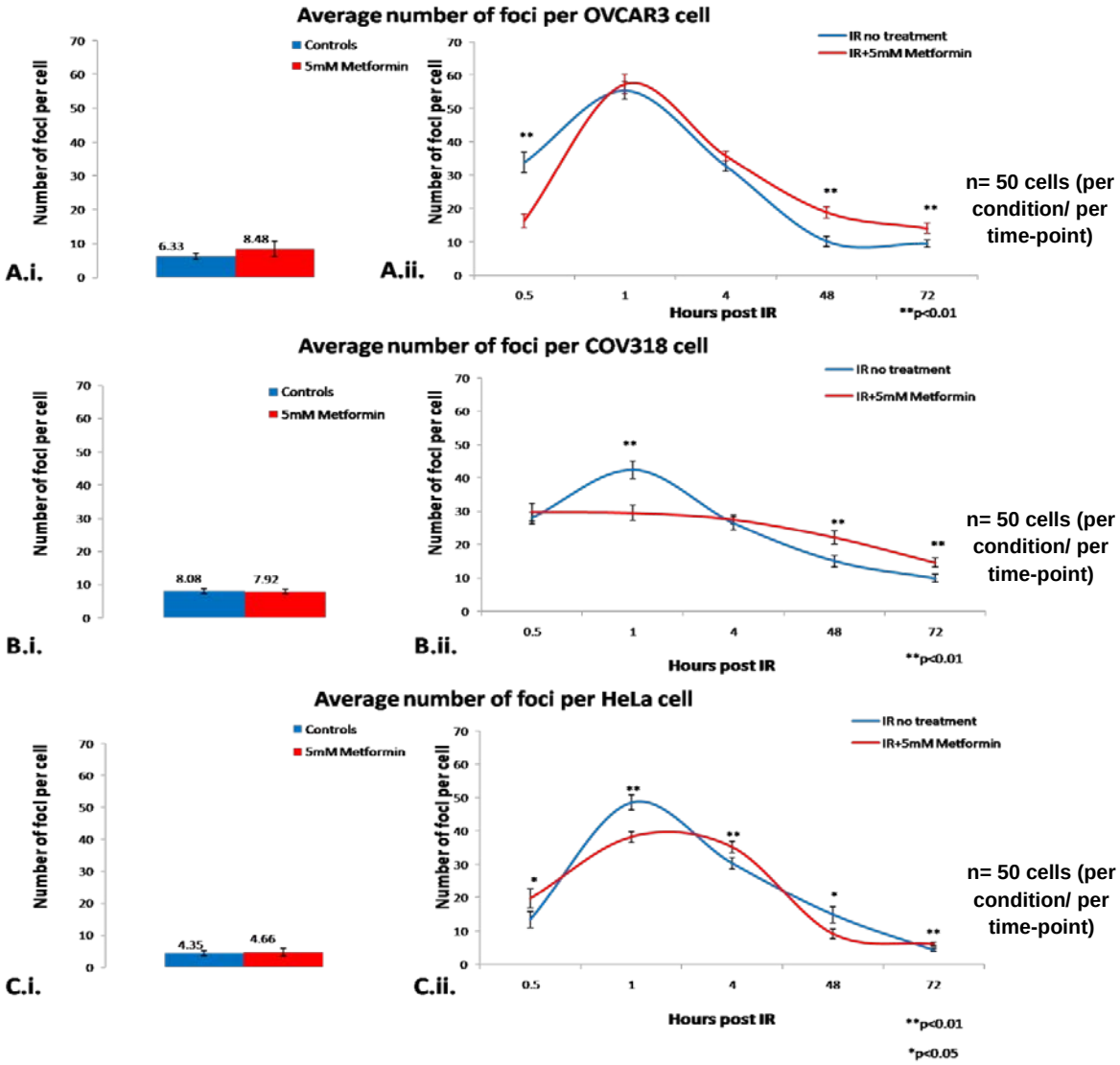

Figure 11. IF experiment comparing of the average number of 53BP1 foci per cell in Metformin- treated and untreated cells suggests similar DNA damage levels.

OVCAR3, COV318 and HeLa cells were treated with Metformin (0 or 5mM) for 24 hours, followed by irradiation (0, 2 ,8 Gy) and fixation at different timepoints after IR (0.5, 1, 4, 24, 48, 72 hours). The cells were stained for 53BP1 and imaged under a Fluorescence Microscope where 53BP1 foci were measured in each cell. 50 cells were analysed for each condition and time-point, and the average number of foci was evaluated. **A) OVCAR3 cells: Ai) without IR.** Control and 5mM metformin-treated cells have similar average of 53BP1 foci per cell **Aii) without IR.** The first hour post IR (0.5 to 1 hour) metformin-treated cells have lower average of 53BP1 foci per cell compared with untreated cells indicating less DNA damage. From 1 to 4 hours post-IR both cell populations have similar average of 53BP1 foci per cell indicating similar DNA damage levels. From 48 to 72 hours post-IR Metformin treated cells have a slightly increased average of 53BP1 foci per cell compared with untreated cell. **B) COV318 cells: Bi) without IR.** Similar observations as Ai (OVCAR3). **Bii) with IR.** Similar observations as Aii (OVCAR3). **C) HeLa cells: Ci) without IR.** Similar observations as Ai and Bi (OVCAR3 and COV318). **Cii) with IR.** Metformin-treated cells have higher average of 53BP1 foci per cell variability in some time-points (0.5, 4 and 72 hours post-IR) while in others (1 and 48 hours post-IR) they have a lower average compared with the untreated cells (statistical significance with p-values : *p<0.05 or **p<0.01)

4.6 Metformin treatment specifically affects levels of DNA damage markers in cells at S-phase

Besides the controversy in the literature surrounding the functions of Metformin in the DNA damage and DNA damage response, little is known about the cell cycle phase in which Metformin exerts its role on DNA damage. Interestingly, a study observed that human lung adenocarcinoma A549 cells have reduced DNA damage after Metformin treatment , when they are undergoing G2/M- or S-phase (as evidenced from reduced DNA damaged signaling i.e. reduced ATM activation and decreased γH2AX expression) [53].

Consequently, we aimed to assess if Metformin's role on DNA damage predominates in a certain phase of the cell cycle, and more specifically in S-phase. To address this issue we conducted an IF experiment in which COV318, OVCAR3 and HeLa cells were treated with Metformin (0 and 5 mM) for 24 hours and were fixed at different time-points after removal of the drug. The cells were fixed and stained for 53BP1 and Cyclin A, followed by imaging in a fluorescence microscope. Cyclin A staining indicates which cells undergo S phase, as Cyclin A expression is at high levels during S (and early G2) phase of the cell cycle. 53BP1 staining was used to determine the cells with probable increased DNA damage (cells with more than 10 53BP1 foci per cell). To analyse the data, we calculated the percentage of damaged cells that are Cyclin A positive (cells in S-phase) and Cyclin A negative in both control (untreated) and 5mM-Metformin treated cells. Fig. 12Ai, 12Bi and 12Ci depict the percentage of Cyclin A positive cells that have increased DNA damage (cells having more than 10 53BP1 foci/cell) in OVCAR3, HeLa and COV318 cell populations respectively. We observed that in OVCAR3 and HeLa cells, the 5 mM metformin-treated cell population has more Cyclin A positive cells (with increased DNA damage) compared to the untreated cell population (controls) (Fig. 12Ai and 12Bi respectively). In contrast to OVCAR3 and HeLa cells, the COV318 cell population treated with Metformin

consists of less Cyclin A positive cells (having increased DNA damage) compared with the untreated cell population (controls) (Fig. 12Ci).

Fig. 12Aii, 12Bii and 12Cii show the percentage of Cyclin A negative cells that have DNA damage markers (cells having more than 10 53BP1 foci/cell) in OVCAR3, HeLa and COV318 cell populations respectively. We observed that OVCAR3 (12Aii), HeLa (12Bii) and COV318 (12Cii) cell populations treated with 5 mM Metformin have a similar percentage of Cyclin A negative cells (with DNA damage markers) compared to the untreated cell population. Even though the graphs show a slight increase, it is less than 3% difference between control and 5mM metformin-treated cells and of a lesser magnitude compared with Fig. 12Ai, 12Bi and 12Ci.

Collectively, this experiment suggested that in OVCAR3 and Hela cells, but not in COV318 cells, Metformin might have a greater negative impact in cells undergoing S-phase. This is indicated from the increased ratio of Cyclin A positive cells with DNA damage markers in metformin-treated versus untreated cells. Also, this hypothesis is supported by the similar ratio of Cyclin A negative cells with DNA damage markers, in metformin-treated versus untreated cells. The fact that an increased percentage of Metformin-treated cells have DNA damage while in S-phase, suggests an enhanced effect of the drug in S-phase cells. This is intriguing, as it may indicate that Metformin causes problems during replication or is affecting the intra-S DNA damage cell cycle checkpoint.

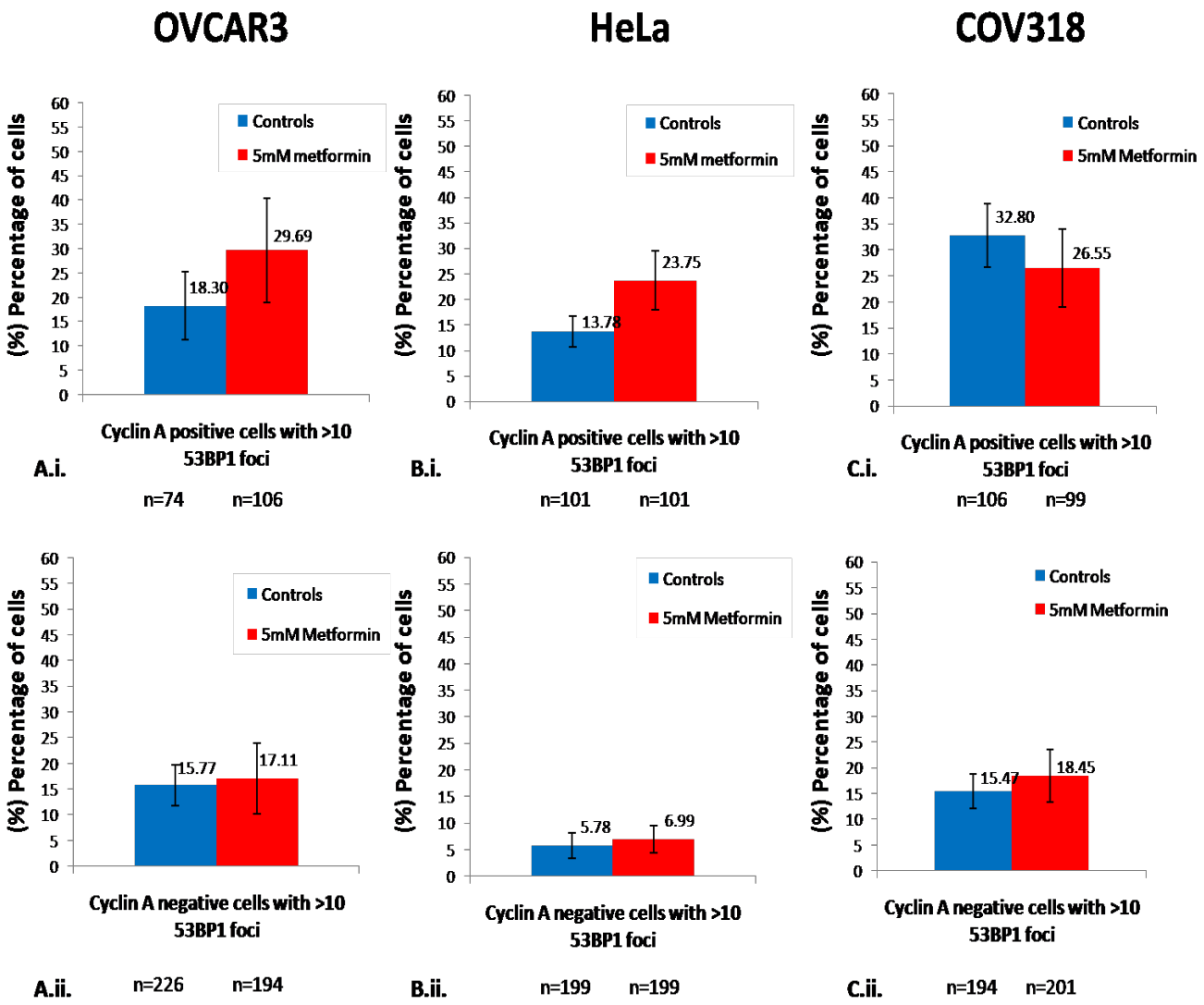

Figure 12. Comparison of Cyclin A positive cells and negative cells with more than 10 53BP1 foci (DNA damage marker) in OVCAR3, HeLa and COV318 cells.
Cells were treated with Metformin (0 or 5 mM) for 24 hours, and were fixed at different time-points after removal of the drug. The cells were stained for 53BP1 and Cyclin A, followed by imaging in a Fluorescence Microscope. Cyclin A positive cells (and negative cells) that had more than 10 53BP1 foci were quantified and presented as a percentage. Note that **n** is the number of analysed cells that are Cyclin A positive or negative respectively. **A) OVCAR3 cells: Ai)** The 5mM Metformin-treated cell population has more Cyclin A positive cells (with DNA damage markers) compared with the control cell population **Aii)** Control and 5mM metformin-treated cell populations have similar percentages of Cyclin A negative cells (with DNA damage markers) **B) HeLa cells: Bi)** Similar observations as Ai. (OVCAR3). **Bii)** Similar observations as Aii. (OVCAR3). **C) COV318 cells: Ci)** The 5mM Metformin-treated cell population has fewer Cyclin A positive cells (with DNA damage markers) compared with the control cell population **Cii)** Similar observations as Aii.(OVCAR3) and Bii.(HeLa).

4.7 Metformin treatment increases cytotoxicity in irradiated OVCAR3 cells, relative to irradiation alone

Based on our previous observations suggesting that Metformin may have a role in DNA damage/repair pathways, we next aimed to evaluate whether Metformin could also have an impact on the survival and proliferation of OVCAR3 and COV318 cells (when combined with IR). Previous studies have shown that Metformin inhibits the proliferation of OVCAR3 cells [26,29] and can also induce apoptosis [37]. Furthermore, a previous study in hepatoma cell lines showed that Metformin combined with IR reduces cell survival [58]. However, the effect of Metformin combined with IR on OC cell proliferation and survival, is still unknown. For these reasons, we conducted Colony Formation Assays on OVCAR3 and COV318 cells to evaluate the effectiveness of Metformin (combined with IR) on the survival and proliferation of these cell lines compared to the effects of IR alone. Consequently, our main aim was to assess if Metformin potentiates the DNA damaging effects of IR.

COV318 and OVCAR3 cells were treated with Metformin (0 or 5 mM) for 24 hours (acute treatment) and then irradiated with various doses of irradiation (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 Gy). Next, the cells were seeded and left to form colonies for 7 days. After fixation and staining, the colonies were counted for each condition and the survival curve was plotted. It was observed that COV318 could not form colonies and their morphology indicated cell senescence. Thus, Colony Formation Assays could not be used any further with these cells. However, Colony Formation Assays in OVCAR3 cells provided valuable data. To begin with, we considered that the colonies formed by non-irradiated non-treated cells represent 100% survival. The colonies formed by non-irradiated Metformin-treated cells, were also considered to represent 100% survival in order to exclude the growth inhibitory or apoptotic effects of Metformin in the final analysis. This is due to the fact that the purpose of this Colony Assay was to evaluate if

Metformin potentiates the DNA damaging effects of IR (focusing on DNA damage/repair pathways); thus having a negative impact on colony survival.

It was observed that IR reduced the percentage of viability in a dose-dependent manner in OVCAR3 untreated cells. When IR was combined with Metformin treatment, the percentage of viable colonies was even lower compared to untreated cells under the same irradiation conditions (Fig.13). Consequently, Metformin treatment combined with IR results in decreased survival of OVCAR3 cells compared to IR alone. At IR doses 2, 3 and 4, 7, 8 Gy this difference in survival between untreated and Metformin-treated cells bears statistical significance ($p<0.01$ and $p<0.05$ respectively). As a result, Metformin possibly potentiates the DNA damaging effects of IR, resulting in reduced survival of OVCAR3 cells.

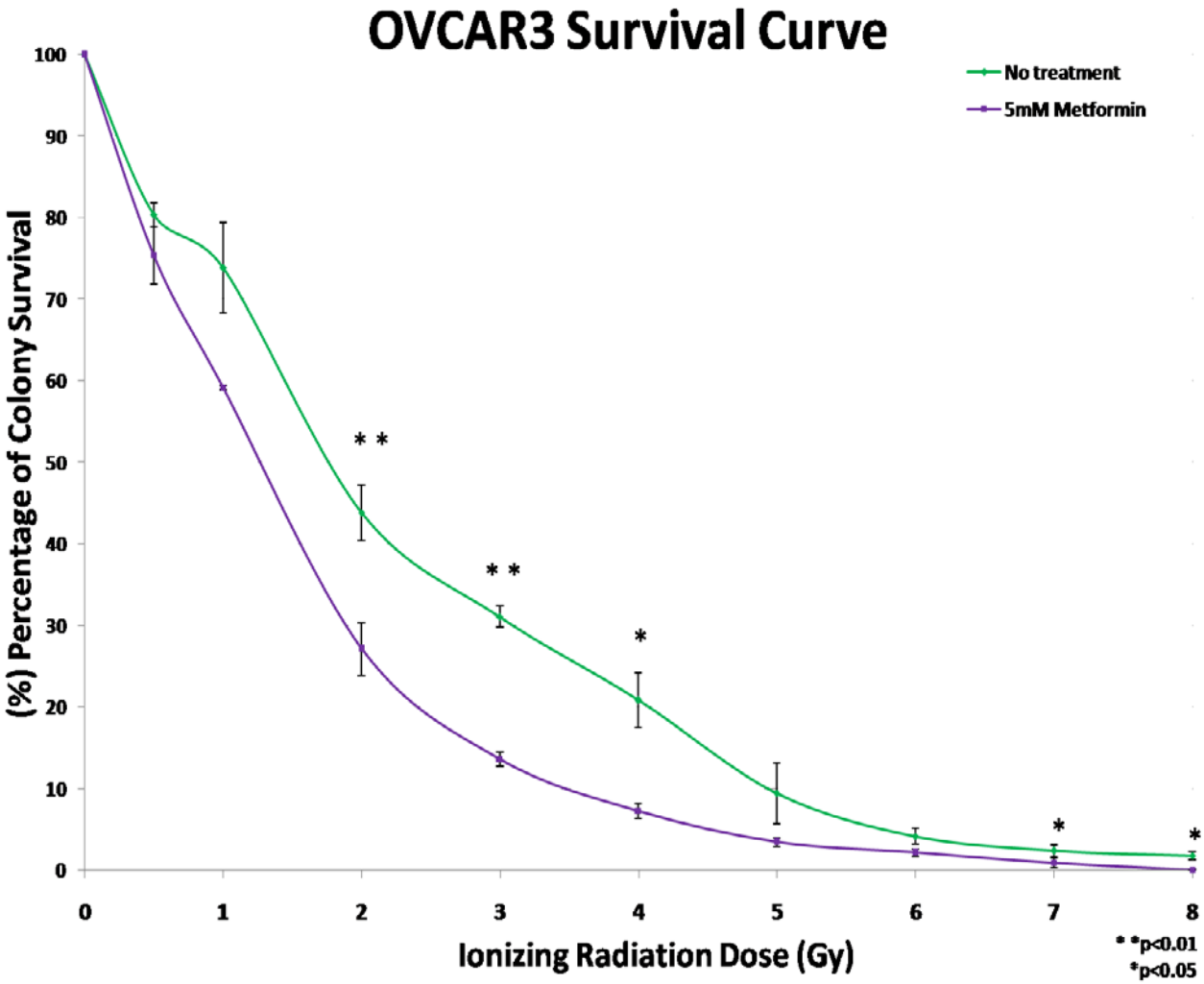

Figure 13. **Colony Formation Assay showing that the combination of Metformin and irradiation reduces the survival of OVCAR3 cells compared to irradiation alone.**

OVCAR3 cells were treated with Metformin (0 or 5mM) for 24 hours and irradiated at various doses (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 Gy). The cells were plated and left to form colonies for 7 days. Metformin-treated cells receiving IR have formed fewer colonies as shown from the decreased percentage of colony survival compared to untreated cells (receiving IR).Reduction in survival is IR-dose dependent for both non-treated and Metformin-treated cells receiving IR. For IR doses 2, 3 and 4, 7, 8 Gy this difference in survival is statistically significant ($p<0.01$ and $p<0.05$ respectively). Graph shows the means of 3 independent experiments.

4.8 OVCAR3 survival is reduced further by combined Metformin and Gemcitabine treatment, relative to Gemcitabine alone

Because we hypothesized that Metformin potentiates the DNA damaging effects of IR, and because it may also have a negative impact during replication, we speculated that it may also potentiate the DNA-damaging effects of Gemcitabine. A previous study in ovarian cancer cells showed that Metformin potentiates the cytotoxic effects of another chemotherapeutic drug (Carboplatin) but not Paclitaxel [30]. So, as Gemcitabine is a drug widely used in ovarian cancer, it would be interesting to assess whether Metformin potentiates its effects in OVCAR3 cells. Gemcitabine is a nucleoside analog, which main function is to incorporate into DNA during replication, leading to DNA synthesis inhibition and apoptosis [65]. Consequently, we wanted to determine whether Metformin potentiates the effects of Gemcitabine by evaluating their effect on proliferation and survival of OVCAR3 cells. To address this issue we performed Colony Formation Assays. OVCAR3 cells where seeded and pre-treated with 5 mM Metformin for 22 hours followed by addition of Gemcitabine in various doses (0, 0.1, 0.5, 0.75 ,1, 2 μM). As a result, OVCAR3 cells were treated with both Metformin and Gemcitabine for 2 hours (acute treatment) and then were left to form colonies for 7 days. Fixation and staining was followed by counting colonies and plotting the survival curve. The colonies formed by Metformin-treated cells only (no Gemcitabine) were considered to represent 100% survival in order to exclude the growth inhibitory or apoptotic effects of Metformin in the final analysis. Fig.14 shows that increasing doses of Gemcitabine resulted in reduced percentage of colony survival. The combination of Metformin and Gemcitabine also reduced the percentage of colony survival; however, their combination induced greater cytotoxicity compared to Gemcitabine treatment alone. These results (which bear statistical significance with p-values $p<0.01$ or $p<0.05$) suggest that Metformin causes a minor increase in the cytotoxic effects of Gemcitabine in OVCAR3 cells.

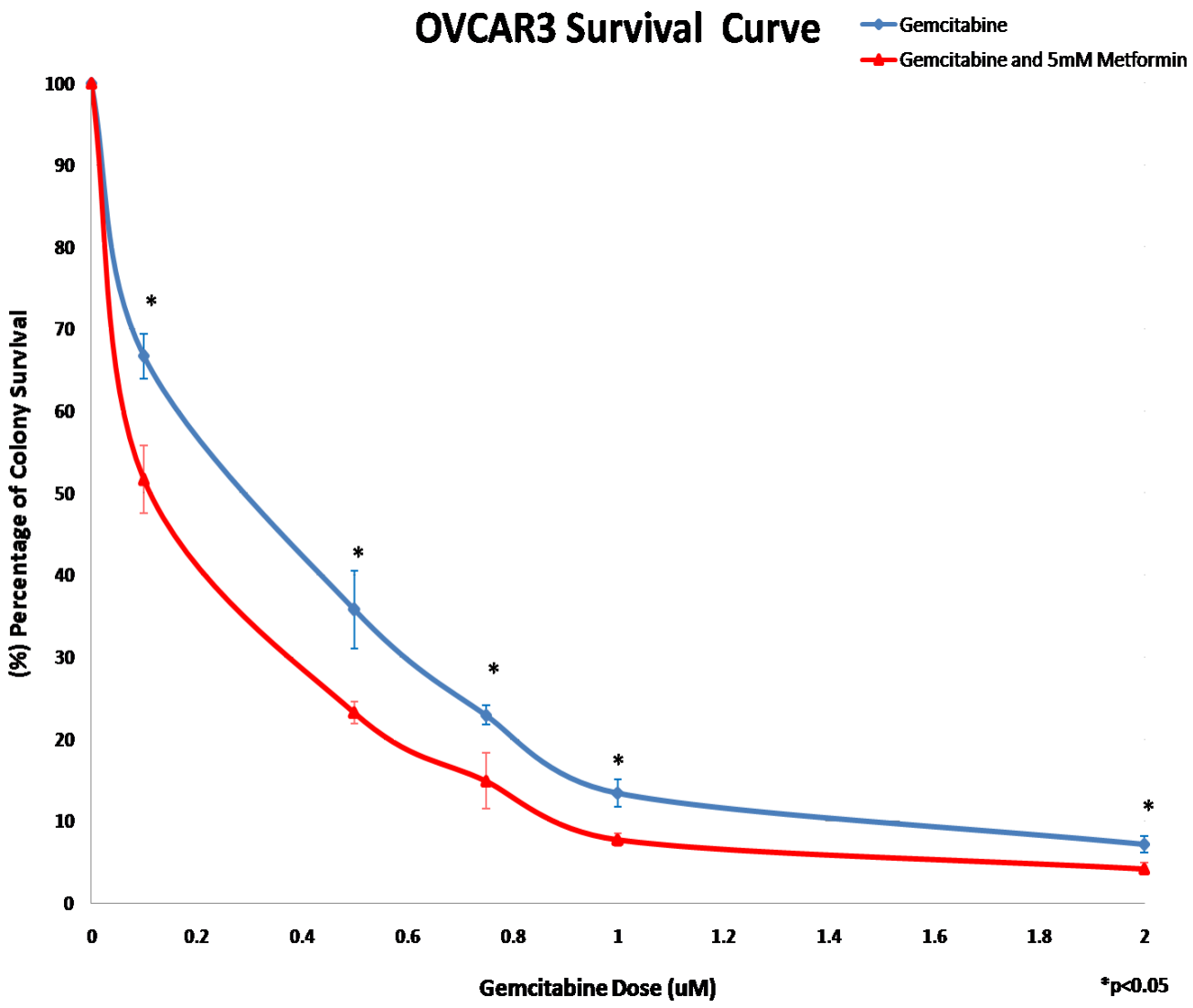

Figure 14. Colony Formation Assay showing that Metformin increases the cytotoxic effects of Gemcitabine in OVCAR3 cells.

OVCAR3 cells were pre-treated with Metformin (0 or 5mM) for 22 hours followed by treatment with Gemcitabine for 2 hours at various doses (0, 0.1, 0.5, 0.75, 1 and 2 μM). The drugs were then removed and the cells were plated and left to form colonies for 7 days. Increasing doses of Gemcitabine combined with Metformin reduce the percentage of colony survival compared with the percentage of colony survival for Gemcitabine treatment alone. Results depicted on the graph bear statistical significance ($p<0.05$). The graph shows the means of 3 independent experiments.

5. DISCUSSION

Metformin is an anti-hyperglycaemic drug which is commonly used for the management of type II diabetes [8].Recent epidemiologic studies have associated Metformin with a decreased risk for several cancers including ovarian, breast, colorectal and pancreatic cancer [6,13,19]. Further studies supported that Metformin treatment shows a better prognosis for ovarian cancer patients [13,19]. These observations were in agreement with following *in vitro* and *in vivo* studies, proposing that this drug has an anti-proliferative effect across various cancer cell lines and animal models [8].Metformin is thought to be associated with oxidative stress, DNA damage and the DDR. However, there are disagreements in the literature concerning these Metformin effects. Some studies suggest that Metformin protects, and others that it causes DNA damage [26,27,55,57,64]. Metformin's involvement in the DDR is also unclear, as a study proposes that Metformin attenuates the activation of the DDR by preventing ATM activation [53], while another study supports the opposite [54]. Intriguingly, even if these two studies presented opposite results, they both suggested that through these mechanisms Metformin exerts a protective role against DNA damage. On the other hand, a recent study proposes that Metformin is involved in the DDR in a negative way, as it impairs DNA repair in hepatoma cells [58].

The controversy surrounding Metformin's role in the DNA damage and DDR, as well as the limited literature concerning its association with OC, has prompted us to investigate the potential role of Metformin in the DNA damage and DDR in the OC cell lines OVCAR3 and COV318.

Studies have expressed concerns about the chosen dose of Metformin in *in vitro* experiments, as it is higher than the concentration used in humans (most studies use 1-50 mM of Metformin, while the therapeutic range in humans is 2.8-15 μM [9]). Recent studies have shown that even a dose as low as 10 uM could have an effect in specific hepatoma and ovarian

cancer cell lines [30,58]. However, in the present study a 5 mM Metformin dose was chosen as the highest dose for cells in Western Blot analysis, IF experiments and Colony Formation Assays, in order to correspond with previous experiments performed in our laboratory. Previous studies using a 5 mM and 8 mM dose of Metformin showed that they are not cytotoxic, as they caused limited apoptosis and necrosis [31 and personal communication with Dr. Sudha Sundar]. Hence, we assume that the 5mM Metformin dose that we use in our experiments is not cytotoxic and the results we obtained are from the functions of the drug and not from adverse side-effects. Future experiments could focus on finding the minimum effective dose of the drug in these particular cell lines.

5.1 Metformin uptake in OVCAR3 and COV318 cells

Our first goal was to provide evidence that Metformin is successfully transported into our cells since some cell lines have been reported as insensitive to the drug (e.g. MDA-MB231 [36]). The reason is unknown; one possibility is the low or variable expression of OCT1 (membrane transporter of Metformin) [21]. Previous studies have shown that Metformin can induce growth inhibition in OVCAR3 cells [26,27,31]; thus we can assume that Metformin can successfully transport into these cells. However, COV318 is a rare OC cell line with limited literature available; thus, it was essential to assess Metformin uptake in these cells.

For the purpose of this dissertation, we used an indirect way to determine if Metformin was transported into our cells; through Metformin effects on the Insulin pathway and more specifically, on one of its downstream targets, IRS-1. We expected that both OVCAR3 and COV318 would express IRS-1, since IRS-1 is widely expressed in various tissues and cell lines [34]. Western Blot analysis on OVCAR3 showed that Metformin-treated cells reduce the expression of IRS-1 (unphosphorylated and phosphorylated IRS-1); thus, they lead to IRS-1 down-regulation. This is in agreement with studies suggesting that Metformin down-regulates the

insulin pathway [9] and inhibits the activation of IRS-1 through AMPK eventually leading to its down-regulation [33]. Our Western Blot analysis in COV318 cells showed that IRS-1 was less phosphorylated in Metformin-treated cells. This is in agreement with a previous study suggesting that Metformin-mediated AMPK activation leads to down-regulation of IRS-1 through serine phosphorylation at Ser794 [33]. It is known that serine phosphorylation exerts an inhibitory role on IRS-1, as it eventually reduces its activation by reducing the levels of phosphorylated IRS-1 or by leading to IRS-1 degradation [35]. These observations support our own since, even if Metformin increases serine phosphorylation, we observed a generic reduction in the phosphorylated form of IRS-1. It is unknown why we observed a slightly different effect of Metformin on IRS-1 in OVCAR3 and COV318. We hypothesise that different effects reflect the differences between cell lines: in their proliferation, their metabolism, as well as the constitution of their cell culture media (OVCAR3 are grown in insulin-rich conditions). Also, one can speculate that the reduced IRS-1 phosphorylation in COV318 cells may eventually lead to reduced IRS-1 through degradation [35] at a later time-point, after the Western Blot analysis took place. To conclude, we suggest that Metformin transports into OVCAR3 and COV318 since it down-regulates IRS-1, either by reducing IRS-1 expression (in OVCAR3 cells) or by reducing IRS-1 phosphorylation (in COV318 cells), likely through AMPK pathways.

5.2 Metformin does not alter the expression of DNA repair proteins Rad51 and PARP1

Next, we evaluated whether Metformin has a role in the DNA damage and DNA damage response through down-regulation of DNA repair proteins. It was observed that Metformin treatment does not affect the expression of the DNA repair proteins Rad51 and PARP1 as evident from the Western Blot analysis. Previous unpublished data supported mRNA down-regulation of Rad51 and PARP1 (personal communication with Dr. Sudha Sundar). However,

currently there are no published studies associating Metformin with Rad51 (gene or protein expression). On the other hand, two studies have shown that PARP1 expression in breast cancer cells [36] and OVCAR3 cells [31] remains un-affected by Metformin treatment, which is in agreement with our observations. It is most likely that the mRNA down-regulation of Rad51 and PARP1, does not correspond to a protein-level down-regulation, as previous studies have shown that mRNA abundance is not always correlated with protein abundance in the cell [62].Taken together, the data suggest that a 24 hour 5mM Metformin treatment does not down-regulate the protein expression of the DNA repair proteins Rad51 and PARP1.

5.3 53BP1 is a more suitable DNA damage marker for OVCAR3 and COV318 cells

To further investigate the potential role of Metformin in the DNA damage and DDR, we firstly had to identify a suitable DNA damage marker for OVCAR3 and COV318 cells. Thus, we studied the foci formation of γH2AX and 53BP1 (in non-irradiated and irradiated conditions) in OVCAR3 and COV318 and compared them with HeLa and U2OS cells. It is known that γH2AX and 53BP1 are markers of probable DNA damage [52,48,66]. These DNA damage markers have comparable kinetics and they co-localize in sites of damage by forming foci [47]. Through our experiments we observed that γH2AX is not an appropriate DNA damage marker for OVCAR3 or COV318, because of the high abundance and persistent formation of γH2AX foci even in the absence of IR-induced DNA damage and at late recovery time-points. The high numbers of γH2AX foci and the possibility that OVCAR3 and COV318 have constitutive DNA damage, makes the analysis of these foci extremely difficult and would probably interfere with the interpretation of any results. On the other hand, 53BP1 foci formation appeared to have similar kinetics in OVCAR3 and COV318 compared with our controls (HeLa and U2OS). 53BP1 foci were relatively reduced or absent in non-irradiated conditions; while in the presence of IR-induced DNA damage, 53BP1 foci formed and disappeared with similar rates as HeLa and

U2OS cells. Furthermore, 53BP1 foci were not of high abundance as γH2AX, hence they are more easily analysed in the context of DNA damage. It was previously shown that the formation and disassociation of 53BP1 foci is representative of the kinetics following DSB damage and repair respectively [47]. Consequently, 53BP1 was used in later experiments to assess the effects of Metformin on DNA damage and DDR.

5.4 Metformin delayed the resolution of 53BP1 foci (associated with DNA double-strand breaks) and reduced cell survival when combined with irradiation

In order to evaluate the role of Metformin on DNA damage and DDR we performed IF in OVCAR3, COV318 and HeLa cells to study the formation and dissociation of DNA damage associated foci. A study has shown that HeLa cells are insensitive to the growth inhibitory effects of Metformin [63]; thus, we originally used this cell line as a negative control for our experiments. In OVCAR3 and HeLa cells (non-irradiated conditions) we observed that the Metformin-treated cell population consisted of a higher amount of cells having probable DNA damage (designated by “more than 10 53BP1 foci/cell”) compared to untreated cells. In COV318 we didn’t observe a significant difference. In irradiated conditions, we showed that in early time-points after IR (30 to 60 minutes post-IR) the population of cells receiving Metformin had less cells with DNA damage markers; after that time-point however, that percentage rises (4 to 24 hours post-IR) but it does not reduce in the same rate as untreated cells (even at 72 hours post-IR). These observations were similar between OVCAR3 and COV318 cells but not HeLa cells. This may potentially relate to the insensitivity of HeLa cells to the growth inhibitory effects of Metformin but the exact reason is unknown. The fact that at early time points after IR both OVCAR3 and COV318 cells treated with the drug, consisted of fewer cells with probable DNA damage was rather intriguing. One hypothesis is that the treatment with Metformin in the presence of IR may delay or impair the DDR. In that scenario, in early time points after IR-induced DNA damage, there would be a

delayed response which might delay the formation of 53BP1 foci at DSBs. We hypothesise that in OVCAR3 and COV318 cells, this is represented by a reduced number of cells having high numbers of 53BP1 foci (early after IR exposure). The hypothesis that Metformin may delay the DDR, might, at least in part, explain the delayed DNA repair events that we observed in OVCAR3 and COV318 cells; evidenced by early absence of 53BP1 foci, but higher levels persisting long after IR exposure. Alternatively, this may be explained by the hypothesis that Metformin treatment may result in impaired DNA repair in OVCAR3 and COV318 cells (hence 53BP1 persisting for longer). The hypotheses of Metformin delaying the DDR or DNA repair may also explain our observation that Metformin-treated cells exhibit a minor increase of DNA damage markers in OVCAR3 cells (in absence of IR).

Our observations in IF experiments were reflected in our results in subsequent colony formation assays, in which we investigated whether Metformin potentiated the DNA-damaging effects of IR by reducing clonogenic survival of ovarian cancer cells. We observed that a 24 hour treatment of Metformin (5 mM) followed by IR, resulted in a statistically significant reduced OVCAR3 colony survival compared with cells treated with IR only. This reduction in colony survival was IR dose-dependent. We considered that colonies produced by Metformin-treated cells (non-irradiated) represent 100% survival, in our attempt to minimize the growth-inhibitory or apoptotic effects of the drug. From our observations we can suggest that Metformin potentiates the effects of IR by reducing the survival of OVCAR3 cells. We hypothesise that Metformin enhances the radiation response by having a negative impact on DNA damage and DNA repair pathways, which eventually leads to reduced cell survival. This hypothesis can be supported by our observations in IF experiments suggesting that Metformin may delay the DDR and/or DNA repair.

Most studies in the literature try to approach the role of Metformin in DNA damage and DDR by assessing γH2AX foci formation and ATM phosphorylation. Given the fact that γH2AX and 53BP1 have similar foci formation kinetics and co-localize in DSBs [47] we can compare the data from these studies to our findings. In absence of IR, one study has shown that a 48 hour treatment with Metformin (1, 5 and 20 mM) leads to reduced H2AX phosphorylation (γH2AX) and reduced ATM activation (reduced Ser1981 -ATM phosphorylation) [53]. On the other hand, another study showed that a 48 hour treatment with 5mM Metformin increased γH2AX foci formation but interestingly, no 53BP1 foci co-localization was observed in IF experiments. Furthermore, they supported that there was increased signal of activated ATM (Ser 1981 phospho-ATM) but it also did not co-localize with the γH2AX foci [54]. Interestingly, Halicka *et al.* (2011) [53] has repeated the experiments of Vazquez-Martin *et al.* (2011) [54] and their results were contradicting. Whereas, another study showed that 10 uM Metformin treatment (for 1 hour without IR) induces a minor increase in γH2AX expression 1 hour after treatment, which is reduced after 24 hours [58]. This agrees with our observations, as in absence of IR, there were minor or no differences in the percentage of cells having high abundance of 53BP1 foci between Metformin-treated and untreated cells in OVCAR3 and COV318 respectively. However, the fact that some studies are contradicting may also reflect a difference in the time scales at which DNA damage signalling was assessed and underlines that further investigation is essential.

In irradiated conditions, Liu *et al.* (2012) [58] showed that 1 hour after a 10 uM Metformin treatment, γH2AX expression was increased and persisted at higher levels even after 24 hours post-IR compared with untreated cells. This was in agreement with our observations in IF experiments when Metformin was used with IR (after 1 hour post-IR). Their hypothesis, that the delayed disappearance of γH2AX is due to inhibition of DNA repair, also agrees (at least in part) with our speculations on Metformin-induced impair of DDR or DNA repair. It is still unknown why

we observed different results in HeLa cells, especially since they are insensitive to Metformin-induced growth inhibition [63]. The minor differences between Metformin-treated and untreated cells (of lesser magnitude than OVCAR3 and COV318) as well as the overlap between certain time-points, suggest that they probably derive from experimental data variation. In any case, it would be interesting to perform future experiments in HeLa cells using Metformin, to evaluate whether it has a role in the DDR and DNA damage response in this cell line, but this currently diverges from our interest in the mechanisms of Metformin in OC cells.

Even though no other studies on OC have assessed the effects of Metformin combined with IR through colony formation assays, other *in vitro* studies made similar observations in hepatoma and pancreatic cancer cells by conducting colony assays combining IR and Metformin [58,59]. Also, an *in vivo* study proposed that Metformin sensitises breast cancer cells to IR (using similar experimental techniques as in the present study) as well as enhances the efficacy of IR to suppress tumour growth [60]. These studies, however, proposed slightly different mechanisms behind their similar observations [58,59,60]. The study on hepatoma cells hypothesised that Metformin may be functioning as a radiation sensitiser by impairing DNA repair [58]; this is similar to our own hypothesis of Metformin-induce impair of DDR or DNA repair. The other studies on breast and pancreatic cancer cells hypothesised that the observed effects may be attributed to Metformin-induced growth inhibition through activation of AMPK and mTOR downregulation [59,60]. We speculate that these two proposed mechanisms do not contradict each other, they rather complement each other. This is supported by studies which have shown that by inhibiting AMPK, the radio-sensitising abilities of Metformin (in IR conditions) were partly reduced in pancreatic and lung cancer cells [59,67]. Further investigation of the exact underlying mechanisms is necessary as the growth-inhibitory effects of the drug and IR

may also play a great part in these effects (even though, in the present study we attempted to exclude the growth inhibitory effects from our analysis).

To conclude, our observations from IF experiments and Colony formation assays suggest that Metformin may delay or impair the DDR or DNA repair when combined with IR which may explain the ability of the drug to enhance the damaging effects of IR reducing clonogenic survival. Furthermore, we do not believe that the observed effects are due to Metformin-induced DNA damage. It has been suggested that the average number of 53BP1 foci in a cell corresponds to the extension of the DNA damage, as it is proportional to the amount of DSBs [47]. We observed that the average number of 53BP1 foci per cell is quite similar between Metformin-treated and control cells in non-IR and IR conditions, suggesting that Metformin does not cause increased DNA damage.

The possible underlying mechanisms behind our observations are not very clear; only speculations can be made, as the DDR and DNA repair networks consist of complicated and interacting pathways [45]. Some studies correlate Metformin with ATM kinase providing a possible candidate that can give more insight into the problem [9,68]. ATM activation is associated with binding to DSB sites and starting a cascade of regulating events, including DNA repair [68]. Halicka *et al.* (2011) [53] suggested that ATM activation is reduced immediately after Metformin treatment. A declined ATM activation may translate into delayed 53BP1 recruitment to the sites of damage, and at later time-points after treatment, it may explain the impaired/delayed DNA repair events that we observed in the present study. Furthermore, since above mentioned studies (which observed reduced clonogenic survival after combining Metformin and IR) hypothesised that the underlying mechanisms are impair in DNA repair and AMPK-dependent inhibition [58,59,60]; ATM provides a cross-talk between these mechanisms. Apart from ATM's participation in the DDR pathway, ATM can also participate in the AMPK pathway since it

phosphorylates and activates LKB1, which in turn activates AMPK leading to mTOR downregulation and growth inhibition [68].

The implication of ATM in the effects caused by Metformin is only a speculation and it is essential to investigate this further; it would be particularly interesting to assess the expression and foci formation of 53BP1, γH2AX and phospho-ATM at several time-points after Metformin-treatment (in IR and non-IR conditions), in order to correlate ATM activation levels with foci formation and DNA repair. Also, it would be interesting to assess DNA damage markers (γH2AX, 53BP1, ATM phosphorylation) together with the expression of AMPK/ mTOR pathway proteins after combining Metformin with IR in order to elucidate the exact mechanism behind the enhanced radiation response. Another experiment could be to inhibit AMPK (e.g. with compound C) and then assess the impact of the drug in radio-sensitisation, in order to evaluate whether it is partly attributable to the role of Metformin on the DDR and DNA repair pathways.

5.5 Metformin results in increased percentage of replicating cells having 53BP1 foci (associated with DNA double-strand breaks) and in increased cell death after combination with Gemcitabine

Another question surrounding Metformin's role in the DNA damage and DDR, is the phase of the cell cycle in which the drug exerts a greater effect. We observed that the Metformin-treated cell population that are Cyclin A positive, a marker of S/early G2 cell cycle phase, has higher percentage of cells with DNA damage markers (more than 10 53BP1 foci) compared to the untreated cell population. This suggests that more cells undergoing S-phase (replication) have probable DNA damage. This was not observed in Cyclin A negative cells where the percentage was similar between untreated and Metformin-treated cell populations.

These observations suggest that cells undergoing S-phase (DNA replication) are more sensitive to the effects of Metformin. In accordance with our previous observations, we may hypothesise that replicating cells are more sensitive to the possible impair of DDR or DNA repair induced by Metformin, which is reflected by an increased population with probable DNA damage.

Due to these observations, we went on to investigate whether Metformin potentiates the DNA-damaging effects of Gemcitabine (which causes problems during replication by inducing DNA synthesis inhibition and apoptosis [65]). No studies have investigated so far whether Metformin potentiates the effects of Gemcitabine in OC, but studies have shown that Metformin potentiates the cytotoxic effects of PEITC (phenethyl isothiocyanate) [31], Carboplatin [30] and Cisplatin [29]. In the present study we performed colony formation assays in OVCAR3 cells treated with Gemcitabine or combination of Gemcitabine and Metformin, and observed that the combination of drugs caused a reduction in the survival of OVCAR3 cells compared to Gemcitabine alone. This result suggests that Metformin increases the damaging effects of Gemcitabine in ovarian cancer. Collectively, in the IF experiment we observed that in the S-phase cell population, a higher percentage of cells has DNA damage markers suggesting that Metformin has an S-phase specific enhanced effect. Furthermore, since Gemcitabine has replication-specific functions (integrates into DNA during S-phase) and Metformin increased the effects of Gemcitabine in the colony assays (minor increase in OVCAR3 cytotoxicity), then this may further support that Metformin has a greater effect in replicating cells. In conclusion, from the observations of the IF experiments and colony assays we hypothesise that Metformin may have a greater effect in S-phase (replicating) cells.

In contrast to our observations in the IF experiment, a study in human lung adenocarcinoma cells, showed that cells undergoing G2/M- or S-phase have reduced DNA damage signaling (as evidenced from reduced γH2AX foci formation) post-Metformin treatment

[53]. However, our observations in colony assays are in agreement with the observations of a study which combined Metformin and Gemcitabine showing reduced survival of pancreatic cancer cells compared to Gemcitabine treatment alone [59].

To conclude, the exact mechanisms behind our observations in the IF experiments and the colony formation assays are still yet to be defined; however they may relate to delayed repair mechanisms, particularly in cells undergoing replication. We speculate that Metformin's effect may relate to its effect on ATP levels. It is known that Metformin causes energy stress by inhibiting mitochondrial complex I which in turn inhibits the oxidative phosphorylation chain. Thus, the AMP: ATP ratio is increased and the cell undergoes energy stress through ATP depletion [6,8]. A previous study hypothesized that ATP depletion caused by Metformin is one of the reasons why DNA repair is impaired, as DNA repair is an energy-demanding process [58]. In addition, DNA replication also requires increased amounts of ATP [69] so the Metformin-induced ATP depletion may further enhance the effects of Metformin during S-phase. Also, if we take our previous hypotheses into account, that Metformin delays/impairs the DDR or DNA repair pathways, one could expect an impact in the DNA damage cell cycle checkpoints. Especially if Metformin resulted in delayed or impaired ATM activation (which regulates cell cycle checkpoints in response to DNA damage among others [68]) a negative impact on the intra S checkpoint may be expected. Furthermore, the role of these checkpoints is to delay or arrest the cell cycle in order to provide more time for DNA repair [45]; if DNA repair is hypothetically delayed, then one might assume that more damaged cells would remain longer in S-phase waiting to be repaired. This hypothesis supports our observations in the IF experiment, in which the population of Cyclin A positive cells has increased numbers of cells with DNA damage markers in Metformin-treated conditions. However, we have to be cautious with these speculations because our hypotheses on the role of Metformin in the DDR and DNA repair were mostly based on Metformin combined with IR. Further studies are also necessary to determine the exact

mechanism behind the combinatorial effect of Gemcitabine and Metformin, as well as to examine the nature of their relationship i.e. whether it is synergistic, additive or epistatic.

Consequently, future experiments would be useful to assess the impact of Metformin in the DDR and DNA repair in each cell cycle phase (in absence or presence of IR). Another experiment could be to introduce ATP to Metformin-treated cells, in order to see if these observed effects are rescued. Furthermore, a future experiment that could evaluate whether Metformin potentiates the DNA-damaging effects of Gemcitabine by delaying the DDR or DNA repair, is to assess DNA damage markers after their combined treatment and compare them with each drug treatment alone. The potential of combining these drugs is promising, as apparent from an undergoing clinical trial, in which Metformin is combined with Gemcitabine as adjuvant therapy for patients suffering from pancreatic cancer [70]. In the present study, when we combined Metformin and Gemcitabine, the size of the effect was not great; however, in our analysis we excluded the anti-proliferative and apoptotic functions of Metformin. If one takes these into consideration, and the fact that Metformin may also have a role in the DDR, DNA repair or an effect during replication, then the combinatorial effects of Metformin and Gemcitabine may be greater. Thus, the combination of Metformin and Gemcitabine treatment may prove useful against ovarian cancer and should undergo further investigation.

6. CONCLUSIONS

The potential role of Metformin on DNA damage and DDR has been the centre of controversy among studies, with contradicting findings depending on the dose of the drug, the treatment strategy and the cell line that was used. The controversy surrounding this drug and the lack of studies in OC , have prompted us to investigate the role of Metformin on DNA damage and DNA repair using the ovarian cancer cell lines OVCAR3 and COV318 (which are representative of ovarian tumours). Through Western Blot analysis we observed that Metformin did not affect the protein expression of the DNA repair proteins Rad51 and PARP1.Through IF experiments and colony formation assays, we observed that Metformin delayed the disappearance of DNA damage markers (associated with DNA double-strand breaks) and reduced cell survival when combined with irradiation. Metformin treatment however, did not increase the average amount of protein foci (associated with DNA double strand breaks) per cell. This evidence strongly suggests that Metformin may potentiate the DNA-damaging effects of irradiation by delaying or impairing the DDR or repair and not by inducing DNA damage. Furthermore, our experiments suggested that replicating cells were more sensitive to Metformin's effects, since a higher percentage of this cell population had DNA damage markers (associated with DNA double-strand breaks). This appears to be reflected in colony formation assays, where Metformin was combined with Gemcitabine, a chemotherapeutic drug that causes problems during replication by inhibiting DNA synthesis and causing apoptosis. Their combination resulted in reduced cell survival which may be explained by Metformin-induced delay or impair of the DDR and the ability of the drug to have an enhanced effect during replication. To conclude, this study was the first to attempt to give insight into the role of Metformin in the DDR in ovarian cancer; however, further investigation to verify these hypotheses and to discover the underlying mechanisms behind these Metformin's effects are of crucial importance.

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