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# Exploring the Role of Cell Signalling and DNA Damage Pathways in Primary Surgically Resected Ovarian Cancer

*By*

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# Abstract


## Background:

Ovarian cancer (OC) is the most lethal gynaecologic malignancy. It is a heterogeneous disease at the molecular, histological and clinical levels. It encompasses a collection of neoplasms, which have distinct molecular profiles and clinicopathological features. Despite there being a wide variety of OC subtypes, all are treated similarly with no categorisation. In addition, although there is initial sensitivity to chemotherapy especially the platinum-based regimens, majority of patients developed resistance to treatments and subsequent recurrence of the disease. Tremendous efforts have been made to identify novel pathways and prognostic biomarkers for potential therapeutic strategies. However, to date these parameters are not sufficient to reflect the degree of heterogeneity and to guide management decisions at individual patient level. Therefore, further refinement of the existing prognostic tools is urgently needed. Several studies have shown that the major histologic subtypes of OC have different risk factors, genetic susceptibilities, and clinical response to therapy. Moreover, they are all different at the molecular level. In this current study, we hypothesised that both cellular and DNA damage signalling pathways can influence ovarian tumour progression and prognosis. This study aimed to investigate a large cohort of surgically treated primary OC patients with their available, electronically recorded follow-up data and test the aberrant expression of different proteins directly related to cell signalling and DNA damage pathways. This is to further understand not only the biology of the tumour, but also to identify their clinical significance. Furthermore, to identify which of these key proteins merits to be dominantly functioning when co-expressed.

## Patients and Methods:

This study included 525 primary OC cases treated surgically at Nottingham University Hospitals (NUH) between 1997 and 2010. Clinicopathological and outcome data was collected. All cases were histologically reviewed, and the representative tumour blocks were retrieved. Tissue microarrays (TMAs) were constructed from the selected and marked tumour tissue sites. Then, assessment of two main pathways with known intimate relations to cancer development and progression, cell signalling and DNA damage repair (DDR) was performed. Key pathways' protein expression status was investigated then, correlated with the clinicopathological variables, platinum sensitivity and patients' outcome. For this purpose, we used an exploratory design for this research, pursuing both the clinical and pre-clinical experiments. Immunohistochemistry (IHC) was utilised to stain a number of key targets: 1) cell signalling pathway proteins (p-AKT, p-mTOR, p-4EBP1, p-P70S6K), and (ATM, PTEN, P85ά, XIAP) and 2) DDR pathway (PARP1, XRCC1, polβ), and (LIG1, LIG3, LIG4), then their prognostic value was analysed. Preclinical analysis by gene knockdown using siRNA, nuclear and cytoplasmic extraction and western blot (WB) in a panel of platinum sensitive and platinum resistant OC cell lines (A2780 and A2780cis, respectively) were also utilised.

## Results:

The majority of cell signalling pathway members (p-AKT, p-mTOR, p-4EBP1, p-P70S6K) and (ATM, P85ά and XIAP) showed positive associations with poor prognostic variables and shorter survival in the whole (unselected) cohort and in platinum sensitive tumours. Interestingly, p-P70S6K negative expression was highly significantly associated with improved sensitivity to platinum agents. On the other hand, p-AKT overexpression showed significance with resistance to chemotherapy and higher risk of residual disease. In addition, p-AKT and p-4EBP1 were independent predictors of unfavourable outcome. Cases with low/negative

expression of both AKT and 4EBP1 exhibited a significantly improved survival.

Interestingly, we have identified PTEN as a marker of improved outcome. Its high expression was correlated with favourable patients' prognosis. Interestingly, ATM and XIAP were additional independent factors for progression free survival (PFS). Importantly, when ATM status was positive, PTEN and p85α lost their significance on the patients' outcome. Similar findings were observed with XIAP positivity. This could imply PTEN and p85α context specific function in relation to the corresponding expression status of ATM and XIAP.

Regarding the DNA signalling pathway proteins, PARP1, XRCC1 and polβ, all showed negative association with favourable outcome in the unselected (whole) cohort and platinum sensitive cases. In addition, PARP1 and XRCC1 positive cases exhibited a higher chance of residual disease after surgery. Interestingly, negative XRCC1 expression improved the response to platinating agents. Importantly, in multivariate analysis we have identified XRCC1 as an independent factor for poor recurrence outcome. Preclinical XRCC1 knockdown increased PARP1 levels, a novel finding that may identify a group of patients who may preferentially benefit from PARP inhibitor therapy.

When the biological significance of the three human ligases was investigated, both LIG1 and cytoplasmic LIG3 were associated with poor prognostic variables and adverse clinical outcome in the unselected and platinum sensitive cohorts. Overexpression of both proteins significantly enhanced the risk of residual tumour following surgery. Interestingly, low/negative cytoplasmic LIG3 improved the response to platinum therapy. LIG4 overexpression was a predictor of prolonged survival and improved response to chemotherapy. Importantly, in this thesis we have identified that LIG3 prognostic impact is dependent on its cellular sub-localisation status. Its cytoplasmic form exhibited tumour-encouraging qualities and

worse patients' outcome, unlike its nuclear isoform. Preclinically, we observed a significant increase in the cytoplasmic fraction of LIG3 after cisplatin treatment. Therefore, we speculate that the cytoplasmic LIG3 can influence the response to platinum therapy and may contribute to the development of chemotherapy resistance. In addition and importantly, LIG1 showed an independent poor prognostic significance in multivariate analysis, another novel finding in this research.

## Conclusions:

To conclude, this thesis illustrates that OC is can exhibit different variations in the expression of tumour key protein markers, which can be utilised to predict tumour behaviour and stratify patients for therapy. This explorative study combined both clinical and pre-clinical experiments, and provided novel findings in primary ovarian tumour cases that can be utilised to improve the disease prognosis. In the future, elaborated and well-designed functional studies may reveal better insights on the contributions for each identified biomarker candidate, and their relevant pathway in driving or inhibiting platinum resistance mechanisms, and improve the outcome in primary OC patients.

# Acknowledgement

Firstly, special thanks and grateful to Allah the Almighty who gave me the power to finish this project.

I cannot express enough thanks to my supervisors: Professors Emad Rakha, Sirinivasan Madhusudan and Steven Chan for their valuable support throughout my PhD. Their enthusiasm, immense knowledge, timed follow up and tremendous mentoring allowed me to grow as an independent researcher. Their advice on both research as well as on my career has been priceless together with their limitless ideas that enlightened my experience in national and international conferences, in addition to publishing scientific papers.

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Words will not be enough to thank my parents, my brothers and sisters for their limitless encouragement. At the same time, I am indebted to my loving, supportive and friendly family; my wife Dr Dena Ahmad and my daughters: Safa, Marwa, Aya and my little son Zakariya, their encouragement and standing by me when the times got rough are much appreciated and duly noted. Their help and support is what sustained me thus far and assisted in finishing my thesis.

# Dedications

I would like to dedicate this thesis to my mother, Etidal Abdullah. She is the light of my life and the strongest and bravest woman I have ever known, and I am very proud to be her son. Mum, you inspire me every day, instilling the strength and determination in me to push through no matter how bad things seem. All that I am and all what I achieved so far is because of your endless support, and all that I do is for you.

# Abstracts and Publications

## Presented abstracts at national and international conferences

**1- PI3K/AKT/mTOR signalling in Ovarian Cancer and the effect of their upstream HER Family Proteins**

**M Alabdullah**, S Chan, P Moseley, E Rakha

Has been presented as oral presentation at the Faculty postgraduate Research Forum (Medicine and Health Sciences Faculty) – university of Nottingham, UK, 24th June /2015.

**2- ATM regulated PTEN degradation is CK2α mediated in p85 deficient cells: unravelling a new ATM–PTEN-PI3K network in cancers**

Reem Ali, Christina Perry, **Muslim Alabdullah**, Islam Miligy, Abdolrahman Shams Nateri, Tarek Abdel Fatah, Paul Moseley, Stephen Chan and Srinivasan Madhusudan

Has been presented as poster presentation at the 6th EU-US Conference on Repair of endogenous DNA damage - University of Udine, Italy, 24th – 28th September /2017. This poster won the best poster prize.

**3- Eukaryotic initiation factor 4E-binding protein 1 (4EBP1) is an independent predictor of poor outcome in ovarian cancer**

**M Alabdullah**, I Miligy, P Moseley, S Madhusudan, S Chan, E Rakha

Has been presented as poster presentation at the joint Royal Society of Medicime and pathological society 2018-winter meeting: epigenetics - understanding disease and guiding therapies - London, UK, 25th – 26th January /2018.

**4- mTORC1 and its downstream effectors predict poor outcome in primary epithelial ovarian cancer**

**M Alabdullah**, I Miligy, P Moseley, S Madhusudan, S Chan, E Rakha

Has been presented as poster presentation at the European Society of Medical Oncology (ESMO) meeting- 2018: international congress on Targeted Anticancer Therapies – Paris, France, 5th – 7th March/ 2018.

**5- Clinicopathological, predictive and prognostic significance of XRCC1-Ligase III heterodimer expression in Ovarian Cancer**

**M Alabdullah**, P Moseley, S Chan, E Rakha, S Madhusudan

Has been presented as poster presentation at the European Society of Medical Oncology (ESMO) meeting- 2018: international congress on Targeted Anticancer Therapies – Paris, France, 5th – 7th March/ 2018.

**6- Ribosomal protein S6 kinase (p70S6K) expression is associated with platinum resistance and poor clinical outcome in Primary Epithelial Ovarian Cancer**

**M Alabdullah**, M Toss, P Moseley, S Madhusudan, S Chan, E Rakha

Has been presented as poster presentation at the 11th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland - Maastricht Pathology 2018 – Maastricht, Netherlands, 19th – 22nd June / 2018.

**7- AKT expression is associated with poor clinical outcome in Ovarian Cancer**

**M Alabdullah**, P Moseley, S Madhusudan, S Chan, E Rakha

Has been presented as poster presentation at the 25th Biennial Congress of the European Association for Cancer Research – Amsterdam, Netherlands, 30th June – 03rd July /2018.

**8- Human Ligase profiling to predict platinum sensitivity and clinical outcome in primary epithelial ovarian cancers**

**M Alabdullah**, R Ali, P Moseley, S Chan, E Rakha, S Madhusudan

Has been presented as poster presentation at the 25th Biennial Congress of the European Association for Cancer Research – Amsterdam, Netherlands, 30th June – 03rd July /2018.

**9- Predictive, prognostic and therapeutic significance of human Ligases (I, III & IV) in epithelial Ovarian Cancers**

**M Alabdullah**, R Ali, I Miligy, A Alblihy, P Moseley, S Chan, E Rakha, S Madhusudan

Has been presented as poster presentation at the CRN (Cancer Research Nottingham) - 2018 annual symposium event of the University of Nottingham - Nottingham, UK, 19th September /2018.

**10- Clinical and outcome significance of positive AKT expression in Epithelial Ovarian Cancer**

**M Alabdullah**, A Alblihy, P Moseley, S Madhusudan, S Chan, E Rakha

Has been presented as poster presentation at the National Cancer Research Institute (NCRI) – 2018 international Conference – Glasgow, UK, 04th -06th November /2018.

**11- Clinicopathological significance of ligase I, III & IV in Epithelial Ovarian Cancers**

**M Alabdullah**, A Alblihy, R Ali, P Moseley, S Chan, E Rakha, S Madhusudan

Has been presented as poster presentation at the National Cancer Research Institute (NCRI) – 2018 international Conference – Glasgow, UK, 04th -06th November /2018.

**12- MRE11 is a novel potential promising biomarker in ovarian cancer management**

A Alblihy, **M Alabdullah**, R Ali, K Mosquita, E Rakha, S Madhusudan

Has been presented as poster presentation at the National Cancer Research Institute (NCRI) – 2018 international Conference – Glasgow, UK, 04th -06th November /2018.

**13- Positive AKT Expression is a Predictive Marker for Tumour Recurrence in Epithelial Ovarian Cancer**

**M Alabdullah**, P Moseley, S Chan, S Madhusudan, E Rakha

Has been presented as combined oral and poster presentations in the second Joint Meeting with the Royal Society of Medicine (RSM) and 211th Scientific Meeting of the Pathological Society of Great Britain & Ireland - Let's Talk Precision Pathology – London, UK, 22nd – 23rd January /2019.

**14- Predictive, prognostic and therapeutic significance of human Ligases (I, III & IV) in epithelial Ovarian Cancers**

**M Alabdullah**, R Ali, I Miligy, A Alblihy, P Moseley, S Chan, E Rakha, S Madhusudan

Has been presented as poster presentations in the 2nd Joint Meeting with the Royal Society of Medicine (RSM) and 211th Scientific Meeting of the Pathological Society of Great Britain & Ireland - Let's Talk Precision Pathology – London, UK, 22nd – 23rd January /2019.

# Publications

I. **The mTOR downstream regulator (p-4EBP1) is a novel independent prognostic marker in ovarian cancer**
**M Alabdullah**, D Ahmad, P Moseley, S Madhusudan, S Chan & E Rakha
Journal of Obstetrics and Gynaecology, IF: 0.807; 39:4, 522-528.

II. **PARP1 blockade is synthetically lethal in XRCC1 deficient sporadic epithelial ovarian cancers**
Reem Ali, **Muslim Alabdullah***, Adel Alblihy, Islam Miligy, Katia A Mesquita, Stephen Chan, Paul Moseley, Emad Rakha, Srinivasan Madhusudan
Cancer letters, IF: 7.360, 31669203.
* First joint author

III. **ATM Regulated PTEN Degradation Is XIAP E3 Ubiquitin Ligase Mediated in p85α Deficient Cancer Cells and Influence Platinum Sensitivity**
Reem Ali, **Muslim Alabdullah**, Islam Miligy, Makhliyo Normatova, Roya Babaei-Jadidi, Abdolrahman Nateri, Emad Rakha, Srinivasan Madhusudan
Cells open access, IF: 4.829, PMC6848936.

IV. **ERCC1-XPF deficiency is a predictor of olaparib induced synthetic lethality and platinum sensitivity in epithelial ovarian cancers**
Katia Mesquita, **Muslim Alabdullah**, Michaela Griffin, Michael Toss, Tarek Abdel Fatah, Adel Alblihy, Paul Moseley, Stephen Chan, Emad Rakha, Srinivasan Madhusudan
Gynecologic Oncology, IF: 4.540, 10.1016

V. **Human DNA ligase profiling and therapeutic targeting in epithelial ovarian cancers**
Reem Ali*, **Muslim Alabdullah***, Adel Alblihy, Islam Miligy, Tarek Abdel-Fatah, Stephen Chan, Pei Wen Chiang, Nigel Mongan, Emad Rakha, Alan Tomkinson, Srinivasan Madhusudan

Submitted and considered for publication after revision in Theranostics, IF: 8.5

* First joint author

**VI. RAD50 deficiency is a predictor of platinum sensitivity in sporadic epithelial ovarian cancers**

Adel Alblihy, **Muslim Alabdullah**, Michael Toss, Mashael algethami, Nigel Mongan, Emad Rakha and Srinivasan Madhusudan

Accepted for publication in molecular biomedicine journal

**VII. Targeting meiotic recombination 11 (MRE11) nuclease for personaliSation of epithelial ovarian cancer therapy**

Adel Alblihy, Reem Ali, Alison Ritchie, Cinzia Allegrucci, Paloma Ordonez Moran, Katia Mesquita, Mashael Algethami, Michael S Toss, **Muslim Alabdullah**, Ian Hickson, Natalie Tatum, Jennie Jeyapalan, Anna Grabowska, Emad Rakha, Nigel Mongan and Srinivasan Madhusudan

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**VIII. Molecular disruption of DNA polymerase β for platinum sensitization and synthetic lethality in epithelial ovarian cancers**

Reem Ali, Adel Alblihy, Islam Miligy, **Muslim Alabdullah**, Mansour Alsaleem, Michael Toss, Tarek Abdel-Fatah, Paul Moseley, Stephen Chan, Nigel Mongan, Satya Narayan, Emad Rakha and Srinivasan Madhusudan

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**IX. Clinicopathological and functional evaluation reveal NBS1 as a predictor of platinum resistance in epithelial ovarian cancers**

Adel Alblihy, **Muslim Alabdullah**, Reem Ali, Mashael Algethami, Michael Toss, Nigel Mongan, Emad Rakha, Srinivasan Madhusudan

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## Abbreviations

4EBP1: 4E-binding protein 1 ..... 35
ACTION: Adjuvant Chemotherapy Trial in Ovarian Neoplasia ..... 28
alt-NHEJ: alternative non-homologous end joining ..... 168
antibody ..... 62
antigen ..... 62
ATM: Ataxia-telegiectasia mutated ..... 37
BER: Base excision repair ..... 43
CA: Cancer antigen ..... 15
Ca125: Cancer Antigen 125 ..... 22
CAP: cyclophosphamide, adriamycin and cisplatin ..... 29
CEA: Carcinoembryonic Antigen ..... 22
Chronic Myeloid Leukaemia ..... 254
CML ..... 254
CT: computed tomography ..... 23
cytokeratin ..... 22
Cytoplasmic Extraction Reagent ..... 73
DFS: Disease Free Survival ..... 29
Dithiothreitol ..... 69
EGFR: Epidermal growth factor receptor ..... 31
EGFR1: epidermal growth factor 1 ..... 4
FFPE Formalin Fixed Paraffin Embedded ..... 55
FIGO: International Federation of Gynaecology and Obstetrics ..... 23
GF: growth factor ..... 3
H&E: Haematoxylin and Eosin ..... 60
HCL: Hydrochloric acid ..... 61
horseradish peroxidase ..... 70
IAP3: inhibitor of apoptosis protein 3 ..... 38
IAPs: Inhibitor of apoptosis proteins ..... 38
ICLs: inter-strand crosslinks ..... 43
ICON1: International Collaborative Ovarian Neoplasm Trial 1 ..... 28
IHC: Immunohistochemistry ..... 22, 33, 60
IMS: Industrial Methylated Spirits ..... 60
IP: Intra-peritoneal ..... 30

IR: Ionising radiation ..... 41
Ligase I ..... 43
Ligase III ..... 48
Ligase IV ..... 44, 48
MDT: multi-disciplinary team ..... 23
MMR: mismatch repair system ..... 29
MRI: Magnetic Resonance Imaging ..... 15
mTOR: mammalian target of rapamycin ..... 34
NER: Nucleotide excision repair ..... 44
NHSB: Nottingham Health Science Biobank ..... 55
NUH: Nottingham University Hospitals ..... 51
OCP: oral contraceptive pill ..... 9
OCS: Ovarian cancer screening ..... 18
OS: Overall Survival ..... 52
Ovarian cancer ..... 3
PARP1: Poly [ADP-ribose] polymerase 1 ..... 44
PET/CT: integrated positron emission tomography and CT ..... 23
PFS: Ovarian Cancer Progression Free Survival ..... 52
PID:Pelvic inflammatory disease ..... 8
PIKK: PI3K-like protein kinases ..... 37
PIP2: phosphatidylinositol (4,5) triphosphate ..... 37
PIP3: phosphatidylinositol (3,4,5)-triphosphate ..... 37
POLβ: DNA polymerase β ..... 44
pRb: retinoblastoma protein ..... 4
PTEN: Phosphatase and tensin homolog ..... 37
QMC: Queen's Medical Centre ..... 65
RCT: randomised controlled trial ..... 18
RMI: Risk of Malignancy Index ..... 17
ROS: Reactive oxygen species ..... 40
S6K1: S6 kinase beta-1 ..... 35
SEER: Surveillance, Epidemiology, and End Results program ..... 18
Short Tandem Repeat ..... 66
Single-Strand Break Repair ..... 168
SPSS: Statistical Package for the Social Sciences ..... 74
TMA: Tissue Microarray ..... 52

TRUST: Trial of Radical Upfront Surgical Therapy ..... 27
UKCTOCS: United Kingdom Trial of OC Screening..... 18
VEGF: vascular endothelial growth factor ..... 6
WB: Western blotting ..... 68
XIAP: X-linked inhibitor of apoptosis protein..... 31, 38
XRCC1: X-ray repair cross-complementing protein 1 ..... 44

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# 1 General Introduction

## 1.1 General introduction to ovaries

### 1.1.1 Background

Ovary is a name that was derived from Latin word 'ovarium'. The ovary is part of the female reproductive system. It produces an ovum, which when released; it travels through the fallopian tube to the uterus. They also secrete hormones that play a role in the menstrual cycle and fertility. The ovary progresses many stages through starting from the prenatal period to the menopause (Colvin and Abdullatif, 2013).

### 1.1.2 Embryology

There is a coordinated interaction between somatic cells and female germ cells in embryonic ovaries. Therefore, this creates a unique microenvironment that facilitates the formation of follicles (Yao, 2005). The ovary development is closely associated with female reproductive tract development. Multiple Genes are essential for the initiation of the ovarian development. The first step of gonadal development is virtually identical in both females and males. However, in the presence of XX chromosome, the ovary begins to develop and a clear difference between male and female genital development becomes evident. Prior to birth, Oocytes proliferate and arrest in meiosis stage (McKey et al., 2019).

### 1.1.3 Structure

Each ovary is located in the corresponding ovarian fossa located alongside the lateral wall of the uterus, which is bounded by the external and internal iliac arteries (Daftary et al., 2011, Hoffman et al., 2012). Moreover, both ovaries are surrounded by a capsule with an outer cortex and an inner medulla (Hoffman et al., 2012). The capsule is composed of a dense connective tissue and is known as the tunica albuginea (Training, 2020). One of the two ovaries releases an egg each menstrual cycle, this when ovulation occurs (Daftary et al., 2011).

### 1.1.4 Microanatomy

The ovarian surface is covered with germinal epithelium, which is lined by simple cuboidal to columnar epithelium (Medicine). The outer cortex consists of ovarian

follicles with intervening stroma (Colvin and Abdullatif, 2013). The primary Oocyte is located in the follicles. In addition, the corpus luteum that is derived from the follicles and it is responsible for production of the hormone progesterone during early pregnancy. It can be hard sometimes to distinguish between both the cortex and medulla histologically, but the medulla usually devoid any follicles. Moreover, follicular cells are flat epithelial cells that originate from surface epithelium covering the ovary. They are surrounded by Granulosa cells (Sadler, 2011).

### 1.1.5 Function

At puberty, the ovaries will secrete increasing levels of sex hormones. As a result, the development of secondary sexual characteristics will begin. In addition, the structure and function of the ovary will change after puberty (Colvin and Abdullatif, 2013). Ovaries they also play an important role in pregnancy and fertility. When Oocytes are released from the Fallopian tube, hormone levels will change due to the effect of a variety of feedback mechanisms that consequently stimulate the endocrine system (Richards and Pangas, 2010). Both the Hypothalamus and Pituitary control these mechanisms. The signals from the hypothalamus are directed to the pituitary gland. In turn, the pituitary gland will secrete hormones to stimulate the ovaries to release their own hormones.

## 1.2 Ovarian cancer (OC)

### 1.2.1 Incidence and mortality of ovarian cancer

OC is considered as one of the most prevalent malignancies in women at adult age group. It accounts for about 2.5% of all cancer diagnoses and because of its poor relative survival rates, it is responsible for 5% of all cancer deaths (Furuya, 2012). This poor prognosis is a direct result of the generally delayed diagnosis whereby the cancer has already metastasised into the abdominal cavity. There are around 7,500 new OC cases in the UK every year, which is 20 every day. Its incidence in the UK is highest in females aged 75 to 79 year. Incidence rates for OC are projected to rise by 15% in the UK to reach 32 cases per 100,000 females by year 2035 (UK, 2019b). 1.3% is the recognised average lifetime risk for OC, which is the highest among older women. Unfortunately, due to the low survival rate, the mortality trends tend to mimic its incidence (Hedemann et al., 2018).

There are around 4,100 OC deaths in the UK every year, which is 11 cases every day. It is the sixth most common cause of cancer deaths in the UK and it accounts for 5% of all females' cancer deaths. If OC is diagnosed at its earliest stage, more than 93% of patients will survive their disease for five years or more, this is compared with almost 13% when the disease is diagnosed at an advanced stage (UK, 2019b).

### 1.2.2 Hallmarks of cancer

Tumour development in humans is a multistep process that may reflect a genetic alteration, which can drive the progressive transformation of normal cells into highly malignant derivatives (Hanahan and Weinberg, 2016). Intermediate steps in a process through which cells evolve progressively from normality to premalignant states have been noticed. This will progress to invasive cancers development (Foulds, 1954). Therefore, dysregulation of the hallmarks of cancer pathways, are necessary for oncogenesis (Hanahan and Weinberg, 2016):

#### 1.2.2.1 Sustaining proliferative signalling

The capacity for uncontrolled growth is one of the most obvious properties of cancer cells. Tumour cells are independence from normal growth factor (GF) control. This is partly due to the generation of their own GFs (Hanahan and

Weinberg, 2016). Tumours achieve growth autonomy by three molecular strategies, alteration in extra cellular growth signals, receptor transducers of these signals and alteration of intracellular circuits. Therefore, receptor over-expression may enable tumour cells to increase their normal response to normal levels of GF. For instance, overexpression of epidermal growth factor 1 (EGFR1) in OC, is associated with a subsequent negative impact on prognosis (Gadducci et al., 2009).

#### 1.2.2.2 Evading growth suppressors

Natural braking mechanisms are essential counterbalance to proliferative signals in normal cells. They either overrule the initiation of, or subsequently block, the cell-division process instigated by such signals. The genes encoding these proteins are often termed tumour suppressor genes. The central coordinator of anti-proliferative signals is retinoblastoma protein (pRb). It blocks proliferation by sequestering and inactivating transcription factors from the E2F group of genes which are involved in cell cycle regulation which control the expression of genes that are essential for progression from G1 into S phase (Weinberg, 1995). As a consequence of any disruption in the pRb pathway, it will liberate E2Fs. As a result, uncontrolled cellular proliferation will develop through making cells unresponsive to anti-proliferative signals. TGFβ is an anti-proliferative factor, which was widely investigated. It prevents pRb phosphorylation and its subsequent activation (Datto et al., 1997). Loss of TGFβ responsiveness has been demonstrated in tumours (Hanahan and Weinberg, 2016).

#### 1.2.2.3 Resisting cell death

Fundamentally distinct barrier to aberrant cell proliferation, which involves intrinsic cellular mechanisms that can orchestrate the programmed death of cells deemed to be either aberrant or, in the case of normal development and homeostasis, superfluous. The most prominent form of programmed cell death is apoptosis, the genetically programmed fragmentation of a cell destined to die. (Hanahan and Weinberg, 2016). The apoptotic program can be triggered by cell intrinsic and non-cell-autonomous signals that detect different forms of cellular abnormality, which leads to cell death. Then, the rapid engulfment of apoptotic cell bodies ensures that their death does not release subcellular components that

would otherwise provoke an immune response. Aberration in this mechanism can enhance cancer development (Hanahan and Weinberg, 2016).

Necroptosis is the second form of programmed cell death. Cellular regulators and effectors distinct from those regulating apoptosis govern it. Oxygen and energy deprivation, viral infection, and inflammation can activate necroptosis (Berghe et al., 2014). Cells dying by necroptosis release immunogenic debris that can activate an immune inflammatory response, which can have both tumour-promoting and tumour-antagonising effects (Hanahan and Weinberg, 2016).

Autophagy is another program that capable of inducing cell death. It serves as a recycling system that can help cells respond to nutrient deprivation. Autophagy-associated cell death is distinct in its characteristics from both apoptosis and necroptosis (Katara et al., 2016).

The above three distinct mechanisms must be variably circumvented or attenuated by cancer cells if they and their descendants are to continue their proliferative expansion and phenotypic evolution to states of heightened malignancy.

#### 1.2.2.4 Enabling replicative immortality

The early work of Hayflick demonstrated that cells in culture have replicative potential (Hayflick, 1997). Once such cell populations have progressed through a certain number of doublings, they stop growing, a process termed senescence. Tumour cells have been shown *in vitro* to be immortalised with limitless replicative potential. Defects in the tumour suppressor systems are thought to be part of the causes (Hayflick, 1997). However, effects on telomere maintenance, represent the end of chromososmes may play a more significant role. Telomeres act as the counting device for cell division. Progressive loss of base pairs from the telomere occurs each cell division. Therefore, loss of protection of the chromosomal DNA occurs. As a result, severe karyotype abnormalities can lead to cell death (Counter et al., 1992). In cancer cells, telomere is maintained via upregulation of telomerase enzymes, which aid in adding base pairs to the telomere. This preservation of the telomere effectively inactivates the cells usual counting mechanism for cell division (Bryan et al., 1995). Thus, cancer cells acquire the capability to maintain their telomeres, avoiding the barrier of intolerably shortened telomeres, thereby enabling the unlimited replicative

potential that is required for continuing proliferation of cancer cells (Hanahan and Weinberg, 2016).

#### 1.2.2.5 Inducing angiogenesis

Angiogenesis is the process by which new vessels formed to provide adequate blood supply for the developing tissue. Stimulatory signals from different pathways, such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) control this process. For adequate cell function and survival, it is crucial for tissue vasculature to provide enough oxygen and nutrients, necessitating that all cells are located within 100μm of a blood capillary. Of note, the tumour-associated neovasculature is usually aberrant both morphologically and functionally. Tumour blood vessels are tortuous, dilated, and leaky, with erratic flow patterns (Hanahan and Weinberg, 2016).

#### 1.2.2.6 Activating invasion and metastasis

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. It is involving both cell-intrinsic programs and assistance from accessory cells in the tissue microenvironment. Unlike normal cells, tumour cells have the capability for invasion and metastasis. This enables cancer cells to escape the primary tumour mass and colonise in a new part of the body where nutrients and space are not limited. The activation of extracellular proteases and the altered binding specificities of cell–cell adhesion molecules, cadherins, and integrins are clearly central to the acquisition of invasiveness and metastatic ability (Hanahan and Weinberg, 2016).

#### 1.2.2.7 Deregulating cellular energetics and metabolism

Although glucose is the primary fuel source used by most cancer cells, glutamine is also emerging as another key blood-borne source of energy and a precursor of lipids and amino acids (Daye and Wellen, 2012). A third player in metabolic fuelling is lactate, which is recently appreciated to have diverse tumour-promoting capabilities (Dhup et al., 2012). Oncogenes such as KRAS and cMYC, as well as the loss of function of p53, can serve to reprogram the energy metabolism of cancer cells and enhance oncogenesis (Hanahan and Weinberg, 2016).

#### 1.2.2.8 Avoiding immune destruction

An efficient body immune system would eliminate aberrantly proliferating premalignant cells. However, antigens expressed by spontaneously arising cancer cells are likely shared with those expressed by their cells-of-origin in normal tissues and thus are ignored by the immune system, reflecting the tolerance of the immune system for self-antigens. Therefore, evading immune destruction seems increasingly to be an important mandate for developing tumours and thus an evident hallmark of cancer (Hanahan and Weinberg, 2016).

### 1.2.3 Risk factors of ovarian cancer

The exact mechanisms of OC development and progression are still not fully recognised. Generally, OC related risk factors could be either hereditary, environmental, or lifestyle related.

#### 1.2.3.1 Demographic factor - Age

The epithelial OC is a postmenopausal age-related disease (Chornokur et al., 2013, Chan et al., 2006). Women over the age of 65 have an increased incidence. Previous studies have identified that the median age at diagnosis of OC is 50–79 years (Chan et al., 2006, Arora et al., 2018, Zheng et al., 2018). Although many researchers have pointed out that younger age patients were associated with the improved outcome (Arora et al., 2018, Chan et al., 2003, Chan et al., 2004), others didn't recognise the age as an independent prognostic factor (Massi et al., 1996). Interestingly, older age is associated with more advanced disease and lower survival rate (Poole et al., 2013, Chan et al., 2003, Ries, 1993, Orskov et al., 2016) when compared with lower age cases. However, OC can also develop in pre-menopausal women, around 1000 women under the age of 50 develop ovarian tumour every year in the UK (Cancer, 2014).

#### 1.2.3.2 Reproductive factors

##### *1.2.3.2.1 Menstrual related factors*

Tung et al (Tung et al., 2003) have stated that ovarian tumours are strongly associated with menstrual periods and ovulation cycles. There is an inverse relationship between ovulation cycles and the risk of OC, this was also recognised by other studies (Mori et al., 1988, Kim et al., 2017). Women who have not had

an ovulation cycle for 8.7 years, the risk of OC was reduced by 4-times, a conclusion of a case-controlled study (Salazar-Martinez et al., 1999). These findings support the theory of "incessant ovulation". Based on this theory, any factor that contributes to the reduction of ovulation can have a protective effect against OC (Fathalla, 1971).

*1.2.3.2.2 Menarche and Menopause*

Some studies showed a relationship between the early onset of menarche and risk of OC (Fujita et al., 2008, Jordan et al., 2005). However, other researchers stated that menarche has no influence on the risk of ovarian tumour development (Titus-Ernstoff et al., 2001, Hankinson et al., 1995). Therefore, no significant correlation was identified.

*1.2.3.2.3 Parity*

Several studies recognised pregnancy as a protective factor against OC (Kim et al., 2017, Kvale et al., 1988, Hankinson et al., 1995, Riman et al., 2001, Soegaard et al., 2007, Huusom et al., 2006). A case-control study showed that increased number of pregnancies could lower the risk of OC (Adami et al., 1994).

*1.2.3.2.4 Pregnancy characteristics*

Case control studies identified that both preterm labour and male infants are factors that could promote the risk of OC development (Jordan et al., 2009, Skold et al., 2018). On the other hand, no relationship deemed significant between birth weight and OC (Skold et al., 2018).

### 1.2.3.3 Gynaecologic factors

#### 1.2.3.3.1 Pelvic inflammatory disease (PID)

PID has a controversial role in development of OC. Ness et al, supported a hypothesis suggesting that pelvic inflammation can contribute to OC development (Ness et al., 2000). Although chlamydia trachomatis inflammation may contribute to the development of OC, this pathogen is very common in the genital area (Wong et al., 2007). On the other hand, in a case-control study conducted by Merritt et al stated that chronic inflammation has no role in the development of ovarian tumours (Merritt et al., 2008). Importantly, recurrent PID can significantly promote the risk of developing OC (Risch and Howe, 1995).

#### 1.2.3.3.2 **Endometriosis**

In 1925, Sampson proposed that there is a link between endometriosis and OC. This was based on the theory of malignant changes of endometriosis (Sampson, 1925). Chronic inflammation, loss of PTEN, CTNNB1 (β-catenin), KRAS, microsatellite instability, and ARID1A genes are all involved in the development of ovarian tumours in endometriosis patients. Endometriosis-associated OC is more commonly detected at a younger age and typically at lower stages (Pavone and Lyttle, 2015). Moreover, endometriosis in nulliparous women can promote OC development by three times (Stewart et al., 2013).

#### 1.2.3.3.3 **Ovarian cysts**

Benign ovarian cysts can predispose to malignant ovarian tumours, a case-control study concluded. However, they tend to be more of borderline subtype (Rossing et al., 2008). Interestingly, surgical management of the ovarian cysts further promoted malignancy risk (Rossing et al., 2008). Complex cysts pose higher risk of progression to malignancy in premenopausal women, unlike their postmenopausal counterparts (Caserta et al., 2001, Hartge et al., 2000).

#### 1.2.3.3.4 **Tubal ligation**

Tubal ligation reduces OC risk (Sieh et al., 2013, Ness et al., 2001, Ness et al., 2011, Madsen et al., 2015). The risk reduction was (20%) in high-grade serous carcinoma, (32%) in invasive mucinous type, (42%) in clear cell carcinoma, and (52%) in endometrioid cancer when tubal ligation was performed (Gaitskell et al., 2016, Sieh et al., 2013, Madsen et al., 2015).

#### 1.2.3.3.5 **Contraceptive methods**

The use of oral contraception reduced OC risk (Tung et al., 2003, Soegaard et al., 2007, Tsilidis et al., 2011, Royar et al., 2001). The use of oral contraceptive pill (OCP) can reduce the risk of OC by 7% each year especially when its first use was at less than 25 years of age (Royar et al., 2001, Soegaard et al., 2007, Moorman et al., 2008). Interestingly, this risk reduction can persist for up to 10–15 years after discontinuing the pills (La Vecchia and Franceschi, 1999, Riman et al., 2001, Mori et al., 1988).

#### 1.2.3.3.6 **Hormone replacement therapy (HRT)**

The use of combined oestrogen-progesterone therapy after menopause does not increase the risk of OC (Rossing et al., 2007). Similarly, another study examined the relation between HRT exposure and OC development found no significant correlation exists between both (Hempling et al., 1997). However, the use of estrogenic methods of contraception for more than 10 years significantly reduced OC risk (Lacey et al., 2002). Similarly, progesterone component of the combined HRT also found to effectively reduce OC risk (Rossing et al., 2008).

#### 1.2.3.3.7 **Infertility treatment**

Nulliparity and infertility are recognised risk factors of OC development (Lerner-Geva et al., 2012). Several studies have indicated an association between the use of clomiphene citrate (Reigstad et al., 2017) and gonadotropin (Brinton et al., 2004) with the increased risk of ovarian tumours. In addition, the use of ovulation-inducing drugs also increased the risk of cancer development (Shushan et al., 1996). However, other studies did not find this risk statistically significant (Kurian et al., 2005, Silva Idos et al., 2009, Calderon-Margalit et al., 2009, Potashnik et al., 1999, Venn et al., 1995, Franceschi et al., 1994).

### 1.2.3.4 Genetic factors

#### 1.2.3.4.1 **Family history**

The presence of positive family history of breast and/or OC is the most important risk factor (Torre et al., 2018). Developing breast cancer can significantly promote the risk of OC (Kazerouni et al., 2006). In addition, a positive family history of uterine cancer, especially in mothers or sisters can significantly promote OC risk (Mori et al., 1988).

#### 1.2.3.4.2 **BRCA mutations**

Mutations in tumour suppressor genes have an association in more than one-fifth of OC cases (Walsh et al., 2011). Moreover, 65–85% of inherited ovarian tumours can happen because of germline mutations in *BRCA* genes (Toss et al., 2015). By the age of 40, it is estimated that the risk of developing OC in BRCA1 and BRCA2 mutations carriers is less than 3%. By the age of 50 this risk increases to more than three times (10%) (Andrews and Mutch, 2017). Breast cancer in BRCA1 and

BRCA2 mutation carriers increased the 10-year risk of developing OC to 12.7% and 6.8%, respectively (Andrews and Mutch, 2017, Kotsopoulos et al., 2018, Metcalfe et al., 2005). Interestingly, planned Salpingo-oophorectomy surgery in BRCA-positive individuals can reduce the risk of OC by 75% (Kauff et al., 2002).

#### 1.2.3.4.3 **Lynch syndrome**

Lynch syndrome is a cancer predisposition syndrome that is responsible for 1–3% of all colorectal cancers. It is inherited by autosomal dominant pattern (Iordache et al., 2018). It occurs due to a mutation in one of the four mismatch repair genes (MHL1, MSH2, MSH6, and PMS2) (Andrews and Mutch, 2017). 10–15% of the inherited OC cases are associated with Lynch syndrome (Nakamura et al., 2014). Women with positive family history of Lynch syndrome have 6–8% lifetime risk of developing OC (Lu and Daniels, 2013, Nakamura et al., 2014, Helder-Woolderink et al., 2016). Both endometrioid and clear cell are the most commonly encountered tumour subtypes in these individuals (Lu and Daniels, 2013).

### 1.2.3.5 Lifestyle factors

#### 1.2.3.5.1 **Nutrition and diet**

Eating fish can increase the risk of developing OC but daily milk intake halted this risk (Goodman et al., 2002, Mori et al., 1988). In addition, high cholesterol diet also increased OC risk; however, this is reduced by consuming more vegetables, beta-carotene, and B-complex vitamins (Pan et al., 2004). Plant-based healthy diet plays an important role in the reduction of hormone-related cancers through their phytoestrogens protective role (McCann et al., 2003, McCann et al., 2001). On the other hand, saturated fat was associated with increased risk of OC, mainly mucinous subtype (Risch et al., 1996, Ong et al., 2016).

#### 1.2.3.5.2 **Obesity and physical activity**

Great controversy exists regarding influence of both obesity and physical activity on the risk of OC development. Obesity reduces survival in OC (Bandera et al., 2017, Delort et al., 2009, Leitzmann et al., 2009). Rodriguez et al reported that both obesity and tallness increased the mortality of OC (Rodriguez et al., 2002, Kim et al., 2017). However, in another study both the weight and height were not prognostic factors for OC (Kotsopoulos et al., 2012. Interestingly, regular physical activity reduced the risk of OC {Cottreau, 2000 #413). However, this risk

reduction deemed non-significant by other studies (Moorman et al., 2008, Huang et al., 2016).

#### 1.2.3.5.3 Caffeine and smoking

Consumption of coffee may increase the risk of OC in women before menopause (Kuper et al., 2000). Cigarette smoking on the other hand, does not change the risk of OC (Kuper et al., 2000, Goodman and Tung, 2003). However, Jordan et al (Jordan et al., 2007) stated that daily smoking of one pack for 20 years doubled the risk of developing benign, borderline, and malignant ovarian tumours.

### 1.2.3.6 Other factors

#### 1.2.3.6.1 Lactation

Duration of breastfeeding and the number of breast-fed children have an inverse relationship with OC risk (Su et al., 2013, Riman et al., 2001). Lactation reduced OC risk by 22% in a case control study (Jordan et al., 2010, Tung et al., 2003).

#### 1.2.3.6.2 Socioeconomic status

The socioeconomic status can predict OC incidence and survival (Booth et al., 2010). Lower social status was associated with the more advanced illness (Brewster et al., 2001). Easier access to healthcare services (Morris et al., 2010), OC symptoms awareness, underlying illnesses and lifestyle justify the link between socioeconomic status and OC (Praestegaard et al., 2016).

## 1.2.4 Mechanisms of ovarian carcinogenesis

There are four main theories that can explain the progression to OC. These theories attempt to explain the risk factors discussed previously and their role to development of OC.

### 1.2.4.1 Incessant ovulation hypothesis

The process of ovulation damages the ovarian epithelium. As a result, erroneous DNA repair might develop and inaccurate DNA repair can lead to the development of cancer (Henderson et al., 1982, Purdie et al., 2003). Therefore, the protective effect of both OCP and pregnancy is explained by this hypothesis, both inhibit ongoing ovulation. However, progesterone only pills does not inhibit ovulation, so their protective effect cannot be explained by this hypothesis (Risch et al., 1994).

#### 1.2.4.2 Gonadotrophin hypothesis

Previous studies showed that exposing mice to high levels of gonadotrophin increased the risk of OC (Capen et al., 1995). However, a different study stated that raised level of gonadotrophin does not cause OC per se. High Gonadotrophins will induce ovarian oestrogen secretion, which in turn induces proliferation and transformation of ovarian epithelium to malignancy (Cramer and Welch, 1983). This hypothesis explains the increased OC incidence seen in postmenopausal women and those affected with Polycystic Ovarian Syndrome (PCOS) as they have significantly elevated gonadotrophin levels (Cramer and Welch, 1983).

#### 1.2.4.3 Androgen hypothesis

As ovaries are known to have androgen receptors, Risch et al suggested that androgens might play a role in ovarian carcinogenesis. Moreover, previous experiments showed that androgens can stimulate the growth of OC cell lines (Ahonen et al., 2000, Risch, 1998). Therefore, this hypothesis might explain the increased risk of OC seen with PCOS as high androgen levels are usually measured in their blood (Schildkraut et al., 1996). Interestingly, as OCP reduce androgen levels, their protective effect could potentially been explained by this hypothesis (Murphy et al., 1990).

#### 1.2.4.4 Progesterone hypothesis

The risk of OC has been reduced by the administration of progesterone (Barnes et al., 2002). Progesterone is an anti-mitotic hormone. It increases the wild type p53 tumour suppressor gene and induced apoptosis in OC cell lines (Risch, 1998). The protective effects of pregnancy, OCP and the progesterone only pill have supported the role of progesterone in reducing OC risk by the increase in circulating levels of progesterone (Rosenberg et al., 1994).

### 1.2.5 Diagnosis of ovarian cancer

#### 1.2.5.1 Symptoms and signs

Most women with OC are diagnosed when the disease is advanced and difficult to cure (Ozols, 1997). Although the natural history of ovarian oncogenesis has been documented poorly, clinical studies of women who had normal-appearing ovaries at hysterectomy and who subsequently developed OC have shown that advanced

disease can evolve within 13–24 months (Sightler et al., 1991, McGowan, 1987). Many retrospective patient questionnaire studies have suggested, even in patients with localised disease, OC symptoms often start many months prior to diagnosis perhaps paralleling the time course of disease progression (Flam et al., 1988, Goff et al., 2000, Vine et al., 2001, Olson et al., 2001, Webb et al., 2004).

Symptoms of OC are typically vague and non-specific. Therefore, this can lead to late presentation and ultimately poor prognosis. For this reason, OC was named as the "silent killer". However, this definition is not strictly true since 95% of patients develop different range of symptoms prior to diagnosis (Goff et al., 2000). The non-severe and vagueness of these symptoms, in the majority of cases, lead to them being ignored by both the patient and the General Practitioner.

In the early stages of tumour, irregular menses may develop in premenstrual women. Urinary frequency or constipation may also develop if a pelvic mass is compressing the bladder or rectum. Acute symptoms are usually rare. This might involve sudden onset of pain secondary to ovarian rupture or torsion.

On the other hand, in advanced stages the symptoms are mainly related to disease metastasis, development of ascites, omental spread and bowel metastases can all lead to symptoms of abdominal distension, bloating, nausea and constipation. A survey involved almost a 2000 women with OC showed that 70% had gastrointestinal symptoms, 58% suffered of abdominal pain, 34% exhibited urinary symptoms and 26% had pelvic discomfort (Goff et al., 2000).

Detection of pelvic mass in postmenopausal women remains the classical sign, which highlights the possibility of OC. This mass is often fixed and in association with ascites and a palpable mass in the upper abdomen (omental cake), omentum that contains bulky tumour. However, in this population, a mass with less than 5cm in diameter is malignant in only 3% of the cases. This risk is obviously higher if ascites and/or omental cake are detected as well (Rulin and Preston, 1987).

#### 1.2.5.2 Investigations

The gold-standard for the final diagnosis and staging of suspected OC is via a laparotomy and tissue biopsies (Lheureux et al., 2019). However, this carries a significant morbidity especially when a number of suspected cancers prove to be

benign. Therefore, further investigations are proposed and divided into three main categories: radiological, haematological and histology.

#### 1.2.5.2.1 **Imaging**

Every women suspected have OC should undergo transvaginal ultrasonography, (Figure 1-1) (Cannistra, 2004). This can initially assess the ovarian architecture and vascularity, differentiate cystic from solid masses, and detect ascites. The sensitivity and specificity for distinguishing benign from malignant adnexal lesions on transvaginal ultrasonography are 86% to 94%, and 94% to 96%, respectively (Timmerman et al., 2010, Ameye et al., 2012). However, in difficult cases pelvic Magnetic Resonance Imaging (MRI) can be very useful as well (Spencer, 2005). In addition, and because of the increasing use of neoadjuvant chemotherapy, radiology guided staging can be utilised via the use of either CT or MRI (Tempany et al., 2000).

#### 1.2.5.2.2 **Laboratory tests**

To start with, a full blood count, blood chemistry including liver function tests and calcium (to assess for paraneoplastic syndromes). Then followed by, measuring serum biomarkers as cancer antigen 125 (CA) is the biomarker commonly tested. However, its diagnostic utility depends on disease risk and stage at the time of presentation. CA 125 is an antigen derived from coelomic epithelium and is the classical serum marker for OC (Verheijen et al., 1999). Overall, it is elevated in about 80% of epithelial OC. However, only 50% of an early-stage disease has high CA 125 (Cannistra, 2004). Furthermore, other benign conditions as endometriosis and fibroids can also increase CA 125 level (Clarke-Pearson, 2009, Cannistra, 2004).Other tumour markers can be also useful if ovarian tumour is secondary from another organ primary. For example, CEA in colorectal tumours (Duffy, 2006), Ca-153 in breast cancer (Duffy, 2006), and Ca-19.9 in pancreatic malignancy (Goonetilleke and Siriwardena, 2007).

#### 1.2.5.2.3 **Tumour tissue histology**

Utilising a tissue specimen is the gold standard for a final OC diagnosis. Therefore, the use of image-guided biopsies has become a standard clinical practice. This can be performed either through ultrasound guided aspiration of ascitic fluid for cytology, or a CT guided biopsy of an omental cake or pelvic mass to provide tissue for histological analysis. In general, a tissue diagnosis is preferred. However, the combination of an elevated CA-125 coupled with the presence of adenocarcinoma cells in ascites cytology deemed adequate for the diagnosis of OC prior to the initiation of neoadjuvant chemotherapy (Spencer, 2005).

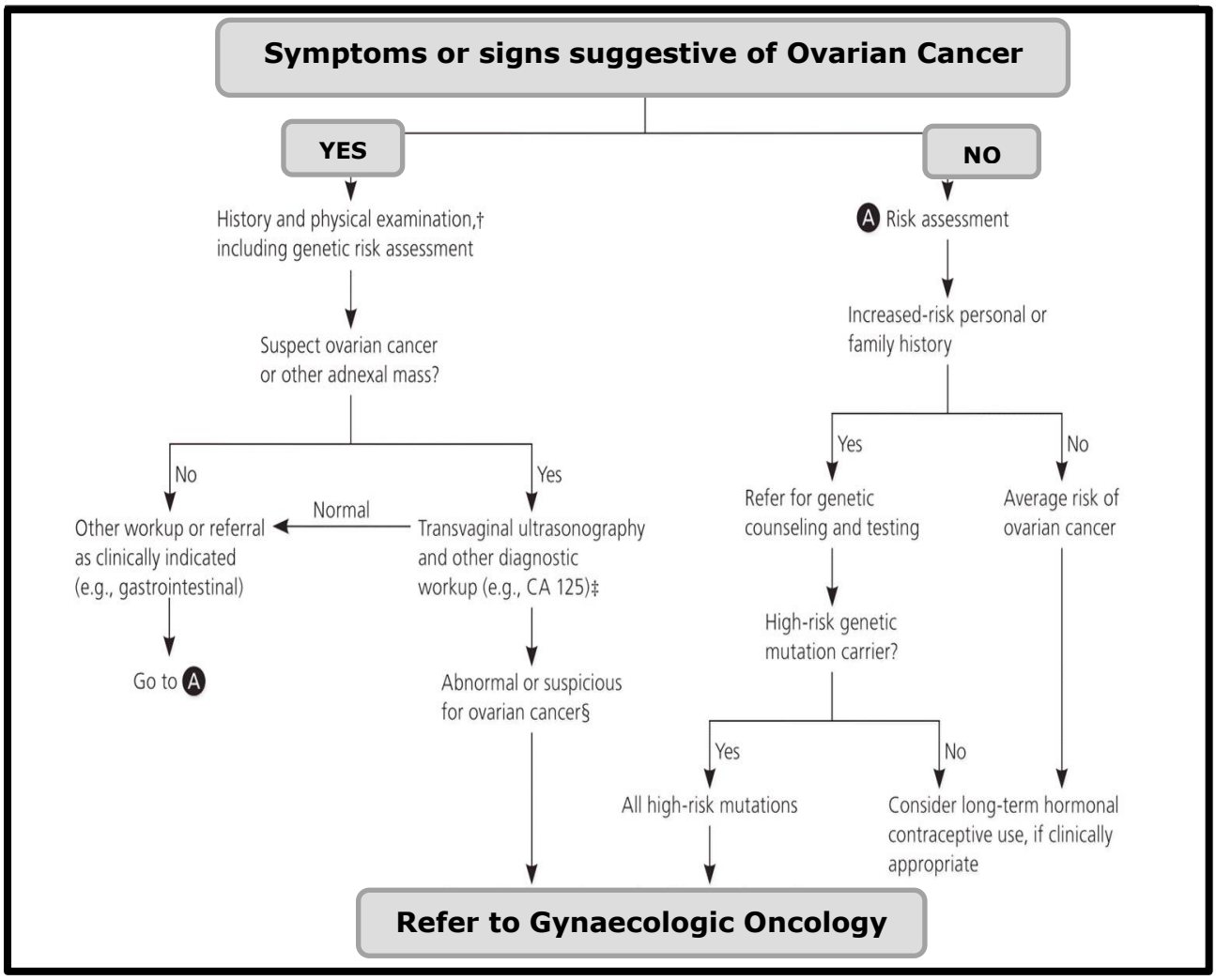



**Figure 1-1: Initial assessment of patients presented with OC, adapted from Ameye et al (Ameye et al., 2012)**

### 1.2.5.3 Risk of malignancy index

As OC is usually associated with elevated Ca-125 level and ultrasound scan of ovarian mass can discover various features to raise suspicion of OC, these features can be used to identify high-risk individuals. The Risk of Malignancy Index (RMI) represents an algorithm, which incorporates a combined assessment of three elements. An ultrasound features of a pelvic mass, the Ca-125 level and the patient's menopausal status. This will produce a quantitative assessment of the risk of an individual mass being malignant (Jacobs et al., 1990). The RMI has been shown to be very useful in clinical practice. It provides 85% sensitivity and 97% specificity for predicting OC if the score is higher than 200 (Jacobs et al., 1990). This has been tested on retrospective and prospective data with very encouraging results (Jacobs et al., 1990, Davies et al., 1993). The model has been subject to some adaptation into the most widely used format at present (Tingulstad et al., 1996). The RMI is measured by (**RMI= ultrasound score x menopausal score x Ca-125),** (Table 1-1)**.** This predictive model has the advantage of allowing an effective planning (Kehoe et al., 1994). This model has been successfully used to triage patients in peripheral units for referral into tertiary cancer centres in the UK (Bailey et al., 2006).

**Table 1-1: The modified risk of malignancy index developed by Tingulstad et al (Tingulstad et al., 1996)**

| ***Parameter*** | ***Score*** |
|---|---|
| **Ultrasound features** | |
| Multilocular cyst | None or one features = 1<br>Two or more features = 3 |
| Solid areas | |
| Bilateral lesions | |
| Ascites | |
| Intra-abdominal metastases | |
| **Menopausal status** | |
| Post-menopausal | 3 |
| Pre-menopausal | 1 |
| **Ca-125 level** | U/ml |

#### 1.2.5.4 Ovarian cancer screening

A late presentation is a typical feature of OC. Since early stage disease is associated with an 85% 5-year survival, OC screening (OCS) becomes a clinical need. It aims to detect cancer cases early in disease course, with subsequent benefits from improved survival rates. As with any screening test, there are a number of hurdles including adequate sensitivity, specificity and acceptability of the test. In addition, cost effectiveness considerations and ultimately the improved outcome at reducing mortality rates need to be regularly assessed. At present, there is no conclusive evidence to suggest that OCS is effective. Preliminary data exists from a randomised controlled trial (RCT) performed by Jacobs et al who found that out of 22000 women within the trial, the patients who developed OC and participated in screening had an improved median survival (Jacobs et al., 1999). Additional evidence from a Japanese OCS study showed an increased proportion of stage I cancers in screened women compared with unscreened ones (59% vs. 30%) (Sato et al., 2000).

Currently, there are large RCTs in the general population to assess the impact of OCS on OC mortality. The United Kingdom Trial of OC Screening (UKCTOCS) is a similar study with 200000 women included. The results showed that a further follow-up is needed before firm conclusions can be reached on the efficacy and cost-effectiveness of screening program (Menon and Jacobs, 2002, Jacobs et al., 2016).

### 1.2.6 Pathological classification of ovarian cancer

#### 1.2.6.1 Histological Types

All OC masses are classified according to the histological appearance of their structures. According to their histology, different aspects of clinical treatment, management, and long-term prognosis will be affected. Regardless of histologic type, the gross pathology of OC is very similar. Usually they have both solid and cystic components (Hoffman et al., 2012). In general, OC cases are classified into two types: Type I cancers, with low histological grade. It includes endometrioid, mucinous, and clear-cell carcinomas. The second one is type II with higher histological grade. This includes serous carcinoma (Jayson et al., 2014). According to Surveillance, Epidemiology, and End Results Program (SEER), the histology

types of OC, percentage and the five year survival rate are summarised below, (Table 1-2) (In Baguio RN, 2007).

As OC is a heterogenous disease (Kossaï et al., 2018, Wang et al., 2005), OC studies become more challenging task. Many factors can contribute to disease prognosis, which is very difficult to predict. Histological type, tumour stage and the degree of cytoreducibility at surgery are all greatly contributing factors to the prognosis. The most common histological types of OC are:

**Table 1-2: pathological classification of Ovarian Cancer (In Baguio RN, 2007)**

| OC (%) | Histology | Five-year Survival |
|---|---|---|
| 26.4 | Serous cystadenocarcinoma | 21.0 |
| 15.9 | Borderline adenocarcinoma | 98.2 |
| 12.6 | Adenocarcinoma,not otherwise specified | 18.3 |
| 9.8 | Endometrioid tumour | 70.9 |
| 5.5 | Papilloma | 21.0 |
| 4.2 | Mucinous cystadenocarcinoma | 77.7 |
| 4.0 | Clear-cell ovarian carcinoma | 61.5 |
| 3.4 | Mucinous adenocarcinoma | 49.1 |
| 1.3 | Cystadenocarcinoma | 50.7 |
| 4.1 | Carcinoma not otherwise specified | 26.8 |
| 1.1 | Sex cord-stromal tumour | 87.8 |
| 1.7 | Mullerian tumour | 29.8 |
| 1.5 | Germ cell tumour | 91.0 |
| 0.8 | Teratoma | 89.1 |
| 0.5 | Dysgerminoma | 96.8 |
| 0.5 | Squamous cell carcinoma | 51.3 |
| 0.2 | Brenner tumour | 67.9 |

#### 1.2.6.1.1 **Serous cystadenocarcinoma**

The serous subtype is the most common type of OC. It accounts for the majority of cases (Heintz et al., 2001, Quirk and Natarajan, 2005). They are mostly lately presented to health care professionals. Hence, they are associated with a poor prognosis compared with other subtypes (Makar et al., 1995).

#### 1.2.6.1.2 **Endometrioid tumour**

Endometrioid tumours have a similar appearance to colonic metastases, which may spread transcoelomically to the ovaries. Endometriod tumours are malignant in 80% of the cases (R:, 1996). Ovarian endometriosis has a strong association with endometrioid malignancy. Some authors suggested that these tumours might in fact represent malignant transformation of endometriosis (DePriest et al., 1992).

#### 1.2.6.1.3 **Mucinous cystadenocarcinoma**

Mucinous OC have a relatively favourable outcome, most probably because it's early presentation. Historically the incidence of this type of OC was higher; however, it is now thought to be at reduced risk (Heintz et al., 2001, Quirk and Natarajan, 2005). A huge pelvic mass is considered as its typical presentation. In addition, they tend to be resistant to platinum chemotherapy (Harrison et al., 2008).

#### 1.2.6.1.4 **Clear cell carcinoma**

Clear cell cancers are also strongly associated with endometriosis (Kennedy et al., 1989). They represent approximately 5% of all OC subtypes (Heintz et al., 2001, Quirk and Natarajan, 2005). They tend to present earlier when compared with the other tumour subtypes. However, they are more likely to display platinum resistance. Ultimately, their prognosis is poor (Heintz et al., 2001, Itamochi et al., 2008).

#### 1.2.6.1.5 **Undifferentiated carcinomas**

Undifferentiated OC usually present at a late stage disease. Their actual incidence is widely variable between studies; it ranges from 5-25% of all OC subtypes. Interestingly, they can display resistance to chemotherapy. Hence, the prognosis is usually very poor (Heintz et al., 2001, Quirk and Natarajan, 2005).

#### 1.2.6.2 Histological Grade

The grade of OC can describe to what degree the tumour cells are differentiated from their normal counterparts. This will provide the Oncologist with additional useful information about the expected behaviour and prognosis of the disease (UK, 2019a).

The OC malignant cells can be classified into three grades: well-differentiated grade 1 (G1), moderately differentiated grade 2 (G2) and poorly differentiated grade 3 (G3) tumours. As OC is a heterogeneous disease, the same grading system does not appear applicable to all tumour subtypes. In clear cell carcinoma, only nuclear atypia can provide prognostic information, whereas grading is useful in serous subtype (Clement, 1987). Overall, the OC grading is not considered as a useful independent prognostic factor, although it can be useful on a sub-stage level, especially in early stage disease (Heintz et al., 2001).

#### 1.2.6.3 Immunohistochemical characteristics

The ovary is a recognised target for many metastatic adenocarcinomas such as colorectal, gastric, breast, pancreas, and gall bladder, in addition, to both renal and other gynaecological malignancies. These tumours may appear very similar to each other morphologically under light microscope. Therefore, when assessing an ovarian tumour a potential metastatic origin should always be considered. This is especially true of mucinous OC (Seidman et al., 2004). As a result and because of the similarities in tumour morphology, light microscopy alone can be unreliable and misleading (Seidman et al., 2004).

Tumour tissue Immunohistochemistry (IHC) provides crucial additional information on a tumour's characteristics and its likely place of origin. This will influence both the management and prognosis of OC (McCluggage and Wilkinson, 2005). Therefore, a routine clinical practice is to perform OC IHC to detect antibodies specific to cytokeratin (CK) 7 and 20, Carcinoembryonic Antigen (CEA) and Cancer Antigen 125 (Ca125). Generally, mucinous subtype of OC tend to be CK7+, Ca125+ and CEA-, CK20- (McCluggage and Wilkinson, 2005, Ji et al., 2002). Wilms Tumour 1 gene is usually negative with pancreatic cancers, but with the majority of OC it is positive (Goldstein et al., 2001). Furthermore, renal clear

cell cancers can also mimic OC. The use of CD10, a renal cell carcinoma antigen can be useful in such circumstances (Cameron et al., 2003).

In clinical practice, the combination of histological morphology and IHC profiles coupled with clinical and radiological information can provide a reliable diagnosis of the tumour site. Therefore, the importance of a cohesive and thorough assessment of all these components highlights one of the major roles of an oncology multi-disciplinary team (MDT) meeting.

#### 1.2.6.4 Stage

It was well recognised that OC is rather a group of diseases each with different morphology and biological behaviour. Approximately 90% of ovarian tumours are carcinomas (Young, 2014, Prat, 2012, Ong et al., 2016). In the modern era of personalised cancer medicine and because the difference in response to chemotherapy between tumour histotypes, the International Federation of Gynaecology and Obstetrics (FIGO) Committee on Gynaecologic Oncology unanimously agreed that histologic type should be identified at staging (Prat, 2015).

The tumour staging is typically performed through a longitudinal midline abdominal incision. However and more recently laparoscopy has been successfully used for staging (Leblanc et al., 2006). Interestingly, with the recent increasing role of neoadjuvant chemotherapy, staging via image-guided techniques is being used successfully. Typically, with the use of computed tomography scan (CT) and integrated positron emission tomography and CT (PET/CT) (Kitajima et al., 2008). However, laparotomy is still the gold standard method for OC staging. The spread of OC is most commonly trans-peritoneally. Therefore, close inspection and biopsies of the peritoneal surfaces including the diaphragm, para-colic gutters and omentum are required to detect macroscopic metastases. In addition, cytological assessment of both ascitic fluid and peritoneal washings is required for accurate staging (Benedetti-Panici et al., 1993, Zanetta et al., 1998, Hand et al., 1993, Holschneider and Berek, 2000, Wolfe et al., 1997, Yancik, 1993, Quirk and Natarajan, 2005).

FIGO system is the most widely used method for OC stage assessment. This was adapted in 1987 (1987). Generally, there are four stages of OC, (Table 1-3) (Institute, 2020b):

#### 1.2.6.4.1 **Stage I**

In this stage, the tumour is confined to the ovaries. Tumour rupture, surface involvement by tumour cells or presence of malignant cells in the ascetic fluid or peritoneal washings warrants a stage of IC.

#### 1.2.6.4.2 **Stage II**

This stage is making up less than 10% of OC. The disease spread is limited to the pelvic organs. All stage II disease is treated with adjuvant chemotherapy, so sub-classification is not clinically essential.

#### 1.2.6.4.3 **Stage III**

These tumours characteristically spread along peritoneal surfaces involving both pelvic and abdominal peritoneum. This stage represents most of the OC cases (Heintz et al., 2006).

#### 1.2.6.4.4 **Stage IV**

It is the stage when distant metastasis beyond the pelvic organs already happened. The spread can affect the liver, spleen and even extra-abdominal metastases including the lungs. 12% to 21% of patients present with stage IV disease (Institute, 2020b).

**Table 1-3: Definitions of FIGO staging system, (Institute, 2020b)**

| Stage | Definition |
|---|---|
| ***Stage I: Tumor confined to ovaries or fallopian tube(s).*** | |
| IA | Tumour limited to one ovary (capsule intact) or fallopian tube; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings |
| IB | Tumour limited to both ovaries with intact capsule or fallopian tubes; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings |
| IC | Tumour limited to one or both ovaries or fallopian tubes, with any of the following:<br>**IC1**: Surgical spill.<br>**IC2**: Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface.<br>**IC3**: Malignant cells in the ascites or peritoneal washings |
| ***Stage II: Tumour involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer.*** | |
| IIA | Extension and/or implants on the uterus and/or fallopian tubes and/or ovaries |
| IIB | Extension to other pelvic intraperitoneal tissues |
| ***Stage III: Tumour involves one or both ovaries, or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes.*** | |
| IIIA1 | Positive retroperitoneal lymph nodes only (cytologically or histologically proven):<br>**IIIA1(i)**: Metastasis up to 10 mm in greatest dimension.<br>**IIIA1(ii)**: Metastasis >10 mm in greatest dimension. |
| IIIA2 | Microscopic extra-pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes |
| IIIB | Macroscopic peritoneal metastases beyond the pelvis ≤2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes |
| IIIC | Macroscopic peritoneal metastasis beyond the pelvis >2 cm in greatest dimension, with or without metastasis to the retroperitoneal nodes |
| ***Stage IV: Distant metastasis excluding peritoneal metastases.*** | |
| IVA | Pleural effusion with positive cytology |
| IVB | Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity) |

### 1.2.7 Management of ovarian cancer

#### 1.2.7.1 Surgical tumour resection

The aim of surgery in OC varies according to the stage of disease. Thorough surgical resection is the aim in early stage disease. However and in advanced disease, optimal debulking of the tumour represents the main surgical goal.

##### 1.2.7.1.1 Early stage disease

Early stage OC can only be firmly recognised if a thorough staging laparotomy was performed (Young et al., 1983). Young et al in his series highlighted the importance of correct operative staging in patents with OC when the tumour appears confined to ovaries. Accurate operative staging improved the patients' survival. Suboptimal staged patients had only 60% 5-year survival, unlike the 90% survival rate when a thorough staging was employed. However, this difference might likely be due to inclusion of patients with more advanced disease and with inadequate staging, where occults metastases could be missed. Clearly, inclusion of these cases would lead to a lower than expected survival rate (Young et al., 1983).

If OC is suspected, a frozen section biopsy of the mass enables an intra-operative diagnosis to be made, although its sensitivity is as low as 65% (Geomini et al., 2005). Then, adequate tumour staging followed by total abdominal hysterectomy and removal of the remaining ovary. Since up to 15% of apparent stage I cancers have nodal metastases, lymph node dissection is of particular importance (Cass et al., 2001). In carefully selected cases, where women wish to retain their fertility, a unilateral oophorectomy can be performed with preservation of the uterus and contralateral ovary. This is followed by a very careful follow up with serial Ca-125 measurements and TVS. Then, completion surgery is usually recommended after child bearing.

##### 1.2.7.1.2 Advanced stage disease

In advanced stage OC, the surgical goal remains to remove of as much disease as possible at the primary laparotomy. This approach was supported by different studies (Covens, 2000). Over decades, the primary cytoreductive surgery followed by chemotherapy has been considered as the standard management for

advanced stage disease. However, an alternative approach of interval debulking surgery following neoadjuvant chemotherapy was subsequently reported. Currently, Trial of Radical Upfront Surgical Therapy (TRUST) is ongoing and results are expected by 2024 to identify the optimal management plan (Reuss et al., 2019).

The definition of maximal cytoreduction varies from no visible macroscopic disease to tumour nodules of <2cm (Bristow et al., 2002, Eisenkop et al., 1998, Eisenkop et al., 1992). However, the residual disease measurement is usually inaccurate and frequently underestimated (Prefontaine et al., 1994). Often patients' symptoms improve after a large volume of tumour and ascites is removed in cytoreductive surgery. Moreover, the nutritional status of the patient prior to further adjuvant treatment will improve due to the reduction in intestinal symptoms. Furthermore, large bulky tumours tend to contain necrotic areas and poorly vascularised. Consequently, this will reduce chemotherapeutic agents' concentrations inside the tumour. In addition large tumour masses tend to contain a high proportion of non-dividing cells, which will be resistant to the mechanisms employed by most chemotherapy. Therefore, debulking surgery produces smaller volumes of disease with improved blood supply and high growth fraction with improved sensitivity to chemotherapy (Skipper, 1978, Bookman and Berek, 1992).

On the other hand, some still question the role of primary surgery in advanced OC (Hunter et al., 1992). The ability to optimally debulk a tumour may be a reflection of the biological aggressiveness of the tumour as opposed to the skill of the surgeon. The morbidity and impact on quality of life may be unacceptability high after radical surgery, especially when newer chemotherapy agents have a greater effect on "log kill" than surgery. Overall, the evidence regarding the optimum surgical approach to OC remains controversial, with evidence and biological hypothesises promoting both radical and conservative approaches.

#### 1.2.7.2 Chemotherapy

Chemotherapy has a vital role in OC management in both early and advanced stage disease.

#### 1.2.7.2.1 **Early stage disease**

Identifying the grade and sub-stage of disease will determine the need for adjuvant treatment in early stage OC. In a study involved 656 early OC patients, Guthrie et al concluded that stages 1A and 1B (grade 1 and 2) had the same prognosis irrespective of adjuvant chemotherapy status (with or without), with a 5 year survival of over 95% for both groups. However, for those patients with poorly differentiated tumours (grade 3), or stage 1C-IV, further treatment was deemed necessary (Guthrie et al., 1984). The evidence for adjuvant treatment of early stage OC is mainly derived from two parallel RCTs, the International Collaborative Ovarian Neoplasm Trial 1 (ICON1) and the Adjuvant Chemotherapy Trial in Ovarian Neoplasia (ACTION) (Trimbos et al., 2003). In the ICON1 trial, optimal surgical staging was not required to enter the trial. In addition, stage I and IIA disease was included in the study. All patients were randomised to platinum-based chemotherapy or observation. The treated group had an improved 5-year survival of 73% compared with 63% of the control arm. In the ACTION trial, about one third of the included cases had optimal staging, and similarly to IOCON1, the randomisation was to either platinum chemotherapy administered or observation. The optimally staged patients had an improved survival compared to those who were not staged optimally. Moreover, the latter group had improved survival with chemotherapy use if compared to the control arm. On the other hand, no difference noted between both groups in optimally staged ones. Therefore, chemotherapy is warranted in non-optimally staged early OC (Trimbos et al., 2003).

#### 1.2.7.2.2 **Advanced stage**

Systemic chemotherapy has become a major component of treatment in advanced stage OC for many years. In 1970s the platinum based compounds were introduced, initially cisplatin followed by carboplatin. Their ability to link themselves between the DNA strands is the reason behind their anticancer activity. This inhibits DNA replication, repair and induce apoptosis. In the 1990s, Paclitaxel was incorporated into platinum regimen. It binds to tubulin, hence inhibits mitosis during the M phase of the cell cycle. Cisplatin as a single agent has shown improved survival and reduced toxicity rates. For this reason, it replaced cyclophosphamide (Young et al., 1978).

A second-generation platinum analogue, carboplatin was then introduced. This was superior to cisplatin with reduced gastrointestinal side effects, especially nausea and vomiting. Furthermore, less nephrotoxicity, neurotoxicity and ototoxicity were encountered, although it was noted to have more myelosuppressive properties (Alberts et al., 1992, Swenerton et al., 1992).

The RCT (ICON 2) compared the triple chemotherapy regimen cyclophosphamide, adriamycin and cisplatin (CAP) with carboplatin. Carboplatin was as effective as CAP with less toxicities (1998).

After the establishment of platinum agents as the main form of chemotherapy in advanced OC, paclitaxel was introduced as a potential adjunct. Numerous studies compared the combinations of paclitaxel with either cisplatin or carboplatin. The toxicity profile was improved in the carboplatin/paclitaxel combination. However, no differences were noted in neither Disease Free Survival (DFS) nor Overall Survival (OS) (du Bois et al., 2003, Ozols et al., 2003). In addition, a second-generation taxane (docetaxel) was produced and showed significantly reduced neurotoxicity and haematological suppression, compared to paclitaxel when used in combination with carboplatin (Vasey et al., 2004).

Overall, the evidence has produced confusion and ultimately variation in clinical practice. Often, the combined approach of carboplatin/ paclitaxel is being favoured in the younger, fitter patients who are more able to tolerate the increased risk of toxicity. The UK national guidelines suggest that carboplatin can be used as a single agent or in combination with paclitaxel (Excellence, 2020).

#### 1.2.7.2.3 **Mechanisms of chemoresistance**

Erroneous DNA replication and subsequent cell death through the DNA mismatch repair system (MMR) is Cisplatin mode of action. During development of platinum resistance components of the MMR system can be inactivated (through methylation) such as the gene hMLH1. Therefore, a demethylation agent, as decitabine may reactivate hMLH1 and restore platinum sensitivity (Plumb et al., 2000). Moreover, reduction in platinum intracellular concentration, through reduced cellular uptake or increased efflux is another mechanism for platinum resistance development. Permeability glycoprotein was found to be over

expressed in platinum resistant cells, resulting in increased efflux of platinum compounds from cancerous cells (Seiden et al., 2002).

#### 1.2.7.3 Novel chemotherapy methods

##### 1.2.7.3.1 Dose dense, maintenance and novel combination

Escalating the dose of chemotherapy improves survival. Kaye et al demonstrated an improved OS with doubling the dose of cisplatin, although this difference disappeared after long term follow up (Kaye et al., 1996). Similarly, weekly, as opposed to three weekly, administration of paclitaxel provides advantages in reducing toxicity and improving response rates, even in platinum refractory disease (Markman et al., 2002). However, extended courses of cisplatin did not alter PFS or OS (Jaaback and Johnson, 2006). On the other hand, prolonged treatment with paclitaxel improves PFS and reduces platinum resistance (Markman, 2008, Bookman et al., 2003).

##### 1.2.7.3.2 Intraperitoneal administration

The theoretical advantage of Intra-peritoneal (IP) administration of chemotherapeutic agents is that it acts directly on the tumour cells. There may be a survival advantage with IP delivery of platinum agents (Jaaback and Johnson, 2006, Markman et al., 2002). However, there are catheter-related problems and potential increased toxicity. This treatment route is yet to become established (Armstrong et al., 2006).

##### 1.2.7.3.3 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy refers to the administration of chemotherapy prior to surgery. This approach improves the complete tumour respectability (Jacob et al., 1991, Vergote et al., 1998). Moreover, a recent study (Pinelli et al., 2020) showed that radical surgical resection after a period of Neoadjuvant therapy improved patients' outcome in advanced OC cases.

#### 1.2.7.4 Novel targeted therapies

The increased understanding of tumour biology provides a number of potential therapeutic targets. These include the use of growth factor inhibitors, anti-angiogenic therapy, gene therapy and immunotherapy.

#### 1.2.7.4.1 Growth factor inhibitors

Epidermal growth factor receptor (EGFR) is one of the most extensively studied growth factors in OC. This pathway is responsible for cell growth and proliferation (Agarwal et al., 2006). Growth factor inhibitors have provided good response in lung cancer but disappointing effects in OC (Agarwal et al., 2006).

The P13K/Akt/mTOR pathway also stimulates cell growth and proliferation. This pathway is frequently amplified in OC cases. Therefore, inhibiting key components of those pathways might improve the OC outcome (Agarwal et al., 2006).

#### 1.2.7.4.2 Apoptosis inducers

Phenoxodiol is a topoisomerase II inhibitor, which induces apoptosis and may have a role in re-sensitising paclitaxel resistant OC cells (Agarwal et al., 2006). This can be achieved by inhibiting the X-linked inhibitor of apoptosis protein (XIAP,) which is shown to be over-activated on paclitaxel resistant tumours (Sapi et al., 2004).

#### 1.2.7.4.3 Anti-angiogenic therapies

Bevacizumab can inhibit angiogenesis. It is a monoclonal antibody, which binds and inactivates all isoforms of VEGF-A (Burger et al., 2007). In addition, Thalidomide, which has anti-angiogenic properties, was shown to stabilise recurrent OC cells (Agarwal et al., 2006).

### 1.2.8 Prognosis of ovarian cancer

A large number of established clinical variables influence the prognosis of patients with OC. Moreover, novel molecular, histological and serum parameters presented as useful makers of tumour behaviour, which can assist in our understanding of carcinogenesis. These parameters may be of use clinically in the planning and development of future treatments to improve patients' outcome.

#### 1.2.8.1 Clinical parameters

Similar to most cancer types, age can predict survival. The prognosis becomes unfavourable with increasing age. Even adjusting for stage, the 5-year survival for patients younger than 50 years is 40%, compared with only 15% for older than 50 (Ford et al., 1998). This might be very likely to reflect additional related

health factors, which are more common in older age group, as the associated co-morbidities and general health.

Tumour stage is another useful clinical prognostic indicator for OC, increase stage correlates with worse outcome (Auersperg, 2013). Typically, stage I tumours has 76 - 93% 5-year survival, stage II 60 - 74%, stage III 25 - 41%. However, the 5-year survival is only 11% for stage IV disease (Heintz et al., 2001).

Moreover, the grade of a tumour correlates with survival especially in early stage disease. Grade I tumours have a 5-year survival of 91% compared to approximately 75% for grades II and III (Heintz et al., 2001).

In general, the 5-year survival for OC affected women remained poor at approximately 30% (Heintz et al., 2001). However, recently the wide use of platinum-based chemotherapy might have improved this figure slightly to 47.6% (Institute, 2020a).

#### 1.2.8.2 Novel prognostic markers

Because OC is a heterogeneous disease, the use of various tumour biological markers can predict the tumour behaviour. Biomarkers can be used for prognostic stratification in order to estimate survival, which can help predicting tumour behaviour and response to chemotherapy. They allow the clinician to provide patients with useful information on the likely outcome of the disease. Since there are clear advantages in accurately identifying tumour prognosis, novel markers are constantly being sought. Discovering molecular markers with independent prognostic power may therefore, reveal the importance of a particular intracellular process, and identify a target to which novel therapies can be developed (Hanahan and Weinberg, 2000). Biomarkers can be derived from of patients' serum or tissue and are related to one key hallmarks of carcinogenesis (Hahn and Weinberg, 2002).

##### 1.2.8.2.1 Serum markers

The most studied tumour marker in OC is Ca-125; however, its usefulness is controversial. Some studies suggest that in stage I disease elevated preoperative Ca-125 gives a relative risk of dying at 6.37 (Nagele et al., 1995). Moreover, postoperative low level of Ca-125 following optimal debulking may be an

independent predictor of favourable prognosis. Furthermore, Ca-125 can be used to guide the chemotherapy response (Gerestein et al., 2009).

Moreover, Low preoperative haemoglobin and elevated platelet count are both poor prognostic factors. These two parameters have been successfully used to produce a normogram, which could predict both PFS and OS in OC patients (Gerestein et al., 2009). In addition, Pro-coagulant and fibrinolytic factors are both released by tumour cells. Subsequently, fibrin split products (D Dimers) will increase. There is some evidence that raised D Dimers may predict poor prognosis (Gadducci et al., 2009).

#### 1.2.8.2.2 **Tissue markers**

Cell cycle and DNA repair pathway proteins, which are known to play vital roles in tumour biology of different organs, have been highlighted as potential prognostic biomarkers in OC. In recent years, a number of studies have evaluated the prognostic significance of specific biomarkers and their influence on cancer progression (Engqvist et al., 2020, Psyrri et al., 2005). Further tissue markers will be discussed in details later in this thesis.

### 1.2.9 Exploring novel prognostic tissue markers and tissue microarray

In general, molecular markers can be found in different body fluids (e.g. urine and blood), excised or biopsied cancer tissue and laboratory grown tumour cell lines. Cancer induces molecular change in genes that can be detected through alterations in DNA, RNA or protein expression.

The genome level is the classical starting point for detection of carcinogenesis and aberrant pathways. However, not all genetic defects result in changes at protein level, the level that is required to exert a biological effect. Therefore, it is often more practical to detect abnormal protein expression, from which genetic abnormalities can be sought. Immunohistochemistry (IHC) is a commonly used tool for assessing protein expression. Moreover and over the recent years, a high throughput platform has been developed to enable rapid and consistent analysis of tissues stained immunohistochemically. This technique is called Tissue Micro Array (TMA) (Kononen et al., 1998). Traditionally IHC studies relied on labour intensive and time-consuming technique by staining the whole sections of tissue.

In addition and since only a limited number of slides could be processed at one time, multiple IHC runs were needed. Therefore, considerable variations in the experimental conditions can be created and may introduce inconsistencies in the analysis. Therefore, to address these drawbacks the concept of transferring small samples of many tissue blocks into a single block was explored, and a number of techniques have been attempted. The most recent technique was developed by Konnen et al who described the use of a custom-built instrument, called a Tissue Microarrayer, which produced cylindrical tissue cores from archived blocks, which could be transferred to holes precisely punched out in a recipient paraffin block (Kononen et al., 1998). Reduction in reagent costs, variability of experimental conditions and being more time efficient are all advantages of the TMA use (Kononen et al., 1998).

Rosen has validated the useful use of TMAs in OC through creating six copies of TMA containing 45 ovarian tumour cores. Then, those cores were stained for ER, Ki-67 and mutant p53. There was a high concordance of protein expression between the TMA cores and the whole tissue sections, which were used as the gold standard (Rosen et al., 2004). Therefore, TMA stained tissue can be utilised for tissue staining as it can reflect the full section OC tissue biology.

### 1.2.10 Cellular pathways associated with ovarian cancer and their related biomarkers

Until date, no single marker is sufficient for diagnosing OC or predict its prognosis (Shin et al., 2020). The combination of multiple tests or testing patterns to achieve high sensitivity and specificity is currently highly encouraged.

#### 1.2.10.1 Mammalian target of rapamycin (mTOR) and its upstream effector and downstream regulators

The signalling cascade involving AKT/mTOR is the most frequently altered pathway in human cancer. Moreover, it controls many of the processes and characteristics that are important for cancer development, e.g., cell cycle, cell survival, metabolism, motility, angiogenesis, chemoresistance, and genomic instability (Fruman and Rommel, 2014, Altomare et al., 2004, Camilleri-Broet et al., 2004, Hsu et al., 2004).

The PI3K-AKT is a signal transduction pathway that promotes survival and growth in response to extracellular signals, AKT (Protein Kinase B/ serine/threonine kinase) is its key protein. Activation of a cell surface receptor and phosphorylation of PI3K occur after stimulation by one of the growth factors. Following this the activated PI3K will phosphorylate plasma membrane lipids in order to form the next messenger, $PIP_3$. Then, AKT is recruited to be fully activated (Osaki et al., 2004). As a result, the activated AKT mediates cellular downstream responses, including cell survival, growth, proliferation, cell migration and angiogenesis through phosphorylating a range of intracellular proteins (Manning and Toker, 2017). Problems with PI3K-AKT signalling regulation was linked to cancer development (Carracedo and Pandolfi, 2008). AKT promotes G1 phase progression and cyclin D1 translation via indirect activation of mTOR, a downstream mediator in the PI3K/AKT pathway. Then, The mTOR increases translation of cyclin D1 by activating ribosomal protein S6K, and inhibiting 4E-BP1, thus increasing eIF4e activity (Hay and Sonenberg, 2004, Nicholson and Anderson, 2002). The mTOR receives inputs from various cellular biochemical pathways, including growth factor signalling molecules, mitogens, hormones, cytokines, intracellular energy status and hypoxia (Bjornsti and Houghton, 2004).

The major downstream effectors of mTOR are eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and Ribosomal protein S6 kinase beta-1 (S6K1), also known as p70S6 kinase (P70S6K), (Figure 1-2). Activated p4EBP1 will enhance the release of eIF4E, which is required for cap-dependent translation (Mamane et al., 2004). Thus, p-4EBP1 can regulate transformation, G1 progression and apoptosis (Lynch et al., 2004). In addition, activated P70S6K, facilitates phosphorylation of the S6 protein of the 40S ribosomal subunit, which is involved in ribosomal biogenesis and translational control (Jastrzebski et al., 2007).

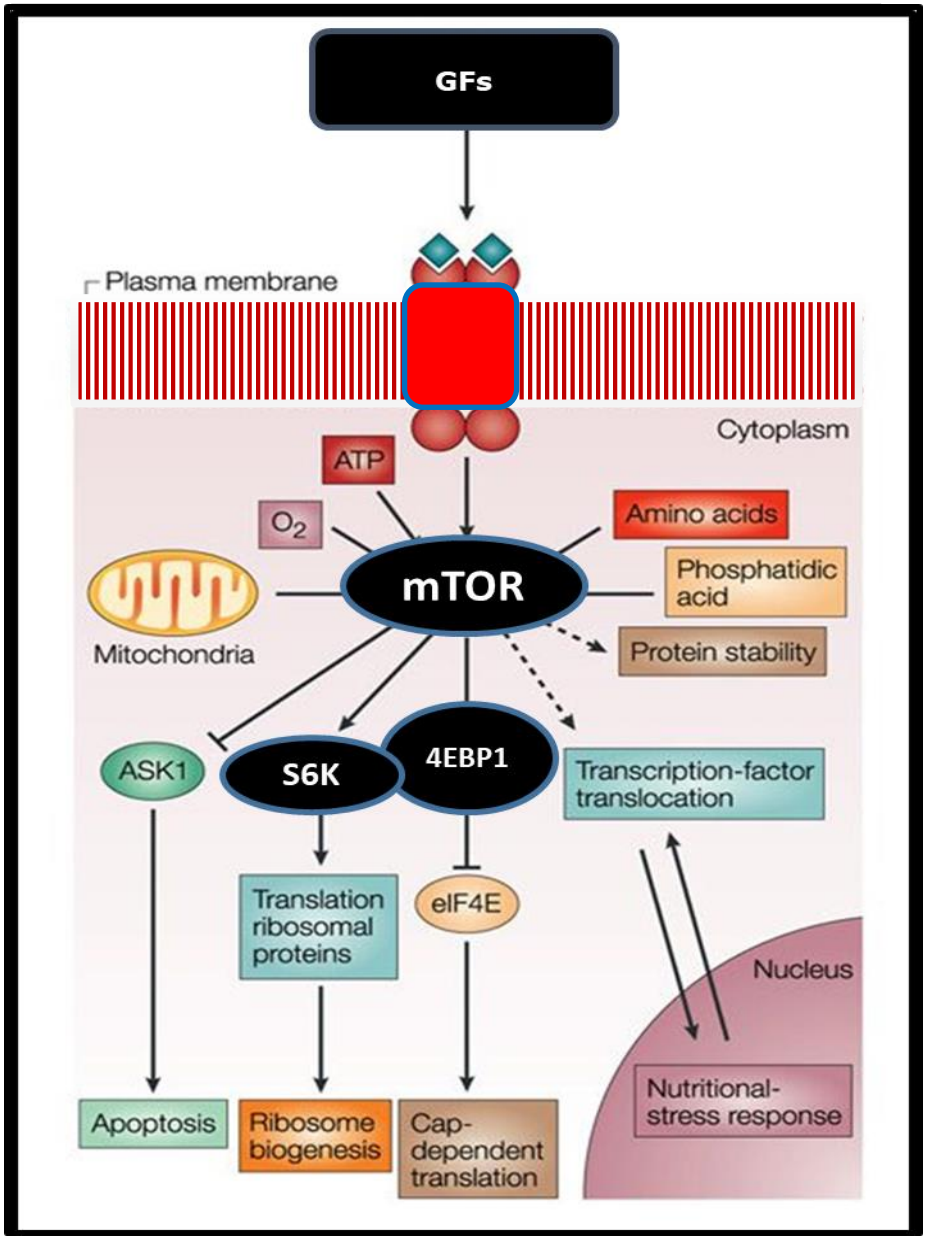



**Figure 1-2: mTOR and its downstream effectors pathway, adapted from Bjornsti et al (Bjornsti and Houghton, 2004)**

#### 1.2.10.2 Phosphatase and tensin homolog (PTEN), Ataxia-telegiectasia mutated (ATM), P85α and X-linked inhibitor of apoptosis protein (XIAP) pathway

Phosphatase and tensin homolog (PTEN) is a protein that, in humans, is encoded by the *PTEN* gene (Steck et al., 1997). Mutation of this gene is a well-recognised step in the development of different organs' cancer. PTEN is a key tumour suppressor and Its lipid phosphatase activity de-phosphorylates PIP3 (phosphatidylinositol (3,4,5)-triphosphate) to PIP2 (phosphatidylinositol (4,5)-triphosphate) and negatively regulates PI3K signalling (Worby and Dixon, 2014). PTEN is critically involved in the regulation of cellular proliferation, survival, energy metabolism, cellular architecture and cell motility (Hopkins et al., 2014, Bassi et al., 2013, Ming and He, 2012b). In addition, PTEN plays a critical role in the repair of damaged DNA through its interaction with ATM-p53 pathways in an AKT-independent manner (Minami et al., 2014, Ming and He, 2012a).

Ataxia-telegiectasia mutated (ATM) is a key member of the PIKK (PI3K-like protein kinases), family of serine/threonine kinases. The gene was first discovered in 1995 then, named as such because its mutations can cause ataxia–telangiectasia disorder (Savitsky et al., 1995). In 1998, both Shiloh and Kastan laboratories independently showed that ATM activity is enhanced by DNA damage (Banin et al., 1998, Canman et al., 1998). ATM is a master regulator of DNA damage response (Juvekar et al., 2016). Following double strand breaks (DSBs) ATM is rapidly recruited then, converted from homodimers to monomers and auto-phosphorylated. Activated ATM, in turn, phosphorylates a plethora of substrates involved not only in cell cycle checkpoints, apoptosis, metabolic and signalling pathways but also in DNA repair (Clouaire et al., 2017, Paull, 2015, Shiloh and Ziv, 2013).

Of the eight mammalian PI3K enzymes, which are grouped into three classes, four class I enzymes (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) have important roles in cancer (Fruman and Rommel, 2014). PI3K heterodimer consists of a p110 catalytic domain (that phosphorylate PIP2 to PIP3) and a p85α regulatory sub-unit (De Santis et al., 2019). In the absence of an activating signal, the interaction of p85α with p110 inhibits p110 kinase activity (De Santis et al., 2019, Fruman and Rommel, 2014, Thorpe et al., 2015). PI3K is recruited to the plasma membrane

after receptor tyrosine kinase activation. Consequently, p85α inhibitory effect on p110 is relieved and p110 phosphorylates PIP2 to PIP3 leading onto PI3K signal activation, a critical regulator of cell proliferation, growth, survival, motility, and metabolism (Thorpe et al., 2015, Fruman and Rommel, 2014, Juvekar et al., 2016).

Inhibitor of apoptosis proteins (IAPs) were first identified in baculoviruses. Their function is to keep the host cell alive during replication process (Birnbaum et al., 1994, Crook et al., 1993). In mammalian cells there are five IAPs: neuronal apoptosis inhibitory protein (Naip) (Roy et al., 1995), X-linked inhibitor of apoptosis protein (XIAP) (Crook et al., 1993, Duckett et al., 1996, Liston et al., 1996), human inhibitor of apoptosis protein-1 (Hiap-1) and Hiap-2 (Liston et al., 1996, Rothe et al., 1995) and survivin (Ambrosini et al., 1997, Li et al., 2001). XIAP, also known as inhibitor of apoptosis protein 3 (IAP3), can stop apoptotic cell death and it is the most potent human IAP protein (Deveraux and Reed, 1999). The XIAP gene, which is located on the X chromosome is responsible for XIAP production (Liston et al., 1996, Duckett et al., 1996). Deregulation of this protein can result in cancer development, neurodegenerative disorders, and autoimmune related diseases (Wilkinson et al., 2004).

PTEN inactivation (Chalhoub and Baker, 2009, Hollander et al., 2011), mutations in PIK3R1 (which encodes p85α) that leading to constitutive activation of p110α (Correia et al., 2014, Cheung et al., 2011, Costa and Engelman, 2014) and ATM inactivation through germ-line mutations (McKinnon, 2012), somatic ATM-inactivating mutations, deletions or epigenetic changes (Cremona and Behrens, 2014, Choi et al., 2016) all predispose to cancer. Emerging data also suggests a potential cross talk between ATM, PI3K, and PTEN pathways. PI3K can activate ATM (Irarrazabal et al., 2006). Similarly, ATM can also phosphorylate PTEN and promote its nuclear translocation following DNA damage (Chen et al., 2015a). In addition, p85α can also bind to and increase PTEN lipid phosphatase activity (Chagpar et al., 2010). Furthermore, ATM has a role in the regulation of PTEN through XIAP in p85α dependent manner, (Figure 1-3) (Ali et al., 2019).

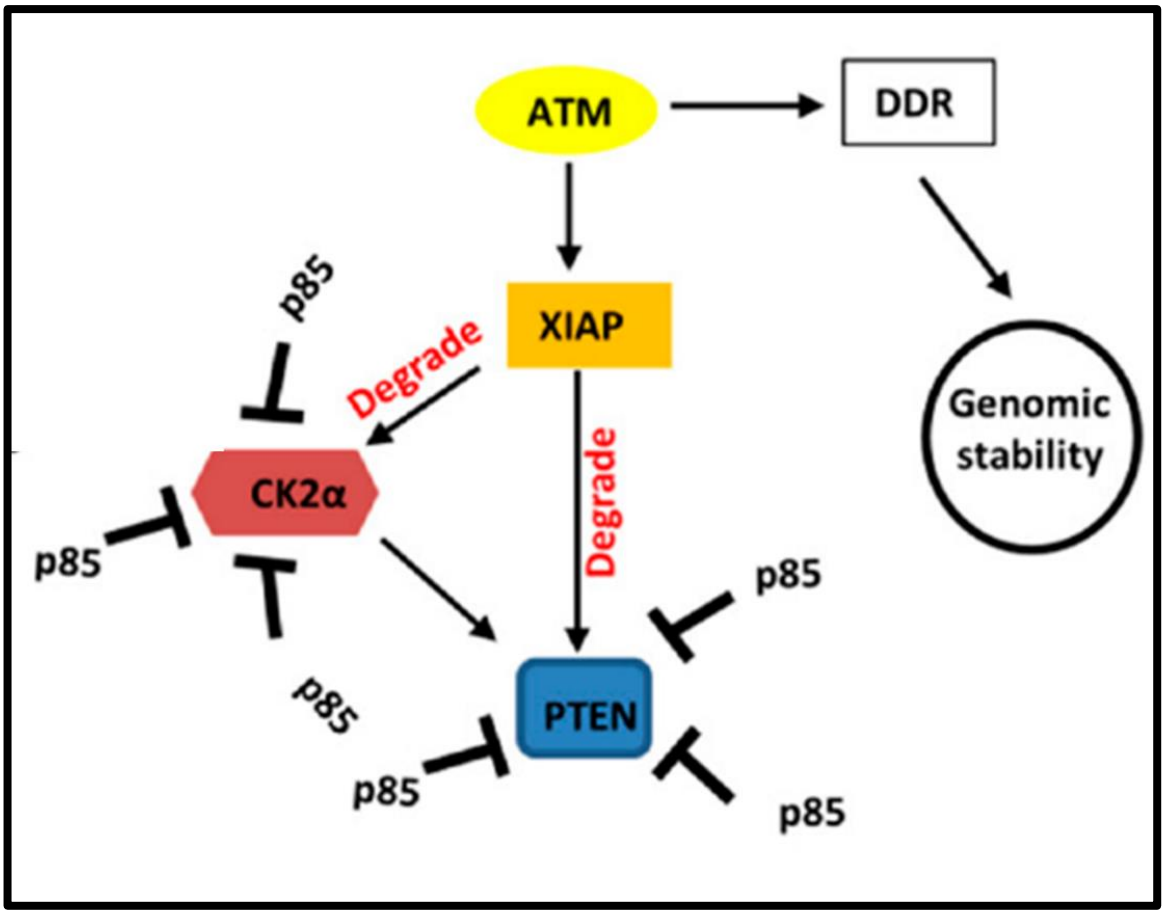



**Figure 1-3: ATM regulated PTEN degradation model, adapted from Ali et al., 2019**

#### 1.2.10.3 DNA damage

There are thousands of exogenous and endogenous daily attacks on every cell of the body. Therefore, DNA is relentlessly assaulted on frequent basis, which causes instability and eventually leading to DNA lesions with ineffective cellular replication. If the affected cells were not effectively and efficiently repaired, they become at risk of mutations and genome aberrations that can transform the cells to cancers (Ali et al., 2017). For this reason, there are groups of co-ordinated multifaceted DNA repair mechanisms to preferentially repair DNA lesions, maintain a balance between genetic diversity in germ cells and reducing harmful mutations to a minimal level. Such mechanisms must remain at equilibrium as consequences of DNA repair defects or mutations in repair genes have a recognisable impact on human health, with their presence leading to premature aging, neurodegeneration, increased cancer risk and immune-deficiency disorders (Ali et al., 2017). Generally, there are two types of DNA damage:

##### 1.2.10.3.1 Endogenous damage

Recently, it was demonstrated that endogenous cell damage could have significant impact on DNA viability (Tubbs and Nussenzweig, 2017). In general, endogenous attacks are responsible for large majority of gene modifications or DNA damage. Spontaneous acid catalysed hydrolysis of the N-glycosidic bond between the deoxyribose DNA bases is one of the simplest forms of endogenous damage. Similarly, DNA base deamination lesions involving the formation of transformation of uracil from cytosine are frequent forms of intrinsic damaging agents. Less commonly, the respective spontaneous deamination of adenine and guanine to hypoxanthine and xanthine can occur (Maugeri-Sacca et al., 2012).

Additionally, accidental cellular endogenous respiration by-products can cause lesions through chemical modification. Reactive oxygen species (ROS) such as O2-, H2O2 and OH, products of normal mitochondrial respiration can readily attack cellular DNA and produce DNA damage (Rowe et al., 2008).

##### 1.2.10.3.2 Exogenous damage

Various environmental sources can lead to exogenous DNA damage. One of the most common physical agents is the UV light (Tubbs and Nussenzweig, 2017). Lesions induced by UV light are repaired primarily by nucleotide DNA excision

repair (Osakabe et al., 2015). Another source of external physical DNA damage is Ionising radiation (IR) that could be produced by nature or from artificial sources. IR can induce a wide variety of DNA damage, double strand breaks is being the most significant. These breaks are repaired by: nonhomologous end-joining (NHEJ) or homologous recombination (HR) DNA repair pathways (Vignard et al., 2013, Santivasi and Xia, 2014).

In addition, chemical agents can also cause DNA damage. Drugs, especially chemotherapeutics can target the cellular DNA and cause damage to produce apoptosis. Both alkylating agents and platinum compounds utilise DNA damage to treat cancer (Maugeri-Sacca et al., 2012). Moreover, cigarette smoking is another exogenous factor that can result in DNA damage (Chen et al., 2015c).

#### 1.2.10.4 DNA repair pathways

Living cells possess the ability to detect and repair DNA damage. DNA lesions can take the form of double strand breaks (DSBs), inter-strand crosslinks, intra-strand cross links, single strand breaks, bulky adducts, modified bases, abasic sites and base mismatches, (Figures 1-4 and 1-5) (Burkhalter et al., 2015, Gee et al., 2018).

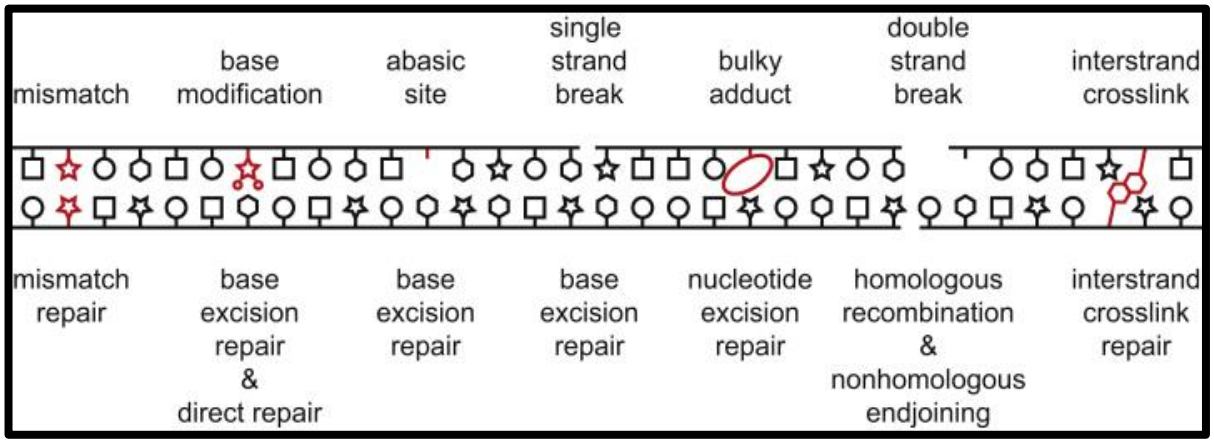



**Figure 1-4: Schem demonstrating types of DNA damage (highlighted in red) and the respective repair mechanisms (indicated below), adapted from Burkhalter et al (Burkhalter et al., 2015)**

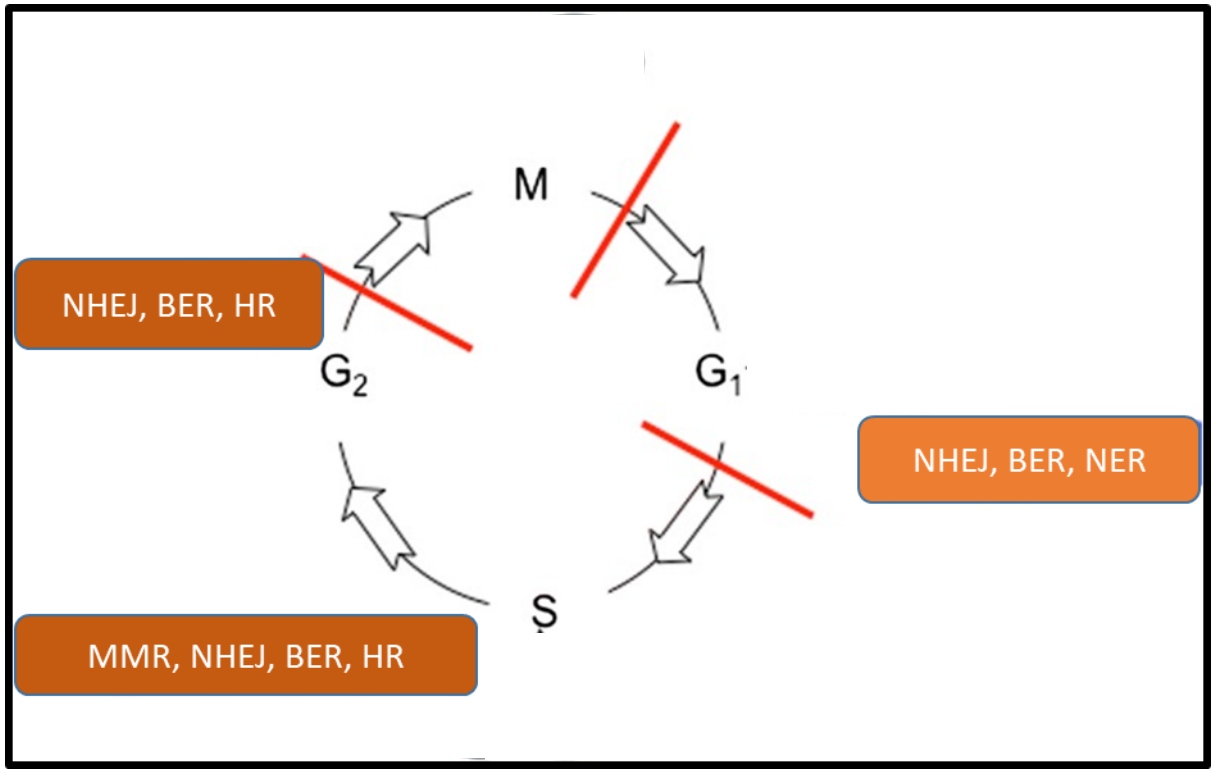



**Figure 1-5: The phases of the cell cycle and the associated DNA repair mechanisms, adapted from Gee et al (Gee *et al*., 2018).**

DNA repair pathways include:

#### 1.2.10.4.1 **Base excision repair (BER)**

BER is a highly co-ordinated pathway with consecutive enzyme reactions. It aids in the excision of damaged DNA bases created mainly from cellular metabolism events i.e. deamination, oxidative damage and alkylation. Several sub-pathways related to BER might be involved depending on the damage recognised. BER has around 12 structurally diverse glycosylases that act on different sites of the lesion. BER relies on the removal of damaged bases by the removal of N-glycosidic bond that links the base to deoxyribose backbone (Burkhalter et al., 2015). Following this, DNA polymerase fills the gap and DNA ligase seals the nick. The synthesis/ligation step is then divided into two sub-pathways; short-patch and long patch BER, which is determined by whether the DNA break has a single or several nucleotides (Wallace, 2014).

#### 1.2.10.4.2 **Miss-match repair (MMR)**

MMR process is highly conserved from E.coli to humans. It works to repair of base-pairing mismatches and/or insertion–deletion loops, which are usually a result of cellular metabolism. A mutator phenotype is the outcome of cells deficient in MMR pathway. This will produce microsatellite instability, elevated mutation frequency and germline mutations. Therefore, this can predispose to variety of cancers (Burkhalter et al., 2015). The MMR pathway is carried out by two primary proteins, MutS (responsible for recognition) and MutL (responsible for coupling recognition complexes to MMR events, leading to error strand removal). This is usually carried out by Ligase I (LIG1) (Dexheimer, 2013).

#### 1.2.10.4.3 **Homologous recombination (HR)**

HR works by exchanging nucleotide sequences between DNA molecules. It is most commonly utilised in the repair of DSBs and inter-strand crosslinks (ICLs). HR divided into three separate phases, which end up by DNA ligation, which is performed by DNA LIG1 (Samadder et al.).

#### 1.2.10.4.4 **Non-homologous end-joining (NHEJ)**

This pathway also repairs the DSBs. It is unique in that break ends are directly ligated without the requirement of a homologous template, unlike HR. NHEJ was

first evolved in bacteria and it is one of the most fundamental mechanisms for sustaining genome stability (Bartlett et al., 2016). The sequential assembly of factors recruited at DSB sites mediate the NHEJ. At the exposed DSB, terminal binding and recognition of Ku70-Ku80 heterodimer, defines the initial recognition step. Access to the DNA terminals happens through the auto-phosphorylation of DNA-PKcs. Then, both Pol μ and Pol λ resynthesise the missing DNA nucleotides. Both DNA Ligase IV (LIG4) and XRCC4 carry out the DNA ligation process (Dexheimer, 2013).

#### 1.2.10.4.5 **Nucleotide excision repair (NER)**

This repair pathway specifically functions in the universal excision of a wide variety of bulky DNA lesions. This is achieved by excising the damage sites and then with the aid of DNA polymerase, the gaps are filled. NER mechanism has various implications in many different biological pathways and disease progression. Moreover, in placental mammals, NER is the only DNA repair pathway that can remove UV-photoproducts (Spivak, 2015). The DNA repair steps include recognition of the DNA lesion, opening of the DNA around the lesion area, excision of the damaged DNA segment, then sequential strand repair and ligation (Gentile et al., 2016).

### 1.2.10.5 The role of Poly [ADP-ribose] polymerase 1 (PARP1), X-ray repair cross-complementing protein 1 (XRCC1), DNA polymerase β (POLβ) in ovarian cancer

The platinum based drugs can cause cellular toxicity directly through the induction of intra-strand and inter-strand DNA adducts. If unrepaired, DSBs can develop during replication. Therefore, DNA repair mechanisms are vital in detecting and processing the damage caused by platinum chemotherapeutic agents (Ali et al., 2020).

PARP1 is one of the PARP family of enzymes. It is encoded by *PARP1* gene (Ha and Snyder, 2000). It is involved in differentiation, proliferation, tumour transformation and normal or abnormal recovery from DNA damage. Moreover, it is linked to type I diabetes pathophysiology (Ha and Snyder, 2000). When a DNA damage happens, PARP1 acts as a first responder that detects and then facilitates the choice of repair pathways (Pascal, 2018). Moreover, PARP1 is implicated in

the regulation of several DNA repair processes including the NER, NHEJ, HR and MMR repair mechanisms (Pascal, 2018). In the absence of PARP1, DSBs accumulates. Importantly, PARP1 binds to DNA repair intermediates such as single-strand breaks and gets activated, which in turn leads to the synthesis of PAR (poly-ADP-ribose) polymers. PARP1 auto-PARylation recruits other DNA repair factors (including XRCC1) at the sites of DNA damage resulting in efficient DNA repair (Lord and Ashworth, 2017).

XRCC1 has a vital role in DNA repair. It is encoded by *xrcc1* gene. XRCC1 acts as a scaffolding protein as it does not have any enzymatic activity. However, it interacts with multiple repair enzymes facilitating them to carry out their vital steps in repairing DNA damage. Similar to PARP1, XRCC1 is involved in BER, NER and single-strand break repair (London, 2015). XRCC1 protein has three globular domains connected by two linker segments. N-terminal domain, which binds to POLβ, the C-terminal BRCT domain that interacts with DNA ligase III and finally, central domain which reacts with PARP1 (London, 2015). Delays in SSB re-joining leading onto SSBs and eventually to DSBs is the outcome of XRCC1 deficiency (Caldecott, 2003, London, 2015). In addition, PARP1 is hyper-activated with XRCC1 deficiency/mutation (Hoch et al., 2017). XRCC1 deficiency potentiates chemotherapy cytotoxicity and platinum sensitivity in cancer cells (Caldecott, 2003).

POLβ is an enzyme that is encoded by *POLβ* gene. It is a distributive polymerase that devoid any proof reading activity. POLβ is incorporated in the BER system to help removing some oxidative DNA damages. Therefore, POLβ is essential not only for DNA maintenance, replication and recombination, but also in drug resistance (Prasad et al., 2017). POLβ interacts with several components of BER machinery such as XRCC1, ligase III and PARP1 to accomplish its biochemical functions (Wallace et al., 2012, Nicolay et al., 2012, Yamtich and Sweasy, 2010). In addition, the sensitivity to chemotherapy was enhanced in POLβ depleted cancer cell lines (Albertella et al., 2005, Yang et al., 2010). On the other hand, POLβ overexpression enhanced the resistance to DNA damaging agents (Canitrot et al., 1998, Nicolay et al., 2012, Abdel-Fatah et al., 2014a), (Figure 1-6) (R.W., 2014).

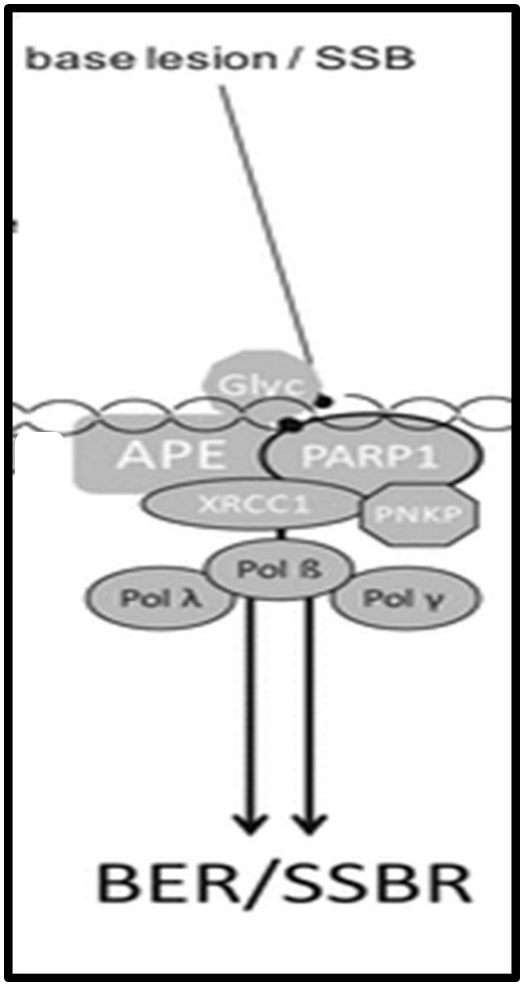



**Figure 1-6: PARP1, XRCC1 and POLB role in DNA repair (R.W., 2014)**

### 1.2.10.6 Human DNA ligases and ovarian cancer

The most serious form of DNA damage is DSBs and if a single DSB is unrepaired, it can lead to cell death (Rich et al., 2000, Liang et al., 2008). DNA ligase encoded by one of the three human *LIG* genes is essential in sealing these breaks in a coordinated three step ligation process (Ellenberger and Tomkinson, 2008, Tomkinson et al., 2020). Human ligases were discovered in 1967 (Lehman, 1974). They are three in number and all related to nucleotidyl transferase family, which are encoded, by human *LIG1*, *LIG3* and *LIG4* genes (Ho et al., 2004, Shuman and Schwer, 1995), (Figure 1-7).

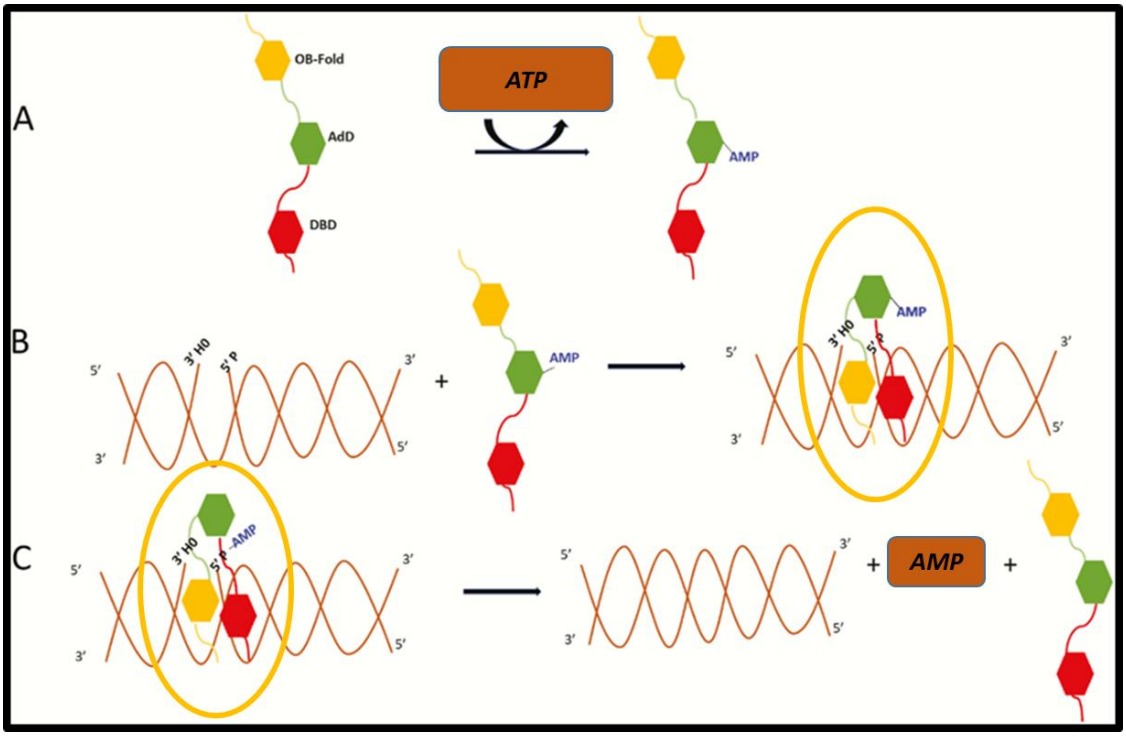



**Figure 1-7: Three-step ligation reaction to DNA breaks, adapted from Tomkinson et al (Tomkinson et al., 2020)**

In addition, DNA ligases can transiently impede DNA repair in tumour cells. Hence, might permit lower dosing with DNA-damaging drugs and reduce their potential toxicity (Torgovnick and Schumacher, 2015).

*LIG1* gene was the first of three human *LIG* genes identified in 1990. DNA LIG1 is composed of 919 amino acid polypeptide with 125 KDa molecular mass (Barnes et al., 1990). In 1992 the first known case of inherited LIG1 deficiency was described. This female individual was complaining of being underweight, anaemic, with growth retardation and developmental delays. In addition, she had sunlight sensitivity and immunodeficiency. She died at 19 year of age from pneumonia (Barnes et al., 1992, Webster et al., 1992). LIG1 overexpression in mouse models increased both their genomic instability and incidence of epithelial tumours (Harrison et al., 2002). Moreover, LIG1 overexpression can enhance the proliferative status of cancer cells (Sun et al., 2001a).

Ligase III (LIG3) is an enzyme that is encoded by *LIG3* gene, which is located on human chromosome 17 at q11.2–q12 (Chen et al., 1995, Wei et al., 1995, Tomkinson and Sallmyr, 2013). Unlike LIG1 and LIG4, LIG3 family members are less widely distributed in eukaryotes. There are three forms of LIG3 and all located in the cell mitochondria and nucleus. They are generated by alternative translation initiation (Lakshmipathy and Campbell, 1999). Preclinical and tissue samples of breast cancer, leukaemia and neuroblastoma showed elevated steady-state levels of LIG3 (Tobin et al., 2013, Tobin et al., 2012, Newman et al., 2015).

The gene encoding DNA Ligase IV (LIG4) was identified around at the same period of *LIG3* gene discovery (Wei et al., 1995). LIG4 can make a stable complex with XRCC4, a DNA DSBs repair protein. Moreover, XRCC4 is essential for LIG4 stability (Grawunder et al., 1997, Grawunder et al., 1998a, Grawunder et al., 1998b, Grawunder et al., 1998c). LIG4 is complexed with the nicked DNA in a similar mechanism to both LIG1 and LIG3 (Pascal et al., 2004, Cotner-Gohara et al., 2010, Tomkinson et al., 2020). Therefore, aberrations in LIG4 can contribute to genomic instability and tumorigenesis (Xie et al., 2014). Kim et al (Kim et al., 2014) concluded that LIG4 overexpression enhanced cells radio-sensitivity in nasopharyngeal cancer. Moreover, LIG4 polymorphism modulated breast cancer and multiple myeloma risk (Kuschel et al., 2002, Roddam et al., 2002).

## 1.3 Hypothesis of the PhD

OC is a heterogeneous set of diseases with approximately 90% of cases being of epithelial origin. Several studies have shown that the major histologic subtypes of OC have different risk factors, genetic susceptibilities, and clinical response to therapy. Moreover, they are all different at the molecular level. It was hypothesised that both cell and DNA damage signalling pathways are vital in OC progression and prognosis.

## 1.4 Aims of the study

The aims of the study were to focus on the expression of different proteins directly related to these two signalling pathways, cell signalling and DNA damage. This is to further understand not only the biology of the tumour but also to identify their clinical significance. Furthermore, to identify which of these key proteins merits to be dominantly functioning when co-expressed.

The specific aims were:

1- Examine a cluster of novel proteins correlated with both signalling pathways utilising IHC methodologies from patients treated primarily with surgical resection of the tumour before the initiation of chemotherapy.

2- Correlate the expression of the signalling pathways' members with clinicopathological variables and patients' outcome.

3- Determine the prognostic and predictive value of the relevant signalling pathways' key members using univariate and multivariate analyses.

4- Assess the effect of carefully selected novel prognostic proteins in preclinical studies utilising tissue culture methodologies.

# 2 Material and Methods

## 2.1 Patients' cohorts' characteristics

### 2.1.1 Patients' series

The study cohort included 950 patients of primary Ovarian Cancer (OC) who were treated at Nottingham University Hospitals (NUH) from 1997 to 2010. All the patients included in this study were confirmed by histology as OC. All included cases were treated primarily by surgery with or without chemotherapy. The available clinicopathological information included tumour histology type, stage, histological grade, chemotherapy regimen used, platinum sensitivity, and tumour relapse with survival status. In addition, the retrieved data included detailed information regarding tumour surgical debulking achievement. All patients' notes and histology reports were reviewed, collected and analysed by the researcher. All the cases who did not meet the designed research selection criteria were excluded. Therefore, the valid cases from the primary series after TMA construction were 525. Cohort characteristics are summarised below, (Table 2-1).

### 2.1.2 Sample size effectivity

Low power studies are less likely to find a true effect. For this reason, in spite of no statistical significant effect recognised in a study, the effect exists at the population level. On the other hand, true effects that are detected might have only inflated effect sizes. Therefore, a true effect is only significant in an underpowered study when the effect obtained in the study is larger than the effect at the population level (Fraley and Vazire, 2014, Brysbaert, 2019). For this reason, we have conduction a power check for this current study, in order to effectively identify a significant finding at the population level in primary OC cases a minimum of 357 samples are required to achieve a 95% confidence rate in analysis.

### 2.1.3 Patients' outcome

Patients were followed up according to a local protocol designed depending on the national guidance, three monthly for two years then, 6 monthly for 5 years and finally, annually for indefinite. The date of latest follow up was recorded in the electronic database to facilitate calculation of survival. Using this data, patient survival status was coded as 0 (alive) or 1 (dead). The precise cause of death was not specifically recorded but anecdotally the majority died of related causes to OC.

The data concerning date of diagnosis, latest follow-up, date of death and survival status were used to generate crude overall survival (OS), and also progression free survival (PFS) similarly, calculated from date of surgery until developing loco-regional recurrence and /or distant metastasis (median= 29 months). The follow up period was calculated for 5 years when any remaining survivors were censored. Patients demographics are summarised below, (Table 2-1).

## 2.2 Patients' clinicopathological aspects

### 2.2.1 Age

The age of the patients at diagnosis was available and ranged from 22-98 years.

### 2.2.2 Pathology

The vast majority of the cases included in the surrogate series developed epithelial OC (96.5%). These showed comparable distribution of histological subtype with literature, (Table 2-1). Tumour grade did appear to have prognostic significance on univariate analysis. However, this was not maintained on multivariate analysis. This suggests that the impact of tumour grade on prognosis is dependent on other factors related to tumour biology. Therefore, tumour grade was not included in the Cox model for assessing different prognostic biological markers.

### 2.2.3 Stage

Data for tumour stage was available for majority of the cases, 504. The distribution of cases was ranged from stage I to IV. Interestingly, 46.8% of the cases were presented at an advanced stage disease (stage III or IV).

**Table 2-1 patients' demographics**

| Characteristics | Number | Percentages % |
|---|---|---|
| **Pathology Type** | | |
| Serous cystadenocarcinoma | 290 | 55.4 |
| Endometrioid | 82 | 15.6 |
| Clear cell carcinoma | 48 | 9.1 |
| Mucinous cystadenocarcinoma | 60 | 11.4 |
| Others | 18 | 3.4 |
| Mixed | 17 | 3.2 |
| Unknown | 10 | 1.9 |
| **FIGO Stage** | | |
| I | 182 | 34.6 |
| II | 76 | 14.4 |
| III | 213 | 40.5 |
| IV | 32 | 6 |
| Unknown | 22 | 4.2 |
| **Grade** | | |
| One | 68 | 12.9 |
| Two | 87 | 16.5 |
| Three | 306 | 58.2 |
| Unknown | 64 | 12.1 |
| **Measurable Disease post-Surgery** | | |
| Non | 329 | 62.7 |
| Measurable | 152 | 29 |
| Unknown | 44 | 8.3 |
| **Chemotherapy Regimen** | | |
| Carboplatin monotherapy | 163 | 31 |
| Carboplatin + Paclitaxel | 177 | 33.7 |
| Other Regimens* | 43 | 8.3 |
| No Chemo | 25 | 4.7 |
| Unknown | 117 | 22.3 |
| **Platinum Sensitivity** | | |
| Sensitive | 376 | 71.6 |
| Resistant | 85 | 16.1 |
| Unknown | 62 | 11.8 |
| **Relapse status** | | |

| | | |
|---|---|---|
| Progression-free | 238 | 45.3 |
| Progressed/relapsed | 243 | 46.2 |
| Unknown | 44 | 8.3 |
| **Survival Status** | | |
| Living | 199 | 37.9 |
| Dead | 286 | 54.4 |
| Unknown | 40 | 7.6 |

*3= CAP (Cyclophosphamide, Adriamycin and Cisplatin) (10 patients)

4 = ICON5 Trial (10 patients)

5 = SOCTROC Trial (16 patient)

6 = Carboplatin and Endoxan (6 patients)

7 = Chlorambucil (1 patient)

## 2.3 Construction of tissue microarray (TMA)

### 2.3.1 Formalin Fixed Paraffin Embedded (FFPE) blocks retrieval from Nottingham Health Science Biobank (NHSB)

All the archival FFPE blocks harbouring OC tissues used in this study were accessible but through a specific requisition application, which was approved and signed by the NHSB. As a mandatory condition, this application form must include the research and development approval number of the project, which allowed us to use NUH patients' specimens in this research. At the end of this process, all the retrieved blocks archived correctly and their related records kept strictly confidential. All the arrangements included in the retrieval process was designed and guided by the researcher.

### 2.3.2 Tissue sectioning

#### 2.3.2.1 Documentations

Tissue sectioning requests were completed once all the requested FFPE blocks were obtained and indexed in special labelled boxes. These forms are specific for human tissue sectioning requests. It must include the Research Ethics Committee approval number, the Health Technology Assessment (HTA) study number, issued by the National Institute for Health Research (NIHR), and the NHSB ACP number. This form applies as well to TMA sectioning for the optimisation and staining of antibodies. This request must be completed and approved, when H&E and IHC staining is required.

#### 2.3.2.2 Sectioning in the microtome

Safety procedures and risk assessment guidelines were followed before sections were cut from the FFPE blocks. An experienced laboratory technicians working at the Histopathology department in Nottingham University Hospitals NHS Trust performed sectioning of the blocks. Instruments and tools used for this method included: a rotary microtome (Leica RM2125, Leica microsystems, United Kingdom); a paraffin section mounting bath (Thermo Scientific, MH8516, United Kingdom), filled with deionised water to eliminate distortions of sectioned tissue; and glass slides, with Leica's X-tra® or Leica's Apex adhesive slides preferred for use in H&E for IHC applications. A plastic box containing crushed ice, disposable

microtome blades (S35 Feather, CellPath, Newtown, Powys, Wales), curved-tip forceps, and xylene-resistant marker pen, or pencil, were also available for use accordingly.

FFPE blocks were placed face up, on ice to be chilled. Then, the chilled blocks were inverted; face down, on ice for five minutes maximum. A block was carefully positioned in a microtome chuck. The movement of the block's face to the blade edge in both axes needed to be parallel. 4µm was the desired sectioning thickness. The block face was trimmed until a 'full-face' section was produced appropriately. The cutting extra sections were used later for optimisation purposes. Then, these sections were laid down carefully on the water surface of the water bath. Single sections were mounted onto microscopic slides by submerging the slides into the water, beneath the tissue, at an angle of approximately 45°. The laid sections were realigned over the slide as necessary. Mounted slides were dried in a rack overnight in an oven at 37°C. Following this, slides were stored in a cold room at 4°C.

### 2.3.3 Preparation of donor blocks and TMA construction

Archival FFPE blocks of primary OC tissue were all collected and retrieved from NHSB. Then, the cut sections were stained by Haematoxylin and Eosin (H&E). In order to confirm the presence of sufficient invasive malignant cells for correct tissue sampling, all the slides were assessed by the researcher and an expert pathologist. Representative tumour areas on both the slide and their corresponding spots on the FFPE blocks were marked alongside. The blocks were loaded onto the TMA Grand Master® (3D HISTECH®, Budapest, Hungary). All the collected TMA samples were loaded into a recipient block. A needle of 0.6 mm wide was used to harvest the tumour tissue from the marked areas of FFPE blocks. All the corresponding TMA maps were prepared as shown below, (Figures 2-1, 2-2, 2-3). Then, the final TMA maps were created by the researcher and made ready for the scoring process, (Figure 2-4).



**Figure 2-1: FFPE blocks of primary OC tissue marked and arranged for TMA**

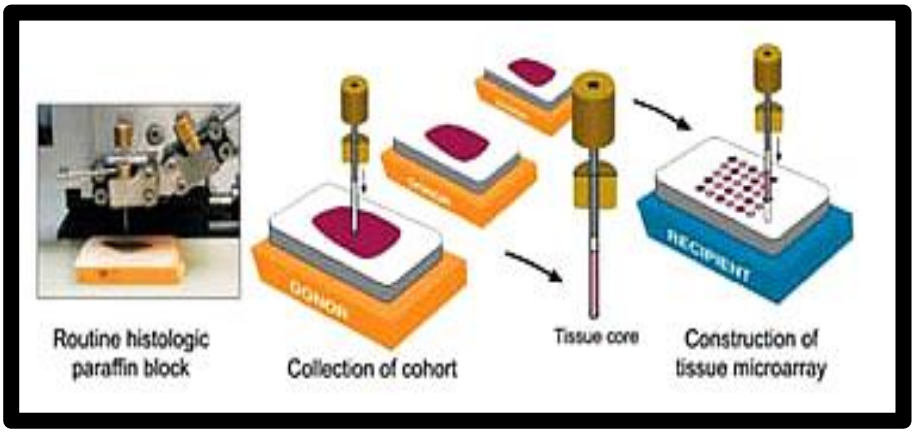



**Figure 2-2: A simplified schematic illustration of the tissue microarray construction. Figure adapted from (Rimm et al., 2001)**

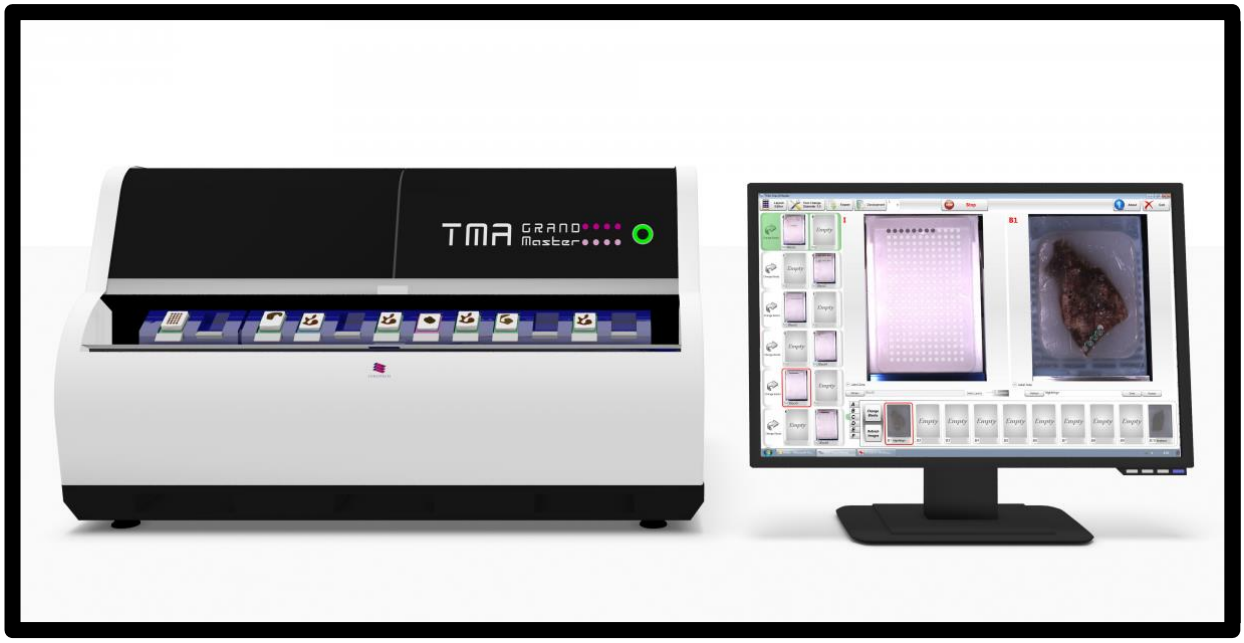
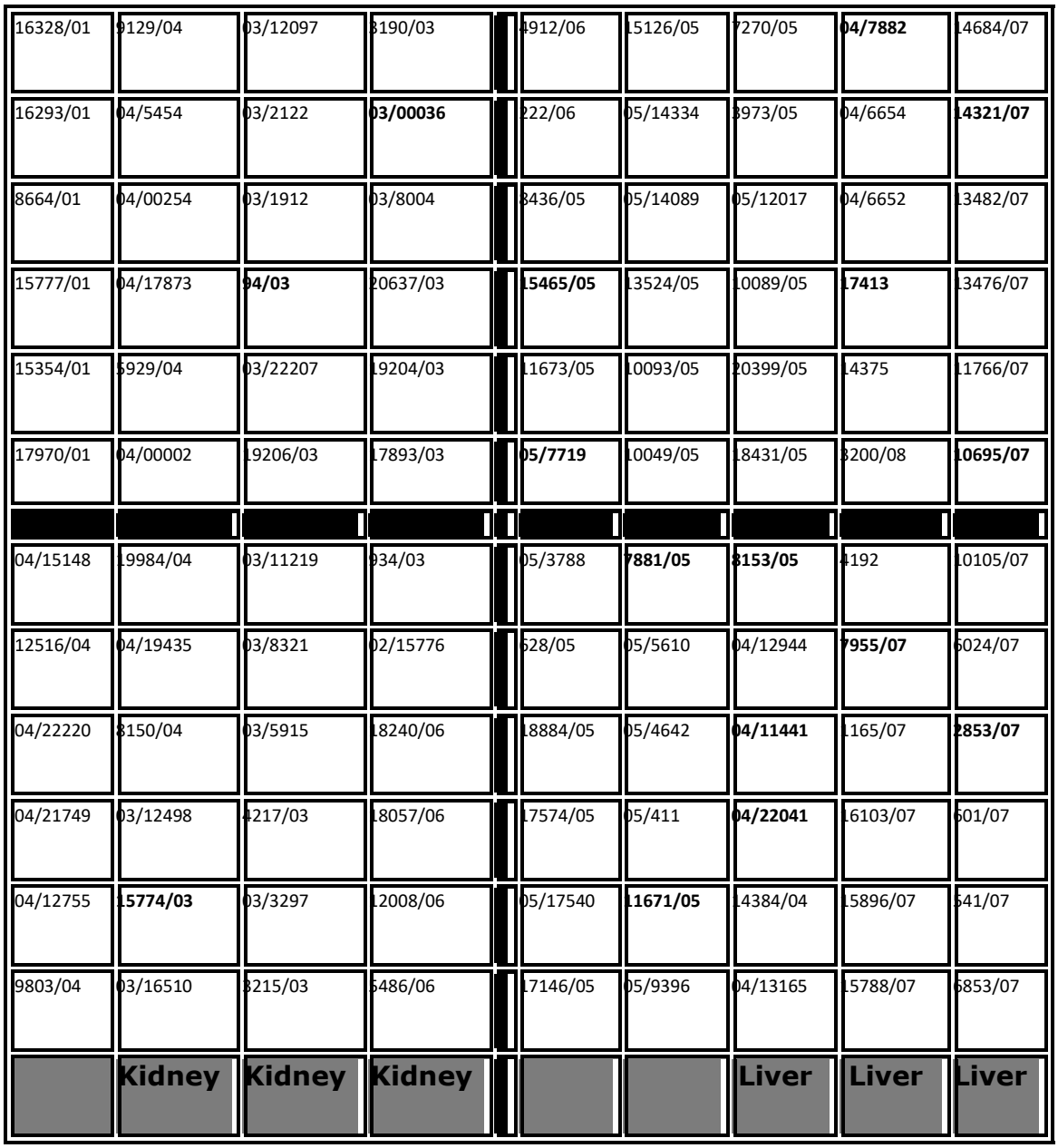



**Figure 2-3: TMA Grand Master Machine used for Construction**



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16328/01 | 9129/04 | 03/12097 | 3190/03 | 4912/06 | 15126/05 | 7270/05 | **04/7882** | 14684/07 |
| 16293/01 | 04/5454 | 03/2122 | **03/00036** | 222/06 | 05/14334 | 3973/05 | 04/6654 | **14321/07** |
| 8664/01 | 04/00254 | 03/1912 | 03/8004 | 8436/05 | 05/14089 | 05/12017 | 04/6652 | 13482/07 |
| 15777/01 | 04/17873 | **94/03** | 20637/03 | **15465/05** | 13524/05 | 10089/05 | **17413** | 13476/07 |
| 15354/01 | 5929/04 | 03/22207 | 19204/03 | 11673/05 | 10093/05 | 20399/05 | 14375 | 11766/07 |
| 17970/01 | 04/00002 | 19206/03 | 17893/03 | **05/7719** | 10049/05 | 18431/05 | 3200/08 | **10695/07** |
| | | | | | | | | |
| 04/15148 | 19984/04 | 03/11219 | 934/03 | 05/3788 | **7881/05** | **8153/05** | 4192 | 10105/07 |
| 12516/04 | 04/19435 | 03/8321 | 02/15776 | 628/05 | 05/5610 | 04/12944 | **7955/07** | 6024/07 |
| 04/22220 | 8150/04 | 03/5915 | 18240/06 | 18884/05 | 05/4642 | **04/11441** | 1165/07 | **2853/07** |
| 04/21749 | 03/12498 | 4217/03 | 18057/06 | 17574/05 | 05/411 | **04/22041** | 16103/07 | 601/07 |
| 04/12755 | **15774/03** | 03/3297 | 12008/06 | 05/17540 | **11671/05** | 14384/04 | 15896/07 | 541/07 |
| 9803/04 | 03/16510 | 3215/03 | 6486/06 | 17146/05 | 05/9396 | 04/13165 | 15788/07 | 6853/07 |
| | **Kidney** | **Kidney** | **Kidney** | | | **Liver** | **Liver** | **Liver** |

**Figure 2-4: An example of a TMA grid used for scoring of tumour cores. The numbers correspond to an individual tumour. Kidney and Liver tissue was used to aid orientation of the slide during staining scoring.**

## 2.4 Histological staining

### 2.4.1 Overview

It is well known that Haematoxylin and Eosin (H&E) staining can be utilised to stain human tissue samples for diagnostic purposes. Violet and blue colours distinctive shades can be seen microscopically. Therefore, histological components and morphologic alterations can be distinguished by pathologists. Haematoxylin has an extreme importance in medical diagnosis, especially in histological diagnosis of malignant neoplasms. It is a positively charged dye, which can stain the basophilic components of a tissue's genetic material. It will stain the large nuclei of cancer cells. On the other hand, the cytoplasmic and extracellular matrix are both counterstained with eosin.

### 2.4.2 Haematoxylin and Eosin procedure

Leica Autostainer XL system (Leica AXL) was used for processing the Immunohistochemistry (IHC) and H&E staining of all tissue sections in this study. Leica AXL is semi-automated machine, its initialisation, programming, and loading/removing tissue slides were performed by the researcher. However, the staining procedure itself was sequentially executed by the machine.

First, tissue sectioning of the FFPE blocks was completed. Secondly, slides deparaffinisation step, which was conducted by placing the tissue slide with the face up on a hot plate at 60°C for ten minutes. This step was essential to melt the hard wax and to prepare it for total dissolving by xylene solution. Following this, all tissue sections were sorted into a slide rack fitting for the xylene vessel, which was placed into a specific station inside the machine in which, the H&E staining programme was selected. Its first step is a five-minute incubation of the slides in xylene. Another five-minute incubation with fresh xylene is followed. After that, three consecutive washes in 100% of Industrial Methylated Spirits (IMS) (product code: M/4450/25, Fisher Scientific, UK) were performed for two minutes each. Then, the tissue sections were washed in water twice, two minutes each.

Haematoxylin (product code: 12658626, Fisher Scientific, UK) was filtered, and then poured into a clean Leica AXL reagent vessel. The washed tissue sections were immersed in Harris's haematoxylin vessel for five minutes followed by 10

seconds wash in acid alcohol, and 1% Hydrochloric acid (HCL) in IMS was applied. Subsequently, another 20 seconds wash with water was conducted. Tissue sections were dipped into a reagent vessel filled with 1% of borax (product code: B9876, Sigma, UK) for 40 seconds, then washed in water for one minute.

Contrast counterstaining of haematoxylin was performed by immersing the tissue section in another reagent vessel filled with 1% Eosin (product code: 12668616, Fisher Scientific, UK) for 30 seconds, then the sections were washed in water for 15 seconds. The remaining water droplets were totally removed with three dehydration baths in 100% isopropyl alcohol (product code: P/7507/PB17, Fisher Scientific, UK), 40 seconds each. Subsequently, two five minutes xylene baths were utilised to clear alcohol. Finally, the tissue sections were mounted with DPX mounting medium to finish with slides covered by clear glass cover slip.

## 2.5 Clinical study (Immunohistochemical staining)

### 2.5.1 Overview

The first IHC experiment was reported in the 1940s (Coons et al., 1941). IHC is considered as an indispensable practice, which critically affects the clinical management of OC patients. From molecular subtyping to detection of validated prognostic biomarkers, IHC plays a critical role in enriching the reliability of pathology reports in many neoplastic diseases, including OC. The IHC procedure is based on a reversible reaction between an antibody (Ab) and an antigen (Ag). Utilising this biochemical bonding between Ab and Ag in the clinical practice certainly improves patients' clinical profiles and prognosis estimation accuracy by combining the observed histological features of a tumour with its protein expression patterns.

### 2.5.2 Procedure

In this study, IHC was used to semi-quantify the levels of protein expression on OC TMA sections. All cut tissues were obtained from invasive FFPE specimens. The IHC procedure was performed by using NovolinkTM Max Polymer Detection System (product code: RE7280-K, Leica, Biosystems, UK), which is suitable for antigen detection by using mouse immunoglobulin G (IgG) and rabbit IgG. It included the following steps:

#### 2.5.2.1 Slides preparation

All tissue sections were loaded onto Leica's X-tra® slides. All the slides were labelled using a marker pen. Date, symbol of targeted protein, user name, applied antibody dilution factor, and specimens' identification number were the basic standard labelling data used for each slide.

#### 2.5.2.2 Rehydration of TMA tissue sections

Starting with dewaxing the tissues, all TMA sections were placed on a hot plate (Leica HI1220 flattening table, product code: 14042321474, Leica Biosystems, UK), face up, at 60°C for 10 minutes. Then, with sorting the slides into the Leica's slide rack (product code: 14047533750, Leica Biosystems, UK) deparaffinisation continued. The rack holding the slides was placed into a reagent vessel filled with

fresh xylene (product code: X/0250/PB17, Fisher Scientific, UK) and contained inside the chamber of the Leica AXL. The dewaxing programme, previously introduced into the machine, was activated and this programme can keep the slides soaked in xylene for five minutes. Automated transfer of the holding rack into another fresh xylene vessel followed this, where it was incubated for another five minutes. Three baths of IMS were applied to all slides, as the first two baths were in 100% IMS for two minutes and the third bath was in 70% IMS. The rack was then rehydrated in a vessel filled with flowing water for another five minutes.

#### 2.5.2.3 Preparation of citrate buffer

The sodium citrate buffer was prepared by the complete dissolving of 2.94 gm of Tris-sodium citrate dihydrate in 1L of distilled water. A pre-calibrated laboratory pH meter was used to adjust the pH to exactly 6.00 by adding few HCL drops. Then, the solution was stored at 4°C. Afterwards, a plastic Leica AXL vessel was filled with 500ml of citrate buffer, the TMA slides rack was immersed in the rack, and a plastic lid with no metal handles was placed on the top of the vessel. The vessel then placed inside a laboratory microwave; the microwave's door was locked and it was operated at maximum power for 20 minutes. Protective glasses were worn during this step; the vessel was removed from the turned-off microwave with a heat-resistant pair of gloves. The vessel lid was removed in the sink and flowing water was applied with a rubber hose into the vessel, displacing the citrate buffer and cooling down the slides for five minutes.

#### 2.5.2.4 Staining procedure

After the cooling stage with tap water, a hydrophobic barrier was marked out on the slide to ensure maximum staining. Sections were washed 2x5 minutes with TBS-Tween and then incubated with 300µl of peroxidase block (Leica Biosystems). Following this, slides were washed 3x5minutes TBS-Tween. The sections were then incubated with 300µl of protein block (Leica Biosystems) and washed in a similar way as mentioned before. Then the primary antibody was added to the slides according to the previously optimised concentration and duration. Slides were then incubated according to each antibody optimal conditions. With each experiment a $\beta$2 microglobulin at 1:2000 staining was used as a control antibody, and appropriate tissue as recommended by the manufacturer or human protein atlas was utilised as a positive control. Once the optimal incubation time with the

primary antibody was finished, a 3x5minute washes were started. Then, an incubation with 300µl of post primary antibody for 30 minutes was performed, followed by 3x5minute washes. A 30-minute incubation with 300µl of Polymer and further wash cycle were the next step. Finally, DAB substrate/buffer was then made of a 1:20 concentration and sections were incubated in 300µl for 5 minutes, which was followed by another 3 washes. Haematoxylin, 300µl was then applied and incubated with the slides for 6 minutes. Then proceeding to the stage of dehydrating and clearing in the Leica Autostainer. The dehydration step included washing the slides in three baths of ethanol (EtOH), 2 minutes each, followed by two washes in xylene, five minutes each. Lastly, the stained tissues were then mounted in DPX, a synthetic resin mountant mixture of distyrene (a polystyrene), a plasticiser (tricresyl phosphate), and xylene medium and sealed with a transparent coverslip to be ready for use.

#### 2.5.2.5 Optimisation of antibodies used for immunohistochemistry

Firstly, all the antibodies were optimised on TMA before the staining on the whole series. This was performed by changing different variables in the protocol of the staining, mainly the antibody concentration and/or the incubation time. The starting point was usually with the dilution recommended by the supplier's datasheet in addition to three or more dilutions above and below the recommended. After the first attempt, estimate was created about the expected concentration/incubation period. Then, the optimisation procedure was repeated. Once optimum result was obtained and properly agreed with an expert histopathologist (no/less background staining, and high degree of expression heterogeneity), this was properly documented and the staining procedure planned accordingly. Full list of antibodies and their optimisation details is included in the supplementary section.

Importantly, in addition to Western blotting, negative controls (with omission of the primary antibody) were used in every IHC experiment to ensure the genuine specificity of the surrogate biomarkers. All negative controls used in this study were applied on OC tissue. In addition, in each experiment, positive controls were included and as instructed by the manufacturer or from the human protein atlas (http://www.proteinatlas.org/), supplementary section.

### 2.5.3 Imaging of tissue sections and histological scoring (H-score) system

#### 2.5.3.1 Whole slide imaging

As time progresses, staining details can fade because of multiple factors as long periods of storage, direct exposure to light or, simply, the slide itself can be damaged for various reasons. Therefore, the quality of IHC and H&E staining intensity is not permanent. For this reason, digitalising histological tissue sections is not only a fancy terminology accompanying modern clinical practices; in fact, it has helped pathologists to keep their patients' highly valued FFPE specimens for a longer time, in a smaller space, with convenient and private sharing and editing options for patients' clinical data.

In the current study, all the IHC-stained tissue sections were scanned using an electron microscopy, which was available at the imaging unit, Cellular Pathology Department, Queen's Medical Centre (QMC), NUH. At the end, high-resolution digital images of the whole slides were generated with the Nanozoomer Digital Pathology scanner (NDP C9600series; Hamamatsu Photonics, Welwyn Garden City, Hertfordshire, UK). A 20-times magnification power was readily applied for all scanned slides. NDP.view2 software (Hamamatsu Photonics, Systems Division) was used to facilitate the storage of the TMA cores using a high-resolution screen (24 inches, 1920x1080 pixels).

### 2.5.4 H-score

Both credibility and reproducibility are required for the selection of the scoring methods used to interpret IHC staining results. Therefore and as a mandatory condition, the semi-quantitative scoring method in IHC (H-score) requires an expert and well-trained observer. H-score was utilised as the sole method to score the IHC-stained tissue sections in this study. This was generated by setting tissue staining intensities into four levels of scoring, negative=0, weak=1, moderate=2, and strong=3. This was achieved by examining various and randomly selected TMA cores from different slides. The sum of individual intensity multiplied by the total percentage of cells expressing it is the mathematical definition of an H-score. All the stained tissue sections in this study were scored twice, the main researcher and a qualified expert pathologist (Dr Islam Abdelaziz, Dr Dena Ahmad). The

selection and scoring of the TMA slides was done blindly and individually. Then, all the scoring results were concealed until the H-scoring for all slides were completed. Intra-class correlation among the scores was calculated and the threshold of H-score agreement was >75%. Any slide with a lower scoring concordance was re-scored and the discrepancies were discussed.

### 2.5.5 mRNA expression and human epithelial ovarian cancers:

In order to compare our results and confirm the findings, the predictive and prognostic significance of the surrogate biomarkers' studied in this thesis mRNA expression was investigated in publicly available ovarian tumour gene expression data sets *(http://kmplot.com/analysis/index.php?p=service&cancer=ovar)* (Győrffy et al., 2012). A total of 1075 Serous cystadenocarcinomas were included in this analysis.

## 2.6 Preclinical methodologies

### 2.6.1 Sub-culturing cell lines

A2780, human ovarian carcinoma, isolated from primary tumours of untreated patient (platinum sensitive) and A2780Cis, Cisplatin-resistant cell line developed by continuous exposure of A2780 to cisplatin doses. Both OC cancer cell lines (obtained from the American Type Culture Collection; ATCC, Manassas, USA) were grown using 1% penicillin/streptomycin + 10% Foetal Bovine Serum (FBS) RPMI (1640) at 37ºC with 5% CO2/95% air, (Table 2-2). Authentication of cell lines were done by ATCC company using Promega Powerplex® 17 Short Tandem Repeat (STR) system to ascertain there was no cross-contamination or misidentification of cell lines. Mycoplasma test was routinely performed almost every six months using MycoProbe Mycoplasma Detection Kit (R&D systems; Abingdon, UK). Cell lines were used up to 16 passages.

To trypsinise cells, each cell line was separately handled under sterile conditions with its own reagents. First, cell media was removed by pipetting, and then cells washed by PBS (without Mg2+ and Ca2+). Subsequently, 0.5mg/ml trypsin- EDTA (Sigma Aldrich, UK) was added to disrupt cell monolayers. Then, all cells were incubated for 3-5 minutes at 37ºC; medium was then, added to the flask 4:1 in order to deactivate trypsin. Centrifuge at 1000 rpm for 5 minutes for cells was the

next step. Followed by, cell pellet was re-suspended in fresh media, which was then mixed well and placed into a new flask.

**Table 2-2: ovarian cancer cell lines used for initial testing, including growth medium, tumour/cell type and source of purchase**

| Cell Line | Growth Media | Primary Tumour | Cell Type | Properties | Purchased |
|---|---|---|---|---|---|
| **A2780** | **RPMI** (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Carcinoma | Ovarian Cancer | Cisplatin Sensitive | Sigma Aldrich (Gillingha, UK) |
| **A2780cis** | RPMI (Thermofisher Scientific) - 10% FBS (F4135, Merck, UK), 1% Penicillin-Streptomycin (P4333, Merck, UK). | Carcinoma | Ovarian Cancer | Cisplatin Resistant | Sigma Aldrich (Gillingha, UK) |

### 2.6.2 Cell lysate preparation

Cell lysates were prepared for western blot. Cells were collected as previously described and counted. A minimum of $1 \times 10^6$ cells per sample were lysed by resuspension in 100 µl of lysis buffer, consisting of RIPA buffer (R0278-500ML, Sigma Aldrich, UK), 1% of Cocktail 2 (P5726-1ML , Sigma Aldrich; UK), 1% of Cocktail 3 (P0044-1ML, Sigma Aldrich; UK) and 1% of protease inhibitor (Sigma; UK). The cell lysate was stored at -20 °C. Before using the lysate for western blot, the lysates were repeatedly aspirated with a fine-gauge needle in order to break cell membranes. Lysates then, were centrifuged at 13000 rpm for 20 minutes at 4 °C to remove cell debris.

### 2.6.3 Protein quantification

The bovine serum albumin (BSA) protein quantification assay was used to calculate protein concentrations in cell lysates by generating protein strand curves, in this case for BSA. The BCA kit (A7906, Sigma Aldrich, UK) is a colorimetric detection kit using the reduction of Cu+2 to Cu+1 by protein in an alkaline medium (the biuret reaction) with high sensitivity. The colorimetric detection of the cuprous cation (Cu+1) using bicinchoninic acid is highly sensitive producing purple coloured product that can be strongly detected at 562 nm absorbance. The colour intensity of the product is nearly linear with increasing protein concentrations over a broad working range (20–2000 µg/mL). BSA was diluted in nuclease-free water to the following concentrations: 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 25 µg/ml and 0. Samples were diluted in nuclease free water in 1:5, and 8 µl of diluted protein samples were pipetted in triplicate in a 96-well plate. Then 200 µl of working solution (reagent A+ regent B) was added to each of the 96 well plate and the plates were then incubated at 37°C for 30 minutes. The 96-well plates were read by FLUOstar OPTIMA, UK/ Infinite® F50 (UK) microplate reader at 590 wavelengths. Adel Alblihy, PhD student in Prof. Madhusudan group performed all protein quantifications in this study.

### 2.6.4 Western blotting

In order to assess the binding specificity of each purchased antibody to its corresponding antigenic sequence, western blotting (WB) was performed. The

qualitative detection of a single protein band in OC cell lysates, was our tool to describe the binding specificity for each one of the utilised antibodies in this study in addition to the preclinical experiments. The surrogate biomarkers' specificity was confirmed before conducting the IHC optimisation step. The term 'blotting' denotes the transferring of the isolated protein from a separation gel medium onto a surface of non-reactive membrane. This procedure included six key steps:

#### 2.6.4.1 Separation of proteins

A ready-made, SDS 4X sample buffer, an anionic detergent that can break the hydrogen bond between molecules and help protein unfolding (B0008, Thermo Fisher, UK) was used. 10x Dithiothreitol (DTT) reducing agent (B0009, Thermofisher, UK) was added to the sample to aid the reduction of disulfide linkages between cysteine molecules. Cell lysates (20 µg) were added to 2.5 µL sample buffer and 1 µL reducing agents. Then, heating at 100°C for 5 minutes was performed for denaturation. Protein samples were loaded into Precast SDS 4-12% gels (NW04125BOX, Thermofisher; UK) at 125 V for 1 hour.

#### 2.6.4.2 Transfer proteins

Proteins were transferred from gels to a nitrocellulose membrane (Whatman, GE Healthcare). Gels were removed from their casing, sandwiched between filter paper and transfer sponges that had pre-soaked in a transfer buffer (20% (v/v) methanol, 50 mM Tris-HCl, and 380 mM glycine), and placed in a transfer model mini bolt Module (B1000, Thermofisher, UK). The transfer process was performed at 11 volts for 90 minutes.

#### 2.6.4.3 Blocking

This step was performed to reduce the non-specific protein binding via blocking unreacted sites on membranes. Blocking was done by soaking membranes 5% non-fat milk in phosphate buffered saline/0.05% tween20 (PBS-T) for 1 hour at room temperature.

#### 2.6.4.4 Incubation with the primary antibody

The primary antibodies, (Table 2-3) were diluted in 1% BSA. Membranes were incubated with the primary antibodies at room temperature for one hour or at 4°C overnight. The membranes were then washed with PBS-T (3 × 5 min) to remove

non-bound primary antibodies. The washed membranes were incubated with a housekeeper antibody (beta-actin or GAPDH) for 1 hour at room temperature to normalise the levels of proteins of interest by determining that protein loading was the same across the gel. After incubation with the housekeeper antibody, the washing procedure was repeated.

#### 2.6.4.5 Incubation with the secondary antibody

The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies to detect the primary antibodies. The secondary antibodies were used as follow: IRDye® 680RD Donkey anti-Mouse IgG (LI-COR, USA) and IRDye® 800CW Donkey anti-Rabbit IgG (LI-COR, USA). Each antibody was diluted 1:10,000 in 1% BSA. Membranes were incubated at room temperature for 1 hour, followed by washing with PBS-T as described above.

#### 2.6.4.6 Band detection and analysis

For protein detection, the LI-COR Odyssey Imaging System was utilised. When quantifying protein bands was needed for some experiments, the Image Studio Lite software (Ver 3.1) (Li-Cor, USA) was used. Once the detected surrogate protein band together with the housekeeper gene band intensities were quantified in LI-COR software. The readings for proteins of interest were normalised to the reading for housekeeper proteins. Western blot for all cell culture experiments was performed in three independent settings. Differences between samples was calculated using student t-test. Graphs and statistical analysis were performed using GraphPad Prism software version 7.
Full list of the antibodies used and their details can be found below, (Table 2-3).

**Table 2-3: Antibodies used in Western blot**

| Antibody | Supplier | Species | Molecular weight( KDa) | Concentration | Incubation period | Purpose |
|---|---|---|---|---|---|---|
| Anti-mTOR phospho (S2448) | Abcam | Rabbit | 289 | 1:1000 | Overnight/ 4°C | Specificity* |
| p-4EBP1 (Thr37/46) | Cell Signaling | Rabbit | 15-20 | 1:1000 | Overnight/ 4°C | specificity |
| p-P70S6K (Thr389) | Cell Signaling | Mouse | 70 | 1:1000 | Overnight/ 4°C | specificity |
| p-Akt (Ser473) | Cell Signaling | Mouse | 60 | 1:1000 | Overnight/ 4°C | specificity |
| Anti-PI3K p85 (ab86714) | Abcam | Mouse | 85 | 1:1000 | Overnight/ 4°C | specificity |
| PTEN (D4.3) | Cell Signaling | Rabbit | 54 | 1:1000 | Overnight/ 4°C | Specificity |
| Anti-ATM (ab32420) | Cell Signaling | Rabbit | 350, 370 | 1:1000 | 60 min/ Room temp | specificity |
| XRCC1 (33-2-5) | Invitrogen | Mouse | 85 | 1:500 | Overnight/ 4°C | Specificity |
| Anti-XIAP (ab21278) | Abcam | Rabbit | 55 | 1:1000 | Overnight/ 4°C | specificity |
| Anti-PARP (551025) | BD Pharmingen | Mouse | 116 | 1:1000 | 60 min/ Room temp | Specificity |
| Anti-DNA polymerase Beta (ab26343) | Abcam | Rabbit | 38 | 1:1000 | Overnight/ 4°C | Specificity |
| Anti-LIG1 (ab177946) | Abcam | Rabbit | 102 | 1:1000 | Overnight/ 4°C | Specificity/ Cell culture |
| Anti-LIG3 (HPA006723) | SIGMA | Rabbit | 48 | 1:100 | 60 min/ Room temp | Specificity/ Cell culture |
| Anti-LIG4 (HPA001334) | SIGMA | Rabbit | 104 | 1:500 | Overnight/ 4°C | specificity |

***Lysate from a mixture of two OC cell lines, A2780 and A2780cis cell lines were used to determine the antibody specificity.**

### 2.6.5 Transient knockdown (siRNA)

#### 2.6.5.1 Introduction

Silencing RNA is a popular method used to transiently knockdown specific genes. siRNA is a class 20-25 base pairs of double stranded RNA that operates within the RNA interface pathway (RNAi). siRNA interferes with the complementary nucleotides sequences and prevents translation by degrading mRNA after transcription. Hence, it interferes with specific genes and reversibly knocking it down.

#### 2.6.5.2 Procedure details

Cell lines and reagent were taken from the group of Prof. Madhusudan as collaborative project. XRCC1 (ID S14942) and LIG1 (ID S8173) siRNAs oligonucleotides were obtained from Thermofisher scientific, UK. Negative scrambled control constructs (catalogue no. 4390843) were purchased from Invitrogen, UK. Constructs were diluted in 50 µL nuclease-free water to make a stock solution of 100 µM, and then diluted 10 µM working solution in the experiment day. Lipofectamine 3000 transfection reagent (L3000015, Thermofisher; UK) was applied to deliver siRNA to the cells in Opti-MEM reduced serum medium (11058021, Thermofisher, UK). The proportions of Lipofectamine used were according to the manufacturer's recommendation. Cells were seeded at 60-80% confluency in T25 flasks and incubated overnight. 20.8 µl Lipofectamine was diluted in 0.75 ml of the Opti-MEM, and 9.4 µl of the working SiRNA solution or negative control was diluted in 0.75 ml of the Opti-MEM for 5 minutes. The diluted lipofectamine was then added to the diluted siRNA to make the transfection master mix, which was incubated for 15 minutes at room temperature. Cells were washed by the Opti-MEM before the transfection master mix was added. The transfected cells were incubated at 37°C overnight. The following day, the Opti-MEM was removed by pipetting and fresh cells-specific media was added. The transfection efficiency was checked at day 3 and 5 using western blot and a suitable window was then selected for each gene. All knocking down procedures were performed in three independent experiments. Differences between samples were calculated using the student t-test. This procedure was performed with the help of Professor Madhusudan's group PhD students (Dr Reem Ali and Dr Adel Alblihy).

### 2.6.6 Cell counting

To count cells, 10µl of the cell suspension was aliquoted onto a haemocytometer chamber. Then, suspended with 10µl of trypan blue. The mean number of all the counted cells within the 5x5 division grids were calculated. The following equation was then used to determine cell number:

*Number of cells/ml = dilution factor (10) x mean cell count x 10*

### 2.6.7 Nuclear and cytoplasmic extraction

#### 2.6.7.1 Introduction

Extracts prepared can be very useful in preclinical research as can be used directly for functional studies and as a starting material for purification of the proteins involved in these processes.

#### 2.6.7.2 Procedure details

Cells from both A2780 and A2780Cis cell lines were seeded in T25 flasks overnight. Cisplatin of 5µM was then added to the cells and incubated for 24 and 48 hours. Then, cells were collected by trypsinization, centrifuged at 1000 rpm for 5 minutes at room temperature. The cells were then transferred to 1.5ml Eppendorf tubes. Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit was utilised for separation of nuclear and cytoplasmic fractions and according to the manufacturer's instructions (78833, Thermo Fisher; UK). The cells were washed with cold PBS leaving the cell pellet as dry as possible, after the PBS was carefully discarded. The cells were then incubated with 200 µl of Cytoplasmic Extraction Reagent (CER) I and vigorously vortex the tubes for 15 seconds, then incubated on ice for 10 minutes. 11 µl of cold-CER II was added to the tubes and the cells were vortexed vigorously for 5 seconds and incubated on ice for 1 minute. After that, the cells were vortexed for another 5 seconds and centrifuged at maximum speed in a microcentrifuge for 5 minutes. The supernatants were immediately transferred to clean Eppendorf tubes and placed on ice. The nuclei in the cell pallet were incubated with 100 µl cold-Nuclear Extraction Reagent (NER), vortexed for 15 seconds and placed on ice. The cell pallet vortexed for 15 seconds every 10 minutes for a total of 40 minutes. The pellets were then centrifuged at a maximum speed in a microcentrifuge for 10 minutes. The nuclear extracts were immediately

transferred to clean tubes and placed on ice. Extracts were quantified using BCA protein quantification kit, and protein levels were checked by utilising western blot technique, previously discussed. Nuclear and cytoplasmic extracts were blotted against YY1 as a nuclear marker and GAPDH as a cytoplasmic abundant marker in order to verify the purity of the extractions. Proteins in the nuclear extractions were normalised to YY1 levels. Protein levels in cytoplasmic extracts were normalised to GAPDH. Professor Madhusudan's research fellow, Dr Katia Mosquita, kindly performed this procedure.

## 2.7 Statistics

### 2.7.1 Introduction

Several statistical parameters were accredited in this study. Nominal variables, including categorical and ordinal variables, continuous scale data, and independent or dependent variables exemplify this variety. Each statistical test requires specific conditions and variable type(s); therefore, multiple statistical analyses were conducted. All IHC statistical tests were performed with the Statistical Package for the Social Sciences (SPSS). However, Graph Pad Prism7 software was utilised to compare between two groups after WB quantification in all preclinical experiments.

### 2.7.2 Descriptive statistics

It was important to identify specific features of a particular dataset; hence, the description of the available data in this study was essential. For example, scale H-score data for a population, mean, median, and mode were investigated and histogram charts illustrating the normal distribution curve were created accordingly. For continuous variables, Cut-off points were decided according to their distribution status. In case of normally distributed data, the mean was used as the optimal cut-off value. However otherwise, median was designated as an optimal cut-off point. Other figures, such as percentage and total number of cases, were also employed in this study using descriptive analyses.

### 2.7.3 Chi-square test (goodness to fit)

The chi-square test was one of the non-parametric statistical tests that was used in this study to determine the statistical relationships between two groups of independent variables. A chi-square test could expect that the difference of rates distribution for a selected and categorised biomarker of interest against valid equivalent data that belongs to the other nominal variable(s), tumour grade, stage, or other stained biomarkers. Output data was presented in rows and columns with a threshold of significance (p-value) was set at $<0.05$.

### 2.7.4 Student's T test

The *t*-test was used to determine if the means of two sets of data are significantly different from each other. This test was mainly utilised in preclinical comparisons between two normally distributed values. The significant p-value was set at $<0.05$.

### 2.7.5 Pearson rank test (model/line of best fit)

In the current study, in order to determine the linear association between two continuous variables, Pearson correlation coefficient (r) was used. The value of r takes a range of values from +1 to -1. The 0 indicates that no association exists between the two investigated variables. The closest value to +1 indicates a strong positive co-expression, whereas the negative values suggest an inverse correlation. A p-value of $<0.05$ was considered as significant.

### 2.7.6 Univariate analysis for patients' outcome

Kaplan-Meier survival curve was drawn in order to determine the differences in the survival influence by the different tested biomarkers. It was performed by plotting survival curves. Log-rank test was used to find the significant associations. Patients were censored after 60 months of follow up.

### 2.7.7 Proportional hazards model (Cox regression model)

To identify the main and independent driving protein in the pathways investigated, cox regression test for multivariate analysis was applied. This can help test for confounders and prognostic / predictive independency of the investigated

biomarkers. For this purpose, standard well-known prognostic / predictive factors were used as platinum sensitivity.

## 2.8 REMARK criteria

This retrospective study adheres to REMARK criteria (McShane et al., 2006).

## 2.9 Ethical approval

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Molecular and genetics classification of Ovarian Cancer" under the ethical approval number (ACP0000297) and in compliance with the current ethical and legal guidelines of the United Kingdom. All basic and operational ethical principles for this study were conducted in accordance with the 1975 Helsinki Declaration, and its later amendments or comparable ethical standards. The identification and health-sensitive information for all patients in this study were kept anonymous and stored safely.

# 3 mTOR Signalling Pathway Activation is Associated with Adverse Outcome in Ovarian Cancer

## 3.1 Introduction

Recently, the research in ovarian cancer (OC) improved our understanding of the molecular processes that occur in cancerous cells, including the receptors and molecular mechanisms involved in carcinogenesis and tumour progression (Marchetti et al., 2015, Leone Roberti Maggiore et al., 2013, Bellati et al., 2013, Marchetti et al., 2012, Bellati et al., 2011, Ruscito et al., 2016, Gasparri et al., 2016, Gasparri et al., 2017). However and in spite of this improvement, unfortunately, OC remains the deadliest gynaecological cancer and a leading cause of cancer mortality (Gasparri et al., 2017, UK, 2019b, Siegel et al., 2019). More than half of the patients with OC are diagnosed at an advanced stage. This is mainly because of its late presentation and aggressive behaviour. In addition, OC has a high relapse rate with being platinum-resistant, which if this is the case, is extremely difficult to treat and often lead to death in a short interval of time (Miller et al., 2009).

One of the most investigated intracellular signalling pathways in cancer is the Akt/mTOR signalling pathway. This pathway is vital in the regulation of cell growth, motility, survival, proliferation, protein synthesis, autophagy, transcription as well as angiogenesis (Hay and Sonenberg, 2004). PI3K/AKT/mTOR is frequently altered in ovarian tumours and it was shown to be activated in about 70% of OC patients (Huang et al., 2011, Mabuchi et al., 2015). As a result, this signalling pathway can influence tumour cellular growth, proliferation and survival (Li et al., 2014). Higher invasive and migratory capacities of the tumour is associated with its activation and it is considered as a potential predictor of OC cells invasiveness (Bai et al., 2015, Tanwar et al., 2011). In mice models, inhibition of PI3K/Akt/mTOR resulted in delaying tumour growth and improved survival, highlighting the important role of this pathway as a new therapeutic strategy (Cheaib et al., 2015).

In response to various extracellular signals, activated PI3K phosphorylates and activates AKT. This in turn can phosphorylate mTOR at Ser 2448 position. In addition, indirectly AKT can activate mTOR by phosphorylating tuberous sclerosis complex 2. Once mTOR is activated, it phosphorylates both S6K-1 and 4EBP-1. Activated S6K-1 leads to the translation of mRNA and enhance the

transition from the G1 to S phase of the cell cycle. Similarly, the phosphorylation of 4EBP-1 also enhances mRNA translation leading to cell cycle progression or angiogenesis (Laplante and Sabatini, 2012, Mabuchi et al., 2015), (Figure 3-1) (Li et al., 2014).

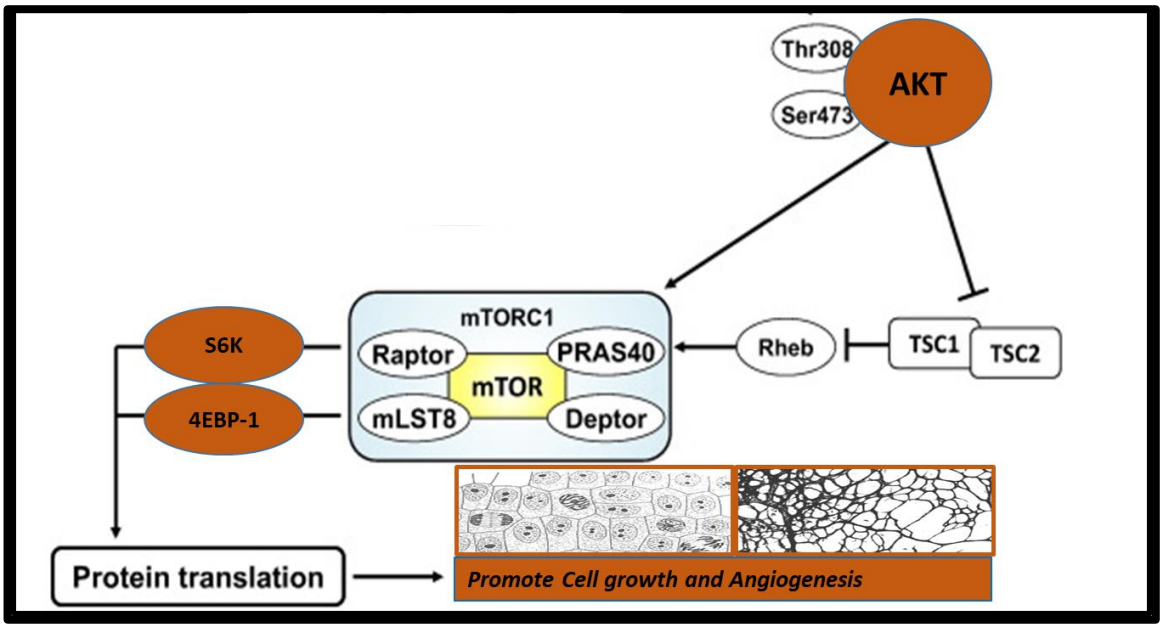



**Figure 3-1: Diagram representation the PI3K/AKT/mTOR signalling pathway showing mTOR its upstream effectors, downstream regulators, adapted from Li et al (Li et al., 2014)**

Until date, the exact mechanisms of invasiveness and metastases of OC are yet to be determined. Considering the primary tumour phenotypes, the intratumoural heterogeneity, and the contribution of the stromal components are examples of how predicting the outcome is a challenging task. Investigation of the components of the mTOR pathway may lead to a better understanding of disease biology, and help prognostic stratification for treatment decisions. Recent studies suggest that the downstream effectors of mTOR, 4EBP1 and P70S6K can influence disease prognosis in breast, cervical, biliary tract, and head-and-neck tumours (Rojo *et al.*, 2007, Herberger *et al.*, 2007, Molinolo *et al.*, 2007). However, to date, only few reports evaluated the expression patterns of the p-mTOR and its upstream regulator, p-AKT and the downstream effectors in patients with primary OC (Altomare *et al.*, 2004, Castellvi *et al.*, 2006).

### 3.1.1 Hypothesis

mTOR signalling pathway has a vital role in OC. Moreover, its association with both the upstream regulator protein, AKT and the downstream effectors, 4EBP1 and P70S6K is evident. It is hypothesised that p-mTOR and the other members of this pathway (upstream and downstream) have associations with adverse clinical outcome in primary ovarian tumour patients.

### 3.1.2 Aims

1. Determine the expression pattern of p-AKT, p-mTOR, p-4EBP1 and p-P70S6K in a well-characterised series of primary OC cases treated primarily by surgical resection. In addition, to correlate their expression with the patients' clinicopathological variables.

2. Determine the effect of these surrogate biomarkers on prognosis not only in the whole patients' cohort but also in the platinum sensitive and resistant patients.

3. Perform multivariate analysis to identify the independent prognostic proteins that have the highest influence on survival. Then, further exploring their isolated effect on influencing the patients' outcome

## 3.2 Methods

### 3.2.1 Study cohort

Investigation of the expression of p-AKT, p-mTOR, p-4EBP1 and p-P70S6K in primary ovarian epithelial cancer was carried out during the first year of this study. It was performed on tissue microarrays of 195 consecutive epithelial OC cases treated at Nottingham University Hospitals (NUH) between 2000 and 2007, already constructed and available for research purposes. Patients were comprehensively staged as per the FIGO staging system. All patients were treated primarily by surgery followed by platinum-based chemotherapy as per tumour stage. Overall survival (OS) was calculated from the operation date until the 1st of October 2012 when any remaining survivors were censored. Progression free survival (PFS) was calculated from the date of the initial surgery to the date of disease progression. Patient demographics are summarised in the supplementary section.

### 3.2.2 Western Blot (WB)

Full details about the WB procedure including the concentrations of antibodies used and the purchased materials can be found in the methods section of this thesis, (Table 2-3).

### 3.2.3 Immunohistochemistry (IHC)

All details regarding the IHC staining including tissue handling and processing of FFPE blocks and TMA sectioning are detailed in the methodology chapter of this thesis. Briefly, four-micron sections of the tissue array block were cut and placed on Surgipath X-tra Adhesive microscope slides for IHC staining. Staining for p-AKT, p-mTOR, p-4EBP1 and p-P70S6K was performed as described previously. Pre-treatment of TMA sections was performed with citrate buffer (pH 6.0, 20 min, microwave). TMA sections were incubated at room temperature with each antibody according to optimal conditions decided after an earlier optimisation procedure performed by the researcher, detailed in the appendix section, supplementary table. Antibody concentration of 1:150 was used for all the tested antibodies with 18 hours incubation period at room temperature except p-AKT,

one hour was the optimal incubation time. Negative controls with no added primary antibody were included in each run, appendix section supplementary figures.

### 3.2.4 Evaluation of immune staining and statistical analysis

The evaluation of IHC stained slides was performed as described previously in the methodology section. The H score was used to score all the tested markers. The statistical analysis methods was described in details in chapter two of this study.

## 3.3 Results

### 3.3.1 Specificity of the antibodies

Before attempting the IHC experiments, the specificity of the investigated antibodies, p-AKT, p-4EBP1, p-mTOR, and p-P70S6K were evaluated using WB methodologies. All images for the p-4EBP1, p-mTOR, and p-P70S6K supported a specific binding affinity to a single protein band for A2780 OC cell lysate around their predicted molecular masses, 15-20 kDa for the p-4EBP1 protein, 289 kDa for the p-mTOR protein, and 70 kDa for the p-P70S6K protein, (Figure 3-2). The specificity of each protein was tested on separate occasion. Similarly, WB analysis for p-AKT showed a specific single band at the corresponding molecular weight, 55-60 KDa in A2780 and A2780Cis OC cell lines, (Figure 3-3).

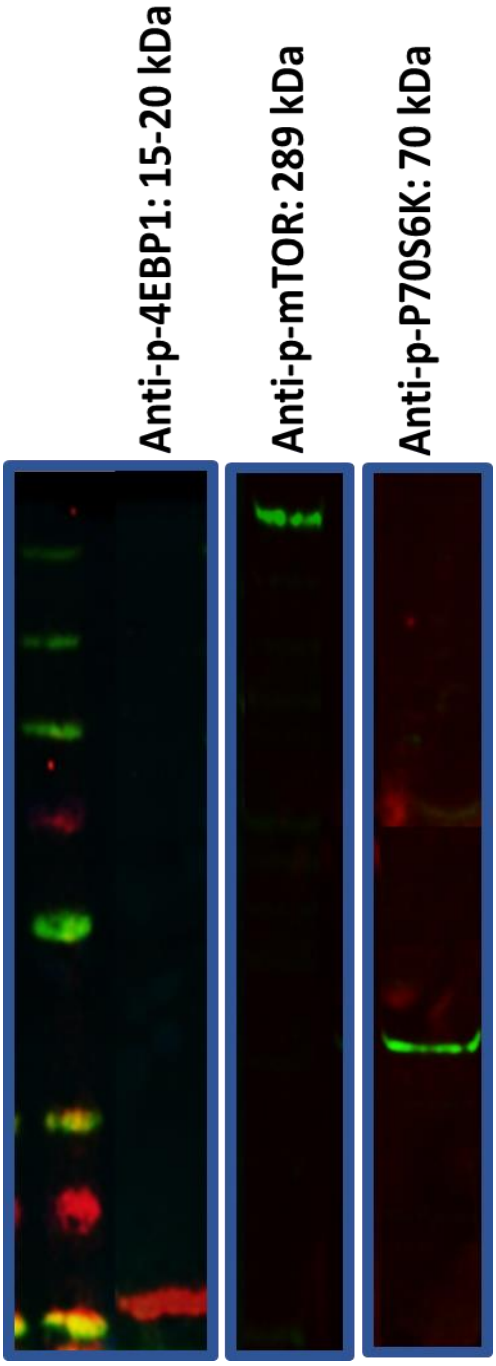



**Figure 3-2: Binding specificity of the anti p-4EBP1, p-mTOR and p-P70S6K antibodies. WB membrane showed specific antigen binding around ~15-20 KDa for p-4EBP1, ~289 KDa for p-mTOR and ~70 KDa for p-P70S6K in A2780 OC cell line lysate.**

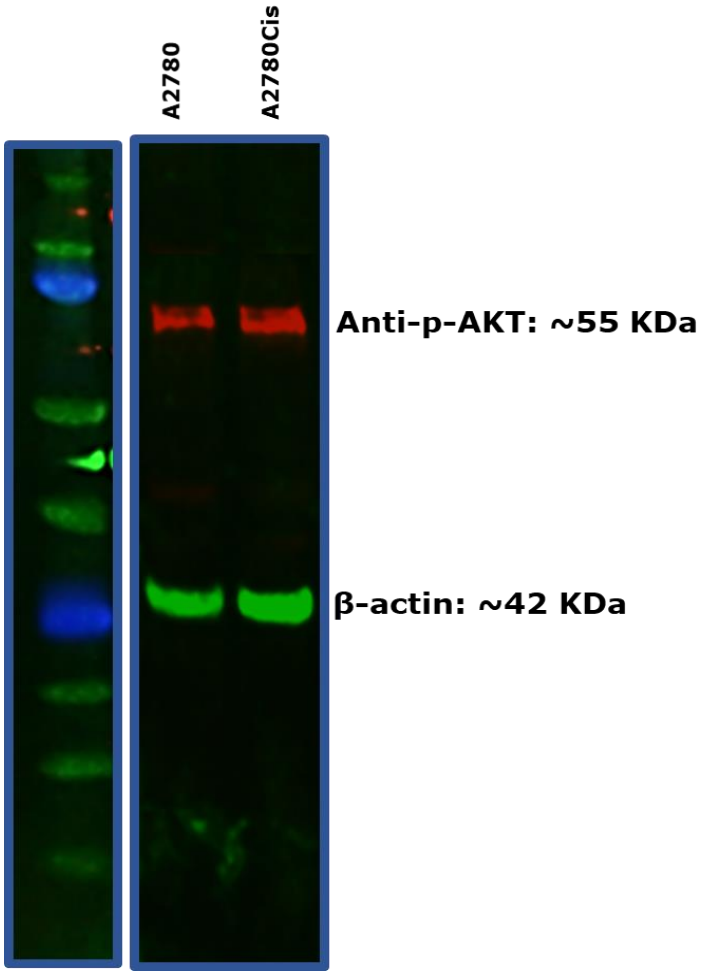



**Figure 3-3: Binding specificity of the anti-p-AKT antibody. WB membrane showed specific antigen binding around ~55 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines**

### 3.3.2 Immunohistochemical results

IHC methodologies have been applied in this study in order to assess the altered protein expression of p-AKT, p-mTOR, p-4EBP1 and p-P70S6K in OC patients and explore their relation to the patients' clinicopathological data and prognosis. Furthermore, identify their interactions and their clinical impact on OC progression. The examination of the immuno-stained TMA sections revealed a unique localised distribution for the proteins mentioned above among the different cellular compartments.

### 3.3.3 The expression pattern of the tested antibodies

#### 3.3.3.1 p-AKT

The expression of this protein was homogenously cytoplasmic in OC cells, (Figure 3-4). The optimum cut-off point which dichotomised the cohort was set using the median at H-score of >120. Out of the total cases, 56.25% showed high expression, while 43.75% had negative/low expression.

#### 3.3.3.2 p-mTOR

p-mTOR expression was mainly located in the cytoplasm of the malignant cells, (Figure 3-5). Importantly, the value of ≤150 (H-score) was set to be the optimal cut-off using the median value. Out of the total valid cases, 56.2% were rendered negative/low, while 43.1% deemed high in their expression.

#### 3.3.3.3 p-4EBP1

The expression of p-4EBP1 was exclusively cytoplasmic in malignant tumour cells, (Figure 3-6). Of worth, the cut-off point was chosen by using the median value and was set at ≤140. Importantly, out of the total valid cases, 53.2% were negative/low and 46.7% had high expression of this protein.

#### 3.3.3.4 p-P70S6K

This biomarker revealed mainly nuclear expression in cancerous cells, (Figure 3-7). In addition, cytoplasmic staining was also noticed but its median of H- score was not representative of a specific distribution that can split the cases into groups, so this staining was not considered. The nuclear expression cut-off was

chosen at ≤180 (H-score) using the median value. 58% of the cases were regarded negative/low and 42% had high expression.

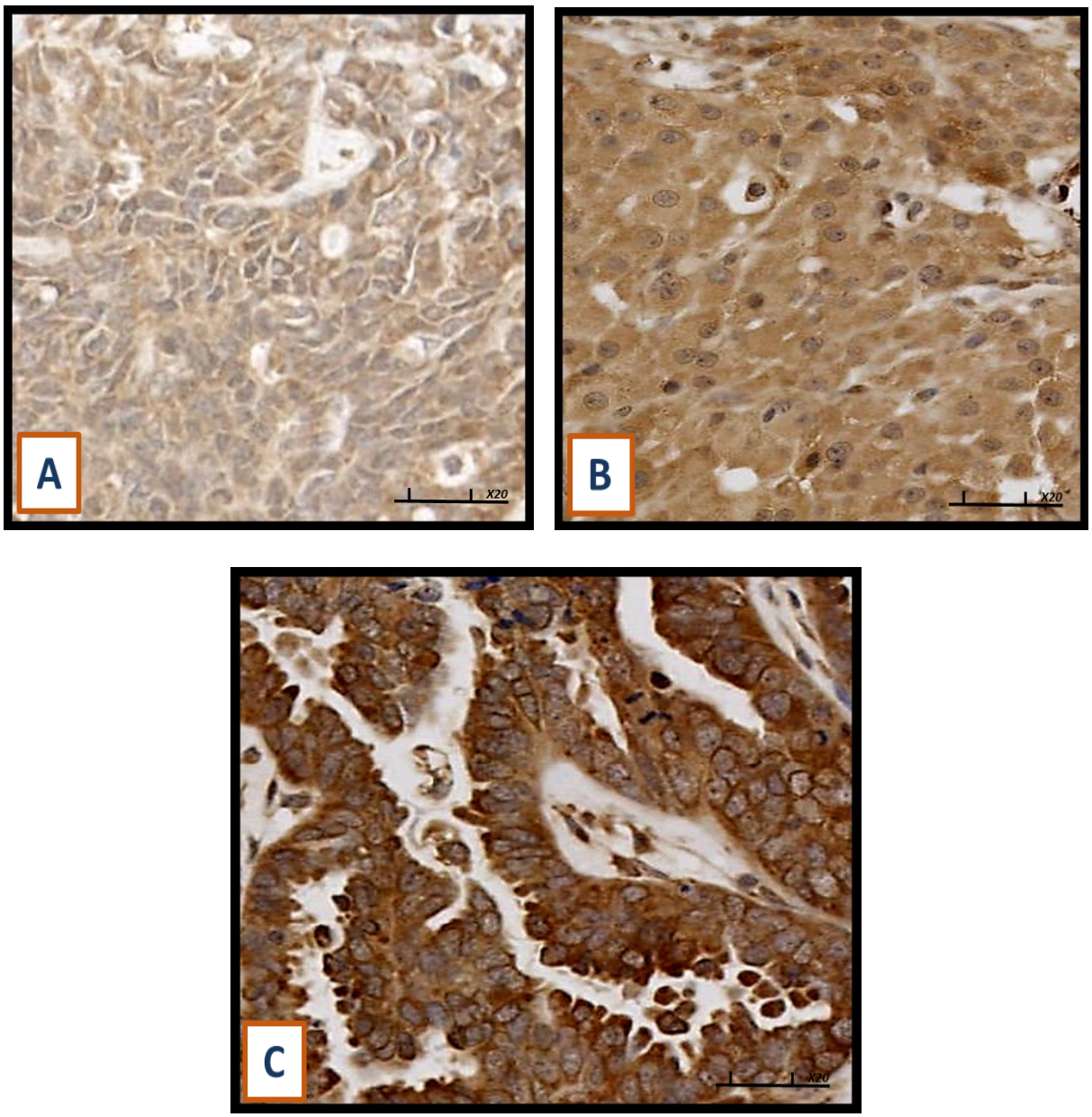



**Figure 3-4: Immunohistochemistry expression of p-AKT in tissue microarray images. Cytoplasmic expression of AKT in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

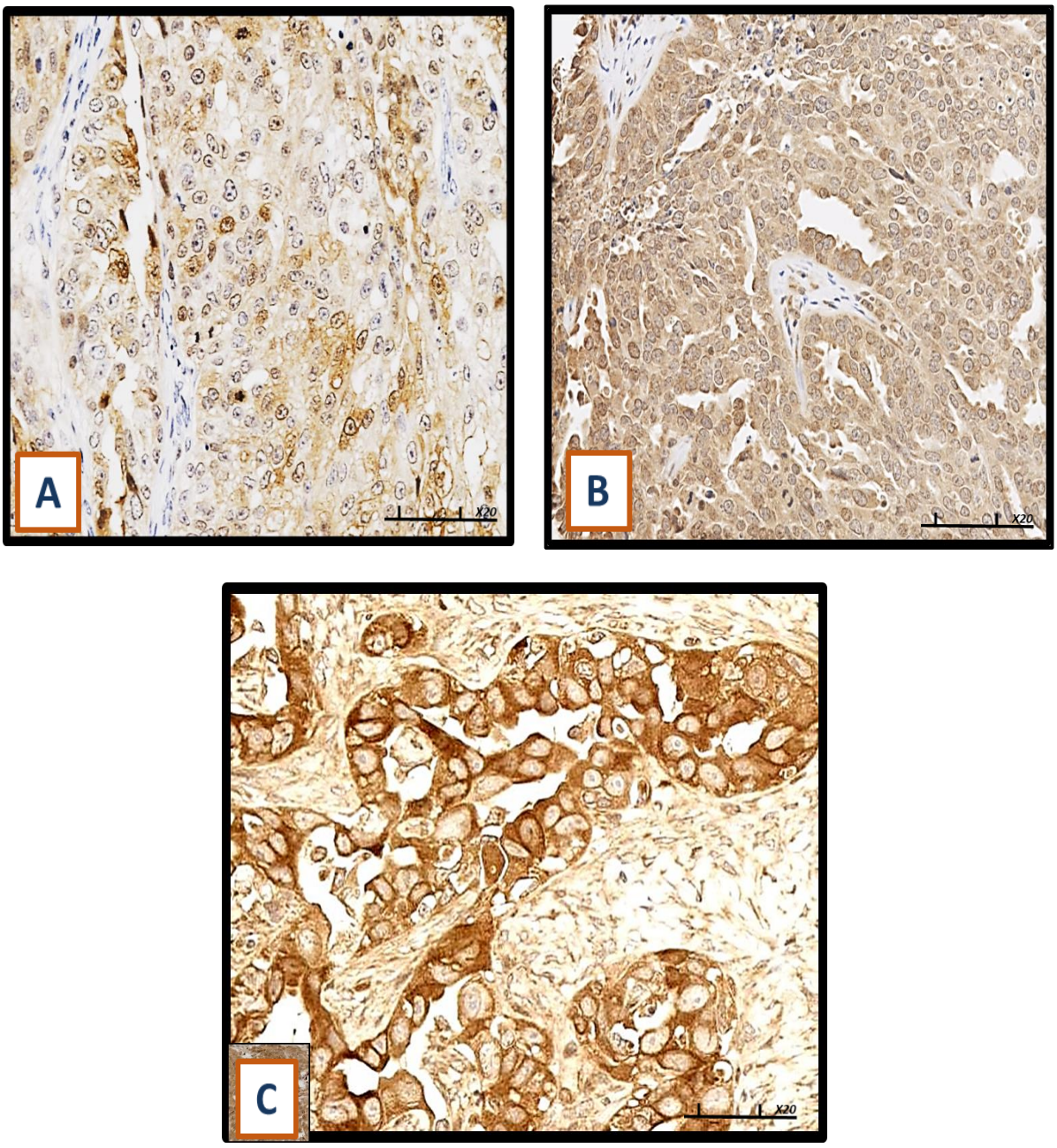



**Figure 3-5: Immunohistochemistry expression of p-mTOR in tissue microarray images. Cytoplasmic expression of p-mTOR in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

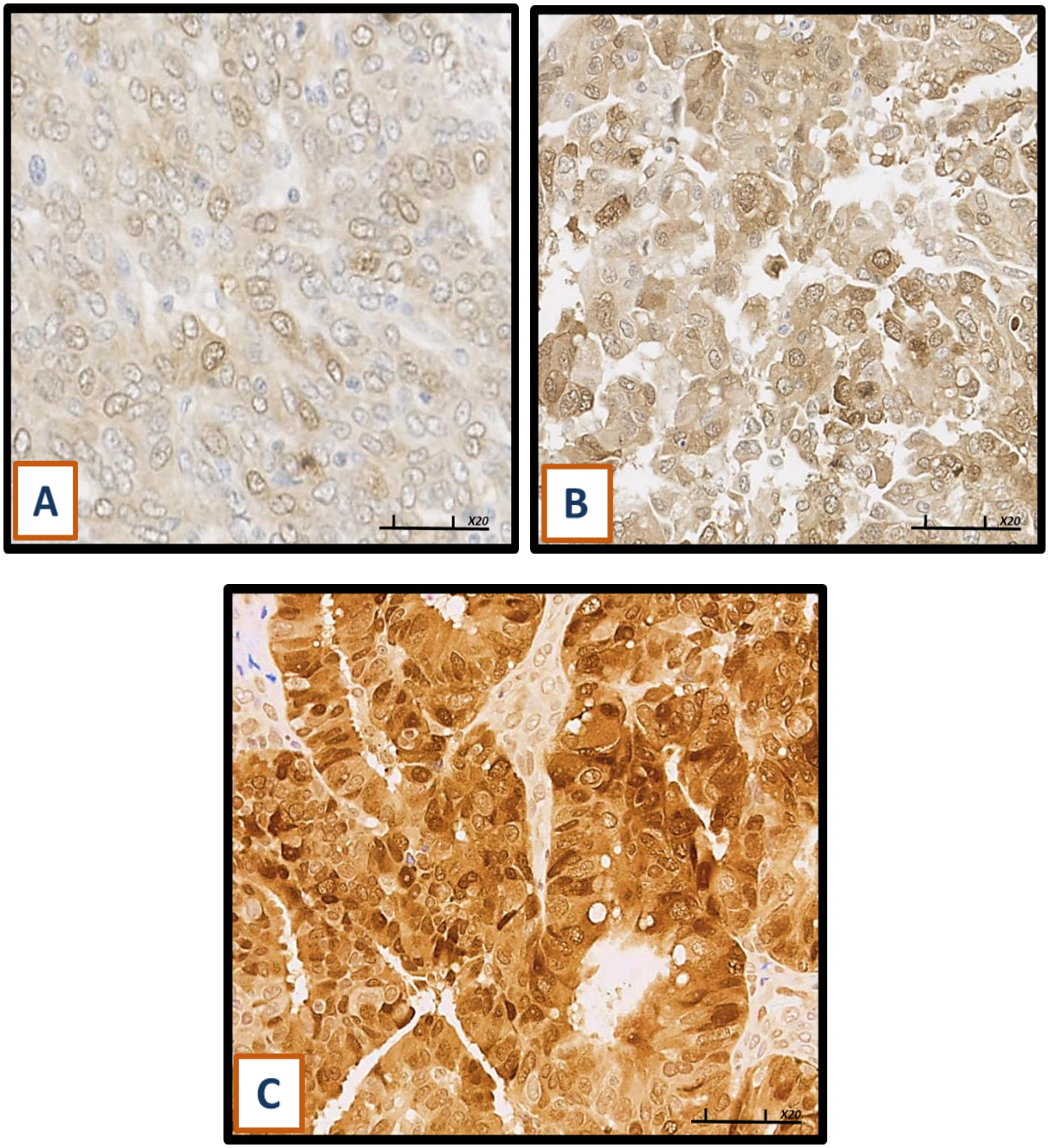



**Figure 3-6: Immunohistochemistry expression of p-4EBP1 in tissue microarray images. Cytoplasmic expression of p-4EBP1 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

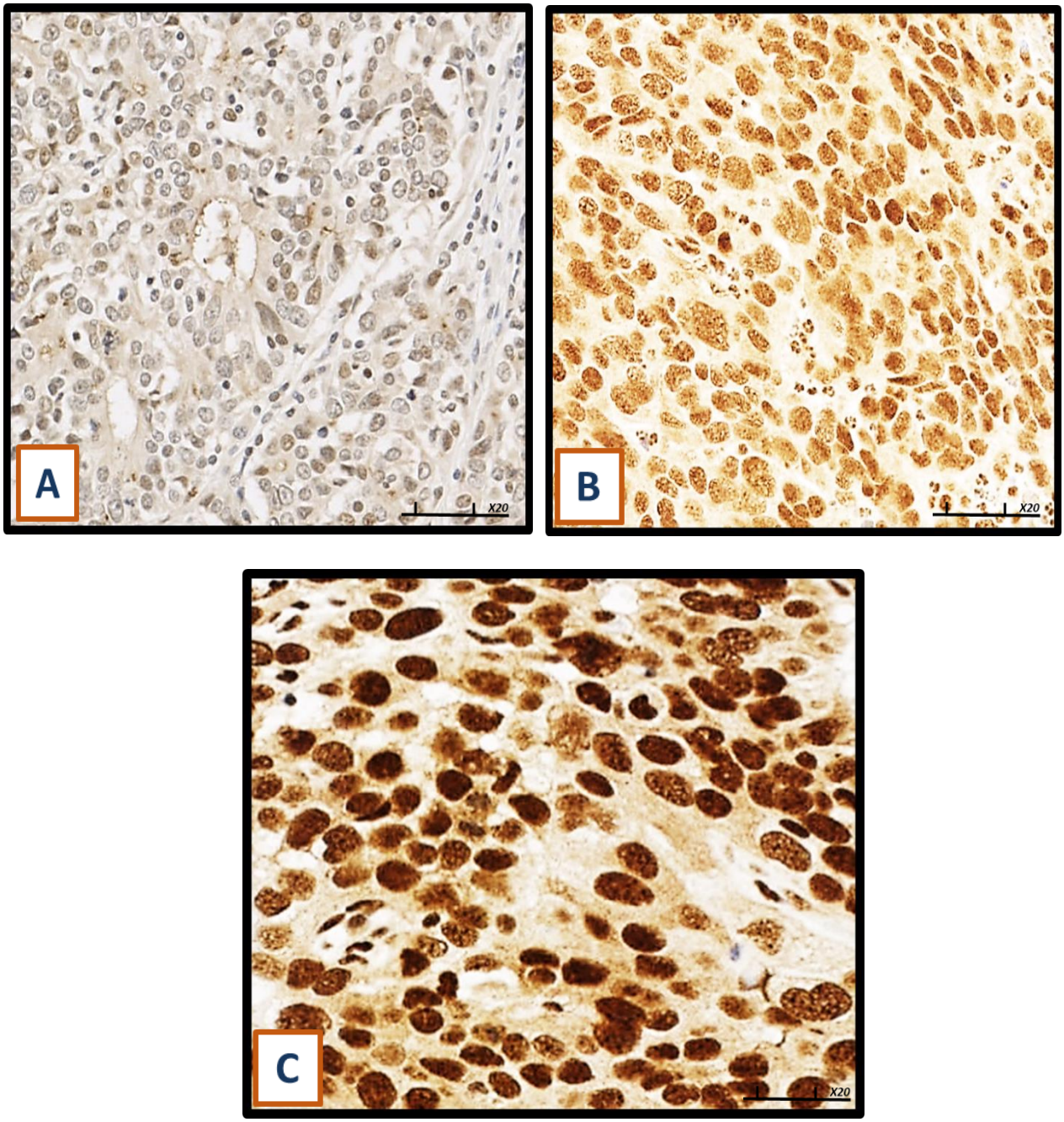



**Figure 3-7: Immunohistochemistry expression of p-P70S6K in tissue microarray images. Cytoplasmic expression of p- P70S6K in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

### 3.3.4 Associations of tested biomarkers with clinicopathological variables

#### 3.3.4.1 p-AKT

High cytoplasmic expression p-AKT was highly positively associated with measurable disease before the initiation of chemotherapy, (p-value =0.001). Moreover, high levels of this protein was inversely associated with the sensitivity to platinum therapeutic agents, (p-value =0.040). However, no significant association deemed significant with the other measured clinicopathological characteristics, (Table 3-1).

Interestingly, analysing the combined expression of p-AKT with the other tested related biomarkers, p-mTOR, p-4EBP1 and p-P70S6K and correlating them with the clinicopathological data showed positive association with measurable disease before chemotherapy, (p-value =0.001, p-value =0.004 and p-value =0.001 respectively) (Tables 3-2, 3-3, 3-4). In addition, serous carcinomas subtype was significantly more common in highly expressed p-AKT and p-P70S6K tumours, (p-value =0.001) (Table 3-4) and in borderline non-significance with p-4EBP1, (p-value =0.051), (Table 3-3). On the other hand, tumours with negative expression of both p-AKT and p-4EBP1 had a significant better response to platinum based chemotherapy, (p-value =0.046) (Table 3-3).

#### 3.3.4.2 p-mTOR

Apart from a positive association between high p-mTOR expression and measurable disease before chemotherapy, (p-value =0.002), high p-mTOR did not reveal other associations with the clinicopathological variables if compared with the low protein expressers, (Table 3-5). However, analysing the combined expressions of p-mTOR with p-4EBP1 and p-P70S6K proteins and correlating them with the clinicopathological data showed that serous carcinomas subtype was significantly more common in p-mTOR and p-4EBP1 positively expressed tumours, (p-value =0.016) (Table 3-6). On the other hand, tumours with negative expression of both p-mTOR with p-4EBP1 and p-P70S6K had less risk of measurable disease, (p-value =0.043) and (p-value <0.0001) respectively, (Table 3-6 and 3-7). Similarly, patients with negative expression of both p-mTOR and p-

P70S6K had a significant better response to platinum based chemotherapy, (p-value =0.030) (Table 3-7).

#### 3.3.4.3 p-4EBP1

High expression of p-4EBP1 was highly associated with the serous tumour histological subtype, (p-value =0.005), and positively linked to increase cancer deposits post-surgery, (p-value =0.035) (Table 3-8).

#### 3.3.4.4 p-P70S6K

p-P70S6K low expression was associated mainly with both Mucinous and Endometrioid tumour histology subtypes in addition its correlation with lower stage disease, (p-value =0.015) and (p-value =0.012) respectively. Moreover, in line correlation was noted between low levels of this antibody and less residual disease after surgery, (p-value =0.019) and improved response to platinum therapy, (p-value =0.0001) (Table 3-9).

**Table 3-1: Clinicopathological significance of p-AKT protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| p-AKT Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **p-AKT Negative No. (%)** | **p-AKT Positive No. No. (%)** | ***P- value* ( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 30 (36.1) | 53 (63.9) | |
| Mucinous | 8 (66.7) | 4 (33.3) | 0.067 |
| Endometrioid | 11 (40.7) | 16 (59.3) | (8.761) |
| Clear cell carcinoma | 10 (58.8) | 7 (41.2) | |
| Mixed | 2 (100.0) | 0 (0) | |
| ***FIGO Stage*** | | | |
| I | 23 (51.1) | 22 (48.9) | 0.643 |
| II | 10 (45.5) | 12 (54.5) | (1.672) |
| III | 25 (40.3) | 37 (59.7) | |
| IV | 5 (35.7) | 9 (64.3) | |
| ***Grade*** | | | |
| G1 | 9 (50.0) | 9 (50.0) | 0.812 |
| G2 | 5 (45.5) | 6 (54.5) | (0.417) |
| G3 | 45 (42.1) | 62 (57.9) | |
| ***Measurable Disease*** | | | |
| Non | 46 (56.8) | 35 (43.2) | ***0.001*** |
| Measurable | 16 (28.6) | 40 (71.4) | **(10.642)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 46 (51.1) | 44 (48.9) | ***0.040*** |
| Resistant | 15 (32.6) | 31 (67.4) | **(4.213)** |

**Table 3-2: Clinicopathological significance of the combined p-AKT and p-mTOR proteins expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| Combined p-AKT/p-mTOR Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **AKT-/mTOR- No. (%)** | **AKT-/mTOR+ No. (%)** | **AKT+/mTOR- No. (%)** | **AKT+/mTOR+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | 0.202 (15.760) |
| Serous | 19(27.1) | 5 (7.1) | 16 (22.9) | 30 (42.9) | |
| Mucinous | 4 (40.0) | 2 (20.0) | 1 (10.0) | 3 (30.0) | |
| Endometrioid | 8 (34.8) | 2 (8.7) | 7 (30.4) | 6 (26.1) | |
| Clear cell carcinoma | 10 (66.7) | 0 (0.0) | 1 (6.7) | 4 (26.7) | |
| Mixed | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | | | 0.771 (5.688) |
| I | 18 (47.4) | 2 (5.3) | 9 (23.7) | 9 (23.7) | |
| II | 7 (36.8) | 2 (10.5) | 3 (15.8) | 7 (36.8) | |
| III | 16 (29.1) | 4 (7.3) | 11 (20.0) | 24 (43.6) | |
| IV | 3 (33.3) | 1 (11.1) | 2 (22.2) | 3 (33.3) | |
| ***Grade*** | | | | | 0.609 (4.501) |
| G1 | 5 (35.7) | 2 (14.3) | 3 (21.4) | 4 (28.6) | |
| G2 | 2 (22.2) | 1 (11.1) | 4 (44.4) | 2 (22.2) | |
| G3 | 33 (36.3) | 6 (6.6) | 18 (19.8) | 34 (37.4) | |
| ***Measurable Disease*** | | | | | ***0.001*** **(16.206)** |
| Non | 35 (51.5) | 6 (8.8) | 12 (17.6) | 15 (22.1) | |
| Measurable | 9 (18.4) | 3 (6.1) | 12 (24.5) | 25 (51.0) | |
| ***Platinum sensitivity*** | | | | | 0.311 (3.576) |
| Sensitive | 32 (41.0) | 7 (9.0) | 14 (17.9) | 25 (32.1) | |
| Resistant | 10 (26.3) | 2 (5.3) | 10 (26.3) | 16 (42.1) | |

**Table 3-3: Clinicopathological significance of the combined p-AKT and p-4EBP1 expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| Combined p-AKT/p-4EBP1 Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **AKT-/4EBP1- No. (%)** | **AKT-/4EBP1+ No. (%)** | **AKT+/4EBP1- No. (%)** | **AKT+/4EBP1+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 16 (22.2) | 8 (11.1) | 18 (25.0) | 30 (41.7) | |
| Mucinous | 4 (50.0) | 0 (0.0) | 4 (50.0) | 0 (0.0) | |
| Endometrioid | 3 (13.0) | 5 (21.7) | 6 (26.1) | 9 (39.1) | 0.051 (16.885) |
| Clear cell carcinoma | 7 (50.0) | 2 (14.3) | 3 (21.4) | 2 (14.3) | |
| Mixed | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | | | |
| I | 13 (37.1) | 4 (11.4) | 9 (25.7) | 9 (25.7) | 0.630 (7.069) |
| II | 4 (23.5) | 2 (11.8) | 7 (41.2) | 4 (23.5) | |
| III | 12 (21.4) | 8 (14.3) | 12 (21.4) | 24 (42.9) | |
| IV | 2 (20.0) | 1 (10.0) | 3 (30.0) | 4 (40.0) | |
| ***Grade*** | | | | | |
| G1 | 2 (16.7) | 2 (16.7) | 6 (50.0) | 2 (16.7) | 0.296 (7.280) |
| G2 | 1 (16.7) | 0 (0.0) | 3 (50.0) | 2 (33.3) | |
| G3 | 25 (26.3) | 13 (13.7) | 21 (22.1) | 36 (37.9) | |
| ***Measurable Disease*** | | | | | |
| Non | 23 (33.8) | 12 (17.6) | 18 (26.5) | 15 (22.1) | ***0.004*** **(13.305)** |
| Measurable | 8 (16.7) | 3 (6.3) | 12 (25.0) | 25 (52.1) | |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 26 (35.6) | 10 (13.7) | 16 (21.9) | 21 (28.8) | ***0.046*** **(8.017)** |
| Resistant | 5 (12.8) | 4 (10.3) | 12 (30.8) | 18 (46.2) | |

**Table 3-4: Clinicopathological significance of the combined p-AKT and p-P70S6K expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| Combined p-AKT/p-P70S6K Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **AKT-/ P70S6K- No. (%)** | **AKT-/ P70S6K+ No. (%)** | **AKT+/ P70S6K- No. (%)** | **AKT+/ P70S6K+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 19 (26.4) | 5 (6.9) | 19 (26.4) | 29 (40.3) | |
| Mucinous | 3 (42.9) | 0 (0.0) | 4 (57.1) | 0 (0.0) | |
| Endometrioid | 8 (33.3) | 2 (8.3) | 9 (37.5) | 5 (20.8) | ***0.001*** |
| Clear cell carcinoma | 8 (47.1) | 2 (11.8) | 1 (5.9) | 6 (35.3) | **(34.307)** |
| Mixed | 0 (0.0) | 2 (100.0) | 0 (0.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | | | |
| I | 13 (33.3) | 6 (15.4) | 11 (28.2) | 9 (23.1) | 0.540 |
| II | 8 (42.1) | 0 (0.0) | 4 (21.1) | 7 (36.8) | (7.945) |
| III | 16 (28.6) | 5 (8.9) | 16 (28.6) | 19 (33.9) | |
| IV | 3 (30.0) | 0 (0.0) | 2 (20.0) | 5 (50.0) | |
| ***Grade*** | | | | | |
| G1 | 5 (41.7) | 0 (0.0) | 2 (16.7) | 5 (41.7) | 0.361 |
| G2 | 2 (22.2) | 1 (11.1) | 5 (55.6) | 1 (11.1) | (6.588) |
| G3 | 30 (31.3) | 9 (9.4) | 25 (26) | 32 (33.3) | |
| ***Measurable Disease*** | | | | | |
| Non | 29 (42.6) | 9 (13.2) | 17 (25.0) | 13 (19.1) | ***0.001*** |
| Measurable | 11 (22.0) | 1 (2.0) | 13 (26.0) | 25 (50.0) | **(16.460)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 29 (36.3) | 9 (11.3) | 18 (22.5) | 24 (30.0) | 0.246 |
| Resistant | 9 (23.1) | 2 (5.1) | 12 (30.8) | 16 (41.0) | (4.147) |

**Table 3-5: Clinicopathological significance of p-mTOR protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| p-mTOR Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **p-mTOR Negative No. (%)** | **p-mTOR Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 37 (49.3) | 38 (50.7) | |
| Mucinous | 5 (45.5) | 6 (54.5) | |
| Endometrioid | 17 (68.0) | 8 (32.0) | 0.205 |
| Clear cell carcinoma | 11 (73.3) | 4 (26.7) | (5.922) |
| Mixed | 37 (49.3) | 38 (50.7) | |
| ***FIGO Stage*** | | | |
| I | 28 (70.0) | 12 (30.0) | 0.216 |
| II | 11 (55.0) | 9 (45.0) | (4.454) |
| III | 29 (49.2) | 30 (50.8) | |
| IV | 5 (50.0) | 5 (50.0) | |
| ***Grade*** | | | |
| G1 | 5 (45.5) | 6 (54.5) | 0.752 |
| G2 | 11 (57.9) | 8 (42.1) | (0.571) |
| G3 | 52 (57.1) | 39 (42.9) | |
| ***Measurable Disease*** | | | |
| Non | 50 (69.4) | 22 (30.6) | ***0.002*** |
| Measurable | 22 (41.5) | 31 (58.5) | **(9.755)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 50 (58.1) | 36 (41.9) | 0.568 |
| Resistant | 20 (52.6) | 18 (47.4) | (0.325) |

**Table 3-6: Clinicopathological significance of the combined p-mTOR and p-4EBP1 expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| Combined p-mTOR/p-4EBP1 Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **mTOR-/ 4EBP1- No. (%)** | **mTOR-/ 4EBP1+ No. (%)** | **mTOR+/ 4EBP1- No. (%)** | **mTOR+/ 4EBP1+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 20 (30.3) | 12 (18.2) | 10 (15.2) | 24 (36.4) | |
| Mucinous | 3 (42.9) | 0 (0.0) | 4 (57.1) | 0 (0.0) | |
| Endometrioid | 5 (25.0) | 7 (35.0) | 2 (10.0) | 6 (30.0) | ***0.016*** **(20.372)** |
| Clear cell carcinoma | 8 (61.5) | 2 (15.4) | 2 (15.4) | 1 (7.7) | |
| Mixed | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | | | |
| I | 15 (48.4) | 6 (19.4) | 6 (19.4) | 4 (12.9) | 0.168 (12.876) |
| II | 7 (46.7) | 0 (0.0) | 2 (13.3) | 6 (40.0) | |
| III | 12 (22.6) | 13 (24.5) | 9 (17.0) | 19 (35.8) | |
| IV | 3 (37.5) | 2 (25.0) | 1 (12.5) | 2 (25.0) | |
| ***Grade*** | | | | | |
| G1 | 2 (33.3) | 0 (0.0) | 2 (33.3) | 2 (33.3) | 0.524 (5.152) |
| G2 | 3 (23.1) | 3 (23.1) | 4 (30.8) | 3 (23.1) | |
| G3 | 29 (34.9) | 18 (21.7) | 11 (13.3) | 25 (30.1) | |
| ***Measurable Disease*** | | | | | |
| Non | 26 (44.1) | 12 (20.3) | 10 (16.9) | 11 (18.6) | ***0.043*** **(7.681)** |
| Measurable | 11 (23.9) | 9 (19.6) | 7 (15.2) | 19 (41.3) | |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 29 (42.0) | 11 (15.9) | 10 (14.5) | 19 (27.5) | 0.173 (4.990) |
| Resistant | 7 (20.6) | 9 (26.5) | 7 (20.6) | 11 (32.4) | |

**Table 3-7: Clinicopathological significance of the combined p-mTOR and p-P70S6K expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| Combined p-mTOR/p-P70S6K Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **mTOR-/ P70S6K- No. (%)** | **mTOR-/ P70S6K+ No. (%)** | **mTOR+/ P70S6K- No. (%)** | **mTOR+/ P70S6K+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 29 (43.9) | 3 (4.5) | 17 (25.8) | 17 (25.8) | |
| Mucinous | 3 (42.9) | 0 (0.0) | 3 (42.9) | 1 (14.3) | |
| Endometrioid | 10 (52.6) | 2 (10.5) | 5 (26.3) | 2 (10.5) | 0.702 |
| Clear cell carcinoma | 10 (71.4) | 0 (0.0) | 2 (14.3) | 2 (14.3) | (9.009) |
| Mixed | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | | | |
| I | 22 (61.1) | 2 (5.6) | 10 (27.8) | 2 (5.6) | 0.123 |
| II | 8 (53.3) | 0 (0.0) | 2 (13.3) | 5 (33.3) | (13.971) |
| III | 22 (43.1) | 2 (3.9) | 15 (29.4) | 12 (23.5) | |
| IV | 3 (42.9) | 1 (14.3) | 0 (0.0) | 3 (42.9) | |
| ***Grade*** | | | | | |
| G1 | 2 (33.3) | 0 (0.0) | 3 (50.0) | 1 (16.7) | 0.880 |
| G2 | 6 (42.9) | 1 (7.1) | 4 (28.6) | 3 (21.4) | (2.393) |
| G3 | 42 (51.9) | 4 (4.9) | 20 (24.7) | 15 (18.5) | |
| ***Measurable Disease*** | | | | | |
| Non | 35 (68.6) | 1 (2.0) | 11 (21.6) | 4 (7.8) | ***<0.0001*** |
| Measurable | 5 (27.8) | 0 (0.0) | 2 (11.1) | 11 (61.1) | **(22.320)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 41 (56.9) | 1 (1.4) | 15 (20.8) | 15 (20.8) | ***0.030*** |
| Resistant | 11 (34.4) | 4 (12.5) | 10 (31.3) | 7 (21.9) | **(8.957)** |

**Table 3-8: Clinicopathological significance of p-4EBP1 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| p-4EBP1 Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **p-4EBP1 Negative No. (%)** | **p-4EBP1 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 35 (47.3) | 39 (52.7) | |
| Mucinous | 8 (100.0) | 0 (0) | ***0.005*** **(12.942)** |
| Endometrioid | 9 (39.1) | 14 (60.9) | |
| Clear cell carcinoma | 12 (75.0) | 4 (25.0) | |
| Mixed | 0 (0) | 0 (0) | |
| ***FIGO Stage*** | | | |
| I | 23 (63.9) | 13 (36.1) | 0.206 |
| II | 11 (64.7) | 6 (35.3) | (4.574) |
| III | 26 (44.1) | 33 (55.9) | |
| IV | 5 (50.0) | 5 (50.0) | |
| ***Grade*** | | | |
| G1 | 8 (66.7) | 4 (33.3) | 0.407 |
| G2 | 4 (66.7) | 2 (33.3) | (1.800) |
| G3 | 49 (49.5) | 50 (50.5) | |
| ***Measurable Disease*** | | | |
| Non | 43 (61.4) | 27 (38.6) | ***0.035*** |
| Measurable | 21 (42.0) | 29 (58.0) | **(4.423)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 44 (57.9) | 32 (42.1) | 0.186 |
| Resistant | 18 (45.0) | 22 (55.0) | (1.751) |

**Table 3-9: Clinicopathological significance of p- P70S6K protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| p-P70S6K Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **p-P70S6K Negative No. (%)** | **p-P70S6K Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 39 (50.6) | 38 (49.4) | |
| Mucinous | 8 (100.0) | 0 (0) | ***0.015*** **(12.382)** |
| Endometrioid | 18 (72.0) | 7 (28.0) | |
| Clear cell carcinoma | 9 (52.9) | 8 (47.1) | |
| Mixed | 0 (0) | 2 (100.0) | |
| ***FIGO Stage*** | | | |
| I | 36 (85.7) | 6 (14.3) | |
| II | 11 (68.8) | 5 (31.3) | ***0.012*** **(12.225)** |
| III | 38 (70.4) | 16 (29.6) | |
| IV | 3 (33.3) | 6 (66.7) | |
| ***Grade*** | | | |
| G1 | 9 (60.0) | 6 (40.0) | 0.442 (1.634) |
| G2 | 7 (77.8) | 2 (22.2) | |
| G3 | 56 (56.0) | 44 (44.0) | |
| ***Measurable Disease*** | | | |
| Non | 49 (67.1) | 24 (32.9) | ***0.019*** **(5.496)** |
| Measurable | 24 (46.2) | 28 (53.8) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 50 (87.7) | 7 (12.3) | ***<0.0001*** **(16.404)** |
| Resistant | 8 (42.1) | 11 (57.9) | |

### 3.3.5 The associations of p-AKT with the other biological markers

High expression of p-AKT within the whole cohort was positively associated with p-mTOR expression, (p-value <0.0001) and its associated proteins: p-4EBP1 and p-P70S6K, (p-value =010) and (p-value <0.0001) respectively. In addition, this association was maintained within the platinum sensitive patients, p-mTOR (p-value <0.0001), p-4EBP1 (p-value =010) and p-P70S6K (p-value <0.0001). In contrast, the correlation between high expression of p-AKT and p-4EBP1 was not statistically significant when platinum resistant cohort was the selection. However, the association between high p-AKT with both p-mTOR and p-P70S6K was maintained at statistically significant value, (p-value =010) and (p-value =0.028) respectively (Table 3-10).

### 3.3.6 The associations between p-mTOR and its downstream regulators

Within the completely studied cohort, high expression of p-mTOR in tumours was in highly significant association with its both downstream regulators, p-4EBP1 and p-P70S6K, (p-value =005) and (p-value =0.002) respectively. Similarly, within platinum sensitive tumours this relation was maintained at statistically significant value, between p-mTOR and p-4EBP1, (p-value =002) and p-P70S6K, (p-value =016). On the other hand, no associations were rendered significant in platinum resistant tumours, (Table 3-11).

**Table 3-10: The associations between p-AKT and the other surrogate biological markers**

| **Whole cohort** | | | |
|---|---|---|---|
| | **low p-AKT No. (%)** | **High p-AKT No. (%)** | ***P- value ( X2 )*** |
| p-mTOR<br>Negative<br>Positive | <br>44 (63.8)<br>9 (17.3) | <br>25 (36.2)<br>43 (82.7) | ***<0.0001***<br>**( 26.003)** |
| p-4EBP1<br>Negative<br>Positive | <br>31 (50.0)<br>15 (26.8) | <br>31 (50.0)<br>41 (73.2) | ***0.010***<br>**(6.666)** |
| p-P70S6K<br>Negative<br>Positive | <br>40 (54.8)<br>11 (21.6) | <br>33 (45.2)<br>40 (78.4) | ***<0.0001***<br>**(13.689)** |
| **Platinum sensitive cohort** | | | |
| | **low p-AKT No. (%)** | **High p-AKT No. (%)** | ***P- value ( X2 )*** |
| p-mTOR<br>Negative<br>Positive | <br>32 (69.6)<br>7 (21.9) | <br>14 (30.4)<br>25 (78.1) | ***<0.0001***<br>***( 17.168)*** |
| p-4EBP1<br>Negative<br>Positive | <br>26 (61.9)<br>10 (32.3) | <br>16 (38.1)<br>21 (67.7) | ***0.012***<br>**(6.272)** |
| p-P70S6K<br>Negative<br>Positive | <br>29 (61.7)<br>9 (27.3) | <br>18 (38.3)<br>24 (72.7) | ***0.002***<br>**(9.216)** |
| **Platinum resistant cohort** | | | |
| | **low p-AKT No. (%)** | **High p-AKT No. (%)** | ***P- value ( X2 )*** |
| p-mTOR<br>Negative<br>Positive | <br>10 (50.0)<br>2 (11.1) | <br>10 (50.0)<br>16 (88.9) | ***0.010***<br>**( 6.631)** |
| p-4EBP1<br>Negative<br>Positive | <br>5 (29.4)<br>4 (18.2) | <br>12 (70.6)<br>18 (81.8) | 0.409<br>(0.681) |
| p-P70S6K<br>Negative<br>Positive | <br>9 (42.9)<br>2 (11.1) | <br>12 (57.1)<br>16 (88.9) | ***0.028***<br>**(4.824)** |

**Table 3-11: The associations between p-mTOR and its downstream effectors**

| **Whole cohort** | | | |
|---|---|---|---|
| | **Low p-mTOR No. (%)** | **High p- mTOR No. (%)** | ***P- value* ( $X^2$ )** |
| p-4EBP1<br>Negative<br>Positive | <br>37 (67.3)<br>21 (40.4) | <br>18 (32.7)<br>31 (59.6) | ***0.005***<br>**(7.785)** |
| p-P70S6K<br>Negative<br>Positive | <br>48 (67.6)<br>18 (39.1) | <br>23 (32.4)<br>28 (60.9) | ***0.002***<br>**(9.205)** |
| **Platinum sensitive cohort** | | | |
| p-4EBP1<br>Negative<br>Positive | <br>29 (74.4)<br>11 (36.7) | <br>10 (25.6)<br>19 (63.3) | ***0.002***<br>**(9.887)** |
| p-P70S6K<br>Negative<br>Positive | <br>32 (69.6)<br>13 (41.9) | <br>14 (30.4)<br>18 (58.1) | ***0.016***<br>**(5.821)** |
| **Platinum resistant cohort** | | | |
| | **Low p- mTOR No. (%)** | **High p- mTOR No. (%)** | ***P- value* ( $X^2$ )** |
| p-4EBP1<br>Negative<br>Positive | <br>7 (50.0)<br>9 (45.0) | <br>7 (50.0)<br>11 (55.0) | 0.774<br>(0.083) |
| p-P70S6K<br>Negative<br>Positive | <br>13 (65.0)<br>5 (33.3) | <br>7 (35.0)<br>10 (66.7) | 0.064<br>(3.441) |

### 3.3.7 Outcome analysis

#### 3.3.7.1 Univariate analysis of the whole cohort

In general, high expression of all the tested proteins showed unfavourable outcome. Compared with low protein expressers, increase p-AKT cytoplasmic expression was associated with shorter both PFS and OS, (p-value =0.004) and (p-value =0.046) respectively (Figure 3-8). Similarly, high p-mTOR cytoplasmic expression showed correlation with adverse clinical outcome in terms of poor PFS, (p-value =0.023) and borderline significance with shorter OS, (p-value =0.079) (Figure 3-9), compared with tumours that had low p-mTOR expression. Tumours with high p-4EBP1 expression showed an adverse clinical outcome with poor OS, (p-value =0.005) and PFS, (p-value =0.005) (Figure 3-10), compared with tumours that had low p-4EBP1 expression. On the other hand, low p-P70S6K nuclear expression in tumours showed an improved clinical outcome in terms of OS, (p-value =0.045) and PFS, (p-value =0.001) (Figure 3-11), compared with tumours that had high p-P70S6K expression. At the transcriptional level, high AKT mRNA was in borderline significance with poor PFS (p-value = 0.0511), but not with OS (p-value = 0.218). In addition, high mTOR mRNA was also associated significantly with poor PFS (p-value = 0.0390); however, no association deemed significant with OS (p-value = 0.914).

Additionally, investigating p-AKT expression with the other proteins revealed that high expression of p-AKT and p-4EBP1 was associated with the worst outcome in terms of both PFS and OS, (p-value =0.002) and (p-value =0.014) respectively (Figure 3-12), compared with low protein expression tumours. In addition, combined high level of both p-AKT and p-mTOR also showed borderline significance with shorter OS, (p-value =0.082) but significant association with lower recurrence outcome, (p-value =0.010) (Figure 3-13), compared with low expressers. However, a trend toward significance with worse both OS and PFS was calculated with the combined high expression of p-AKT and p-P70S6K, if compared with low expression samples, (p-value =0.060) and (p-value =0.054) respectively (Figure 3-14).

Moreover, testing p-mTOR expression with both p-4EBP1 and p-P70S6K proteins together we found that tumours who had low protein expression of both p-mTOR and p-4EBP1 had better OS, (p-value =0.004) and PFS, (p-value =0.002) (Figure

3-15), compared to tumours with high protein expressers. Similarly, we found that tumours with low protein expression of both p-mTOR and p-P70S6K have better OS, (p-value =0.008) and PFS, p-value =0.011) (Figure 3-16), compared to tumours with high protein expressers of both p-mTOR and p-P70S6K.

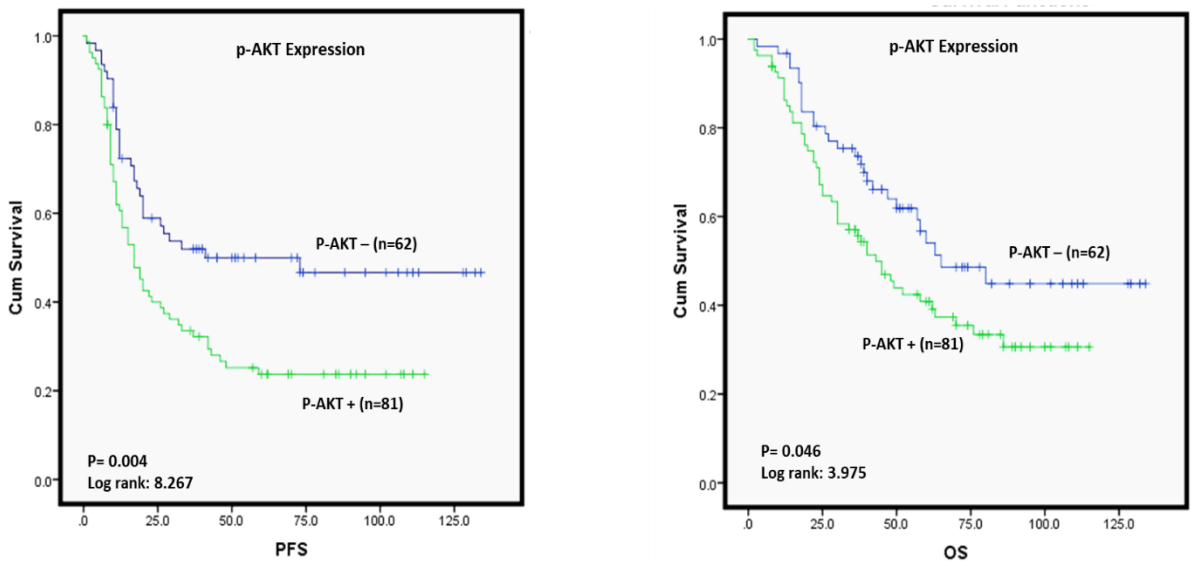



**Figure 3-8: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT protein with PFS and OS in primary ovarian carcinoma – whole series**

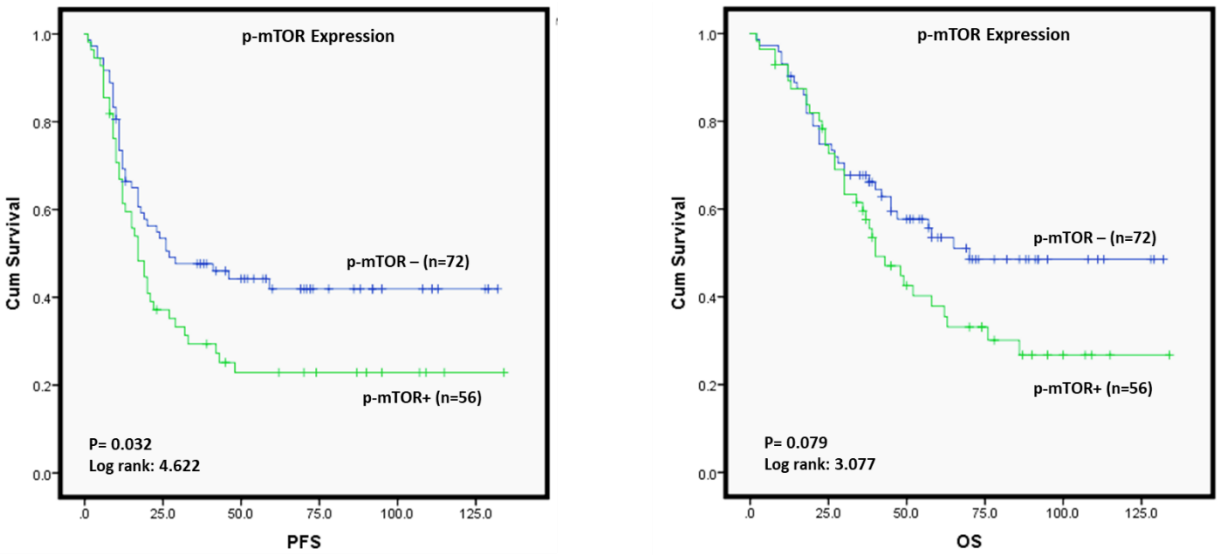



**Figure 3-9: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR protein with PFS and OS in primary ovarian carcinoma – whole series**

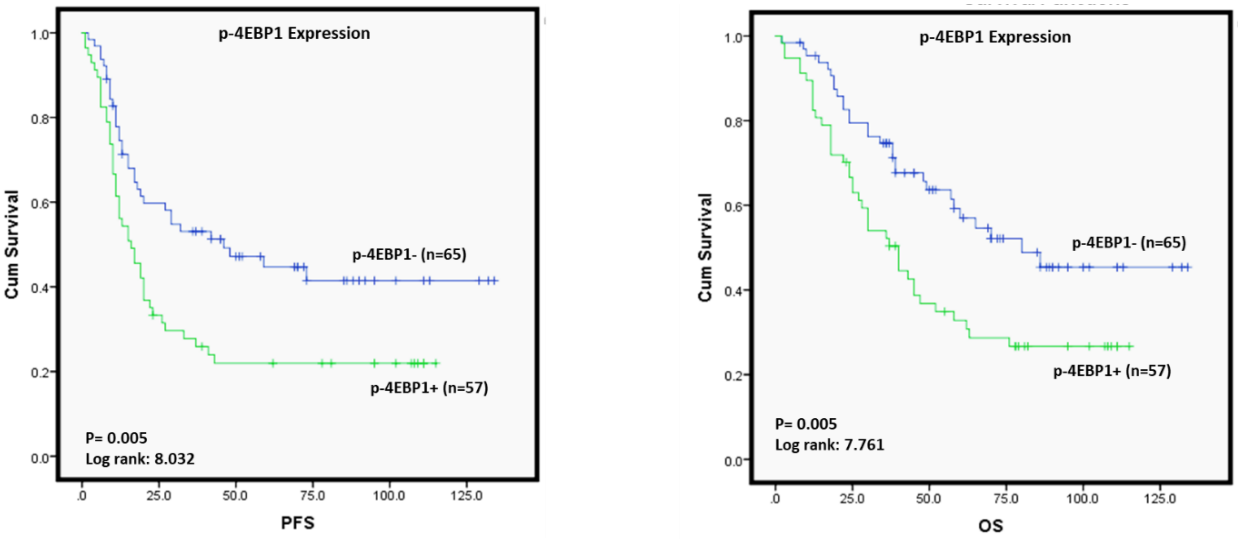



**Figure 3-10: Kaplan Meier plots illustrating the associations between the altered expression of p-4EBP1 protein with PFS and OS in primary ovarian carcinoma – whole series**

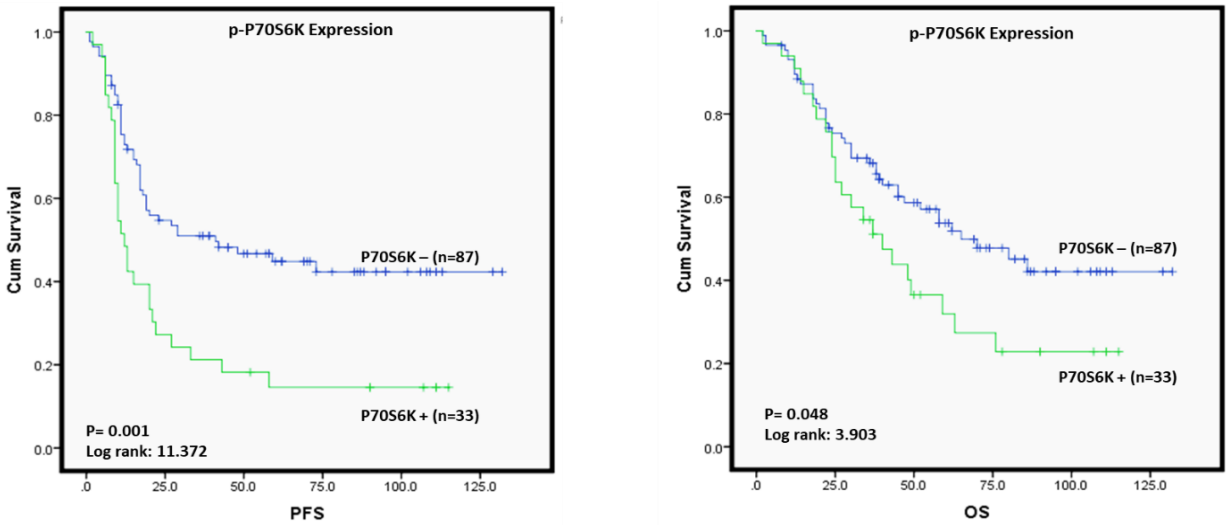



**Figure 3-11: Kaplan Meier plots illustrating the associations between the altered expression of p-P70S6K protein with PFS and OS in primary ovarian carcinoma – whole series**

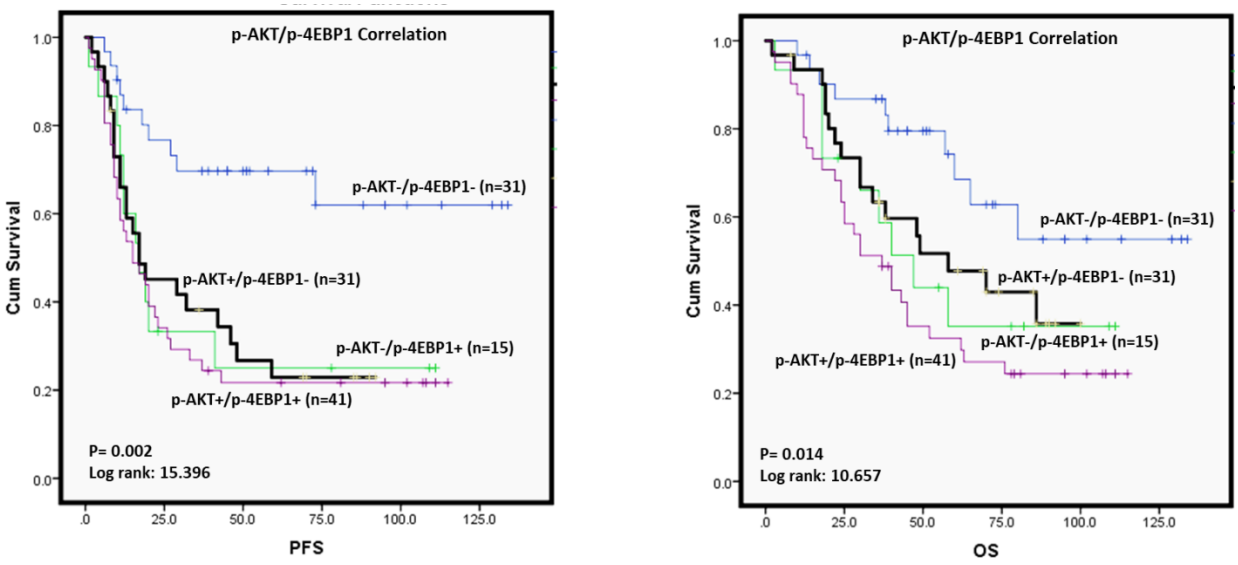



**Figure 3-12: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

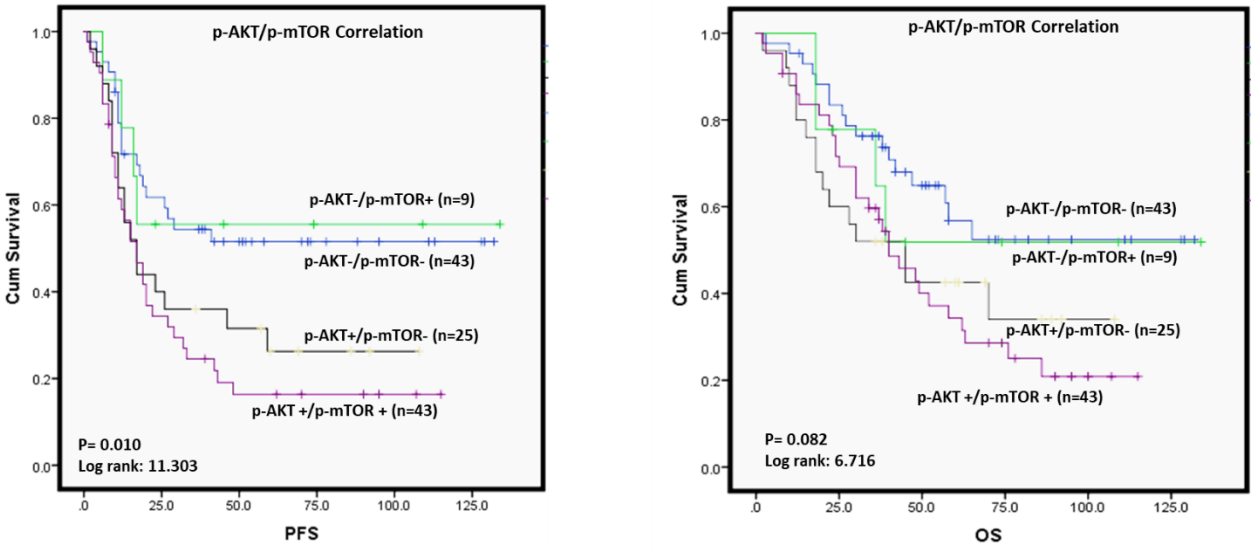



**Figure 3-13: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-mTOR proteins with PFS and OS in primary ovarian carcinoma – whole series**

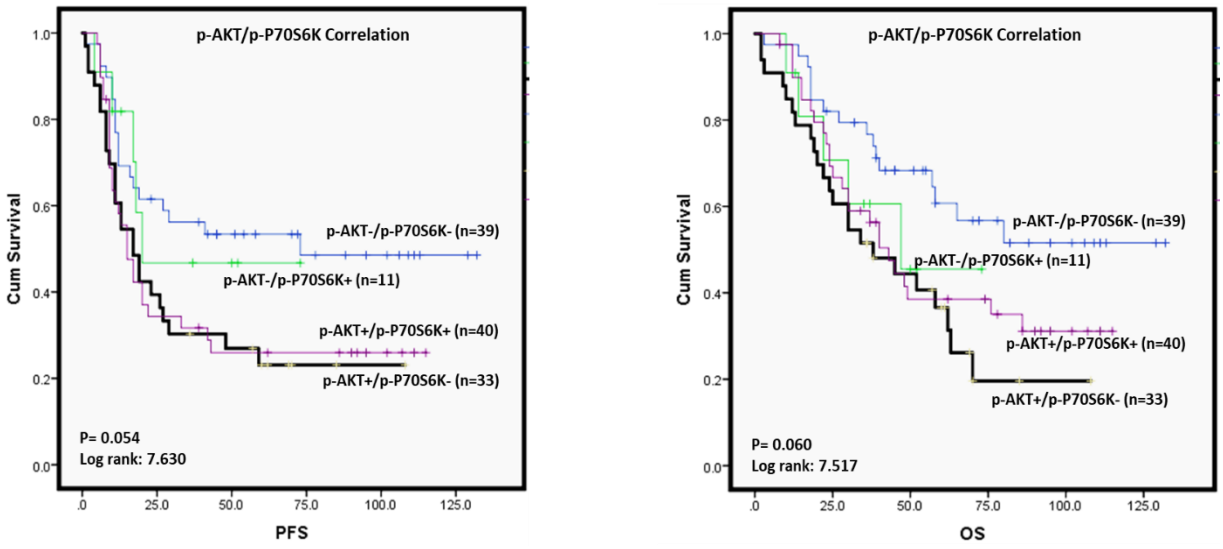



**Figure 3-14: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-P70S6K proteins with PFS and OS in primary ovarian carcinoma – whole series**

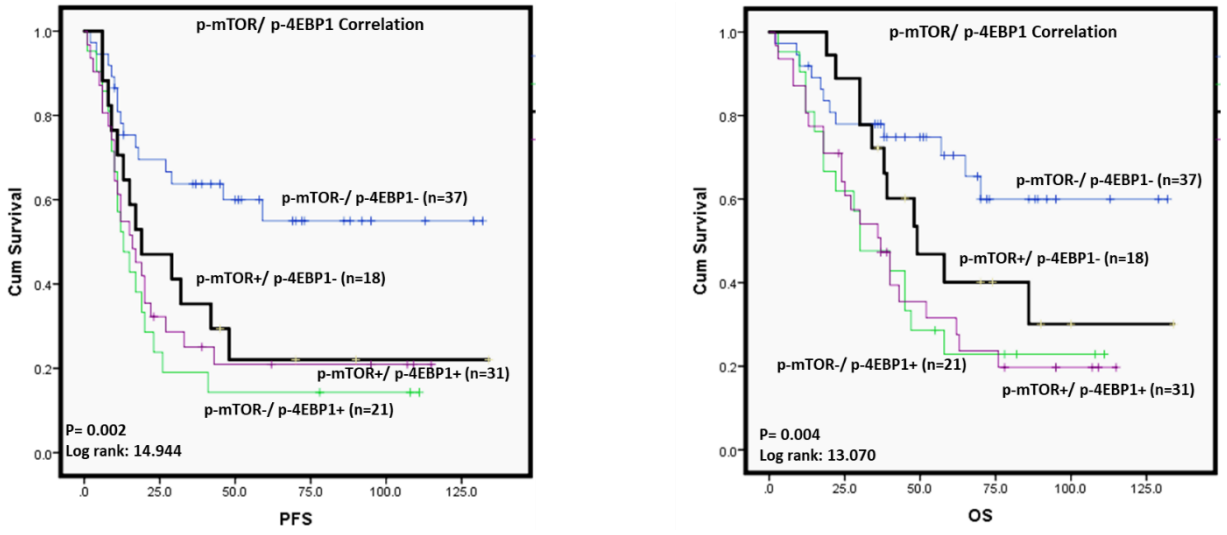



**Figure 3-15: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

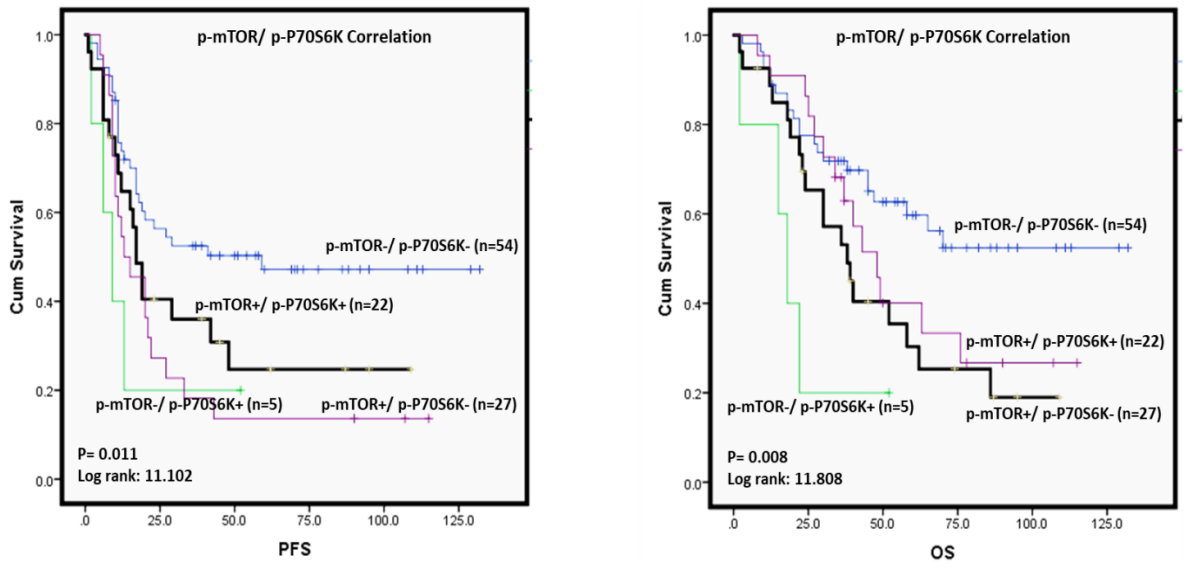



**Figure 3-16: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR and p-P70S6K proteins with PFS and OS in primary ovarian carcinoma – whole series**

#### 3.3.7.2 Univariate analysis - platinum sensitive cohort

The KM estimates of the PFS for the platinum sensitive patients showed that the overexpression of p-AKT had a significant worse outcome if compared with the low expressers, (p-value =0.033) (Figure 3-17). Similarly, high p-mTOR and p-4EBP1 cytoplasmic expression in tumour tissues showed adverse clinical outcome with poor recurrence outcome, (p-value =0.016) (Figure 3-18), (p-value =0.009) (Figure 3-19) respectively, and worse OS for both proteins (p-value =0.048) (Figure 3-18) and (p-value =0.021) (Figure 3-19) respectively. In addition, borderline significance was noted between lower PFS and p-P70S6K overexpression, (p-value =0.078), compared with tumours that had low p-P70S6K expression. However, the correlation with OS was non-significant, (p-value =0.201) (Figure 3-20).

Furthermore, investigating the effect of the combined expression of the surrogate proteins in this chapter with the positive p-AKT expression revealed interestingly that high expression of p-AKT and p-4EBP1 was associated with the worst PFS outcome, (p-value =0.023) (Figure 3-22), compared with low protein expression tumours. In addition, combined high level of both p-AKT and p-mTOR also showed significant association with less PFS, (p-value =0.025) (Figure 3-21), compared with low expressers. However, the combined high expression of p-AKT and p-P70S6K had non-significance with both OS and PFS when compared with low expression malignant samples, (p-value =0.242) and (p-value =0.201) respectively (Figure 3-23).

Similarly, investigating p-mTOR/ p-4EBP1/ p-P70S6K proteins together we importantly found that, tumours who had low protein expression of both p-mTOR and p-4EBP1 have a significant better PFS, (p-value =0.010) and a trend toward significance on extending the OS, (p-value =0.051) (Figure 3-24), compared to tumours with high p-mTOR and p-4EBP1 protein expression. Similarly, we found that tumours with low protein expression of both p-mTOR and p-P70S6K have improved PFS, (p-value =0.013), compared with high protein expressers, (Figure 3-25).

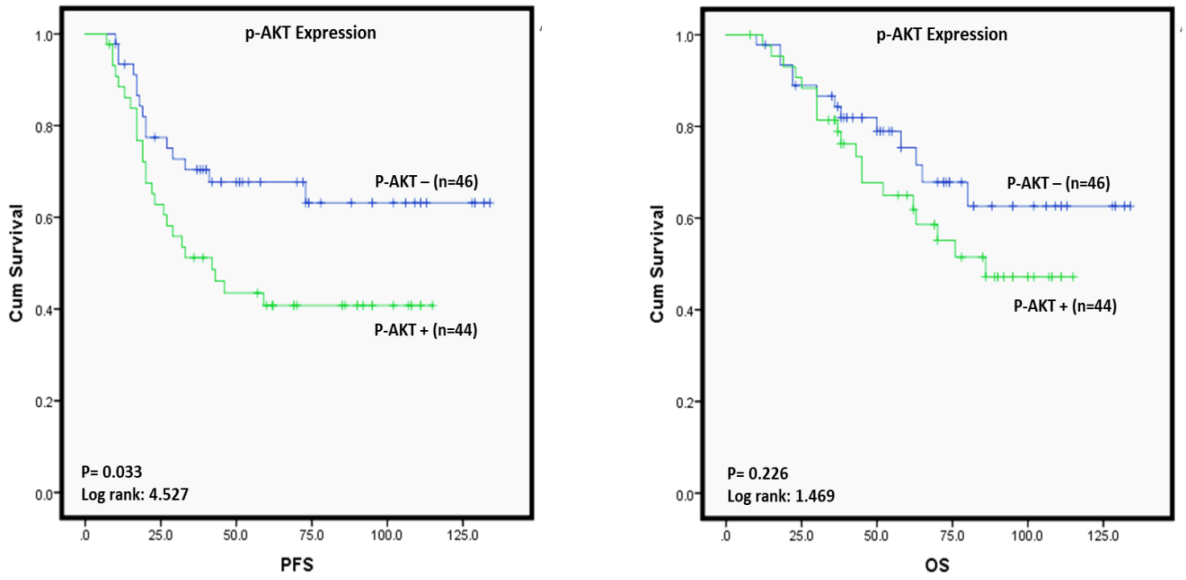



**Figure 3-17: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

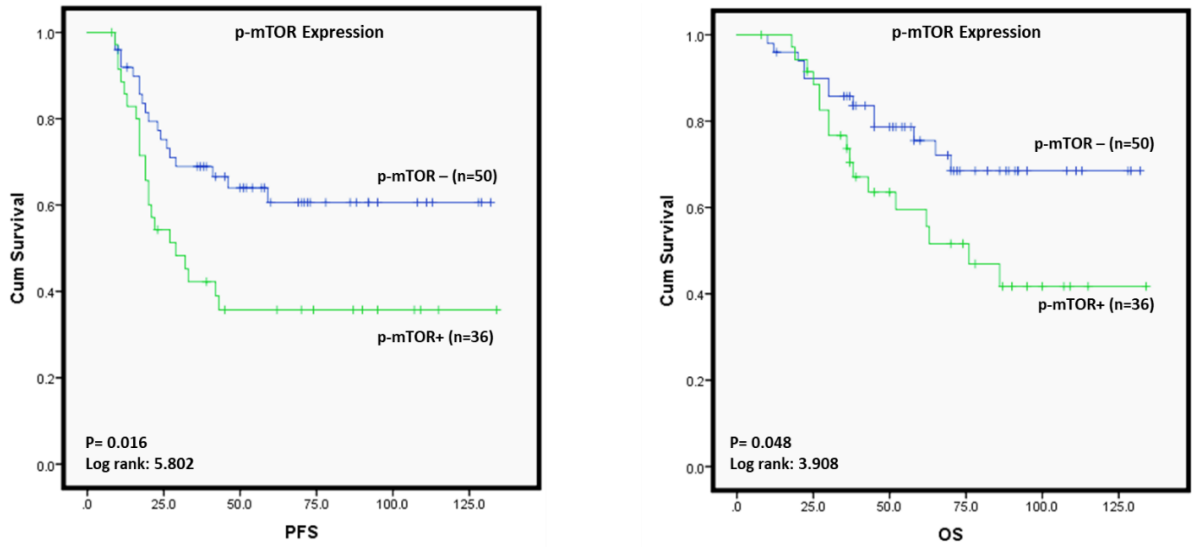



**Figure 3-18: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

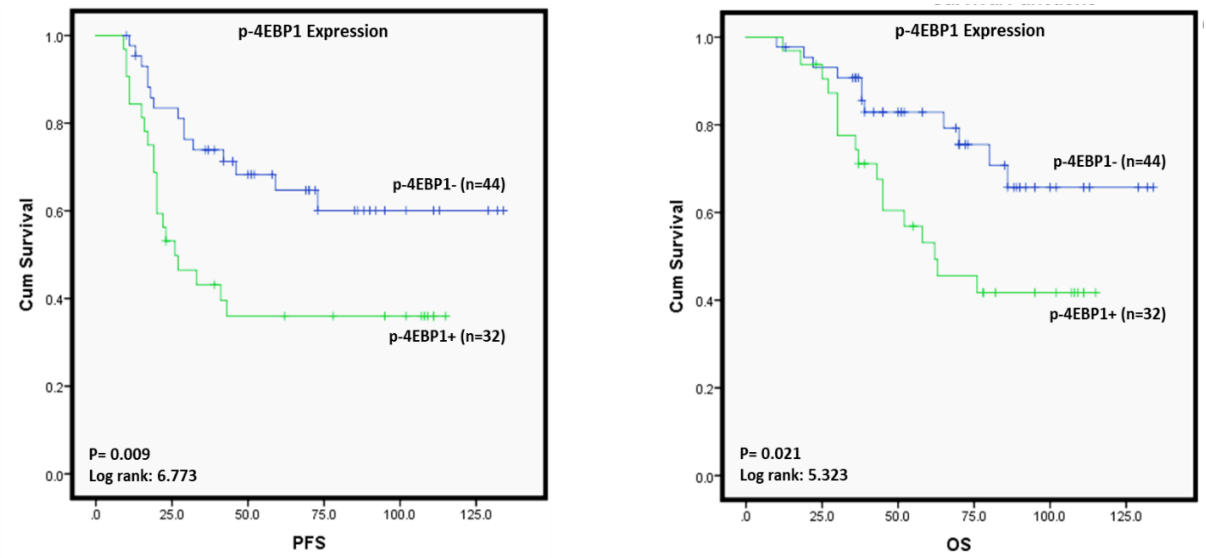



**Figure 3-19: Kaplan Meier plots illustrating the associations between the altered expression of p-4EBP1 protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

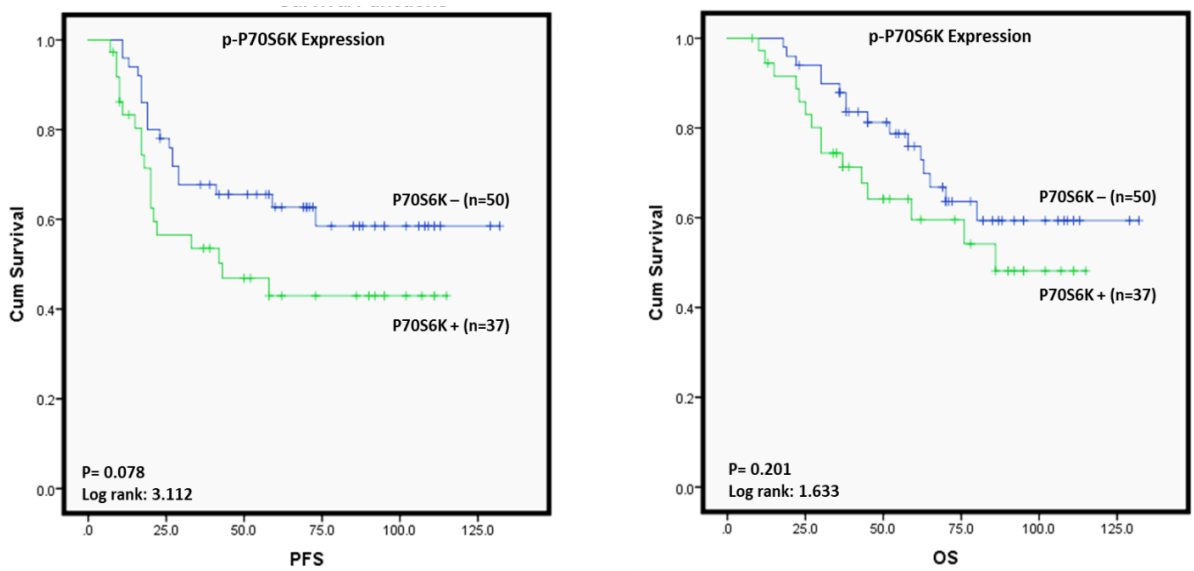



**Figure 3-20: Kaplan Meier plots illustrating the associations between the altered expression of p-P70S6K protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

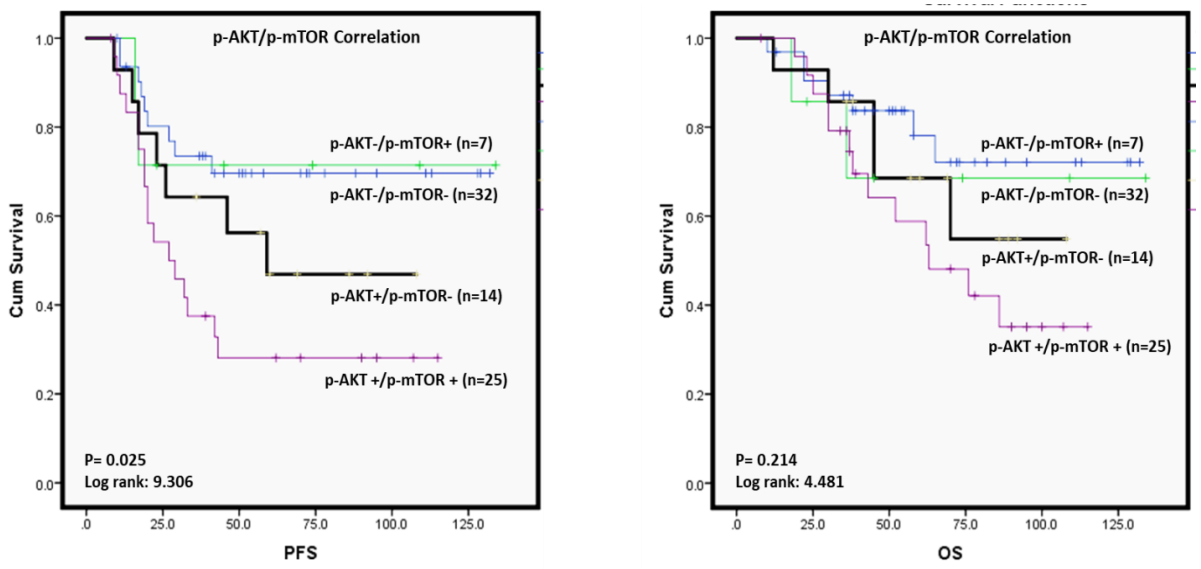



**Figure 3-21: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-mTOR proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

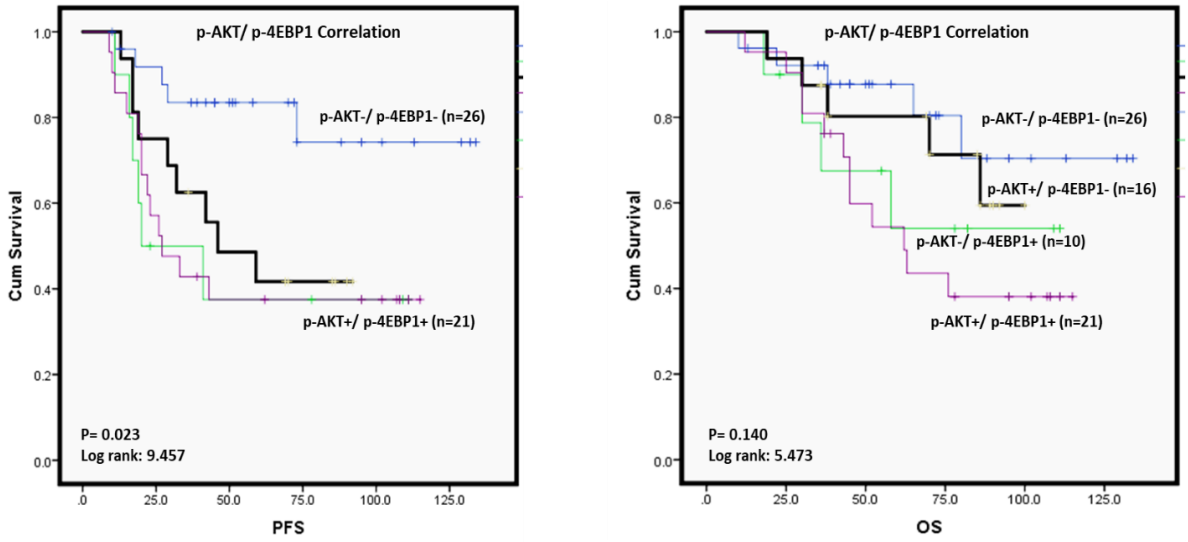



**Figure 3-22: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

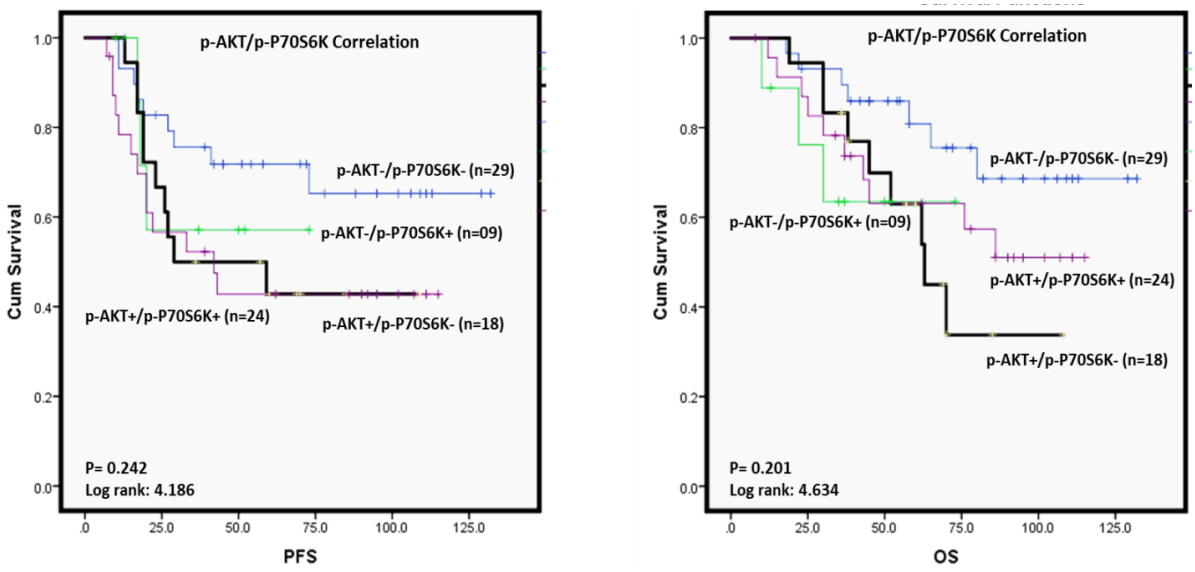



**Figure 3-23: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-P70S6K proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

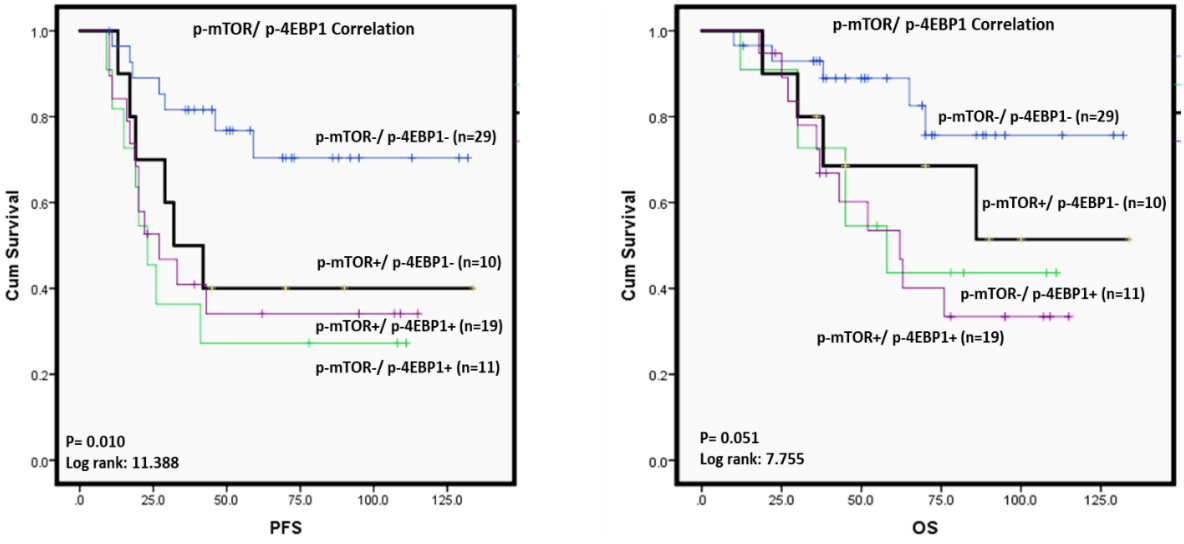



**Figure 3-24: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

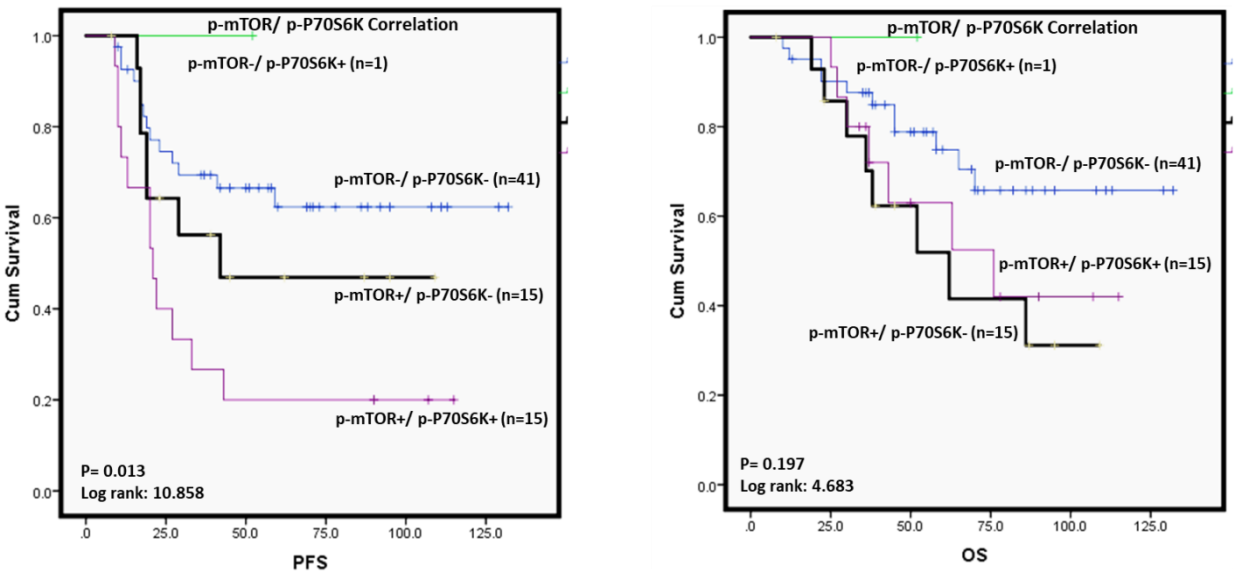



**Figure 3-25: Kaplan Meier plots illustrating the associations between the altered expression of p-mTOR and p-P70S6K proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

#### 3.3.7.3 Univariate analysis - platinum resistant cohort

When the survival was analysed in platinum resistant cases, we found no statistically significant correlation between patients' outcome and all the surrogate markers (p-AKT, p-mTOR, p-4EBP1 and p-P70S6K), (Supplementary table).

#### 3.3.7.4 Multivariate analysis

In addition to platinum sensitivity, p-AKT, p-mTOR, p-4EBP1 and p-P70S6K protein expressions were investigated in a Cox multivariate model for both PFS and OS. In the whole cohort of OC patients (unselected), High p-AKT, (p-value =0.012) and p-4EBP1, (p-value =0.021) were independently associated with poor PFS, (Table 3-12). Similarly, both high p-AKT, (p-value =0.018) and p-4EBP1, (p-value =0.013) were independent factors associated with poor OS, (Table 3-13).

**Table 3-12: Cox multivariate Regression model for the PFS in the whole (unselected), platinum sensitive and resistant ovarian cancer series**

| Whole cohort | | | | |
|---|---|---|---|---|
| | ***p-value*** | **HR** | **95% CI** | |
| **p-AKT** | ***0.012*** | 0.774 | 1.187 | 3.964 |
| **p-mTOR** | 0.997 | 0.001 | 0.576 | 1.732 |
| **p-4EBP1** | ***0.021*** | 0.623 | 1.099 | 3.160 |
| **p-p70S6K** | 0.281 | -0.298 | 0.431 | 1.276 |
| **Platinum sensitivity** | ***<0.0001*** | 17.790 | 16.990 | 32.979 |

**Table 3-13: Cox multivariate Regression model for the OS in the whole (unselected), platinum sensitive and resistant ovarian cancer series**

| Whole cohort | | | | |
|---|---|---|---|---|
| | ***p-value*** | **HR** | **95% CI** | |
| **p-AKT** | ***0.018*** | 0.788 | 1.142 | 4.236 |
| **p-mTOR** | 0.970 | 0.011 | 0.558 | 1.835 |
| **p-4EBP1** | ***0.013*** | 0.682 | 1.152 | 3.397 |
| **p-p70S6K** | 0.160 | -0.414 | 0.371 | 1.177 |
| **Platinum sensitivity** | ***<0.0001*** | 18.790 | 12.450 | 30.249 |

#### 3.3.7.5 Survival outcome in only p-AKT/p-4EBP1 selected tumours

The KM estimates when only both positive and negative p-AKT and p-4EBP1 tumours were selected. In the whole cohort, the combined overexpression of both p-AKT and p-4EBP1 had a significant shorter PFS and OS, (p-value <0.001) and (p-value =0.001) respectively (Figure 3-26). Similarly, in platinum sensitive patients, unfavourable PFS was highly significantly correlated with p-AKT and p-4EBP1 overexpression, (p-value =0.004) (Figure 3-24). However, this association lost its significance for OS, (p-value =0.313) (Figure 3-27) and in platinum resistant cases, (Supplementary table).

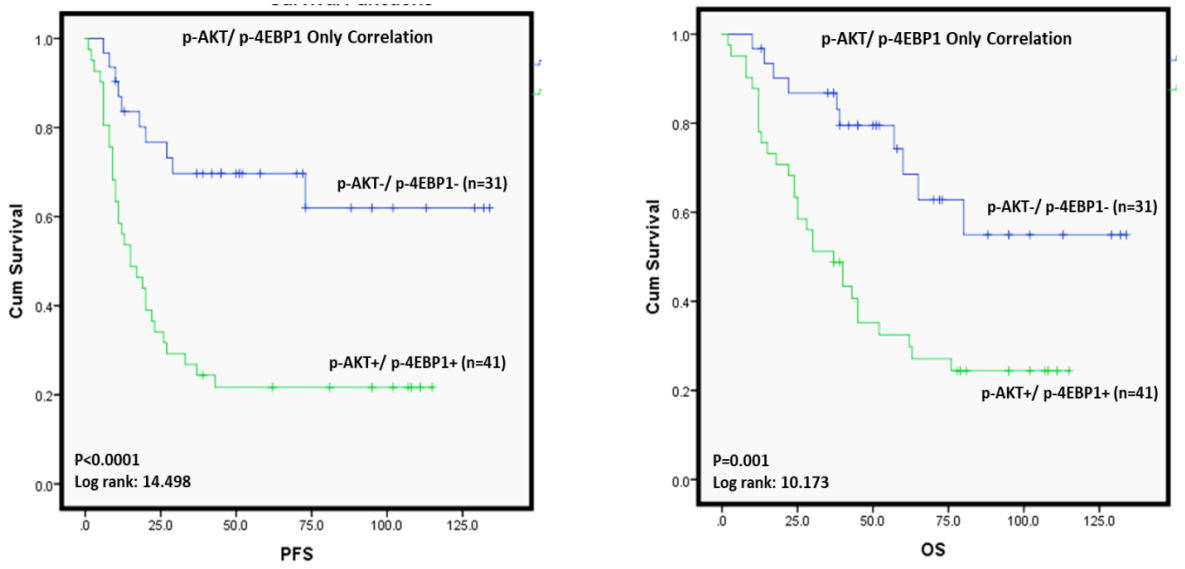



**Figure 3-26: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

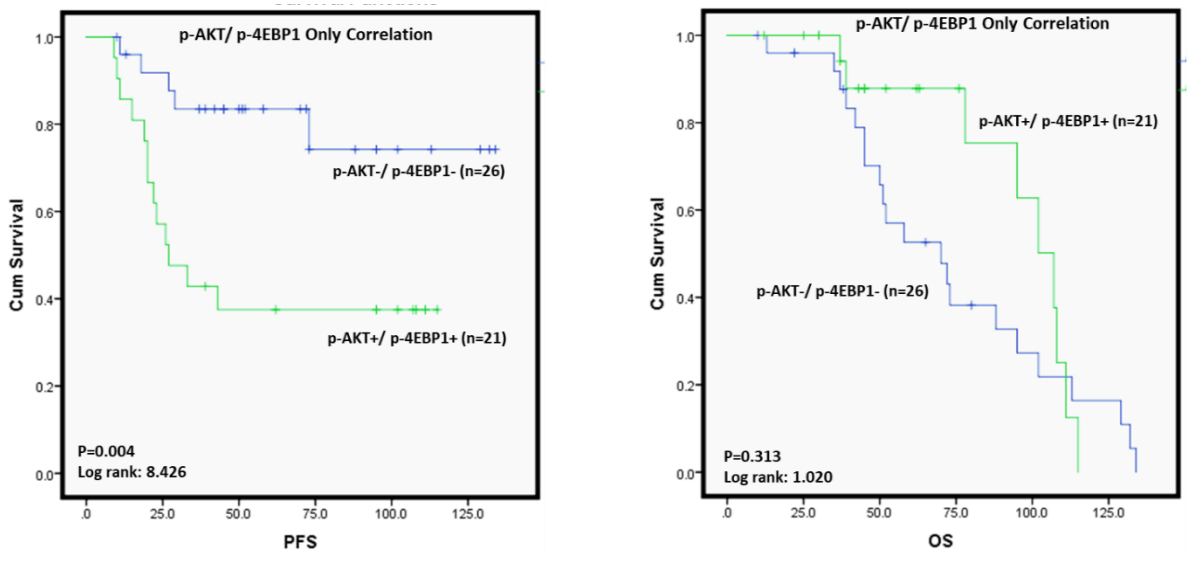



**Figure 3-27: Kaplan Meier plots illustrating the associations between the altered expression of p-AKT and p-4EBP1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

## 3.4 Discussion

Human carcinogenesis can happen as a consequence of different alterations in nearly 300 genes which encode for cell proteins that are vital in regulating cell proliferation, DNA repair, differentiation, and apoptosis. However, among gene mutations, RNA levels, and protein expression unfortunately there is no linear correlation that is always evident. Recent studies suggest that dysregulation of translational machinery also has a crucial role in carcinogenesis (No et al., 2011b). Moreover, in developed countries, OC has still the highest mortality rate among all other gynaecologic malignancies (Jemal et al., 2008). Its diagnosis is still at advanced stage and the prognosis, in spite of successful surgery and platinum based chemotherapy is still poor (Yap et al., 2009).

PI3K/Akt/mTOR pathway proteins altered expression is a well-known observation in several cancers (Markman et al., 2010). Although mutation of *Akt* is rare (< 5%) in serous OC, previous IHC analyses have shown that this pathway is activated in half of OC tumours (Altomare et al., 2004, Verhaak et al., 2013, Lee et al., 2015). In addition, there is an increasing evidence that mTOR; the p-AKT downstream marker is a central regulator of translation initiation, which is a rate-limiting step in the translational process. p-mTOR, a serine/threonine kinase, regulates translation via phosphorylation of its effectors including 4EBP1 and p70S6K.

In this study, we reported novel findings for the altered protein expression in AKT/mTOR pathway in OC patients. However, different protein localisation and molecular subtypes showed different observations, in keeping with the heterogeneity of OC, not only in morphology but also in the evolutionary molecular changes through its primary and recurrent phases after surgery and chemotherapy. We hypothesized that the protein members in the AKT/mTOR pathway are important factors for the progression of OC.

A previous study performed by Lee et al (Lee et al., 2015) concluded that AKT activation is present in over half of OC patients'. However and except for tumour grade, this study did not demonstrate a significant association between p-AKT overexpression and any of the other clinicopathological parameters. Moreover, the results of previous studies regarding the prognostic value of p-AKT expression in

OC patients are conflicting. In one study, the expression of p-AKT was found to be negatively correlated with the prognosis of OC patients (Jia et al., 2014), which is in contrast to the results demonstrated by other researchers (Woenckhaus et al., 2007, Rahman et al., 2012). These studies demonstrated that p-AKT expression was neither associated with PFS nor OS. However, one of those studies assessed the nuclear form of p-AKT and the other one demonstrated the effect of p-AKT on clear cell carcinoma subtype only (Woenckhaus et al., 2007, Rahman et al., 2012).

In the current study, we have shown that p-AKT was adversely associated with platinum sensitivity in OC patients. This finding is in line with another IHC study, which was performed on 118 paraffin tissue sections of patients with OC and borderline malignancy. Patients who had p-AKT over-expression exhibited higher chemoresistance to platinum agents (Jia et al., 2014). In addition and in preclinical studies, Kim et al (Kim et al., 2007) and Craig et al (Carden et al., 2012) also reported that over-expression of p-AKT in OC cell lines enhanced malignant cells' chemoresistance.

Interestingly, we have found that increase expression of p-AKT was positively associated with poor patients' outcome. In addition, p-AKT overexpression was always a predictive of worse outcome when combined with the other surrogate markers. This finding was also concluded in another study (Jia et al., 2014). Their results showed that p-AKT over-expressers had poorer prognosis, if compared with low expressers. In addition, in the same study, p-AKT over-expression was associated with advanced-stage tumours (Jia et al., 2014) and, p-AKT was positively correlated with an increased risk of tumour relapse (Lee et al., 2015). Moreover, in gastric cancer and despite the negative association between overexpression of p-AKT and the clinicopathological parameters, p-AKT high protein level was associated with significantly poor prognosis (Sasaki et al., 2014). Interestingly, this study illustrated that the majority of those associations between p-AKT overexpression and survival were maintained within the platinum sensitive cohort. High level of p-AKT on its own or if combined with p-mTOR, p-4EBP1 and p-P70S6K was inversely associated with the recurrence outcome. Thus, we can emphasise that over-expression of p-AKT protein in primary OC may predict poor survival.

Previous study showed that overexpression of p-4EBP1 was significantly associated with poor prognostic factors, including advanced FIGO stage, higher histologic grade, and size of residual mass at the time of surgery (No et al., 2011b). These results come also in agreement with other studies (Castellvi et al., 2006, Noske et al., 2008). In addition, No et al linked p-4EBP1 overexpression in tumours with a higher risk of developing chemoresistance (No et al., 2011b).

Our data showed that overexpression of p-4EBP1 was significantly associated with aggressive tumour features. Moreover, survival analysis illustrated that patients with p-4EBP1 overexpression had significantly poor outcome in both univariate and multivariate analysis. A finding, which comes in line with No et al, who stated that, the patients with p-4EBP1 overexpression, had significantly poor disease-free survival in univariate analysis (No et al., 2011b). Importantly, p-4EBP1 has maintained its poor prognostic behaviour within the platinum sensitive cohort, reflecting its prognostic role in such group of patients. In addition, isolated high level of p-4EBP1 or when combined with the other investigated proteins in this chapter, was inversely associated with the patients' outcome. Taken together, these findings suggest that increased protein translation through the phosphorylation of 4EBP1 could be associated with the progression of ovarian tumours by promoting the invasiveness of disease.

In general, we have identified that p-mTOR is a marker of aggressive disease. In the current study, p-mTOR overexpression was associated with higher residual disease post-surgery. In addition, shorter PFS was the outcome for high p-mTOR expression cases. Overall, our results come generally in agreement with previous other study (No et al., 2011b).

Several publications on cervical and hepatocellular carcinomas reported that p-mTOR expression was linked to poor prognosis. On the other hand, other study conducted on gastric carcinoma patients identified that p-mTOR as a good prognostic factor (Xiao *et al.*, 2009) (Bachmann et al., 2006). Moreover, a previous study in OC identified a non-significant correlation between p-mTOR and survival (No et al., 2011a), unlike the current study finding. These discrepancies might be attributed to the negative feedback loop of mTOR regulated by p-AKT or molecular crosstalk of the mTOR signalling pathway.

Regarding p-P70S6K, in a previous IHC study performed on OC patients, p-P70S6K showed mainly a nuclear localisation, which was positive in 30% of tumour specimens. Lesser significant cytoplasmic expression was also noted (Castellvi et al., 2006). Interestingly, malignant tumours showed higher expression of P70S6K than their benign or borderline counterparts did. Although in this study (Castellvi et al., 2006) p-P70S6K overexpression did not show significant correlation with histologic grade, surgical stage, or survival, another study conducted on breast cancer concluded that p-P70S6K overexpression was correlated with increased tumour size, higher histologic grade, and presence of lymph node metastasis, in addition to increased risk of local recurrence (Rojo et al., 2007).

The above findings are in partial consistency with the current study. High nuclear expression of p-P70S6K was noted in around 40% of tumour cells. In addition, overexpression of the phosphorylated form of this protein showed significant association with poor prognostic clinicopathological factors. On the other hand, low P70S6K expressers had better response to platinum chemotherapeutic agents, when compared with high expression tumours. Moreover, overexpression of this protein was associated with adverse clinical outcome.

Interestingly, in the current study we have provided the evidence for the close correlation between p-AKT expression and p-mTOR, p-4EBP1 and p-P70S6K in primary OC patients. A consistent finding with Altomare et al (Altomare et al., 2004), in which they illustrated a preclinical evidence for the close association between p-AKT activation and mTOR. Hence, a subset of human ovarian tumours may be strongly dependent on AKT/mTOR signalling for tumour progression. Therefore, OC patients with aberrant AKT/mTOR signalling may benefit from pharmacologic inhibition of mTOR or one of its regulators or effectors to improve disease prognosis (Altomare et al., 2004, Neshat et al., 2001, Podsypanina et al., 2001).

In multivariate Cox model, importantly high p-AKT and p-4EBP1 expression were independently associated with poor recurrence and OS survival, providing a further evidence for the prognostic and predictive significance of those two proteins on the patients' outcome. Furthermore, we have illustrated that combined overexpression of both p-AKT and p-4EBP1 in OC cases was highly significantly associated with worse outcome. This provides further evidence for the importance

of these two markers and their impact on OC prognosis. Taken together, to the best of our knowledge we have provided for the first time the evidence that both p-AKT and p-4EBP1 have a crucial impact on OC outcome. However, this data was based on a retrospective cohort, and is in need of prospective validation in larger multicentre study.

Selection of patients who may benefit from molecular-targeted therapy is based on identifying specific molecular targets in tumours. Although the current techniques require standardisation, our results show p-AKT and p-4EBP1 overexpression have clinical significance in OC. Therefore, this may provide a platform for phenotypic profiling and personalized therapeutic strategies with inhibitors to target the mTOR pathway and/or its upstream / downstream effectors.

In summary, p-mTOR is a downstream signalling molecule that has shown differential associations with its key biomarkers including its upstream regulators and downstream effectors in primary OC patients. Our study reveals that overexpression of p-AKT and p-4EBP1 in OC is highly correlated with unfavourable outcome and resistance to platinum therapy. OC patients who positively expressed p-AKT and p-4EBP1 demonstrated lower survival rate, higher risk of residual disease, and higher recurrence events than their counterparts with negative expression of those proteins. Our study also showed that the altered p-AKT and p-4EBP1 proteins expression could be differentially interpreted between platinum sensitive and resistant patients. Moreover, the interactions of p-AKT and p-4EBP1 with other proteins suggested that both of them could be novel key players in the OC outcome. Our results suggest a pivotal multi-factorial role of p-AKT and p-4EBP1 in OC, and this potentiates further scientific and clinical approaches in prognostic biomarkers and therapeutics. In addition, this finding could be used for future further research that might improve the patients' outcome and their response to platinum therapy.

## 3.5 Study limitations related to this chapter

1. The OC series used in this chapter is a historic and retrospective.

2. For this historical patient cohort, relatively old paraffin blocks with missing cases were used to study the expression of the surrogate tissue markers. These tissues could possibly be potentially different from the recently fixed and prepared tissue specimens.

3. The very limited number of cases available of platinum sensitive and resistant cases made their study very challenging.

# 4 Exploring ATM and XIAP Novel Role in Ovarian Cancer

## 4.1 Introduction

In a standard management of Ovarian Cancer (OC), platinating chemotherapeutic agents are frequently used (Tsibulak et al., 2019, van Zyl et al., 2018). The estimated response rates to platinum-based chemotherapy can be up to 60–80%. Despite the initial good clinical response to standard therapy, the prognosis of patients is still very poor (Hashiguchi et al., 2004). Although DNA repair deficient tumour cells will be sensitive to platinum therapy, enhanced DNA repair capacity in tumours is a frequent cause of resistance to therapy, which in turn impacts negatively on the clinical outcomes (Dasari and Tchounwou, 2014, Ali et al., 2019). Therefore, the development of additional prognostic indicators, closely related to tumour cell biology, is essential for identification of patients with a particularly poor prognosis.

Ataxia-telegiectasia mutated (ATM) is a master regulator of DNA damage response (DDR) following double strand breaks (DSBs) (Jin and Oh, 2019). It is involved not only in DNA repair but also in cell cycle checkpoints, apoptosis, various metabolic and signalling pathways (Clouaire et al., 2017, Paull, 2015, Shiloh and Ziv, 2013). ATM Mutations can contribute to lymphoid malignancies (Shiloh and Kastan, 2001) and to familial breast and ovarian tumours (Chenevix-Trench et al., 2002, Thorstenson et al., 2003). In addition, emerging data also suggests a role for ATM in sporadic tumours' pathogenesis (Prokopcova et al., 2007, Ahmed and Rahman, 2006, Khanna and Chenevix-Trench, 2004, Nevanlinna and Bartek, 2006).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a key tumour suppressor (Worby and Dixon, 2014). Moreover, PTEN plays a key role in cell cycle regulation, induction of cell apoptosis and transduction of multiple signalling pathways of cells (Gil et al., 2006, Tang and Eng, 2006, Ogier-Denis and Codogno, 2003, Gozuacik and Kimchi, 2004, Wang et al., 2015). In many human tumour specimens, PTEN deletion or mutation have been identified (Maehama and Dixon, 1999). Moreover, recent studies have suggested a link between PTEN deletion and development of drug resistant tumours (Song et al., 2012, Zhuang et al., 2009, Wang et al., 2015). In OC, PTEN can negatively regulate PI3K/Akt pathway, hence suppress the cancer cell growth and provoke a

G1 arrest of the cell cycle (Minaguchi et al., 1999, Weng et al., 1999, Choi et al., 2002, Cai et al., 2014).

The phosphatidylinositol-3-kinase (PI3K) pathway has been recognised as the most commonly activated signalling pathway in human tumours. It is a key player in tumour progression (Fruman and Rommel, 2014, Liu et al., 2009, Yang et al., 2016). Genetic mutations of components of this pathway have been shown to lead to tumour progression in numerous cancer types, including both breast (Sun et al., 2001b) and ovarian (Shayesteh et al., 1999). PI3K heterodimer consists of a p110 catalytic domain (that phosphorylates PIP2 to PIP3) and a p85α regulatory sub-unit (De Santis et al., 2019). The correlation between p85 isoforms and cancer development has been reported previously. In addition, p85α mutants promote cell survival, AKT activation, and oncogenesis (Jaiswal et al., 2009). In addition, Sun et al (Sun et al., 2010) showed that mutant p85 protein expression in chicken embryonic fibroblasts increased cell proliferation and induced oncogenic transformation. Moreover, p85α levels was noted to be higher in ovarian tumour tissue specimens, when compared with the normal ovaries (Zhang and Sun, 2007). On the other hand, in breast cancer, *in vivo* inhibition of p85 activity significantly reduced the proliferation of tumour cells (Folgiero et al., 2012, Zhou et al., 2014).

X-linked inhibitor of apoptosis protein (XIAP) is a member of the Inhibitor of apoptosis protein (IAP) family (Gyrd-Hansen et al., 2008, Mehrotra et al., 2010, Zhang et al., 2018a). It is involved in regulating innate immune responses (Krieg et al., 2009). Moreover, XIAP has been shown to be overexpressed in several cancers, including ovarian tumours (LaCasse et al., 1998, Tamm et al., 2000, Yang et al., 2003). Interestingly, XIAP confers resistance to some chemotherapeutic agents utilised in different types of cancers, including OC (He et al., 2012, Farrand et al., 2013, Miyamoto et al., 2018, Zhang et al., 2013, Lin et al., 2013, Zhang et al., 2018a). Overexpression of XIAP attenuated the ability of cisplatin to induce apoptosis in OC (Li et al., 2001). On the other hand, its pre-clinical downregulation increased sensitivity to cisplatin in resistant serous OC cells (Sasaki et al., 2000, Ma et al., 2009).

A potential cross talk between ATM, p85α, and PTEN does exist. PI3K could activate ATM (Irarrazabal et al., 2006). Moreover, ATM can also phosphorylate

PTEN (Chen et al., 2015a). Similarly, p85α can also bind to and increase PTEN lipid phosphatase activity (Chagpar et al., 2010). XIAP has functional association with AKT (Prabhu et al., 2017) and ATM can also phosphorylate AKT (Halaby et al., 2008). In addition, ATM also can regulate XIAP (Ali et al., 2019).

### 4.1.1 Hypothesis

ATM, PTEN, XIAP and p85α are important signalling transduction molecules that have diverse and interrelated function in OC. The employment of those biomarkers' implications in OC prognosis could lead to the identification of subsets of high-risk invasive OC patients that may require critical consideration. It is hypothesised that both ATM and XIAP interactions with PTEN and p85α can create a divergent associations and change their influence on outcome in primary OC.

### 4.1.2 Aims

1- To examine ATM, PTEN, XIAP and p85α at the protein level by applying IHC procedures to a large cohort of OC patients treated primarily by surgery. Then, correlate their expression with the patients' clinicopathological variables.

2- To determine the role of those biomarkers in modulating the response to platinum chemotherapeutic agents.

3- To assess the prognostic potential of ATM, PTEN, XIAP and p85α.

4- To identify the key independent prognostic factors and examine their influence on patients' outcome.

## 4.2 Methods

Tissue microarray (TMA) from primary surgically treated OC cases (525 in number) was prepared and immunohistochemical (IHC) staining was performed as described previously in chapter two of this thesis. The expression of ATM, PTEN, p85α and XIAP proteins was determined in the stained tumour tissue. Before the IHC staining, Western blot (WB) methodology was utilised to check the specificity of the purchased antibodies. The full details of WB procedure including the concentrations of antibodies used and the different used materials can be found in the methodology chapter.

Before performing IHC staining, optimisation of the surrogate antibodies' was performed to identify the optimal concentration and incubation for representative staining, detailed in the appendix chapter. Then, after selecting the optimal antibody concentration with the help of an expert pathologist, all primary antibodies were diluted in a specific volume of BondTM primary antibody diluent to reach the optimum working dilution: 1:100 anti-ATM rabbit monoclonal antibody, 1:50 anti-PTEN rabbit monoclonal antibody, 1:3000 of anti-PI3K p85α mouse monoclonal antibody, and 1:500 anti-XIAP rabbit polyclonal antibody. All primary antibodies were applied and incubated with tumour tissues for 60 minutes at room temperature, except anti-PI3K p85α antibody, which was incubated for 18 hours at 4C. The detailed procedures of IHC, digital imaging, and scoring are referred to in the methodology chapter.

## 4.3 Results

### 4.3.1 Specificity of the surrogate antibodies

The evaluation of WB images for the anti-ATM, anti-PTEN, anti-PI3K p85α and anti-XIAP primary antibodies supported a specific binding affinity to a single protein band for each cell lysate around the predicted molecular masses as instructed by the supplying company. 350 KDa for the ATM protein, (Figure 4.1), 54 KDa for the PTEN protein (Figure 4.2), 85 KDa for the p85α protein (Figure 4.3) and 55 KDa for the XIAP protein (Figure 4.4).

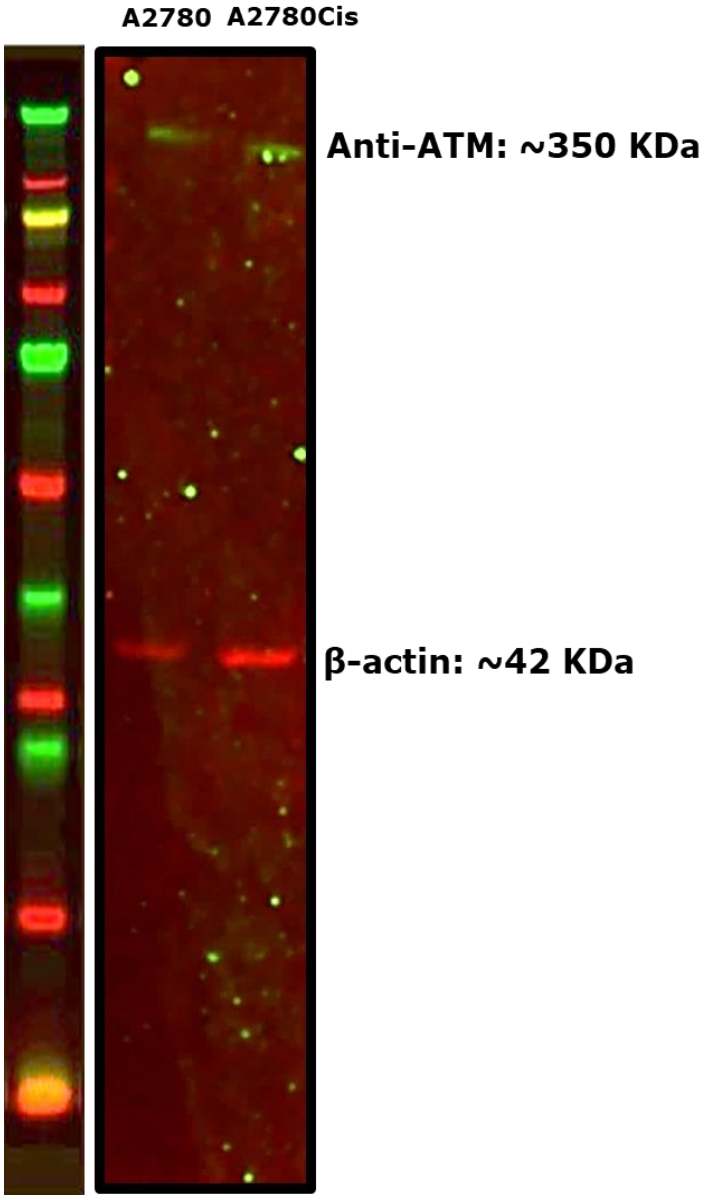



**Figure 4-1: Binding specificity of the anti-ATM antibody.**
**WB membrane showed specific antigen-binding of for anti-ATM antibody around ~350 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

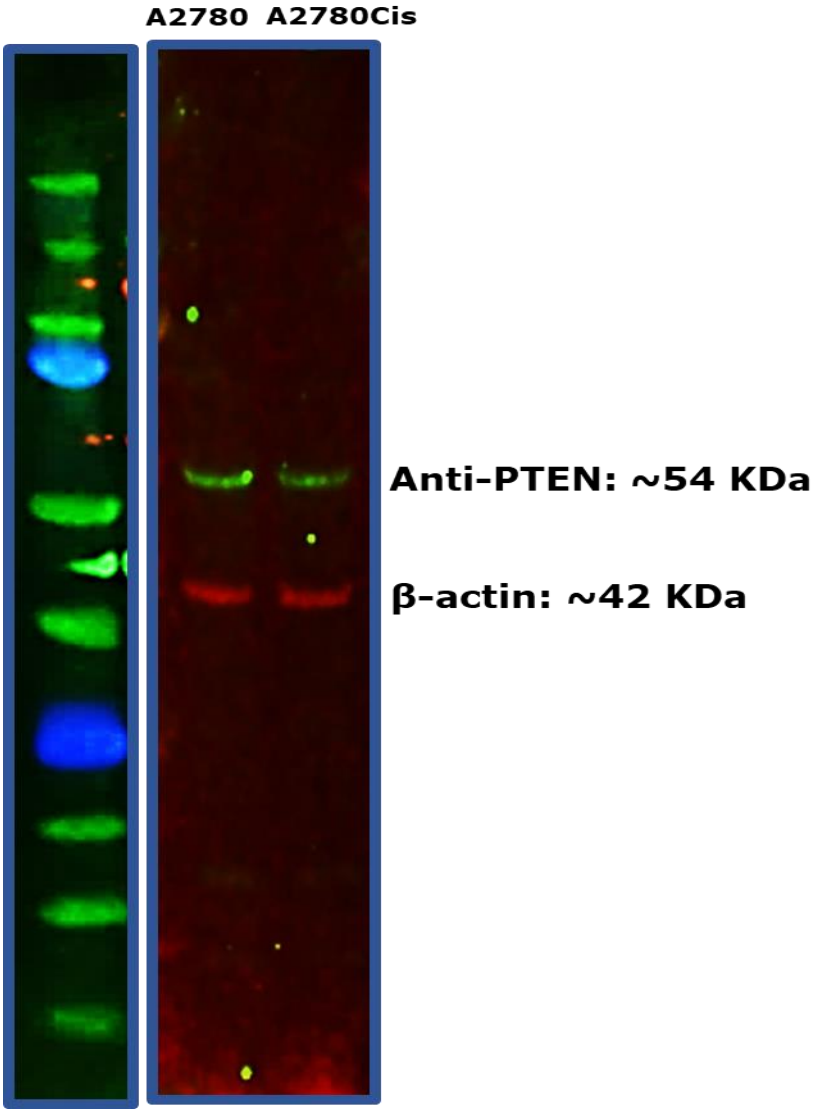



**Figure 4-2: Binding specificity of the anti-PTEN antibody.**
**WB membrane showed specific antigen-binding of for anti-PTEN antibody around ~54 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

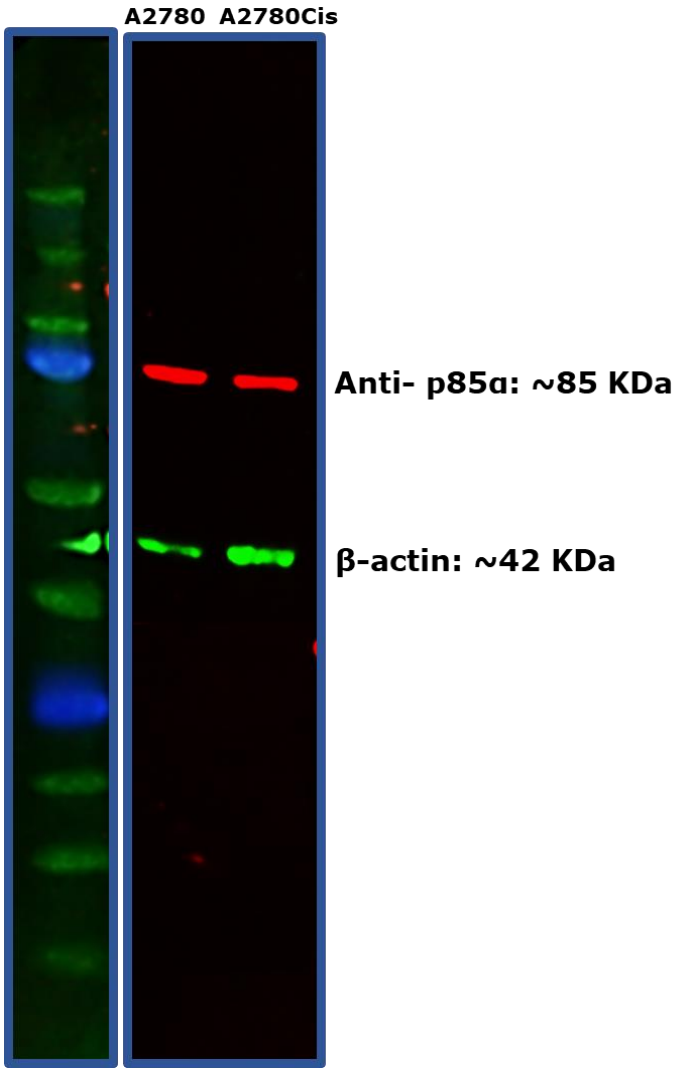



**Figure 4-3: Binding specificity of the anti- p85α antibody.**
**WB membrane showed specific antigen-binding of for anti- p85α antibody around ~85 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

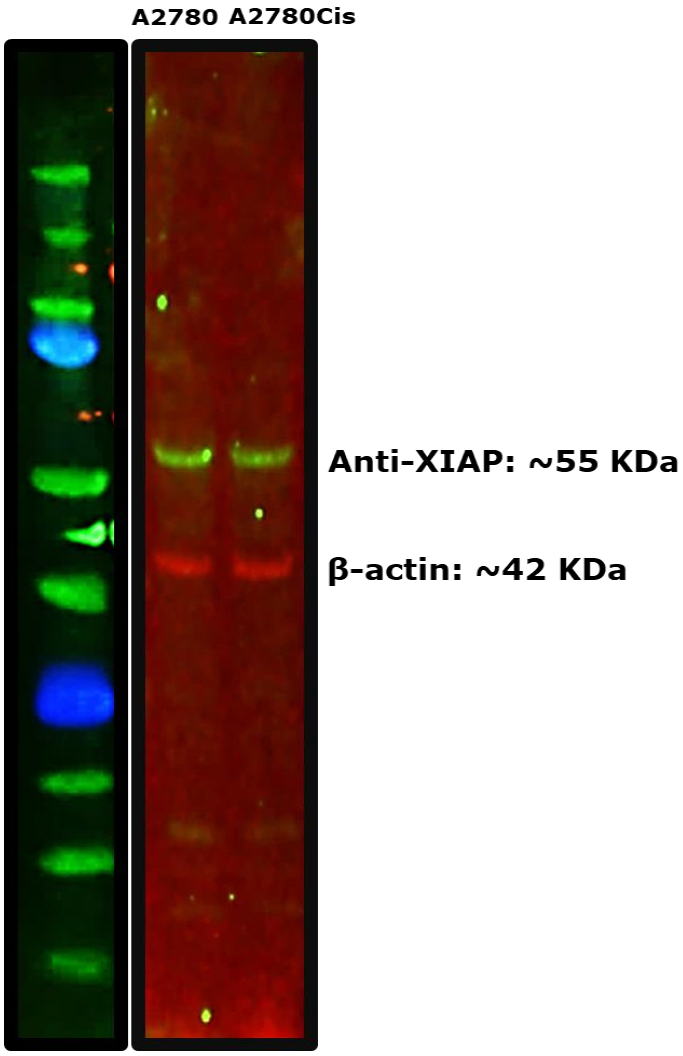



**Figure 4-4: Binding specificity of the anti-XIAP antibody.**
**WB membrane showed specific antigen-binding of for anti- XIAP antibody around ~55 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

### 4.3.2 Immunohistochemistry results

IHC methodology has been utilised to assess the altered expression of ATM, PTEN, p85α and XIAP antibodies in primary resectable OC cases. All the surrogate markers mentioned above have shown a unique localised distribution among the different cellular compartments. The protein expressions of ATM was exclusively nuclear, (Figure 4.5). The protein expression of PTEN and XIAP were mainly cytoplasmic, (Figure 4.6) and (Figure 4.7), respectively. P85α showed both cytoplasmic and membranous protein expression, (Figure 4.8). Its membranous expression was weak and non-significant, therefore was not taken into consideration. The median value was used to obtain the designated cut-off points. They were at <25 H-score for nuclear ATM, <80 H-score for cytoplasmic PTEN, ≤140 H-score for cytoplasmic P85α and <100 H-score for cytoplasmic XIAP. The IHC staining intensities showed negative / weak, moderate, and strong staining for the four interrogated proteins.

Positive ATM protein expression was observed in 54.5% of patients, while negative expression of ATM was confirmed in 45.5%. Similarly, positive PTEN expression was seen in 52.1%, while the remaining patients, 47.9% showed negative expression for PTEN. Positive XIAP was observed in 49.3%, and negative cytoplasmic XIAP expression was confirmed in 50.7%. Finally, the positive expression of P85α protein was observed in 29.2% of patients, while negative expression of P85α was confirmed in 70.8%. In the following subsections, clinical relevance for these four molecular regulators of OC are presented.

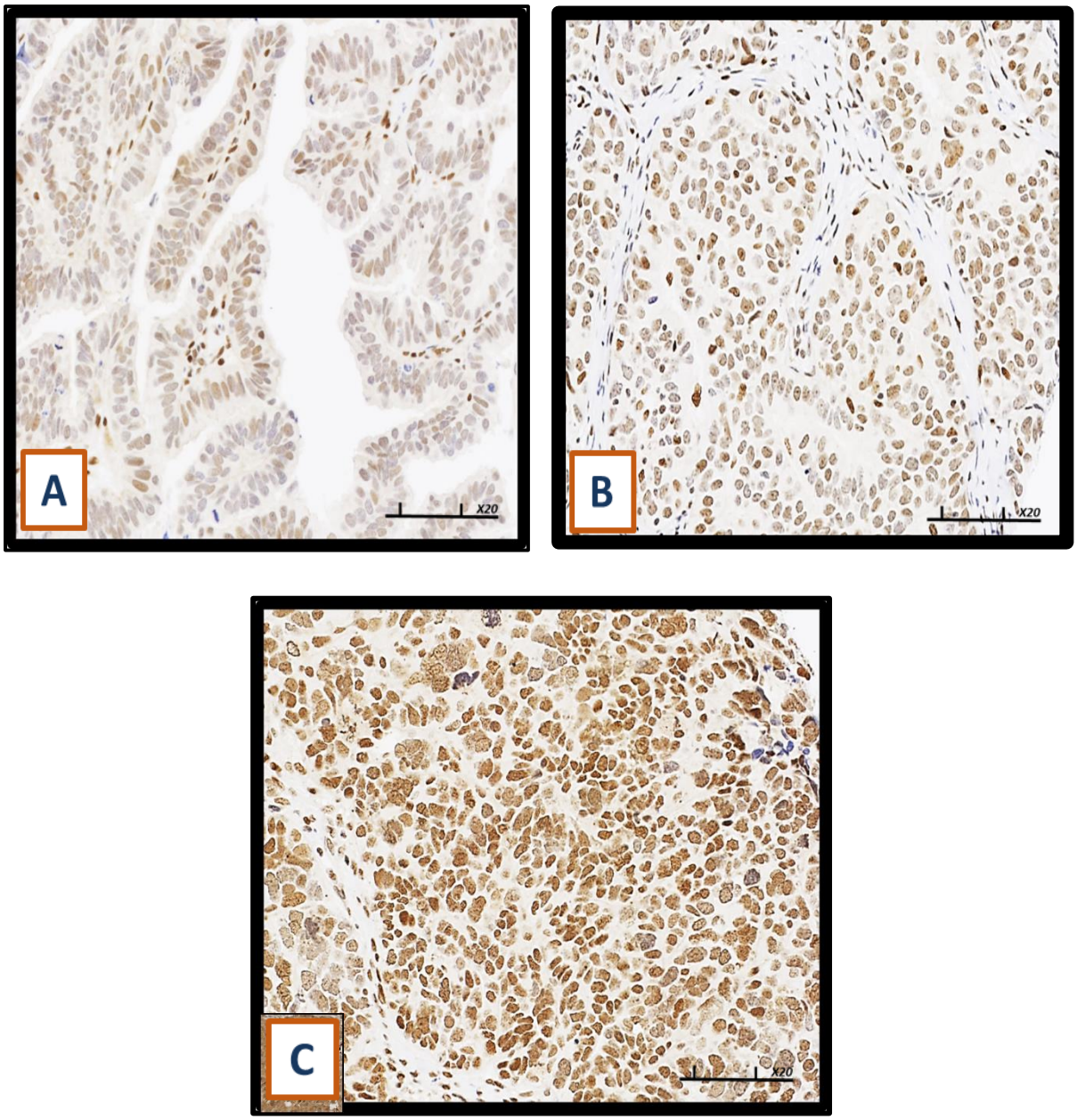



**Figure 4-5: Immunohistochemistry expression of ATM in tissue microarray images. Nuclear expression of ATM in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

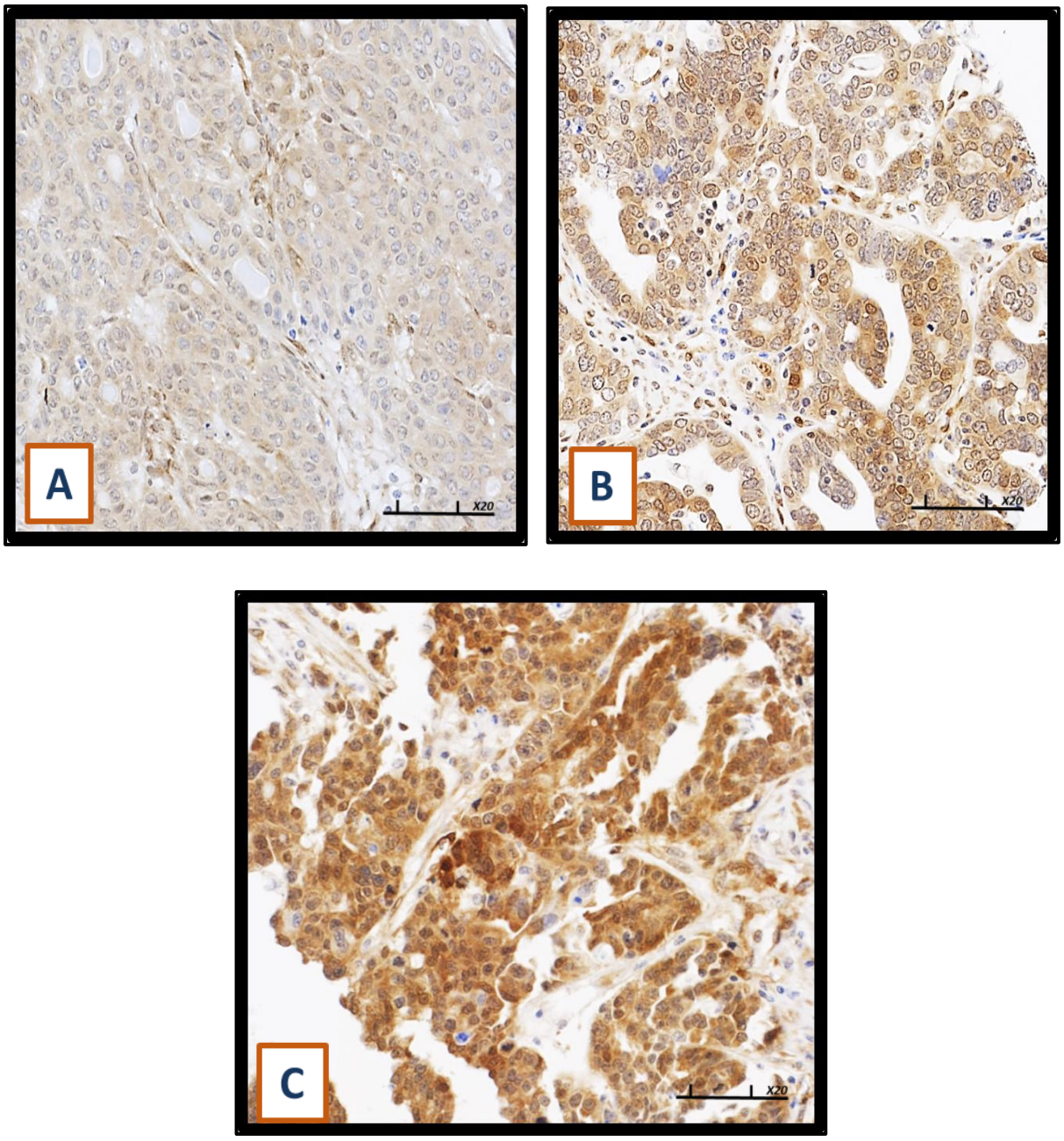



**Figure 4-6: Immunohistochemistry expression of PTEN in tissue microarray images. Cytoplasmic expression of PTEN in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

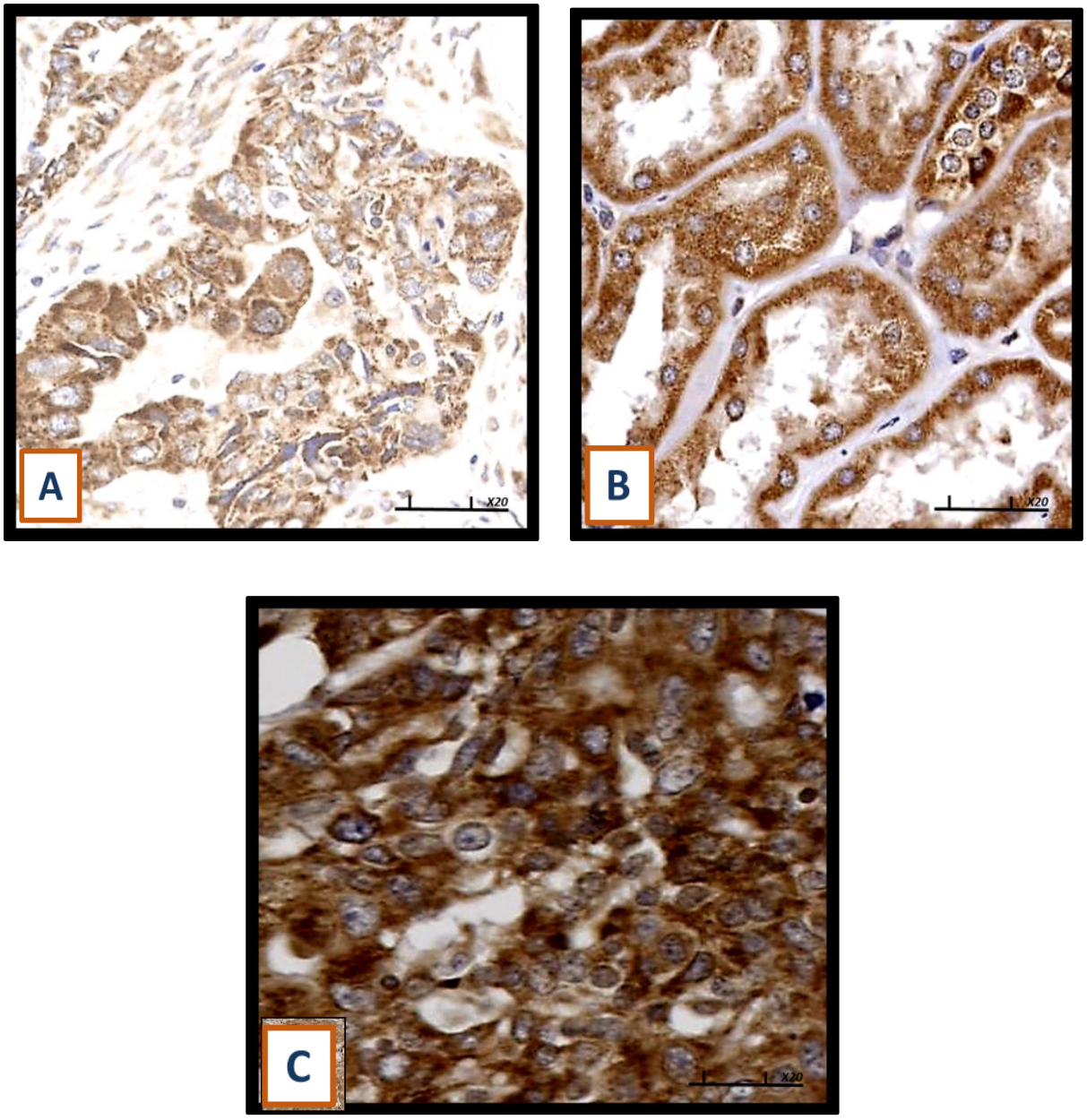



**Figure 4-7: Immunohistochemistry expression of XIAP in tissue microarray images. Cytoplasmic expression of XIAP in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

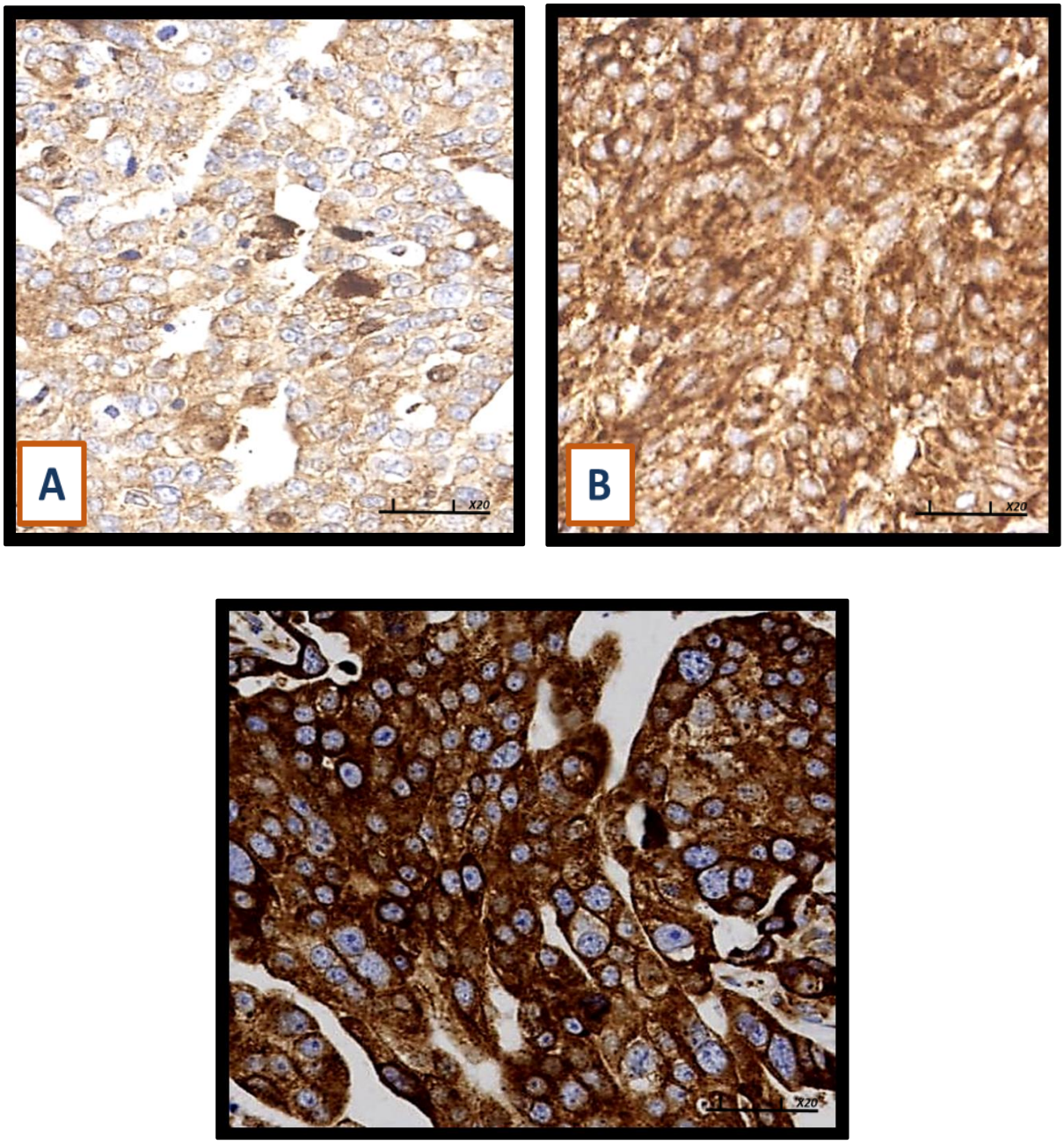



**Figure 4-8: Immunohistochemistry expression of P85α in tissue microarray images. Cytoplasmic expression of P85α in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

#### 4.3.2.1 Clinicopathological relevance of the selected proteins

##### 4.3.2.1.1 **ATM**

Although ATM is crucial for DDR process, clinically, apart from the significant association with serous tumour histology subtype, (p-value =0.006), the nuclear overexpression of ATM had no significant clinical impact on OC patients (Table 4-1).

##### 4.3.2.1.2 **PTEN**

The altered protein expression of PTEN in the cytoplasm did not appear to be a promising prognostic indicator for invasive OC in our investigated patients' cohort. However, the loss of cytoplasmic PTEN protein was correlated with serous ovarian tumours, (p-value =0.004) (Table 4-2).

##### 4.3.2.1.3 **p85α**

Our results show that the p85α cytoplasmic negative expression was strongly associated with Clear cell, Mucinous and Endometrioid histology subtypes, (p-value =0.002). On the other hand, patients with cytoplasmic protein overexpression of p85α showed high significance with residual tumour following surgery, (p-value =0.002). Cases with downregulated p85α had improved response to platinum therapy, (p-value =0.026) (Table 4-3).

##### 4.3.2.1.4 **XIAP**

For XIAP protein, the upregulated expression of cytoplasmic XIAP was an indicator for poor prognosis. Positive expression of this protein was associated with serous and clear cell histology tumour subtypes, (p-value =0.023). However, XIAP negative expression significantly improved complete tumour resection with less residual disease following surgery, (p-value =0.047) (Table 4-4).

**Table 4-1: Clinicopathological significance of ATM protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| ATM Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **ATM Negative No. (%)** | **ATM Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 102 (39.4) | 157 (60.6) | |
| Mucinous | 29 (58.0) | 21 (42.0) | |
| Endometrioid | 35 (50.7) | 34 (49.3) | ***0.006*** |
| Clear cell carcinoma | 26 (66.7) | 13 (33.3) | **(16.176)** |
| Mixed | 6 (37.5) | 10 (62.5) | |
| Other | 5 (33.3) | 10 (66.7) | |
| ***FIGO Stage*** | | | |
| I | 70 (44.6) | 70 (44.6) | 0.746 |
| II | 27 (41.5) | 38 (58.5) | (1.229) |
| III | 93 (48.7) | 98 (51.3) | |
| IV | 12 (44.4) | 15 (55.6) | |
| ***Grade*** | | | |
| G1 | 28 (46.7) | 32 (53.3) | 0.681 |
| G2 | 31 (41.9) | 43 (58.1) | (0.769) |
| G3 | 130 (47.6) | 143 (52.4) | |
| ***Measurable Disease*** | | | |
| Non | 122 (44.0) | 155 (56.0) | 0.188 |
| Measurable | 66 (49.3) | 68 (50.7) | (1.133) |
| ***Platinum sensitivity*** | | | |
| Sensitive | 152 (46.6) | 174 (53.4) | 0.527 |
| Resistant | 38 (50.7) | 37 (49.3) | (0.399) |

**Table 4-2: Clinicopathological significance of PTEN protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| PTEN Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **PTEN Negative No. (%)** | **PTEN Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 136 (55.3) | 110 (44.7) | |
| Mucinous | 32 (60.4) | 21 (39.6) | |
| Endometrioid | 41 (64.1) | 23 (35.9) | ***0.004*** **(19.270)** |
| Clear cell carcinoma | 27 (71.1) | 11 (28.9) | |
| Mixed | 7 (43.8) | 9 (56.3) | |
| Other | 9 (64.3) | 5 (35.7) | |
| ***FIGO Stage*** | | | |
| I | 83 (54.2) | 70 (45.8) | 0.644 (1.192) |
| II | 35 (56.5) | 27 (43.5) | |
| III | 89 (48.9) | 93 (51.1) | |
| IV | 13 (56.5) | 10 (43.5) | |
| ***Grade*** | | | |
| G1 | 30 (52.6) | 27 (47.4) | 0.974 (0.023) |
| G2 | 39 (53.4) | 34 (46.6) | |
| G3 | 133 (52.0) | 123 (48.0) | |
| ***Measurable Disease*** | | | |
| Non | 136 (51.1) | 130 (48.9) | 0.113 (2.509) |
| Measurable | 55 (42.6) | 74 (57.4) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 172 (54.8) | 142 (45.2) | 0.407 (0.302) |
| Resistant | 34 (49.3) | 35 (50.7) | |

**Table 4-3: Clinicopathological significance of P85α protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| P85α Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **P85α Negative No. (%)** | **P85α Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 156 (63.4) | 90 (36.6) | |
| Mucinous | 42 (79.2) | 11 (20.8) | |
| Endometrioid | 48 (75.0) | 16 (25.0) | ***0.002*** **(19.284)** |
| Clear cell carcinoma | 33 (86.8) | 5 (13.2) | |
| Mixed | 11 (68.8) | 5 (31.3) | |
| Other | 14 (100.0) | 0 (0.0) | |
| ***FIGO Stage*** | | | |
| I | 112 (73.2) | 41 (26.8) | 0.356 (3.181) |
| II | 47 (75.8) | 15 (24.2) | |
| III | 120 (65.9) | 62 (34.1) | |
| IV | 16 (69.6) | 7 (30.4) | |
| ***Grade*** | | | |
| G1 | 42 (73.7) | 15 (26.3) | 0.406 (1.802) |
| G2 | 54 (74.0) | 19 (26.0) | |
| G3 | 172 (67.2) | 84 (32.8) | |
| ***Measurable Disease*** | | | |
| Non | 203 (76.3) | 63 (23.7) | ***0.001*** **(11.635)** |
| Measurable | 77 (59.7) | 52 (40.3) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 229 (72.9) | 85 (27.1) | ***0.026*** **(4.964)** |
| Resistant | 41 (59.4) | 28 (40.6) | |

**Table 4-4: Clinicopathological significance of XIAP protein expression in the patients of primary ovarian carcinoma. Significant values are in bold**

| XIAP Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **XIAP Negative No. (%)** | **XIAP Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 105 (47.9%) | 114 (52.1%) | |
| Mucinous | 30 (68.2%) | 14 (31.8%) | |
| Endometrioid | 30 (51.7%) | 28 (48.3%) | ***0.023*** **(13.053)** |
| Clear cell carcinoma | 9 (32.1%) | 19 (67.9%) | |
| Mixed | 10 (71.4%) | 4 (28.6%) | |
| Other | 7 (63.6%) | 4 (36.4%) | |
| ***FIGO Stage*** | | | |
| I | 73 (54.5%) | 61 (45.5%) | 0.683 |
| II | 26 (47.3%) | 29 (52.7%) | (1.498) |
| III | 77 (48.4%) | 82 (51.6%) | |
| IV | 11 (55.0%) | 9 (45.0%) | |
| ***Grade*** | | | |
| G1 | 28 (60.9%) | 18 (39.1%) | 0.111 |
| G2 | 36 (58.1%) | 26 (41.9%) | (4.399) |
| G3 | 105 (47.1%) | 118 (52.9%) | |
| ***Measurable Disease*** | | | |
| Non | 125 (54.3) | 105 (45.7) | ***0.047*** |
| Measurable | 49 (43.0) | 65 (57.0) | **(3.939)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 133 (48.5%) | 141 (51.5%) | 0.091 |
| Resistant | 33 (61.1%) | 21 (38.9%) | (2.852) |

#### 4.3.2.2 Outcome analysis

##### 4.3.2.2.1 Univariate analysis of survival

The KM survival analyses of both progression free (PFS) and overall survival (OS) with ATM, PTEN, p85α and XIAP within the whole series of OC patients revealed that downregulated expression of p85α and XIAP were associated with favourable outcomes with prolonged PFS, (p85α: p-value =0.003, XIAP: p-value =0.012), (Figure 4-11) and (Figure 4-12), respectively. However, upregulated expression of PTEN significantly improved patients' PFS, (p-value =0.044) (Figure 4-10). Interestingly, ATM upregulation did not influence the recurrence risk of ovarian tumour cases, (p-value =0.151) (Figure 4-9).

At the designated cut-off points, no statistical significance was calculated for the positive expression of ATM, PTEN, p85α and XIAP with OS, (ATM: p-value =0.345, PTEN: p-value =0.546, p85α: p-value =0.381, XIAP: p-value =0.194), (Figure 4-9), (Figure 4-10), (Figure 4-11) and (Figure 4-12), respectively.

At the transcriptional level, similarly, high PTEN mRNA was in borderline significance with improved PFS (p-value = 0.0636). On the other hand, high XIAP mRNA was associated significantly with poor PFS (p-value = 0.0278) and OS (p-value = 0.0456).

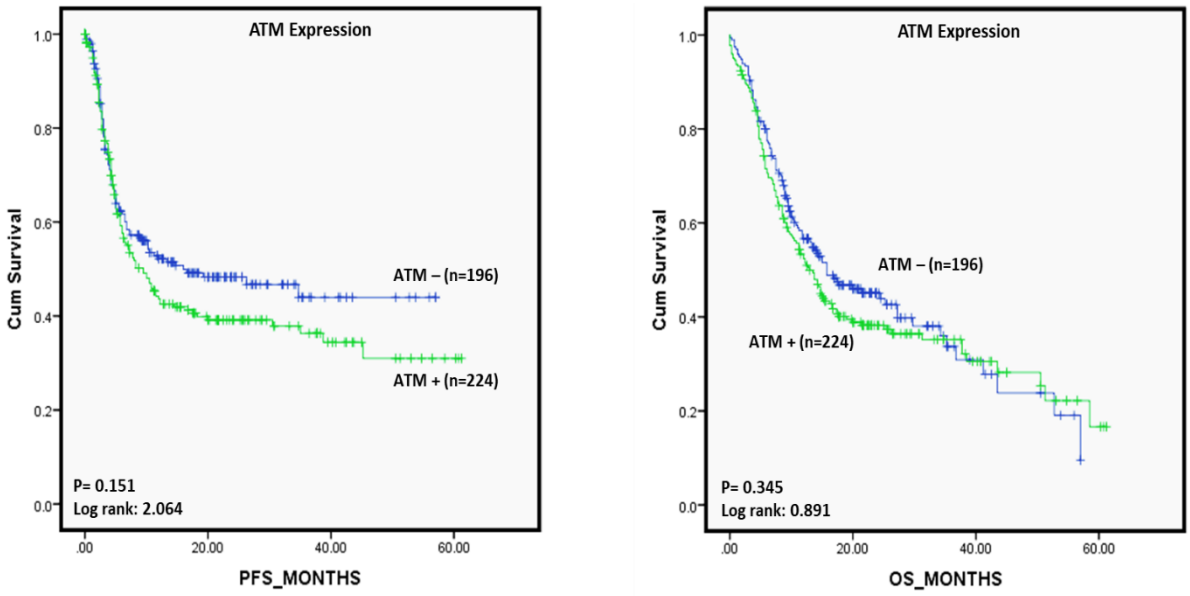



**Figure 4-9: Kaplan Meier plots illustrating the associations between the altered expression of ATM protein with PFS and OS in primary ovarian carcinoma**

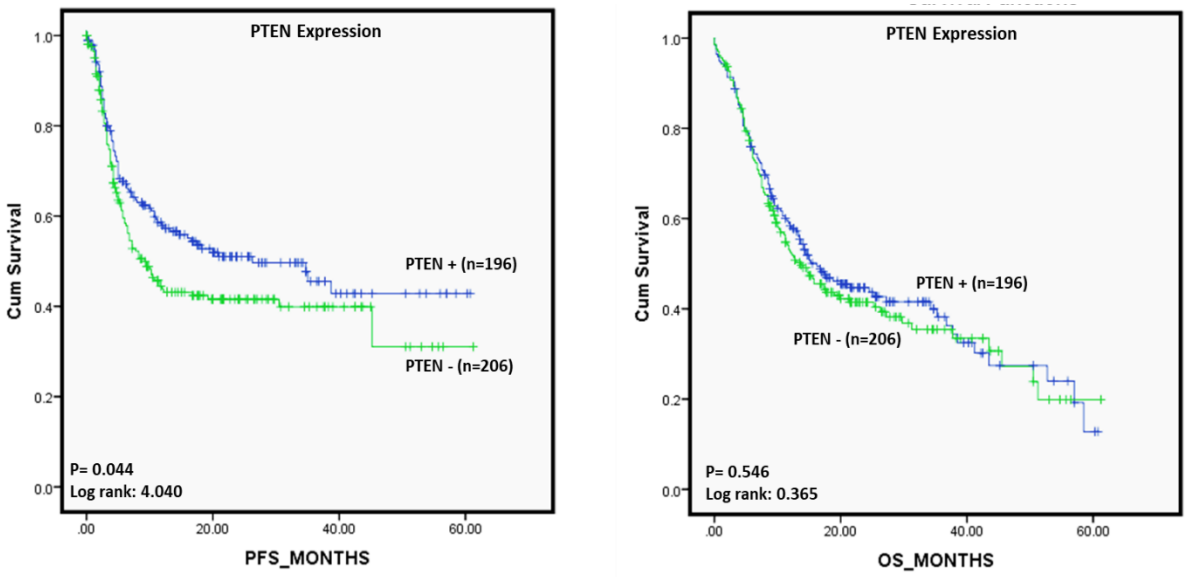



**Figure 4-10: Kaplan Meier plots illustrating the associations between the altered expression of PTEN protein with PFS and OS in primary ovarian carcinoma**

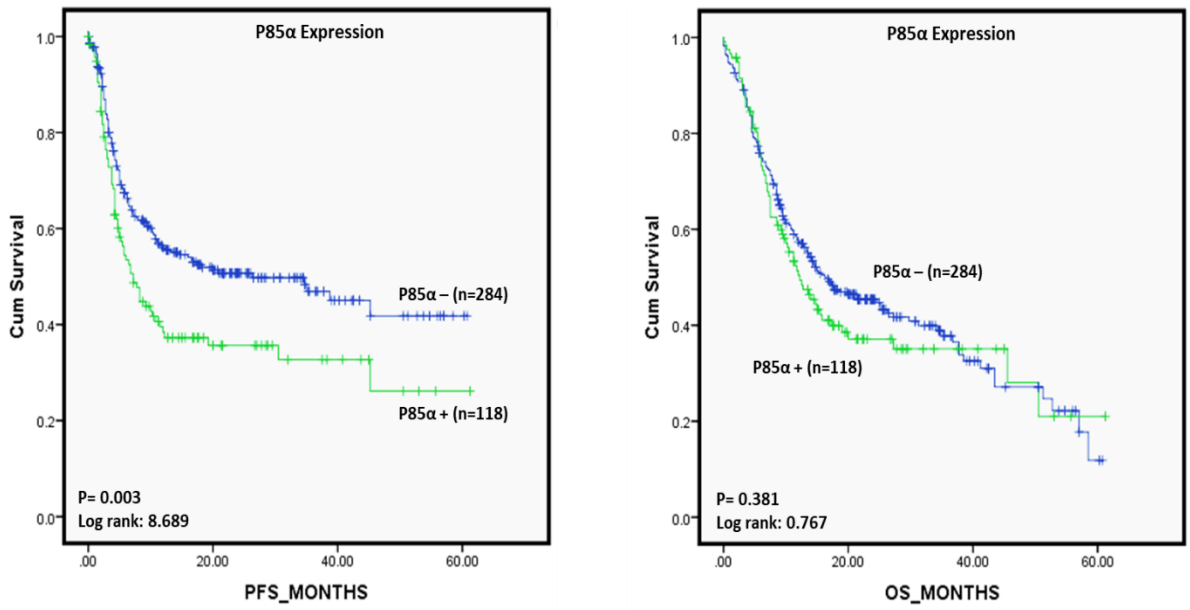



**Figure 4-11: Kaplan Meier plots illustrating the associations between the altered expression of p85α protein with PFS and OS in primary ovarian carcinoma**

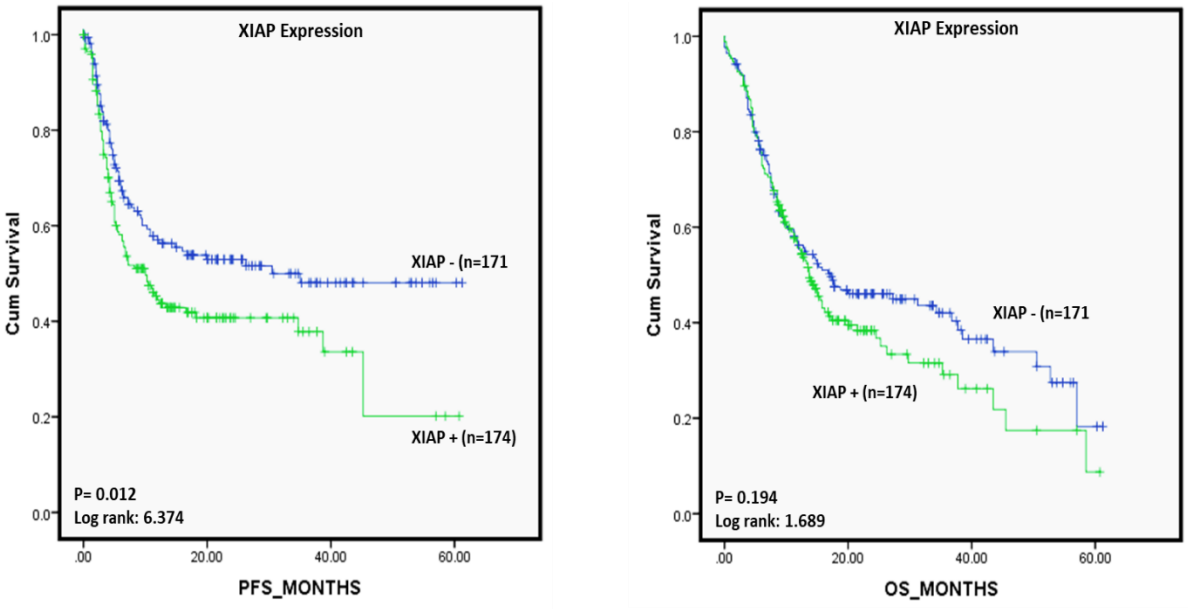



**Figure 4-12: Kaplan Meier plots illustrating the associations between the altered expression of XIAP protein with PFS and OS in primary ovarian carcinoma**

#### 4.3.2.2.2 **Multivariate analysis of survival**

Using cox regression model, in addition to platinum sensitivity, ATM, PTEN, p85α and XIAP were tested. In recurrence outcome, the multivariate analysis revealed that the platinum sensitivity together with downregulated expression of ATM and XIAP exhibited independent prognostic features that may classify a subset of OC patients as a high-risk group: ATM, (p-value =0.017) and XIAP, (p-value =0.036) (Table 4-5). However, analysing the surrogate proteins with regards to OS showed that, in addition to platinum sensitivity, only ATM was an independent predictor of unfavourable outcome, (p-value =0.028) (Table 4-6).

**Table 4-5: Multivariate Cox regression analysis for prediction of ovarian cancer progression free survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | *P-value* | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **ATM** | ***0.017*** | 1.514 | 1.077 | 2.127 |
| **XIAP** | ***0.036*** | 1.429 | 1.024 | 1.995 |
| **PTEN** | 0.388 | 1.214 | 0.782 | 1.885 |
| **P85α** | 0.704 | 0.912 | 0.566 | 1.470 |
| **Platinum sensitivity** | ***<0.0001*** | 19.897 | 12.580 | 31.469 |

**Table 4-6: Multivariate Cox regression analysis for prediction of ovarian cancer overall survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | *P-value* | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **ATM** | ***0.028*** | 1.420 | 1.038 | 1.944 |
| **XIAP** | 0.439 | 1.127 | 0.832 | 1.527 |
| **PTEN** | 0.854 | 0.964 | 0.652 | 1.425 |
| **P85α** | 0.338 | 0.806 | 0.518 | 1.254 |
| **Platinum sensitivity** | ***<0.0001*** | 7.454 | 5.105 | 10.883 |

Given the novel role of ATM and XIAP in multivariate analysis, we investigated their altered expression influence on both PTEN and p85α in terms of cases' outcome. This was proposed in order to identify ATM and XIAP influence on modifying the isolated previously explained prognostic potential of both PTEN and p85α.

#### 4.3.2.2.3 **Outcome analysis in ATM selected cohort**

The KM survival analyses of both PFS and OS for PTEN and p85α within the ATM negative series of OC patients revealed that, upregulated expression of PTEN was associated with favourable outcomes with prolonged PFS and OS, (p-value =0.002) and (p-value =0.031) respectively (Figure 4-13). On the other hand, p85α overexpression was highly significantly correlated with poor PFS, (p-value =0.005) and a trend towards significance with worse OS (p-value =0.075), (Figure 4-14).

Interestingly, the significant effect of both PTEN and p85α on patients' outcome in ATM negative cases was lost in ATM positive selected cohort, PFS (PTEN: p-value =0.660, p85α: p-value =0.173), OS (PTEN: p-value =0.290, p85α: p-value =0.905), (Figure 4-15) and (Figure 4-16) respectively.

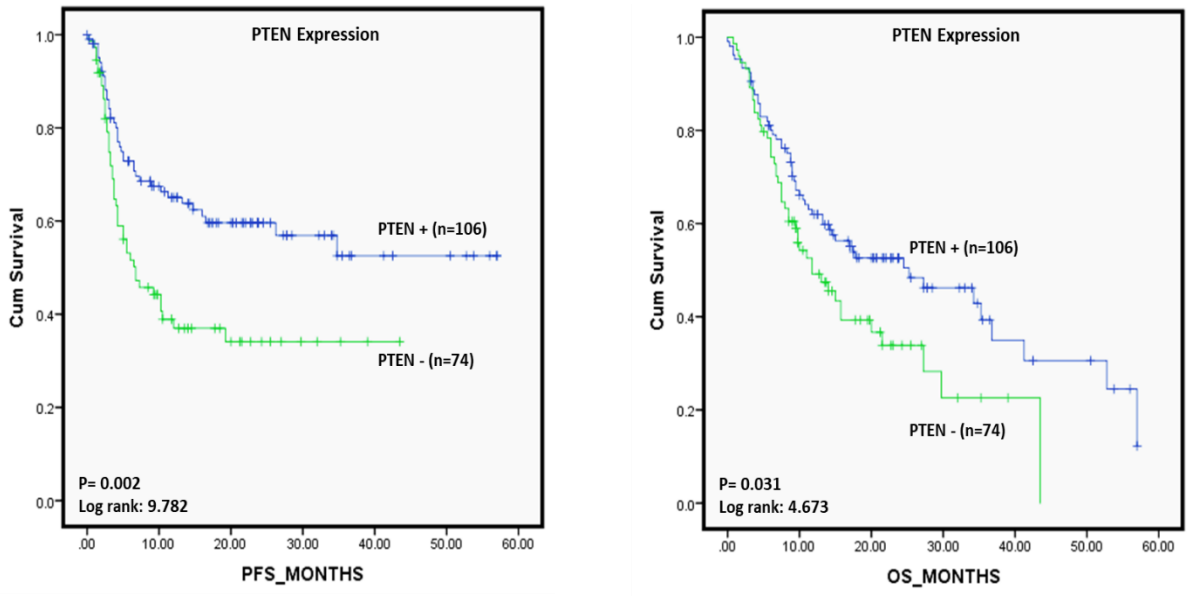



**Figure 4-13: Kaplan Meier plots illustrating the associations between the altered expression of PTEN protein with PFS and OS in primary ovarian carcinoma – ATM negative selection**

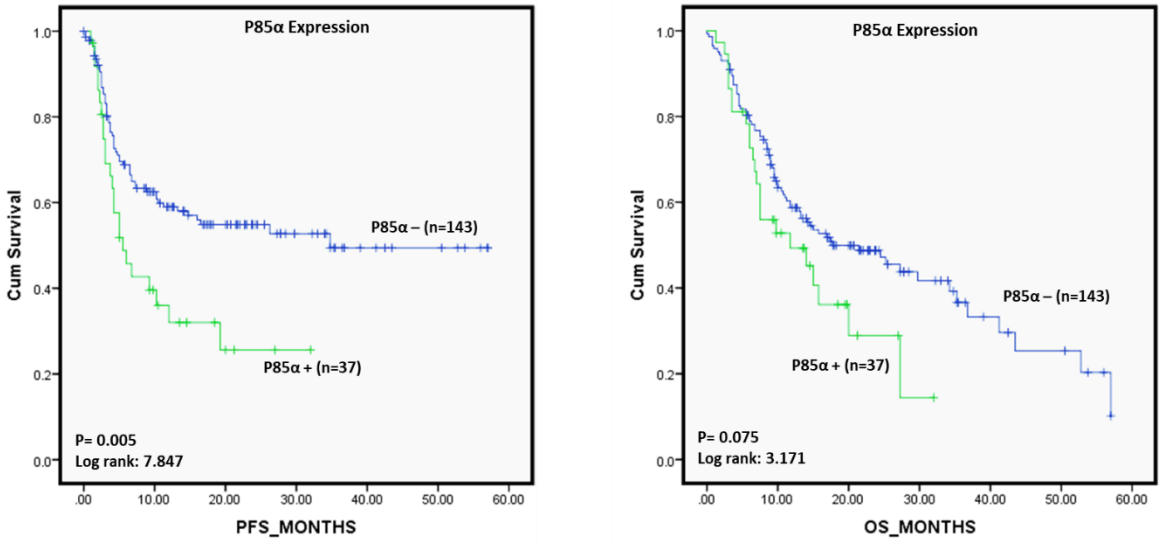



**Figure 4-14: Kaplan Meier plots illustrating the associations between the altered expression of p85α protein with PFS and OS in primary ovarian carcinoma – ATM negative selection**

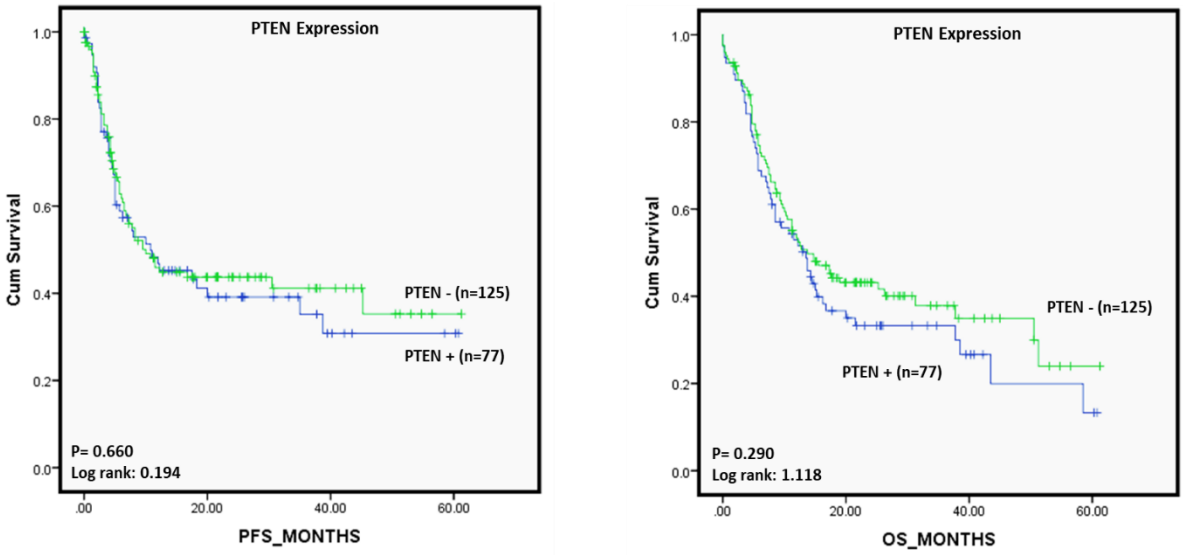



**Figure 4-15: Kaplan Meier plots illustrating the associations between the altered expression of PTEN protein with PFS and OS in primary ovarian carcinoma – ATM positive selection**

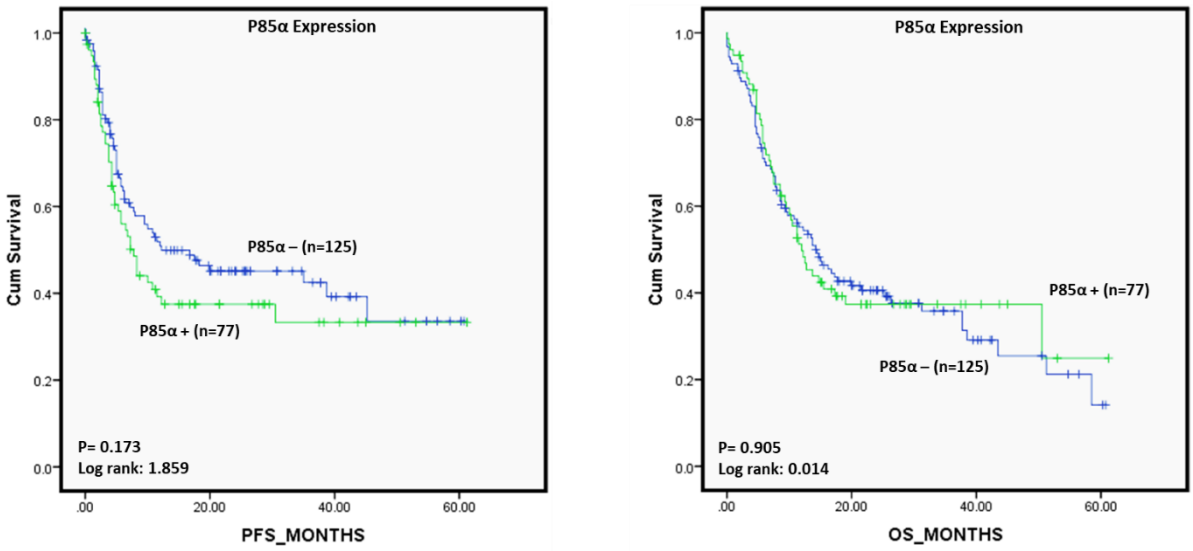



**Figure 4-16: Kaplan Meier plots illustrating the associations between the altered expression of p85α protein with PFS and OS in primary ovarian carcinoma – ATM positive selection**

#### 4.3.2.2.4 **Outcome analysis in XIAP selected cohort**

Similarly to ATM, when cases were tested with the KM survival analyses for both PTEN and p85α within the XIAP negative ovarian tumour cohort revealed that, upregulated expression of PTEN was in trend towards significance with favourable outcomes in terms of both the PFS and OS, (p-value =0.058) and (p-value =0.090) respectively (Figure 4-17). P85α overexpression; however, was significantly correlated with poor PFS (p-value =0.019). On the other hand, the association with OS was not statistically significant (p-value =0.138), (Figure 4-18).

Notably, when XIAP positive cases were the selection, the effect of both PTEN and p85α on patients' survival lost its statistical significance, PFS (PTEN: p-value =0.559, p85α: p-value =0.149), OS (PTEN: p-value =0.392, p85α: p-value =0.707), (Figure 4-19) and (Figure 4-20) respectively.

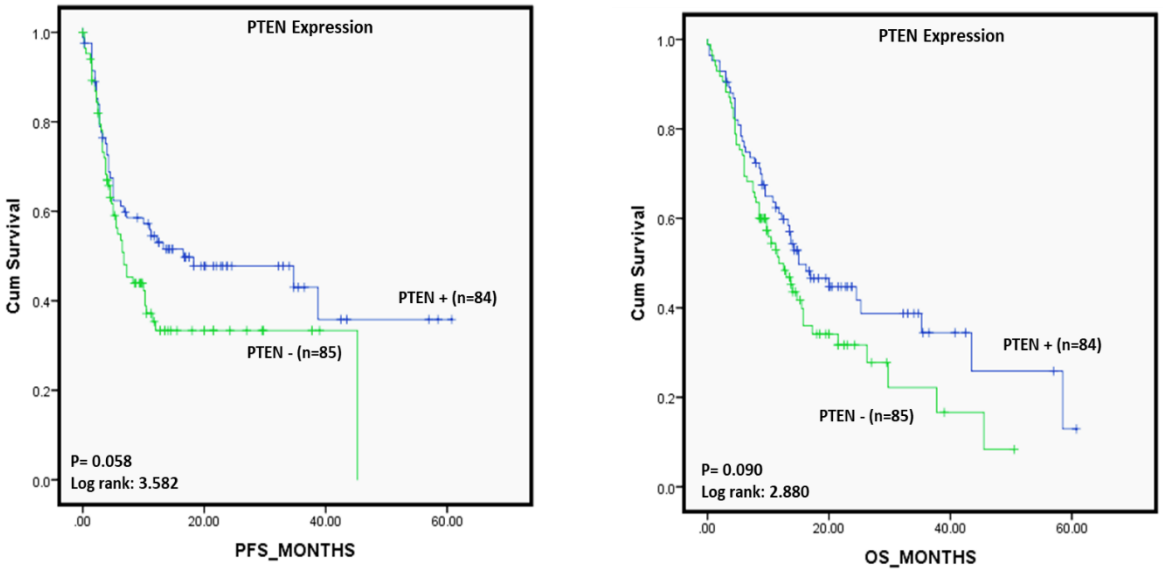



**Figure 4-17: Kaplan Meier plots illustrating the associations between the altered expression of PTEN protein with PFS and OS in primary ovarian carcinoma – XIAP negative selection**

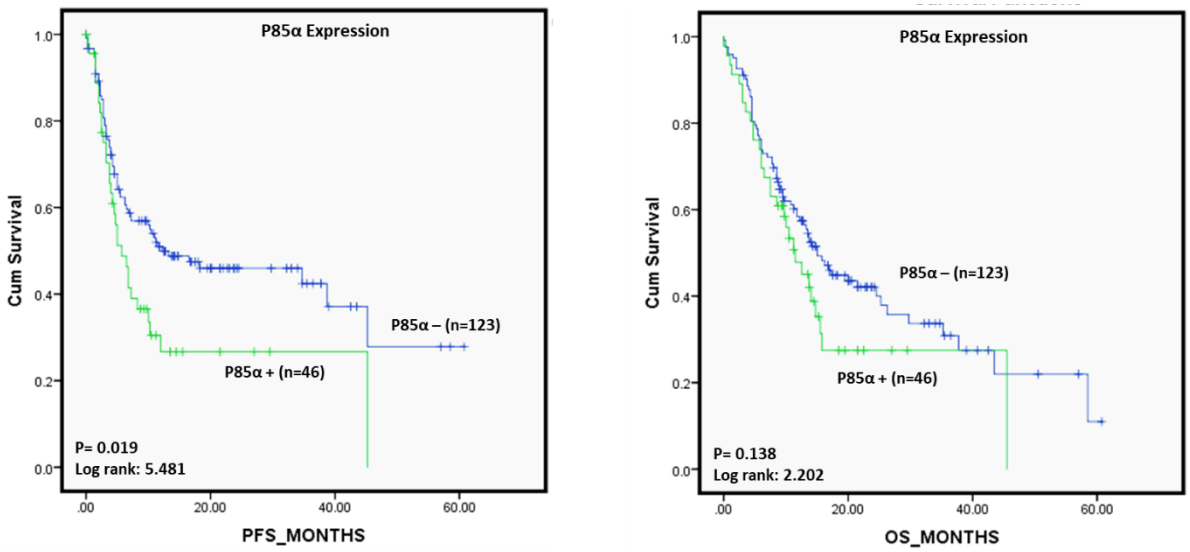



**Figure 4-18: Kaplan Meier plots illustrating the associations between the altered expression of p85α protein with PFS and OS in primary ovarian carcinoma – XIAP negative selection**

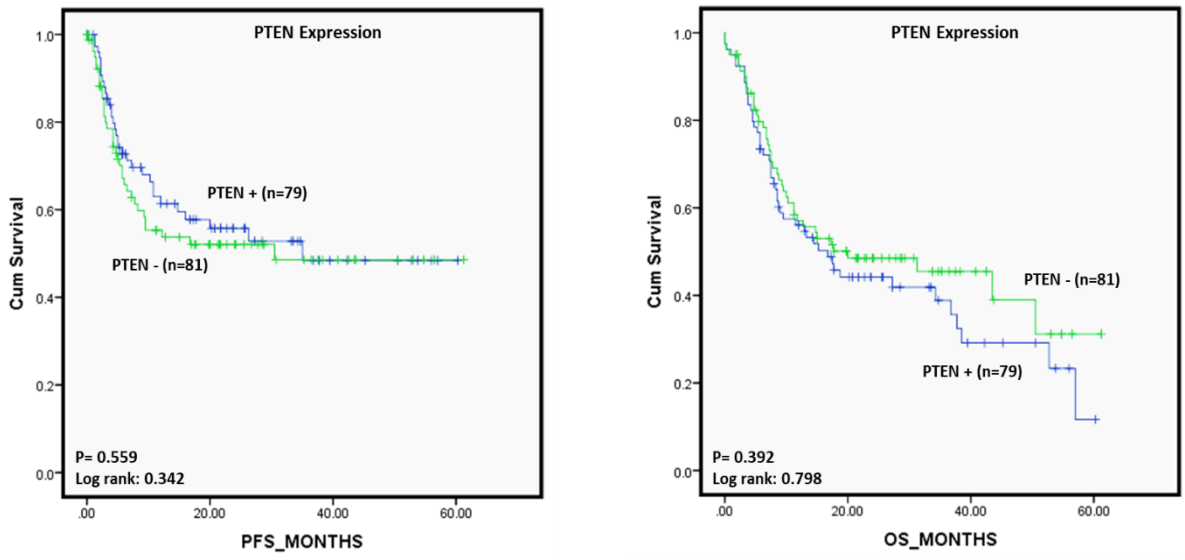



**Figure 4-19: Kaplan Meier plots illustrating the associations between the altered expression of PTEN protein with PFS and OS in primary ovarian carcinoma – XIAP positive selection**

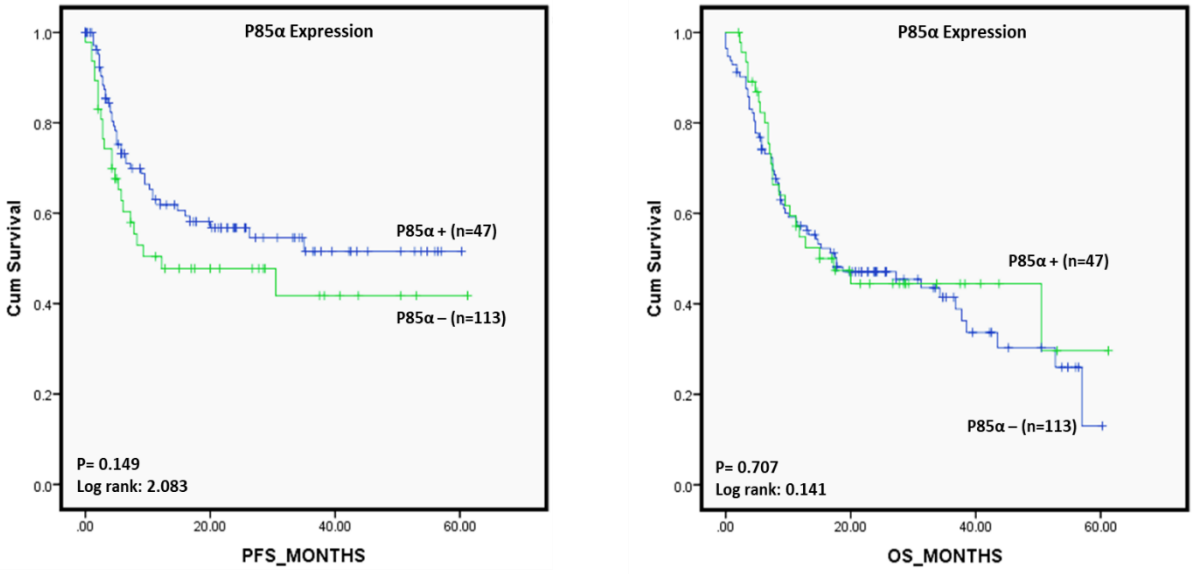



**Figure 4-20: Kaplan Meier plots illustrating the associations between the altered expression of p85α protein with PFS and OS in primary ovarian carcinoma – XIAP positive selection**

#### 4.3.2.2.5 **Outcome analysis for combined ATM and XIAP expression**

After conducting spearman's correlation coefficient between both ATM and XIAP, as expected, it revealed that both proteins are highly associated with each other, (p-value <0.00001). Therefore, the KM estimates for the combined ATM and XIAP expression was also tested. Interestingly, tumours with ATM-/XIAP- had a highly significantly favourable PFS, (p-value =0.005). Similarly, ATM-/XIAP- patients showed improved OS but it did not reach the statistical significance, (p-value =0.113) (Figure 4-21).

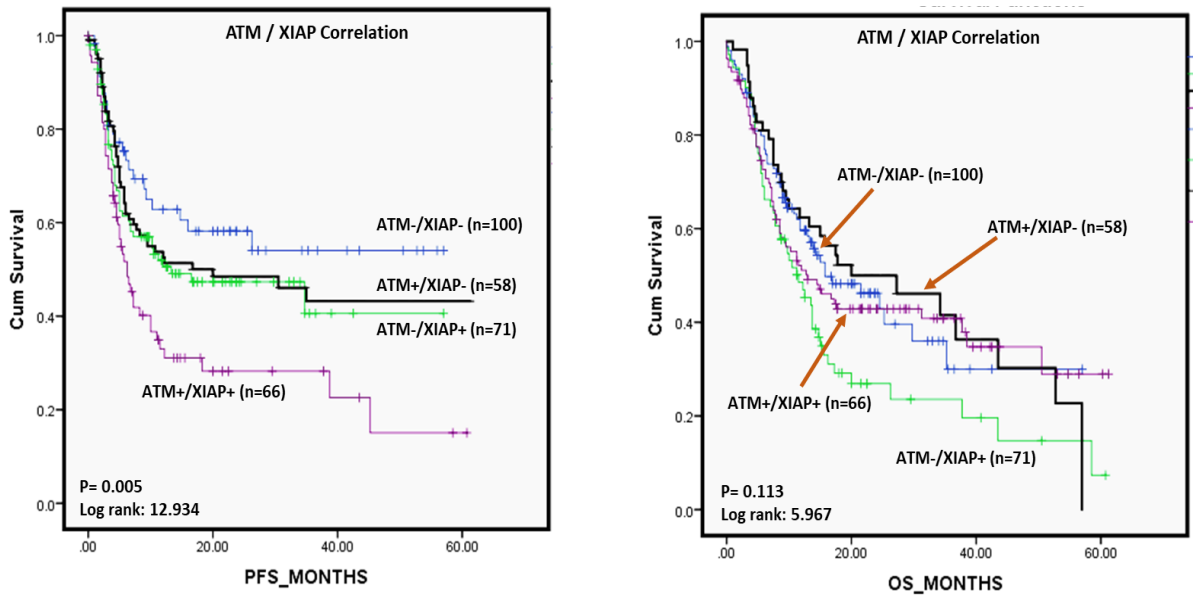



**Figure 4-21: Kaplan Meier plots illustrating the associations between the altered expression of the combined ATM and XIAP proteins with PFS and OS in primary ovarian carcinoma**

## 4.4 Discussion

Despite the previous extensive investigation of various biomarkers in both early and advanced stage OC, the differential gene/protein expression associated with disease progression and platinum sensitivity remains poorly characterised. Although the wide use of platinum-based chemotherapy has significantly improved clinical outcomes with 60-80% initial response rate, most ovarian tumours will develop platinum resistance and patients eventually succumb to the disease (Tsibulak et al., 2019, van Zyl et al., 2018). Therefore, the development of biomarkers to predict the cases' survival is highly desirable. In the current study, we provide evidence that a network including the ATM, PTEN, p85α, and XIAP has a vital role in influencing cancer progression and patients' survival in ovarian tumour cases treated primarily by surgical resection. Moreover, we have shown how the differential expression status of ATM and XIAP might influence PTEN and p85α function indicating distinctive clinically relevant groups of cancer patients.

ATM is a crucial signalling protein that activates a complex network of DDR pathways that coordinate the cell cycle checkpoint and DNA repair functions (Abraham, 2001). ATM mutation can result in cell sensitivity to DNA damage and cancer predisposition (Helleday, 2010, Kastan and Bartek, 2004, McCabe et al., 2006). ATM protein is exclusively expressed in the nucleus (Abdel-Fatah et al., 2014c, Abdel-Fatah et al., 2014b, Ye et al., 2014, Hashiguchi et al., 2004, Grabsch et al., 2006). This comes in agreement with our findings, as this protein was exclusively expressed in the nucleus of ovarian malignant cells.

Multiple studies on different types of cancers have reported the prognostic effect of the ATM positive protein expression. However, different views have been described regarding the ATM function and its role in influencing tumours' behaviour and patients' outcome (Abdel-Fatah et al., 2014c, Ye et al., 2014, Hashiguchi et al., 2004, Grabsch et al., 2006, Abdel-Fatah et al., 2013, Abdel-Fatah et al., 2014b). One view by Abdel-fatah et al in a study performed on breast cancer, ATM high expression was linked to smaller tumour size, lower histology grade, less invasiveness and improved survival (Abdel-Fatah et al., 2014b). Similarly, in colonic cancer ATM overexpression was shown to correlate with improved OS; however, no statistical association could be established between this protein expression and tumour stage or lymph node metastasis (Grabsch et

al., 2006). On the other hand, a study performed on 97 cases of serous OC concluded that ATM overexpression had no relation with the clinical stage, tumour grade and chemotherapy status (Ye et al., 2014). However, Hashiguchi et al identified a significant association between loss of ATM protein and non-serous ovarian tumours when compared with the serous histology subtype. In contrast, no correlation was identified with the cases' survival (Hashiguchi et al., 2004). Abdel-fatah et al identified a significant positive link between high ATM expression with serous carcinomas and platinum resistance. Furthermore, in univariate analysis ATM high expression in the nucleus was linked to adverse clinical outcome (Abdel-Fatah et al., 2014c).

In our study, ATM high expression was significantly associated with serous tumour histology type. On the flip side, it did not show a reliable prognostic relevance or significant association with the other clinicopathological variables in OC. Our results come partially in line with the previous mentioned studies. It is evident that there is some controversy on the role of ATM in influencing cellular fates and outcome in patients with cancer, including ovarian tumours. Different studies have not confirmed this and importantly, the results in this chapter are supported by similar findings from other studies which identified no correlation between ATM overexpression and clinicopathological characteristics or cases' survival (Ye et al., 2014, Hashiguchi et al., 2004).

PTEN is a tumour-suppressor gene (Wang et al., 2015, Ali et al., 2019). It has been emphasised by different studies that PTEN is involved in the conduction of multiple cellular signalling pathways, regulation of cell cycle, apoptosis and autophagy. This protein has a vital role in cell adhesion, migration and angiogenesis. PTEN abnormal expression and genetic mutations were observed in glioma, breast, prostatic cancer and other tumours. This altered expression of PTEN has an important role in the occurrence, development and migration of tumour cells (Li et al., 1997, Wang et al., 2015).

Interestingly in ovarian tumours, PTEN down-regulation can predispose to the development and progression of cancer. The expression level of PTEN in normal ovarian tissue, benign ovarian tumours, borderline ovarian tumours and ovarian cancer gradually decreased, a previous study concluded (Laudański et al., 2011, Skírnisdóttir and Seidal, 2011). PTEN is mainly expressed in the cytoplasm of the

malignant ovarian cells, with occasionally stained nuclei (Wang et al., 2015, Abe et al., 2013). Wu et al showed that, transfecting PTEN gene in platinum resistant OC cell lines significantly enhanced cisplatin sensitivity (Wu et al., 2007). Moreover, Yan et al (Yan et al., 2006) demonstrated that PTEN protein could make the cisplatin-resistant OC cell lines sensitive to chemotherapy. Remarkably, a previous study demonstrated that not just PTEN expression is decreased in ovarian tumours but also a more significant decrease in chemotherapy resistant cells, suggesting the existence of a correlation between PTEN low expression with OC progression and resistance to treatment (Ying et al., 2015). Similarly, in endometrial cancer reduced levels of PTEN protein was observed when compared with the normal endometrial tissue specimens, further highlighting the protective role of this protein (Gao et al., 2009). Cai et al also linked the reduced PTEN expression detected by IHC in OC specimens to poor survival (Cai et al., 2014).

The above findings come in agreement with the current study, the examination of the stained TMA sections showed that PTEN protein was predominately sub-localised in the cytoplasmic part of the malignant cells. Moreover, the reduced biological activity of PTEN protein in primary ovarian tumour tissue specimens was in positive association with serous histology type. On the other hand, PTEN overexpression significantly improved patients' outcome. The high cytoplasmic expression of PTEN did not appear to significantly affect the response to platinum treatment in the studied cohort.

In the light of some differences found with other studies regarding the relationship between PTEN overexpression and response to platinum therapy, it seems that the constitution of the cohort itself could have an impact on the expression and behaviour of these proteins. In this context, a study conducted by Abe et al (Abe et al., 2013) on clear cell carcinoma demonstrated no association between the aberrant PTEN expression and development of platinum resistance, an inline observation with the current research.

Plethora of studies focused on different methods in order to evaluate the expression of p85α subunit in several tissues' tumour specimens in order to highlight its role in the regulation of the fundamental cellular processes, such as the cell cycle and cell survival (Chamberlain et al., 2004, De Santis et al., 2009, Luo et al., 2005, Aziz et al., 2009). To the best of our knowledge, there is very

limited information on the immunohistochemical expression of p85α protein in primary OC. Here, our results show that the aberrant localisation of p85α protein in the cytoplasm of tumour cells has implications on both the clinicopathological patients' characteristics and their outcome. Downregulated p85α expression showed significant association with improved surgical resection, platinum sensitivity and patients' survival. These results comes in agreement with a study conducted by Zhang et al (Zhang and Sun, 2007). In this study, p85α protein was evaluated by WB methodology and its level was compared between normal ovarian tissue, benign tumours, borderline malignancy and OC. A significantly elevated expression of p85α was observed in cancerous tissue. Similarly, higher ovarian tumour grade was associated with upregulated p85α protein expression (Zhang and Sun, 2007). Interestingly, preclinical inhibition of p85α activity enhanced platinum sensitivity in malignant cells (Zhang and Sun, 2007).

Similarly, Zhou et al conducted IHC study on one hundred and twenty six primary invasive breast carcinoma tissue specimens, p85α was predominantly sub-localised in the cytoplasm and it was associated with aggressive tumour characteristics and unfavourable outcome of the patients (Zhou et al., 2014). Yang et al (Yang et al., 2016 816) also concluded similar findings.

This current study revealed that the downregulated expression of p85α protein is a significant favourable prognostic factor for primary OC. This protein may play an important role in the development of platinum resistance and cancer progression. Further functional assessment of p85α to determine its mechanistic roles and therapeutic potential in invasive OC is warranted.

A member of the IAP family, XIAP prevents apoptosis through direct inhibition of effector caspases (caspases 3, 6, 7, and 9) (Gyrd-Hansen and Meier, 2010, Zhang et al., 2018a). It has been well-documented that XIAP plays a crucial role in tumour maintenance in different organs malignancies (Lin et al., 2013, Gu et al., 2009, Tosello et al., 2016, Schile et al., 2008). Lack of XIAP in ovarian tumour cells promoted their sensitisation to apoptosis (Sasaki et al., 2000). Moreover, high expression of this protein in OC cells can be used as a determinant of the tumour prognosis and response to therapy (Fraser et al., 2003, Asselin et al., 2001, Li et al., 2001, Sasaki et al., 2000). Currently, there is increasing evidence

focusing on XIAP suppression to develop new strategies for cancer prevention and treatment (Chaudhary et al., 2016).

Our investigation of the clinicopathological impact of the altered protein expression of XIAP protein in the primary OC cases unveiled strong positive association with aggressive tumour phenotype. XIAP positive expression in the cytoplasm enhanced the aggressive characteristics of OC, such as clear cell and serous histology subtypes. In addition, increased the likelihood of residual malignancy following surgical excision. On the other hand, low/negative XIAP level improved patients' recurrence free outcome. Zhang et al supported the findings here; highlighting a high expression of cytoplasmic XIAP was associated with shorter PFS (Zhang et al., 2018a). Similarly, XIAP overexpression significantly reduced PFS in clear cell carcinoma cases (Miyamoto et al., 2018). In addition, Hussain et al linked XIAP low/negative expression to favourable tumour characteristics in breast cancer (Hussain et al., 2017).

Clarification of the crucial biomarkers that can influence patients' survival is vital. In general and among all the investigated proteins, with the exception of PTEN, all the markers in this chapter were associated with worse patient's outcome. However, only ATM and XIAP were independent poor prognostic indicators for PFS. This comes in line with Grabsch et al, in which ATM was independent poor prognostic protein in colorectal cancer (Grabsch et al., 2006). Moreover, a trend towards significance was identified for high ATM expression in breast cancer as independent marker of prognosis (Abdel-Fatah et al., 2014b). ATM inhibition has recently emerged as a promising anti-cancer strategy (O'Connor, 2015). Moreover, in OC ATM high expression was correlated with aggressive clinicopathological parameters and poor outcome; however, it did not show significance as an independent prognostic protein in multivariate analysis (Abdel-Fatah et al., 2014c), this may be related to the limited number of cases included in the study.

Similarly, in this study XIAP was an independent factor for poor PFS, a finding which comes in agreement with Miyamoto et al (Miyamoto et al., 2018) who showed that XIAP was independent factor for poor outcome in clear cell carcinoma. Moreover, XIAP knockdown not just retarded ovarian tumour cells proliferation but also it promoted carboplatin-induced cell apoptosis (Zhang et al., 2018a). In

another study on breast cancer, multivariate analysis revealed XIAP as an independent poor prognostic factor (Hussain et al., 2017).

The above observations suggest that ATM and XIAP high expression tumours may indicate a high-risk subpopulation of invasive OC patients. Therefore and further splitting the studied cohort according to their corresponding ATM and XIAP expression, interestingly, when both biomarkers were positive, PTEN and p85α lost their significance on patients' outcome, a cardinal observation in the current study. Importantly, patients with combined low/negative ATM and XIAP had improved recurrence free outcome. Given the previously described role for ATM and XIAP in influencing patients' outcome (Ali et al., 2019), we speculate a novel role for both proteins in modulating the effect of both PTEN and p85α. However, further detailed functional studies are required to confirm this hypothesis.

In conclusion, ATM, PTEN, p85α and XIAP expression are predictors of patients' outcome in primary ovarian tumours. The data presented here reveal a novel network between the investigated proteins with vital clinical applications in OC. In addition, PTEN and p85α are more likely to behave in cell specific context with regard to ATM and XIAP expression status. We propose that both latter biomarkers have a master role in orchestrating the clinical influence of the other network proteins. Therefore, their expression revealed a distinct clinical group with different biological features from others with a remarkable driving effect on outcome. Future evaluation utilising functional and preclinical studies is essential to further explore the role of this network in primary ovarian tumours.

## 4.5 Study limitations related to this chapter

1- The data was assessed in one centre only and no multicentre validation was considered.

2- All the biomarkers studied in this chapter showed either nuclear or cytoplasmic expression; however, this does not exclude the possibility that they are expressed in the other compartments of OC cells. Therefore, future studies can highlight the importance of subcellular localisation of these proteins, in addition to the role of their positive expression in the tumour surrounding areas or the tumour stromal tissues.

3- Data assessed only through the utilisation of IHC methodology. Future preclinical functional studies are required to confirm our findings.

# 5 DNA Damage Repair Proteins (PARP1, XRCC1 and polβ) have Unfavourable Potential Prognostic Role in Primary Ovarian Cancer

## 5.1 Introduction

To date and in spite of the availability of personalised cancer therapeutic agents and the advances in platinum-based chemotherapy, the survival in advanced ovarian cancer (OC) patients remains poor. Platinum agents' cytotoxicity is directly related to their ability in induction of intra-strand and inter-strand DNA adducts in cells, which if unrepaired, can promote the development of DNA double strand breaks (DSBs) during replication. This induced DNA damage, is detected and processed through the DNA repair mechanisms (Lord and Ashworth, 2017, Ali et al., 2020).

The enzyme Poly-(ADP)-ribose polymerase 1 (PARP1) is critically involved in DNA repair. The formation of PAR (poly-ADP-ribose) polymers occurs when PARP1 is activated through binding to DNA single-strand breaks. As a result, other DNA repair factors (including XRCC1) will be recruited at the DNA damage sites resulting in efficient DNA repair (Lord and Ashworth, 2017, Veuger et al., 2004, Parsons et al., 2005). Interestingly, in solid tumours, it has been demonstrated that PARP1 is vital and strongly related to promoting platinum chemo-resistance and it gets upregulated in different types of cancers (Mylavarapu et al., 2018, Tangutoori et al., 2015, Zhang et al., 2020). Therefore, this might indicate that PARP1 can contribute to resistance to DNA-damaging anticancer treatments (Hirai et al., 1983, Nomura et al., 2000, Zaremba et al., 2011). For these reasons, PARP1 has become an important target in cancer therapy. Therefore, this led to the development of PARP1 inhibitors as anticancer drugs (Curtin, 2013b, Javle and Curtin, 2011, Underhill et al., 2011, Curtin, 2013a). Furthermore, in both breast and OC, the preclinical studies, and clinical trials confirmed PARP1 inhibitors effectiveness in the management of those tumours (Curtin, 2013a, Underhill et al., 2011, Bryant et al., 2005, Hartwell et al., 1997, Curtin, 2005, Nijman, 2011, Coskun et al., 2019). However, resistance to PARP inhibitors can develop because of multiple acquired or intrinsic factors (Lord and Ashworth, 2017, Lord et al., 2015).

X-ray repair cross-complementing protein 1 (XRCC1) has essential roles in DNA repair. It is a scaffolding protein and its N-terminal domain binds to DNA strand breaks (Caldecott, 2003, London, 2015, Ali et al., 2020). XRCC1 is composed of three domains, BRCT I domain that interacts with PARP1, BRCT II domain which

binds to Ligase III and the N-terminal domain which links to POLβ (London, 2015). XRCC1 is involved in coordination of DNA Base Excision Repair (BER) and Single-Strand Break Repair (SSBR). In addition, XRCC1 also has roles in alternative non-homologous end joining (alt-NHEJ) pathway for double strand breaks (DSBs). Delays in SSB re-joining leading onto SSBs and ultimately if unrepaired, to double DSBs (Moser et al., 2007, Hoch et al., 2017). Moreover, XRCC1 deficiency potentiates chemotherapy cytotoxicity (Moser et al., 2007) including platinum sensitivity in OC cells (Abdel-Fatah et al., 2013). Furthermore, studies in OC patients have demonstrated a significant association between XRCC1 positive expression and clinical outcome (Cheng et al., 2012, Li and Li, 2013, Abdel-Fatah et al., 2013). Interstingly, XRCC1 polymorphisms can have a recognised impact on risk and prognosis of different cancerous tissues including ovarian tumours, findings concluded by numerous studies (Chen et al., 2017, Wang et al., 2016a, Li et al., 2016, Yuan et al., 2015, Chen et al., 2015b, Du et al., 2015, Zhang et al., 2018b, Li and Li, 2013).

It is worth mentioning that, Mitochondria in mammalian cells contains an efficient BER system that can employ DNA polymerase β (POLβ) in order to remove some of the oxidative DNA damage products (Prasad et al., 2017). Therefore, POLβ has a key role in maintaining the genomic stability (Prasad et al., 2017). Polβ is a distributive polymerase that devoid any proof reading activity. It interacts with several components of the BER machinery such as XRCC1, PARP1 and ligase III to accomplish its biochemical functions (Yamtich and Sweasy, Wallace, Nicolay et al.). About 30% of human tumours appear to express polβ variant proteins, which are associated with aggressive tumour phenotype (Sobol et al., 1996). Moreover, an increase level of polβ was measured in different types of cancer, including prostate, breast, and colonic (Srivastava et al., 1999). Nevertheless, both Polβ levels and activity were noted to increase in chronic myeloid leukaemia patients (Sobol et al., 1996). Similarly, in OC, higher levels of POLβ was also detected in cancerous tissue specimens (Canitrot et al., 2000). It has been reported by different researchers that POLβ protein deficiency can promote the hypersensitivity of tumour cells to alkylating agents, induced apoptosis, and chromosomal breaking (Canitrot et al., 1998, Bergoglio et al., 2002, Bergoglio et al., 2001, Srivastava et al., 1999).

### 5.1.1 Hypothesis

Inactivation of DNA repair mechanisms is an important oncogenic event in most human cancers. It is hypothesised that PARP1, XRCC1 and Polβ interact with each other resulting in divergent associations and outcome in OC patients. Here, we aimed to explore the prognostic and biological significance of PARP1, XRCC1 and POLβ proteins expression in primary OC tissues using the large and well-characterised primary OC series. Our interpretation of the prognostic and altered expression of PARP1, XRCC1 and POLβ may reveal new insights into the prognostic knowledge of ovarian tumours.

### 5.1.2 Aims

1- Determine the immunohistochemical expression of PARP1, XRCC1 and POLβ proteins in a large well-characterised cohort of primary operable OC cases.

2- Correlate PARP1, XRCC1 and POLβ expression with the clinicopathological variables. In addition, testing the effect of the combined expression of the above biomarkers with clinicopathological data in order to illustrate their joint biological influence on OC patients.

3- Assess the isolated and combined prognostic and predictive potential of PARP1, XRCC1 and POLβ in the whole patients' cohort, platinum sensitive and resistant series.

4- Explore the independent prognostic marker that can influence the patients' outcome and pre-clinically, will identify the impact of its knockdown on PARP1 level in both platinum sensitive and resistant OC cell lines.

## 5.2 Methods

### 5.2.1 Clinical studies

Tissue microarray from 525 primary OC cases was prepared and then immunohistochemical staining was performed as described previously in chapter two of this thesis. Before that, the specificity of the tested antibodies was confirmed using WB technique. The full details of WB procedure including the concentrations of antibodies used and the purchased materials can be found in the methodology section, (Table 2-3). The expression of PARP1, XRCC1 and POLβ antibodies used in IHC was determined in the whole, platinum sensitive and resistant series. The optimised concentration of the recombinant Ag solution required us to totally neutralise the binding capacity of the primary mouse anti-human PARP1 antibody was 1:600 with 60 min incubation at room temperature. TMA sections were incubated for 30 min at room temperature with 1:250 of the second antibody, anti-XRCC1 mouse monoclonal. Finally, the optimised dilution factors for the anti-polβ rabbit polyclonal antibody was 1:200 with 60 minutes incubation time at room temperature. Optimisation procedures performed prior to selecting the ideal antibody concentrations, full details in supplementary section.

### 5.2.2 Preclinical studies

Both platinum sensitive and resistant OC cell lines, A2780 and A2780Cis respectively were used, detailed in (Table 2-2). The well-described platinum sensitive A2780 cell line was established from a patient with previously untreated OC. However, A2780Cis was developed by chronic exposure of the parent cisplatin-sensitive A2780 cell line to increasing concentrations of cisplatin (Beaufort et al., 2014). Cells were cultured in RPMI medium supplemented with 10% FBS and 1% penicillin streptomycin. To maintain cisplatin resistance in A2780Cis, cells were exposed to 1 μM cisplatin every 2–3 passages. In all XRCC1 KD experiments, cell lysate sample was retrieved on day 3 in order to ensure that the XRCC1 KD will persist for the rest of the experiments.

For XRCC1 transient SiRNA transfection, cells were seeded at 60–80% confluency in t25 flasks overnight. Then using Lipofectamine 3000 reagent, XRCC1 siRNA were delivered to the cells in opti-mem and as per the manufacturer's protocol.

RIPA buffer supplemented with phosphatase and protease inhibitors cocktails was used for collecting all cell lysates, full details of the procedure can be found in the methodology chapter. The efficiency of transfection was confirmed using WB technique as detailed in (section 2.6.4).

## 5.3 Results

### 5.3.1 Specificity of the tested antibodies

The evaluation of WB images for the anti-PARP1, anti-XRCC1, and anti-POLβ primary antibodies supported a specific binding affinity to a single protein band for both ovarian cell lysates, A2780 and A2780Cis around their predicted molecular masses as instructed by the supplying company. It was 116 kDa for the PARP1 protein, (Figure 5.1), 85 kDa for the XRCC1 protein, (Figure 5.2), and 38 kDa for the POLβ, (Figure 5.3).

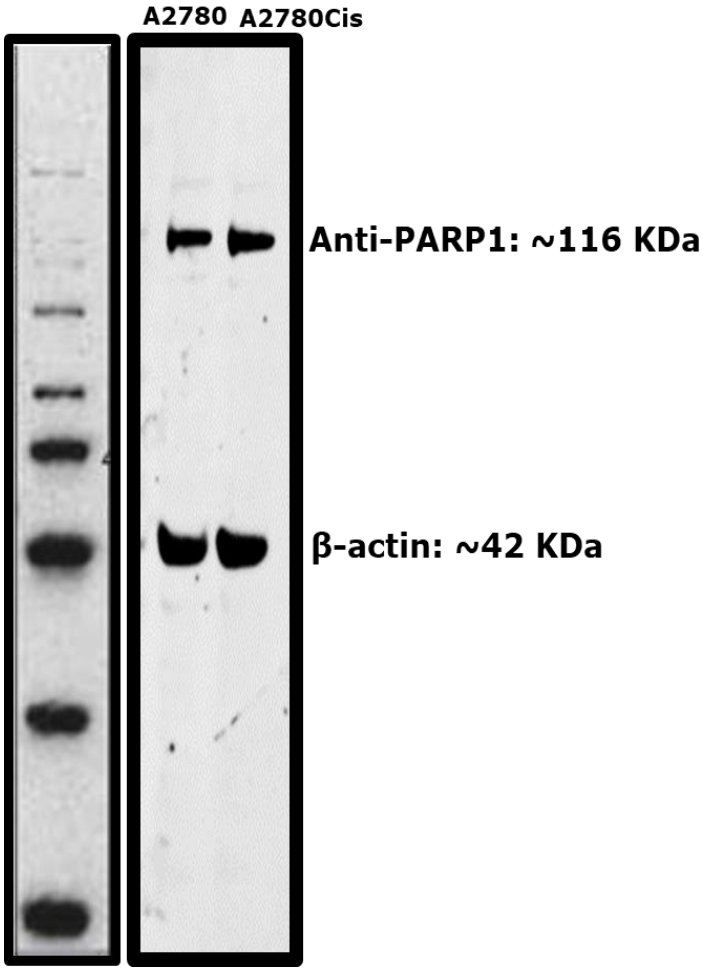



**Figure 5-1: Binding specificity of the anti-PARP1 antibody.**
**WB membrane showed specific antigen-binding of for. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

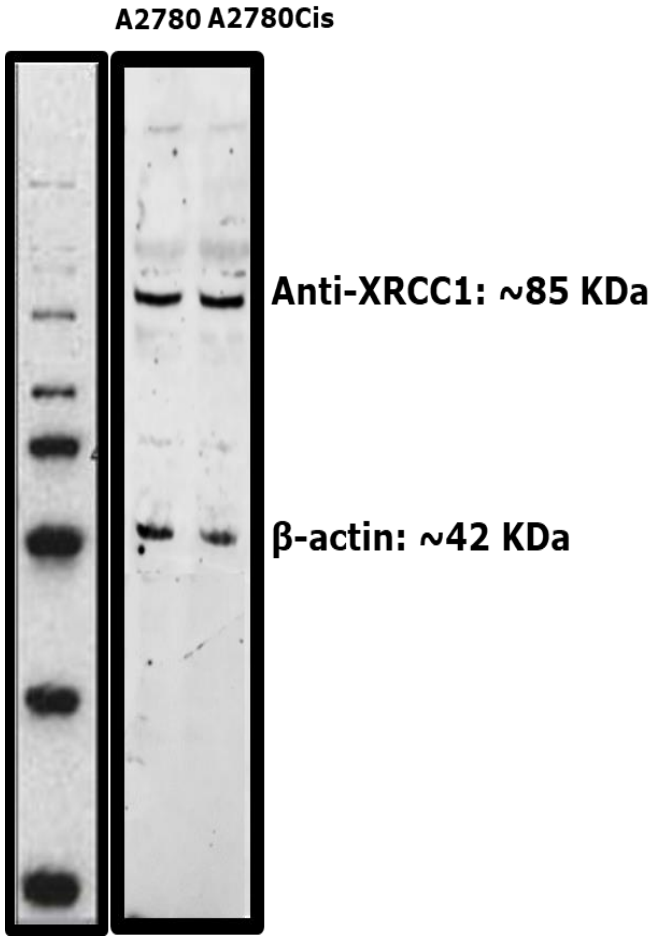



**Figure 5-2: Binding specificity of the anti-XRCC1 antibody.**
**WB membrane showed specific antigen-binding of for anti-XRCC1 antibody around ~85 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

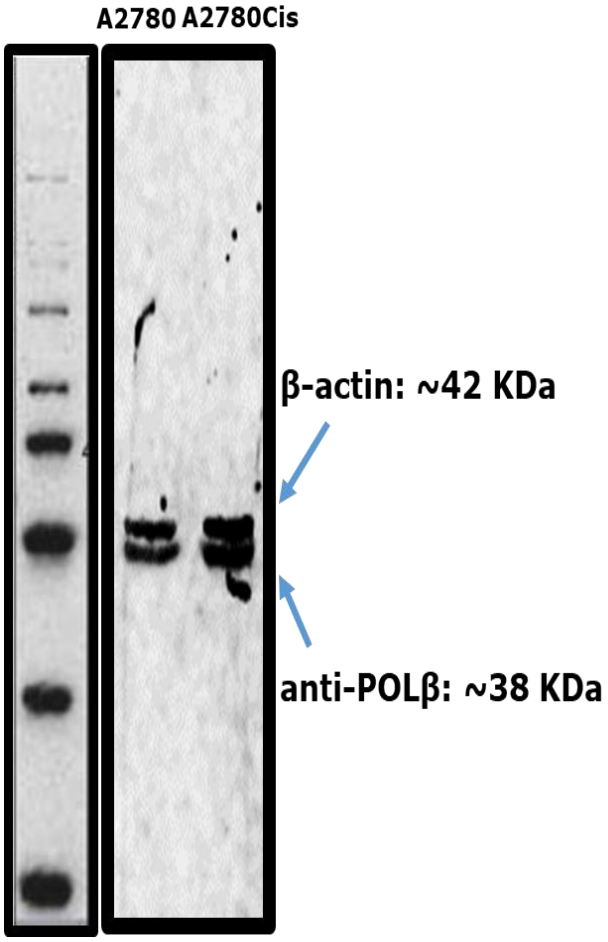



**Figure 5-3: Binding specificity of the anti-POLβ antibody.**
**WB membrane showed specific antigen-binding of for anti-POLβ antibody around ~38 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

### 5.3.2 Immunohistochemical results

Altered protein expression of PARP1, XRCC1 and POLβ in OC patients have been assessed using IHC methodologies. Minimal of two cores were assessed for each patient in different settings by the researcher and an expert histopathologist. Then, this expression was analysed and correlated with the patients' clinicopathological variables to identify their prognostic importance. Furthermore, testing the protein interactions and its clinical impact with OC progression was performed. The examination of the immuno-stained TMA sections revealed a unique localised distribution for the proteins mentioned above among the different cellular compartments.

#### 5.3.2.1 The expression pattern of the studied antibodies

##### 5.3.2.1.1 PARP1

The expression of this protein was mainly nuclear in the tumour cells with variable intensities, although there were some faint staining cytoplasmic expression; it was not taken into consideration, (Figure 5-4). The cut-off point (H-score) using the median value was set at < 80, where 47.9% of the cases were classified as negative/low, while the remaining 52.1% had high expression.

##### 5.3.2.1.2 XRCC1

Similarly, this biomarker revealed mainly nuclear expression in invasive OC cells, (Figure 5-5). The cut-off for its positive nuclear expression was chosen at >100 (H-score) using the median distribution value. 33.3% of the specimens showed negative/low staining, and 66.7% were rendered high in expression.

##### 5.3.2.1.3 Polβ

In a similar staining localisation to the above two proteins, polβ expression was mainly nuclear with faint cytoplasmic perinuclear staining, which was not counted into consideration, (Figure 5-6). Importantly, Low/negative nuclear polβ expression was defined by median H-score of ≤180. Out of the total valid cases, 56.6% were rendered negative/low, while 43.4% deemed high in their expression.

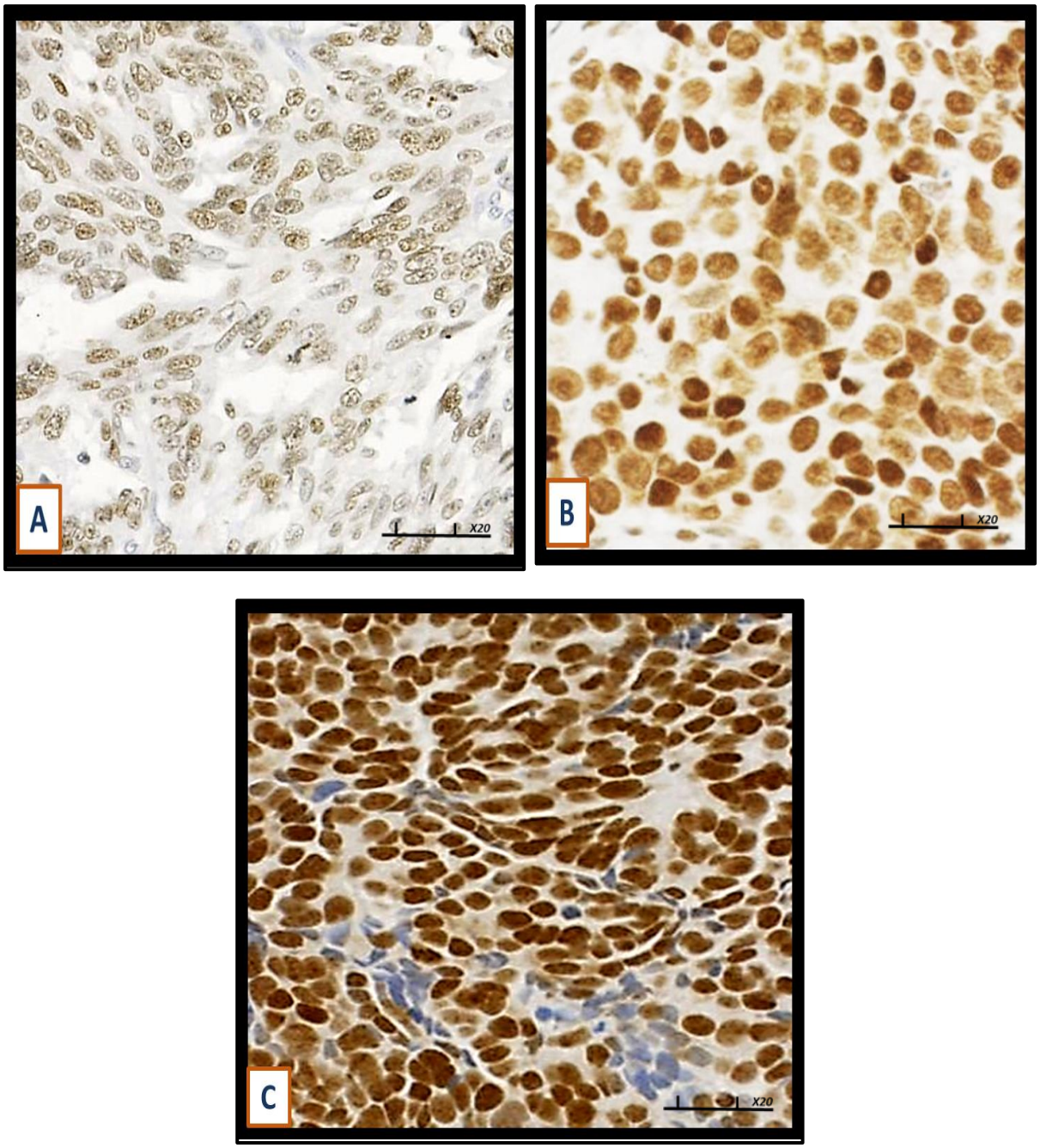



**Figure 5-4: Immunohistochemistry expression of PARP1 in tissue microarray images. Nuclear expression of PARP1 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

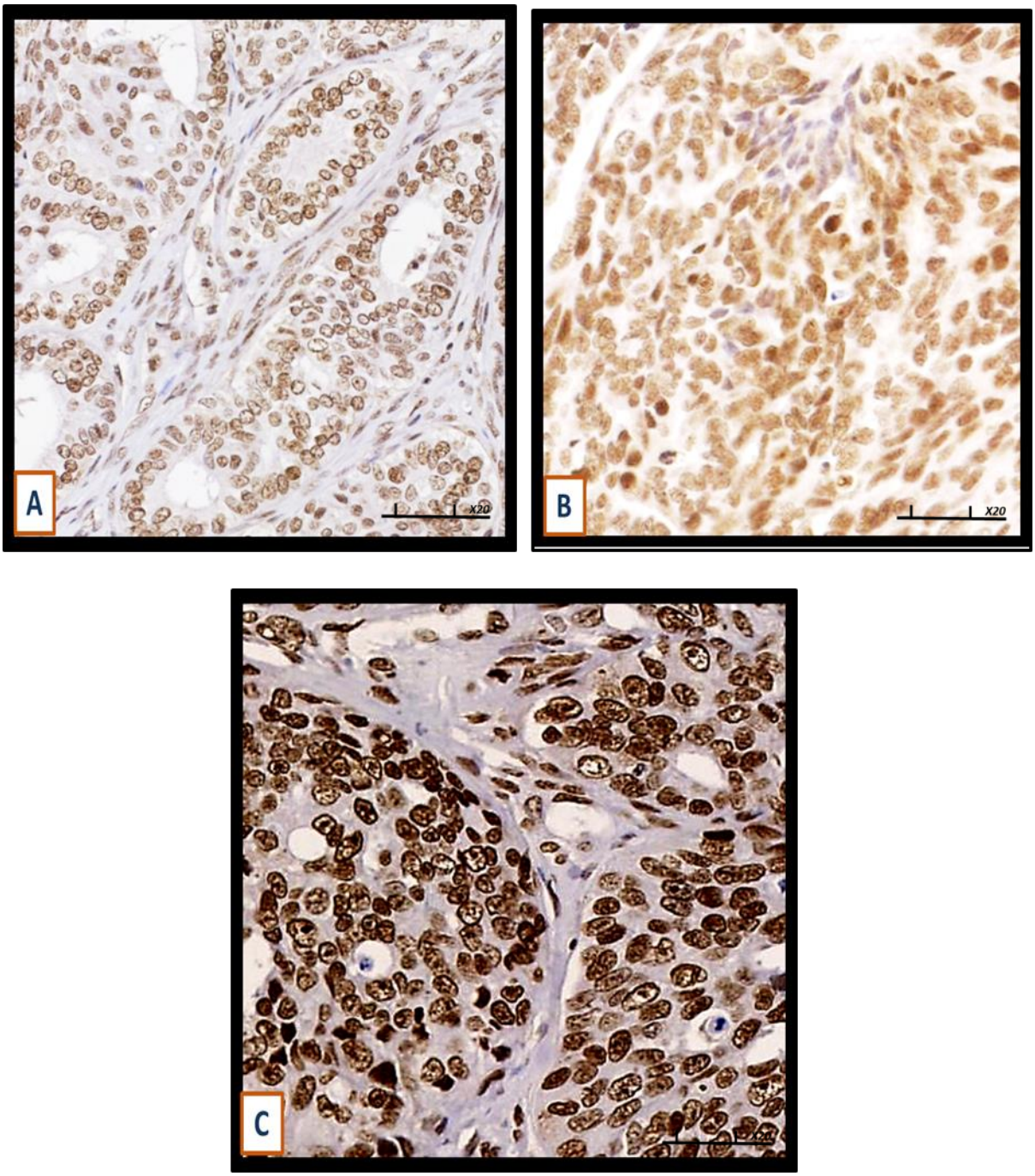



**Figure 5-5: Immunohistochemistry expression of XRCC1 in tissue microarray images. Nuclear expression of XRCC1 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

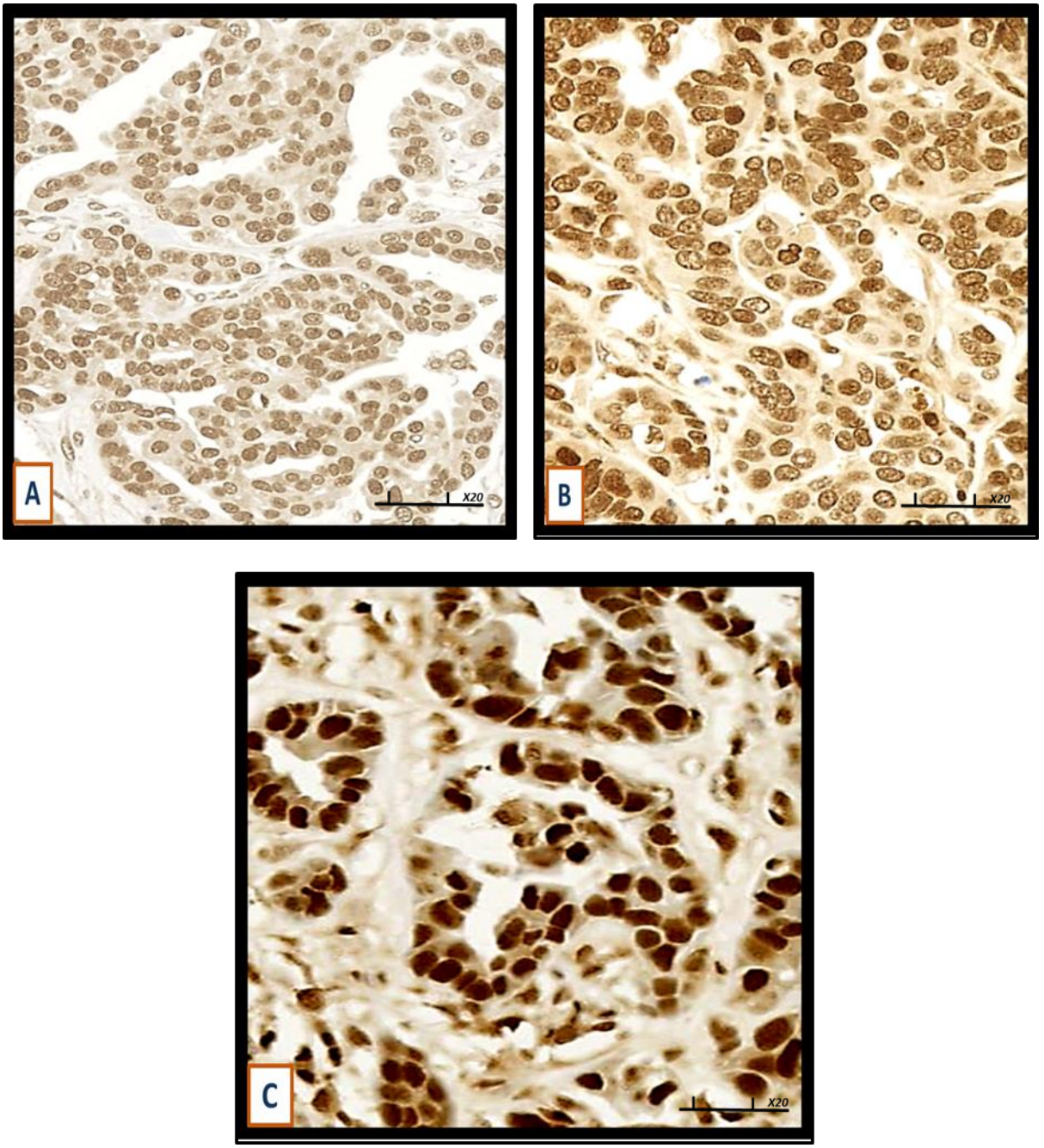



**Figure 5-6: Immunohistochemistry expression of polβ in tissue microarray images. Nuclear expression of polβ in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

### 5.3.3 Correlation of PARP1, XRCC1 and polβ positive expression with clinicopathological variables

#### 5.3.3.1 PARP1

High PARP1 nuclear expression was highly positively associated with serous adenocarcinomas, (p-value < 0.0001). On the other hand, low level of this protein was very significantly associated with lower grade disease, (p-value < 0.0001) compared with high expressers. In addition, if compared with low expression cases, high levels of this protein was associated with higher risk of post-surgical residual disease, (p-value = 0.024) (Table 5-1).

Moreover, and by further analysing the combined expression of PARP1 with both XRCC1 and polβ proteins and then, correlating their positive expression with the clinicopathological data. Tumours with combined high expression of both PARP1 and XRCC1 had a highly positive significant association with serous adenocarcinomas histology subtype, (p-value < 0.0001), higher-grade disease, (p-value = 0.002) and increased risk of measurable tumour after surgery, (p-value = 0.004), if compared with low expressers of both biomarkers, (Table 5-4). Similarly, high expression of both polβ and PARP1 was correlated with serous tumour histology and higher grade, (p-value < 0.0001) and (p-value =0.005) respectively. On the other hand, low/negative expression tumours of both biomarkers had lower chance of post-operative residual disease, (p-value = 0.009) when compared with the high expression cases, (Table 5-5).

#### 5.3.3.2 XRCC1

XRCC1 protein expression showed strong features as a poor prognostic biomarker for OC patients. Upregulated protein expression of XRCC1 was strongly associated with serous histology type, (p-value <0.0001). Moreover, the positive expression of same biomarker was also correlated with advanced tumour stage, (p-value = 0.001) and an increased likelihood of having post-operative measurable disease, (p-value =0.002) indicating sub-optimal debulking at surgery when compared with low XRCC1 expressers. On the other hand, the absence/low expression of XRCC1 protein significantly improved the response to platinum chemotherapeutic agents, (p-value =0.005) (Table 5-2).

In addition, when the both XRCC1 and polβ high co-expression was analysed together with the clinicopathgological variables, high expression of both proteins was highly significantly correlated with serous histology type, (p-value <0.0001). Furthermore, a positive association between high expression of both biomarkers and advanced tumour stage and grade was noted, (p-value =0.004) and (p-value =0.007) respectively. On the other hand, XRCC1/polβ negative/low expression significantly improved the response to platinum agents, (p-value =0.023). Furthermore, polβ/XRCC1 overexpression was in borderline significance with higher residual disease post-surgery, (p-value =0.068) (Table 5-6).

#### 5.3.3.3 polβ

Similar to PARP1 and XRCC1, Polβ overexpression was linked to aggressive epithelial OC characteristics. higher levels of polβ protein expression was significantly correlated with serous tumour histology, (p-value =0.001). However, low level of this protein was significantly associated with lower stage disease, (p-value = 0.003) when compared with high tumour expressers. In addition, both platinum resistance and higher-grade disease was more common in tumours with high polβ expression, although it was in borderline significance, (p-value =0.076) and (p-value =0.076) respectively, (Table 5-3).

**Table 5-1: Clinicopathological significance of PARP1 positive expression in primary OC, significant values are in bold**

| PARP1 Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **PARP1 Negative No. (%)** | **PARP1 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 84 (35.7) | 151 (64.3) | |
| Mucinous | 37 (78.7) | 10 (21.3) | |
| Endometrioid | 40 (58.0) | 29 (42.0) | ***<0.0001*** **(54.305)** |
| Clear cell carcinoma | 29 (80.6) | 7 (19.4) | |
| Mixed | 3 (21.4) | 11 (78.6) | |
| Other | 6 (40) | 9 (60) | |
| ***FIGO Stage*** | | | |
| I | 78 (52.3) | 71 (47.7) | 0.443 (2.685) |
| II | 29 (44.6) | 36 (55.4) | |
| III | 74 (43.5) | 96 (56.5) | |
| IV | 12 (48.0) | 13 (52.0) | |
| ***Grade*** | | | |
| G1 | 43 (75.4) | 14 (24.6) | ***<0.0001*** **(21.148)** |
| G2 | 31 (46.3) | 36 (53.7) | |
| G3 | 104 (41.8) | 145 (58.2) | |
| ***Measurable Disease*** | | | |
| Non | 138 (52.1) | 127 (47.9) | ***0.024*** **(5.121)** |
| Measurable | 48 (39.7) | 73 (60.3) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 154 (49.7) | 156 (50.3) | 0.397 (0.717) |
| Resistant | 29 (43.9) | 37 (56.1) | |

**Table 5-2: Clinicopathological significance of XRCC1 positive expression in primary OC, significant values are in bold**

| XRCC1 Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **XRCC1 Negative No. (%)** | **XRCC1 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 66 (24.7) | 201 (75.3) | |
| Mucinous | 23 (47.9) | 25 (52.1) | |
| Endometrioid | 30 (41.7) | 42 (58.3) | ***<0.0001*** **(28.104)** |
| Clear cell carcinoma | 24 (58.5) | 17 (41.5) | |
| Mixed | 4 (28.6) | 10 (71.4) | |
| Other | 3 (25) | 9 (75) | |
| ***FIGO Stage*** | | | |
| I | 70 (44.9) | 86 (55.1) | ***0.001*** **(15.581)** |
| II | 18 (26.5) | 50 (73.5) | |
| III | 58 (29.7) | 137 (70.3) | |
| IV | 5 (16.7) | 25 (83.3) | |
| ***Grade*** | | | |
| G1 | 27 (46.6) | 31 (53.4) | 0.105 (4.515) |
| G2 | 24 (31.6) | 52 (68.4) | |
| G3 | 90 (32.5) | 187 (67.5) | |
| ***Measurable Disease*** | | | |
| Non | 138 (52.1) | 127 (47.9) | ***0.002*** **(9.362)** |
| Measurable | 48 (39.7) | 73 (60.3) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 127 (38.6) | 202 (61.4) | ***0.005*** **(7.790)** |
| Resistant | 17 (21.8) | 61 (78.2) | |

**Table 5-3: Clinicopathological significance of Polβ positive expression in primary OC, significant values are in bold**

| Pol β Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **Pol β Negative No. (%)** | **Pol β Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 113 (48.5) | 120 (51.5) | |
| Mucinous | 32 (74.4) | 11 (25.6) | |
| Endometrioid | 49 (69.0) | 22 (31.0) | ***0.001*** **(21.876)** |
| Clear cell carcinoma | 26 (70.3) | 11 (29.7) | |
| Mixed | 6 (50.0) | 6 (50.0) | |
| Other | 4 (33.3) | 8 (66.7) | |
| ***FIGO Stage*** | | | |
| I | 101 (67.3) | 49 (32.7) | ***0.003*** **(14.273)** |
| II | 25 (41.0) | 36 (59.0) | |
| III | 87 (52.4) | 79 (47.6) | |
| IV | 15 (55.6) | 12 (44.4) | |
| ***Grade*** | | | |
| G1 | 30 (61.2) | 19 (38.8) | 0.072 (5.259) |
| G2 | 45 (64.3) | 25 (35.7) | |
| G3 | 124 (50.4) | 122 (49.6) | |
| ***Measurable Disease*** | | | |
| Non | 148 (57.6) | 109 (42.4) | 0.522 (0.410) |
| Measurable | 66 (54.1) | 56 (45.9) | |
| ***Platinum sensitivity*** | | | |
| Sensitive | 157 (52.0) | 145 (48.0) | 0.076 (3.157) |
| Resistant | 25 (39.7) | 38 (60.3) | |

**Table 5-4: Clinicopathological significance of combined PARP1 and XRCC1 positive expression in primary OC, significant values are in bold**

| Combined PARP1/XRCC1 Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **PARP1-/ XRCC1- No. (%)** | **PARP1-/ XRCC1+ No. (%)** | **PARP1+/ XRCC1- No. (%)** | **PARP1+/ XRCC1+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 32 (14.3) | 45 (20.2) | 27 (12.1) | 119 (53.4) | |
| Mucinous | 18 (45.0) | 14 (35.0) | 1 (2.5) | 7 (17.5) | |
| Endometrioid | 21 (34.4) | 14 (23.0) | 6 (9.8) | 20 (32.8) | ***≤0.0001*** |
| Clear cell carcinoma | 19 (59.4) | 7 (21.9) | 1 (3.1) | 5 (15.6) | **(69.891)** |
| Mixed | 1 (7.7) | 2 (15.4) | 3 (23.1) | 7 (53.8) | |
| Other | 0 (0.0) | 3 (27.3) | 2 (18.2) | 6 (54.5) | |
| ***FIGO Stage*** | | | | | |
| I | 46 (34.3) | 22 (16.4) | 13 (9.7) | 53 (39.6) | 0.100 |
| II | 11 (18.3) | 14 (23.3) | 7 (11.7) | 28 (46.7) | (14.761) |
| III | 30 (18.9) | 40 (25.2) | 20 (12.6) | 69 (43.4) | |
| IV | 4 (16.7) | 7 (29.2) | 1 (4.2) | 12 (50.0) | |
| ***Grade*** | | | | | |
| G1 | 22 (44.0) | 15 (30.0) | 1 (2.0) | 12 (24.0) | ***0.002*** |
| G2 | 15 (24.2) | 13 (21.0) | 7 (11.3) | 27 (43.5) | **(20.273)** |
| G3 | 48 (20.6) | 47 (20.2) | 30 (12.9) | 108 (46.4) | |
| ***Measurable Disease*** | | | | | |
| Non | 73 (30.5) | 47 (19.7) | 23 (9.6) | 96 (40.2) | ***0.004*** |
| Measurable | 15 (13.0) | 30 (26.1) | 16 (13.9) | 54 (47.0) | **(13.179)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 77 (27.4) | 58 (20.6) | 34 (12.1) | 112 (39.9) | 0.107 |
| Resistant | 9 (14.8) | 17 (27.9) | 5 (8.2) | 30 (49.2) | (6.101) |

**Table 5-5: Clinicopathological significance of combined Polβ and PARP1 positive expression in primary OC, significant values are in bold**

| Combined Polβ/PARP1 Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **Polβ-/ PARP1- No. (%)** | **Polβ-/ PARP1+ No. (%)** | **Polβ+/ PARP1- No. (%)** | **Polβ+/ PARP1+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 44 (21.3) | 55 (26.6) | 27 (13.0) | 81 (39.1) | |
| Mucinous | 22 (61.1) | 5 (13.9) | 4 (11.1) | 5 (13.9) | |
| Endometrioid | 27 (42.9) | 16 (25.4) | 8 (12.7) | 12 (19.0) | ***≤0.0001*** |
| Clear cell carcinoma | 22 (66.7) | 2 (6.1) | 4 (12.1) | 5 (15.2) | **(60.959)** |
| Mixed | 1 (9.1) | 5 (45.5) | 1 (9.1) | 4 (36.4) | |
| Other | 3 (27.3) | 1 (9.1) | 0 (0.0) | 7 (63.6) | |
| ***FIGO Stage*** | | | | | |
| I | 54 (40.3) | 34 (25.4) | 12 (9.0) | 34 (25.4) | 0.077 |
| II | 16 (28.6) | 7 (12.5) | 7 (12.5) | 26 (46.4) | (15.525) |
| III | 39 (26.9) | 38 (26.2) | 22 (15.2) | 46 (31.7) | |
| IV | 8 (33.3) | 5 (20.8) | 3 (12.5) | 8 (33.3) | |
| ***Grade*** | | | | | |
| G1 | 23 (51.1) | 5 (11.1) | 9 (20.0) | 8 (17.8) | ***0.005*** |
| G2 | 21 (36.2) | 15 (25.9) | 4 (6.9) | 18 (31.0) | **(18.340)** |
| G3 | 58 (26.1) | 54 (24.3) | 30 (13.5) | 80 (36.0) | |
| ***Measurable Disease*** | | | | | |
| Non | 88 (38.3) | 42 (18.3) | 27 (11.7) | 73 (31.7) | ***0.009*** |
| Measurable | 24 (22.4) | 34 (31.8) | 14 (13.1) | 35 (32.7) | **(11.552)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 92 (34.1) | 63 (23.3) | 35 (13.0) | 80 (29.6) | 0.636 |
| Resistant | 15 (26.3) | 14 (24.6) | 7 (12.3) | 21 (36.8) | (1.706) |

**Table 5-6: Clinicopathological significance of combined Polβ and XRCC1 positive expression in primary OC, significant values are in bold**

| Combined Pol β/XRCC1 Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **Polβ-/ XRCC1- No. (%)** | **Pol β-/ XRCC1+ No. (%)** | **Polβ+/ XRCC1- No. (%)** | **Polβ+/ XRCC+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 37 (16.3) | 73 (32.2) | 20 (8.8) | 97 (42.7) | |
| Mucinous | 18 (45.0) | 13 (32.5) | 1 (2.5) | 8 (20.0) | |
| Endometrioid | 22 (32.8) | 24 (35.8) | 5 (7.5) | 16 (23.9) | ***≤0.0001*** |
| Clear cell carcinoma | 18 (54.5) | 5 (15.2) | 2 (6.1) | 8 (24.2) | **(44.099)** |
| Mixed | 3 (25.0) | 3 (25.0) | 1 (8.3) | 5 (41.7) | |
| Other | 1 (9.1) | 3 (27.3) | 2 (18.2) | 5 (45.5) | |
| ***FIGO Stage*** | | | | | |
| I | 53 (37.6) | 43 (30.5) | 8 (5.7) | 37 (26.2) | ***0.004*** |
| II | 12 (20.3) | 12 (20.3) | 6 (10.2) | 29 (49.2) | **(24.301)** |
| III | 32 (20.0) | 52 (32.5) | 15 (9.4) | 61 (38.1) | |
| IV | 3 (11.5) | 11 (42.3) | 2 (7.7) | 10 (38.5) | |
| ***Grade*** | | | | | |
| G1 | 21 (43.8) | 9 (18.8) | 1 (2.1) | 17 (35.4) | ***0.007*** |
| G2 | 18 (26.5) | 26 (38.2) | 3 (4.4) | 21 (30.9) | **(17.570)** |
| G3 | 52 (22.2) | 66 (28.2) | 27 (11.5) | 89 (38.0) | |
| ***Measurable Disease*** | | | | | |
| Non | 74 (30.2) | 68 (27.8) | 23 (9.4) | 80 (32.7) | 0.068 |
| Measurable | 22 (18.6) | 41 (34.7) | 8 (6.8) | 47 (39.8) | (7.128) |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 88 (30.6) | 81 (28.1) | 23 (8.0) | 96 (33.3) | ***0.023*** |
| Resistant | 7 (11.5) | 23 (37.7) | 7 (11.5) | 24 (39.3) | **(9.525)** |

### 5.3.4 Outcome analysis

#### 5.3.4.1 Univariate analysis in the whole cohort

In general, high expression of all the surrogate proteins, XRCC1, PARP1 and polβ showed unfavourable prognosis. Compared with low protein expression ovarian tumours, high nuclear expression of XRCC1 was associated with shorter both Progression Free Survival (PFS), (p-value =0.001) and Overall Survival (OS), (p-value =0.001) (Figure 5-7). Similarly, high PARP1 expression showed an adverse clinical outcome with poor recurrence outcome, (p-value =0.004) and OS, (p-value =0.001) (Figure 5-8), compared with tumours that had low PARP1 expression. In addition, cases with high polβ expression showed an adverse clinical outcome with poor OS, (p-value =0.047) and PFS, (p-value =0.005) (Figure 5-9), when compared with tumours that had low polβ level. At the transcriptional level, similarly, high XRCC1 and polβ mRNA was associated with poor OS, (p-value = 0.0116) and (p-value = 0.0341) respectively.

Additionally, investigating XRCC1 expression with the other proteins revealed that low combined expression of both XRCC1 and PARP1 was associated with better outcome in terms of both recurrence and overall survival, (p-value =0.010) and (p-value =0.002) respectively, (Figure 5-10), compared with high protein expression tumours. Similarly, combined negative/low level of both XRCC1 and polβ also showed improved outcome with longer both OS, (p-value =0.002) and PFS, (p-value =0.004), (Figure 5-11), compared with overexpressers. On the other hand, high significance with worse outcome was calculated with the combined high expression of polβ and PARP1, when compared with low expression samples, PFS (p-value =0.032) and OS (p-value =0.012), (Figure 5-12).

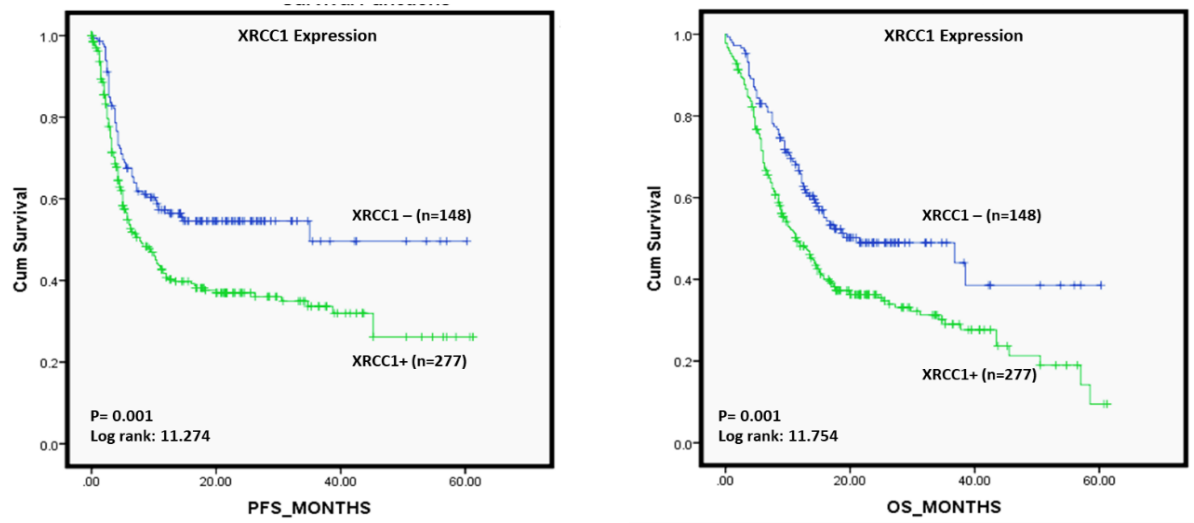



**Figure 5-7: Kaplan Meier plots illustrating the associations between the altered expression of XRCC1 protein with PFS and OS in primary ovarian carcinoma – whole series**

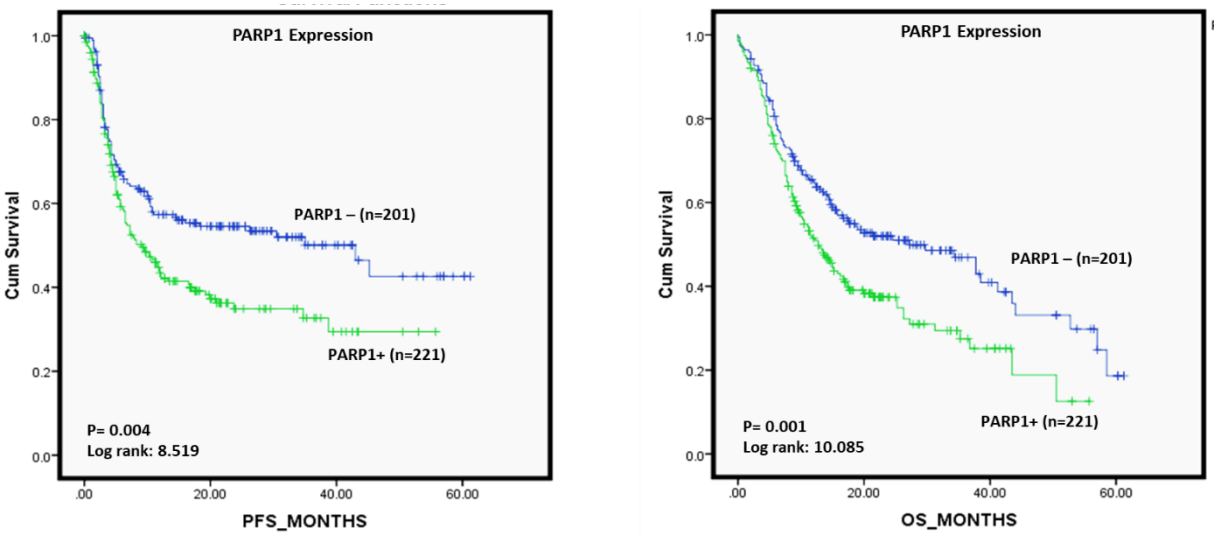



**Figure 5-8: Kaplan Meier plots illustrating the associations between the altered expression of PARP1 protein with PFS and OS in primary ovarian carcinoma – whole series**

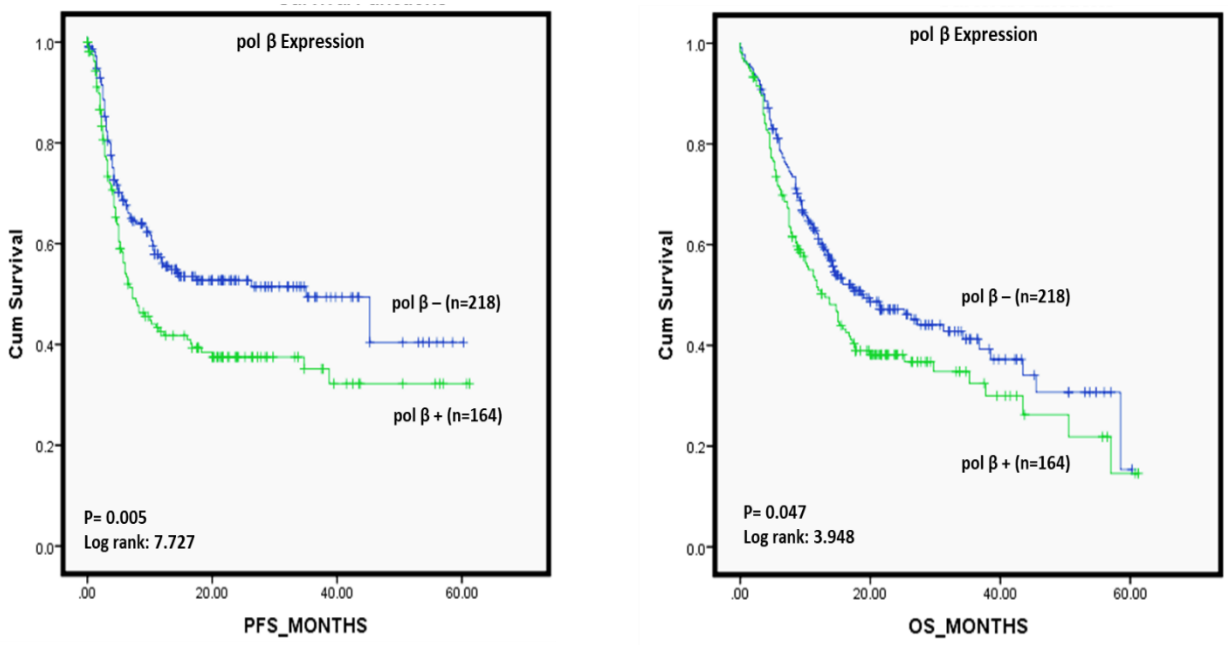



**Figure 5-9: Kaplan Meier plots illustrating the associations between the altered expression of polβ protein with PFS and OS in primary ovarian carcinoma – whole series**

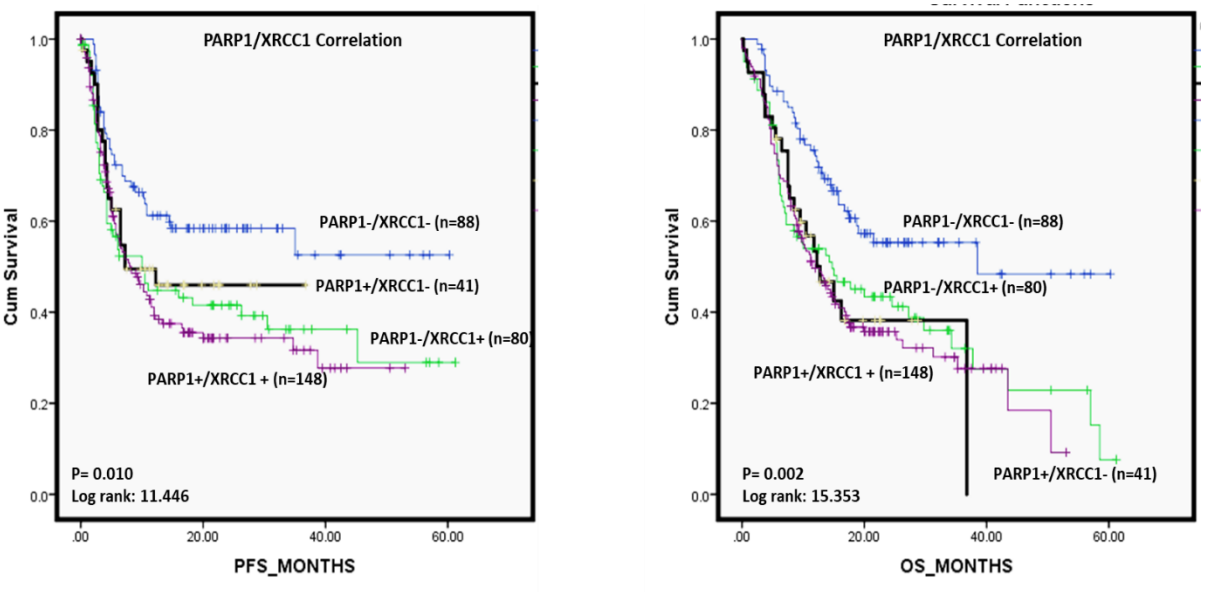



**Figure 5-10: Kaplan Meier plots illustrating the associations between the combined altered expression of PARP1 and XRCC1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

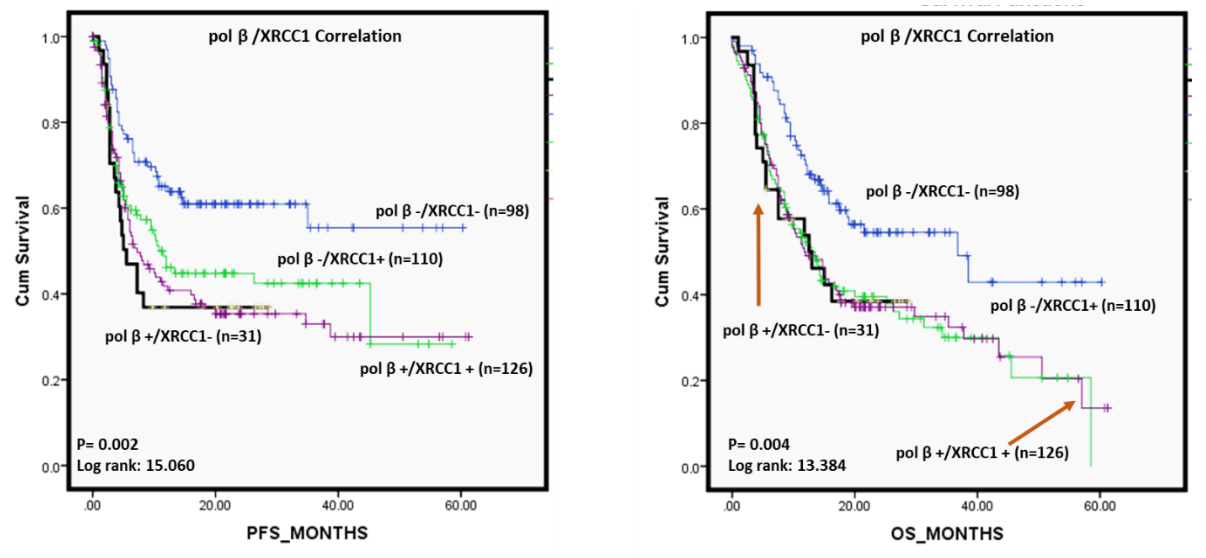



**Figure 5-11: Kaplan Meier plots illustrating the associations between the combined altered expression of polβ and XRCC1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

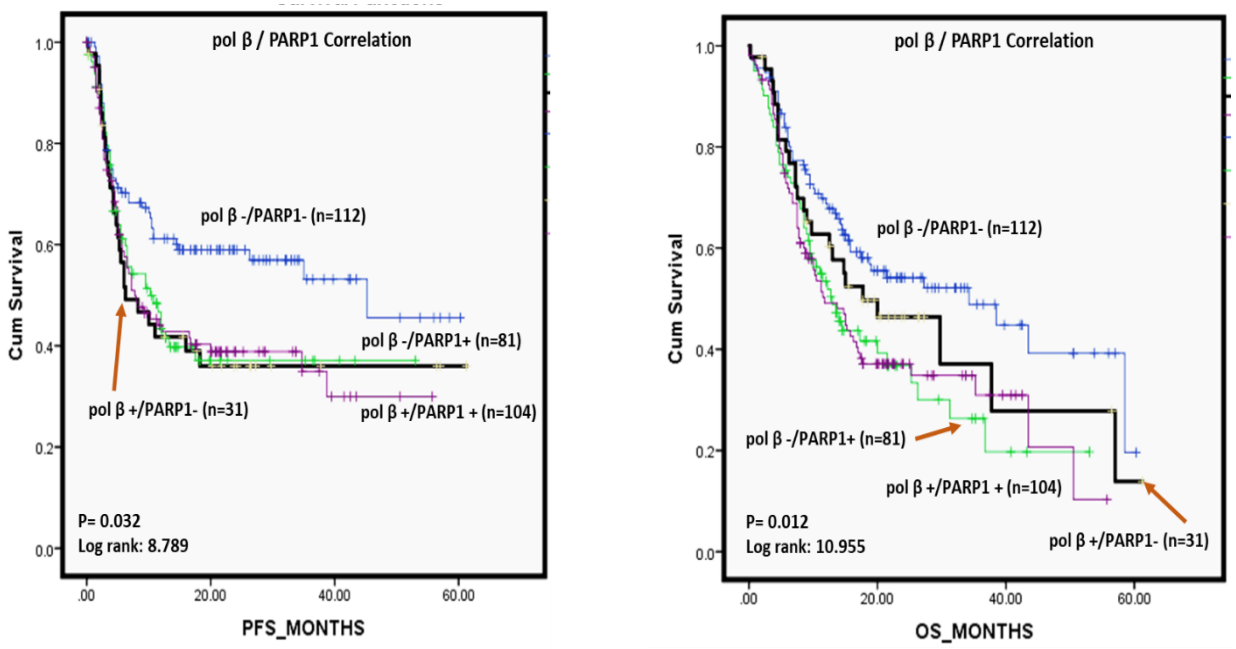



**Figure 5-12: Kaplan Meier plots illustrating the associations between the combined altered expression of polβ and PARP1 proteins with PFS and OS in primary ovarian carcinoma – whole series**

#### 5.3.4.2 Univariate survival analysis in platinum sensitive cohort

The KM estimates of both the PFS and OS for patients with OC who were treated primarily by surgery showed that the overexpression of XRCC1 had a significant worse outcome if compared with low expressers, (p-value = 0.030) and (p-value = 0.015) respectively, (Figure 5-13). Similarly, high PARP1 and polβ nuclear expression in tumours showed adverse clinical outcome with poor PFS, (p-value =0.005) (Figure 5-14), (p-value =0.014) (Figure 5-15) and OS, (p-value =0.001) (Figure 5-14) and (p-value =0.028) (Figure 5-15), respectively, compared with tumours that had low expression.

Furthermore, investigating the effect of the combined expression of all the three tested biomarkers with each other on the survival outcome, combined high level of both XRCC1 and PARP1 showed inverse significant relation with OS, (p-value =0.014) and borderline significance with worse PFS, (p-value =0.068) (Figure 5-16). On the other hand, combined negative/low expression of XRCC1 and polβ revealed interestingly, that it was associated with the improved outcome in terms of both PFS, (p-value =0.022) and OS, (p-value =0.049) compared with protein overexpression tumours, (Figure 5-17). Moreover, combined low expression of both PARP1 and polβ showed significant association with improved PFS, (p-value =0.030) and OS, (p-value =0.018) (Figure 5-18), compared with high protein expressers.

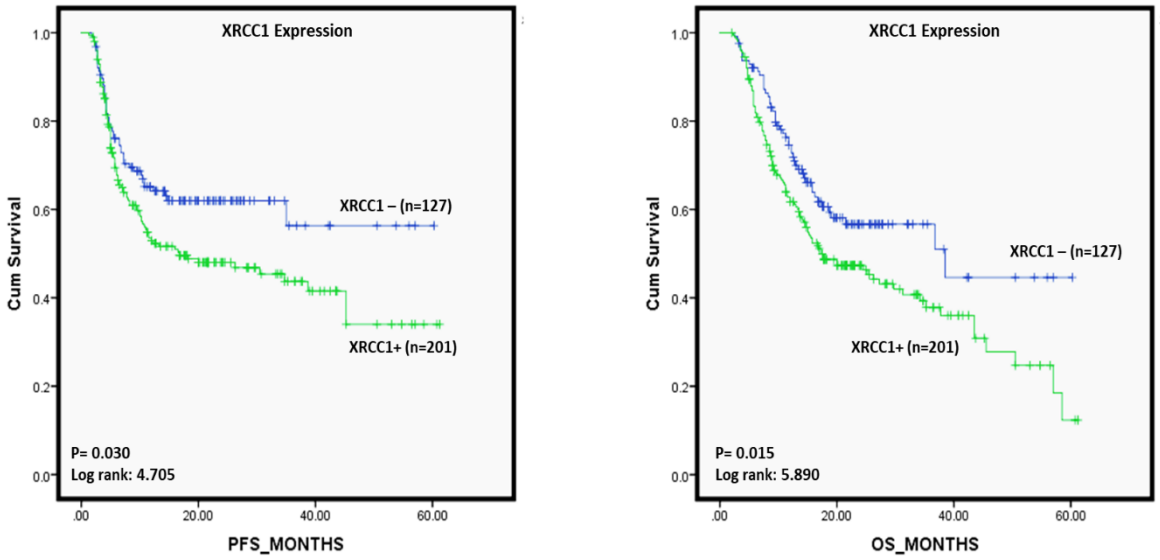



**Figure 5-13: Kaplan Meier plots illustrating the associations between the altered expression of XRCC1 protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

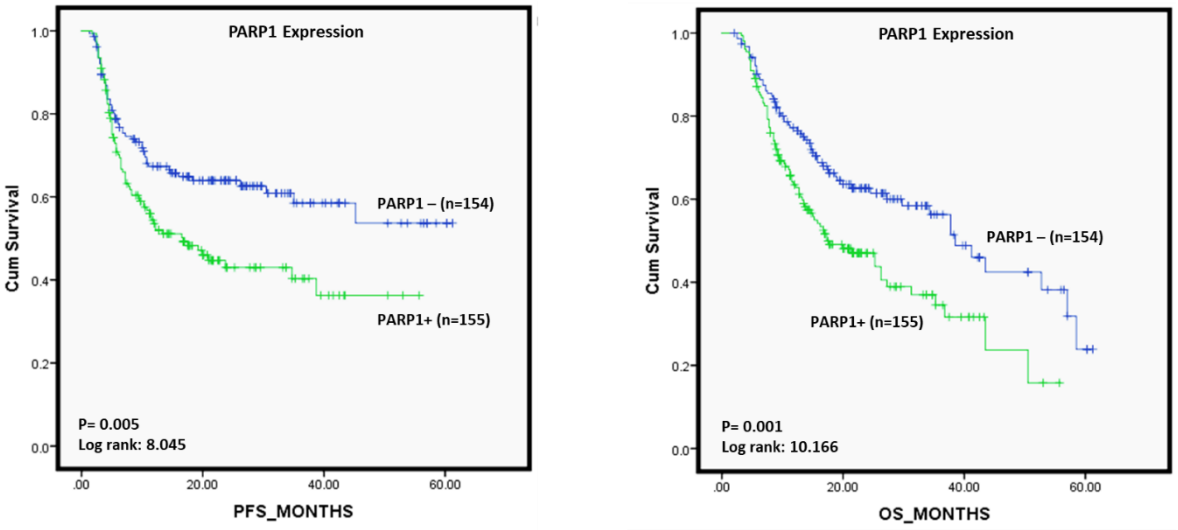



**Figure 5-14: Kaplan Meier plots illustrating the associations between the altered expression of PARP1 protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

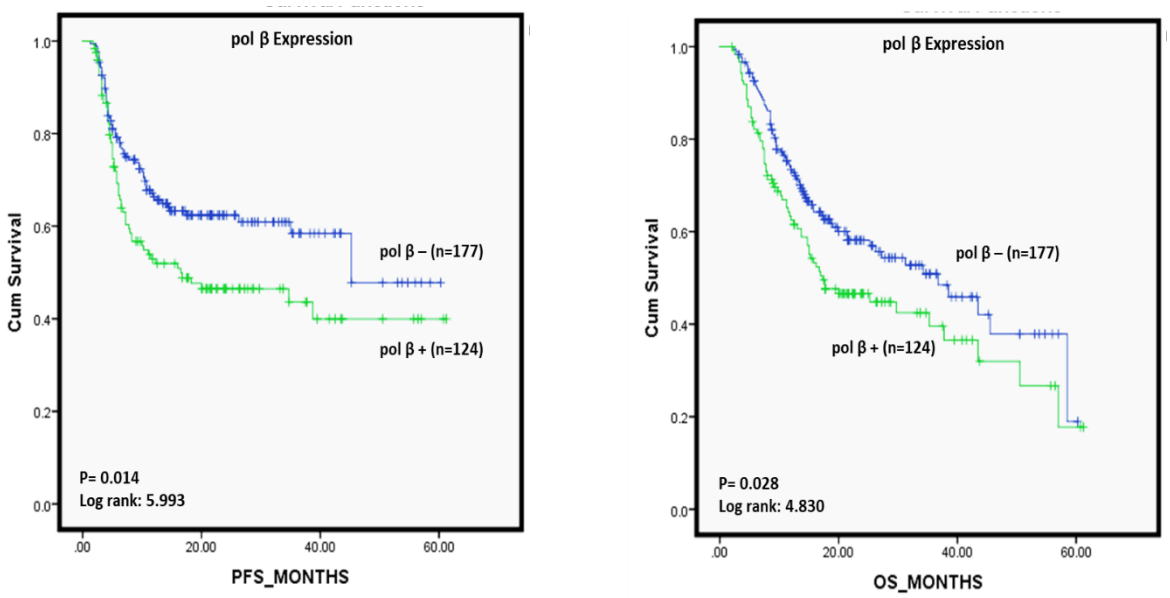



**Figure 5-15: Kaplan Meier plots illustrating the associations between the altered expression of polβ protein with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

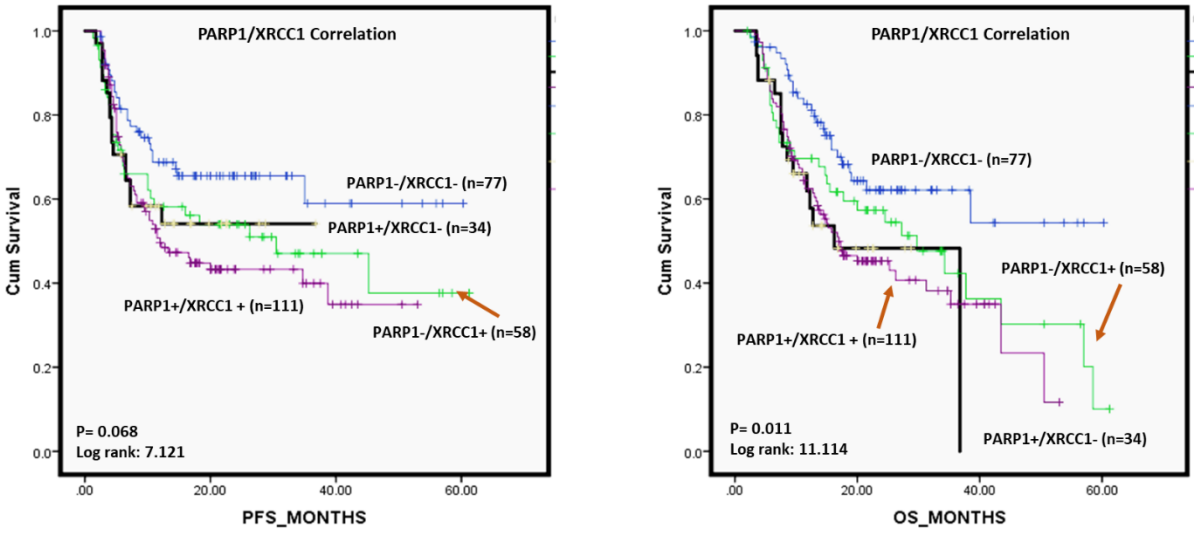



**Figure 5-16: Kaplan Meier plots illustrating the associations between the combined altered expression of PARP1 and XRCC1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

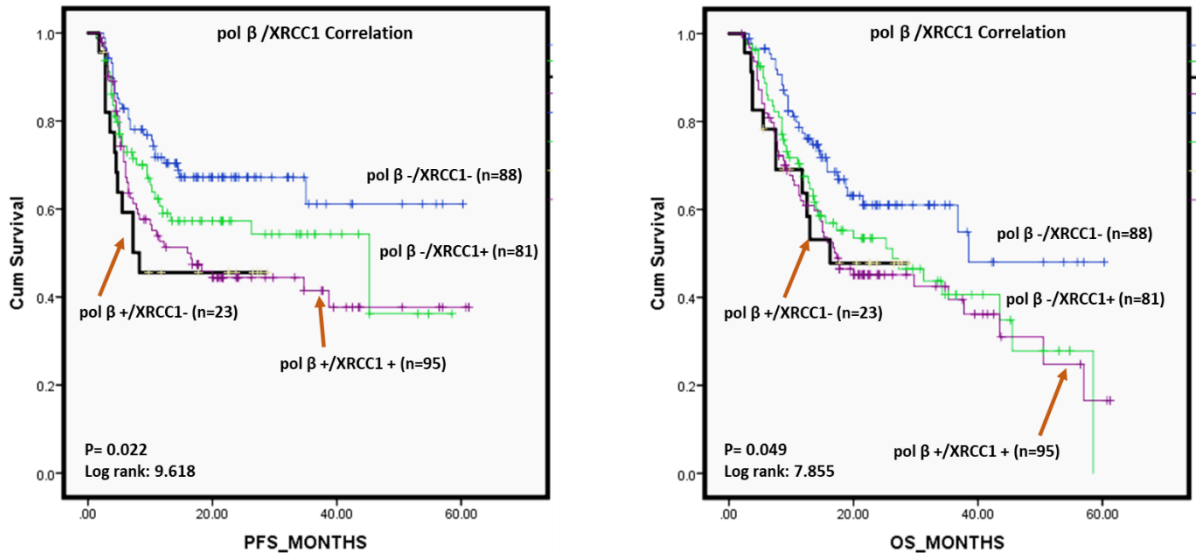



**Figure 5-17: Kaplan Meier plots illustrating the associations between the combined altered expression of polβ and XRCC1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

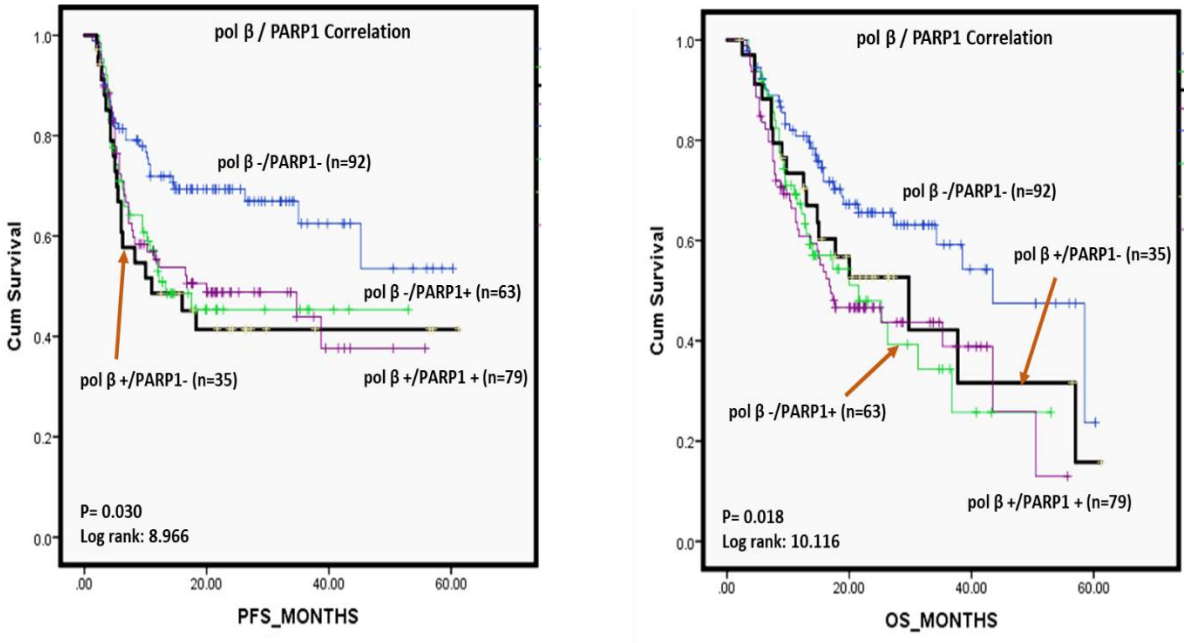



**Figure 5-18: Kaplan Meier plots illustrating the associations between the combined altered expression of polβ and PARP1 proteins with PFS and OS in primary ovarian carcinoma – platinum sensitive selection**

#### 5.3.4.3 Univariate survival analysis in platinum resistant cohort

Apart from a significant correlation of increased XRCC1 expression with worse PFS, (p-value =0.040), there was no significance with patients' outcome noted with the other biomarkers in platinum resistant ovarian tumours, (Supplementary Table).

#### 5.3.4.4 Multivariate analysis

To further test the influence of XRCC1, PARP1 and polβ proteins overexpression on OC patients' prognosis, a multivariate analysis (Cox multivariate model) of the above biomarkers positive expression with the platinum sensitivity were tested. The results revealed that XRCC1 together with platinum sensitivity were independent prognostic factors for worse PFS, (p-value =0.028) and (p-value <0.0001), respectively, (Table 5-7). Concerning OS, both PARP1, (p-value =0.015) and platinum sensitivity, (p-value <0.0001) were independent factors associated with poor survival, (Table 5-8).

**Table 5-7: Multivariate Cox regression analysis for prediction of ovarian cancer progression free survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | *p-value* | HR | 95% CI | |
|---|---|---|---|---|
| **XRCC1** | ***0.028*** | 0.402 | 1.046 | 2.136 |
| **PARP1** | 0.910 | 0.020 | 0.725 | 1.434 |
| **Polβ** | 0.345 | 1.167 | 0.842 | 1.616 |
| **Platinum sensitivity** | ***<0.0001*** | 2.683 | 9.837 | 21.738 |

**Table 5-8: Multivariate Cox regression analysis for prediction of ovarian cancer overall survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | *p-value* | HR | 95% CI | |
|---|---|---|---|---|
| **XRCC1** | 0.237 | 0.213 | 0.869 | 1.761 |
| **PARP1** | ***0.015*** | 0.415 | 1.082 | 2.118 |
| **Polβ** | 0.788 | -0.045 | 0.690 | 1.326 |
| **Platinum sensitivity** | ***<0.0001*** | 1.917 | 4.782 | 9.677 |

Taken together the clinical data provides evidence that XRCC1 deficiency is associated with platinum sensitivity. In addition, as XRCC1 knockdown increased PARP1 activity (Hoch et al., 2017). Therefore, PARP1 targeting could be a promising approach in XRCC1 deficient OC cases. For this reason, we proceeded to perform pre-clinical evaluation and XRCC1 knockdown in OC cell lines.

### 5.3.5 Preclinical experiments results

#### 5.3.5.1 Background

As low XRCC1 protein level, improved platinum sensitivity in OC cases (Abdel-Fatah et al., 2013). In addition and at the preclinical level, XRCC1 deficient OC cell lines were also sensitive to platinum treatment (Abdel-Fatah et al., 2013). Furthermore, as PARP1 inhibitors are standard maintenance therapies to prolong PFS in platinum sensitive sporadic OC patients. We therefore, tested the hypothesis that PARP inhibitor therapy could improve patients' outcome and reduce recurrence rate in XRCC1 deficient OC cells.

#### 5.3.5.2 XRCC1 knockdown and its effect on PARP1 level

XRCC1 deficiency influence on PARP1 expression level was tested through depletion of XRCC1 using siRNAs. Following the transfection, cell lysates were sampled on both day 3 and 5 for the detection of the XRCC1 level. The depletion of XRCC1 in both A2780 and A2780Cis cell lines was confirmed via WB technique, (Figure 5-19) and (Figure 5-20) respectively. Scrambled siRNA was utilised as a control. Robust XRCC1 KD was observed following the transfection process. Interestingly and when compared to control cells, a significant increase in PARP1 protein levels was observed in XRCC1 deficient OC cells, (Figure 5-21).

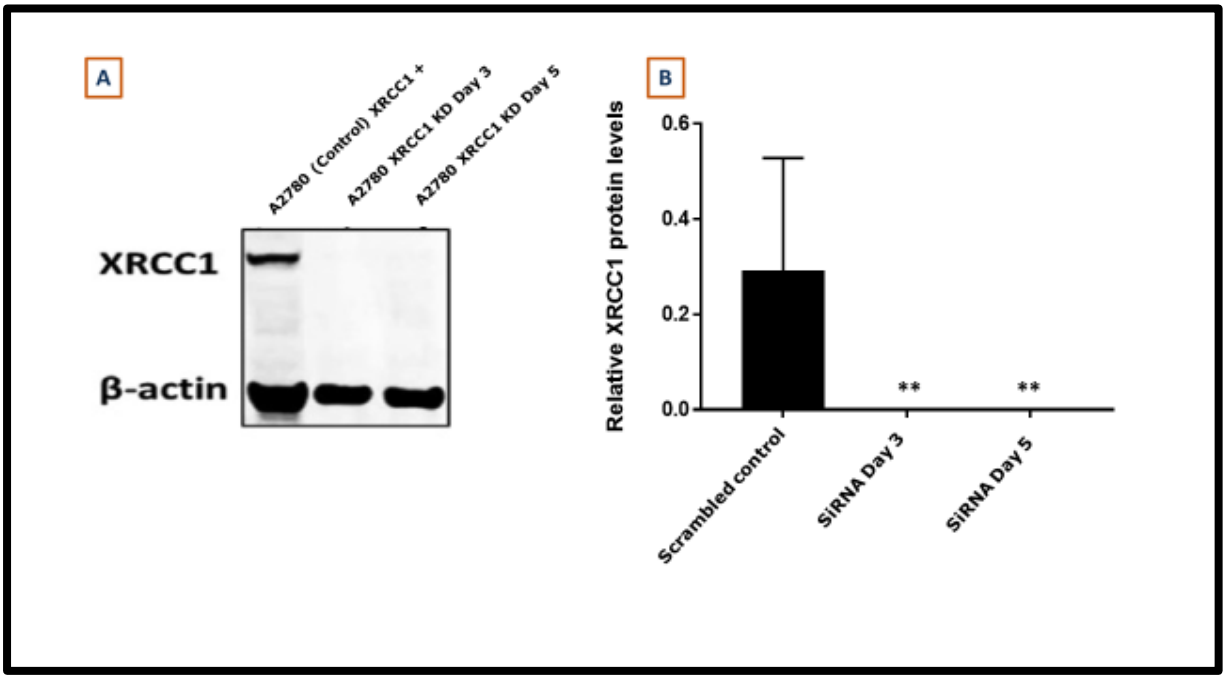



**Figure 5-19: A) Representative immunoblots of XRCC1 levels in A2780 transfected with XRCC1 SiRNA or scrambled SiRNA control. B) Quantification of XRCC1 levels. B-actin was used as a loading control**

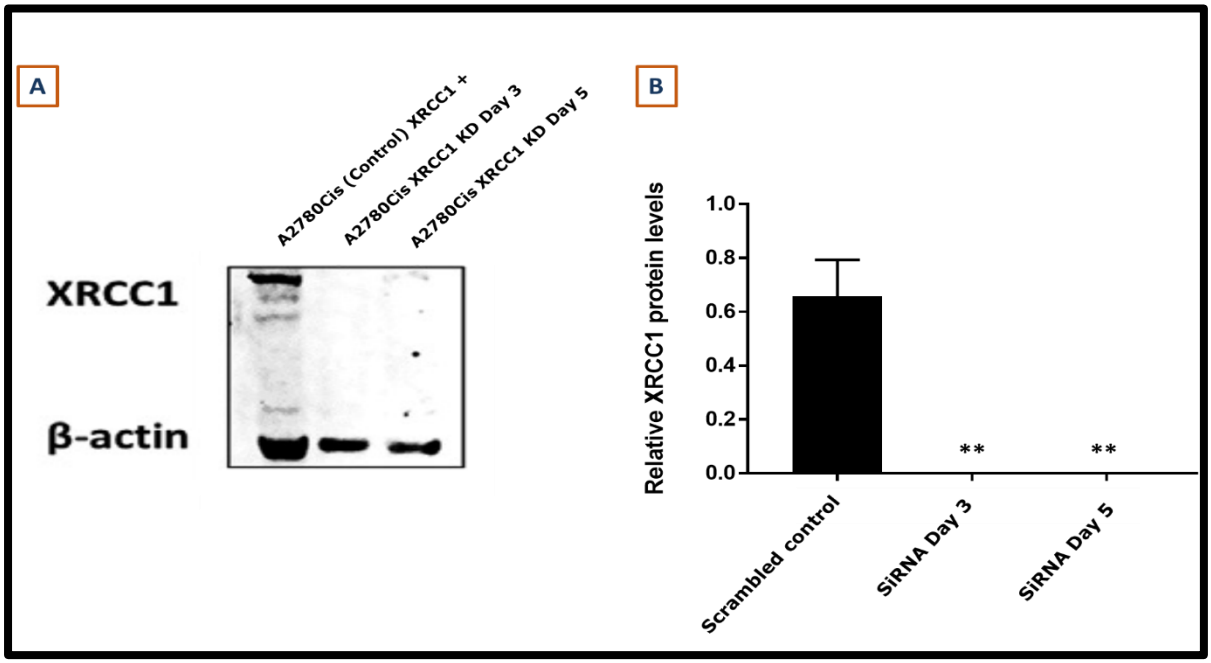



**Figure 5-20: A) Representative immunoblots of XRCC1 levels in A2780Cis transfected with XRCC1 SiRNA or scrambled SiRNA control. B) Quantification of XRCC1 levels. B-actin was used as a loading control**

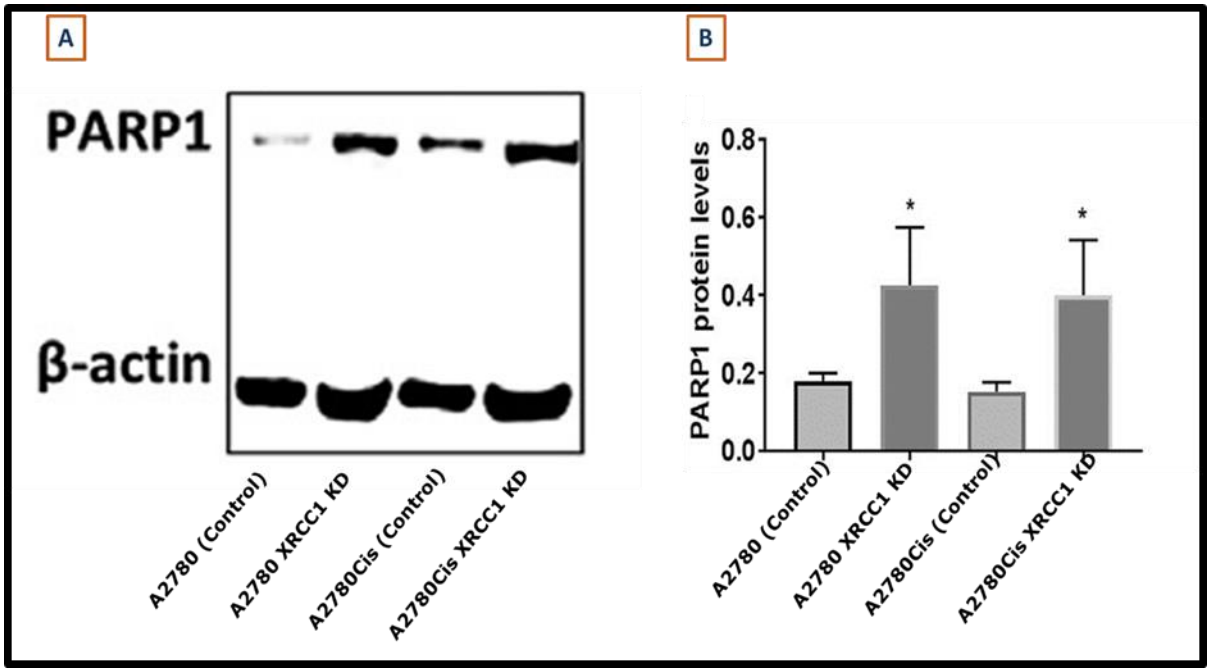



**Figure 5-21: A) Representative immunoblots of PARP1 levels in A2780 and A2780Cis XRCC1 KD and control ovarian cancer cells, B) Quantification of PARP1 levels. B-actin was used as a loading control**

## 5.4 Discussion

Different studies have emphasised the role of DDR proteins (XRCC1, PARP1 and polβ) in cancer progression. Moreover, they linked their positive expression to altered response to platinum therapeutic agents (Kaur et al., 2020, Singh et al., 2019b, Caldecott, 2003, Hegedus et al., 2019, Xiao et al., 2020, Sobczak et al., 2019, Wang et al., 2016b, Zhao et al., 2008, Sliwinski et al., 2007). However, the clinical and biological implications of the positive expression of those biomarkers in human primary OC tissue have remained largely unexplored.

In this study, the role of XRCC1, PARP1 and polβ was investigated in a big cohort of primary OC cases in order to determine the influence of the high expression of those proteins not only on the patients' pathological variables and their response to chemotherapeutic agents, but also on patients' survival. In our IHC data, in general we showed that XRCC1, PARP1 and polβ are positively associated with poor tumour prognostic variables, chemo-resistance and poor outcome.

The scaffolding protein, XRCC1 is a key player in BER, SSBR, and alt-NHEJ. XRCC1 deficiency in tumour cells delays the SSB rejoining and results in elevated levels of sister chromatid exchanges (Caldecott, 2003, London, 2015). Interestingly, XRCC1 deficiency or mutation can also result in PARP1 hyper-activation (Hoch et al., 2017). Moreover, depletion of XRCC1 increases not only the effect of ionizing radiation on tumour cells, but also the sensitivity of the cancer cells to chemotherapy agents (Caldecott, 2003, London, 2015). Furthermore, genetic polymorphisms in XRCC1 may influence cancer risk and the response to platinum-based chemotherapy (Wang and Zheng, 2012, Yang et al., 2017a, Zhang et al., 2018b, Zhu et al., 2015). These polymorphisms in XRCC1 may also have an effect on patients' clinical outcome in OC through the modifying role on the response to chemotherapeutic agents (Zhang et al., 2018b).

In a previous IHC study on 225 primary OC cases, XRCC1 positive expression was mainly sub-localised in the Nucleus of the tumour cells. In addition, XRCC1 overexpression was linked to platinum resistance and increased risk of tumour recurrence (Abdel-Fatah et al., 2013). Moreover, serous histology type, higher stage disease and residual tumour post-surgery were all linked to XRCC1 overexpression in the same study. In addition and regarding the patients'

outcome, XRCC1 low expressers showed a significant relation to prolonged survival (Abdel-Fatah et al., 2013). Similarly, a study conducted by Ang et al (Ang et al., 2011) on primary head and neck squamous carcinoma, in which, 138 tumour cases were recruited. Both poor patients' outcome and advanced stage disease had significant association with increased XRCC1 level (Ang et al., 2011). Moreover, in patients who were treated with chemo/radio therapy, low XRCC1 improved their survival (Ang et al., 2011). These results come in line with our findings.

In our large cohort of primary ovarian tumours, XRCC1 high expression was linked to poor prognostic clinicopathological variables, significantly increased tumour relapse rate, and reduced survival. On the flip side, tumours with low XRCC1 level showed improved response to platinum chemotherapy. Importantly, in platinum sensitive patients the inverse influence of high XRCC1 on patients' survival was maintained. Similarly, although the poor prognostic role of XRCC1 on disease progression remained significant in platinum resistant tumours, its relation to worse OS was maintained, but did not reach the statistical significance.

PARP1 upregulation together with other DNA repair proteins may affect cancer phenotype and lead to resistance not just to the conventional anticancer therapy, but also to monotherapy using PARP inhibitors. Accurate knowledge of the PARP expression levels in cancerous tissues will provide a broad understanding of the DNA repair capacity and help guiding the treatment strategies of cancer (Coskun et al., 2019). Currently, IHC methodology is used in vivo for PARP1 protein level estimation (Coskun et al., 2019, Gagné et al., 2018, Bonfiglio et al., 2017, Fontana et al., 2017). Preclinical studies and clinical trials in both breast and ovarian cancers have showed the efficacy of PARP inhibitors in clinical practice (Curtin, 2013a, Bryant et al., 2005, Ledermann et al., 2014, Jones, 2012, Mateo et al., 2015).

In the current study, we investigated PARP1 expression in primary OC patients. Schreibe et al stated that PARP1 is mostly localised in the nucleus and accounts for approximately 75 % of the overall PARP enzymatic activity (Schreiber et al., 2006). Similarly, Gan et al also identified PARP1 in OC patient to be mainly expressed in the nucleus (Gan et al., 2013). This finding comes in agreement with the current study, PARP1 showed predominant nuclear staining in PARP1 positive

cases. Our results also complied with several publications in breast cancer, in which the nucleus was the prevalent localisation of the PARP protein (Rojo et al., 2012, Green et al., 2015, Aleskandarany et al., 2015, Domagala et al., 2011). Moreover, in this current study we have shown that overexpression of PARP1 protein was in significant relation with serous tumour histology, higher-grade and residual disease post-surgery. Similarly, Resta et al concluded from his study on 111 cases of OC tissue specimens that, high PARP1 expression was associated with higher-grade tumours (Resta et al., 2018). Similarly and in another study, positive PARP expression was significantly linked to higher-grade ovarian tumours (Godoy et al., 2011). Although tumours with increased expression of PARP1 were associated with resistance to chemotherapy in a study performed by Gan et al (Gan et al., 2013), this was not identified in this current study. However, our results comes in line with Wysham et al who concluded that combined high expression of PARP1, FANCD2, and p53 proteins was associated not only with a doubled three year risk of recurrence, but also with platinum resistance (Wysham et al., 2012); However, the isolated high PARP level had no effect on platinum sensitivity. In addition, PARP1 overexpression also impacted negatively on both PFS and OS, a finding concluded by the same study (Wysham et al., 2012). Interestingly, in this current study the effect of PARP1 overexpression on outcome was also maintained in platinum sensitive patients, a finding that would concur with the previous studies that confirmed the predictive significance of PARP1 in OC. They demonstrated that increased expression of PARP is associated with poor outcome (Wysham et al., 2012, Gan et al., 2013, Resta et al., 2018).

Polβ is a key player in DNA BER and it promotes genomic stability (Abdel-Fatah et al., 2014a). There is emerging data on the role of polβ in human carcinogenesis as increased level of this protein was measured in cancer cells. Higher levels of polβ have been identified in colonic and prostatic tumours, when compared with the normal tissue levels (Srivastava et al., 1999, Su et al., 2013). In addition, mutations in polβ was also observed in solid tumours. These mutations can influence the response to chemotherapy and promote aggressive tumour phenotype (Starcevic et al., 2004). Polβ overexpression is being particularly more frequent in uterine, breast, ovary, stomach and skin tumours (Albertella et al., 2005). In OC, mutations in polβ was also recognised, although their clinical significance remained unclear (Khanra et al., 2012).

In the current study, polβ overexpression in the unselected whole cohort was associated with poor outcome in terms of both PFS and OS. In addition, polβ high level was also in borderline significance with platinum resistance. On the other hand and in platinum sensitive sub-group, low polβ expression was linked to better outcome. Our data implies that polβ is a useful predictor of outcome in ovarian tumour cases.

To date, a very limited information is available about the clinical influence of Polβ high expression on OC patients. High levels of Polβ protein was found in preclinical created high-grade serous ovarian carcinoma (Guffanti et al., 2018). Similarly, in IHC study of primary Gastric carcinoma, Polβ overexpression was in association with aggressive tumour characteristics (Tan et al., 2015). This finding comes in agreement with the current study. In addition, Polβ deficiency in mice is embryonically lethal (Gu et al., 1994) and embryonic fibroblasts derived from such mice are hypersensitive to alkylating agents (Sobol et al., 1996, Yang et al., 2010). On the other hand, polβ overexpression in pre-clinical models enhanced the relative resistance to DNA-damaging agents (Canitrot et al., 1998). Moreover, polβ depletion in colorectal cancer cell lines promoted their platinum sensitivity (Iwatsuki et al., 2009).

PARP1, XRCC1 and Polβ interact with each other (Fan et al., 2004, Kubota et al., 1996, Bhattacharyya and Banerjee, 2001, Masson et al., 1998, Vidal et al., 2001). Therefore, we performed XRCC1, PARP1 and Polβ co-expression analyses. Overall, we demonstrated that patients with tumours that have low expression of those proteins develop less aggressive tumour in terms of both clinicopatghological characteristics and better survival outcome. On the other hand, high expression of both XRCC1 and Polβ was associated with significant resistance to platinum agents. Similarly and in platinum sensitive patients, PARP1, XRCC1 and Polβ high protein levels was correlated with a significant unfavourable outcome when compared with low protein expressers. This provides further evidence to the synergistic clinical influence of the surrogate biomarkers on primary OC patients.

Importantly, in multivariate Cox model, XRCC1 overexpression was independent predictor of worse cancer recurrence. Therefore, this provides further evidence for the significance of positive XRCC1 in negatively influencing patients' outcome. This finding comes in line with another study, XRCC1 protein expression was assessed

by IHC methodology in 138 consecutive head and neck cancer specimens. High XRCC1 expression was independently associated with increased risk of disease progression (Ang et al., 2011).

Given the important clinical role of XRCC1 in influencing ovarian tumours progression and its intimate relation to PARP1, which could be targeted by chemotherapy, we proceeded to preclinical experiments in order to identify the effect of XRCC1 depletion on PARP1 level and hence the potential benefit of PARP1 inhibitors in XRCC1 depleted tumours.

Hoch et al demonstrated that XRCC1 mutations in cells reduced the rates of SSB repair and elevated the levels of PARP protein. In addition, preclinical XRCC1 knockdown increased PARP1 activity (Hoch et al., 2017). Moreover, in a recent study on breast cancers, an increased PARP1 expression was noted in XRCC1 deficient cancer cells, when compared with the control wild type cells (Ali et al., 2018).

A very interesting observation in the current study is that, in XRCC1 knocked down cells we observed a significant increase in PARP1 expression. Therefore, we postulate that increased PARP1 level may also contribute to a hyperactive PARP1 status seen in XRCC1 deficient cells. Ali et al concluded similar findings, induced XRCC1 deficiency in breast cancer cell lines, activated PARP1 status and consequently potentiated the response to therapy (Ali et al., 2018). Therefore, we postulate that XRCC1 deficient OC cells might be hypersensitive to PARP inhibitor therapy, a novel observation in this study that could be used in the future to improve patients' outcome.

To conclude this chapter, DNA damage repair process is a key player in OC progression. In this study, we aimed to identify the driver proteins that are significantly associated and differentially expressed in OC patients. The present study explored the clinical effect of XRCC1, PARP1 and Polβ in a large well-characterised cohort of primary OC patients. Moreover, preclinical investigation of XRCC1 knockdown influence on PARP1 level. In general, all the surrogate biomarkers were linked to unfavourable tumour characteristics and poor outcome. Clinically, our study provided novel findings and useful prognostic indications. Importantly, we provided the evidence that XRCC1, PARP1 and Polβ work

simultaneously on primary OC patients to exhibit poor prognostic behaviour. In addition, XRCC1 overexpression acted as an independent factor for poor cancer progression and its preclinical deficiency increased PARP1 protein level, a feature that might be useful in PARP1 personalised targeting in XRCC1 deficient tumours. In this study, we identified several promising protein candidates that were intimately associated with tumour prognosis. Therefore, these novel findings may potentiate further scientific and clinical approaches in prognostic biomarkers and therapeutics.

## 5.5 Study limitations related to this chapter

1- The number of platinum resistant cases was limited, which made their assessment a challenging task.

2- Only two OC cell lines (platinum sensitive and resistant) were used in preclinical XRCC1 knockdown experiment.

3- XRCC1 knockdown is a temporary gene silencing method that needs further confirmation via utilising gene knockout.

# 6 The Role of Human DNA Ligases in Primary Epithelial Ovarian Cancer

## 6.1 Introduction

The environmental factors often repeatedly cause damage to the DNA (Li et al., 2015). Carcinogenesis will be the outcome if such DNA damage is not efficiently repaired by the host DNA repair system. Thus, the DNA restorative ability plays a critical role in maintaining the stability of the human genome (Li et al., 2015). DNA ligases play an essential role in maintaining genomic integrity (Çağlayan, 2019). There are three human genes, which encode ATP-dependent DNA ligases that functions in nuclear DNA metabolism: Ligase 1 (LIG1), Ligase III (LIG3) and Ligase IV (LIG4) (Ellenberger and Tomkinson, 2008). In contrast, the mitochondria contains a single species of DNA ligase that is encoded by the *LIG3* gene, which is essential for mitochondrial DNA replication and repair (Lakshmipathy and Campbell, 1999, Lakshmipathy and Campbell, 2001, Gao et al., 2011, Simsek et al., 2011, Tomkinson et al., 2020).

DNA LIG1 plays an essential role in DNA Nucleotide excision repair (NER), Long-patch Base Excision Repair (LP-BER), Single-Strand Break Repair (SSBR) and Microhomology-Mediated End Joining (MMEJ), also known as Alternative Nonhomologous End-Joining (alt-NHEJ) DNA repair (Nocentini, 1999, Levin et al., 2000). In addition, LIG1 is essential for DNA replication and recombination processes (Lindahl and Barnes, 1992, Soderhall and Lindahl, 1975, Barnes et al., 1992, Chan and Becker, 1985, Montecucco et al., 1995). Dysfunction of the immune system, which may predispose for cancer development, can be the outcome of altered LIG1 expression (Bazrgar et al., 2014, Han et al., 2014). Moreover, in a study performed on nasopharyngeal cancer patients, LIG1 genetic polymorphisms modified not only tumour susceptibility, but also cancer radiosensitivity (Tian et al., 2015). Moreover, another study measured LIG1 levels in different organs tumour specimens obtained from patients and preclinical human tumour cell lines, ovarian tumours were included. Then, they compared their LIG1 level with the benign tissues; LIG1 was considerably up regulated in cancer specimens. Nevertheless, LIG1 presence is essential for survival of tumour cells, a conclusion of a previous study conducted by Sun et al (Sun et al., 2001a).

The *LIG3* gene encodes for DNA LIG3. Unlike LIG1, the LIG3 gene encodes three different DNA ligase polypeptides, Mitochondrial and Nuclear versions (Lakshmipathy and Campbell, 1999). DNA LIG3 dependent repair of DNA breaks

in the nucleus is critical for neuronal cell viability. Thus, mutations in LIG3 can be causative factor in inherited neurodegenerative syndromes (Yoon and Caldecott, 2018). Moreover, LIG3 deletion resulted in embryonic lethality in mice (Puebla-Osorio et al., 2006). In cancerous tissue, an elevated steady-state level of DNA LIG3 was identified as biomarker of altered DNA DSB repair. Breast cancer, leukaemias and neuroblastoma were among the evaluated tumour types (Tobin et al., 2013, Tobin et al., 2012, Newman et al., 2015). In addition, LIG3 deficient cell lines were hypersensitive to DNA-alkylating agents (Ellenberger and Tomkinson, 2008). Interestingly, both LIG1 and LIG3 can complement each other to efficiently repair the DNA damage (Sleeth et al., 2004, Çağlayan, 2019).

LIG4 interacts with human XRCC4 and is involved in DSB DNA repair by NHEJ mechanism (van Gent and van der Burg, 2007, Grawunder et al., 1997). Deletion of *LIG4* gene caused embryonic lethality in mice (Frank et al., 2000, Frank, Barnes et al., 1998). Mutations in LIG4 gene are associated with a rare autosomal syndrome that is characterised by microcephaly, immunodeficiency, genomic instability and a higher susceptibility to cancer development (Pollard and Gatti, 2009, Chistiakov et al., 2008, O'Driscoll and Jeggo, 2006). Aberrations in human LIG4 can predispose to immunodeficiency and cancer development (O'Driscoll et al., 2001).

### 6.1.1 Hypothesis

Human DNA ligases are important signalling transduction molecules that have diverse function in different cancerous organs. Given the key roles played by LIG1, LIG3 and LIG4 in genomic integrity, we evaluated their expression in a large cohort of primary epithelial ovarian cancer (OC). Our interpretation of the prognostic and molecular impact of ligases altered protein expression may reveal new insights in management of ovarian tumour patients.

### 6.1.2 Aims

The aim of this study is to identify the novel role of human ligases (LIG1, LIG3 and LIG4) positive expression on the patients' clinicopathological variables and outcome in primary OC cases, and to pinpoint potential prognostic and drug-targeted proteins, for future examination. The utilisation of both IHC methodology

and pre-clinical laboratory methods allowed us to identify surrogate markers that can be utilised for direct targeting to improve disease prognosis.

## 6.2 Methods

### 6.2.1 Clinical studies

At first, western blot (WB) methodology was utilised to confirm the specificity of the tested proteins, LIG1, LIG3 and LIG4. The full details of WB procedure including the concentrations of antibodies used and the purchased materials can be found chapter two, (Table 2-3). Then, tissue microarray (TMA) from 525 primary OC tissue specimens was prepared and immunohistochemical (IHC) staining was performed as described previously in chapter two, (section 2.5). Before the staining stage, optimisation of all the surrogate proteins was performed to identify the optimal antibody concentration, detailed in the supplementary section. LIG1, LIG3 and LIG4 expression was determined in the whole, platinum sensitive and resistant series as explained in the methodology chapter. Pre-treatment of TMA sections was performed with citrate buffer (pH 6.0, 20 min, Microwave). Then tissue tumour specimens were incubated at room temperature with 1:25 anti-LIG1 rabbit monoclonal antibody (ab177946, ABCAM) and 1:100 anti-LIG4 rabbit polyclonal antibody (HPAA001334, SIGMA) for 60 minutes. However, the incubation time was overnight at room temperature with 1:100 concentration of anti-LIG3 rabbit polyclonal antibody (HPA006723, SIGMA). The detailed procedure of IHC, digital imaging, and scoring are referred to in Chapter 2.

### 6.2.2 Preclinical methods

#### 6.2.2.1 Nuclear and cytoplasmic extraction

LIG3 nuclear and cytoplasmic extraction was performed as explained in details at chapter 2, (Section 2.6.7). A2780 and A2780Cis OC cell lines were utilised for this procedure and LIG3 level was measured after 48hours of 5μM cisplatin treatment.

#### 6.2.2.2 LIG1 knockdown using SiRNA

SiRNA were obtained from Invitrogen, UK. Knock down procedure was performed as described previously in details in chapter two, (Section 2.6.5). Transfection efficiency was confirmed at day 3 and day 5 using WB, detailed in (Section 2.6.4). Both A2780 and A2780Cis OC cell lines were chosen for this experiment.

## 6.3 Results

### 6.3.1 Antibodies' specificity

The evaluation of WB images for the anti-LIG1, anti-LIG3, and anti-LIG4 primary antibodies supported a specific binding affinity to a single protein band for both A2780 and A2780Cis OC cell lysates around their predicted molecular weight as instructed by the supplying company. It was 120 kDa for the LIG1 protein, (Figure 6-1), 113 kDa for the LIG3 protein, (Figure 6-2), and around 109 kDa for the LIG4, (Figure 6-3).

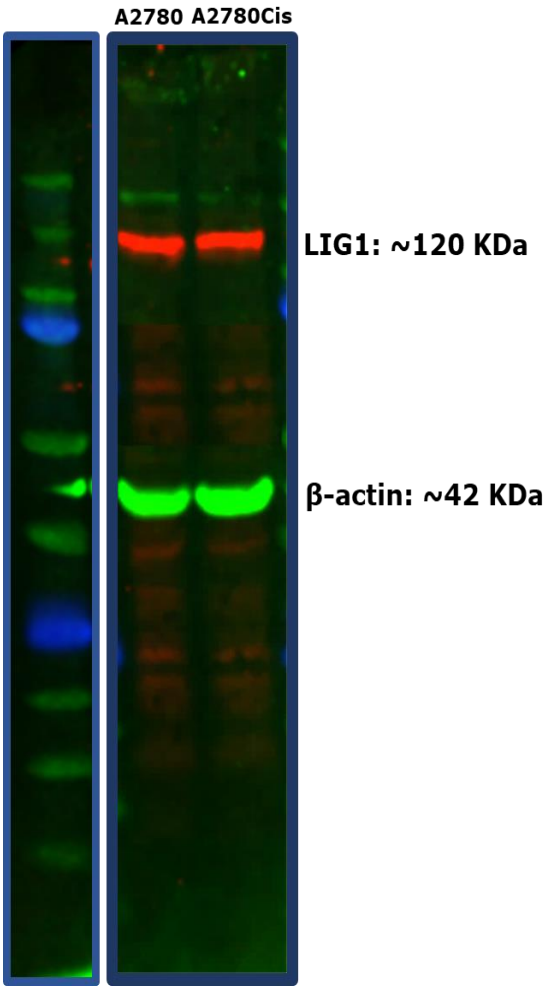



**Figure 6-1: Binding specificity of the anti-LIG1 antibody.**
**WB membrane showed specific antigen-binding of for anti-LIG1 antibody around ~120 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

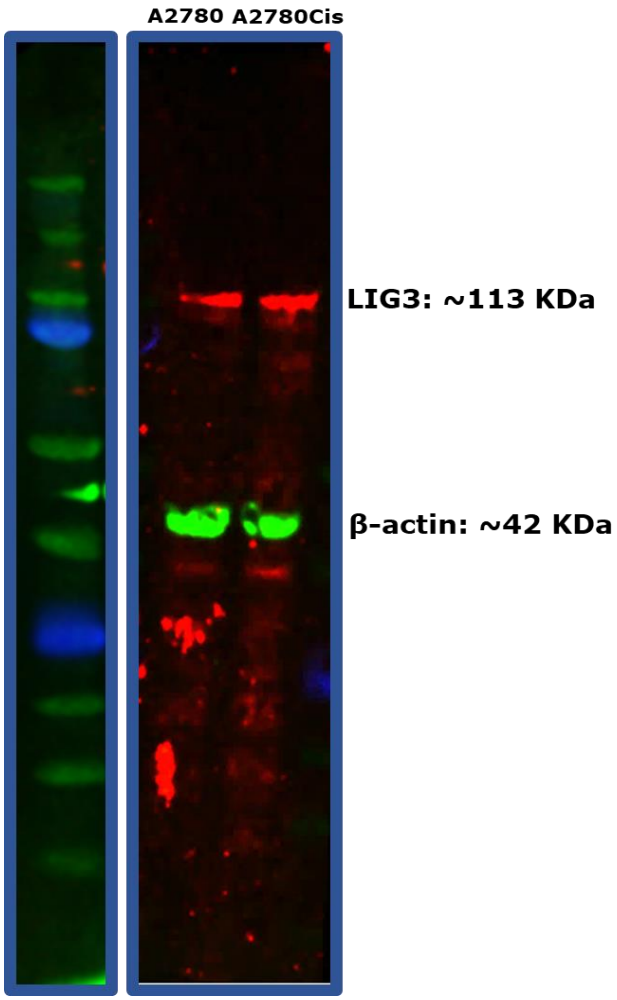



**Figure 6-2: Binding specificity of the anti-LIG3 antibody.**
**WB membrane showed specific antigen-binding of for anti-LIG3 antibody around ~113 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

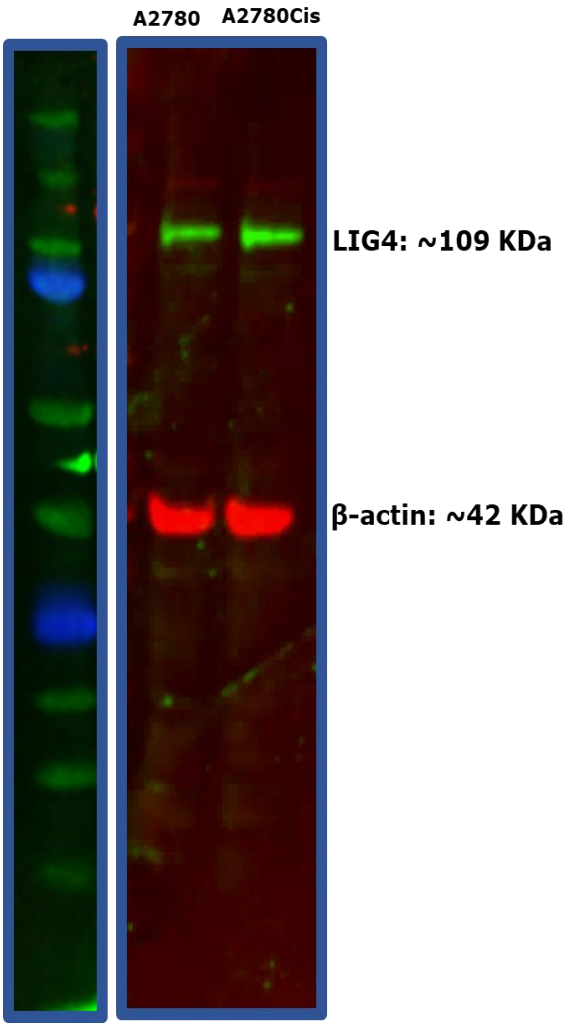



**Figure 6-3: Binding specificity of the anti-LIG4 antibody.**
**WB membrane showed specific antigen-binding of for anti-LIG4 antibody around ~109 KDa. The positive protein expression of β-actin, as endogenous control, was detected around ~42 KDa. Protein ladder, lane 1, A2780 and lane 2, A2780Cis OC cell lines.**

### 6.3.2 Immunohistochemical results

#### 6.3.2.1 The expression pattern of the surrogate proteins

In this study, we applied IHC methodologies to assess the altered protein expression of LIG1, LIG3 and LIG4 and to investigate their prognostic importance in primary OC, including their association with platinum sensitivity and their correlation with patients' clinicopathological parameters. The examination of the immuno-stained TMA sections revealed a unique localised distribution for the proteins mentioned above among the different cellular compartments. The protein expressions of LIG1 and LIG4 were mainly nuclear, (Figure 6-4) and (Figure 6-6) respectively. On the other hand, LIG3 positive expression was identified in both the nucleus and cytoplasm, (Figure 6-5). The designated cut-off points were calculated using the median value. They were chosen at <60 H-score for nuclear LIG1, ≤190 H-score for nuclear LIG4. Moreover and regarding LIG3, ≤ 100 and ≤ 0 H-score were utilised as the optimal cut-off for cytoplasmic and nuclear positive LIG3 expression, respectively. The IHC staining intensities showed negative/ weak, moderate, and strong staining for the three interrogated proteins.

In terms of LIG1 nuclear expression, 46.8% of tumours were considered low/negative and 53.2% of the tumours were deemed high in their LIG1 expression, (Figure 6-4). However, positive LIG3 expression was localised to both the nucleus and cytoplasm in malignant cells. Positive cytoplasmic expression was seen in 47.1% of tumours, while the remaining patients, 52.9% showed negative/low cytoplasmic expression for LIG3. Similarly, positive nuclear LIG3 was observed in 47.6%, and negative/low nuclear expression was confirmed in 52.4%, (Figure 6-5). The combined LIG3 staining was as follows: cytoplasmic-/nuclear- in 25.3%, cytoplasmic-/nuclear+ in 27.5%, cytoplasmic+/nuclear- in 27.0%, and cytoplasmic+/nuclear+ in 20.2% of the specimens. Finally, the positive nuclear expression of LIG4 was observed in 30.5% of the cases, while the remaining patients, 69.5% showed negative/low expression of LIG4, (Figure 6-6).

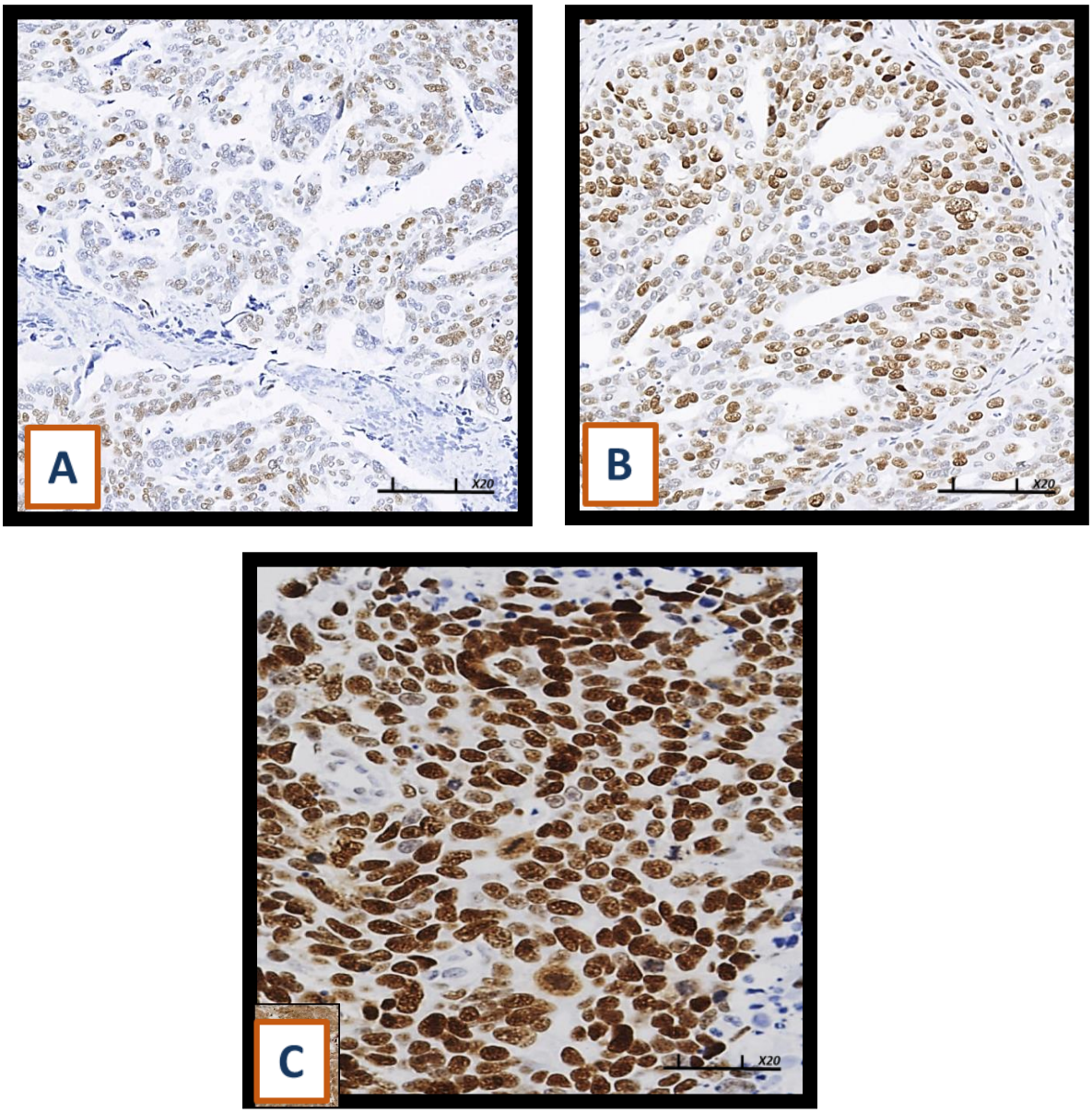



**Figure 6-4: Immunohistochemistry expression of LIG1 in tissue microarray images. Nuclear expression of LIG1 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

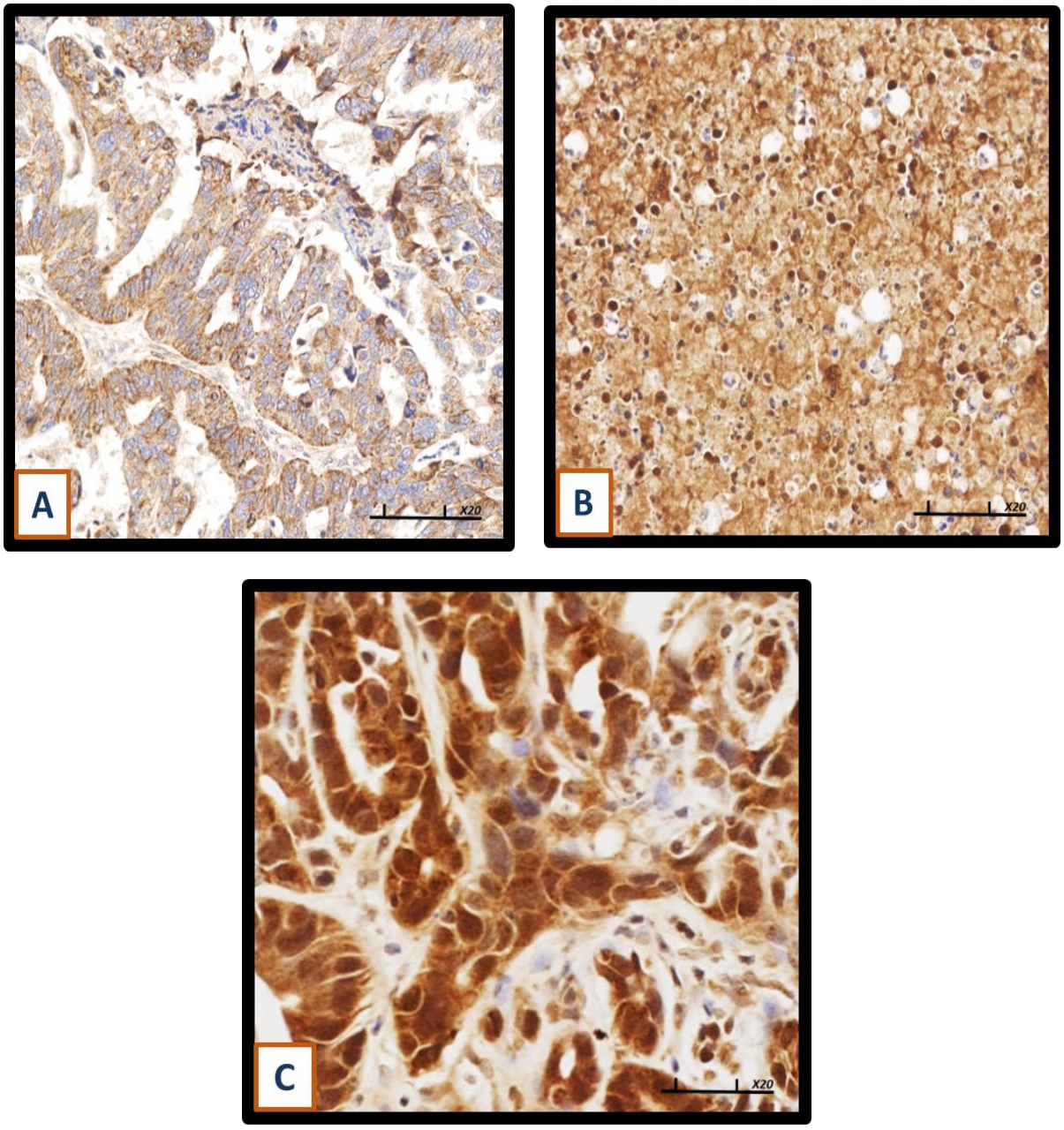



**Figure 6-5: Immunohistochemistry expression of LIG3 in tissue microarray images. Nuclear expression of LIG3 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

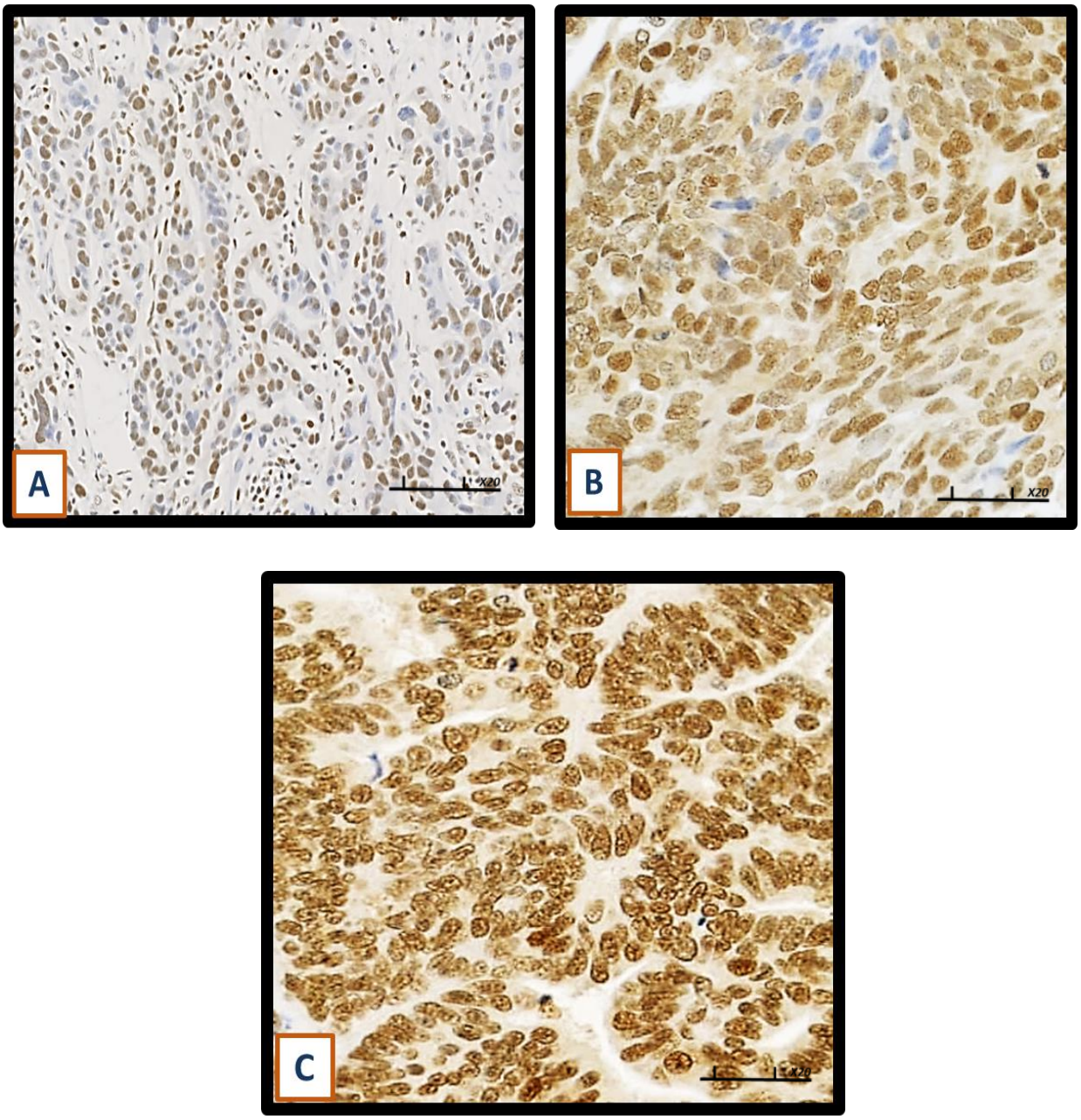



**Figure 6-6: Immunohistochemistry expression of LIG4 in tissue microarray images. Nuclear expression of LIG4 in cancer cells was distributed as follows; A) weak staining, B) moderate staining, and C) strong staining. Images were captured at 20-times magnifications.**

#### 6.3.2.2 The clinicopathological impact for LIG1 protein

Protein expression of LIG1 showed strong features as a poor prognostic biomarker for invasive OC patients. Upregulated protein expression of LIG1 was strongly associated with serous histology tumour subtype, (p-value< 0.0001). Moreover, the positive expression of LIG1 was also correlated with advanced stage disease, (p-value< 0.0001), higher tumour grade, (p-value < 0.0001), and higher risk of measurable disease before chemotherapy, (p-value= 0.011) compared to tumours with low LIG1 expression. On the other hand, tumours with negative/low LIG1 nuclear expression were sensitive to platinum therapy but with borderline non-significance, (p-value= 0.063) (Table 6-1).

Moreover and as expected, LIG1 protein was interrelated with LIG3 isoforms, the nuclear LIG3 protein, (p-value <0.0003) and cytoplasmic LIG3, (p-value =0.049). However, no correlation was identified between both LIG1 and LIG4 (p-value = 0.583), (Table 6-1).

Moreover, investigating the combined expression of LIG1 with both the cytoplasmic and nuclear LIG3 protein. Then, correlating their positive expression with the clinicopathological data, tumours with combined high expression of both LIG1 and cytoplasmic LIG3 had a significant association with serous adenocarcinomas and advanced stage disease, (p-value< 0.0001). On the other hand, negative/low expression of both proteins was significantly correlated with lower histology grade, (p-value< 0.0001) and reduced risk of measurable disease after surgery, (p-value= 0.004), if compared with high expressers of both biomarkers. Importantly, cases with high LIG1/LIG3 protein levels illustrated significant resistance to platinum therapy, (p-value= 0.027) (Table 6-5). Similarly, high expression of both LIG1 and nuclear LIG3 proteins was highly significantly correlated with serous tumour histology and advanced stage disease, (p-value< 0.0001). However, low/negative expression tumours of both biomarkers showed significant association with lower histology grade and reduced chance of post-operative residual disease, (p-value= 0.001) and (p-value= 0.036) respectively when compared with high expression cases, (Table 6-6). However, no correlation deemed significant with platinum sensitivity.

#### 6.3.2.3 The clinicopathological impact of LIG3 protein

##### 6.3.2.3.1 Cytoplasmic expression of LIG3

In general, the upregulated expression of cytoplasmic LIG3 was an indicator for poor prognosis and resistance to platinum therapy. Positive cytoplasmic expression of LIG3 was associated with the advanced stage disease, (p-value= 0.002), higher histology grade, (p-value= 0.028) and residual tumour post-surgery, (p-value= 0.006). On the other hand, negative/low expression tumours of cytoplasmic LIG3 showed a significantly improved response to platinum chemotherapy with lower resistance compared with high expression cases, (p-value= 0.025) (Table 6-2).

##### 6.3.2.3.2 Nuclear expression of LIG3

Clinically, apart from the significant association with mucinous and endometroid histology type, (p-value= 0.017), the nuclear expression of LIG3 had no clinical impact on OC cases' other clinicopathological variables, (Table 6-3).

##### 6.3.2.3.3 Combined cytoplasmic and nuclear expression of LIG3

When the combined cytoplasmic and nuclear expression of LIG3 was tested and correlated with the patients' clinicopathgological variables, we observed that tumours with high cytoplasmic/high nuclear LIG3 co-expression was significantly associated with serous histology type, (p-value= 0.042). On the other hand, when both proteins were low expressed a significant association was noted with low stage tumour, (p-value= 0.015), if compared with high expressers. On the other hand, cases showed less residual disease following surgery when LIG3 cytoplasmic/nuclear expression was negative/low, (Table 6-4).

#### 6.3.2.4 The clinicopathological impact of LIG4 protein

In general, the upregulated expression of nuclear LIG4 was an indicator for improved response to platinum therapy. We observed in the current study that low nuclear protein expression of LIG4 has a significant correlation with poor response to platinum based chemotherapy, (p-value= 0.049), if compared with over expressers of this biomarker. However, no other significant correlation was noted between high expression of LIG4 and the rest of the clinicopathological variables, (Table 6-7).

**Table 6-1: Clinicopathological significance of LIG1 protein with Protein-protein interactions between ligases in the patients of primary ovarian carcinoma. Significant values are in bold.**

| LIG1 Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **LIG1 Negative No. (%)** | **LIG1 Positive No. (%)** | ***P- value* ( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 80 (32.5) | 166 (67.5) | |
| Mucinous | 37 (71.2) | 15 (28.8) | |
| Endometrioid | 49 (70.0) | 21 (30.0) | ***<0.0001*** |
| Clear cell carcinoma | 23 (65.7) | 12 (34.3) | **(52.682)** |
| Mixed | 8 (47.1) | 9 (52.9) | |
| Other | 7 (46.7) | 8 (53.3) | |
| ***FIGO Stage*** | | | |
| I | 96 (61.5) | 60 (38.5) | ***<0.0001*** |
| II | 33 (48.5) | 35 (51.5) | **(25.778)** |
| III | 62 (35.4) | 113 (64.6) | |
| IV | 7 (29.2) | 17 (70.8) | |
| ***Grade*** | | | |
| G1 | 34 (63.0) | 20 (37.0) | ***<0.0001*** |
| G2 | 45 (61.6) | 28 (38.4) | **(18.920)** |
| G3 | 99 (38.7) | 157 (61.3) | |
| ***Measurable Disease*** | | | |
| Non | 140 (52.0) | 129 (48.0) | ***0.011*** |
| Measurable | 50 (38.5) | 80 (61.5) | **(6.483)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 155 (48.9) | 162 (51.1) | 0.063 |
| Resistant | 24 (36.4) | 42 (63.6) | (3.446) |
| ***LIG3 Nuclear*** | | | |
| Negative | 116 (61.7) | 72 (38.3) | ***<0.0001*** |
| Positive | 95 (43.8) | 122 (56.2) | **(12.967)** |
| ***LIG3 Cytoplasmic*** | | | |
| Negative | 110 (58.5) | 78 (41.5) | ***0.042*** |
| Positive | 105 (48.4) | 112 (51.6) | **(4.415)** |
| ***LIG4*** | | | |
| Negative | 116 (68.6) | 53 (31.4) | 0.583 |
| Positive | 139 (71.3) | 56 (28.7) | (0.301) |

**Table 6-2: Clinicopathological significance of cytoplasmic LIG3 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| LIG3 Cytoplasmic Expression | | | |
|---|---|---|---|
| **Parameter** | **LIG3 Negative No. (%)** | **LIG3 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 114 (48.5) | 121 (51.5) | |
| Mucinous | 30 (61.2) | 19 (38.8) | |
| Endometrioid | 36 (57.1) | 27 (42.9) | 0.458 |
| Clear cell carcinoma | 20 (60.6) | 13 (39.4) | (4.666) |
| Mixed | 10 (58.8) | 7 (41.2) | |
| Other | 7 (53.8) | 6 (46.2) | |
| ***FIGO Stage*** | | | |
| I | 94 (64.8) | 51 (35.2) | ***0.002*** |
| II | 26 (41.3) | 37 (58.7) | **(14.677)** |
| III | 84 (49.7) | 85 (50.3) | |
| IV | 9 (37.5) | 15 (62.5) | |
| ***Grade*** | | | |
| G1 | 35 (67.3) | 17 (32.7) | ***0.028*** |
| G2 | 29 (43.3) | 38 (56.7) | **(7.131)** |
| G3 | 124 (50.4) | 122 (49.6) | |
| ***Measurable Disease*** | | | |
| Non | 149 (58.7) | 105 (41.3) | ***0.006*** |
| Measurable | 53 (43.4) | 69 (56.6) | **(7.678)** |
| ***Platinum sensitivity*** | | | |
| Sensitive | 163 (54.2) | 138 (45.8) | ***0.025*** |
| Resistant | 23 (38.3) | 37 (61.7) | **(5.013)** |

**Table 6-3: Clinicopathological significance of nuclear LIG3 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| LIG3 Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **LIG3 Negative No. (%)** | **LIG3 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 113 (48.1) | 122 (51.9) | |
| Mucinous | 34 (69.4) | 15 (30.6) | |
| Endometrioid | 38 (60.3) | 25 (39.7) | ***0.017*** |
| Clear cell carcinoma | 19 (57.6) | 14 (42.4) | **(13.782)** |
| Mixed | 6 (35.3) | 11 (64.7) | |
| Other | 4 (30.8) | 9 (69.2) | |
| ***FIGO Stage*** | | | |
| I | 82 (56.6) | 63 (43.4) | 0.278 |
| II | 27 (42.9) | 36 (57.1) | (3.853) |
| III | 91 (53.8) | 78 (46.2) | |
| IV | 11 (45.8) | 13 (54.2) | |
| ***Grade*** | | | |
| G1 | 29 (55.8) | 23 (44.2) | 0.947 |
| G2 | 36 (53.7) | 31 (46.3) | (0.109) |
| G3 | 131 (53.3) | 115 (46.7) | |
| ***Measurable Disease*** | | | |
| Non | 131 (51.6) | 123 (48.4) | 0.338 |
| Measurable | 66 (55.0) | 54 (45.0) | (2.172) |
| ***Platinum sensitivity*** | | | |
| Sensitive | 164 (54.5) | 137 (45.5) | 0.757 |
| Resistant | 34 (56.7) | 26 (43.3) | (0.096) |

**Table 6-4: Clinicopathological significance of the combined cytoplasmic/nuclear LIG3 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| LIG3 Cytoplasmic/Nuclear Coexpression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **C-/ N- No. (%)** | **C-/ N+ No. (%)** | **C+/ N- No. (%)** | **C+/ N+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 48 (20.4) | 66 (28.1) | 65 (27.7) | 56 (23.8) | |
| Mucinous | 20 (40.8) | 10 (20.4) | 14 (28.6) | 5 (10.2) | |
| Endometrioid | 19 (30.2) | 17 (27.0) | 19 (30.2) | 8 (12.7) | ***0.042*** **(25.599)** |
| Clear cell carcinoma | 13 (39.4) | 7 (21.2) | 6 (18.2) | 7 (21.2) | |
| Mixed | 2 (11.8) | 8 (47.1) | 4 (23.5) | 3 (17.6) | |
| Other | 1 (7.7) | 6 (46.2) | 3 (23.1) | 3 (23.1) | |
| ***FIGO Stage*** | | | | | |
| I | 48 (33.1) | 46 (31.7) | 34 (23.4) | 17 (11.7) | ***0.015*** **(20.502)** |
| II | 10 (15.9) | 16 (25.4) | 17 (27.0) | 20 (31.7) | |
| III | 42 (24.9) | 42 (24.9) | 49 (29.0) | 36 (21.3) | |
| IV | 4 (16.7) | 5 (20.8) | 7 (29.2) | 8 (33.3) | |
| ***Grade*** | | | | | |
| G1 | 19 (36.5) | 16 (30.8) | 10 (19.2) | 7 (13.5) | 0.235 (8.047) |
| G2 | 13 (19.4) | 16 (23.9) | 23 (34.3) | 15 (22.4) | |
| G3 | 58 (23.6) | 66 (26.8) | 73 (29.7) | 49 (19.9) | |
| ***Measurable Disease*** | | | | | |
| Non | 72 (28.3) | 77 (30.3) | 59 (23.2) | 46 (18.1) | ***0.038*** **(8.400)** |
| Measurable | 25 (20.5) | 28 (23.0) | 43 (35.2) | 26 (21.3) | |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 84 (27.9) | 79 (26.2) | 80 (26.6) | 58 (19.3) | 0.158 (5.191) |
| Resistant | 13 (21.7) | 10 (16.7) | 21 (35.0) | 16 (26.7) | |

**Table 6-5: Clinicopathological significance of the combined LIG1 and cytoplasmic LIG3 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| Combined LIG1 / LIG3 Cytoplasmic Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **LIG1-/ LIG3- No. (%)** | **LIG1-/ LIG3+ No. (%)** | **LIG1+/ LIG3- No. (%)** | **LIG1+/ LIG3+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 36 (16.0) | 34 (15.1) | 75 (33.3) | 80 (35.6) | |
| Mucinous | 23 (47.9) | 11 (22.9) | 6 (12.5) | 8 (16.7) | |
| Endometrioid | 26 (41.3) | 17 (27.0) | 10 (15.9) | 10 (15.9) | ***<0.0001*** |
| Clear cell carcinoma | 14 (43.8) | 8 (25.0) | 5 (15.6) | 5 (15.6) | **(55.548)** |
| Mixed | 6 (35.3) | 2 (11.8) | 4 (23.5) | 5 (29.4) | |
| Other | 4 (30.8) | 3 (23.1) | 3 (23.1) | 3 (23.1) | |
| ***FIGO Stage*** | | | | | |
| I | 59 (41.3) | 29 (20.3) | 35 (24.5) | 20 (14.0) | ***<0.0001*** |
| II | 15 (24.2) | 14 (22.6) | 10 (16.1) | 23 (37.1) | **(41.464)** |
| III | 30 (18.5) | 25 (15.4) | 51 (31.5) | 56 (34.6) | |
| IV | 1 (4.5) | 5 (22.7) | 7 (31.8) | 9 (40.9) | |
| ***Grade*** | | | | | |
| G1 | 26 (52.0) | 6 (12.0) | 9 (18.0) | 9 (18.0) | ***<0.0001*** |
| G2 | 22 (33.8) | 18 (27.7) | 5 (7.7) | 20 (30.8) | **(38.591)** |
| G3 | 45 (18.8) | 44 (18.4) | 77 (32.2) | 73 (30.5) | |
| ***Measurable Disease*** | | | | | |
| Non | 80 (32.1) | 49 (19.7) | 66 (26.5) | 54 (21.7) | ***0.004*** |
| Measurable | 20 (17.1) | 23 (19.7) | 31 (26.5) | 43 (36.8) | **(13.402)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 85 (29.3) | 55 (19.0) | 73 (25.2) | 77 (26.6) | ***0.027*** |
| Resistant | 7 (11.9) | 12 (20.3) | 16 (27.1) | 24 (40.7) | **(9.162)** |

**Table 6-6: Clinicopathological significance of the combined LIG1 and nuclear LIG3 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| Combined LIG1 / LIG3 Nuclear Expression | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **LIG1-/ LIG3- No. (%)** | **LIG1-/ LIG3+ No. (%)** | **LIG1+/ LIG3- No. (%)** | **LIG1+/ LIG3+ No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | | | |
| Serous | 43 (19.1) | 27 (12.0) | 63 (28.0) | 92 (40.9) | |
| Mucinous | 25 (52.1) | 9 (18.8) | 8 (16.7) | 6 (12.5) | |
| Endometrioid | 26 (41.3) | 17 (27.0) | 12 (19.0) | 8 (12.7) | ***<0.0001*** |
| Clear cell carcinoma | 14 (43.8) | 8 (25.0) | 5 (15.6) | 5 (15.6) | **(63.723)** |
| Mixed | 3 (17.6) | 5 (29.4) | 3 (17.6) | 6 (35.3) | |
| Other | 2 (15.4) | 5 (38.5) | 2 (15.4) | 4 (30.8) | |
| ***FIGO Stage*** | | | | | |
| I | 57 (39.9) | 31 (21.7) | 24 (16.8) | 31 (21.7) | ***<0.0001*** |
| II | 14 (22.6) | 15 (24.2) | 13 (21.0) | 20 (32.3) | **(31.553)** |
| III | 37 (22.8) | 18 (11.1) | 49 (30.2) | 58 (35.8) | |
| IV | 4 (18.2) | 2 (9.1) | 5 (22.7) | 11 (50.0) | |
| ***Grade*** | | | | | |
| G1 | 19 (38.0) | 13 (26.0) | 8 (16.0) | 10 (20.0) | ***0.001*** |
| G2 | 27 (41.5) | 13 (20.0) | 8 (12.3) | 17 (26.2) | **(22.277)** |
| G3 | 58 (24.3) | 31 (13.0) | 69 (28.9) | 81 (33.9) | |
| ***Measurable Disease*** | | | | | |
| Non | 79 (31.7) | 50 (20.1) | 49 (19.7) | 71 (28.5) | ***0.036*** |
| Measurable | 29 (24.8) | 14 (12.0) | 35 (29.9) | 39 (33.3) | **(8.546)** |
| ***Platinum sensitivity*** | | | | | |
| Sensitive | 89 (30.7) | 51 (17.6) | 68 (23.4) | 82 (28.3) | 0.125 |
| Resistant | 14 (23.7) | 5 (8.5) | 19 (32.2) | 21 (35.6) | (5.738) |

**Table 6-7: Clinicopathological significance of LIG4 protein expression in the patients of primary ovarian carcinoma. Significant values are in bold.**

| LIG4 Nuclear Expression | | | |
|---|---|---|---|
| **Parameter** | **LIG4 Negative No. (%)** | **LIG4 Positive No. (%)** | ***P- value*** **( $X^2$ )** |
| ***Pathological Type*** | | | |
| Serous | 142 (67.3) | 69 (32.7) | |
| Mucinous | 32 (72.7) | 12 (27.3) | |
| Endometrioid | 43 (75.4) | 14 (24.6) | 0.758 |
| Clear cell carcinoma | 21 (72.4) | 8 (27.6) | (2.620) |
| Mixed | 9 (64.3) | 5 (35.7) | |
| Other | 7 (58.3) | 5 (41.7) | |
| ***FIGO Stage*** | | | |
| I | 95 (69.9) | 41 (30.1) | 0.971 |
| II | 39 (67.2) | 19 (32.8) | (0.239) |
| III | 106 (70.7) | 44 (29.3) | |
| IV | 12 (70.6) | 5 (29.4) | |
| ***Grade*** | | | |
| G1 | 29 (60.4) | 19 (39.6) | 0.325 |
| G2 | 42 (68.9) | 19 (31.1) | (2.249) |
| G3 | 155 (71.4) | 62 (28.6) | |
| ***Measurable Disease*** | | | |
| Non | 161 (68.8) | 73 (31.2) | 0.371 |
| Measurable | 78 (73.6) | 28 (26.4) | (0.799) |
| ***Platinum sensitivity*** | | | |
| Sensitive | 193 (69.7) | 84 (30.3) | **0.049** |
| Resistant | 37 (84.1) | 7 (15.9) | **(3.885)** |

#### 6.3.2.5 Outcome analysis

##### 6.3.2.5.1 Univariate analysis

###### *6.3.2.5.1.1 Whole cohort*

Compared with low protein expression tumours, the KM survival analyses of Progression Free Survival (PFS) and Overall Survival (OS) showed unfavourable outcomes for patients with the upregulated expression of LIG1 protein, (p-value= 0.001) and (p-value= 0.037) respectively, (Figure 6-7). Similarly, overexpression of cytoplasmic LIG3 was correlated with shorter PFS, (p-value< 0.001) and OS, (p-value=0.003) (Figure 6-8). However, high LIG3 nuclear expression showed a non-significant correlation with PFS and OS, (p-value= 0.517) and (p-value= 0.254) respectively (Figure 6-9), compared with tumours that had low LIG3 expression. In addition, when LIG3 cytoplasmic and nuclear combined expression was tested with survival, we observed that tumours with high cytoplasmic/low nuclear LIG3 co-expression had worse PFS and OS compared to tumours with low cytoplasmic/low nuclear LIG3 co-expression, who exhibited the highest survival, (p-value= 0.001) and (p-value= 0.024) respectively (Figure 6-10). Similarly, analysing the combined expression of both LIG1 and LIG3 revealed that tumours with low LIG1/low cytoplasmic LIG3 had highly significantly favourable PFS, (p-value= 0.00002) and OS, (p-value= 0.007) (Figure 6-11). On the other hand, upregulated expression of LIG1/nuclear LIG3 was correlated with the worst outcome in terms of PFS, (p-value= 0.001) but it was in borderline non-significance with OS, (p-value= 0.054) (Figure 6-12). The KM survival analyses of LIG4 showed favourable outcomes for patients with upregulated expression of nuclear LIG4 protein, PFS (p-value =0.001) and OS (p-value =0.024) (Figure 6-13). At the transcriptional level, similarly, high LIG1 mRNA was associated with poor PFS (p-value = 0.029) and OS (p-value = 0.019). In addition, high LIG3 mRNA was also associated significantly with poor PFS (p-value = 0.023), but not with OS (p-value = 0.11). On the other hand, low LIG4 mRNA was in borderline non-significant with poor PFS (p-value = 0.091), but not with OS (p-value = 0.26).

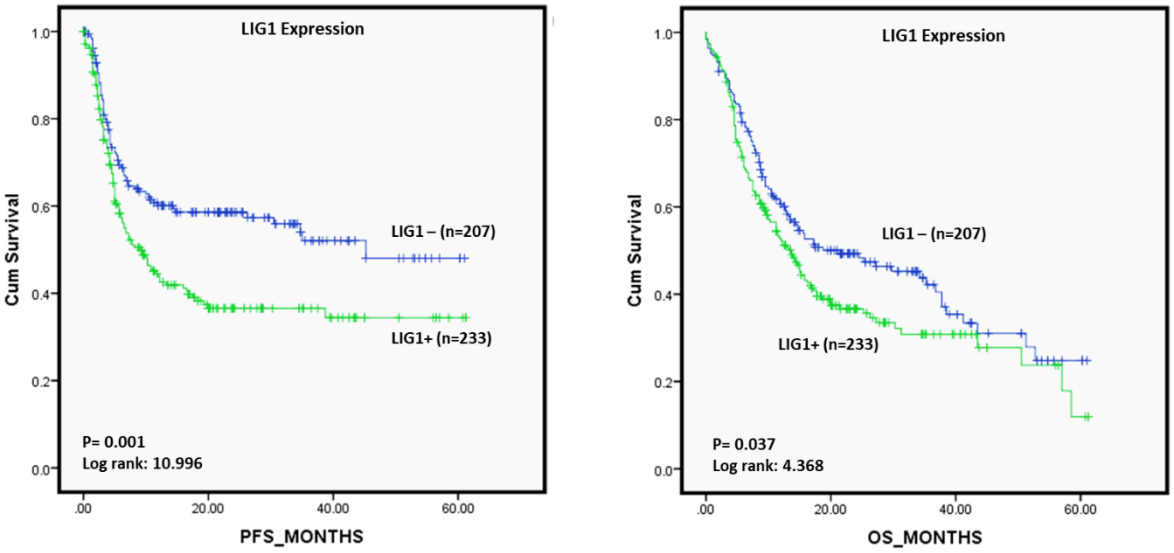



**Figure 6-7: Kaplan Meier plots illustrating the associations between the altered expression of LIG1 protein with PFS and OS in primary ovarian carcinoma – whole series**

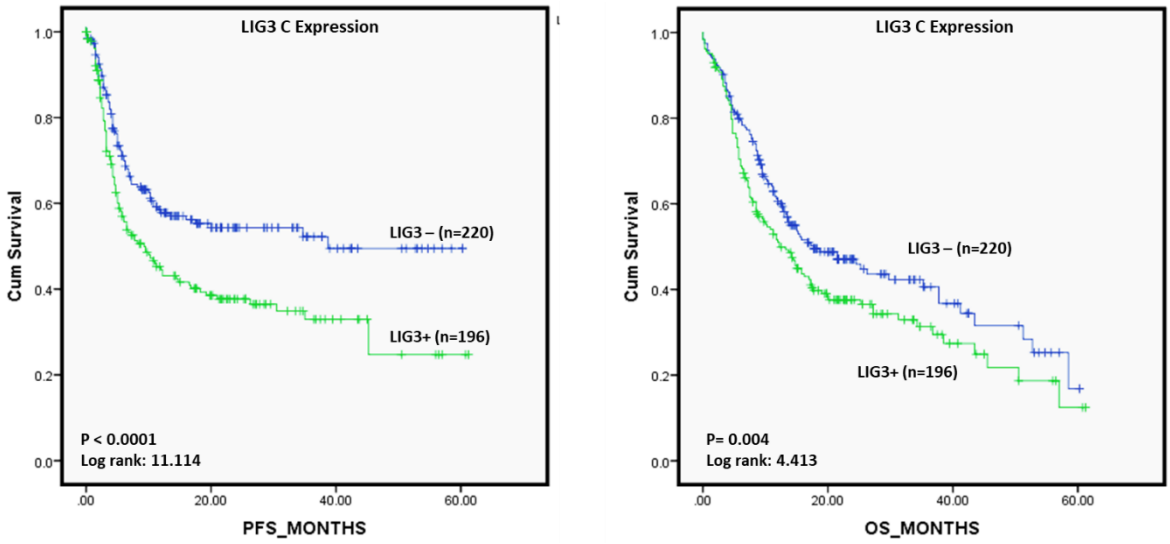



**Figure 6-8: Kaplan Meier plots illustrating the associations between the altered expression of cytoplasmic LIG3 protein with PFS and OS in primary ovarian carcinoma – whole series**

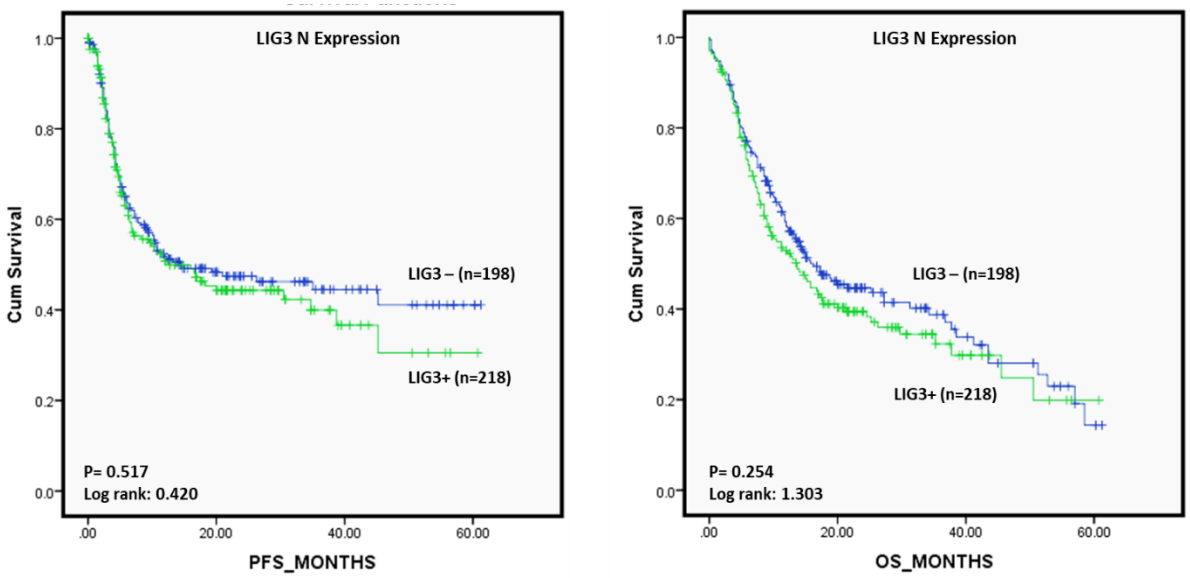



**Figure 6-9: Kaplan Meier plots illustrating the associations between the altered expression of nuclear LIG3 protein with PFS and OS in primary ovarian carcinoma – whole series**

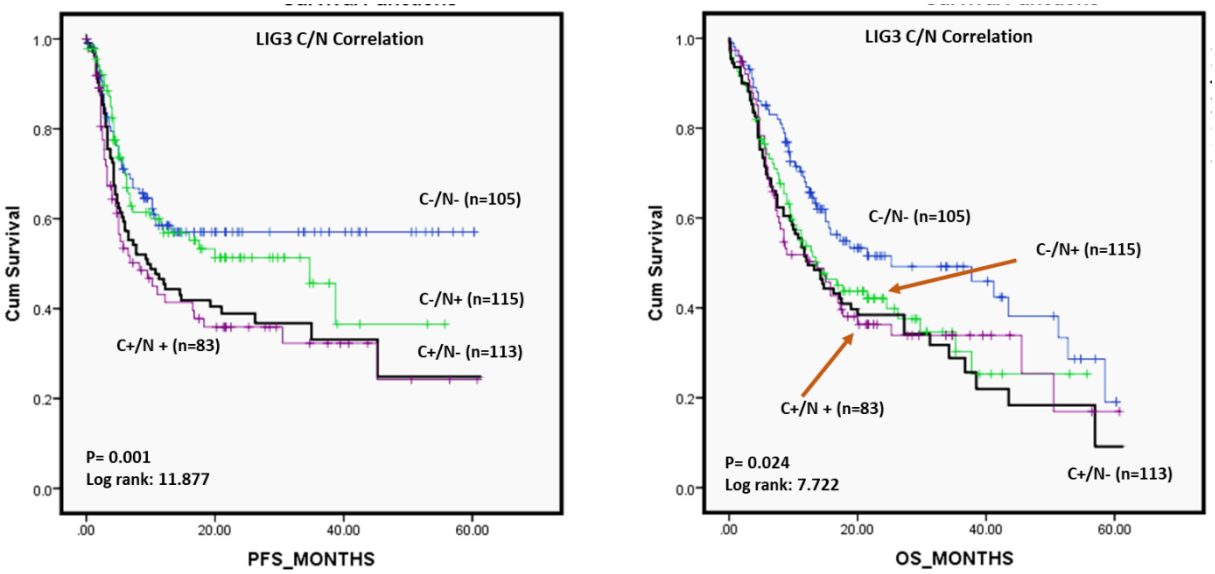



**Figure 6-10: Kaplan Meier plots illustrating the associations between the altered expression of the combined cytoplasmic and nuclear LIG3 protein with PFS and OS in primary ovarian carcinoma – whole series**

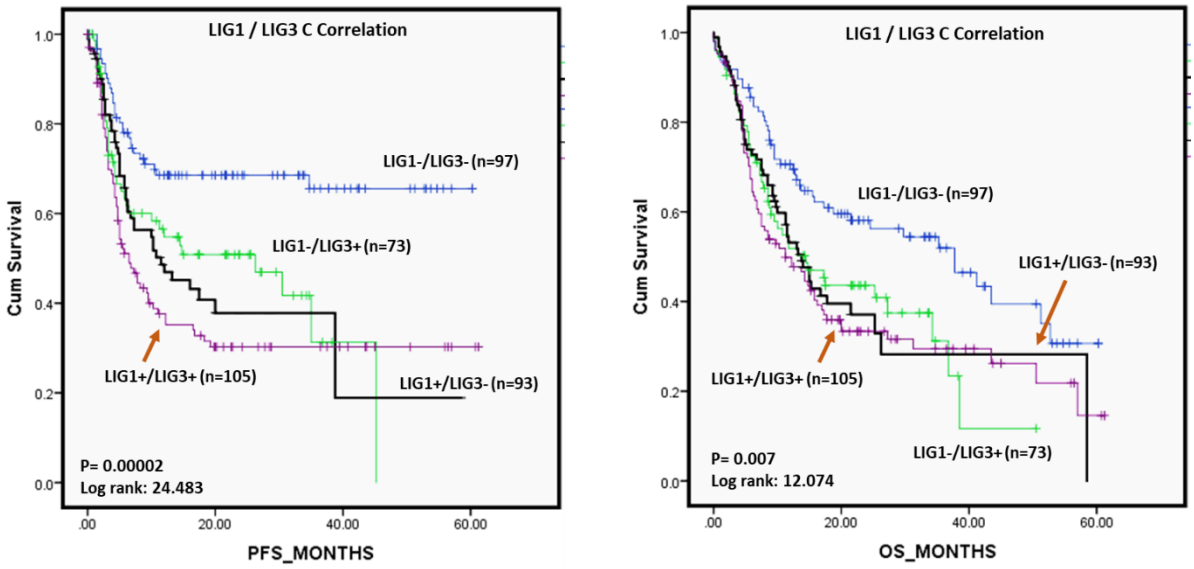



**Figure 6-11: Kaplan Meier plots illustrating the associations between the altered expression of the combined LIG1 and cytoplasmic LIG3 proteins with PFS and OS in primary ovarian carcinoma – whole series**

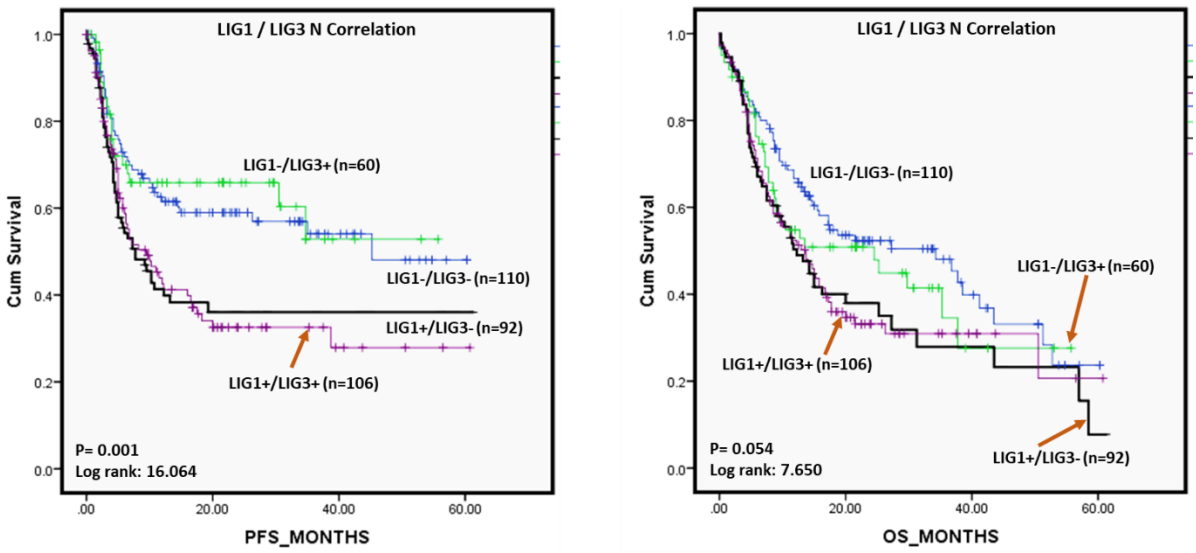



**Figure 6-12: Kaplan Meier plots illustrating the associations between the altered expression of the combined LIG1 and nuclear LIG3 proteins with PFS and OS in primary ovarian carcinoma – whole series**

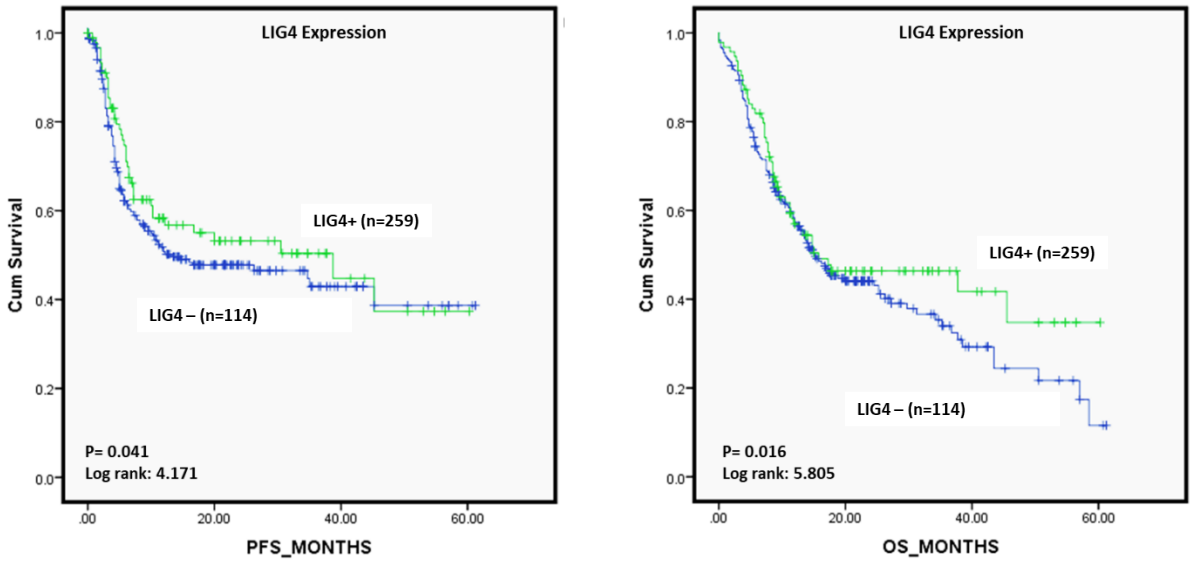



**Figure 6-13: Kaplan Meier plots illustrating the associations between the altered expression of LIG4 protein with PFS and OS in primary ovarian carcinoma – whole series**

#### *6.3.2.5.1.2 Platinum sensitive cohort*

When KM survival analyses of both PFS and OS was conducted in only the platinum sensitive cases, it revealed that overexpression of LIG1 was associated with worse PFS, (p-value= 0.008) and OS, (p-value= 0.034) than its negative/low expression cases, (Figure 6-14). Similarly, positive cytoplasmic expression of LIG3 showed a shorter PFS (p-value= 0.018) than its negative expressers. Although it did not reach statistical significance, LIG3 cytoplasmic overexpression showed unfavourable OS, (p-value= 0.117), (Figure 6-15). On the other hand, nuclear LIG3 expression did not significantly influence PFS (p-value= 0.196) or OS (p-value= 0.132), (Figure 6-16). Interestingly, similar to the whole cohort results when LIG3 cytoplasmic and nuclear expression was combined together, we observed that tumours with high cytoplasmic/low nuclear LIG3 co-expression had worse PFS and OS compared to tumours with low cytoplasmic/low nuclear LIG3 co-expression, but with a trend towards significance, (p-value= 0.052) and (p-value= 0.072) respectively (Figure 6-17). When the combined expression of both LIG1 and LIG3 was investigated, low LIG1/low cytoplasmic LIG3 conferred an improved outcome, PFS (p-value= 0.002) and OS (p-value= 0.020), (Figure 6-18). Similarly, low LIG1/low nuclear LIG3 had improved survival when compared with the high expressers, PFS (p-value= 0.013), and OS (p-value= 0.040), (Figure 6-19). On the other hand, KM survival analyses of LIG4 showed a non-significant association between both PFS (p-value =0.887) and OS (p-value =0.423) with the upregulated expression of nuclear LIG4 protein, (Figure 6-20).

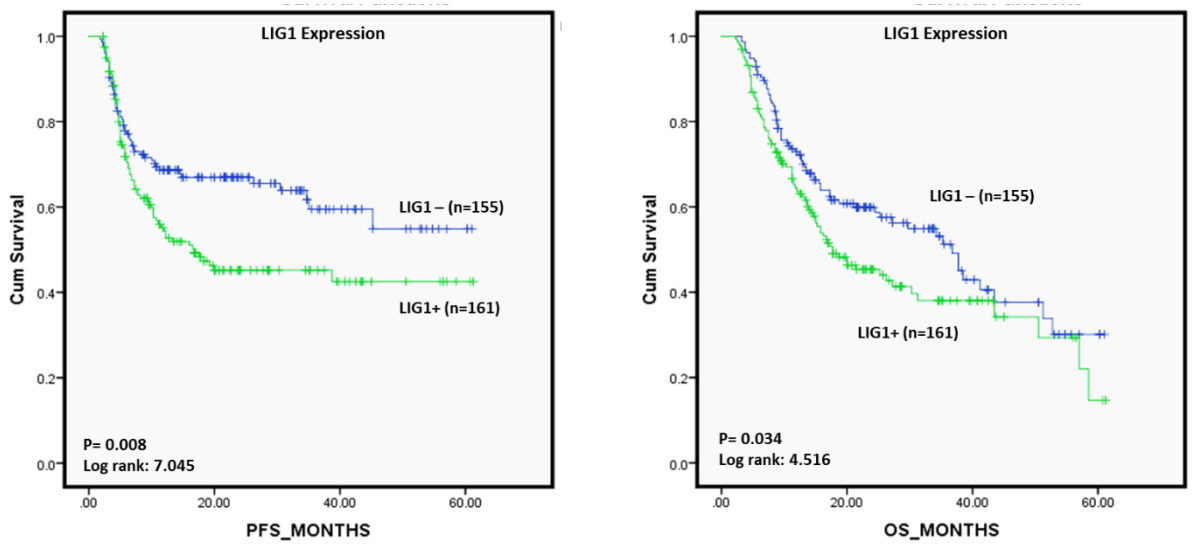



**Figure 6-14: Kaplan Meier plots illustrating the associations between the altered expression of LIG1 protein with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

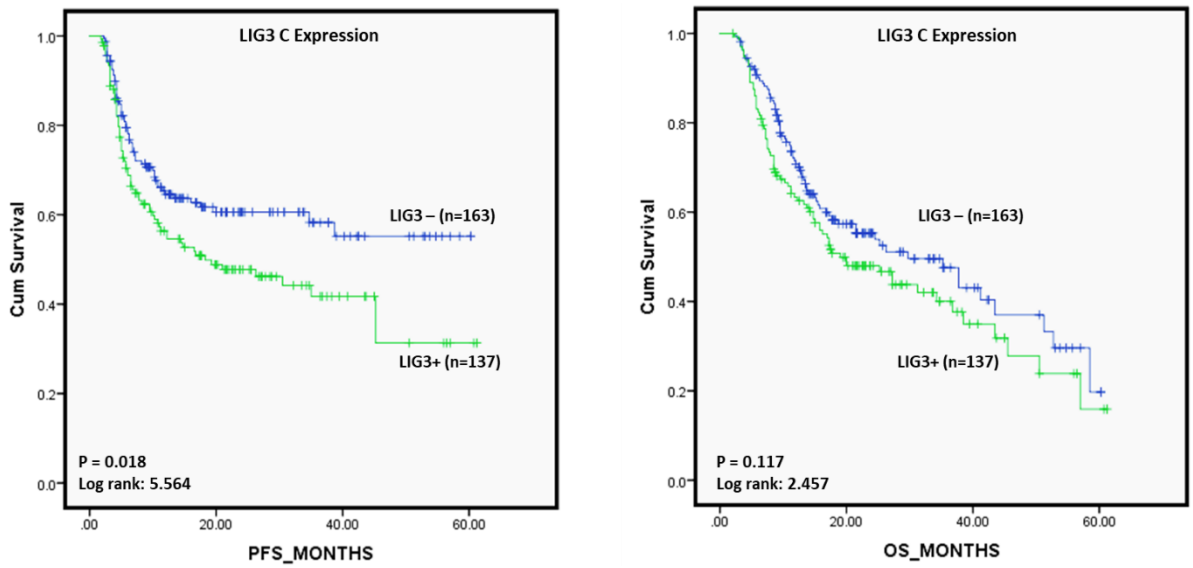



**Figure 6-15: Kaplan Meier plots illustrating the associations between the altered expression of cytoplasmic LIG3 protein with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

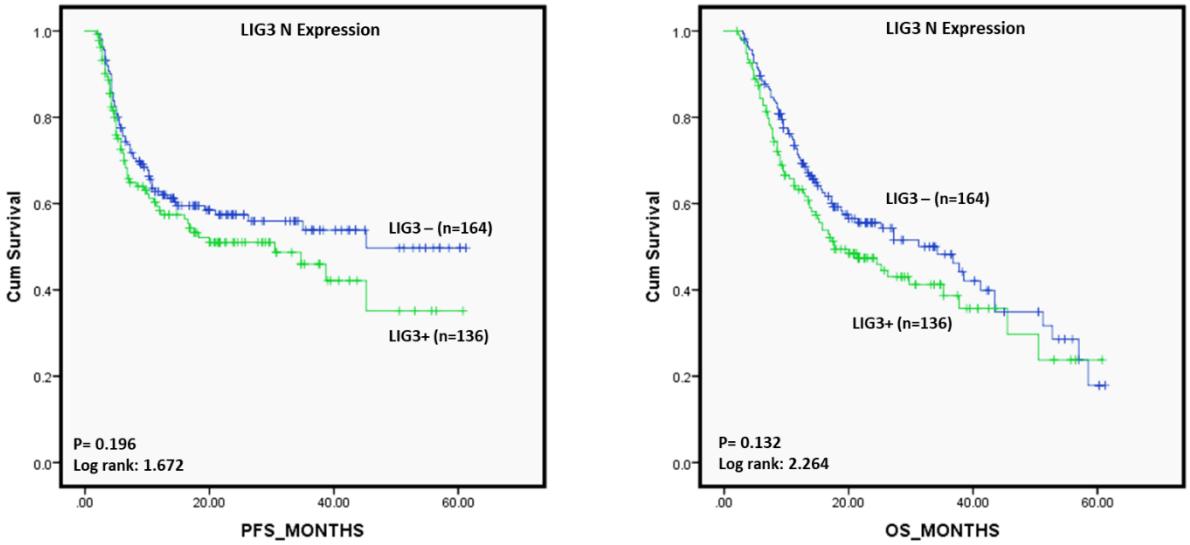



**Figure 6-16: Kaplan Meier plots illustrating the associations between the altered expression of nuclear LIG3 protein with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

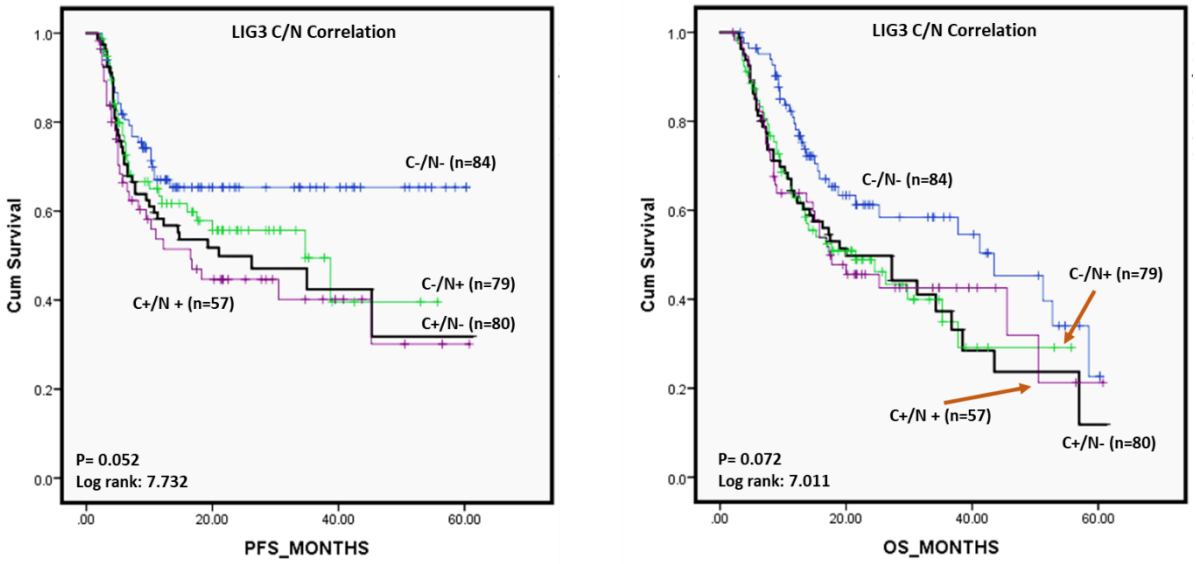



**Figure 6-17: Kaplan Meier plots illustrating the associations between the altered expression of the combined cytoplasmic and nuclear LIG3 protein with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

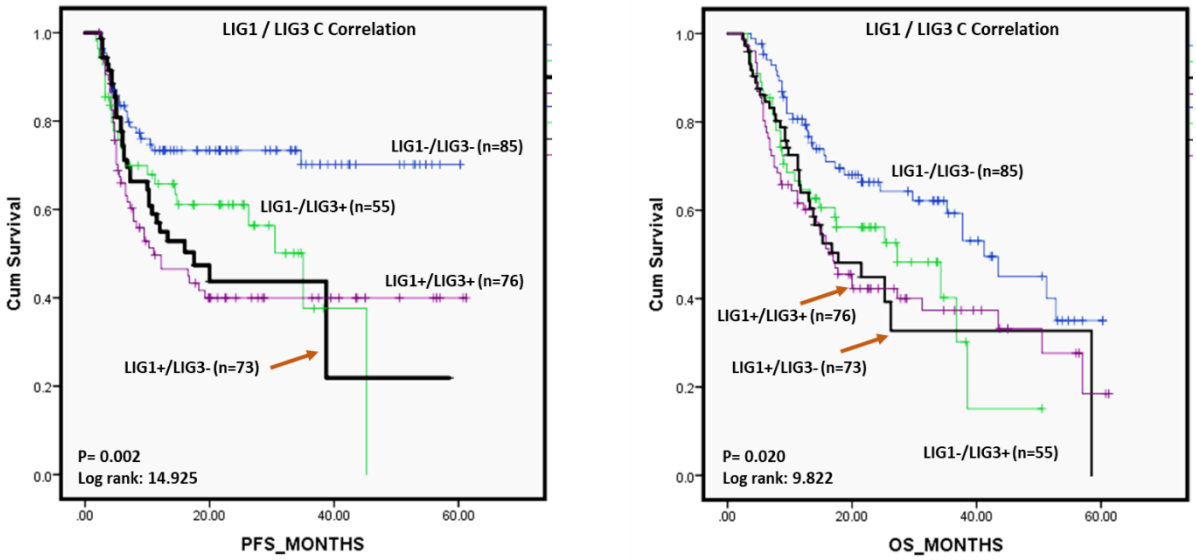



**Figure 6-18: Kaplan Meier plots illustrating the associations between the altered expression of the combined LIG1 and cytoplasmic LIG3 proteins with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

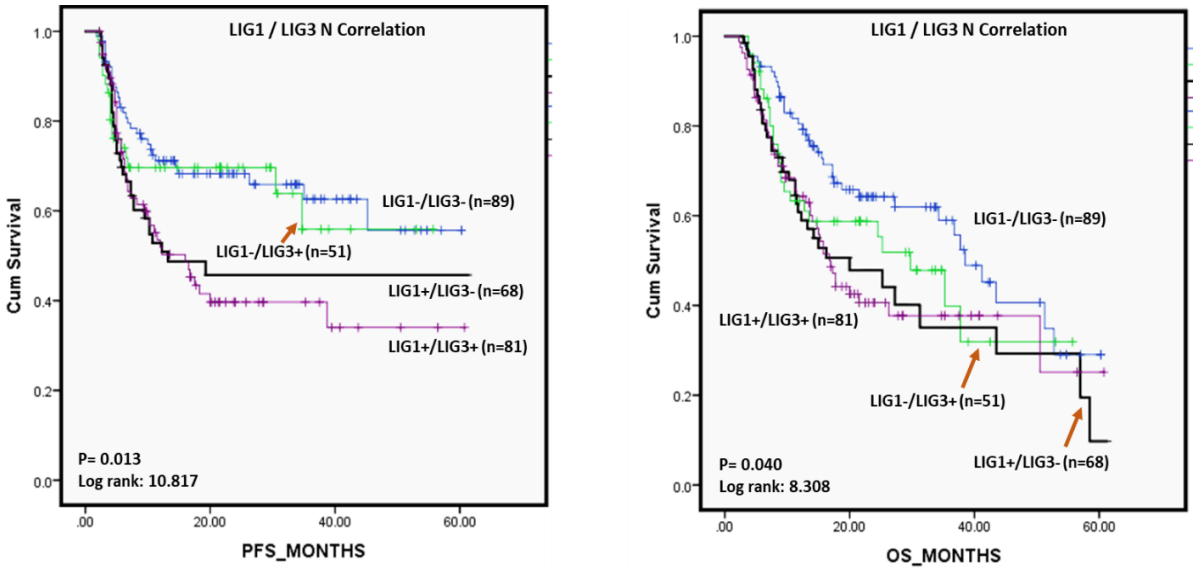



**Figure 6-19: Kaplan Meier plots illustrating the associations between the altered expression of the combined LIG1 and nuclear LIG3 proteins with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

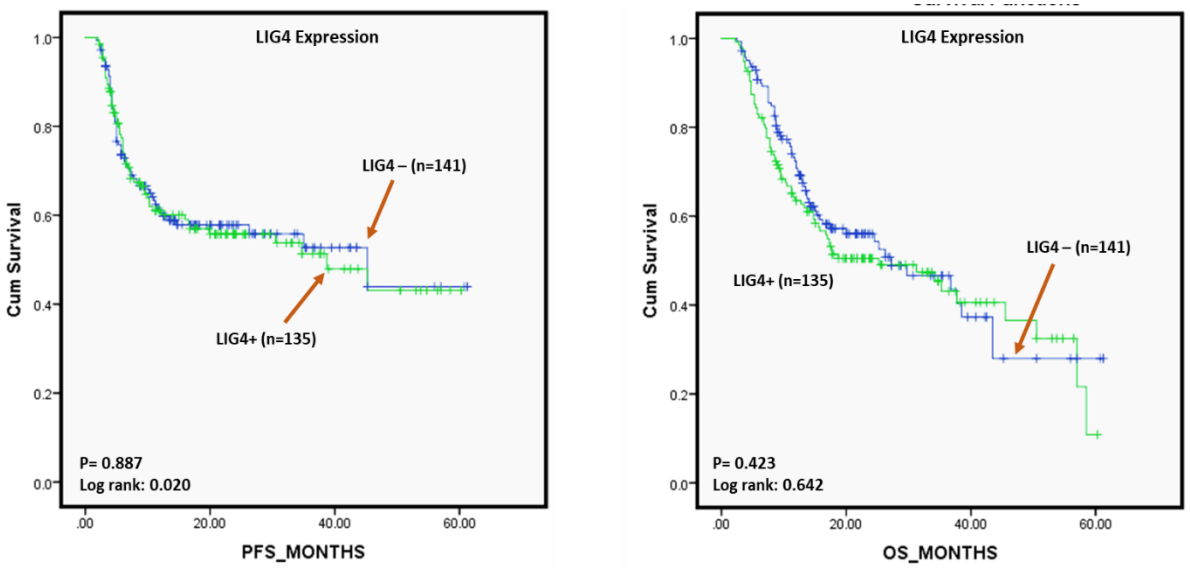



**Figure 6-20: Kaplan Meier plots illustrating the associations between the altered expression of LIG4 protein with PFS and OS in primary ovarian carcinoma – Platinum sensitive selection**

#### *6.3.2.5.1.3 Platinum resistant cohort*

By analysing the outcome of the patients who showed resistance to platinum chemotherapy and positively expressed human ligases, no statistically significant correlation was deemed significant, (Supplementary Table).

#### 6.3.2.5.2 **Multivariate analysis**

The multivariate analysis (Cox multivariate model) for the positive expression of nuclear LIG1, LIG3 (cytoplasmic and nuclear isoforms) and LIG4 proteins together with the platinum sensitivity, interestingly, revealed that platinum sensitivity and upregulated nuclear expression of LIG1 exhibited independent prognostic features for both PFS, (p-value= 0.001) as well as the OS, (p-value= 0.029). Therefore, this may classify a subset of invasive OC patients as a high-risk group. In addition, although high cytoplasmic LIG3 influenced the recurrence free survival, (p-value= 0.045), it did not influence the OS, (p-value= 0.167). On the other hand, LIG4 did not show an independent prognostic characteristics, (Table 6-8) and (Table 6-9) respectively.

Taken together the clinical data provides evidence that LIG3 has a different clinical influence on OC cases depending on its subcellular localisation, i.e. cytoplasmic or nuclear. Moreover, LIG1 high protein expression was independently correlated with poor outcome. Therefore, we proceeded to pre-clinical evaluation of LIG3 nuclear and cytoplasmic extraction and LIG1 knock down in platinum sensitive and resistant OC cell lines.

**Table 6-8: Multivariate Cox regression analysis for prediction of ovarian cancer progression free survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | ***P-value*** | **HR** | **95% CI** | |
|---|---|---|---|---|
| | | | **Lower** | **Upper** |
| **LIG1-Nuclear** | ***0.001*** | 1.820 | 1.285 | 2.578 |
| **LIG3-Cytoplasmic** | ***0.045*** | 1.409 | 1.007 | 1.971 |
| **LIG3-Nuclear** | 0.200 | 0.796 | 0.561 | 1.129 |
| **LIG4-Nuclear** | 0.365 | 0.833 | 0.561 | 1.237 |
| **Platinum sensitivity** | ***<0.0001*** | 1.817 | 4.782 | 9.677 |

**Table 6-9: Multivariate Cox regression analysis for prediction of ovarian cancer overall survival with altered protein expression and platinum sensitivity, significant values are in bold**

| | ***P-value*** | **HR** | **95% CI** | |
|---|---|---|---|---|
| | | | **Lower** | **Upper** |
| **LIG1-Nuclear** | ***0.029*** | 1.407 | 1.036 | 1.910 |
| **LIG3-Cytoplasmic** | 0.167 | 1.237 | 0.915 | 1.671 |
| **LIG3-Nuclear** | 0.747 | 0.950 | 0.695 | 1.298 |
| **LIG4-Nuclear** | 0.094 | 0.737 | 0.516 | 1.053 |
| **Platinum sensitivity** | ***<0.0001*** | 1.717 | 4.882 | 8.677 |

### 6.3.3 Preclinical results

#### 6.3.3.1 LIG3 level in nuclear and cytoplasmic extraction

In this experiment, we have evaluated LIG3 levels in nuclear and cytoplasmic extracts from platinum sensitive and resistant OC cell lines, A2780 and A2780Cis respectively before and after 48 hours of cisplatin treatment. Although after 24 hours we haven't noted a significant difference in LIG3 level, at 48 hours, in platinum sensitive cell lines (A2780), this research revealed a significant increase in the cytoplasmic fraction of LIG3, (p-value= 0.0072) compared with the untreated cells. Similarly, an increase in nuclear fraction of LIG3 was observed but it did not reach a statistically significant value, (p-value= 0.1014) (Figure 6-21). On the other hand, in A2780Cis cells both cytoplasmic and nuclear levels of LIG3 showed no significant changes after cisplatin treatment, (p-value= 0.2854) and (p-value= 0.7510) respectively (Figure 6-22).

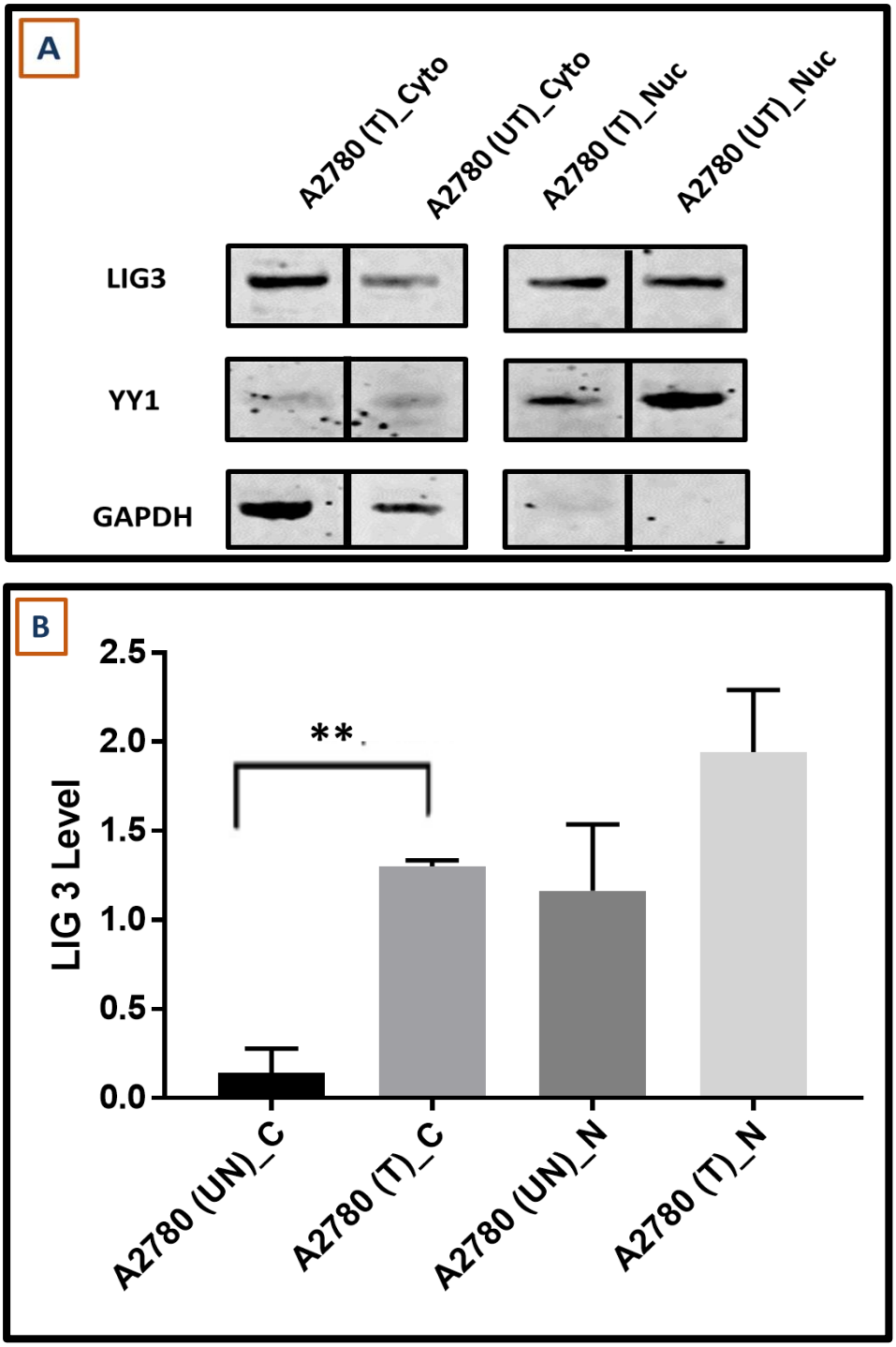



**Figure 6-21: A) Representative immunoblots of nuclear and cytoplasmic levels of LIG3 in A2780 ovarian cancer cells, B) Quantification of cytoplasmic and nuclear levels of LIG3 after adjustment with the loading control. GAPDH was used as a loading control for the cytoplasmic fractions and YY1 as a loading control to the nuclear fractions.**

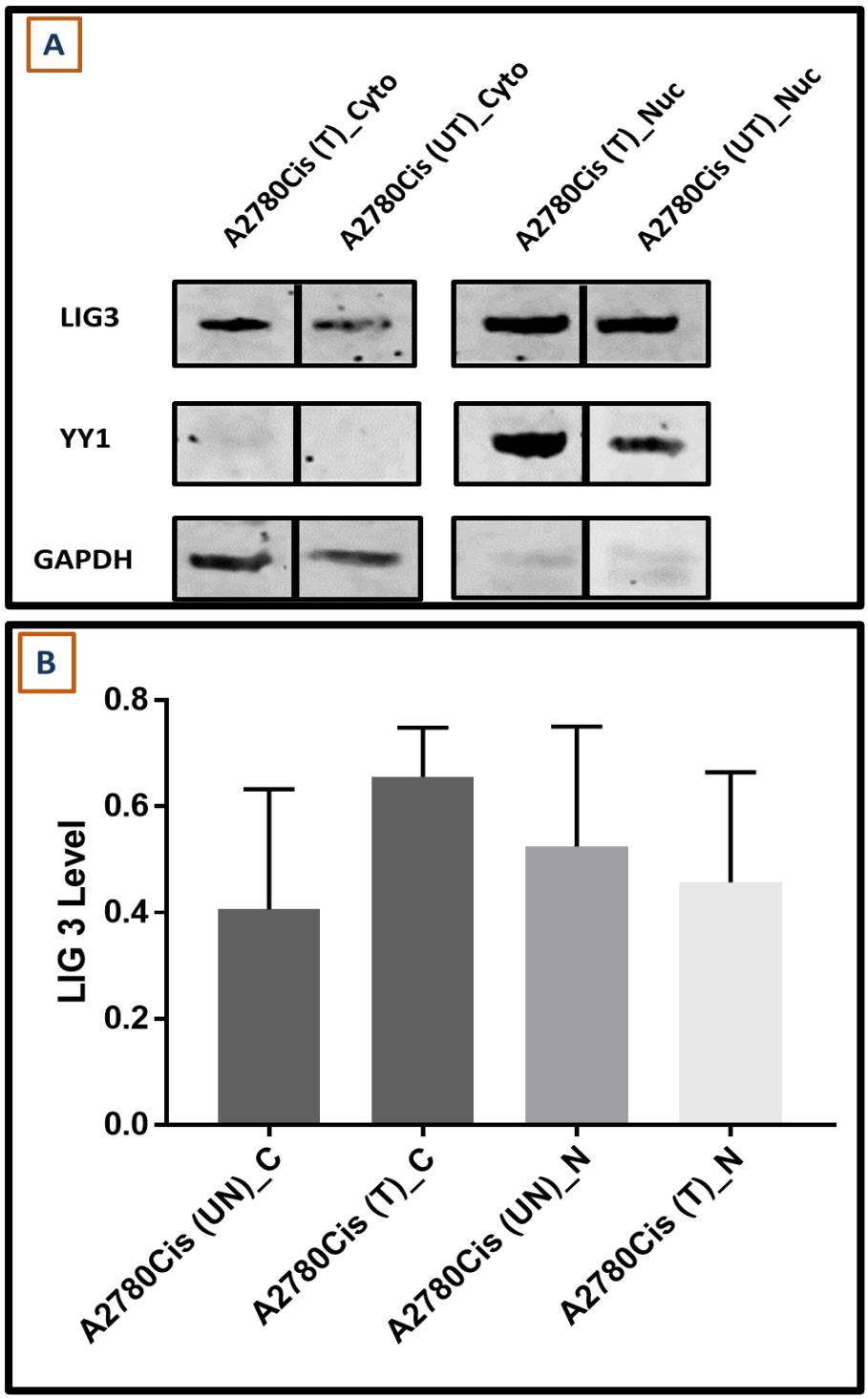



**Figure 6-22: A) Representative immunoblots of nuclear and cytoplasmic levels of LIG3 in A2780Cis ovarian cancer cells, B) Quantification of cytoplasmic and nuclear levels of LIG3 after adjustment with the loading control. GAPDH was used as a loading control for the cytoplasmic fractions and YY1 as a loading control to the nuclear fractions.**

#### 6.3.3.2 LIG1 knock down

The above clinical data provided the required evidence that deficiency of LIG1 in primary OC patients was predictive of improved not just the patients' outcome but also the sensitivity to platinum chemotherapy, borderline significance. Therefore, it was speculated that the transient knock down of the LIG1 gene utilising SiRNA would produce similar preclinical results. Depletion of LIG1, in theory can lead to effective killing of the tumour cell by chemotherapeutic agents, and improve the tumour response to platinum compounds. Transfection efficiency was confirmed at day 3 and day 5 using western blotting as it showed significant reduction in LIG1 level in both A2780 and A2780Cis OC cell lines, compared to scrambled SiRNA which was utilised as a control, (Figure 6-23) and (Figure 6-24) respectively. LIG1 knocked down cell lysate was provided to the rest of the group to perform further preclinical functional studies.

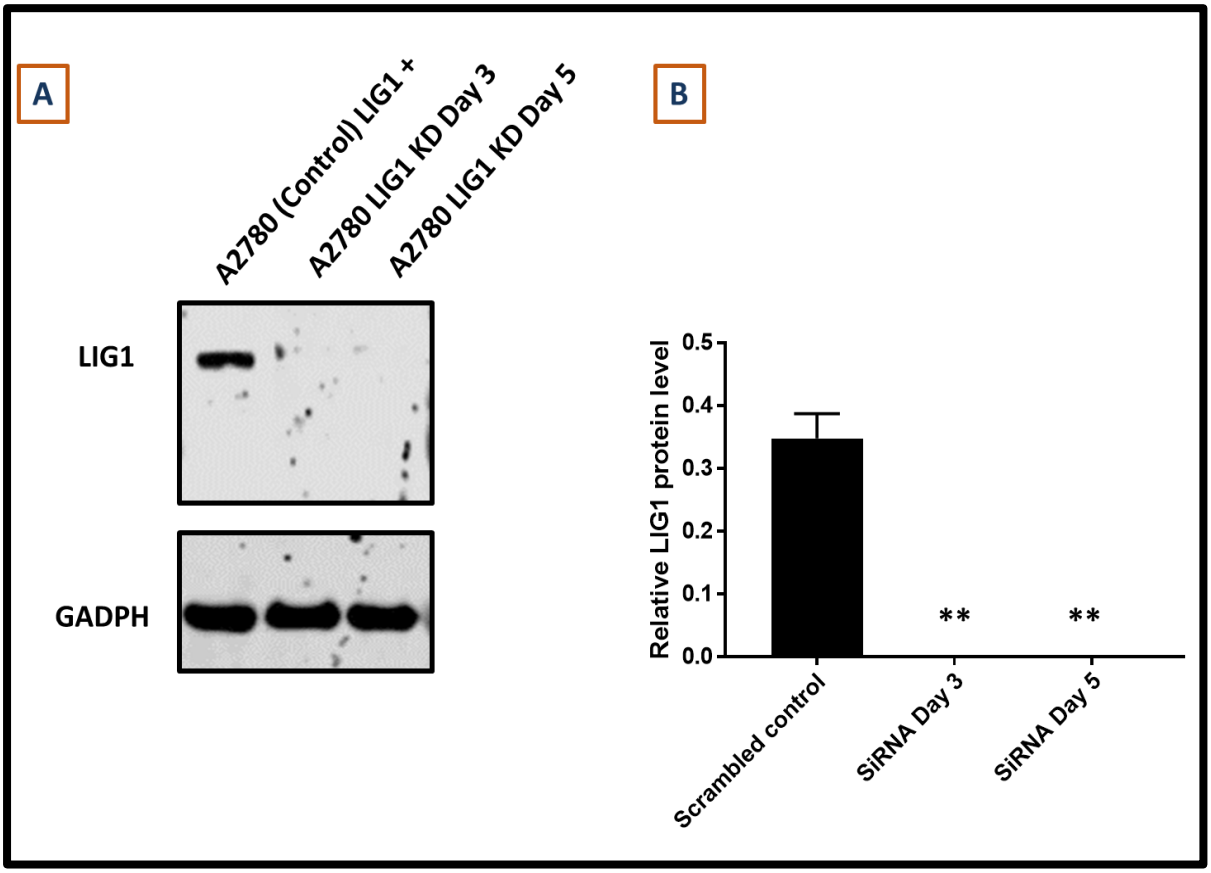



**Figure 6-23: A) Representative immunoblots of LIG1 levels in A2780 transfected with LIG1 siRNA or scrambled siRNA control. B) Quantification of LIG1 levels. GADPH was used as a loading control**

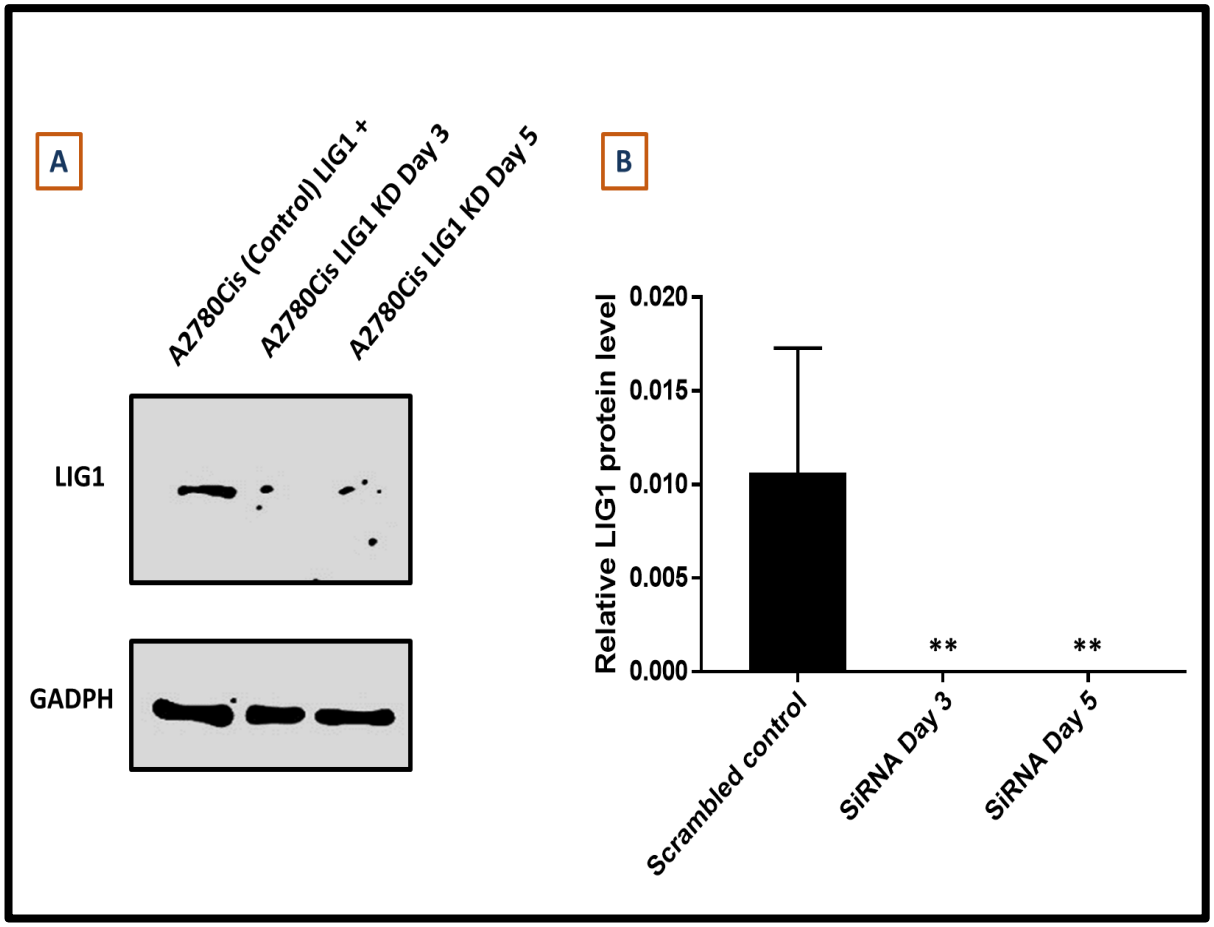



**Figure 6-24: A) Representative immunoblots of LIG1 levels in A2780Cis transfected with LIG1 siRNA or scrambled siRNA control. B) Quantification of LIG1 levels. GADPH was used as a loading control**

## 6.4 Discussion

Normal cellular metabolic processes in mammals can result in byproducts with potential cellular toxicity (Katyal and McKinnon, 2011). These can result in the production DNA damage (Caldecott, 2003, Lindahl, 1993) which; if left unrepaired, can cause cell death. However, if this DNA damage is misrepaired, it can lead to genomic instability and in turn facilitate cancer development or progression (Jeggo and Löbrich, 2007, Herrero et al., 2015). The three human ligases, LIG1, LIG3 and LIG4 have critical roles in maintaining the genomic integrity (Tomkinson et al., 2020). They are evolutionary related protein family that can repair the DNA damage. They are involved in DNA replication, genetic recombination and DNA repair (Ellenberger and Tomkinson, 2008, Timson et al., 2000, Martin and MacNeill, 2002). Despite the major role of DNA ligases in rapidly proliferating cells, we are not aware of any attempt to investigate their clinical expression in ovarian tumour cases. Therefore, this is the first comprehensive study of human ligases in epithelial ovarian tumours. We provided evidence that LIG1, LIG3 and LIG4 high protein expression have crucial prognostic significance in primary OC patients.

Human DNA LIG1 is an important component in DNA replication and DDR pathways (Prigent et al., 1994, Waga et al., 1994, Li et al., 1994, Barnes et al., 1992, Chan and Becker, 1985, Montecucco et al., 1995, Sun et al., 2001a). Moreover, LIG1 expression is modulated during cell differentiation and proliferation (Signoret and David, 1986, Montecucco et al., 1992, Teraoka et al., 1992, Jessberger et al., 1997, Sun et al., 2001a). Sun et al have examined the level of LIG1 in primary and metastatic tumour tissue specimens, including ovarian tumours. A significant rise in LIG1 protein concentration was observed in tumours when compared with the normal tissue controls (Sun et al., 2001a). In addition, DNA LIG1 level was very high in human tumour cells grown in mice (Sun et al., 2001a) providing us with the evidence that LIG1 is overexpressed in tumours. Moreover, LIG1 appears to be not only essential in rapidly proliferating tumour cells but also has unique functions in DDR (Liddiard et al., 2019, Tomkinson et al., 2020). This is not surprising, since studies in cell culture models showed that cancer cells that positively express LIG1 gene tend to be highly proliferative. Therefore, LIG1 promotes tumour cell proliferation and enhances their invasiveness (Sun et al., 2001a, Montecucco et al., 1992, Tomkinson et al., 2020).

Furthermore, in previous clinical studies, LIG1 genetic polymorphisms was associated with increased risk of gastro intestinal tract malignancy (Lee et al., 2008) and head and neck tumours (Michiels et al., 2007).

In the current study, IHC analysis and consequent scoring of ovarian TMAs revealed various significant associations between LIG1 overexpression and patients' clinicopathological variables. Over 400 tumours were deemed suitable for analysis, presenting the largest cohort of primary OC cases that has been assessed in studies. In general, LIG1 high expression was significantly correlated with aggressive tumour characteristics. Its overexpression showed significant correlation with serous histology subtype, advanced stage disease, higher tumour grade and increased risk of residual malignancy after surgery. On the other hand, low LIG1 was in borderline association with improved sensitivity to platinum therapy. Similarly, negative LIG1 expressers showed improved outcome in terms of both PFS and OS in both the whole cohort and when platinum sensitive patients were selected.

An interplay and correlation exists between both LIG1 and LIG3 in order to finalise the DNA BER at the end. DNA LIG3 can complement LIG1 during LP-BER, and similarly LIG1 is able to efficiently substitute LIG3α/XRCC1 complex during SP-BER. On the other hand, and as expected no correlation deemed significant between both LIG1 and LIG4 (Sleeth et al., 2004, Çağlayan, 2019). In agreement with the above findings, in this cohort we have performed cross-comparison between the distribution of the LIG1 expression profile among the categorical subgroups with LIG4 and LIG3, cytoplasmic and nuclear isoforms. This revealed a very highly significant positive association of LIG1 with nuclear and cytoplasmic LIG3 isoform. On the other hand, the correlation between LIG1 and LIG4 proteins was not statistically significant.

Interestingly, in multivariate analysis, LIG1 was independent poor prognostic marker for both PFS and OS in OC patients. Therefore, when we depleted LIG1 in ovarian tumour cells, we observed increased platinum sensitivity, which was associated with DSB accumulation, S-phase cell cycle arrest and increased apoptosis, findings that were not included in this research but under publication. In agreement with our findings, knockdown of DNA LIG1 inhibited the growth of MCF-7 cancer cells, a study on breast cancer (Sun et al., 2001a). In addition, DNA

LIG1 appears to have vital functions in a human cancer cell lines (Liddiard et al., 2019).

This current study revealed that the upregulated expression of LIG1 protein is a significant prognostic factor for primary ovarian tumours. LIG1 overexpression might play an important role in OC progression, metastasis and the development platinum resistance. Therefore, LIG1 may be a good target for the development of cancer therapeutics to improve patients' survival.

LIG3 is a biomarker of altered DDR. Compared with the other human ligases, DNA LIG3 has the most robust intermolecular DNA joining activity (Chen et al., 2000). Interestingly, high levels of this protein have been identified in cancerous tissues, when compared with non-tumour specimens (Tobin et al., 2013, Tobin et al., 2012). The *LIG3* gene encodes three distinct DNA ligase polypeptides including nuclear LIG3α, mitochondrial LIG3α and a germ cell-specific LIG3β (Tomkinson and Sallmyr, 2013). Although elevated level DNA LIG3β has been measured in testes, its mRNA has not been detected in the ovaries (Mackey et al., 1997).

Here in this study, we have identified LIG3 as one of the differentially expressed proteins that showed high association with the cases' clinicopathological characteristics, platinum sensitivity and patients' prognosis. Our results indicate a significant difference in the prognostic impact between the cytoplasmic and nuclear isoforms LIG3 protein in invasive OC patients. The cytoplasmic LIG3 expression showed pivotal impact on OC patients. Its high expression was inversely correlated with reduced tumour stage, lower tumour grade, less residual tumour following surgery, and improved platinum sensitivity. On the other hand, patients who expressed lower cytoplasmic levels of LIG3 exhibited better survival in terms of both PFS and OS. Similarly, in platinum sensitive cases, negative/low LIG3 expressers was associated with lower recurrence risk. Importantly, in multivariate analysis, the positive cytoplasmic expression of LIG3 was an additional independent prognostic factor to poor recurrence free survival, reflecting its important role in modifying patients' outcome.

On the other hand, the nuclear overexpression of LIG3 did not show a reliable prognostic relevance or any association with the patients' clinicopathological variables and outcome in the unselected whole cohort, platinum sensitive and

resistant cases. Similarly, unlike the cytoplasmic LIG3, nuclear LIG3 high expression in multivariate analysis was not an independent factor for patients' outcome. Interestingly, when we performed the combined analysis of both cytoplasmic and nuclear LIG3 isoforms, we have demonstrated that tumours with high cytoplasmic /low nuclear LIG3 expression was significantly correlated with adverse clinicopathological factors, including resistance to platinum therapeutic agents. Similarly, cytoplasmic LIG3 overexpression significantly predicted poor patients' outcome.

Interest in the underlying mechanisms regarding the role LIG3 cytoplasmic and nuclear subcellular localisation in cancer progression is ongoing. A study on patients with Alzheimer's disease concluded that LIG3 deficiency in mitochondria can result in mitochondrial malfunction (Canugovi et al., 2014, Akbari et al., 2014). Moreover, Caldecott et al stated that the nuclear localisation of LIG3 is dependent upon complex formation with XRCC1, a partner protein that has a nuclear localisation signal (Caldecott, 2003, Parsons et al., 2010). Recently, it was reported that four isoforms of LIG3, which are produced by alternative splicing and alternative initiation, were identified. They were recognised in both the nucleus and cytoplasm mitochondria of the cells. Its mitochondrial isoform functions in mitochondrial base-excision DNA repair. On the other hand, its nuclear counterpart interacts with the nuclear XRCC1 protein (Uniprot.org, 2002-2020). Importantly, in this study when we added cisplatin to OC cell lines, we observed a significant increase in cytoplasmic LIG3 level in platinum sensitive (A2780) cells, compared to its nuclear samples. In A2780Cis, although cytoplasmic LIG3 protein level did increase, it did not reach the statistical significance. This provides further preclinical evidence to the importance of cytoplasmic LIG3 in modulating platinum sensitivity in OC patients.

Moreover, focusing on the effects of protein-protein interactions and their role in OC progression and prognosis. Our results show that the combined LIG1/LIG3 protein overexpression is remarkably associated with more aggressive tumour characteristics. On the other hand, low/negative expression of both biomarkers significantly improved the patients' outcome. Interestingly, cases who had combined low/negative expression of both LIG1/ cytoplasmic LIG3 exhibited a significantly improved response to platinum therapeutic agents. Therefore,

according to our findings, the downregulated LIG1/cytoplasmic LIG3 proteins was associated with longer PFS and OS.

From these above studies, and from our results, we propose that the molecular regulation of LIG3 in OC is differentiated according to the subcellular localisation of the LIG3 protein. We speculate that the cytoplasmic staining of LIG3 observed in tumours could represent mitochondrial form of LIG3α. Altered mitochondrial DNA repair and replication capacity may influence response to platinum therapy, and increased LIG3α in the mitochondria could contribute to platinum resistance. However, further detailed mechanistic studies are required to confirm this hypothesis.

*LIG4* genetic polymorphisms have been linked to reduced risk of breast cancer development (Kuschel et al., 2002) and influenced myeloma risk (Roddam et al., 2002). Furthermore, LIG4 enzymatic variations can affect DNA stability with variable sensitivity to DNA damaging agents (Assis et al., 2013). Similarly, reduced levels of LIG4 have been described in different organs' cancer cell lines (Assis et al., 2013).

In this study, the examination of the stained TMA sections showed that the nucleus was the dominant localisation for LIG4 protein. In addition, importantly, we show that low LIG4 is a marker of poor prognosis and reduced response to therapy in OC. On the other hand, LIG4 nuclear overexpression was positively associated with improved patients' outcome and chemotherapy sensitivity.

Our data would concur with another study conducted on primary nasopharyngeal carcinomas, where LIG4 low level was associated with unfavourable recurrence and cumulative survival (Kim et al., 2014). In addition, in the same study, high expressers of this protein showed improved response to radiotherapy treatment (Kim et al., 2014). Moreover, LIG4 positivity was exclusively localised in the nucleus of the malignant cells, in another study performed on malignant OC specimens (Assis et al., 2013), a finding which comes in agreement with the current study. In addition, reduced expression of LIG4 has been shown to contribute to abnormal DSB repair in Chronic Myeloid Leukaemia (CML) cell lines (Roddam et al., 2002). Furthermore, in humans, germ-line mutation and

inactivation of LIG4 was found to enhance cancer predisposition and immunodeficiency syndromes (Tomkinson et al., 2020).

Our study revealed that the upregulated expression of LIG4 protein is a significant prognostic factor for primary OC. Low expression of LIG4 protein might play an important role in the development of OC progression, metastasis as well as reduced response to platinum compounds. However, further detailed functional studies are warranted to determine the detailed cellular roles and LIG4 therapeutic potentials in ovarian tumour patients.

To conclude this chapter, DNA ligation is the last step in the process of DNA repair. It is a key event in finalising the DNA repair cascade. In this study, we aimed to identify the driver proteins, human ligases that are significantly associated and differentially expressed in OC. The present study tested the effect of the three human ligases, which revealed a highly significant association with OC. High expression of both LIG1 and LIG3 were strongly associated with unfavourable outcomes. On the other hand, LIG4 showed correlation with improved prognosis. Moreover, we have identified LIG1 as an independent factor for poor outcome in primary OC patients. Clinically, our study provided novel findings and useful prognostic indications. However, it appeared that the underlying mechanisms of OC progression are contextualised with myriad biophysical and biochemical factors. More work is needed to elucidate the roles and relative contributions of the DNA ligases on ovarian tumour patients' prognosis and survival. Unfortunately, the current pandemic had a huge negative impact on this research progression. Therefore, LIG3 sub-localisation was not investigated further. This will be addressed in future study designs.

## 6.5 Study limitations related to this chapter

1- For the exploratory research approach, the limited number of published studies interrogating human ligases' clinicopathological role in primary OC required us to apply different experimental methods.

2- Only two cell lines were utilised in preclinical experiments.

3- COVID19 pandemic halted the further preclinical study of the impact of LIG3 subcellular localisation on OC patients.

# 7 General Discussion

## 7.1 General discussion

As we described and discussed in the previous chapters, ovarian cancer (OC) is an uncommon disease compared with the other diagnosed malignancies among women. However, OC is the fourth most common cause of cancer-related death of women in the UK (UK, 2019). Hence, enhancement of the current diagnosis, prognosis, and therapeutic management of OC is of crucial significance for female health and well-being. This task is challenging because ovarian tumours are heterogeneous diseases that manifest myriad morphological and molecular entities.

The main and definitive treatment of OC is complete surgical resection combined with platinum-based chemotherapy (Wang et al., 2015). Over the last 3 decades, there have been significant improvements in the surgical management and postoperative care received by women with OC. In addition, the wide use of platinum-based compounds has improved dramatically the management of these cases (Lee et al., 2015). Chemotherapy has become an important part of OC treatment as they can terminate the growth of tumour cells through the inhibition of DNA replication and transcription. However, resistance against platinum-based drugs can develop easily (Lee et al., 2015, Lee et al., 2005, Yunos et al., 2013, Wang et al., 2015). Therefore and in spite of the above advancements in treating and understanding the molecular diversity of OC, drug resistance can still limit their therapeutic effects, and can lead to poor prognosis with a current 30% 5-year survival rate (Wang et al., 2015).

Ovarian tumours rapid growth, non-specific clinical symptoms at early stage of the disease, and lack of early diagnostic methods make prompt and early diagnosis challenging to clinicians. As a result, most patients are typically diagnosed at an advanced stage disease, when the tumour has already spread beyond the pelvis. Consequently, complete removal of tumour by surgery will become extremely unlikely and challenging (Singh et al., 2019a). On the other hand, early diagnosis at the localised stage of OC is highly curable with more than 95% 5-year survival rate (Siegel et al., 2011). The key molecular players of OC progression can modulate tumour growth, metabolism, cellular adhesion, migration, and invasion. Therefore and in order to improve the outcome of women diagnosed with OC, the identification of key molecular biomarkers with intimate relation to cancer

progression and prognosis is critically needed. A better understanding of the associated genome portrait will help in the identification of novel promising biomarkers of clinical utility that can be targeted in the future to improve the disease prognosis.

OC patients are influenced by specific molecular expression profiles that can be associated with distinctive classes according to their molecular biology. Cell signalling and DNA damage repair (DDR) pathways are both critically involved in OC development and progression (Yang et al., 2017b, Singh et al., 2019a). Therefore, the exploration of these pathways' related proteins is crucial and can produce novel prognostic factors and therapeutic candidates for OC patients. We used an exploratory design for this research, pursuing both the clinical and pre-clinical experiments. We designed a list of experimental objectives. Then, we tested a meticulously well-described primary epithelial OC cohort. For this purpose, many proteins of the surrogate pathways known to be in close relation were studied. These included: 1) cell signalling pathway (p-AKT, p-mTOR, p-4EBP1, p-P70S6K) and (ATM, PTEN, P85ά, XIAP), and 2) DDR pathway (PARP1, XRCC1, polβ) and (the three human ligases: LIG1, LIG3 and LIG4). The selection of the panel of proteins was based on literature reviews (*in vivo* and *in vitro* findings) that reported clear associations of those biomarkers with either ovarian or other organs malignancies. The expression of the investigated biomarkers' panel was studied with relevance to their biological significance in OC in order to assess the alterations in the expression of these proteins, which might help reveal distinct patterns. Furthermore, the correlation with patients' outcome was also considered in the whole cohort, platinum sensitive and resistant subgroups.

For this purpose, we constructed a large cohort of primary OC patients with accurately recorded clinicopathlogical data, platinum sensitivity and survival status. This cohort consisted of 525 patients treated at the University Hospitals of Nottingham NHS Trust. To the best of our knowledge, this cohort included the largest number of primary ovarian tumour cases worldwide. Following this, integrated immunohistochemical staining was utilised to test the expression of the surrogate proteins in our series. Preclinical methodologies were also employed to quantify the expression of these proteins in two OC cell lines, platinum sensitive

and resistant. Finally, nuclear and cytoplasmic extraction was performed to assess the impact of LIG3 subcellular localisation on platinum sensitivity.

## 7.2 Cardinal findings of this study

To recap, the first method we followed to identify OC related genes was based on the results of a thorough literature review. We identified the surrogate markers because of the limited information available for their biological and clinical relevance in OC. However, all have critical roles in regulating cellular and DNA processes.

### 7.2.1 AKT-mTOR pathway has a critical role in ovarian cancer

The expression of AKT-mTOR pathway proteins (p-AKT, p-mTOR, p-4EBP1 and p-P70S6K) revealed that these proteins are associated with poor prognostic variables and shorter survival in the whole (unselected) cohort and within the platinum sensitive tumours. Moreover, all the tested proteins in this pathway were in intimate association with AKT and mTOR, in both the unselected and platinum sensitive tumours (Alabdullah et al., 2019). Interestingly, both p-AKT and p-4EBP1 were independent factors for poor outcome. Tumours with combined AKT/4EBP1 overexpression was highly positively associated with unfavourable clinical variables, higher risk of post-surgery residual disease and resistance to platinum based chemotherapy. Importantly, cases with low/negative expression of both AKT and 4EBP1 exhibited an improved survival in the whole cohort and platinum sensitive one. Therefore and from a clinical perspective, this appears to indicate that p-AKT and p-4EBP1 could be a target for future therapeutic potential in ovarian tumours.

Previous studies have conflicting findings regarding the influence of AKT expression on OC patients. This is because they could not address AKT actual function either due to type of tumour studied, small cohort sizes and techniques for assessment. After a median follow up of 35 months, Lee et al showed that the isolated AKT overexpression was not correlated with patients' outcome, neither PFS nor OS (Lee et al., 2015). On the other hand, when IHC study for p-AKT was performed on 100 primary ovarian tumours, high level of p-AKT was correlated with worse PFS and OS. Moreover, when the influence of AKT expression on

outcome was tested in combination with the Regulated in development and DNA damage response 1 (REDD1) protein, it revealed that negative AKT expressers had a statistically significant better survival, if compared with the high expression tumours (Jia et al., 2014).

Additionally and regarding p-4EBP1 expression, Castellvi et al showed that ovarian malignant tumours significantly expressed higher 4EBP1 levels than their benign and borderline counterparts, an indirect observation for the aggressive role of this protein (Castellvi et al., 2006). In addition, patients with low/negative expression p-4EBP1 had a significantly improved OS and a borderline significance of better PFS (Castellvi et al., 2006).

We can conclude from the above-mentioned studies, patients with altered protein expression of both p-AKT and p-4EBP1 in primary ovarian tumours demonstrated a strong association with worse outcome. Interestingly, we are not aware of any study that tested the combined role of the above both surrogate biomarkers in primary ovarian tumours. Therefore, based on the limited number of studies available and our findings, this pathway appears to be an interesting prognostic target in primary ovarian tumour patients.

### 7.2.2 PTEN and p85α clinical impact on ovarian cancer is ATM and XIAP dependant

In this study, we have examined the clinical implications for the positive expression of ATM, PTEN, P85α and XIAP in primary OC cases. Each one of the surrogate proteins showed variable associations with the clinicopathological characteristics and outcome. In general, XIAP and P85α high levels were correlated with poor prognosis; however, PTEN overexpression was associated with improved outcome (Ali et al., 2019). Interestingly, in multivariate analysis we have identified that both ATM and XIAP were independent factors for PFS. Then, after dividing the patients according to their corresponding status of ATM and XIAP expression, importantly, when ATM was positive, PTEN and p85α lost their significance on the patients' outcome. Similar observation was concluded when XIAP was overexpressed. This is a very interesting observation in the current study. In addition, interestingly, patients with combined ATM and XIAP overexpression exhibited a highly significant unfavourable recurrence free

outcome, this further illustrating the clinical influence of those two biomarkers on OC outcome.

Plethora of studies have examined the influence of the above proteins on cancer. ATM was a significant independent prognostic factor in colorectal cancer (Grabsch et al., 2006), and in borderline significance in another study on breast cancer (Abdel-Fatah et al., 2014b). Similarly, XIAP showed significance as independent protein for poor outcome in clear cell ovarian tumours (Miyamoto et al., 2018) and breast cancer (Hussain et al., 2017).

Therefore, in this study, we speculate a novel role for ATM and XIAP in modulating the clinical impact of PTEN and p85α on the outcome of primary ovarian tumour cases. This finding could represent a different biological class of OC patients depending on their ATM and XIAP expression status. However further functional studies are required to confirm this hypothesis.

### 7.2.3 XRCC1 is a key prediction marker for ovarian cancer recurrence

DNA damage repair (DDR) is a multifaceted process. It depends on the interplay between different related proteins to repair the damaged DNA (Ali et al., 2017). XRCC1, PARP1 and polβ are important driving biomarkers that could enhance OC development and progression. They are key proteins in DDR. Different studies have investigated their role in different cancerous organs (Kaur et al., 2020, Singh et al., 2019b, Caldecott, 2003, Hegedus et al., 2019, Xiao et al., 2020, Sobczak et al., 2019, Wang et al., 2016b, Zhao et al., 2008, Sliwinski et al., 2007). However, there remains a lack of knowledge on how the interaction between these proteins can influence the ovarian tumour cases. Moreover, to identify the biomarker that has the dominating/driver function in primary OC patients.

In this study, we have shown that all the above surrogate proteins were correlated with poor prognostic features in both the unselected and platinum sensitive cohorts. Moreover, XRCC1 has significant biological contribution in OC progression and prognosis, and its overexpression was a marker of resistance to platinum therapy. In addition, as these proteins interact with each other (Fan et al., 2004, Kubota et al., 1996, Bhattacharyya and Banerjee, 2001, Masson et al., 1998, Vidal et al., 2001), we have performed a multivariate analysis, which interestingly revealed that XRCC1 is an independent factor for poor PFS (Ali et al., 2020).

Importantly, preclinical knockdown of XRCC1 increased PARP1 level in ovarian tumour cells, an interesting finding that could be used as a future target by PARP inhibitors to improve patients' prognosis.

This above finding comes in agreement with a previous study in breast cancer in which, XRCC1 deficiency increased PARP1 activity (Ali et al., 2018). In addition and in OC, reduced rates of single-strand break repair and elevated levels of PARP protein was identified in cells that sustained XRCC1 mutation; moreover, XRCC1 knockdown similarly, increased PARP1 activity (Hoch et al., 2017). Therefore, this current study highlighted the critical role of XRCC1 in OC. In addition, PARP1 could be used as a future therapeutic target in XRCC1 deficient OC cells (Ali et al., 2018). However, further detailed mechanistic studies are required to proof this.

### 7.2.4 Human ligases have diverse critical roles in primary ovarian cancer

The three human ligases: LIG1, LIG3 and LIG4 are essential proteins in maintaining the DNA genomic integrity (Tomkinson et al., 2020). In this study, we have investigated the difference in the biological and prognostic significance of the three human ligases on primary OC. In general, apart from LIG4, which its overexpression showed good prognostic features and improved response to platinum therapy, the expression of both LIG1 and LIG3 revealed that these proteins are associated with poor prognostic variables and shorter survival in the unselected and platinum sensitive cohorts. Interestingly in this study, the multivariate analysis revealed that LIG1 was an independent poor prognostic marker for both PFS and OS, a novel finding in this research.

In addition and regarding LIG3, our results indicate a significant difference in its prognostic impact according to the protein cellular sub-localisation. Overexpression of LIG3 in the cytoplasmic compartment of the cell exhibited tumour-encouraging qualities, and the comparison between nuclear and cytoplasmic LIG3 expressions was extremely different. The tested clinicopathological parameters and survival were inversely and significantly associated with the cytoplasmic positive expression of LIG3, but it was not with the nuclear expressed LIG3. Importantly, in our preclinical experiments we observed a significant increase in the cytoplasmic fraction of LIG3 after cisplatin

treatment. Therefore, we speculate that the cytoplasmic staining of LIG3 observed in tumours could represent mitochondrial form of LIG3α, which when altered can negatively affect the capacity of DNA repair and replication. As a result, this can influence the response to platinum therapy and contribute to resistance development. However, further detailed mechanistic studies will be required to illustrate the impact of this observation.

Based on the very limited number of studies available, LIG1 and LIG3 appear to be interesting prognostic targets in OC. Previous studies have recognised the essential role of LIG1 in malignancy. Liddiard et al stated that DNA LIG1 has vital functions in human cancer (Liddiard et al., 2019). In addition, knockdown of LIG1 in breast cancer inhibited tumour cells growth (Sun et al., 2001a). Furthermore, existing studies that interrogated the altered expression of LIG3 in different human cancers also identified the differential expression for this protein in cellular compartments and its impact on the patients. The cytosolic expression of LIG3 was found to predispose for Alzheimer's disease development, unlike its nuclear isoform (Canugovi et al., 2014, Akbari et al., 2014). Moreover, LIG3 was identified to be expressed with higher levels in tumour tissues, when compared with normal tissue specimens (Tobin et al., 2013, Tobin et al., 2012), an indirect observation illustrating the aggressive role of this biomarker in cancer.

Therefore, from the results of the IHC experiments and preclinical studies, the present study identified the pivotal role of human ligases not just on the clinicopathological aspect but also the important and strong association with chemotherapy resistance and patients' outcome. This is the first study, which highlights the importance of and differences in the subcellular localisation of LIG3 and its clinical impact and the effect on patients' outcomes. Moreover, LIG1 and as we discussed in detail in the previous discussion section, could be used as a future target to improve the disease prognosis.

## 7.3 Final Conclusions

This thesis addresses two main areas of tumour biology: cellular and DNA damage pathways, through the combined use of TMA immunohistochemical techniques and preclinical experiments. The work illustrates that OC can be extremely heterogeneous. Therefore, variations in the expression of different protein

markers between individual tumours can be used to predict tumour behaviour and patients' prognosis. Future management of ovarian tumour cases will need to be individualised and based mainly upon the molecular signature of the tumour assessed through utilising immunohistochemistry and preclinical methods. Eminently, predictive and prognostic biomarkers of the particular pathway being targeted need to be included in the design of future clinical trials for novel treatments. The pathways tested in this thesis have direct links to potential targeted therapies, and variations in the functionality of these pathways may exist between individual tumours. Importantly, the methods utilised in this thesis are all easily applicable in clinical practice, the use of IHC is easy and cost effective, with if required, further validation of the results by the preclinical methods can be used routinely for future OC risk stratification.

## 7.4 Potential strength and limitation of this study

### 7.4.1 Potential strengths

1. The use of the largest and well-characterised newly constructed cohort of primary OC allowed different reliable comparisons with the studied biomarkers.

2. The identification of novel prognostic proteins associated directly with platinum sensitivity and patients' outcome.

3. The inclusion of controls (positive and negative) with each IHC run to ensure genuine staining.

4. The determination of antibody-antigen binding specificity with WB.

5. Utilisation of the combined IHC and preclinical methodologies to identify the role of the surrogate markers in primary OC patients.

6. Several published studies were derived from this work.

### 7.4.2 Potential limitations

1. The ovarian cancer series used in this study is based on a retrospective data, and no prospective cases were included. In addition, some TMA cores were missing.

2. Lack of multicentre validation, so the data was assessed in one centre only.

3. The influence of the utilised IHC method by the subjectivity of scorers. However, a well-standardised approach was followed to minimise these limitations together with blinded double scoring (Oyama et al., 2007).

4. Although the protein expression patterns for the stained proteins were homogenous, and the use of TMA on a large scale was acknowledged by different studies on OC (Rosen et al., 2004, Hecht et al., 2008), it is worth using at least three cores per case for an enhanced more accurate assessment of these proteins.

5. The limited number of platinum resistant cases available made their assessment a very challenging task.

6. Preclinical experiments were performed on only two OC cell lines, platinum sensitive and resistant.

## 7.5 Future work

1. Multicentre assessment of the OC cases will be more robust. This will give the opportunity to rely on the obtained results if deemed parallel with the possibility for their clinical application.

2. Prospective assessment of OC cases together with the current series will add more value and confirm further our results.

3. Investigate more key markers that characterise different pathways of DDR to provide a comprehensive profiling of DDR in OC.

4. To highlight the role of subcellular localisation, especially for LIG3 and p85α and its impact on both the patients' clinicopathological characteristics and outcome is warranted.

5. Designing and performing functional studies for the tested proteins in this thesis and explore their role in platinum resistance development.

6. Expansion of the platinum resistant series to support better evaluation especially in terms of treatment related outcome.

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# 9 Appendix

**Supplementary table 1: Patient demographics and pathological features in OC of the old cohort**

| Characteristics | Number | Percentages |
|---|---|---|
| ***Pathology*** | | |
| Serous cystadenocarcinoma | 111 | 58.4 |
| Endometrioid | 38 | 20.0 |
| Clear cell carcinoma | 24 | 12.6 |
| Mucinous cystadenocarcinoma | 14 | 7.4 |
| Others | 7 | 3.6 |
| ***Residual tumour*** | | |
| None | 61 | 32 |
| Microscopic | 48 | 25 |
| <1cm | 14 | 7 |
| >1-2 cm | 17 | 9 |
| >2cm | 53 | 27 |
| ***FIGO Stage*** | | |
| I | 58 | 30 |
| II | 27 | 14 |
| III | 85 | 43 |
| IV | 25 | 13 |
| ***Chemotherapy*** | | |
| Carboplatin monotherapy | 83 | 42.7 |
| Carboplatin + Paclitaxel | 111 | 49.5 |
| ***Platinum sensitivity*** | | |
| Sensitive | 126 | 65 |
| Resistant | 60 | 31 |
| Unknown | 9 | 4 |
| ***Relapse status*** | | |
| Progression-free | 64 | 39 |
| Progressed/relapsed | 130 | 61 |
| ***Survival status*** | | |
| Living with no disease | 83 | 42.6 |
| Dead of disease | 90 | 46.1 |
| Dead of other causes | 22 | 11.3 |

**Optimisation of Antibodies Used for IHC**

As previously described in Chapter 2 Section. In addition to Western blotting, specificity of staining was confirmed by application of negative (with omission of the primary antibody) and positive controls. Positive controls were used according to the manufacturer's datasheet and/or from the human protein atlas available at http://www.proteinatlas.org/. This helped not only to test the specificity of staining but also to assess the pattern and intensity of protein expressions in the appropriate tissue. Details of the negative and positive controls used are summarised in the below table. Moreover, some control TMA slides containing a variety of OC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

**Supplementary table 2: Optimisation conditions for the tested biomarkers**

| Antibody | Optimization concentration | Incubation periods | Positive control | Negative control | Reference |
|---|---|---|---|---|---|
| Anti-mTOR phospho (S2448) | 1:25, 1:50, 1:100, 1:150, 1:200 | 60 min and 18 hours | Testis | OC tissue | Abcam/http://www.proteinatlas.org/ |
| p-4EBP1 (Thr37/46) | 1:50, 1:100, 1:150, 1:200, 1:250 | 60 min and 18 hours | Colon | OC tissue | Cell signalling/ http://www.proteinatlas.org/ |
| p-P70S6K (Thr389) | 1:50, 1:100, 1:150, 1:200, 1:250 | 60 min and 18 hours | Testis | OC tissue | Cell signalling/ http://www.proteinatlas.org/ |
| p-Akt (Ser473) | 1:25, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300 | 60 min and 18 hours | Testis | OC tissue | Cell signalling/ http://www.proteinatlas.org/ |
| Anti-PI3K p85 (ab86714) | 1:100, 1:150, 1:200, 1:300, 1:500, 1:750, 1:1000, 1:2000, 1:3000, 1:4000 | 60 min and 18 hours | Kidney | OC tissue | Abcam/http://www.proteinatlas.org/ |
| PTEN (D4.3) | 1:50, 1:100, 1:200, 1:300, 1:400 | 60 min and 18 hours | Colon | OC tissue | Cell signalling/ http://www.proteinatlas.org/ |
| Anti-ATM (ab32420) | 1:25, 1:50, 1:100, 1:150, 1:200, 1:250 | 60 min and 18 hours | Colon | OC tissue | Cell signalling/ http://www.proteinatlas.org/ |
| XRCC1 (33-2-5) | 1:50, 1:150, 1:200, 1:250, 1:300 | 30, 60 min and 18 hours | Testis | OC tissue | Invitrogen/http://www.proteinatlas.org/ |
| Anti-XIAP (ab21278) | 1:300, 1:500, 1:750, 1:1000 | 60 min and 18 hours | Kidney | OC tissue | Abcam/http://www.proteinatlas.org/ |
| Anti-PARP1 (551025) | 1:400, 1:600, 1:800, 1:1000 | 60 min and 18 hours | Kidney | OC tissue | BDPharmingen/http://www.proteinatlas.org/ |
| Anti-DNA polB (ab26343) | 1:100, 1:150, 1:200, 1:300, 1:400, | 60 min and 18 hours | Kidney | OC tissue | Abcam/http://www.proteinatlas.org/ |
| Anti-LIG1 (ab177946) | 1:25, 1:50, 1:100, 1:200, 1:300 | 60 min and 18 hours | Breast | OC tissue | Abcam/http://www.proteinatlas.org/ |
| Anti-LIG3 (HPA006723 | 1:50, 1:100, 1:150, 1:200, 1:250, 1:300 | 60 min and 18 hours | Testis | OC tissue | SIGMA/http://www.proteinatlas.org/ |
| Anti-LIG4 (HPA001334) | 1:50, 1:100, 1:150, 1:200, 1:300, 1:400 | 60 min and 18 hours | Testis | OC tissue | SIGMA/http://www.proteinatlas.org/ |

**Supplementary table 2: Kaplan Meier analysis outcome illustrating the associations between the altered expression of p-AKT, p-mTOR, p-4EBP1 and p-P70S6K proteins with PFS and OS in primary ovarian carcinoma – platinum resistant selection**

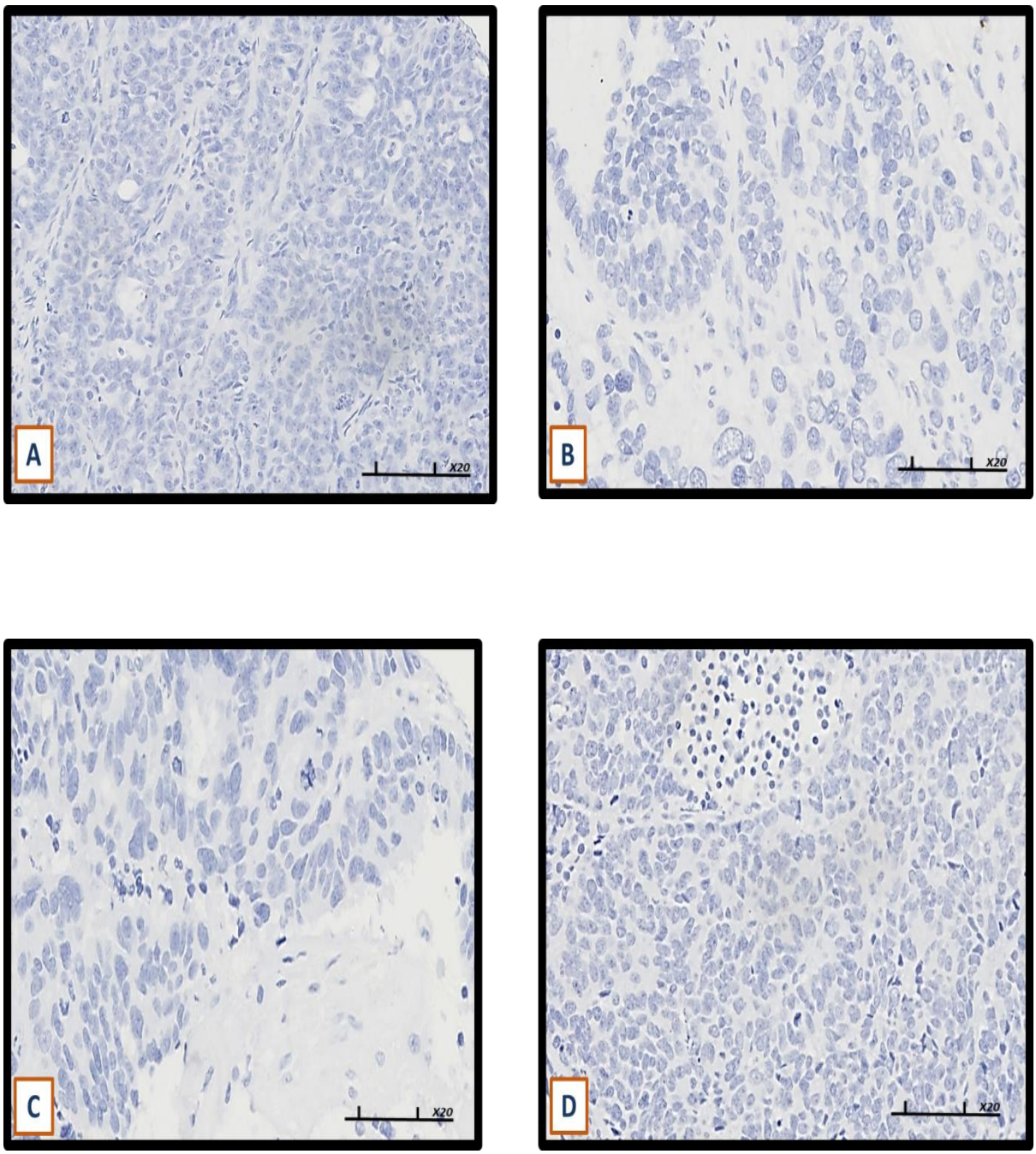
| Biological marker | Number of cases | | PFS P/Value Log Rank | OS P/Value Log Rank |
|---|---|---|---|---|
| | Positive | Negative | | |
| **p-AKT** | 14 | 30 | 0.426 | 0.371 |
| | | | 0.635 | 0.800 |
| **p-mTOR** | 19 | 17 | 0.423 | 0.454 |
| | | | 0.642 | 0.560 |
| **p-4EBP1** | 17 | 22 | 0.994 | 0.190 |
| | | | 0.000 | 1.178 |
| **p-P70S6K** | 20 | 17 | 0.153 | 0.110 |
| | | | 2.044 | 2.561 |
| **p-AKT and p-4EBP1 only tumours** | | | | |
| | **+/+** | **-/-** | | |
| **p-AKT/p-4EBP1** | 18 | 5 | 0.632 | 0.263 |
| | | | 0.229 | 1.253 |

**Supplementary table 3: Kaplan Meier analysis outcome illustrating the associations between the altered expression of XRCC1, PARP1 and polβ proteins with PFS and OS in primary ovarian carcinoma – platinum resistant selection**

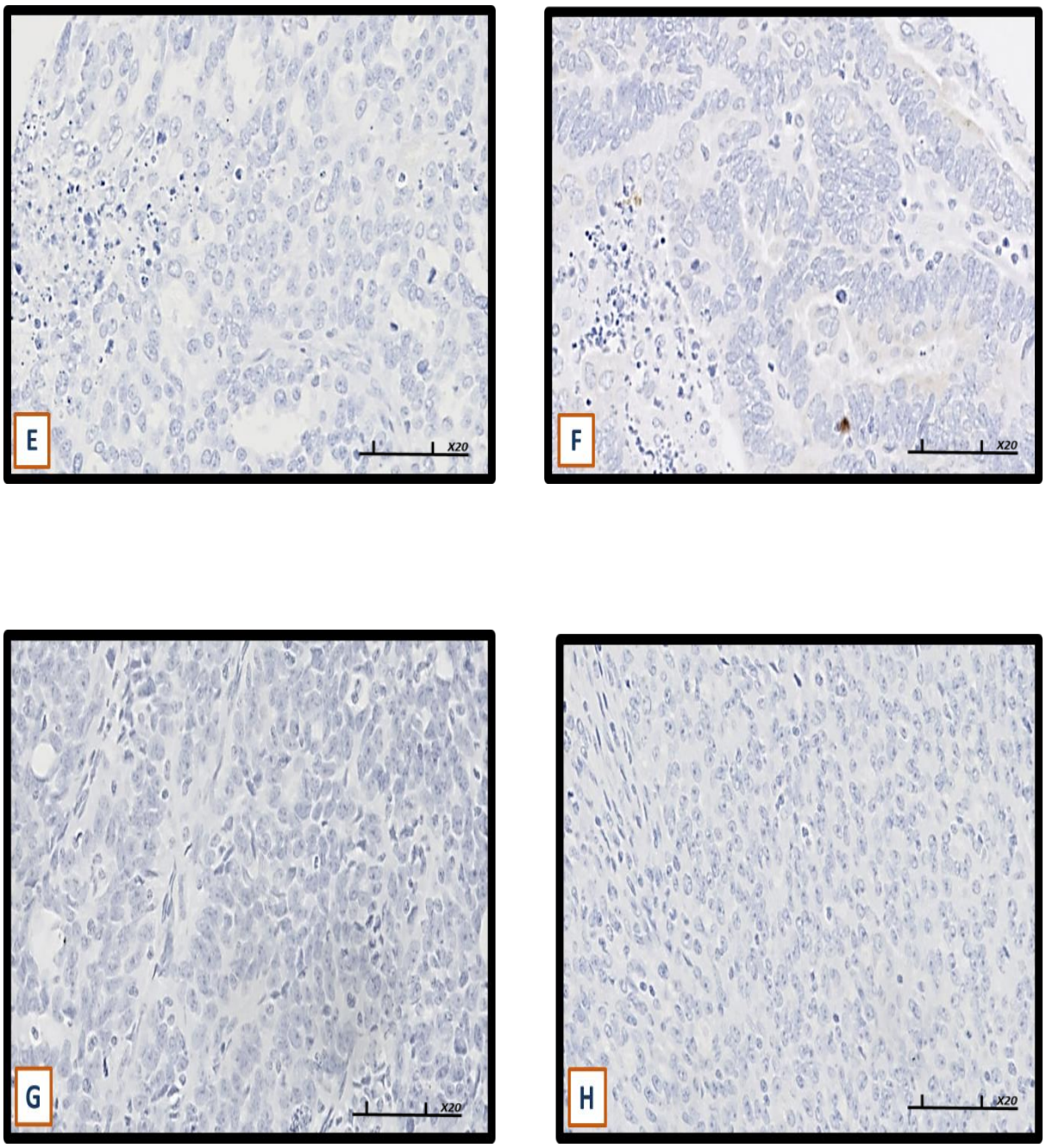
| Biological marker | Number of cases | | PFS | OS |
|---|---|---|---|---|
| | Positive | Negative | P/Value Log Rank | P/Value Log Rank |
| **XRCC1** | 61 | 17 | **0.040** | 0.644 |
| | | | **4.209** | 0.213 |
| **PARP1** | 37 | 29 | 0.773 | 0.360 |
| | | | 0.083 | 0.837 |
| **Pol β** | 31 | 32 | 0.408 | 0.349 |
| | | | 0.686 | 0.877 |

**Supplementary table 4: Kaplan Meier analysis outcome illustrating the associations between the altered expression of LIG1, LIG3 and LIG4 proteins with PFS and OS in primary ovarian carcinoma – platinum resistant selection**

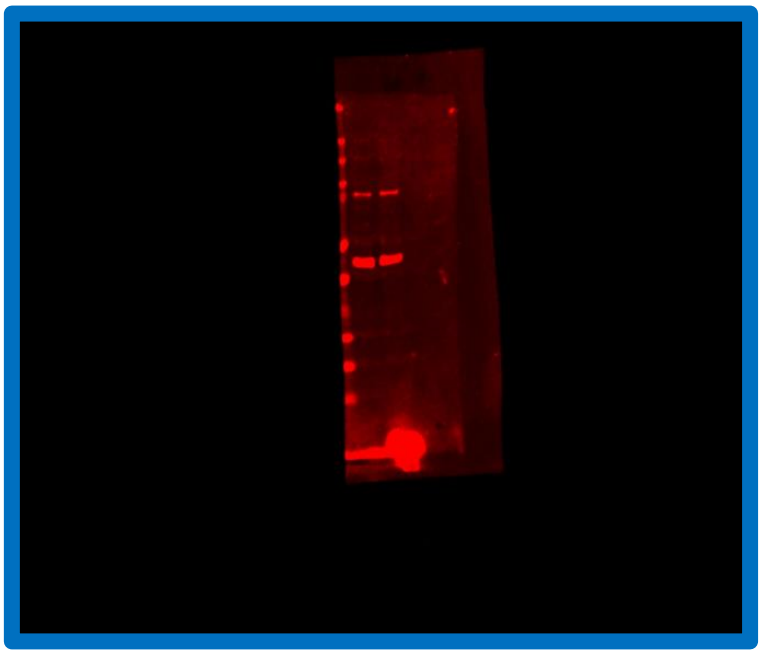
| Biological marker | Number of cases | | PFS P/Value Log Rank | OS P/Value Log Rank |
|---|---|---|---|---|
| | Positive | Negative | | |
| **LIG1** | 24 | 42 | 0.759 | 0.209 |
| | | | 0.094 | 1.578 |
| **LIG3** | 37 | 23 | 0.978 | 0.186 |
| **Cytoplasmic** | | | 0.001 | 1.753 |
| **LIG3 Nuclear** | 26 | 34 | 0.565 | 0.780 |
| | | | 0.331 | 0.078 |
| **LIG4** | 15 | 29 | 0.868 | 0.871 |
| | | | 0.028 | 0.039 |



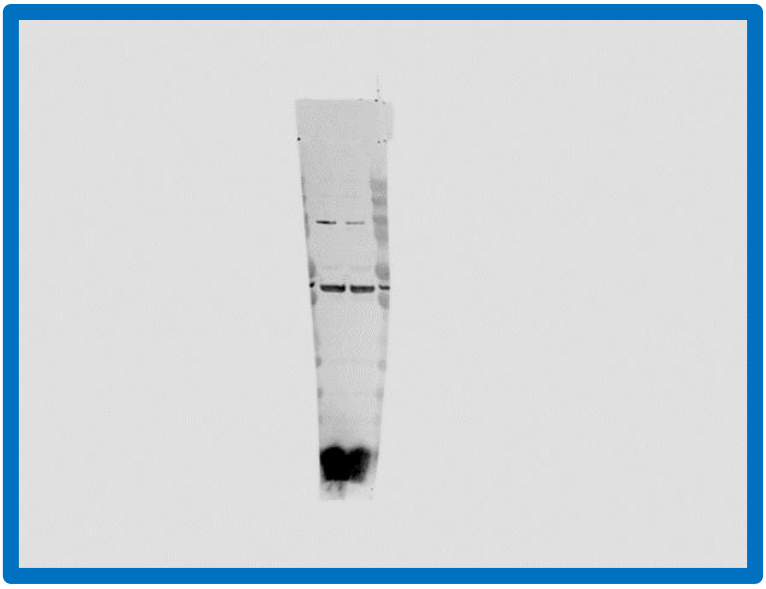

**Supplementary figure: Negative control included in each IHC experiment on ovarian carcinoma**



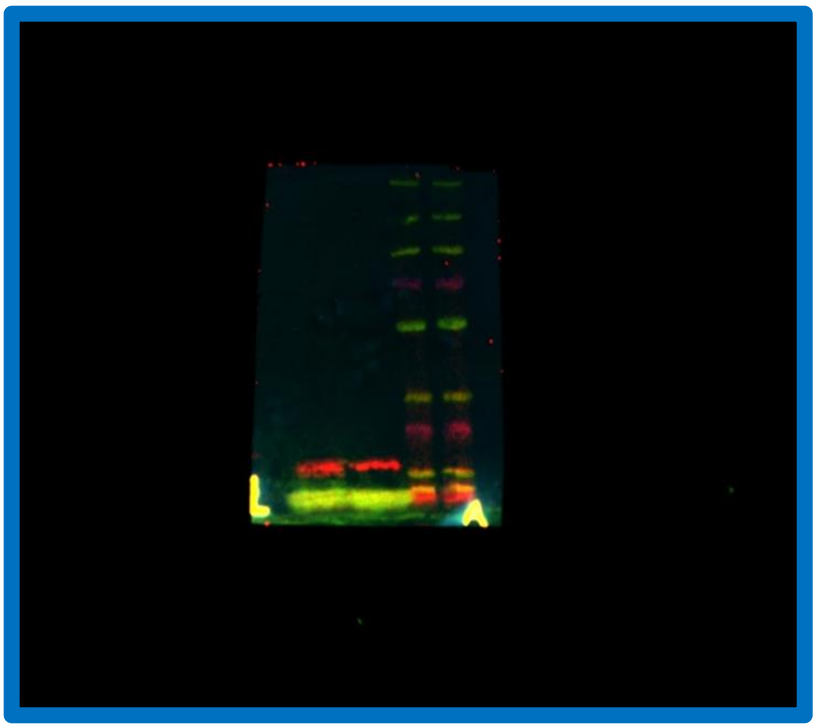

**Supplementary figure: Negative control included in each IHC experiment on ovarian carcinoma**



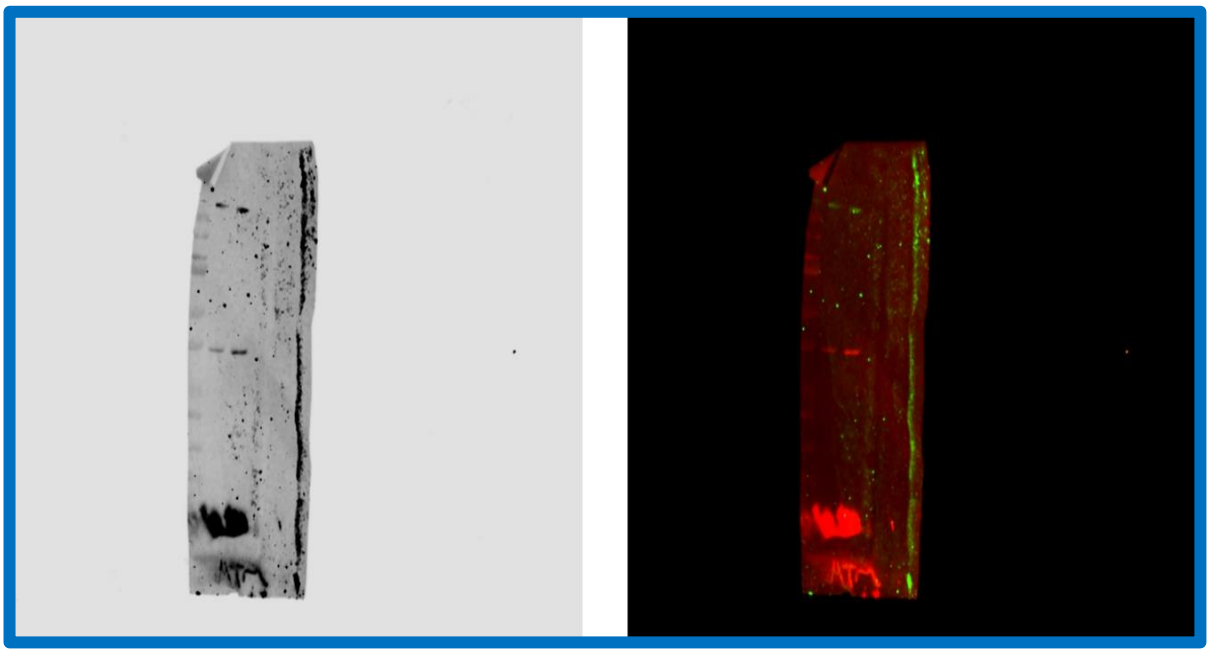
**Supplementary figure: Original uncropped Western Blot picture of anti-p-AKT antibody, Figure (3-3 in thesis). Picture as obtained from the camera**



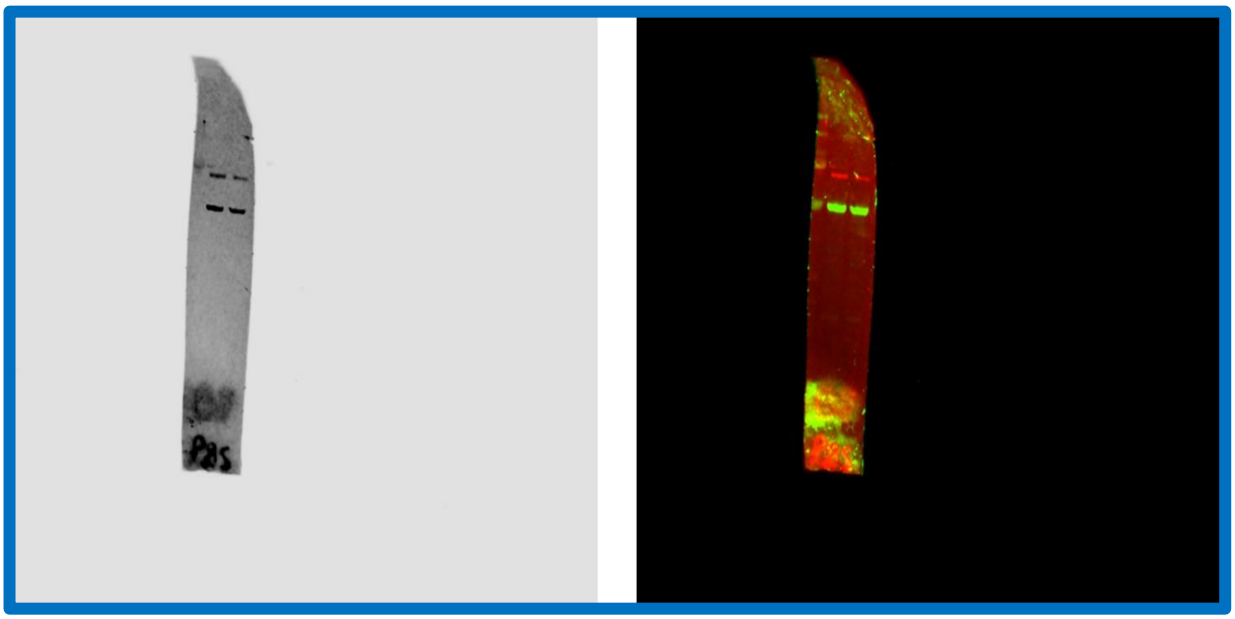
**Supplementary figure: Original uncropped Western Blot picture of anti-p-P70S6K antibody, Figure (3-2 in thesis). Picture as obtained from the camera**



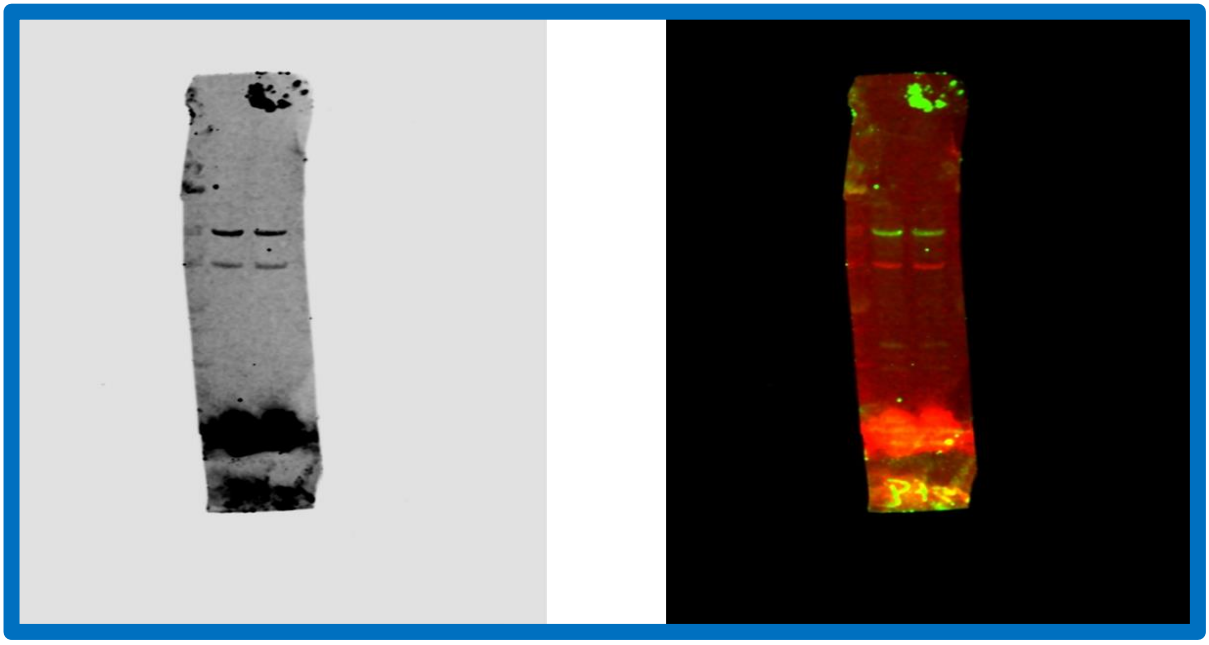
**Supplementary figure: Original uncropped Western Blot picture of anti-p-4EBP1 antibody, Figure (3-2 in thesis). Picture as obtained from the camera**



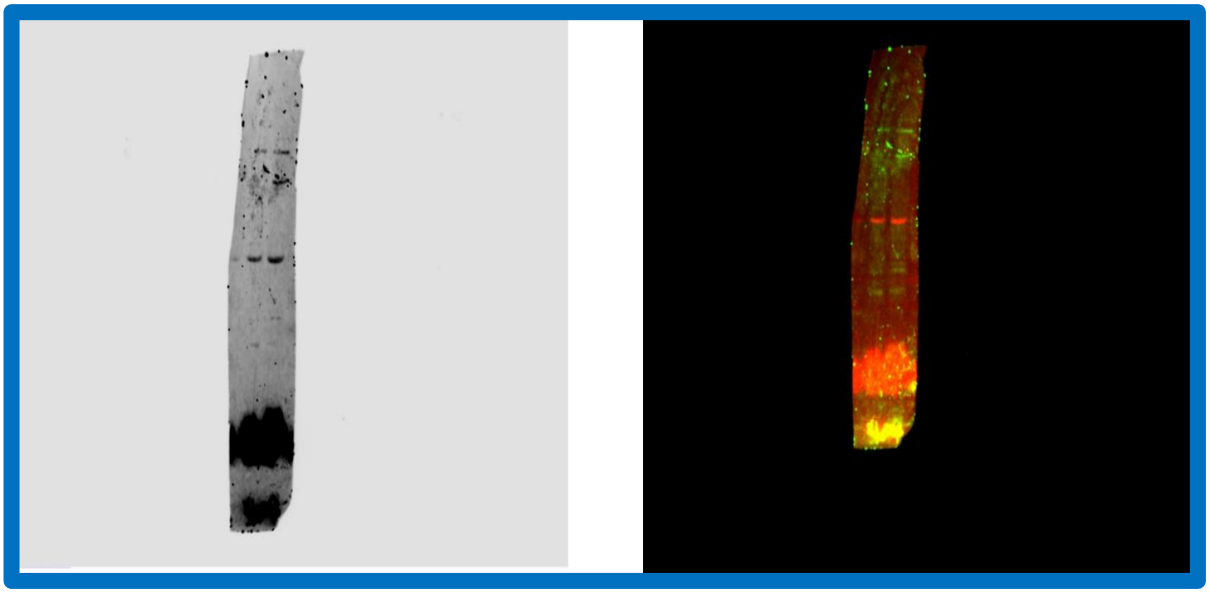
**Supplementary figure: Original uncropped Western Blot picture of anti-ATM antibody, Figure (4-1 in thesis). Picture as obtained from the camera**



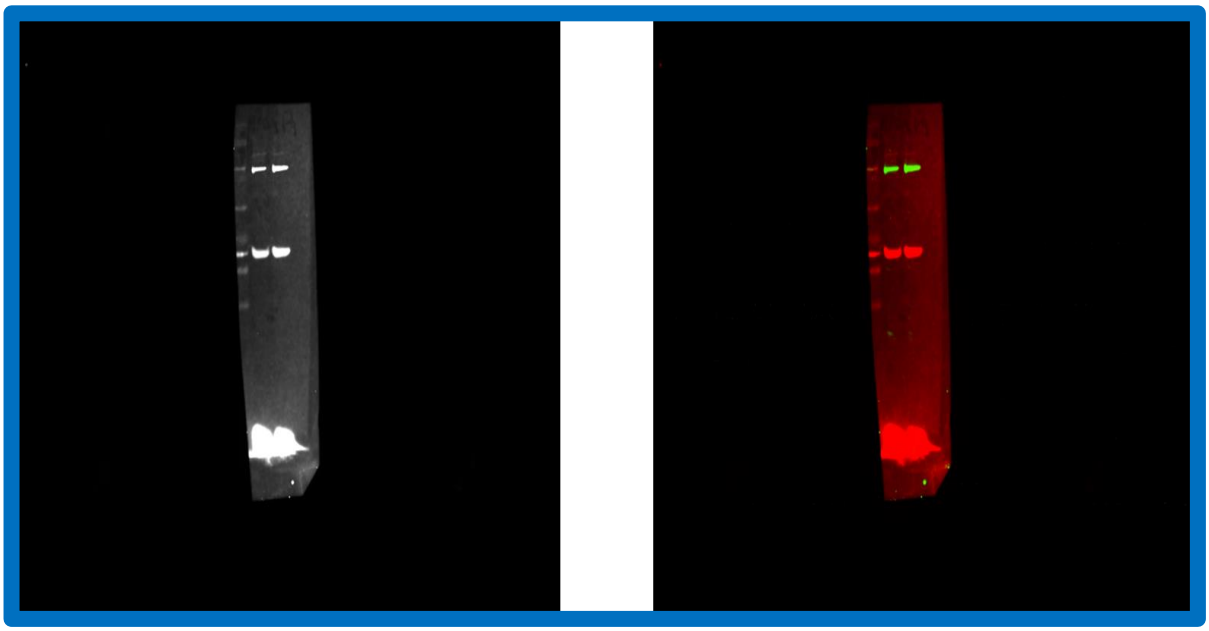
**Supplementary figure: Original uncropped Western Blot picture of anti-P85α antibody, Figure (4-3 in thesis). Picture as obtained from the camera**



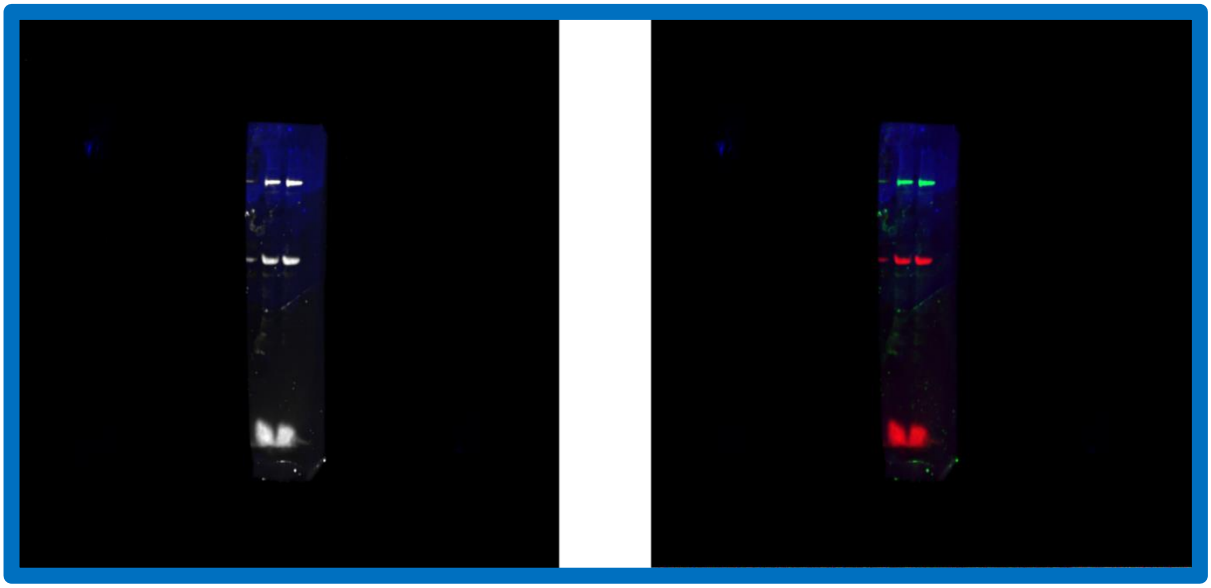
**Supplementary figure: Original uncropped Western Blot picture of anti-PTEN antibody, Figure (4-2 in thesis). Picture as obtained from the camera**



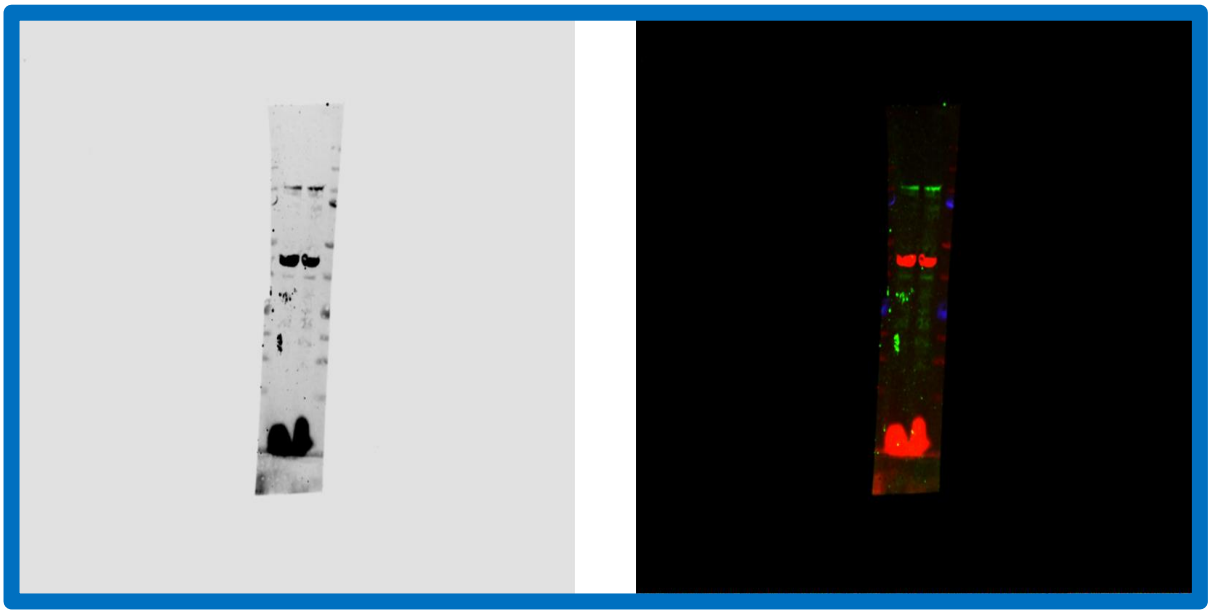
**Supplementary figure: Original uncropped Western Blot picture of anti-XIAP antibody, Figure (4-4 in thesis). Picture as obtained from the camera**



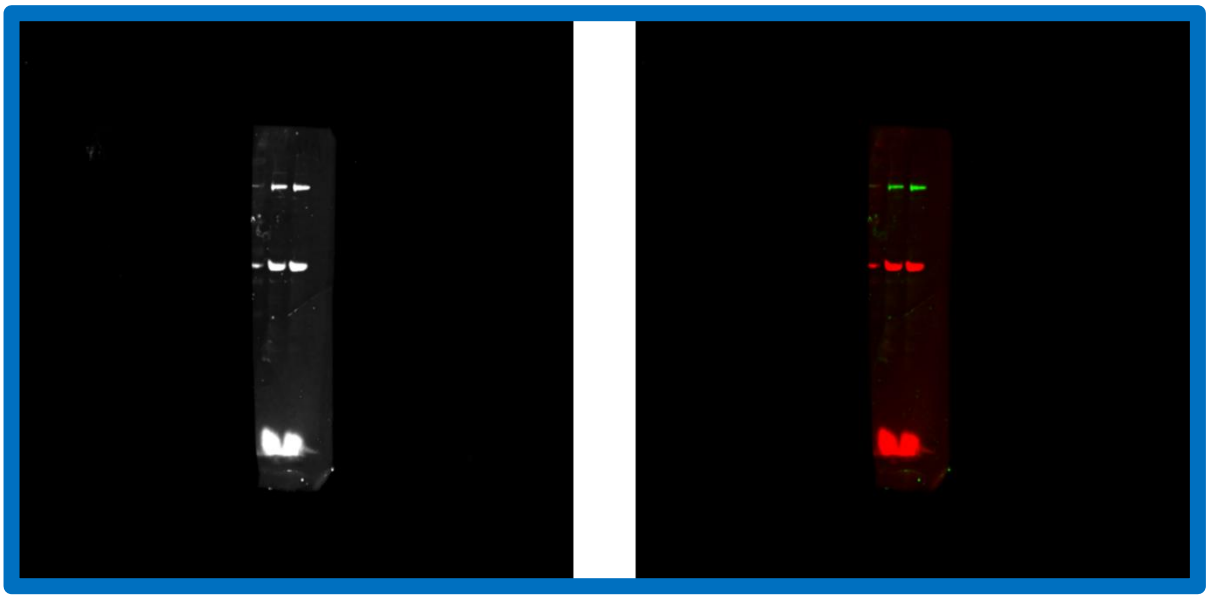
**Supplementary figure: Original uncropped Western Blot picture of anti-PARP1 antibody, Figure (5-1 in thesis). Picture as obtained from the camera**



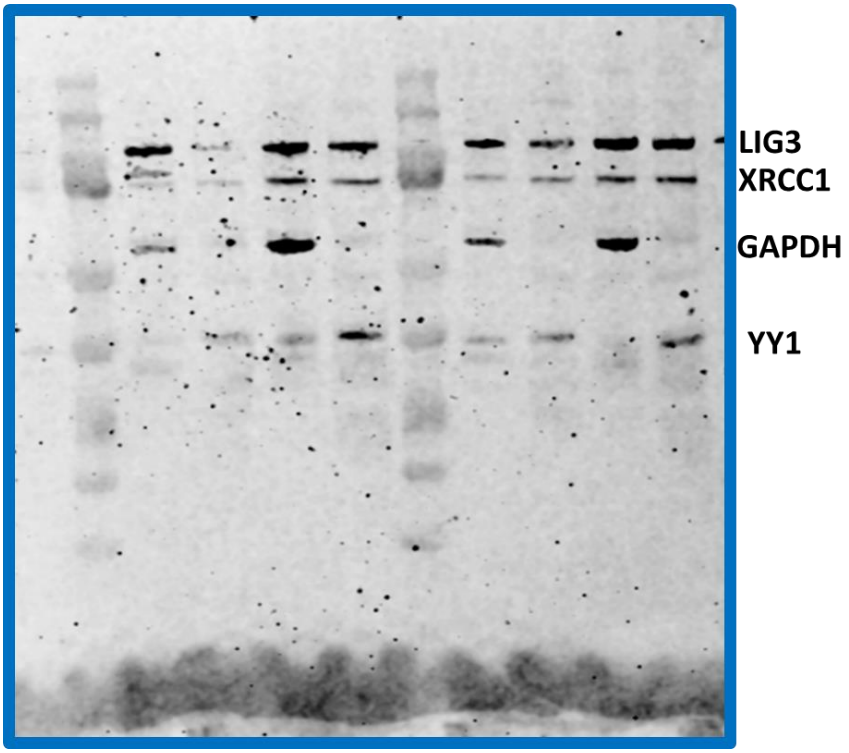
**Supplementary figure: Original uncropped Western Blot picture of anti-LIG1 antibody, Figure (6-1 in thesis). Picture as obtained from the camera**



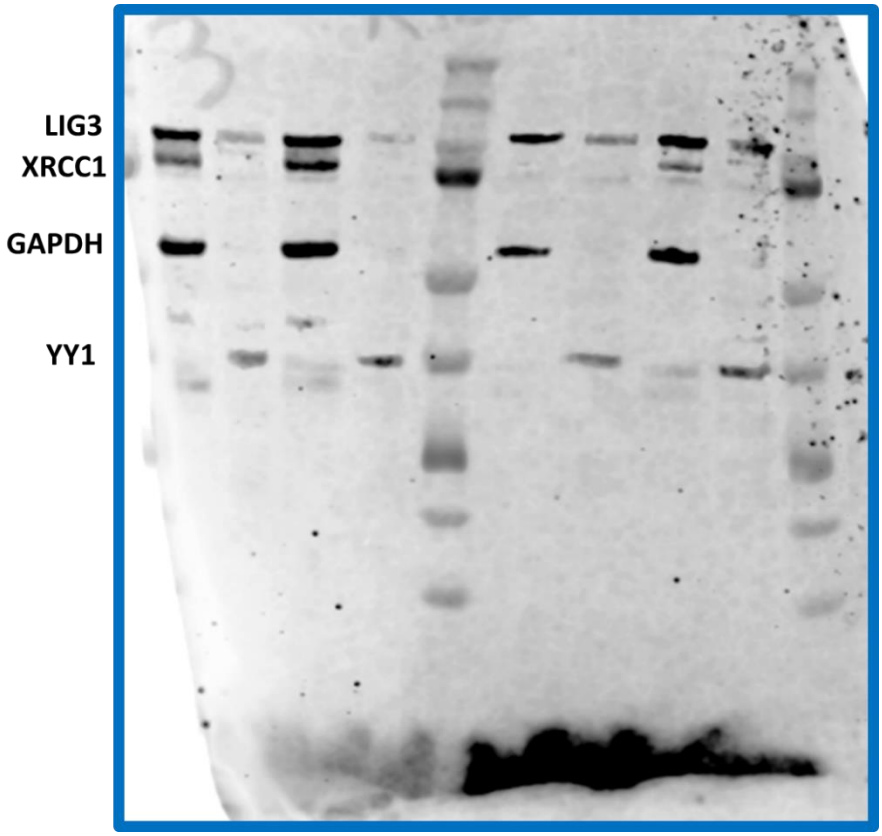
**Supplementary figure: Original uncropped Western Blot picture of anti-LIG3 antibody, Figure (6-2 in thesis). Picture as obtained from the camera**



**Supplementary figure: Original uncropped Western Blot picture of anti-LIG4 antibody, Figure (6-3 in thesis). Picture as obtained from the camera**




**Supplementary figure: Original uncropped Western Blot picture of LIG3 antibody nuclear and cytoplasmic extraction in A2780 cell lysates after 24 and 48 hours of cisplatin treatment, Figure (6-21 in thesis). Picture as obtained from the camera**




**Supplementary figure: Original uncropped Western Blot picture of LIG3 nuclear and cytoplasmic extraction in A2780Cis cell lysates after 24 and 48 hours of cisplatin treatment, Figure (6-22 in thesis). Picture as obtained from the camera**