Role of Annexin A2 in Ovarian Cancer Metastasis

A Thesis Submitted for the Degree of Doctor of Philosophy by

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Mak and Ayah, this is for you, I love you so much.

This thesis is dedicated to my loving parents, Norma Muhammad and Lokman Abdul Hamid.
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Abstract

Introduction

Materials and methods

Patient's tissue microarray cohort

Immunohistochemistry

Immunohistochemical assessment

Statistical analyses

Results

Increased stromal annexin A2 and S100A10 expression in metastatic ovarian cancer tissues compared with matching primary ovarian tumours

High stromal annexin A2 expression in the cancer associated stroma predicts clinical outcome

S100A10 cytoplasmic positivity is associated with reduced survival

Stromal annexin A2 positivity and cytoplasmic S100A10 expression is an independent predictor of clinical outcome

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Summary

Ovarian cancer is the most lethal gynaecological cancer. We identified annexin A2 to be modulated in the co-culture of ovarian cancer and peritoneal cells in vitro. Annexin A2, a calcium phospholipid binding protein, has been characterized in many malignancies and mediates various cellular functions such as cell motility, invasion, proliferation, angiogenesis and cell adhesion. Annexin A2 heterotetramer consists of annexin A2 and S100A10 monomers on the cell surface and plays an important role in the plasminogen activator system that enhances cancer cell invasion and metastasis. The aim of this Ph D thesis was to investigate the role of annexin A2 in ovarian cancer metastasis using in vitro and in vivo ovarian cancer models.

Annexin A2 expression was characterized in serous ovarian cancer cell lines and human serous ovarian cancer tissues. Annexin A2 inhibitors were used to evaluate the effects of annexin A2 on ovarian cancer cell motility, invasion and adhesion to the peritoneal cells. Furthermore, annexin A2 neutralizing antibodies were used to examine the role of annexin A2 in tumour invasion and metastasis using a chick chorioallantoic membrane (CAM) assay and an intraperitoneal xenograft mouse model. We evaluated whether annexin A2 can be used as a diagnostic marker by measuring blood annexin A2 levels in serous ovarian cancer patients. Moreover, annexin A2 and its binding protein, S100A10 expression were assessed using immunohistochemistry to determine their relationship with clinical outcome in a cohort of stage III serous ovarian cancers.

We showed that annexin A2 immunostaining was significantly increased in cancer-associated stromal cells compared with non-malignant ovarian tissues. Annexin A2 siRNAs significantly inhibited ovarian cancer cell motility, invasion and adhesion to peritoneal cells. Moreover, annexin A2 neutralizing antibodies significantly inhibited OV-90 cell motility and invasion in vitro and in the in vivo CAM assay. Furthermore, we also demonstrated that annexin A2 neutralizing antibodies significantly inhibited the invasion of primary ovarian cancer cell lines in the CAM assay. The growth of SKOV-3/GFP Luc cells and peritoneal dissemination in nude mice was significantly inhibited by annexin A2 neutralizing antibodies. Our findings suggested that reduced tumour burden and metastatic spread was a result of reduced cell survival.

Blood annexin A2 levels were increased in early stage and advanced stage ovarian cancer patients compared with women without malignancy (normal ovaries and benign ovarian tumours). We showed an improved sensitivity for detecting early stage ovarian cancer by combining annexin A2 and CA125 at the 95% and 98% specificity level. Kaplan-Meier analyses of stage III serous ovarian cancers showed that high stromal annexin A2 expression was significantly associated with
reduced progression-free survival and overall survival. Moreover, we also showed high cytoplasmic S100A10 in the cancer cells to be associated with reduced overall survival. Both, high stromal annexin A2 and high cytoplasmic S100A10, were independent predictors of overall survival in a multivariate analysis which included positive residual disease.

In conclusion, our findings indicate that annexin A2 plays an important role in ovarian cancer tumourigenesis and metastasis is therefore a potential novel therapeutic target against ovarian cancer. We also demonstrated that annexin A2 has both diagnostic and prognostic significance and may be useful for serous ovarian cancer diagnosis and patient management.
Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for the award of any degree or diploma in any university; and that to the best of my knowledge and belief, this work does not contain any material previously published or written by any other person except where due reference is made in the text.

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Noor Alia Lokman
February 2014
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Publications Arising During Ph D Candidature


Publications Contributing to This Thesis


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Lokman NA, Elder ASF, Ween MP, Pyragius CE, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis, Matrix Biology Society of Australia and New Zealand (MBSANZ), 20-23rd October 2013, McCracken Country Club, South Australia, Australia. (oral and poster)

Lokman NA, Elder ASF, Ween MP, Pyragius CE, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis, Postgraduate Research Expo, Faculty of Health Science, University of Adelaide, 29th August 2013, National Wine Centre, Adelaide, South Australia, Australia. (poster)


2012

Lokman NA, Ween MP, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 released during ovarian cancer-peritoneal cell interaction promotes a pro-metastatic cancer cell behaviour in ovarian cancer metastasis, Robinson Institute Research Symposium, 12th December 2012, National Wine Centre, Adelaide, South Australia, Australia. (poster)

Lokman NA, Ween MP, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 released during ovarian cancer-peritoneal cell interaction promotes a pro-metastatic cancer cell behaviour in ovarian cancer metastasis, Cold Spring Harbor Asia/International Cancer Microenvironment
Society Joint Conference on Tumour Microenvironment, 13th to 17th November 2012, Suzhou Dushu Lake Conference Center, Suzhou, China. (poster)


Lokman NA, Ween MP, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 released during ovarian cancer-peritoneal cell interaction promotes a pro-metastatic cancer cell behaviour in ovarian cancer metastasis, Matrix Biology Society of Australia and New Zealand (MBSANZ) 2012, 5th to 8th September 2012, Mantra Legends, Gold Coast, Queensland, Australia. (oral and poster)

Lokman NA, Ween MP, Hoffmann P, Oehler MK and Ricciardelli C, Annexin A2 released during ovarian cancer-peritoneal cell interaction promotes a pro-metastatic cancer cell behaviour in ovarian cancer metastasis, 14th International Biennial Congress of the Metastasis Research Society, 2nd to 5th September 2012, Brisbane Convention Centre, Brisbane, Queensland, Australia. (poster)


2011

Lokman NA, Ween MP, Hoffmann P, Oehler MK. and Ricciardelli C, Role of Annexin A2 in ovarian cancer metastasis. Australian Society for Medical Research (ASMR), SA Scientific Meeting, Adelaide Convention Center, 7th June 2011, South Australian Division (poster)

2010

meeting with the 5th Australian Health & Medical Research Congress 2010, 14th-18th November 2010, Melbourne Convention and Exhibition Centre, Victoria, Australia. (oral and poster)

**Lokman NA, Ween MP, Hoffmann P, Oehler MK and Ricciardelli C**, Role of Annexin A2 in ovarian cancer metastasis, Postgraduate Research Expo, Faculty of Health Science, University of Adelaide, 1st September 2010, The National Wine Centre, Adelaide, South Australia, Australia. (poster)

**Lokman NA, Ween MP, Hoffmann P, Oehler MK. and Ricciardelli C**, Role of Annexin A2 in ovarian cancer metastasis. Australian Society for Medical Research (ASMR), SA Scientific Meeting, Adelaide Entertainment Centre, 9th June 2010, South Australian Division. (oral)
Awards Arising Out of This Thesis

1. High Commended - Student Poster Award, 2013 Robinson Institute Research Symposium, 4th November 2013, National Wine Centre, Adelaide, South Australia, Australia.

2. Vice-Chancellor’s Prize for Best Poster, Faculty of Health Sciences Postgraduate Research Conference, 29th August 2013, National Wine Centre, Adelaide, South Australia, Australia.


4. Best Student Poster Award 2012, Matrix Biology Society of Australia and New Zealand (MBSANZ), 5th to 8th September 2012, Mantra Legends, Gold Coast, Queensland, Australia.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>E-ACA</td>
<td>6-Aminocaproic Acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ANXA2</td>
<td>Annexin A2</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer Antigen 125</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick Chorioallantoic Membrane</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of Differentiation 44</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Media</td>
</tr>
<tr>
<td>CMI</td>
<td>Cancer Cells and Matrigel Implant</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine Tetrahydrochloride</td>
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<tr>
<td>ECT</td>
<td>Ectoderm</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelium-Mesenchymal Transition</td>
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<td>END</td>
<td>Endoderm</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynaecologist and Obstetricians</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
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<td>GM6001</td>
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<td>Matrix Metalloproteinases</td>
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<td>N-terminal domain</td>
<td>Amino Terminus Domain</td>
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<tr>
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<td>Receiver Operating Curve</td>
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<td>ROMA</td>
<td>Risk of Ovarian Malignancy Algorithm</td>
</tr>
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<td>RR</td>
<td>Relative Risk</td>
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<td>RT-PCR</td>
<td>Real-time Polymerase Chain Reaction</td>
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<tr>
<td>u-PA</td>
<td>Urokinase-Type Plasminogen Activator</td>
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<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>siRNAs</td>
<td>Small Interfering RNAs</td>
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<td>SKOV-3/GFP Luc</td>
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<td>t-PA</td>
<td>Tissue-Type Plasminogen Activator</td>
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<td>TGFBIp</td>
<td>Transforming Growth Factor Inducible Protein</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
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Chapter 1: The Role of Annexin A2 in Tumourigenesis and Cancer Progression

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Chapter 1: The Role of Annexin A2 in Tumourigenesis and Cancer Progression

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Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Development and wrote the manuscript.

Certification that the statement of contribution is accurate

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Manuscript evaluation.

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1.1 Abstract

Annexin A2 is a calcium-dependent, phospholipid-binding protein found on various cell types. It is up-regulated in various tumour types and plays multiple roles in regulating cellular functions, including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion. Annexin A2 binds with plasminogen and tissue plasminogen activator on the cell surface, which leads to the conversion of plasminogen to plasmin. Plasmin is a serine protease which plays a key role in the activation of metalloproteinases and degradation of extracellular matrix components essential for metastatic progression. We have recently found that both annexin A2 and plasmin are increased in conditioned media of co cultured ovarian cancer and peritoneal cells. Our studies suggest that annexin A2 is part of a tumour-host signal pathway between ovarian cancer and peritoneal cells which promotes ovarian cancer metastasis. Accumulating evidence suggest that interactions between annexin A2 and its binding proteins play an important role in the tumour microenvironment and act together to enhance cancer metastasis. This article reviews the current knowledge on the biological role of annexin A2 and its binding proteins in solid malignancies including ovarian cancer.

Keywords: annexin A2, p11 protein, t-PA, plasmin and metastasis
1.2 Introduction
The plasminogen activation system known to be involved in thrombolysis and wound healing plays a major role in cancer progression. The inactive enzyme plasminogen is converted to the active serine protease plasmin by plasminogen activators; tissue-type plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA). Plasmin is involved in many biological processes besides vascular thrombolysis including angiogenesis, tissue remodelling and also activates metalloproteinases (MMPs) and latent growth factors and the degradation of extracellular matrix (ECM), processes which all contribute to tumour development and metastasis [1]. A well-studied protein which is an important mediator in the plasminogen activator system is annexin A2 [2]. The interaction between annexin A2 and t-PA mediates the conversion of plasminogen to plasmin and increases the catalytic efficiency of plasmin generation by up to 60-fold [3].
Annexin A2, a calcium-binding cytoskeletal protein is localized at the extracellular surface of endothelial cells and various types of tumour cells [4, 5]. The increased expression of annexin A2 has been reported in cancers of the breast, liver, prostate and pancreas [6-9]. Additionally, annexin A2 has also been demonstrated to play a role in cancer cell migration, invasion [7, 10] and adhesion [11, 12], processes which are essential for cancer metastasis. This article reviews the current knowledge on annexin A2 expression in various malignancies including ovarian cancer and its role in regulating cancer cell behavior.

1.3 The structure and function of annexin A2
Annexin A2 belongs to the annexin family of calcium binding proteins which consists of 12 members (A1-A11 & A13). Each family member shares the conserved calcium binding motifs in the carboxyl domain. However, the individual annexin members have diverse functions due to the highly variable amino-terminal domain [13]. Annexin family members have been reported to play multiple roles in cancer including signal transduction, angiogenesis, tumour invasion and metastasis and apoptosis (reviewed in [14]). Studies have shown annexin A1, A2, A3, A4, A6, A8 and A11 to be up-regulated whilst annexin A1, A2, A7, A8, A10 and A11 are decreased in different cancer types [14]. Moreover, annexins have also been associated with chemo resistance in breast and ovarian cancer cells [15-18].
The human annexin A2 gene spans approximately 40kb on the long arm of chromosome 15 (15q21) [19]. This region has been shown to be associated with allelic imbalance in advanced breast cancer [20]. However, no annexin A2 mutations have been described to date in cancer or other diseases. Annexin A2 has a functional N-terminal domain with p11 proteins and t-PA binding
sites and a C-terminal domain consist of binding sites for calcium, phospholipids and actin filaments (Figure 1.1). Annexin A2 exits both as a monomer and a heterotetramer. The annexin A2 monomer is an intracellular 38 kDa protein whilst, annexin A2 heterotetramer consisting of two subunits of annexin A2 monomers and two subunits of p11 proteins (also known as S100A10) is localized to the plasma membrane [21]. The translocation of annexin A2 monomers from the cytoplasm to the cell surface occurs as a result of heat stress [22], tyrosine phosphorylation [23], and interaction with heat shock protein 90 alpha [24, 25]. In endothelial cells, translocation of annexin A2 to the plasma membrane due to heat stress is dependent on the expression of p11 proteins and phosphorylation of tyrosine 23 [22]. Annexin A2 also assists in the rearrangement of the actin cytoskeleton, maintaining the plasticity of the actin cytoskeleton and regulating membrane trafficking events including endocytosis and exocytosis [26-28].

1.4 Expression of annexin A2 in cancer
Many studies have reported increased expression of annexin A2 in cancer tissues compared with normal tissues [6, 7, 29-31] (Table 1.1). The up-regulation of annexin A2 expression in pancreatic, colorectal, and brain tumours was directly correlated with advanced clinical stage [31-33]. Higher annexin A2 expression was also observed in metastatic breast cancer and colon cancer cells compared with the non-metastatic cells [7, 34]. In prostate cancer however, reports regarding annexin A2 have been conflicting. Whilst several studies reported a reduction of annexin A2 expression in prostate cancer tissues [35-38], a study by Banerjee et al. (2003) showed high focal membrane staining of annexin A2 in high grade prostate cancers and the prostate carcinoma cell lines, PC3 and DU145 [39]. Furthermore, a more recent study demonstrated a correlation between high annexin A2 levels and a more aggressive prostate cancer phenotype [9].

Conflicting results on annexin A2 expression have also been observed in head and neck cancers. A study by Wu et al. (2002) using 2D gel-electrophoresis of two head and neck squamous cell carcinoma cell lines derived from primary and metastatic tumours from the same patient, found annexin A2 to be up-regulated in the cell line established from the metastatic cancer cell line [40]. In contrast, Pena-Alonso et al. (2008) observed a significant down-regulation of annexin A2 mRNA and protein levels in poorly differentiated carcinomas of the head and neck by real time-PCR and immunohistochemistry [41]. These contradictory findings may be explained by different experimental techniques utilised to examine annexin A2 expression or differences between primary tumours and metastatic lesions. The different annexin A2 levels identified by Wu et al. (2002) may reflect annexin A2 protein modifications detected by mass spectrometry which are not
detectable by real-time PCR or immunohistochemistry [40]. Despite various studies having confirmed either an up or down regulation of annexin A2 in various tumour types, the exact mechanism that regulate annexin A2 expression at both mRNA and protein level are poorly understood.

Annexin A2 apart from being a cell surface protein, it has also been shown to be secreted. Deora et al. (2003) reported that annexin A2 is secreted via a non-classical secretory pathway as it does not have a signal secretion sequence [22]. Secreted annexin A2 was also found in the conditioned media of co-cultured human keratinocytes and fibroblasts [42]. Annexin A2 secretion has been also reported for various tumour types. Davis et al. (1995) demonstrated high levels of secreted annexin A2 in a human pancreatic adenocarcinoma cell line (Capan-2) [43]. Zhao et al. (2003) have reported that annexin A2 secretion by rat adrenal pheochromocytoma (PC12) cells in vitro is induced by activation of both the insulin and insulin like growth factor receptor followed by reduction of intracellular annexin A2 [44]. A study by Ji et al. (2009) reported that annexin A2 was up-regulated in the serum of hepatocellular carcinoma patients compared with the serum of healthy patients by ELISA [45]. A recent study using a proteomic approach investigating the secretome of the gastric cancer cell line, SGC7901 identified annexin A2 as a secreted phosphoprotein [46]. Together these studies suggest that annexin A2 has potential diagnostic and prognostic value for several different carcinomas which needs to be further examined.

1.5 Role of annexin A2 and its binding proteins in cancer

The co-localization of annexin A2 with its binding proteins including t-PA, p11 protein, tenascin C and cathepsin B facilitates the proteolytic cascade leading to the activation of pro-enzymes and selective degradation of ECM components which regulate cancer cell properties and enhance metastasis (reviewed in [5]). Several studies have demonstrated the co-expression of annexin A2 and p11 protein in tumour and endothelial cells. Annexin A2 co-localizes with p11 protein at the surface of tumour cells where plasminogen activation occurs [47]. Puisieux et al. (1996) showed a co-expression of annexin A2 and p11 protein in colon, renal and liver carcinoma cell lines [48]. Moreover, over-expression of annexin A2 and p11 protein was observed in renal cell carcinoma tissues when compared with normal kidney [31]. Zhang et al. (2010) also reported over-expression of annexin A2 and p11 protein in the invasive breast cancer cell lines compared with the non-invasive breast cancer cell lines [47]. Annexin A2 also binds to tenascin C (reviewed in [5]), a large ECM molecule which is expressed in the tumour microenvironment and plays an important role in tumourigenesis (reviewed in [49]).
Esposito et al. (2006) reported annexin A2 and tenascin C to be over-expressed at both the mRNA and protein levels in pancreatic cancers compared with normal pancreas tissues [50]. Moreover, the interaction between annexin A2 and tenascin C has been shown to regulate cell migration and to enhance cell proliferation of endothelial cells [51]. Tenascin C has also been shown to be over-expressed in malignant ovarian tumour and to regulate adhesion and migration of ovarian cancer cells [52].

Cathepsin B, a lysosomal cysteine protease on the cell surface of various tumour cells (reviewed in [5]) also binds to annexin A2. Mai et al. (2000) reported that human pro-cathepsin B interacts with the annexin A2 heterotetramer which is co-localized on the surface of human breast carcinoma and glioma cells [53]. Cavallo-Medved et al. (2009) recently reported that the co-localization of cathepsin B and annexin A2 heterotetramer were involved in ECM degradation in endothelial cells during in vitro tube formation [54].

There are also other proteins which have been reported in the literature to bind to annexin A2. Annexin A2 receptor protein which has been characterized in osteoclasts [55] plays an important role in promoting prostate cancer progression [11]. Annexin A2 binds to collagen I in epithelial cells in a calcium dependent manner [56] and annexin A2 also interacts with the transcription factor, STAT6 in the metastatic prostate cancer cells [57]. Annexin A2 also binds to progastrin in colon cancer cells [58] and the interaction between annexin A2 and progastrin mediate growth effects of progastrin in gastrointestinal cancers (reviewed in [59]). Furthermore, annexin A2 has also been reported to form complexes with other S100 proteins such as S100A4 in endothelial cells [60] and S100A6 in pancreatic cancer cells [61].

1.6 Role of annexin A2 in the plasminogen activator system

Emerging evidence indicates that annexin A2 plays an important role in the plasminogen activator system which regulates cancer metastasis and angiogenesis (reviewed in [62, 63]). The interaction between annexin A2 and t-PA mediates the conversion of plasminogen to plasmin [3, 64, 65] which facilitates MMP activation and ECM degradation leading to enhanced cancer cell migration and invasion [10, 66]. These findings suggest that annexin A2 is likely to play an important role in the early dissemination model which has been recently been described by Coghlin and Murray [67].

Kinetic studies have demonstrated that binding of annexin A2 to t-PA on the cell surface of endothelial cells increases the catalytic efficiency of plasmin generation by up to 60-fold [3]. The co-localization of annexin A2 and t-PA has been demonstrated in breast and pancreatic cancer
cells [10, 66, 68]. Roda et al. (2003) reported that the annexin A2 binding site for t-PA, the LCKLSL peptide motif (amino acid 8-13), is vital for the interaction between annexin A2 and t-PA to mediate plasmin production [69]. This was confirmed by Diaz et al. (2004) who reported activation of plasminogen via specific interaction of t-PA and annexin A2 in pancreatic cancer cells using the LCKLSL annexin A2 peptide [10]. Over-expression of annexin A2 resulted in higher levels of plasmin production in acute promyelocytic leukaemia cells [70] and increased plasmin levels were also observed in invasive breast cancer cell lines when compared with non-invasive cell lines [47, 66]. Recently, the interaction between heat shock protein 90 alpha (Hsp90a), annexin A2, t-PA and plasminogen in the exosomes of human breast, glioma and fibrosarcoma cancer cell lines resulted in increased cancer cell motility due to increased plasmin production [24]. Thus, these studies suggest increased annexin A2 expression enhances plasmin generation in the cancer cells via the t-PA dependent plasminogen activation pathway.

1.7 Role of annexin A2 in cancer cell adhesion, invasion, and migration
Several studies suggest that annexin A2 plays an essential role in regulating cancer cell adhesion, invasion, proliferation and migration [10, 36, 66, 71]. The effects of annexin A2 on different cancer cells are summarised in Table 1.2. Sharma et al. (2006) demonstrated that the highly invasive breast cancer cell line, MDA-MB231 expressed high annexin A2 levels and in the presence of plasminogen resulted in plasmin generation which could be blocked by a monoclonal antibody to annexin A2 [7]. However, neither plasmin production nor annexin A2 expression was observed in the non-metastatic MCF-7, breast cancer cell line [7]. Additionally, annexin A2 expression was shown to be up-regulated in MDA-MB231 breast cancer cells by basic fibroblast growth factor (bFGF) which is known to accelerate tumour growth and angiogenesis [7]. A later study by Sharma et al. (2009) confirmed the importance of annexin A2 and t-PA interactions on the cell surface of MDA-MB231 cells and demonstrated that the t-PA dependent plasmin generation was essential for cancer cell migration and neo-angiogenesis [66]. In contrast, Gillette et al. (2004) showed that over-expression of annexin A2 in osteosarcoma cells did not alter their metastatic properties such as motility, adhesion, or proliferation. This study suggested however that annexin A2 decreases osteosarcoma aggressiveness by inducing a more differentiated state [72]. Furthermore, Jung et al. (2007) demonstrated using both in vivo and in vitro studies that the annexin A2 heterotetramer regulates adhesion of hematopoietic stem cells to osteoblasts and bone marrow endothelial cells.
Recently, Shiozawa *et al.* (2008) also reported that annexin A2 regulates adhesion and migration of prostate cancer cells to osteoblasts and endothelial cells [11]. Annexin A2 has also been demonstrated to play an important role in regulating cytoskeleton structures and actin remodeling which both are essential for cancer cell migration [74, 75]. A recent study by Pu *et al.* (2009) reported that annexin A2 interaction with HAb18G/CD187 (a member of immunoglobulin family) promotes invasion and migration of human hepatocellular carcinoma cells (FHCC-98 cells) *in vitro* [76]. siRNA studies have also confirmed that knockdown of annexin A2 expression decreases cell migration in human glioma cells [77] and decreases the invasive ability of multiple myeloma cells [71]. The silencing of annexin A2 expression in hepatocellular carcinoma cells results in a significant decrease of both MMP-2 and MMP-9 levels which decreases cancer cell invasiveness [76]. Bao *et al.* (2009) also showed that the loss of annexin A2 expression leads to reduced levels of pro-angiogenic molecules including MMP-2, MMP-9, MTI-MMP, and TIMP2 in multiple myeloma cell lines (U266 and RPMI18226) [71]. These pro-angiogenic molecules is essential for tumour cells properties such as angiogenesis, proliferation and invasion [71]. Annexin A2 and heat shock protein 90 alpha (Hsp90a) released in the exosomes of the tumour cells activates plasminogen and pro-MMP2 which increase plasmin production to enhance ECM remodeling and increased tumour cell motility and invasion in the tumour microenvironment [24]. These studies demonstrate that annexin A2 is a key factor in regulating MMP secretion and activation which are essential for ECM degradation and metastatic progression. Hou *et al.* (2008) reported that annexin A2 regulates p11 protein and plasmin levels in a mouse lymphoma (L5178Y) cell line [78]. Annexin A2 knockout in L5178Y cells exhibited reduced cell motility and invasion *in vitro* and *in vivo* [78]. In a recent study by Zhang *et al.* (2010) showed that the silencing of annexin A2 expression resulted in an inhibition of breast cancer cell proliferation and invasion which was also associated with a down-regulation of p11 protein and plasmin levels [47]. Together these studies indicate that annexin A2 plays an important role in promoting adhesion, migration and subsequent metastasis of cancer cells.

### 1.8 Post translational modifications and proteolytic cleavage of annexin A2 in cancer cells

Several studies have reported post-translational modification and proteolysis of annexin A2 in cancer. Multiple isoforms of annexin A2 were identified by 2D gel electrophoresis and mass spectrometry in colorectal and oral carcinomas [79, 80]. Phosphorylation at tyrosine 23 in the N-terminal domain of annexin A2, has been shown to be present in liver [8] and pancreatic cancer
tissues but not in corresponding normal tissues [32]. Takano et al. (2008) also observed post-translational modifications of annexin A2 in a chemotherapy-resistant pancreatic cancer cell line compared with chemo-sensitive cells [81]. Zheng et al. (2009) recently reported that phosphorylation of annexin A2 at tyrosine 23 was essential for localisation of annexin A2 at the cell surface and its contribution to the invasive properties of pancreatic cancer cells [32]. Eustace et al. (2011) recently reported increased phosphorylated forms of annexin A2 in dasatinib sensitive melanoma cell lines (WM-115) compared with the dasatinib resistant cell line (WM-2660-4) [82]. Annexin A2 can also undergo proteolysis. N-terminal processing of annexin A2 in human peripheral monocytes [83] and endothelial cells [84] induced by plasmin resulted in the loss of the first 27 amino acid residues. Tomonaga et al. (2007) reported cleavage of annexin A2 in the C-terminal end of annexin A2 in the healthy bowel mucosa but not in the colorectal cancer samples [80]. Tsunezumi et al. (2008) demonstrated that treatment of human colon cancer and breast cancer cell lines with MMP-7 (matrilysin) can result in specific cleavage of annexin A2 at the N-terminal region between Lys9 and Leu10 within the t-PA binding motif [85]. This study additionally demonstrated that the first nine amino acid residues in the N-terminal domain of annexin A2 bound preferentially to t-PA over intact annexin A2 on the surface of colon cancer cells [85]. Annexin A2 cleavage induced by glycogen synthase-3 was also observed in a time-dependent manner in serum-free conditioned media of human lung epithelial cells and human epidermoid cells [86]. Whilst several studies have demonstrated post-translational modifications of annexin A2, including phosphorylation changes and annexin A2 cleavage by cancer cells, the regulation of these modifications is poorly understood. Further studies are required to evaluate the significance of these modified forms of annexin A2 in cancer.

1.9 Annexin A2 and ovarian cancer

To date, there is limited knowledge about the importance of annexin A2 in ovarian cancer. A proteomic ovarian cancer biomarker study by Wang et al. (2004) using 2D liquid phase separation followed by reverse-phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption or ionisation-time of flight (MALDI-TOF) mass spectrometry, identified annexin A2, amongst other proteins at three different pI values. Annexin A2 at pI 7.10, was markedly elevated in ovarian cancer cell lines when compared with normal ovarian surface epithelial cells, suggesting that a post-translational modification of annexin A2 occurs in ovarian cancer cells [87]. Furthermore, Gagne et al. (2007) also found annexin A2 to be up-regulated in the highly metastatic, TOV-112D epithelial ovarian cancer cell line compared with the TOV-81D
ovarian cancer cells with low malignant potential, using iTRAQ (isobaric tag for relative and absolute quantitation) and 2D gel electrophoresis coupled with liquid chromatography and tandem mass spectrometry [88]. A more recent microarray study by Tchagang et al. (2008) reveal increased annexin A2 gene expression in ovarian cancer tissues when compared with normal ovarian tissue [89]. Moreover, several proteomic studies have also identified annexin A2 in the secretome of human ovarian cancer cell lines [90, 91].

We have recently explored the interaction between ovarian cancer and peritoneal cells using an in vitro co culture system [92]. We compared proteins in the secretome of ovarian cancer (OVCAR-5) and peritoneal cells (LP-9) cultured separately (Figure 1.2a) and co-cultured together (Figure 1.2b). We identified annexin A2 to be up-regulated in the secretome of co-cultured ovarian cancer and peritoneal cells by 2D-gel electrophoresis and mass spectrometry (Figure 1.2b). Furthermore, we have recently reported increased plasmin levels during the co culture of ovarian cancer and peritoneal mesothelial cells in vitro [92]. Preliminary findings have demonstrated high annexin A2 immunostaining in serous ovarian cancer tissues compared with benign serous cystadenomas (Figure 1.3). Our studies suggest that increased plasmin production and increased annexin A2 secretion is part of a tumour-host signal pathway between ovarian cancer and peritoneal cells which promotes ovarian cancer metastasis. Ongoing studies in our laboratory are currently investigating the importance of annexin A2 in ovarian cancer metastasis and its potential as a novel therapeutic target for this malignancy.

1.10 Annexin A2 as a potential therapeutic target in cancer

Several research groups have targeted annexin A2 to inhibit cancer progression and metastasis. Monoclonal antibodies against annexin A2 have been reported to be an effective therapeutic approach against Lewis lung carcinoma xenografts [93]. Jacovina et al. (2001) reported that a polyclonal antibody against the N-terminal domain of annexin A2 was able to block plasmin production in rat adrenal pheochromocytoma (PC-12) cells in vitro [94]. Annexin A2 has also been shown to bind to angioatin, a powerful anti-angiogenic molecule that is generated from plasminogen processing [95]. Tuszynski et al. (2002) reported that angioatin binds to the lysine binding domain of annexin A2 in endothelial cells results in anti-angiogenic effect [95]. Moreover, the interaction between annexin A2 and angioatin resulted in reduced plasmin generation in Lewis lung carcinoma cells [93]. These findings suggest that plasminogen and angioatin bind to the same annexin A2 binding site and antiangiogenic action of angioatin may be mediated via interactions with annexin A2 [95]. The identification of angioatin-like compounds that block
annexin A2 binding to plasminogen could be promising cancer therapeutics. More recently, Braden et al. (2009) showed that polymeric nanoparticles combined with annexin A2 siRNA vector to allow long term annexin A2 silencing could inhibit prostate cancer growth in mice [96]. Annexin A2 has also been identified as a molecular target for TM601 (a peptide with tumour-targeting and anti-angiogenic effects) in glioma, lung and pancreatic cancer cells [97]. Together these studies indicate that annexin A2 has a great potential as therapeutic target to inhibit cancer progression and metastasis.

1.11 Conclusions and future directions

In conclusion, annexin A2 has been shown to play an important role in cancer cell migration, invasion, adhesion and angiogenesis processes which are essential for cancer metastasis. Emerging evidence indicates that annexin A2 plays an important role in the plasminogen activator system. The interaction between annexin A2 and t-PA mediates the conversion of plasminogen to plasmin which facilitates ECM degradation leading to enhanced cancer cell migration and invasion (Figure 1.4). Annexin A2 over-expression and proteolytic cleavage has also been demonstrated in different malignancies. The processing of annexin A2 by MMPs and other proteases results in several annexin A2 isoforms but no studies so far have investigated the significance of these isoforms in tumour progression. Additional studies are needed to further explore the interactions between annexin A2 and its binding proteins and the importance of post-translational modifications of annexin A2 to promote tumour invasion and metastasis. A greater understanding of these mechanisms could potentially lead to the development of novel therapeutics to inhibit annexin A2 function and tumour progression.

1.12 Acknowledgement

This research was supported by the Ovarian Cancer Research Foundation (OCRF).
The N-terminal domain consists of the p11 proteins and tissue plasminogen activator (t-PA) binding sites. The C-terminal domain consists of four repeating domains which each contains annexin A2 consensus sequence and the binding sites for calcium, phospholipids and F-actin.

**Figure 1.1 Annexin A2 domain structure.**
Figure 1.2 2D-gel electrophoresis of proteins in co-cultured ovarian cancer and peritoneal cell.

A. 2D-gel electrophoresis of proteins in the secretome of ovarian cancer (OVCAR-5) and peritoneal cells (LP-9) cultured separately. B. 2D-gel electrophoresis of proteins in the secretome of OVCAR-5 and LP-9 co-culture. Circled spots that were up-regulated in the ovarian cancer-peritoneal co culture but absent in the single culture were identified to be annexin A2 by mass spectrometry.
Figure 1.3 Annexin A2 expression in human ovarian tumour tissues.

A. Benign serous cystadenoma  B. Serous ovarian carcinoma. Annexin A2 immunohistochemistry using mouse anti-annexin A2 (BD Biosciences, 1/500), with a citrate buffer microwave retrieval. Magnification bar = 100 μm for all images.
Figure 1.4 Proposed mechanism of annexin A2 promoting cancer metastasis in the plasminogen activation system.

Annexin A2 heterotetramer on the cell surface binds to t-PA and activates plasminogen conversion to plasmin. Plasmin results in activation of MMPs and lead to ECM degradation. Increased annexin A2 expression results in increased plasmin generation and enhances cancer invasion and metastasis.
Table 1.1 Annexin A2 expression in cancer.

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<th>Cancer</th>
<th>Annexin A2 Expression</th>
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<td>Five human pancreatic cell lines had 5-15 fold higher levels of annexin A2 compared with normal cells</td>
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<td>Increased annexin A2 expression in metastatic tumour of clear cell renal carcinoma compared with the primary tumour</td>
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<td>Expression annexin A2 is reduced in prostate cancer cells</td>
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<td>Down-regulation of annexin A2 at mRNA and protein level in metastatic prostate cancer</td>
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<td>Reduced annexin A2 levels in high grade tumour</td>
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<td>Increased expression of annexin A2 in high grade tumour and metastatic cell lines</td>
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<td>The interaction of annexin A2 and t-PA induced cell migration in human breast cancer cell line</td>
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<td>Interaction of annexin A2 and t-PA promotes pancreatic cancer cell invasion <em>in vitro</em></td>
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<td>Annexin A2 promotes invasion of hepatocellular carcinoma via interaction with HAb18G/CD187 <em>in vitro</em></td>
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<td>Annexin A2 enhances invasiveness of multidrug resistant breast cancer cell lines</td>
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<td>Adhesion</td>
<td>Annexin A2 regulates the adhesion of prostate cancer cells</td>
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<td>Annexin A2 regulates cell adhesion of RAW 117 cells (large cell lymphoma cells) to the endothelial cells</td>
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<td>Phosphorylation of annexin A2 mediates baby hamster kidney cell adhesion</td>
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<td>Annexin A2 plays a role in p53 induced apoptosis</td>
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<td>Annexin A2 cleavage leads to cell apoptosis</td>
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<td>Annexin A2 induces proliferation of hepatocytes</td>
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1.13 Statement of hypotheses, aims and research plan

Annexin A2 is one of the proteins modulated in the conditioned media of co-cultured serous ovarian cancer cells (OVCAR-5) and peritoneal cells (LP-9), identified by mass spectrometry and 2D gel electrophoresis. Annexin A2, a multifunctional calcium phospholipid binding protein has been characterized in many malignancies and reported to play an important role in tumourigenesis. Annexin A2 exists on the cell surface as annexin A2 heterotetramer with its binding protein, S100A10. To date, annexin A2 expression and its functional role has not been characterized in ovarian cancer. This study aimed to characterize annexin A2 expression and investigate the functional role of annexin A2 in serous ovarian cancer using in vitro and in vivo ovarian cancer models. We also investigated whether annexin A2 can be used as a diagnostic marker serous ovarian cancer and assessed the efficacy of annexin A2 together with ovarian cancer biomarker, CA125 in serous ovarian cancer patients. Moreover, annexin A2 and S100A10 expression were examined in a uniform cohort of stage III serous ovarian cancers to investigate its potential role as a prognostic marker for ovarian cancer.

1.13.1 Hypotheses

1. Annexin A2 expression plays an important role in ovarian cancer tumourigenesis
2. Annexin A2 inhibitors decrease ovarian cancer cell motility, invasion and adhesion to the peritoneal cells in vitro and in vivo
3. Annexin A2 can be used as a tumour marker for ovarian cancer diagnosis and prognosis

1.13.2 Aims

1. To characterize annexin A2 expression in human serous ovarian cancer tissues, serous ovarian cancer cell lines and primary ovarian cancer cell lines
2. To investigate the effects of annexin A2 inhibitors in ovarian cancer cell motility, invasion and adhesion to the peritoneal cells in vitro
3. To determine whether annexin A2 inhibitors can block ovarian cancer invasion and metastasis in in vivo model
4. To evaluate whether annexin A2 can be used as a tumour marker for ovarian cancer diagnosis
5. To assess whether either annexin A2 or S100A10 can be used as a prognosis marker for ovarian cancer
1.13.3 Research plan

Annexin A2 expression in human normal ovarian surface epithelium, benign cystadenomas, borderline ovarian tumours and serous ovarian cancer tissues (stage I to IV) were characterized using annexin A2 immunohistochemistry. Annexin A2 expression was also examined in the omental tissues, matching primary and metastatic ovarian cancer tissues. Real-time PCR and western immunoblotting were performed to assess annexin A2 expression in human serous ovarian cancer cell lines (OVCAR-3, OVCAR-5, OV-90 and SKOV-3), peritoneal cell line (LP-9) and primary ovarian cancer cell lines derived from stage III serous ovarian cancer patients ascites (n=11). To examine the functional role of annexin A2, annexin A2 inhibitors including annexin A2 siRNAs and neutralizing annexin A2 antibodies were used in cell motility, invasion and adhesion assay to the peritoneal cells in vitro in OVCAR-3, OVCAR-5, OV-90 and SKOV-3 cells. Moreover, we investigated the invasion of OVCAR-3, OV-90, SKOV-3 and two primary ovarian cancer cell lines using the CAM model in vivo. In this study, we also examined the effects of neutralizing annexin A2 antibody using SKOV-3/GFP Luc cells in an intraperitoneal mouse model. Tumour development and metastasis in the nude mice were evaluated using bioluminescence imaging over a period of 36 days (n=5 per treatment group).

Western immunoblotting and commercially available annexin A2 ELISA were used to examine annexin A2 urine levels in serous ovarian cancer patients and control patients. We also utilized annexin A2 ELISA to examine blood annexin A2 levels in serous ovarian cancer patients (n=86) and controls which are patients with normal ovaries (n=27) and benign ovarian tumours (n=30). We performed ROC curve analysis to determine the sensitivity and the specificity of annexin A2 and CA125 for serous ovarian cancer detection. We also investigated the expression of annexin A2 and S100A10 using immunohistochemistry in a cohort of stage III serous ovarian cancer patients (n=91) using TMAs. Kaplan Meier and Cox Regression analyses were performed to investigate if either annexin A2 or S100A10 is associated with progression-free survival and overall survival. This study assessed whether annexin A2 expression plays a role in ovarian cancer metastasis and can be used as a novel diagnosis and prognosis marker for serous ovarian cancer.
Chapter 2: Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis

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STATEMENT OF AUTHORSHIP

Chapter 2: Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis

Noor Alia Lokman (candidate)

Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Performed analysis on all samples presented, interpreted the data and wrote the manuscript.

Certification that the statement of contribution is accurate

Signed: …………………………………………………………………………Date:…………………..

Alison S.F. Elder (co-author)

Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Interpreted the data and manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Carmela Ricciardelli (co-author)
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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: …………………………………………………………………………Date:……………………

Martin K. Oehler (co-author)
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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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2.1 Abstract

The majority of ovarian cancer patients present with advanced disease and despite aggressive treatment, prognosis remains poor. Significant improvement in ovarian cancer survival will require the development of more effective molecularly targeted therapeutics. Commonly, mouse models are used for the in vivo assessment of potential new therapeutic targets in ovarian cancer. However, animal models are costly and time consuming. Other models, such as the chick embryo chorioallantoic membrane (CAM) assay, are therefore an attractive alternative. CAM assays have been widely used to study angiogenesis and tumour invasion of colorectal, prostate and brain cancers. However, there have been limited studies that have used CAM assays to assess ovarian cancer invasion and metastasis. We have therefore developed a CAM assay protocol to monitor the metastatic properties of ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) and to study the effect of potential therapeutic molecules in vivo. The results from the CAM assay are consistent with cancer cell motility and invasion observed in in vitro assays. Our results demonstrate that the CAM assay is a robust and cost effective model to study ovarian cancer cell metastasis. It is therefore a very useful in vivo model for screening of potential novel therapeutics.

Keywords: ovarian cancer; invasion; metastasis; chick chorioallantoic membrane
2.2 Introduction

Ovarian cancer is the most lethal gynaecological malignancy. Most patients are diagnosed at an advanced stage when the cancer cells have already metastasized to the abdominal cavity. Ovarian cancer metastasis is characterized by the ability of ovarian cancer cells to detach from the ovary and to adhere and invade the peritoneal cell layer, which lines the organs in the abdominal cavity [112]. The development of new therapies with the aim to disrupt ovarian cancer metastasis requires the in vivo study of novel targets and molecules. However, commonly used murine models are costly and require a large number of animals as well as a long experimental time frame. An attractive alternative is the chick chorioallantoic membrane (CAM) assay.

CAM assays have been widely used to study angiogenesis [113], tumour cell invasion and metastasis [114-117]. The CAM model has many advantages, such as (a) the highly vascularized nature of the CAM greatly promotes the efficiency of tumour cell grafting; (b) high reproducibility; (c) simplicity and cost effectiveness, and finally (d) as the CAM assay is a closed system, the half-life of many experimental molecules such as small peptides tends to be much longer in comparison to animal models, allowing experimental study of potential anti-metastatic compounds that are only available in small quantities [115, 118]. The CAM is composed of a multilayer epithelium; the ectoderm at the air interface, mesoderm (or stroma) and endoderm at the interface with the allantoic sac [119]. Furthermore, the CAM contains extracellular matrix proteins (ECM) such as fibronectin, laminin, collagen type I and integrin αvβ3 [120]. The presence of these extracellular matrix proteins mimics the physiological cancer cell environment.

Although the CAM assay is a well-established model for studying tumour angiogenesis and invasion in malignancies such as bowel cancer [121, 122], glioma [123-125], prostate cancer [126-128], leukemia [129] and osteosarcoma [130], there has only been one study to date that has used a CAM assay to assess ovarian cancer invasion and metastasis [131]. We recently investigated the ovarian cancer-peritoneal cell interaction and identified several novel proteins that may be involved in ovarian cancer metastasis [92, 132]. To effectively determine their function, we developed a CAM assay protocol using a range of ovarian cancer cell lines to allow the monitoring of candidate molecules on ovarian cancer cell invasion in vivo. The in vivo CAM assay data was compared with results from in vitro assays.
2.3 Materials and methods

2.3.1 Cell culture

The human ovarian cancer cell lines OVCAR-3, SKOV-3 and OV-90 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All ovarian cancer cell lines were maintained in RPMI 1640 medium supplemented with 4 mM L-glutamine, antibiotics (100 U penicillin G, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B), (Sigma-Aldrich, St. Louis, MO, USA). OVCAR-3 and SKOV-3 cells were supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich) and OV-90 cells were supplemented with 10% FBS. All cell lines were maintained at 37°C in an environment of 5% CO₂.

2.3.2 Cell motility and invasion assays

Cell motility and invasion assays were performed as previously described [133]. Briefly, the ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) were diluted to a cell concentration of 1 × 10⁶ cells per ml and labeled with calcein-AM (1 µg/ml, Life Technologies, VIC, Australia). Ovarian cancer cells were mixed at room temperature for 1 hour in the dark on an oscillating platform. Ovarian cancer cells (50 µl, 40,000 cells) were loaded on top of uncoated 12 µm filter inserts (Disposable, 96-well plate, ChemoTx, Neuro Probe Inc, Gaitherburg, MD, USA) for motility assays or 12 µm filters coated with Geltrex (0.6 µl per well, 9 mg/ml, Life Technologies) for invasion assays. 10% FBS RPMI media was used as a chemo attractant. The cells were allowed to migrate and invade to the lower chamber for 6 hours. Non-migratory cells on the top of the filter were removed and the fluorescence was measured at 485-520 nm.

2.3.3 Chick chorioallantoic membrane assay (CAM Assay)

Fertilized white leghorn chicken eggs were obtained from Hi-Chick, South Australia, Australia. Eggs were incubated in a MultiQuip Incubator (E2) at 37°C with 60% humidity. Ethics approval was obtained by the University of Adelaide Animal Ethics Committee. A small window was made in the shell on day 3 of chick embryo development under aseptic conditions. The window was resealed with adhesive tape and eggs were returned to the incubator until day 11 of chick embryo development. On day 11, OVCAR-3, SKOV-3 and OV-90 cell suspensions (9 × 10⁵) were mixed with growth factor reduced matrigel (8.9 mg/ml, BD Biosciences, NSW, Australia) in a total volume of 30 µl. Control anti-mouse IgG (20 µg/ml) (Sigma Aldrich) and neutralizing antibody to protein A (20 µg/ml) (BD Biosciences) were mixed together with the OV-90 cancer cells and matrigel. Matrigel grafts were placed on top of the CAM and eggs were resealed and returned to the
incubator for 72 hours until day 14 (n=6 chicken embryos per cell line). Matrigel grafts with surrounding CAM were harvested from each embryo and fixed with 4% paraformaldehyde for 24 hours and embedded in paraffin. Serial sections (6 µm) were stained with haematoxylin and eosin. Slides were digitally scanned using the NanoZoomer (Hamamatsu Photonics K.K., Japan).

2.3.4 Immunohistochemistry
CAM paraffin sections (6 µm) were incubated on a heat plate at 60°C for 2 hours. Tissue sections were dewaxed with xylene and ethanol, followed by PBS washes. Antigen retrieval was performed by using 1% protease (Sigma-Aldrich) for 10 minutes on a heat plate at 37°C. The endogenous peroxidase activity of the sections was quenched with 0.3% H₂O₂. Each tissue sample was blocked with 5% goat serum for 30 minutes before incubation with monoclonal mouse anti-human cytokeratin clone AE1/AE3 (1/50, Dako, VIC, Australia) at 4°C overnight. Subsequently, the tissues sections were incubated sequentially with biotinylated goat anti-mouse (1/400, Dako), followed by streptavidin-HRP conjugated (1/500, Dako) for 1 hour at room temperature. Immunoreactivity was detected using diaminobenzidine/H₂O₂ substrate (Sigma-Aldrich). The sections were counterstained with 10% haematoxylin (Sigma-Aldrich), dehydrated and mounted in Pertex (Medite Medizintechnik, Germany). Slides were digitally scanned using the NanoZoomer (Hamamatsu Photonics K.K.). For quantitative analysis of ovarian cancer cell invasion into the mesoderm layer, 8 to 12 CAM images from each embryo were assessed by two independent researchers [127].

2.3.5 Statistical analysis
All analyses were performed using SPSS 15.0 for Windows software (SPSS, Chicago, IL, USA). The student’s t-test and one-way ANOVA were used to determine statistical significance of ovarian cancer cell motility and invasion in vitro, and ovarian cancer cell invasion in the CAM model. Statistical significance was accepted at P < 0.05.
2.4 Results

2.4.1 Human ovarian cancer cell motility and invasion in vitro
We compared the motility and invasion of three ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) using *in vitro* assays. We found OV-90 cells to be most invasive through an extracellular matrix and migrated faster through 12 μm pores towards a chemo attractant, compared to SKOV-3 and OVCAR-3 cells (Figure 2.1). OVCAR-3 cells were the least motile and invasive cell line in our study.

2.4.2 Human ovarian cancer cell invasion into the chick chorioallantoic membrane (CAM)
We initially utilized an *ex ovo* method and incubated the chick embryos in plastic weigh boats as described previously [134]. The *ex ovo* method has the advantage of allowing the application of a larger number of matrigel grafts as a wider area of the CAM is accessible. However, the survival rate for the *ex ovo* method was very low and only 10% of embryos survived to day 14. The *in ovo* method had a survival rate of 70% on day 14. In the *in ovo* method, a small window is made in the shell on day 3 of chick embryo development to detach the CAM layer from the egg shell (Figure 2.2a). Ovarian cancer cells (9 x 10^5 cells) were mixed with matrigel to form a gel and grafted on top of the CAM of day 11 chick embryos. The chick embryos were incubated with the matrigel grafts until day 14 of development. An example of a matrigel graft on day 14 is shown in Figure 2.2b. The CAM layers; ectoderm (ET), mesoderm (M) and endoderm (ED) can be seen in Figure 2.3a. Cytokeratin immunohistochemistry was used to identify the CAM layer integrity and presence of ovarian cancer cells in the mesodermal layer.

The invasion of the ovarian cancer cells through the ectoderm into the mesoderm was assessed on day 14 of chick embryo development. OVCAR-3 cytokeratin immunohistochemistry showed some damage to the ectoderm layer but minimal invasion into the CAM mesoderm (Figure 2.3b). The SKOV-3 cells showed invasion into the mesoderm layer and minimal destruction of the ectoderm layer (Figure 2.3c). OV-90 cells were the most invasive cells in the CAM assay (Figure 2.3d), which agrees with the results of the *in vitro* assays (Figure 2.1). Figure 2.3d shows a large number of OV-90 cells invading into the mesoderm of the CAM, as well as the destruction of the CAM ectoderm layer.

Quantitative analysis of ovarian cancer cell invasion into the CAM was performed by determining the number of images (8 to 12 sections per chick embryo) with cancer cell invasion into the CAM.
mesoderm of day 14 chick embryos. Our results showed a significantly higher invasion of OV-90 and SKOV-3 cells into the CAM mesoderm, when compared with OVCAR-3 cells (Figure 2.4).

2.4.3 The effects of protein A neutralizing antibody on OV-90 cancer cell invasion into the CAM

We used a neutralizing antibody against one of the novel proteins identified in our previous study that investigated ovarian cancer-peritoneal cell interactions [92, 132]. OV-90 cancer cells (9 x 10^5 cells) were mixed with matrigel and the control anti-mouse IgG or the neutralizing antibody against protein A before grafting onto day 11 chick embryos. Neutralizing antibody against protein A effectively inhibited OV-90 cancer cell invasion into the mesoderm of the CAM, compared with the control anti-mouse IgG, where OV-90 cancer cells invaded the mesoderm of the CAM and a destruction of the ectoderm layer was observed (Figure 2.5).
Figure 2.1 Motility and invasion of human ovarian cancer cell lines (OVCAR-3, SKOV-3 and OV-90) in vitro.

The fluorescence reading represents the cancer cells that have migrated through the pores or invaded through the extracellular matrix (Geltrex). Data represents the mean ± SEM from two independent experiments performed in quadruplicate. (a) Indicates significant difference from OVCAR-3 cells; and (b) indicates significant difference from SKOV-3 cells, $P < 0.05$. 
Figure 2.2 Chick embryo development.

A. Day 3 chick embryo  
B. Ovarian cancer cells and matrigel graft on the chick chorioallantoic membrane (CAM) on day 14.
Figure 2.3 Invasion of ovarian cancer cells in the chick chorioallantoic membrane (CAM).

A. Control showing the normal structure of the CAM layers; ectoderm (ET), mesoderm (M) and endoderm (ED); OVCAR-3 (B); SKOV-3 (C); and OV-90 (D) cancer cell matrigel grafts (CM) were placed on top of the ectoderm layer and cancer cell invasion into the CAM mesoderm was assessed in day 14 chick embryos. CAM paraffin sections (6 µm) were immunostained with cytokeratin antibody. Original magnification: x200. Magnification bar = 100 µm for all images.
Figure 2.4 Chick chorioallantoic membrane (CAM) invasion by ovarian cancer cells in day 14 chick embryos.

Data generated from 48–60 images from 6 chicken embryos per cell line. Data represents the percentage of images with invasion into the mesoderm, mean ± SEM from two independent experiments. (a) Indicates significant difference from OVCAR-3 cells; and (b) indicates significant difference from SKOV-3 cells, $P < 0.05$. 
Figure 2.5 Effects of protein A neutralizing antibody on OV-90 cancer cell invasion into the chick chorioallantoic membrane (CAM).

OV-90 cancer cells were mixed with matrigel and (A) control anti-mouse IgG (20 µg/ml); or (B) neutralizing antibody against protein A (20 µg/ml). CAM paraffin sections (6 µm) were immunostained with a pan-cytokeratin antibody. ET = ectoderm. M = mesoderm. ED = endoderm. CM = cancer cell matrigel grafts. Original magnification: x200. Magnification bar = 100 µm for all images.
2.5 Discussion

The CAM assay is a frequently applied model to study ovarian cancer angiogenesis [135-138]. However, there is only one study which has used CAM assays to assess ovarian cancer cell invasion and metastasis [131]. Chang et al. described IGROV-1 ovarian cancer cell invasion and metastasis to the posterior CAM and lungs of chick embryos [131]. We have developed a CAM assay protocol to monitor the metastatic properties of ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) and have successfully used it to study the effect of newly identified molecules in vivo. Our results show that the CAM assay is an effective model to study ovarian cancer metastasis. Importantly, our CAM model closely mimics the mode of ovarian cancer metastasis which involves ovarian cancer cell attachment and invasion into the peritoneum. The ectodermal layer of the CAM has many similarities with the peritoneum, which consists of a single layer of mesothelial cells covering the organs in the abdominal cavity.

We observed a higher survival rate with the in ovo method in comparison to the ex ovo method for monitoring of ovarian cancer cell growth in the chick embryos. Various methods have been used to graft cancer cells in the CAM model; such as collagen onplants [134], plastic rings [130], and matrigel grafts [139]. Furthermore, cancer cells can also be inoculated by dropping the cell suspension on top of the CAM [140], or administered intravenously to study metastasis of cancer cells in the chick embryos [129]. Matrigel is one of the most suitable scaffolds used for implantation and grafting of cancer cells onto the CAM. In our model, ovarian cancer cells and matrigel were mixed with or without neutralizing antibodies before grafting onto the CAM of the chick embryos to assess ovarian cancer cell invasion. The grafting of the matrigel in the CAM model allows continuous visualization of the test site. Moreover, other studies have reported visible and solid tumours on the CAM of chick embryos a few days after cancer cell inoculation [122, 130]. We used histological assessment by means of a pan-cytokeratin antibody, to allow the visualization of cancer cells invading into the mesoderm.

The CAM model has been previously employed to assess cancer metastasis [114, 140]. In some studies quantitative alu PCR was used to assess the presence of metastatic human cancer cells in chick embryo organs [141]. Several studies have compared both CAM assays and mouse models to assess tumour growth and metastasis. Colorectal cancer cells were reported to colonize the CAM similarly to the mouse model [121]. Strojnik et al. conducted a study to compare the histological and immunohistochemical characteristics of glioma tumour protein expression in the CAM and an established rat model. They reported a similar profile of proteins expressed in both
models [123]. In addition, the CAM model has also been used concurrently with the nude mice model to assess tumour growth of fibrosarcoma (HT1080 cells) and human squamous carcinoma (Hep3 cells) cells [142]. Lyu et al. also showed that over expression of urokinase-type plasminogen activator receptor (u-PAR) in Hep3 cells leads to an increase in cancer cell invasion in the CAM model as well as accelerated tumour growth in the SCID mice model [143]. These studies demonstrate the validity of the CAM model for in vivo analysis of cancer cell invasion and metastasis.

The CAM model has many advantages. It is cost effective, allows large scale screening and is an easily reproducible in vivo model [115, 118]. A comparison of the advantages and limitations of the CAM against the mouse model are summarized in Table 2.1.

The CAM model has also been used in pre-clinical screening to assess the efficacy of drugs and inhibitors on tumour growth. Hagedorn et al. reported that treatment of human glioma cells with receptor tyrosine kinase inhibitors inhibited tumour growth in a CAM model [125]. Bekes et al. demonstrated that treatment of prostate cancer (PC3 cells) with u-PA activation blocking antibody mAb-112 significantly inhibited cancer cell invasion in the CAM model [144]. Additionally, the CAM model has been used to test the efficacy of chemotherapy agents (such as doxorubicin) in human leukemia cell lines and has been shown to reduce cancer cell growth in the CAM [129]. An important limitation of the CAM model is the inability to assess cancer–immune cell interactions. Examination of cancer-immune cell interactions requires the use of transgenic ovarian cancer models [145], however, these models are not widely available, are not suitable for high throughput screening, and cannot be used with primary ovarian cancer cells derived from clinical samples.

The CAM assay is therefore an attractive model to rapidly assess the effectiveness of novel candidate therapeutic drugs and the in vivo inhibition of specific tumour types and subtypes in one consistent model.

We have shown that OV-90 ovarian cancer cells invade into the mesoderm of the CAM within three days of implantation, therefore making the OV-90 CAM model ideal for studying ovarian cancer invasion and metastasis. In contrast, the OVCAR-3 cells showed limited invasion in the CAM over the three days of our assays, and would therefore be suitable for studying the role of molecules that promote ovarian cancer invasion. In conclusion, the CAM model provides a high throughput in vivo model for the assessment and evaluation of candidate pro-invasive molecules as well as potential therapeutic targets for ovarian cancer.
Table 2.1 Comparison of the advantages and limitations of the chick chorioallantoic membrane (CAM) and mouse model.

<table>
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<tr>
<th>In vivo Model</th>
<th>Advantages</th>
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<th>Limitations</th>
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<tr>
<td>CAM</td>
<td>Short experimental assay (days)</td>
<td>Short observation period (days)</td>
<td>Cannot examine cancer-immune cell interactions</td>
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<td></td>
<td>Inexpensive</td>
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<td>Easily reproducible and high throughput</td>
<td>Rapid morphological changes</td>
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<td></td>
<td>Closed system - allows assessment of small quantities of therapeutic agents</td>
<td>Limited antibodies to chicken tissues for characterization</td>
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<td>Naturally immunodeficient</td>
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<td>Multiple tests per individual CAM</td>
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<td>Allows large scale screening</td>
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<td>Biology and physiology well known</td>
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<td>Availability of <em>in vivo</em> imaging</td>
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<td>Allows direct visualization</td>
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<td>Animals do not have to be restrained</td>
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<td>Can be used with primary human cell lines</td>
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<tr>
<td>Mouse</td>
<td>Longer observation period (weeks to months)</td>
<td>Long experimental length (months to years)</td>
<td>Costly</td>
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<td>Mature immune system</td>
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<td>Availability of <em>in vivo</em> imaging</td>
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<td>Reproducibility is expensive</td>
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<td>Defined genetic background</td>
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<td>Large number of animals required</td>
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<td></td>
<td>Animals have to be restrained</td>
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2.6 Conclusions
The CAM assay is a robust technique that can be used to monitor invasion of ovarian cancer cell lines and to assess the role of novel molecules and potential therapeutic targets. It is a valuable alternative to murine in vivo models for the study of ovarian cancer invasion and metastasis.

2.7 Acknowledgments
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Chapter 3: Annexin A2 is regulated by Ovarian Cancer-Peritoneal Cell Interactions and Promotes Metastasis

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Chapter 3: Annexin A2 is regulated by Ovarian Cancer-Peritoneal Cell Interactions and Promotes Metastasis

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Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Performed analysis on all samples presented, interpreted the data and wrote the manuscript.

Certification that the statement of contribution is accurate

Signed: …………………………………………………………………………Date:…………………..

Alison S.F. Elder (co-author)
Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Performed the intraperitoneal xenograft mice study, interpreted the data and manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: …………………………………………………………………………Date:…………………..
Miranda P. Ween (co-author)
Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Manuscript evaluation

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: ……………………………………………………………………………………………Date:…………………………

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Supervised development of work and manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Martin K. Oehler (co-author)
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Supervised development of work and manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed: …………………………………………………………………………………Date:……………………
Carmela Ricciardelli (co-author)
Statement of contribution (in terms of conceptualization of the work, its realization and its documentation)

Supervised development of work, interpreted the data, manuscript evaluation and acted as corresponding author.

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Chapter 4: Annexin A2 as a Diagnostic Marker for Serous Ovarian Cancer

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Manuscript in Preparation
4.1 Abstract

Up to 70% of ovarian cancers are diagnosed at advanced stage due to unavailable screening methods and limited biomarkers to detect early stage disease. Our group recently identified annexin A2 to be secreted in the conditioned media of co-cultured ovarian cancer and peritoneal cells in vitro. Annexin A2, a calcium phospholipid binding protein plays an important role in serous ovarian cancer metastasis. This study examined the value of annexin A2 as a diagnostic biomarker in serous ovarian cancer. Annexin A2 levels were measured using an ELISA in urine samples from women with normal ovaries (n=6) and benign ovarian tumours (n=19) and serous ovarian cancer (n=36) and in blood samples from women with normal ovaries (n=27) and benign ovarian tumours (n=30) and serous ovarian cancer patients (n=86). ROC curves were generated with annexin A2 alone and in combination with the ovarian cancer marker, CA125. Annexin A2 and CA125 levels were also analyzed together using logistic regression models. There was no difference in urine annexin A2 levels of serous ovarian cancer patients compared with controls. However, blood annexin A2 levels were increased in early stage and advanced stage ovarian cancers compared with controls (P = 0.0001). Using a cutoff point of 300 ng/ml for annexin A2 and the established cutoff point of 35 U/ml for CA125, we observed an improved sensitivity of 100% and specificity of 70% when annexin A2 and CA125 measurements were combined in the controls and early stage ovarian cancer samples. By logistic regression analysis in the early stage and benign ovarian tumours, an AUC of 0.857 (95% CI: 0.753-0.960) was observed when annexin A2 and CA125 measurements combined and an improved sensitivity of 56.5% compared to either annexin A2 (21.7%) or CA125 (21.7%) alone at the 95% specificity level. Annexin A2 has potential as a useful biomarker for serous ovarian cancer diagnosis that warrants further investigation.

Keywords: Annexin A2, CA125, ovarian cancer, diagnosis, biomarker
4.2 Introduction

Epithelial ovarian cancer is the leading cause of death from gynaecological cancer and each year it is responsible for 120,000 deaths worldwide [148]. The majority of epithelial ovarian cancers are of serous histology, followed by clear cell, endometrioid, mucinous and mixed cell types [163]. The International Federation of Gynaecologist and Obstetricians (FIGO) characterized the staging of ovarian cancer as stage I - growth limited to the ovaries, stage II - growth involving one or both ovaries with pelvic extension, stage III - tumour growth with peritoneal implants in the upper abdomen and stage IV - distant metastasis to organs such as the liver, lung or brain [164]. Despite advances in surgery and in chemotherapies, no significant improvement in ovarian cancer survival has been observed over the last two decades [165]. Up to 70% of ovarian cancer patients are diagnosed at advanced stage and to date, there is no effective early detection test for ovarian cancer.

CA125 is widely used as a diagnostic tool and for follow up after ovarian cancer treatment [166]. However, while CA125 is elevated in 70% advanced stage disease, it is elevated in less than 50% of early stage ovarian cancer [167] and also increased in patients with benign ovarian lesions [168, 169]. Consequently, CA125 is not a useful tumour marker for early detection of ovarian cancer.

The diagnostic performance of an early diagnostic test is based on the specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) in detecting the disease [170]. The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial reported that annual screening with CA125 and transvaginal ultrasonography (TVU) compared with standard usual treatment care has no benefit on ovarian cancer mortality rate [171]. Combinations of serum biomarkers which have a high specificity and sensitivity are required for early detection of ovarian cancer and to improve ovarian cancer survival rate.

Our group utilized a proteomic approach to explore the molecules modulated in ovarian cancer and peritoneal cells interactions. From 2D gel electrophoresis and mass spectrometry, we identified novel molecules modulated in the secretome of co-cultured ovarian cancer and peritoneal cells in vitro [92] and one of the proteins was annexin A2 [132]. Annexin A2 is a calcium phospholipid binding protein that exists on the cell surface of various tumour cells [5]. We recently reported that annexin A2 plays an important role in ovarian cancer metastasis (Chapter 3) [172]. Several studies have recently investigated the potential use of annexin A2 as a biomarker. Serum annexin A2 levels was found to be elevated in hepatocellular carcinoma [45, 158, 173], breast cancer [174]...
and multiple myeloma [175]. However, to date, no studies have investigated the potential of using annexin A2 for the diagnosis of ovarian cancer.

In this study, we assessed blood annexin A2 levels in serous ovarian cancers and controls and compared its diagnostic efficacy with CA125. We utilized two established methods to evaluate the efficacy of annexin A2 and CA125 in our patient cohort; i) optimal cutoff values determined by the highest accuracy from the ROC curve and ii) logistic regression model combining annexin A2 and CA125 measurements. The diagnostic performances of either annexin A2 or CA125 alone and in combination were compared in non-cancer controls and serous ovarian cancer patients. We also compared the efficacy of the both biomarkers in benign ovarian tumours versus early stage and advanced stage serous ovarian cancers.
4.3 Materials and methods

4.3.1 Urine, serum and plasma samples

Urine samples were collected from patients with normal ovaries (n=6), patients with benign ovarian tumours (n=19), and serous ovarian cancer patients at time of diagnosis (n=36). Urine samples were centrifuged at 3000 rpm for 10 minutes at room temperature and stored at -80°C until assayed. The clinicopathological characteristics for urine cohorts are detailed in Supplementary Table 4.1. Serum samples were collected from patients with normal ovaries undergoing surgery for benign gynaecological conditions (n=27), patients with benign ovarian tumours (n=30), early stage serous ovarian cancer patients at time of diagnosis (stage I and II) (n=13) and advanced stage serous ovarian cancer patients at time of diagnosis (stage III and IV) (n=63). Blood samples were collected into vacutainer plain tubes, the blood was allowed to clot, centrifuged at 3000 rpm for 10 minutes at room temperature and serum was stored at -80°C until assayed. An additional 10 early stage serous ovarian cancer plasma samples were obtained from Prince Henry’s Institute. All samples were collected with approval from the Royal Adelaide Hospital or Prince Henry’s Institute Human Ethics Committees and written consent was obtained from all patients involved in this study. The clinicopathological characteristics for the serum and plasma cohorts are detailed in Supplementary Table 4.2.

4.3.2 Urine sample preparation for western blot and annexin A2 ELISA

For normalization of the urine samples, urine creatinine values were measured using Hitachi 912 Analysis with a CREA kit (Roche Diagnostics GmbH, Germany). Urine samples were normalized to creatinine values in Tris-buffered saline (0.5 M Tris and 1.5 M sodium chloride, pH 7.4) with the presence of protease inhibitors (Sigma-Aldrich, MO, USA) in a total volume of 2 ml and precipitated using 4-fold volume of cold acetone overnight at -20°C, then spun at 1500 rpm for 5 minutes. The protein pellet was washed twice with 80% cold acetone, dissolved in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M sodium chloride, 50 mM Tris-HCl and 1 mM EDTA, pH 8.0) for western immunoblotting and in 200 μl of PBS (with 0.1% Nonidet P-40, pH 7.4) and sonicated for 30 minutes (Soniclean Pty. Ltd., SA, Australia) before assayed for annexin A2 ELISA.

4.3.3 1D-western immunoblotting

Protein concentration of the urine samples was determined using the Bradford assay (Bio-Rad) and samples were stored at -20°C until assayed. Western immunoblotting was conducted as
previously described [92] with a mouse monoclonal antibody to annexin A2 (1/2000, Clone 5, BD Biosciences) and anti-mouse IgG peroxidase-conjugated secondary antibodies (1/2000, Dako), visualized by enhanced chemiluminescence and autoradiography (ECL Hyperfilm, GE Healthcare).

4.3.4 Annexin A2 ELISA and detection of CA125
Annexin A2 levels were measured using a commercial human annexin A2 ELISA kit as per the manufacturer's instructions (USCN Life Science Inc., Wuhan, China). Briefly, 100 μl of standards, urine samples and 10-fold dilutions of serum or plasma samples in PBS (pH 7.0) were added into each 96 well in duplicates. The absorbance was measured at 450 nm using the Triad series multimode detector (Dynex technologies, VA, USA). The detection limit of this assay was 0.321 ng/ml and the intra assay and inter assay coefficient was 6.1% and 3.3% respectively. CA125 measurements in the serum were determined using the Siemens Advia Centaur XP automated analyzer by the Institute of Medical Veterinary Science (IMVS) (SA Pathology, Adelaide, SA, Australia).

4.3.5 Statistical analyses
Statistical analyses were performed using SPSS for Windows (Version 20.0, SPSS Inc., Chicago, IL, USA), GraphPad Prism for Windows (Version 5.0, La Jolla, CA, USA) and SigmaPlot for Windows (Version 12.5, Systat Software Inc., Chicago, IL, USA). Receiver operating characteristics (ROC) curve were performed for annexin A2 and CA125 measurements with the controls and serous ovarian cancers to obtain the optimal cutoff values. The highest Youden index \( J \) calculated from equation, \( J = (\text{sensitivity} + \text{specificity} - 1) \) was used to calculate the annexin A2 cutoff point. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated as proportion of true positive and true negative patients. The blood measurements for annexin A2 and CA125 were log (natural) transformed due to skewness in the logistic regression analysis and the predictive probability values for annexin A2 and CA125 alone and combined annexin A2 and CA125 were used to perform the ROC curve analyses. The statistical significance between the patient's diagnosis groups was determined with Kruskal-Wallis test and Dunnett C post-hoc test. Spearman's correlation test was used to determine the correlation between blood CA125 and annexin A2 levels. SigmaPlot was used to compare area under the curve (AUC) of the ROC curves for annexin A2 and CA125. Statistical significance was accepted at \( P < 0.05 \).
4.4 Results

4.4.1 Level of annexin A2 in the urine of ovarian cancer patients
Urine annexin A2 level of serous ovarian cancer patients (P1 to P4) and benign ovarian tumour patients (P5 to P8) was examined by western immunoblotting after normalization to urine creatinine measurements (Figure 4.1a). Our findings showed strong annexin A2 bands at 37 kDa in the urine of ovarian cancer patients (P1 and P2) and weak annexin A2 bands for P3 and P4. However, we did not observe any annexin A2 bands at 37 kDa in the urine of benign ovarian tumour patients (P5 to P8). Figure 4.1b shows annexin A2 measurements in the urine of patients with normal ovaries, benign ovarian tumours and serous ovarian cancers using an annexin A2 ELISA. We did not observe any significant difference in the annexin A2 levels in the urine of serous ovarian cancer patients compared with the patient’s with benign ovarian tumours or normal ovaries ($P = 0.521$, Kruskal-Wallis test). Moreover, we also observed no significant difference in urine annexin A2 levels between the early stage (stage I and II) and advanced stage (stage III and IV) ovarian cancer patients ($P = 0.711$, Kruskal-Wallis test). These findings indicate that urine annexin A2 levels cannot be used to diagnose serous ovarian cancer.

4.4.2 Blood annexin A2 levels are elevated in serous ovarian cancer patients compared with non-cancer controls
Blood annexin A2 levels were significantly increased in serous ovarian cancer patients (stage I-IV) compared with patients with normal ovaries and benign ovarian tumours (Figure 4.2a, $P < 0.0001$). However, no significant difference in annexin A2 levels was observed between advanced stage ovarian cancers patients compared with patients with benign ovarian tumours. We also observed that annexin A2 levels were significantly increased in stage I ovarian cancer patients compared to those with stage III and IV ovarian cancers (Figure 4.2c, $P = 0.0001$). We performed a ROC curve of the non-cancer controls and serous ovarian cancers to determine the optimal cutoff value for annexin A2 (Figure 4.2e, AUC=0.670, $P = 0.001$, 95% CI: 0.579-0.760) and the best cutoff point, 300 ng/ml had a sensitivity of 51.2% and a specificity of 78.9%.

4.4.3 CA125 levels in serous ovarian cancer patients
The established tumour marker for ovarian cancer, CA125 levels was significantly increased in serous ovarian cancer patients (stage I-IV) compared to patients with normal ovaries and benign ovarian tumours (Figure 4.2b, $P < 0.0001$). Moreover, a significant increase in CA125 levels was observed in stage III ovarian cancer patients compared with stage I ovarian cancer patients (Figure
4.2d, \( P = 0.0132 \)). Similar to annexin A2, we performed a ROC curve of the non-cancer controls and serous ovarian cancers. Using the established cutoff value for CA125, of 35 U/ml, we observed a sensitivity of 88.4\% and specificity of 89.5\% (Figure 4.2f, AUC=0.925, \( P < 0.0001 \), 95\% CI: 0.879-0.972).

**4.4.4 Annexin A2 levels are weakly correlated with CA125 and able to distinguish serous ovarian cancer patients with normal CA125 levels from non-cancer controls**

We observed a weak correlation between annexin A2 and CA125 levels (Figure 4.3a, \( r = 0.180, P = 0.032 \), Spearman’s correlation test). Annexin A2 was able to distinguish between the serous ovarian cancers with normal (<35 U/ml) CA125 levels and non-cancer controls (Figure 4.3b, AUC: 0.803, \( P = 0.004 \), 95\% CI: 0.674-0.932). Moreover, annexin A2 was also able to distinguish between the serous ovarian cancers with normal CA125 levels compared with benign ovarian tumours (Figure 4.3c, AUC: 0.730, \( P = 0.039 \), 95\% CI: 0.560-0.899).

**4.4.5 Annexin A2 and CA125 diagnostic performance in ovarian cancer patients compared with non-cancer controls using specified cut points**

Using the optimal cutoff values for annexin A2 and CA125, 300 ng/ml and 35 U/ml respectively, we determined the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for elevated annexin A2 (\( \geq 300 \) ng/ml) or elevated CA125 alone (\( \geq 35 \) U/ml) and either elevated annexin A2 or elevated CA125 (Table 4.1). We observed an improvement in the sensitivity of detecting ovarian cancer when annexin A2 and CA125 were combined (98\%) compared with either annexin A2 (52\%) or CA125 (90\%) alone, but no improvement in the specificity. Moreover, we showed no improvement in the PPV when the markers were combined (83\%) but a better NPV (95\%) compared with annexin A2 (52\%) or CA125 (85\%) alone (Table 4.1a). We also compared blood annexin A2 and CA125 levels in the non-cancer controls versus early stage ovarian cancers and observed a sensitivity of 100\% by combining annexin A2 or CA125 (Table 4.1b). We showed an NPV of 100\%, but no improvement in the specificity or PPV when annexin A2 and CA125 measurements were combined (Table 4.1b). Moreover, we also compared the controls with the advanced stage ovarian cancers and observed a small increase in the sensitivity of detecting ovarian cancer by combining annexin A2 and CA125 (97\%) compared with annexin A2 (40\%) and CA125 (95\%) alone. However, we showed no improvement in the specificity or PPV by combining annexin A2 and CA125 measurements but a small improvement in the NPV (Table 4.1c).
4.4.6 Annexin A2 and CA125 diagnostic performance in serous ovarian cancers compared with benign ovarian tumour using specified cutoff points

We also investigated whether annexin A2 could be a potential biomarker for diagnosing ovarian cancer in patients presenting with pelvic masses. Table 4.2 summarized the sensitivity, specificity, PPV and NPV of annexin A2 and CA125 in benign ovarian tumours compared with serous ovarian cancers. We observed an improved sensitivity by combining annexin A2 and CA125 measurements (98%) compared with annexin A2 (52%) or CA125 (90%) alone, but no improvements in the specificity or PPV. The NPV (89%) was also improved when annexin A2 and CA125 measurements were combined compared to annexin A2 alone (34%) and CA125 alone (73%) (Table 4.2a). When we compared benign ovarian tumours only with early stage ovarian cancers, the sensitivity and NPV of combining annexin A2 and CA125 measured was increased to 100%. We showed no improvement in the specificity or PPV by combining annexin A2 and CA125 measurements (Table 4.2b). In comparison, only a small improvement in sensitivity was observed when annexin A2 and CA125 measurements were combined (97%) in the benign ovarian tumours and advanced stage serous ovarian cancer. However, there was no improvement in the specificity, PPV or NPV of the combine measurements compared with the individual markers alone (Table 4.2c). In summary, we have demonstrated that combining blood annexin A2 and CA125 can improve the sensitivity but not the specificity in discriminating benign ovarian tumours from serous ovarian cancer in patients with ovarian lesions.

4.4.7 Logistic regression model to evaluate the efficacy of combining annexin A2 and CA125 measurements in ovarian cancer patients and non-cancer controls

The blood annexin A2 and CA125 levels were log transformed, for the logistic regression model. The ROC curve analysis with non-cancer controls and all ovarian cancer patients (stage I-IV) is shown in Figure 4.4a. The AUC was slightly improved when annexin A2 and CA125 values were combined (AUC=0.94, \(P < 0.0001\), 95% CI: 0.900-0.979) compared with annexin A2 alone (AUC=0.67, \(P = 0.001\), 95% CI: 0.579-0.760) or CA125 alone (AUC=0.93, \(P < 0.0001\), 95% CI: 0.879-0.972). At the 95% specificity level, the combined annexin A2 and CA125 measurements resulted in a sensitivity of 61.6% compared with annexin A2 (17.4%) or CA125 (48.8%) alone. At the 98% specificity level, the sensitivity of combining annexin A2 and CA125 was 44.2% (Table 4.3a).

Non-cancer controls were also analyzed with only the early stage ovarian cancers (Figure 4.4b). ROC curve analysis showed an improved AUC when both annexin A2 and CA125 were combined
We observed an improvement in the sensitivity of detecting early stage ovarian cancer at the 95% specificity level when annexin A2 and CA125 were combined (56.5%) compared with annexin A2 (47.8%) or CA125 (26.1%) alone. The sensitivity at the 98% specificity was also increased to 52.2% when annexin A2 and CA125 measurements were combined (Table 4.3b).

Figure 4.4c showed the ROC curve of the controls and advanced stage ovarian cancer. We observed a slightly improved AUC when annexin A2 and CA125 measurements were combined (AUC=0.96, P < 0.0001, 95% CI: 0.920-0.991) compared with either annexin A2 (AUC=0.61, P < 0.043, 95% CI: 0.505-0.709) or CA125 (AUC=0.95, P < 0.0001, 95% CI: 0.916-0.989) alone. The sensitivity at the 95% specificity was only slightly increased when annexin A2 and CA125 measurements were combined but there was no improvement at the 98% specificity level (Table 4.3c). This study has shown that combining annexin A2 and CA125 measurements using logistic regression can improve the sensitivity of detecting ovarian cancer.

4.4.8 Logistic regression model to evaluate the efficacy of combining annexin A2 and CA125 measurements in ovarian cancer patients versus patients with benign ovarian tumours

We also utilized logistic regression model and ROC curve analysis to combine annexin A2 and CA125 measurements in patients detected with ovarian cancer (stage I to IV) and benign ovarian tumours alone (Figure 4.4d). From the ROC curve, we observed a slightly increased AUC when annexin A2 and CA125 measurements were combined (AUC=0.894, P < 0.0001, 95% CI: 0.826-0.963) compared with annexin A2 (AUC=0.597, P = 0.115, 95% CI: 0.479-0.715) and CA125 (AUC=0.880, P < 0.0001, 95% CI: 0.805-0.955) alone. At the 90% specificity level, we observed an improvement in the sensitivity combining both biomarkers (61.6%) compared with either annexin A2 (22.1%) or CA125 (48.8%) alone. However, at the 95% specificity level, combining annexin A2 and CA125 did not improve the sensitivity (44.2%) compared to CA125 alone which was 45.3% (Table 4.4a). Figure 4.4e shows the ROC curve analyses for the benign ovarian tumours and early stage ovarian cancers. We showed an improved AUC when both biomarkers were combined (AUC=0.857, P < 0.0001, 95% CI: 0.753-0.960) compared to either annexin A2 (AUC=0.788, P < 0.0001, 95% CI: 0.661-0.919) or CA125 alone (AUC=0.790, P < 0.0001, 95% CI: 0.661-0.919). The sensitivity of detecting early stage ovarian cancer was improved at both the 90% and 95% specificity levels when both biomarkers were combined (Table 4.4b).
The ROC curve analysis comparing benign ovarian tumours with advanced stage ovarian cancers demonstrates a slightly improved AUC when both biomarkers were combined (AUC=0.917, $P < 0.0001$, 95% CI: 0.854-0.980) compared to annexin A2 (AUC=0.527, $P = 0.675$, 95% CI: 0.398-0.656) or CA125 (AUC=0.913, $P < 0.0001$, 95% CI: 0.848-0.978) alone (Figure 4.4f). The sensitivity of detecting ovarian cancer was slightly improved at both the 90% and 95% specificity level when annexin A2 and CA125 measurements were combined (Table 4.4c). In summary, we have shown that combining annexin A2 and CA125 measurements using logistic regression can improve the sensitivity of discriminating benign ovarian tumours from serous ovarian cancers and particularly early stage ovarian cancer.
Figure 4.1 Annexin A2 expression in controls and ovarian cancer patient’s urine.

A. Western immunoblotting shows annexin A2 band at 37 kDa in the urine of patients with advanced stage serous ovarian cancer (P1-P4) and low annexin A2 in the urine of patients with benign ovarian tumours (P5-P8). B. Urine annexin A2 levels measured from patients with normal ovaries (n=6), benign ovarian tumours (n=19) and serous ovarian cancers (n=36) by ELISA. The bars represent the median values for annexin A2 levels for normal ovaries (38.8 ng/ml, range: 15.2 - 45.3 ng/ml), benign ovarian tumours (26.7 ng/ml, range: 6.0 - 59.3 ng/ml) and advanced stage ovarian cancer (30.2 ng/ml, range: 12.6 - 175.5 ng/ml). (P = 0.521, Kruskal-Wallis test).
Figure 4.2 Annexin A2 and CA125 levels in normal ovaries, benign ovarian tumours and serous ovarian cancers.

Serum and plasma annexin A2 (A) and CA125 (B) levels measured from patients with normal ovaries (n=27), benign ovarian tumours (n=30), early stage serous ovarian cancers (n=23) and advanced stage serous ovarian cancers (n=63). The bars represent the median values for annexin A2 in the normal ovaries (240.7 ng/ml, range: 143.7 - 400.7 ng/ml), benign ovarian tumours (271.5 ng/ml, 137.3 - 562.8 ng/ml), early stage (400.5 ng/ml, range: 145.6 - 668.8 ng/ml) and advanced stage (275.6 ng/ml, range: 140.4 - 558.7 ng/ml). The bars represent median values for CA125 levels in the normal ovaries (10 U/ml, range: 5 - 26 U/ml), benign ovarian tumours (13 U/ml, range: 4 - 749 U/ml), early stage (122 U/ml, range: 6 - 2274 U/ml) and advanced stage (431 U/ml, range: 16 - 8456 U/ml). The comparison between the patient groups was performed by Kruskal-Wallis test and Dunnett C post-hoc test. * P < 0.0001. Annexin A2 (C) and CA125 (D) levels from patients with stage I (n=15), stage II (n=8), stage III (n=56) and stage IV (n=7) of serous ovarian cancers. The bars represents median values for annexin A2 in stage I (418.6 ng/ml, range: 255.7 - 668.8 ng/ml), stage II (334.4 ng/ml, range: 145.6 - 420.1 ng/ml), stage III (277.7 ng/ml, range: 152.4 - 558.7 ng/ml) and stage IV (274.5 ng/ml, range: 140.4 - 395.1 ng/ml). The bars represents median values for CA125 in stage I (94 U/ml, range: 9 - 2274 U/ml), stage II (126.5 U/ml, range: 6 - 1274 U/ml), stage III (417.5 U/ml, range: 16 - 8456 U/ml) and stage IV (758 U/ml, range: 17 - 2715 U/ml). The comparison between the patient groups was performed by Kruskal-Wallis test and Dunnett C post-hoc test. * P < 0.05. ROC curve and area under the curve (AUC) for annexin A2 (E) and CA125 (F) of controls (normal ovaries and benign ovarian tumours, n=57) and serous ovarian cancers (n=86).
Figure 4.3 Correlation of annexin A2 and CA125 levels.

A. Scatterplot of blood annexin A2 and CA125 correlation in control and ovarian cancer patients (n=143) (r = 0.180, P = 0.032, Spearman’s correlation test). Dotted lines represent the established cutoff for CA125 which is 35 U/ml. B. ROC curve for ovarian cancer patients with normal CA125 levels (<35 U/ml, n=9, AUC: 0.803, 95% CI: 0.674-0.932) and control cases (normal ovaries and benign ovarian tumours, n=57). C. ROC curve for ovarian cancer patients with normal CA125 levels (<35 U/ml, n=9, AUC: 0.730, 95% CI: 0.56-0.899) and benign ovarian tumours, n=30).
Figure 4.4 ROC curves from logistic regression analysis for annexin A2, CA125 and combined annexin A2 and CA125 measurements.

A. ROC curve and area under the curve (AUC) for non-cancer controls (normal ovaries and benign ovarian tumours, n=57) versus serous ovarian cancers (n=86). B. ROC curve and AUC for non-cancer controls (normal ovaries and benign ovarian tumours, n=57) versus early stage serous ovarian cancer (n=23). C. ROC curve and AUC for non-cancer controls (normal ovaries and benign ovarian tumours, n=57) versus advanced stage serous ovarian cancers (n=63). D. ROC curve and AUC for benign ovarian tumours (n=30) versus serous ovarian cancers (n=86). E. ROC curve and AUC for benign ovarian tumours (n=30) versus early stage serous ovarian cancer (n=23). F. ROC curve and AUC for benign ovarian tumours (n=30) versus advanced stage serous ovarian cancers (n=63).
Table 4.1 Comparison of annexin A2 and CA125 marker sensitivity, specificity, PPV and NPV for controls (normal ovaries and benign ovarian tumours) and serous ovarian cancers using specified cutoff points.

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<td>c) Controls (n=57) and advanced stage ovarian cancer (n=63)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin A2</td>
<td>≥300 ng/ml</td>
<td>40</td>
<td>79</td>
<td>68</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>CA125</td>
<td>≥35 U/ml</td>
<td>95</td>
<td>89</td>
<td>91</td>
<td>94</td>
<td>84</td>
</tr>
<tr>
<td>Annexin A2 or CA125</td>
<td>≥300 ng/ml or ≥35 U/ml</td>
<td>97</td>
<td>70</td>
<td>78</td>
<td>95</td>
<td>84</td>
</tr>
</tbody>
</table>
Table 4.2 Comparison of annexin A2 and CA125 markers sensitivity, specificity, PPV and NPV for benign ovarian tumours and serous ovarian cancers using specified cutoff points.

<table>
<thead>
<tr>
<th>a) Benign ovarian tumours (n=30) and serous ovarian cancers (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Annexin A2</td>
</tr>
<tr>
<td>CA125</td>
</tr>
<tr>
<td>Annexin A2 or CA125</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Benign ovarian tumours (n=30) and early stage ovarian cancer (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Annexin A2</td>
</tr>
<tr>
<td>CA125</td>
</tr>
<tr>
<td>Annexin A2 or CA125</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c) Benign ovarian tumours (n=30) and advanced stage ovarian cancer (n=63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Annexin A2</td>
</tr>
<tr>
<td>CA125</td>
</tr>
<tr>
<td>Annexin A2 or CA125</td>
</tr>
</tbody>
</table>
Table 4.3 Comparison of annexin A2 and CA125 markers in controls (normal ovaries and benign ovarian tumours) and serous ovarian cancers using logistic regression and ROC curve analyses.

### a) Controls (n=57) and serous ovarian cancers (n=86)

<table>
<thead>
<tr>
<th>Markers</th>
<th>AUC (95%CI)</th>
<th>P-value *</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>InlogAnnexin A2</td>
<td>0.670 (0.579-0.760)</td>
<td>&lt; 0.0001</td>
<td>17.4 (9)</td>
</tr>
<tr>
<td>InlogCA125</td>
<td>0.925 (0.879-0.972)</td>
<td>-</td>
<td>48.8 (45.3)</td>
</tr>
<tr>
<td>InlogAnnexin A2 and InlogCA125</td>
<td>0.939 (0.900-0.979)</td>
<td>0.6546</td>
<td>61.6 (44.2)</td>
</tr>
</tbody>
</table>

### b) Controls (n=57) and early stage ovarian cancer (n=23)

<table>
<thead>
<tr>
<th>Markers</th>
<th>AUC (95%CI)</th>
<th>P-value *</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>InlogAnnexin A2</td>
<td>0.841 (0.740-0.943)</td>
<td>0.9001</td>
<td>47.8 (21.7)</td>
</tr>
<tr>
<td>InlogCA125</td>
<td>0.850 (0.750-0.951)</td>
<td>-</td>
<td>26.1 (21.7)</td>
</tr>
<tr>
<td>InlogAnnexin A2 and InlogCA125</td>
<td>0.908 (0.835-0.980)</td>
<td>0.3673</td>
<td>56.5 (52.2)</td>
</tr>
</tbody>
</table>

### c) Controls (n=57) and advanced stage ovarian cancer (n=63)

<table>
<thead>
<tr>
<th>Markers</th>
<th>AUC (95%CI)</th>
<th>P-value *</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>InlogAnnexin A2</td>
<td>0.607 (0.505-0.709)</td>
<td>&lt; 0.0001</td>
<td>6.3 (4.8)</td>
</tr>
<tr>
<td>InlogCA125</td>
<td>0.953 (0.916-0.989)</td>
<td>-</td>
<td>57.1 (54)</td>
</tr>
<tr>
<td>InlogAnnexin A2 and InlogCA125</td>
<td>0.955 (0.920-0.991)</td>
<td>0.9185</td>
<td>65.1 (54)</td>
</tr>
</tbody>
</table>

* p-value for comparison of ROC-AUC to CA125
Table 4.4 Comparison of annexin A2 and CA125 markers in benign ovarian tumours and serous ovarian cancers using logistic regression and ROC curve analyses.

<table>
<thead>
<tr>
<th>a) Benign ovarian tumours (n=30) and serous ovarian cancers (n=86)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
<td>AUC (95%CI)</td>
<td>P-value</td>
<td>Sensitivity (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90% specificity</td>
<td>95% specificity</td>
</tr>
<tr>
<td>lnlogAnnexin A2</td>
<td>0.597 (0.479-0.715)</td>
<td>&lt; 0.0001</td>
<td>22.1</td>
<td>9.3</td>
</tr>
<tr>
<td>lnlogCA125</td>
<td>0.880 (0.805-0.955)</td>
<td>-</td>
<td>48.8</td>
<td>45.3</td>
</tr>
<tr>
<td>lnlogAnnexin A2 and lnlogCA125</td>
<td>0.894 (0.826-0.963)</td>
<td>0.7988</td>
<td>61.6</td>
<td>44.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Benign ovarian tumours (n=30) and early stage ovarian cancer (n=23)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
<td>AUC (95%CI)</td>
<td>P-value</td>
<td>Sensitivity (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90% specificity</td>
<td>95% specificity</td>
</tr>
<tr>
<td>lnlogAnnexin A2</td>
<td>0.788 (0.661-0.916)</td>
<td>0.9685</td>
<td>52.2</td>
<td>21.7</td>
</tr>
<tr>
<td>lnlogCA125</td>
<td>0.790 (0.661-0.919)</td>
<td>-</td>
<td>26.1</td>
<td>21.7</td>
</tr>
<tr>
<td>lnlogAnnexin A2 and lnlogCA125</td>
<td>0.857 (0.753-0.960)</td>
<td>0.4409</td>
<td>56.5</td>
<td>56.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c) Benign ovarian tumours (n=30) and advanced stage ovarian cancer (n=63)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
<td>AUC (95%CI)</td>
<td>P-value</td>
<td>Sensitivity (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90% specificity</td>
<td>95% specificity</td>
</tr>
<tr>
<td>lnlogAnnexin A2</td>
<td>0.527 (0.398-0.656)</td>
<td>&lt; 0.0001</td>
<td>11.1</td>
<td>4.8</td>
</tr>
<tr>
<td>lnlogCA125</td>
<td>0.913 (0.848-0.978)</td>
<td>-</td>
<td>57.1</td>
<td>54</td>
</tr>
<tr>
<td>lnlogAnnexin A2 and lnlogCA125</td>
<td>0.917 (0.854-0.980)</td>
<td>0.9408</td>
<td>61.9</td>
<td>55.6</td>
</tr>
</tbody>
</table>

* P-value for comparison of ROC-AUC to CA125
4.5 Discussion
To the best of our knowledge, this is the first study that has investigated whether annexin A2 can be used as a diagnostic marker for serous ovarian cancer. In this study, we showed that i) blood levels of annexin A2 are increased in serous ovarian cancer compared with the patients with normal ovaries and benign ovarian tumours, ii) blood levels of annexin A2 are increased in early stage serous ovarian cancer compared with patients with benign ovarian tumours and iii) combining annexin A2 and CA125 measurements improves the sensitivity of detecting early stage serous ovarian cancer.

Urine is one of the sources for non-invasive biomarkers for cancer detection and we investigated whether annexin A2 level is elevated in the urine of ovarian cancer patients. Western immunoblotting showed elevated annexin A2 in advanced stage ovarian cancer patients compared to benign ovarian tumours. However, we showed using ELISA that annexin A2 urine levels were not elevated in serous ovarian cancers compared to non-cancerous controls. A previous proteomic study identified annexin A2 in the urine of pancreatic cancer patients but failed to validate annexin A2 expression by western immunoblotting. It was suggested that this was due to annexin A2 post-translational modifications in the urine samples [176]. Moreover, another reason for limited or no detection of annexin A2 may also be due to the degradation of proteins in urine samples as no protease inhibitors were added during sample collections [177]. As no protease inhibitors were added to our urine samples at the time of storage, degradation of annexin A2 may also have affected our measurements.

We then investigated whether blood levels of annexin A2 could be utilized as tumour biomarker in ovarian cancer. Several studies have investigated the clinical significance of annexin A2 as a diagnostic tool in cancer and evaluated blood annexin A2 levels in cancers of the liver [45, 158], breast [174] and multiple myeloma [175]. Annexin A2 was significantly elevated in the plasma of breast cancer patients [174] and in the bone marrow aspirate of multiple myeloma patients [175] compared with controls. Moreover, serum annexin A2 was also elevated in hepatocellular carcinoma (HCC) patients compared with normal individuals [45]. In a large cohort study (n=175), serum annexin A2 was significantly elevated in hepatocellular carcinoma patients compared with non-cancerous controls and cancers of the breast, lung, esophageal, gastric and colorectal [158]. We showed that blood levels of annexin A2 were elevated in early and advanced stage ovarian cancer patients compared with healthy controls and patients with benign ovarian tumours. We also observed increased blood annexin A2 levels in stage I ovarian cancers compared with advanced
ovarian cancer patients. The mechanism that results in a higher annexin A2 levels in the early stage serous ovarian cancers compared to advanced stage ovarian cancers is not known. Further investigations are required to examine whether blood annexin A2 levels can reflect the bulk of ovarian cancer disease and is reduced following debulking surgery and chemotherapy like CA125 levels and can be used to monitor disease progression. Previous studies reported annexin A2 having a role in chemoresistance in cancers of the breast [157, 178], pancreas [160, 179] and Lewis Lung Carcinoma [149]. It will be important to determine whether blood annexin A2 levels are also associated with chemoresistance in ovarian cancer.

To evaluate the efficacy of combining both annexin A2 and CA125 to detect ovarian cancer, we utilized both specified cutoffs and logistic regression models followed by ROC curve analysis [158, 180]. Using a cutoff point of 300 ng/ml for annexin A2 and the established cutoff point of 35 U/ml for CA125, we observed an improved sensitivity of 100% and a specificity of 70% when annexin A2 and CA125 measurements were combined in the controls and early stage ovarian cancer samples. Importantly, annexin A2 was also able to distinguish between the serous ovarian cancers with normal (<35 U/ml) CA125 levels and non-cancer controls. By logistic regression, we showed that at high specificity levels (95% and 98%), there was an improvement in the sensitivity of detecting early stage ovarian cancer when annexin A2 and CA125 measurements were combined. Sun et al. showed that combining serum annexin A2 and alpha-fetoprotein (AFP) levels improved the sensitivity of detecting early stage hepatocellular carcinoma to 87.4% compared with either annexin A2 (83.2%) or AFP alone (54.7%) [158]. We also showed that combining annexin A2 and CA125 improved the sensitivities of diagnosing early stage serous ovarian cancer compared with the benign ovarian tumours at 90% and 95% specificity levels.

The strength of this study is that annexin A2 levels were assessed only in serous ovarian cancer patients. However, the limitation of this study to assess the potential clinical use of annexin A2 as a biomarker for ovarian cancer is that the sample size of patients was small. These preliminary findings require further validation in a larger cohort of early and advanced stage ovarian cancer patients with other histological ovarian cancer types such as endometriod, clear cell and mucinous. Several studies have reported established methods for the improvement in ovarian cancer diagnosis such as Risk of Ovarian Malignancy Algorithm (ROMA), which includes CA125 and Human Epididymis Protein 4 (HE4) values together with menopausal status [181, 182] and Risk Malignancy Index (RMI) which incorporated the CA125, ultrasound and menopausal status [183]. Therefore, clinical information such as pre or post-menopausal status and ultrasound assessment
together combined annexin A2 and CA125 biomarkers may be useful to improve the sensitivity, specificity, PPV and NPV. Moreover, the potential clinical utility of annexin A2 as a diagnostic tool in high risk women, particularly post-menopausal women and women with pelvic masses needs to be further investigated together with these clinical assessments similar to ROMA and RMI to improve the diagnostic performance of annexin A2.

In conclusion, we showed that combining annexin A2 and CA125 can improve the sensitivity of detecting serous ovarian cancer and particularly early stage ovarian cancer. Annexin A2 shows promise as a diagnostic biomarker for serous ovarian cancer and its potential clinical significance warrants further investigation.

4.6 Acknowledgements

We thank Suzanne Edwards for her help with the logistic regression model and ROC curve analysis. The authors thank Dr Carmen Pyragius and Dr Izza Tan for their help with the annexin A2 ELISA. This research was supported by the Ovarian Cancer Research Foundation (OCRF) Australia, Cancer Council SA, Australia and the South Australian Health and Medical Research Institute (SAHMRI).
### Supplementary Table 4.1 Clinicopathological characteristics of urine patient cohort.

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>n</th>
<th>Age (Years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (range)</td>
</tr>
<tr>
<td>Controls (n=6)</td>
<td></td>
<td>51 (35-67)</td>
</tr>
<tr>
<td>Normal ovaries</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Simple ovarian cyst</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Benign ovarian tumours (n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous cystadenoma</td>
<td>8</td>
<td>58 (25-90)</td>
</tr>
<tr>
<td>Serous cystadenofibroma</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Ovarian fibroma</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ovarian endometrioma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ovarian leiomyoma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ovarian mucinous cystadenoma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Serous ovarian cancers (n=36)</td>
<td></td>
<td>65 (37-84)</td>
</tr>
<tr>
<td>Stage I</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>29</td>
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</tr>
<tr>
<td>Stage IV</td>
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</tr>
</tbody>
</table>
Supplementary Table 4.2 Clinicopathological characteristics of serum or plasma patient cohort.

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>n</th>
<th>Age (Years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (range)</td>
</tr>
<tr>
<td><strong>Controls (n=27)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal ovaries</td>
<td>9</td>
<td>50 (30-82)</td>
</tr>
<tr>
<td>Simple ovarian cyst</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Menorrhagia</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Endometrial polyp</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Benign ovarian tumours (n=30)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous cystadenoma</td>
<td>18</td>
<td>58 (25-90)</td>
</tr>
<tr>
<td>Serous cystadenofibroma</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ovarian fibroma</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ovarian endometrioma</td>
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<td></td>
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<tr>
<td>Ovarian leiomyoma</td>
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<td>Ovarian mucinous cystadenoma</td>
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<tr>
<td><strong>Serous ovarian cancers (n=86)</strong></td>
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<tr>
<td>Stage I</td>
<td>15</td>
<td>65 (37-88)</td>
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<tr>
<td>Stage II</td>
<td>8</td>
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</tr>
<tr>
<td>Stage III</td>
<td>56</td>
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<tr>
<td>Stage IV</td>
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</tr>
</tbody>
</table>
Chapter 5: Prognostic Significance of Annexin A2 and S100A10 in Serous Ovarian Cancer

Noor A Lokman¹, Carmen E Pyragius¹, Andrew Ruszkiewicz², Martin K Oehler¹, ³ and Carmela Ricciardelli¹

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² Centre of Cancer Biology, University of South Australia and Department of Anatomical Pathology, SA Pathology, SA, Australia
³ Department of Gynaecological Oncology, Royal Adelaide Hospital, Adelaide, SA 5005, Australia

Manuscript in Preparation
5.1 Abstract

Annexin A2, a calcium phospholipid binding protein has been shown to play an important role in ovarian cancer metastasis. This study examined whether annexin A2 can be used as a prognostic marker in serous ovarian cancer. Expression of annexin A2 and its binding protein; S100A10 was assessed using immunohistochemistry in a uniform tissue microarray cohort of stage III serous ovarian cancers (n=91) and matching metastatic lesions (n=30). Kaplan-Meier and Cox Regression analyses were performed to assess the relationship between annexin A2 or S100A10 expression and clinical outcome. Kaplan-Meier and Cox Regression analyses showed high stromal annexin A2 immunostaining to be significantly associated with reduced progression-free survival and overall survival. Moreover, high cytoplasmic S100A10 staining was associated with reduced overall survival but not progression-free survival. Multivariate Cox Regression analysis showed stromal annexin A2 and cytoplasmic S100A10 levels were independent predictors of overall survival in the patients with residual disease. Annexin A2 and S100A10 are novel prognostic biomarkers for serous ovarian cancer patients could aid patient management.

Keywords: ovarian cancer, annexin A2, S100A10, prognosis
5.2 Introduction

Ovarian cancer is the most lethal gynaecological cancer worldwide. It has been estimated that there will be 22,240 new cases of ovarian cancer and 14,030 deaths due to ovarian cancer in the United States in 2013 [148]. Up to 70% of ovarian cancer patients are diagnosed at advanced stage and the 5-year survival rate for advanced stage ovarian cancer is 27% compared to 92% for patients with early stage disease [148]. The standard treatment options for ovarian cancer are radical debulking surgery and chemotherapy such as carboplatin and paclitaxel. However, despite improvements in ovarian cancer treatment, there is a high mortality rate and poor treatment outcome for ovarian cancer due to ovarian cancer recurrence and chemoresistance. Therefore, identifying prognostic markers are important for predicting survival, to guide in the management of ovarian cancer patients and aid in the development of better therapeutic targets.

Annexin A2, a calcium phospholipid binding protein was reported to be expressed in many malignancies and play an important role in tumourigenesis [132]. Previously, we have characterized annexin A2 expression in epithelial cells and cancer associated stromal cells of serous ovarian cancer tissues and in serous ovarian cancer metastatic implants [172]. Annexin A2 expression was significantly increased in the stromal cells of serous ovarian cancer tissues compared with stromal cells of normal ovaries, benign serous cystadenomas and non-invasive borderline ovarian tumours [172]. Annexin A2 has been reported to be a potential prognostic marker in various malignancies. Increased annexin A2 expression was associated with poor prognosis in multiple myeloma [175], non-small cell lung cancer [184, 185], colorectal carcinoma [101, 186], cholangiocarcinoma [187], gastric carcinoma [188] and glioma [189]. However, no studies have investigated whether annexin A2 can be used as a prognostic marker for ovarian cancer.

Annexin A2 and its binding protein, S100A10 exist as an annexin A2 heterotetramer on the cell surface of various tumour cells [190]. S100A10, also known as p11 protein is a member of S100 protein family which has 2 EF-hand calcium binding motifs [191]. Moreover, S100A10 is a cytosolic protein and upon binding to annexin A2, annexin A2 heterotetramer complex translocate to the plasma membrane [192]. S100A10 has been shown to play an important role in oncogenesis [193] and S100A10 expression has been characterized in cancers of the bowel [194, 195], thyroid [196] and kidney [31]. In a recent study, S100A10 protein expression was identified in a gene signature predicting overall survival in primary serous ovarian carcinoma [197]. Moreover, high S100A10 expression has been shown to correlate with poor prognosis in Barrett’s adenocarcinoma [198].
S100A10 was also identified using a proteomic approach to be a potential serum biomarker for gallbladder cancer. Furthermore, increased S100A10 expression in late stage gallbladder cancer was associated with poor clinical outcome [199]. To date, no studies have reported S100A10 expression in ovarian malignancies and its relationship with ovarian cancer prognosis. In this study, we investigate whether annexin A2 and S100A10 expression has a prognostic relevance in stage III serous ovarian cancer, the most common and lethal ovarian cancer subtype.
5.3 Materials and methods

5.3.1 Patient’s tissue microarray cohort
Archived formalin fixed paraffin tissue blocks from stage III serous primary ovarian tumours (n=91) and matching metastatic tissues (n=30), diagnosed between 1994 and 2004 were obtained from the IMVS (SA Pathology, Adelaide, SA, Australia). Tissue microarrays (TMAs) were constructed from formalin fixed paraffin tissue blocks and each tissue block was represented by triplicate 1.0 mm diameter tissue cores, with the approval of ethics committee from the Royal Adelaide Hospital. The clinicopathological characteristics of TMAs cohort are detailed in Supplementary Table 5.1.

5.3.2 Immunohistochemistry
Immunohistochemistry was performed as previously described [172]. Briefly, tissue sections (5 µm) underwent microwave antigen retrieval for 10 minutes at 100⁰C in a steam microwave (Sixth Sense, Whirlpool, VIC, Australia) in 10 mM citric acid buffer (pH 6.0). Sections were incubated overnight with mouse monoclonal antibody against annexin A2 (1/500, BD Biosciences, CA, USA) or mouse monoclonal antibody against S100A10 (1/2000, BD Biosciences) in blocking buffer (5% normal goat serum) at 4⁰C. Visualization of immunoreactivity was achieved using biotinylated anti-mouse immunoglobulins (1/400, Dako, NSW, Australia) and streptavidin-peroxidase conjugate (1/500, Dako). Known positive tissues were used as positive controls [172] and negative controls included tissues incubated with no primary antibody or anti-mouse immunoglobulins.

5.3.3 Immunohistochemical assessment
Slides were digitally scanned using the NanoZoomer Digital Pathology System (Hamamatsu Photonics, SZK, Japan) and images were collected using NDP view imaging software (NDP scan software v2.2, Hamamatsu Photonics). The percentage of positive cancer cells with cytoplasmic and membrane staining and stromal intensity score of strong (3+), moderate (2+), weak (1+), or negative (0) were assessed by two experienced researchers (NL and CR). Positivity ≥10% was defined as high membrane or cytoplasmic immunostaining and <10% was defined as low. A score of 2+ or 3+ was defined as high stromal immunostaining and 1+ or 0 was defined as low stromal immunostaining, as described previously [172].

5.3.4 Statistical analyses
All statistical analyses were performed using the SPSS for Windows software (Version 20.0, SPSS Inc., Chicago, IL, USA). Wilcoxon sign rank test was conducted to compare annexin A2 or...
S100A10 expression in the primary ovarian cancer tissues with matching metastatic lesions. Chi-squared test was performed to determine the correlation of annexin A2 and S100A10 immunostaining in primary ovarian tumour tissues. Kaplan-Meier, univariate Cox Regression and multivariate Cox Regression analyses were performed to assess the association of annexin A2 and S100A10 expression in the stage III ovarian cancer TMA cohort with progression-free survival and overall survival. Statistical significance was accepted at $P < 0.05$. 
5.4 Results

5.4.1 Increased stromal annexin A2 and S100A10 expression in metastatic ovarian cancer tissues compared with matching primary ovarian tumours

Annexin A2 immunostaining patterns in primary ovarian tumours are shown in Figure 5.1a-d. Similar staining patterns to annexin A2 were observed in matching tissues samples immunostained for S100A10 (Figure 5.1e-h). A significant correlation between annexin A2 and S100A10 immunostaining in the membrane ($P = 0.002$), cytoplasm ($P = 0.003$) and stroma ($P < 0.0001$) were observed (Table 5.1a-c). Moreover, we showed a significant increase in stromal annexin A2 expression in metastatic ovarian cancer tissues (26/30) compared with matching primary ovarian cancer tissues (Figure 5.2a-b, $P < 0.0001$). Increased stromal S100A10 expression was also observed in metastatic ovarian cancer tissues (16/31) compared with matching primary tissues (Figure 5.2c-d, $P = 0.001$). However, our findings showed no difference in both annexin A2 or S100A10 expression in epithelial cells between the primary ovarian tumours and matching metastatic tissues (data not shown).

5.4.2 High stromal annexin A2 expression in the cancer associated stroma predicts clinical outcome

Kaplan-Meier analyses showed that annexin A2 positivity in the cancer cell membrane or cytoplasm was not associated with progression-free survival (Figure 5.3a, $P = 0.994$ & Figure 5.3c, $P = 0.568$) or overall survival (Figure 5.3b, $P = 0.437$ & Figure 5.3d, $P = 0.196$). However, high annexin A2 immunostaining in the cancer associated stroma was significantly associated with reduced progression-free survival (Figure 5.3e, $P = 0.009$) and reduced overall survival (Figure 5.3f, $P = 0.04$). The 5-year progression-free survival rate for patients with high stromal annexin A2 expression was 7% compared to 29% for patients with low stromal annexin A2. Moreover, the 5-year overall survival rate for patients with high stromal annexin A2 was 16.2% compared to 41.3% for patients with low stromal annexin A2. No correlations were observed between membrane, cytoplasmic or stromal annexin A2 immunostaining and clinical and pathological parameters including patient’s age ($P = 0.276$), tumour grade ($P = 0.449$) or residual disease status ($P = 0.792$) (Table 5.2).

5.4.3 S100A10 cytoplasmic positivity is associated with reduced survival

Kaplan-Meier analyses showed no significant relationship between S100A10 membrane positivity with progression-free survival (Figure 5.4a, $P = 0.610$) nor overall survival (Figure 5.4b, $P = 0.431$).
However, high S100A10 positivity in the cytoplasm was marginally associated with reduced overall survival (Figure 5.4d, $P = 0.054$), but not with progression-free survival (Figure 5.4c, $P = 0.108$). The 5-year overall survival rate for high cytoplasmic S100A10 expression was 19.1% compared to 28.6% for patients that have low S100A10 cytoplasmic expression. In contrast to stromal annexin A2, stromal S100A10 immunostaining was not associated with either progression-free survival (Figure 5.4e, $P = 0.254$) or overall survival (Figure 5.4f, $P = 0.178$).

5.4.4 Stromal annexin A2 positivity and cytoplasmic S100A10 expression is an independent predictor of clinical outcome

Univariate Cox Regression analysis showed no significant relationship between patient age or tumour grade with progression-free survival or overall survival (Table 5.3a). However the presence of residual disease was associated with a 2.17 fold increased risk of disease progression (95% CI: 1.02-4.63, $P = 0.043$) and 2.9 fold increased risk of death due to serous ovarian cancer (95% CI: 1.28-6.57, $P = 0.011$). Cox Regression analysis showed annexin A2 expression in the membrane is not significantly associated with increased risk of disease progression (RR=1.02, 95% CI: 0.55-1.75, $P = 0.994$) and increased risk of death (RR=1.28, 95% CI: 0.69-2.37, $P = 0.438$). Similarly, cytoplasmic annexin A2 was not associated with increased risk of disease progression (RR=1.2, 95% CI: 0.64-2.25, $P = 0.569$) or an increased risk of death (RR=1.6, 95% CI: 0.78-3.25, $P = 0.200$). However, Cox Regression analysis found that patients with high stromal annexin A2 had a 1.96 fold increased risk of disease progression (95 %CI: 1.17-3.25, $P = 0.010$) and 1.72 fold increased risk of ovarian cancer death (95 %CI: 1.02-2.9, $P = 0.043$; Table 5.3a).

S100A10 membrane expression was not associated with increased risk of disease progression (RR=1.16, 95% CI: 0.66-2.05, $P = 0.610$) and increase risk of death (RR=1.27, 95% CI: 0.70-2.32, $P = 0.423$). High cytoplasmic S100A10 was associated with a 2.3 fold increase of ovarian cancer death, although this only reached a borderline significance (95%CI: 0.97-5.22, $P = 0.06$). In comparison to stromal annexin A2 expression, stromal S100A10 expression was not significantly associated with an increased risk of disease progression (RR=1.33, 95% CI: 0.81-2.20, $P = 0.256$) nor increased risk of ovarian cancer death (RR=1.42, 95% CI: 0.85-2.37, $P = 0.180$). In the multivariable Cox Regression analysis for progression-free survival, which included positive residual disease and stromal annexin A2 immunostaining, stromal annexin A2 remained an independent predictor of progression-free survival (RR=1.94, 95% CI: 1.15-3.28, $P = 0.013$). Moreover, multivariable Cox Regression analysis for overall survival also showed that stromal annexin A2 immunostaining overall survival (RR= 2.07, 95%CI: 1.20-3.56, $P = 0.009$) (Table 5.3b)
and cytoplasmic S100A10 were independent predictors of overall survival (RR = 2.75, 95%CI: 1.09-6.94, P = 0.032, Table 5.3b).
Figure 5.1 Annexin A2 and S100A10 immunostaining in stage III serous ovarian cancers

A representative image of low (A) and high (B) annexin A2 immunostaining in the membrane and cytoplasm of the epithelial cells and cancer associated stroma. Low (C) and high (D) annexin A2 immunostaining in the cancer associated stroma. A representative image of low (E) and high (F) S100A10 expression in the membrane and cytoplasm of the epithelial cells and in the cancer associated stroma. Low (G) and high (H) S100A10 expression in the cancer associated stroma. Ep = cancer cells and St = peritumoural stroma. Magnification bar = 100 μm for all images.
Figure 5.2 Annexin A2 and S100A10 immunostaining in primary ovarian tumour and matching metastasis tissues.

A representative image of low stromal annexin A2 immunostaining (A) in primary ovarian tumour and high stromal annexin A2 immunostaining in matching metastatic tissue (B). Low stromal S100A10 immunostaining is present in primary ovarian tumour (C) compared with high stromal S100A10 immunostaining in matching metastatic tissue (D). Ep = cancer cells and St = peritumoural stroma. Magnification bar = 100 μm for all images.
Figure 5.3 High stromal annexin A2 expression is associated with poor prognosis in stage III serous ovarian cancer patients.

Kaplan Meier analyses showing the association of annexin A2 expression in membrane (A-B) and cytoplasm of epithelial cells (C-D) and cancer associated stroma (E-F) with progression-free survival (n=83) and overall survival in stage III serous ovarian cancer patients (n=87). Patients with high annexin A2 stroma expression had a significantly reduced progression-free survival ($P = 0.009$) and overall survival ($P = 0.04$). Positivity of $\geq$10% was defined as high membrane or cytoplasmic immunostaining and $<10\%$ was defined as low. Annexin A2 stromal immunostaining score of 0 and 1+ was defined as low annexin A2 expression and 2+ and 3+ represents high annexin A2 expression.
Figure 5.4 S100A10 cytoplasmic expression is associated with reduced overall survival for stage III serous ovarian cancer patients.

Kaplan Meier analyses showing association of S100A10 expression in membrane (A-B) and cytoplasm of epithelial cells (C-D) and cancer associated stroma (E-F) with progression-free (n=83) and overall survival in stage III serous ovarian cancer patients (n=88). Patients with high S100A10 cytoplasm expression had a significantly reduced overall survival ($P = 0.054$). Positivity of $\geq 10\%$ was defined as high membrane or cytoplasmic immunostaining and $\lt 10\%$ was defined as low. S100A10 stromal immunostaining score of 0 and 1+ was defined as low S100A10 expression and 2+ and 3+ represents high S100A10 expression.
Table 5.1 Correlation of annexin A2 and S100A10 immunostaining in the membrane and cytoplasm of cancer cells and cancer associated stroma in stage III primary serous ovarian cancer.

<table>
<thead>
<tr>
<th></th>
<th>Annexin A2 membrane &lt;10% a</th>
<th>Annexin A2 membrane ≥10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A10 membrane &lt;10% b</td>
<td>12/22 (54.5%)</td>
<td>12/68 (17.6%)</td>
</tr>
<tr>
<td>S100A10 membrane ≥10%</td>
<td>10/22 (45.5%)</td>
<td>56/68 (82.3%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td>P = 0.002</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Annexin A2 cytoplasm &lt;10% c</th>
<th>Annexin A2 cytoplasm ≥10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A10 cytoplasm &lt;10% d</td>
<td>7/15 (46.7%)</td>
<td>8/75 (10.7%)</td>
</tr>
<tr>
<td>S100A10 cytoplasm ≥10%</td>
<td>8/15 (53.3%)</td>
<td>67/75 (89.3%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td>P = 0.003</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Annexin A2 stroma low e</th>
<th>Annexin A2 stroma high f</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A10 stroma low</td>
<td>29/39 (74.4%)</td>
<td>11/51 (21.6%)</td>
</tr>
<tr>
<td>S100A10 stroma high</td>
<td>10/39 (25.6%)</td>
<td>40/51 (78.4%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

a = Annexin A2 membrane (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
b = S100A10 membrane (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
c = Annexin A2 cytoplasm (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
d = S100A10 cytoplasm (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
e = Annexin A2 stroma as dichotomous variable, cut point intensity < 0 or 1 (low) vs ≥ 2 or 3 (high)
f = S100A10 stroma as dichotomous variable, cut point intensity < 0 or 1 (low) vs ≥ 2 or 3 (high)
Table 5.2 Correlation of annexin A2 stromal intensity with age, tumour grade and residual disease in stage III primary serous ovarian cancer.

<table>
<thead>
<tr>
<th></th>
<th>Annexin A2 stroma low (a)</th>
<th>Annexin A2 stroma high</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55</td>
<td>18/39 (46.2%)</td>
<td>17/51 (33.3%)</td>
</tr>
<tr>
<td>≥55</td>
<td>21/39 (53.8%)</td>
<td>34/51 (66.7%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td></td>
<td>(P = 0.276)</td>
</tr>
<tr>
<td><strong>Tumour grade (b)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well or moderate</td>
<td>7/36 (19.4%)</td>
<td>14/50 (28%)</td>
</tr>
<tr>
<td>Poor</td>
<td>29/36 (80.6%)</td>
<td>36/50 (72%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td></td>
<td>(P = 0.449)</td>
</tr>
<tr>
<td><strong>Residual disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>30/37 (81%)</td>
<td>38/49 (77.6%)</td>
</tr>
<tr>
<td>Yes</td>
<td>7/37 (18.9%)</td>
<td>11/49 (22.4%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td></td>
<td>(P = 0.792)</td>
</tr>
</tbody>
</table>

\(a\) = Annexin A2 stroma as dichotomous variable, cut point intensity < 0 or 1 (low) vs ≥ 2 or 3 (high)

\(b\) = Well or moderate = grade 1 or 2 and poor = grade 3
### Table 5.3 Cox Regression analyses of stage III serous ovarian cancer patient cohort.

**a) Univariate Cox Regression analyses for progression-free survival and overall survival.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative risk</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age a</td>
<td>1.31</td>
<td>0.79-2.15</td>
</tr>
<tr>
<td>Tumour grade b</td>
<td>1.24</td>
<td>0.69-2.21</td>
</tr>
<tr>
<td>Residual disease c</td>
<td>2.17</td>
<td>1.02-4.63</td>
</tr>
<tr>
<td>Annexin A2 mem d</td>
<td>1.02</td>
<td>0.55-1.75</td>
</tr>
<tr>
<td>Annexin A2 cyto e</td>
<td>1.20</td>
<td>0.64-2.25</td>
</tr>
<tr>
<td>Annexin A2 st f</td>
<td>1.96</td>
<td>1.17-3.25</td>
</tr>
<tr>
<td>S100A10 mem g</td>
<td>1.16</td>
<td>0.66-2.05</td>
</tr>
<tr>
<td>S100A10 cyto h</td>
<td>1.77</td>
<td>0.87-3.60</td>
</tr>
<tr>
<td>S100A10 st i</td>
<td>1.33</td>
<td>0.81-2.20</td>
</tr>
</tbody>
</table>

**b) Multivariate Cox Regression analyses for progression-free survival and overall survival. All variables are significant in univariate analysis (n=82).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative risk</td>
<td>95% CI</td>
</tr>
<tr>
<td>Residual disease</td>
<td>1.95</td>
<td>0.92-4.24</td>
</tr>
<tr>
<td>Annexin A2 st</td>
<td>1.94</td>
<td>1.15-3.28</td>
</tr>
<tr>
<td>S100A10 cyto</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**NOTE:** P values highlighted in bold indicate P < 0.05

- a = Age a dichotomous variable, cut point <55 vs ≥55
- b = Tumour grade (well or moderate vs poor)
- c = Residual disease status (negative vs positive)
- d = Annexin A2 membrane (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
- e = Annexin A2 cytoplasm (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
- f = Annexin A2 stroma as dichotomous variable, cut point intensity < 0 or 1 vs ≥ 2 or 3
- g = S100A10 membrane (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
- h = S100A10 cytoplasm (% positive cells) as a dichotomous variable, cut point <10 vs ≥10
- i = S100A10 stroma as dichotomous variable, cut point intensity < 0 or 1 vs ≥ 2 or 3
5.5 Discussion

To date, this is the first study that has investigated the prognostic relevance of annexin A2 in stage III serous ovarian cancer. We demonstrate that high stromal annexin A2 expression is an independent prognostic factor for progression-free and overall survival in patients with ovarian cancer.

In this study, a strong correlation of annexin A2 and S100A10 expression immunostaining was observed in stage III primary serous ovarian cancer. Annexin A2 and S100A10 co-expression has been reported in renal carcinomas [31] and thyroid neoplasms [196]. Moreover, we observed increased annexin A2 and S100A10 immunostaining in stromal cells of metastatic ovarian cancer tissues compared with the matching primary ovarian tumours. Previous studies also showed increased annexin A2 expression in the stroma of breast cancer tissues [200] and in the stroma of cervical cancer tissues after neoadjuvant chemotherapy [201]. Annexin A2 has also been shown to be overexpressed in patients of metastatic renal carcinomas tissues compared with the primary tissues [29]. Moreover, increased annexin A2 expression in the cancer of bile ducts (cholangiosarcoma) was significantly associated with lymphatic invasion, metastasis and perineural invasion [187].

Our findings showed high stromal annexin A2 expression in stage III serous ovarian cancer was significantly associated with reduced progression-free survival and overall survival. However, no relationship between annexin A2 in the membrane or cytoplasm of cancer cells and patients outcome was observed. Moreover, our results showed that high stromal annexin A2 expression independently predicted an increased risk of death for serous ovarian cancer patients with residual disease. Previous studies showed high annexin A2 expression correlates with poor prognosis and reduced survival outcome in multiple myeloma [175], non-small cell lung cancer [184, 185] and colorectal carcinoma [101, 186]. Kagawa et al. also reported that high annexin A2 expression in pancreatic cancer was associated with rapid recurrence and reduced disease free survival in pancreatic cancer patients after being treated with gemcitabine as an adjuvant chemotherapy [202]. Ohno et al. showed that high annexin A2 expression in clear-cell renal carcinomas was associated with a lower 5 year metastasis free rate and an independent predictor for metastasis [29]. Annexin A2 expression in head and neck squamous carcinomas correlates with tumour grade, however, no significant association with higher tumour stages and disease recurrence was observed [41]. Contrary to those results, reduced annexin A2 tissue expression was reported in advanced disease with regional lymph node and distant metastasis in prostate cancer. Moreover,
low annexin A2 expression in prostate cancer was also associated with risk of recurrence and presented as an independent factor for overall survival [203]. The discrepancies of these findings may be explained due to different histological origin of the tumour and diverse role of annexin A2 in different tumour types.

Our previous findings suggest that the extracellular form of annexin A2 found in the cancer associated stroma in the ovarian cancer tissues may represent a cleaved secreted form of annexin A2, which may assist ovarian cancer progression and metastasis [172]. This notion is supported by the report that cleavage of annexin A2 at lysine 10 by MMP-7 can assist tumour invasion and metastasis of colorectal and breast cancer cell lines [85]. Moreover, increased stromal annexin A2 and tenascin C expression in colorectal carcinomas was shown to be an independent factor of poor prognosis [101]. However annexin A2 lacks a signal peptide and is not secreted via the endoplasmic reticulum pathway, the mechanisms that regulate increased stromal annexin A2 are unknown.

We observed high cytoplasmic S100A10 expression in stage III serous ovarian cancer was associated with overall survival (borderline significant) but not progression-free survival. Tan et al. showed high S100A10 expression in advanced stage cancer of the gall bladder to be associated with poor prognosis [199]. A large cohort study (n=228) demonstrated S100A10 gene expression was an independent predictor for tumour recurrence in stage II and III colon carcinoma patients after 5’ fluorouracil-based adjuvant chemotherapy [204]. Moreover, high S100A10 expression in patients with invasive Barrett's adenocarcinomas (n=73) was associated with reduced progression-free survival and was found to be an independent prognostic factor in Barrett’s adenocarcinoma [198]. Our multivariate analysis showed cytoplasmic S100A10 expression in stage III serous ovarian cancer patients to be an independent factor of poor clinical outcome together with patients that have high stromal annexin A2 expression and positive for residual disease. This is the first study to demonstrate that S100A10 expression in serous ovarian cancer tissues.

The strength of this study is that we examined annexin A2 and S100A10 expression in a uniform cohort of stage III serous ovarian cancer patients. It is not known whether annexin A2 and S100A10 are also expressed in other subtypes of epithelial ovarian cancers such as endometrioid, clear cell and mucinous carcinomas. Previous studies a showed correlation of annexin A2 expression with increasing tumour stage in clear cell renal carcinomas [29], colorectal carcinomas [30] and squamous cell carcinomas of the lung [205]. However, further studies need to address whether annexin A2 or S100A10 expression is associated with increasing stage and ovarian
cancer progression in a larger patient cohort. Future studies should also address whether annexin A2 or S100A10 are potential predictors of response to treatment such as chemotherapy in ovarian cancer.

5.6 Acknowledgements
We thank Mrs Wendy Bonner for her help with tissue sectioning. This study was supported by Ovarian Cancer Research Foundation (OCRF) Australia, Cancer Council SA and South Australian Health and Medical Research Institute (SAHMRI).
Supplementary Table 5.1 Clinicopathological characteristics of stage III serous ovarian cancer patient tissue cohort.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO Stage</td>
<td>91</td>
</tr>
<tr>
<td>Histological type</td>
<td>91</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>91</td>
</tr>
<tr>
<td>Median ± SD</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>Range</td>
<td>24-86</td>
</tr>
<tr>
<td>Histological grade a</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>4</td>
</tr>
<tr>
<td>Moderate</td>
<td>17</td>
</tr>
<tr>
<td>Poor</td>
<td>70</td>
</tr>
<tr>
<td>Residual disease</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>68</td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
</tr>
<tr>
<td>No data</td>
<td>5</td>
</tr>
</tbody>
</table>

a = Well = grade 1, moderate = grade 2 and poor = grade 3
Chapter 6: General Discussion and Conclusions
6.1 General discussion

Ovarian cancer is a complex and heterogeneous disease with a late stage presentation [112]. Despite advancements in surgical interventions, chemotherapies and novel drug developments, ovarian cancer patients have a poor survival outcome and a high mortality rate [165]. Ovarian cancer cell implantation onto the peritoneal lining is a vital step in ovarian cancer metastasis. However, the mechanisms involved in this process are poorly characterized. Our group has recently explored the interactions between ovarian cancer and peritoneal cells using an in vitro co-culture system [92]. One of the proteins identified by 2D-gel electrophoresis and mass spectrometry to be regulated by ovarian cancer-peritoneal cell interactions was annexin A2 [132]. Annexin A2, a multifunctional calcium phospholipid binding protein exists as an annexin A2 heterotetramer with S100A10 proteins on the cell surface [21] and has been shown to mediate cancer progression through the plasminogen activation system [2].

The aims of this study were to characterize annexin A2 expression in human serous ovarian cancer tissues, cell lines and primary ovarian cancer cells and to investigate the effects of annexin A2 siRNAs and neutralizing annexin A2 antibody in ovarian cancer cell motility, invasion and adhesion to the peritoneal cells in vitro. This study also determined whether annexin A2 inhibitors could block ovarian cancer invasion and metastasis in in vivo models. Moreover, we also aimed to evaluate whether annexin A2 can be used as a tumour marker for ovarian cancer diagnosis and to assess whether annexin A2 or S100A10 can be used as prognostic markers.

Annexin A2 has been characterized in various malignancies and had been shown to play an important role in tumourigenesis [132]. This is the first study which characterizes and investigates the role of annexin A2 in ovarian cancer and provides further validation on annexin A2, as one of the novel proteins modulated from co-cultured ovarian cancer and peritoneal cells in vitro. Previously, we have identified Transforming Growth Factor Inducible Protein (TGFBIp) in the co-culture system to play a role in ovarian cancer cell motility, invasion and adhesion to the peritoneal cells [92].

Chapter 1 is a published literature review which summarizes annexin A2 expression in various malignancies and its functional role in cancer cell motility, invasion, adhesion, proliferation and apoptosis [132]. Chapter 2 details the development of the chick chorioallantoic membrane (CAM) model to assess the invasion of OVCAR-3, SKOV-3 and OV-90 cells in vivo which was published as a research paper [156]. The CAM model has been used previously to study cancer cell invasion and metastasis [114, 126], however, limited studies have used the CAM model to assess ovarian
cancer cell invasion [131]. We utilized the CAM model not only to assess the invasion of established ovarian cancer cell lines, but also to examine invasion of primary ovarian cancer cell lines as detailed in chapter 3. Moreover, this work highlights that the CAM model is a robust model to investigate the effects of novel inhibitors on ovarian cancer cell invasion.

6.1.1 Annexin A2 plays an important role in ovarian cancer invasion and metastasis

Chapter 3 is a published research paper on annexin A2 expression in ovarian cancer tissues and cell lines and summarizes the results on the role of annexin A2 in ovarian cancer invasion and metastasis using in vitro and in vivo ovarian cancer models [172]. We reported annexin A2 was expressed in serous ovarian cancer cells (Figure 3.1d) and a significant increase of annexin A2 expression in ovarian cancer associated stromal cells compared with normal ovaries, benign ovarian cystadenomas and borderline ovarian tumours (Supplementary Table 3.1). Interestingly, we also observed increased annexin A2 expression in ovarian cancer cells located adjacent to the peritoneal cells in the metastatic omental implants (Figure 3.1f). Annexin A2 siRNAs significantly inhibited ovarian cancer cell motility, invasion and adhesion to the peritoneal cells (Figure 3.5).

Previous studies have shown that annexin A2 plays a role in cancer cell motility and invasion [76, 150, 151] and cell adhesion [11]. We demonstrated that annexin A2 neutralizing antibodies could significantly inhibit invasion of OV-90 cells and two primary ovarian cancer cell lines in the in vivo CAM model. We reported a 3.6-fold reduction of invasion into the CAM mesoderm with the annexin A2 antibody treated OV-90 cells compared to control (Figure 3.6g) and inhibition of primary ovarian cancer cells invasion into the CAM mesoderm by 4-fold compared with the control mouse IgG (Figure 3.7e).

Moreover, we utilized an intraperitoneal xenograft mouse model using SKOV-3/GFP Luc cells to assess annexin A2 role in ovarian cancer invasion and metastasis in vivo. Our findings showed that mice treated with annexin A2 neutralizing antibodies had a reduced tumour burden and peritoneal metastasis compared with control mice treated with mouse IgG (Figure 3.8 & Supplementary Figure 3.3). These observations were consistent with previous studies as mice treated with annexin A2 inhibitors showed reduced tumour growth and progression mediated by the plasminogen activation system in gliomas [150] and breast cancer [162]. We found that the reduced tumour burden in annexin A2 antibody treated mice was due to an increase in cell apoptosis and reduced cell survival (Figure 3.9). The mechanism of cell apoptosis mediated by annexin A2 is poorly characterized, although previous studies have also reported that reduced annexin A2 levels resulted in increased cancer cell apoptosis [71, 86, 150]. Sharma et al. have
shown reduced tumour burden in breast cancer cells of annexin A2 treated mice is a result of inhibition of neoangiogenesis [162] and Zhai et al. showed a reduced gliomas tumour growth is a result of reduced cell proliferation [150]. However, we did not observe any significant changes in either cancer cell proliferation or angiogenesis in the tumour tissues of the annexin A2 treated mice compared with the control mice. This discrepancy may be due to differences in cell lines used between these studies.

A secreted form of annexin A2 was identified by mass spectrometry in the conditioned media of co-cultured ovarian cancer and peritoneal cells missing the N-terminal domain (Supplementary Figure 3.1). Sato et al. also reported annexin A2 to be secreted in the conditioned media of pancreatic cancer cell lines [160]. However, the molecular mechanisms involved in regulating annexin A2 secretion are poorly characterized. Our findings suggest that the secreted form of annexin A2 may be both cleaved and/or modified by post translational mechanisms as we observed changes in size and pl of annexin A2 isoforms (Figure 3.4c-d). We showed that plasmin cleaved recombinant annexin A2 (Figure 3.4e) and previously we observed an increase of plasmin level in the conditioned media of co-cultured ovarian cancer and peritoneal cells [92]. Therefore, we proposed that annexin A2 mediates cancer cell invasion via the plasminogen activation pathway (Figure 1.4).

 Zheng et al. recently reported that tyrosine 23 phosphorylation of annexin A2, was important in enhancing pancreatic ductal adenocarcinoma cells invasion and metastasis in vitro and in vivo and promotes epithelium-mesenchymal transition (EMT) via the TGFβ-Rho-mediated [32]. Annexin A2 also was shown to mediate breast cancer cells invasion and metastasis via t-PA dependent plasmin generation pathway [66]. Moreover, annexin A2 knockdown in glioma cell lines decreases glioma cells invasion and tumourigenesis which is associated with decreased membrane-bound plasmin activity [150]. Together, these findings highlight that annexin A2 plays a vital role in promoting tumourigenesis via the plasminogen activation pathway.

### 6.1.2 Role of annexin A2 in tumour microenvironment

Annexin A2 heterotetramer localizes with CD44 [26] and t-PA [3] which promotes cancer progression via the plasminogen activation pathway. Oliferenko et al. reported interaction of annexin A2 with CD44 in the lipid rafts of mammary epithelial cells [26] and CD44 expression was also observed in serous ovarian cancer tissues and plays a role in ovarian cancer metastasis (reviewed in [206]). Annexin A2 heterotetramer mediates malignant progression i) via interactions with t-PA, cathepsin B, tenascin C and collagen I and ii) its role in regulating plasmin generation which result in proteolysis of basement membrane that releases and activate growth factors,
activation of proteases such as u-PA, MMPs and degradation of ECM proteins (reviewed in [5]). The extracellular matrix remodeling mediated by annexin A2 in the plasminogen activation system may be critical for cancer cell invasion, angiogenesis and metastasis [1, 62, 207].

Our findings highlight that annexin A2 plays an important role in ovarian cancer tumourigenesis in vitro and in vivo and we proposed a model of the mechanisms of annexin A2 which promotes cancer cell invasion, metastasis and tumour growth (Figure 6.1). Annexin A2 heterotetramer localizes with cell surface receptors and extracellular matrix (ECM) proteins such as CD44 [26], tenasin-C [51], cathepsin-B [53] and tissue-plasminogen activator (t-PA) [3]. Annexin A2 and t-PA interactions mediated conversion of plasminogen to plasmin [3] and increases plasmin levels in the tumour microenvironment which results in the activation of growth factors, activation of proteases such as MMP-2 and MMP-9 [151] and degradation of ECM proteins [5] that enhances cancer cell invasion, metastasis and tumour growth. Annexin A2 increases cancer cell migration through the regulation of actin cytoskeletal rearrangement [151]. Moreover, Shiozawa et al. reported that annexin A2 increases cancer cell proliferation and cell survival via the ERK1/2 and MAPK pathway [11]. Annexin A2 is also a substrate for src kinase which regulates phosphorylation of annexin A2 at tyrosine 23 and phosphorylated annexin A2 have been shown to enhance cancer cell invasion [32].

In this study, we found highly abundant annexin A2 and S100A10 expression in the cancer associated stroma of serous ovarian cancer. Stromal annexin A2 and S100A10 may interact with other ECM proteins and proteases such as cathepsin B [53], plasminogen [65] and tenascin C [208] in the tumour microenvironment. Ovarian cancer stroma consists of various ECM components and pro-inflammatory factors that play an important role in the development and progression of ovarian cancer (reviewed in [209]). A recent study identified S100A10 as one of the proteins increased in co-culture of ovarian tumour-stromal cells suggesting that it plays a role in the stromal-epithelial cell crosstalk which is important for ovarian cancer progression [210]. Moreover, the tumour stroma has been shown to be a novel source of cancer biomarkers associated with poor prognosis [211].

Figure 6.2 summarized the effects of annexin A2 inhibitors such as annexin A2 siRNAs and neutralizing annexin A2 antibodies on cancer cell invasion and metastasis. Down-regulation of annexin A2, decreases plasmin levels consequently decreases extracellular matrix remodeling and cancer cell invasion and metastasis. Zhao et al. reported that decreased annexin A2 levels resulted in decreased MMP-2 and MMP-9 levels and reduced hepatocellular carcinoma cell
invasion and metastasis [151]. Moreover, reduced levels of annexin A2 resulted in decreased cancer cell migration [151] and increased cancer cell apoptosis [150]. Therefore, annexin A2 has important roles in cancer cell invasion, migration and apoptosis.

6.1.3 Clinical significance of annexin A2 in serous ovarian cancer patients

In chapter 4, we assessed whether annexin A2 can be used as a diagnostic marker for ovarian cancer. We observed that blood levels of annexin A2 were significantly elevated in serous ovarian cancer patients compared with non-cancer controls. Importantly, blood levels of annexin A2 were elevated in early stage ovarian cancer compared to patients with benign ovarian tumours. We showed that combining annexin A2 and CA125 measurements improved the sensitivity of detecting early stage ovarian cancer at the 95% specificity level. Sun et al. reported that combined serum annexin A2 and alpha-fetoprotein (AFP) measurements improved the sensitivity of hepatocellular carcinoma (HCC) diagnosis [158]. Our preliminary findings in a small cohort of patients also showed that annexin A2 has the clinical potential to improve the sensitivity of distinguishing early serous ovarian cancer from benign ovarian tumours and in cancers with normal CA125 levels. However, this observation needs to be further validated in a larger cohort to assess the utility of combined annexin A2 and CA125 measurements.

Chapter 5 demonstrated the potential use of either annexin A2 or S100A10 as a prognostic marker for ovarian cancer. We found that stromal annexin A2 expression was associated with reduced progression-free and overall survival in stage III serous ovarian cancer cohort. Emoto et al. reported annexin A2 expression in the stromal cells of colorectal cancer [101] and secreted form of annexin A2 has been shown in pancreatic cancer cell lines [43]. However, the association of annexin A2 expression in the ovarian cancer associated stromal cells and the secreted form of annexin A2 in the conditioned media [172] is poorly understood. Therefore, future studies are required to determine the significance of annexin A2 and S100A10 expression with other extracellular matrix proteins in the ovarian cancer associated stromal cells and importance of cleaved or secreted annexin A2 in ovarian cancer progression. Our findings suggest that annexin A2 and S100A10 could be used as novel prognostic marker for serous ovarian cancer patients and targeting stromal annexin A2 has a potential clinical application in the treatment of stage III ovarian cancer.

We showed no significant difference of annexin A2 expression with increasing clinical stages of ovarian cancer (stage I to IV) (Supplementary Table 3.1). Characterization of annexin A2 expression should also be performed on type I (low grade tumours) and type II (high grade
tumours) ovarian cancer subtypes [212]. However, in our patient cohort (n=91), we did not observe any relationship between annexin A2 stromal intensity in the well or moderate tumour grade versus the poor differentiated high grade tumours (Table 5.2). Ovarian cancer is a heterogeneous disease and poor survival rate of ovarian cancer patients demands a better approach and treatment strategies [213]. Classification of ovarian cancer into molecular subtypes and profile through high-throughput genomic approaches [214, 215] may enable us to understand better the pathogenesis of the disease and assist in the development of effective novel therapies.

6.1.4 Co-localization of annexin A2 and S100A10 proteins in ovarian cancer cells

A significant correlation between annexin A2 and S100A10 expression in the epithelial cells and stromal cells was observed in stage III serous ovarian cancer tissues (Figure 5.1 & Supplementary Table 5.1). The expression of annexin A2 on the cell surface is dependent on the co-localization with S100A10 protein [22] and previous studies have showed depletion of annexin A2 resulted in loss of S100A10 expression at the mRNA levels in breast cancer cells [47] and murine lymphoma cells [78]. He et al. showed the cytoplasmic level of S100A10 would determine the levels of cell surface of annexin A2 heterotetramer, as binding of annexin A2 stabilized intracellular S100A10 and masking autonomous S100A10 polyubiquitination signal that led to proteasomal degradation [216]. S100A10 protein is also required for src kinase mediated tyrosine phosphorylation of annexin A2 which translocate both annexin A2 and S100A10 proteins on the cell surface [22, 216]. Moreover, a recent study reported no changes in annexin A2 levels but increased in phosphorylated annexin A2 levels associated with src kinase and S100A10 expression in prednisolone resistance acute lymphoblastic leukemia cells compared with prednisolone sensitive cells [217]. Myrvang et al. also showed protein interactions between annexin A2 and S100A10 in MDA-MB-231 cells mediates breast cancer cell adhesion to the microvascular endothelial cells [218]. Therefore, these studies showed it is important to characterize the expression and roles of both proteins as annexin A2 and S100A10 as they may have synergistic functions in mediating cancer cells behavior.
6.2 Future directions

This study highlights annexin A2 playing an important role in ovarian cancer progression; however, the mechanisms whereby annexin A2 interacts with other ECM proteins and proteases in mediating ovarian cancer metastasis are poorly understood. Future studies are required to investigate the mechanism of annexin A2 mediated t-PA dependent plasmin generation and the importance of tyrosine phosphorylated annexin A2 in ovarian cancer invasion and metastasis. Moreover, S100A10 has been shown to play an important role in invasion of bowel [195] and lung cancers [219]; therefore, the functional role of S100A10 in ovarian cancer motility, invasion and adhesion needs to be further evaluated.

Recent studies showed that SKOV-3 cell line, which has been extensively used in ovarian cancer studies was unlikely to represent high-grade serous ovarian cancer based on their molecular profile characterization [220]. Therefore, other high-grade serous ovarian cancer cell lines and primary ovarian cancer cell lines should also be used in future preclinical research to examine annexin A2 expression and its functional role in serous ovarian cancer invasion and metastasis.

Our findings show that annexin A2 has clinical significance in ovarian cancer diagnosis and prognosis. However, our findings are only limited to serous ovarian cancer patients. Annexin A2 expression remains to be characterized in other ovarian cancers such as the endometrioid, clear cell and mucinous subtypes. Moreover, the sensitivity of combined annexin A2 and CA125 blood levels in ovarian cancer diagnosis could be improved with additional tumour biomarkers or patients' clinical information such as ultrasound assessment and menopausal status. It will be also important to determine whether blood annexin A2 levels can be used to detect ovarian cancer recurrence and associated with the development of chemoresistance. Several studies reported annexin A2 playing a role in chemoresistance in pancreatic cancer cells [81, 160, 179] and breast cancer cells [108, 178, 221]. Limited studies have reported the relationship between annexin A2 with ATP binding cassette (ABC) transporters in chemoresistance and warrants further investigation [221]. Moreover, the association between annexin A2 or S100A10 expression with increasing clinical stages of ovarian cancer warrants further investigation in larger patient cohort.

Future studies should investigate the effect of annexin A2 neutralizing antibodies on ovarian cancer cell survival together with ovarian cancer chemotherapy drugs including carboplatin or paclitaxel.
6.3 Conclusions

Altogether, our findings strongly suggest that annexin A2 plays an important role in ovarian cancer invasion and metastasis. Annexin A2 is expressed in primary and metastatic serous ovarian cancer tissues, serous ovarian cancer cell lines and primary ovarian cancer cell lines. We identified a cleaved and secreted form of annexin A2 in the co-cultured ovarian cancer-peritoneal cells conditioned media. Moreover, annexin A2 siRNAs inhibited ovarian cancer cell motility, invasion and adhesion to the peritoneal cells in vitro. We also showed neutralizing annexin A2 antibodies inhibited OV-90 cells and primary ovarian cancer cells invasion in the CAM model. Moreover, neutralizing annexin A2 antibodies inhibited SKOV-3 cells tumour growth and peritoneal metastasis in the intraperitoneal xenograft mice model by increasing cancer cell apoptosis.

Annexin A2 has an important clinical significance in ovarian cancer diagnosis and prognosis. Blood annexin A2 levels were elevated in serous ovarian cancer compared with patients with normal ovaries and benign ovarian tumours. Moreover, combined annexin A2 and CA125 levels improved the sensitivity of early stage ovarian cancer detection. High stromal annexin A2 expression was associated with reduced progression-free survival and overall survival in stage III serous ovarian cancer. Moreover, multivariate analysis showed high stromal annexin A2 expression to be an independent predictor of progression-free survival and overall survival in stage III serous ovarian cancer patient cohort together with the presence of residual disease. Annexin A2 plays an important role in ovarian cancer invasion and metastasis and has diagnostic and prognostic significance; and is therefore a novel promising biomarker that warrants further evaluation.
Annexin A2 with p11 protein (S100A10) forms an annexin A2 heterotetramer complex on the plasma membrane which co-localize with CD44 [26], tenascin-C [51], cathepsin-B [53] and tissue-plasminogen activator (t-PA) [3]. Annexin A2 mediated t-PA dependent plasmin generation leads to activation of growth factors, activation of proteases including MMPs [151] and degradation of extracellular matrix (ECM) proteins [5] which increases cancer cell invasion, metastasis and tumour growth. Annexin A2 increases cancer cell proliferation and cell survival via the ERK1/2 and MAPK pathway [11]. Annexin A2 is a substrate for src kinase and regulates tyrosine 23 phosphorylation of annexin A2 and enhances cancer cell invasion [32]. Annexin A2 also plays a role in actin cytoskeletal rearrangement and regulates cancer cell migration [151].
Annexin A2 with p11 protein (S100A10) forms an annexin A2 heterotetramer complex on the plasma membrane which co-localized with CD44 molecule. Annexin A2 siRNAs and neutralizing annexin A2 antibodies (ANXA2 Ab) in which inhibit annexin A2 levels or function and inhibit the conversion of tissue-plasminogen activator (t-PA) dependent plasminogen activation on the cancer cells resulting in reduced plasmin levels. Consequently, decrease extracellular matrix remodelling due to inactivation of growth factors, proteases and MMPs result in decrease cancer cell invasion and metastasis. Inhibiting annexin A2 also decreases cancer cell migration and increases cancer cell apoptosis.
References


Appendix

Publications Contributing to This Thesis

1. The Role of Annexin A2 in Tumourigenesis and Cancer Progression
2. Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis
3. Annexin A2 is regulated by Ovarian Cancer-Peritoneal Cell Interactions and Promotes Metastasis
_Cancer Microenvironment, v. 4(2), pp. 199-208_
Chick Chorioallantoic Membrane (CAM) Assay as an *In Vivo* Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis

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**Abstract:** The majority of ovarian cancer patients present with advanced disease and despite aggressive treatment, prognosis remains poor. Significant improvement in ovarian cancer survival will require the development of more effective molecularly targeted therapeutics. Commonly, mouse models are used for the *in vivo* assessment of potential new therapeutic targets in ovarian cancer. However, animal models are costly and time consuming. Other models, such as the chick embryo chorioallantoic membrane (CAM) assay, are therefore an attractive alternative. CAM assays have been widely used to study angiogenesis and tumor invasion of colorectal, prostate and brain cancers. However, there have been limited studies that have used CAM assays to assess ovarian cancer invasion and metastasis. We have therefore developed a CAM assay protocol to monitor the metastatic properties of ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) and to study the effect of potential therapeutic molecules *in vivo*. The results from the CAM assay are consistent with cancer cell motility and invasion observed in *in vitro* assays. Our results demonstrate that the CAM assay is a robust and cost effective model to study ovarian cancer cell metastasis. It is therefore a very useful *in vivo* model for screening of potential novel therapeutics.
1. Introduction

Ovarian cancer is the most lethal gynecological malignancy. Most patients are diagnosed at an advanced stage when the cancer cells have already metastasized to the abdominal cavity. Ovarian cancer metastasis is characterized by the ability of ovarian cancer cells to detach from the ovary and to adhere and invade the peritoneal cell layer, which lines the organs in the abdominal cavity [1]. The development of new therapies with the aim to disrupt ovarian cancer metastasis requires the in vivo study of novel targets and molecules. However, commonly used murine models are costly and require a large number of animals as well as a long experimental time frame. An attractive alternative is the chick chorioallantoic membrane (CAM) assay.

CAM assays have been widely used to study angiogenesis [2], tumor cell invasion and metastasis [3–6]. The CAM model has many advantages, such as (a) the highly vascularized nature of the CAM greatly promotes the efficiency of tumor cell grafting; (b) high reproducibility; (c) simplicity and cost effectiveness, and finally (d) as the CAM assay is a closed system, the half-life of many experimental molecules such as small peptides tends to be much longer in comparison to animal models, allowing experimental study of potential anti-metastatic compounds that are only available in small quantities [4,7]. The CAM is composed of a multilayer epithelium; the ectoderm at the air interface, mesoderm (or stroma) and endoderm at the interface with the allantoic sac [8]. Furthermore, the CAM contains extracellular matrix proteins (ECM) such as fibronectin, laminin, collagen type I and integrin α×β3 [9]. The presence of these extracellular matrix proteins mimics the physiological cancer cell environment.

Although the CAM assay is a well established model for studying tumour angiogenesis and invasion in malignancies such as bowel cancer [10,11], glioma [12–14], prostate cancer [15–17], leukemia [18] and osteosarcoma [19], there has only been one study to date that has used a CAM assay to assess ovarian cancer invasion and metastasis [20]. We recently investigated the ovarian cancer-peritoneal cell interaction and identified several novel proteins that may be involved in ovarian cancer metastasis [21,22]. To effectively determine their function, we developed a CAM assay protocol using a range of ovarian cancer cell lines to allow the monitoring of candidate molecules on ovarian cancer cell invasion in vivo. The in vivo CAM assay data was compared with results from in vitro assays.

2. Results

2.1. Human Ovarian Cancer Cell Motility and Invasion In Vitro

We compared the motility and invasion of three ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) using in vitro assays. We found OV-90 cells to be most invasive through an extracellular matrix and migrated faster through 12 µm pores towards a chemo attractant, compared to SKOV-3 and OVCAR-3 cells (Figure 1). OVCAR-3 cells were the least motile and invasive cell line in our study.
2.2. Human Ovarian Cancer Cell Invasion into the Chick Chorioallantoic Membrane (CAM)

We initially utilized an *ex ovo* method and incubated the chick embryos in plastic weigh boats as described previously [23]. The *ex ovo* method has the advantage of allowing the application of a larger number of matrigel grafts as a wider area of the CAM is accessible. However, the survival rate for the *ex ovo* method was very low and only 10% of embryos survived to day 14. The *in ovo* method had a survival rate of 70% on day 14. In the *in ovo* method, a small window is made in the shell on day 3 of chick embryo development to detach the CAM layer from the egg shell (Figure 2a). Ovarian cancer cells (9 x 10^5 cells) were mixed with matrigel to form a gel and grafted on top of the CAM of day 11 chick embryos. The chick embryos were incubated with the matrigel grafts until day 14 of development. An example of a matrigel graft on day 14 is shown in Figure 2b.

**Figure 2.** (a) Day 3 chick embryo; (b) Ovarian cancer cells and matrigel graft on the chick chorioallantoic membrane (CAM) on day 14.

The CAM layers; ectoderm (ET), mesoderm (M) and endoderm (ED) can be seen in Figure 3a. Cytokeratin immunohistochemistry was used to identify the CAM layer integrity and presence of ovarian cancer cells in the mesodermal layer.
The invasion of the ovarian cancer cells through the ectoderm into the mesoderm was assessed on day 14 of chick embryo development. OVCAR-3 cytokeratin immunohistochemistry showed some damage to the ectoderm layer but minimal invasion into the CAM mesoderm (Figure 3b). The SKOV-3 cells showed invasion into the mesoderm layer and minimal destruction of the ectoderm layer (Figure 3c). OV-90 cells were the most invasive cells in the CAM assay (Figure 3d), which agrees with the results of the in vitro assays (Figure 1). Figure 3d shows a large number of OV-90 cells invading into the mesoderm of the CAM, as well as the destruction of the CAM ectoderm layer.

Quantitative analysis of ovarian cancer cell invasion into the CAM was performed by determining the number of images (8 to 12 sections per chick embryo) with cancer cell invasion into the CAM mesoderm of day 14 chick embryos. Our results showed a significantly higher invasion of OV-90 and SKOV-3 cells into the CAM mesoderm, when compared with OVCAR-3 cells (Figure 4).

2.3. The Effects of Protein a Neutralizing Antibody on OV-90 Cancer Cell Invasion into the CAM

We used a neutralizing antibody against one of the novel proteins identified in our previous study investigating ovarian cancer-peritoneal cell interactions [21,22]. OV-90 cancer cells \((9 \times 10^5 \text{ cells})\) were mixed with matrigel and the control anti-mouse IgG or the neutralizing antibody against protein A before grafting onto day 11 chick embryos. Neutralizing antibody against protein A
effectively inhibited OV-90 cancer cell invasion into the mesoderm of the CAM, compared with the control anti-mouse IgG, where OV-90 cancer cells invaded the mesoderm of the CAM and a destruction of the ectoderm layer was observed (Figure 5).

**Figure 4.** Chick chorioallantoic membrane (CAM) invasion by ovarian cancer cells in day 14 chick embryos. Data generated from 48–60 images from 6 chicken embryos per cell line. Data represents the percentage of images with invasion into the mesoderm, mean ± SEM from two independent experiments. (a) Indicates significant difference from OVCAR-3 cells; and (b) indicates significant difference from SKOV-3 cells, $p < 0.05$.

**Figure 5.** Effects of protein A neutralizing antibody on OV-90 cancer cell invasion into the chick chorioallantoic membrane (CAM). OV-90 cancer cells were mixed with matrigel and (a) control anti-mouse IgG (20 µg/mL); or (b) neutralizing antibody against protein A (20 µg/mL). CAM paraffin sections (6 µm) were immunostained with a pan-cytokeratin antibody. ET = ectoderm. M = mesoderm. ED = endoderm. CM = cancer cell matrigel grafts. Original magnification ×200, scale bar 100 µm.

3. **Discussion**

The CAM assay is a frequently applied model to study ovarian cancer angiogenesis [24–27]. However, there is only one study which has used CAM assays to assess ovarian cancer cell invasion and metastasis [20]. Chang et al. described IGROV-1 ovarian cancer cell invasion and metastasis to
the posterior CAM and lungs of chick embryos [20]. We have developed a CAM assay protocol to
monitor the metastatic properties of ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) and have
successfully used it to study the effect of newly identified molecules in vivo. Our results show that the
CAM assay is an effective model to study ovarian cancer metastasis. Importantly, our CAM model
closely mimics the mode of ovarian cancer metastasis which involves ovarian cancer cell attachment
and invasion into the peritoneum. The ectodermal layer of the CAM has many similarities with
the peritoneum, which consists of a single layer of mesothelial cells covering the organs in the
abdominal cavity.

We observed a higher survival rate with the in ovo method in comparison to the ex ovo method for
monitoring of ovarian cancer cell growth in the chick embryos. Various methods have been used to
graft cancer cells in the CAM model; such as collagen onplants [23], plastic rings [19], and matrigel
goats [28]. Furthermore, cancer cells can also be inoculated by dropping the cell suspension on top
of the CAM [29], or administered intravenously to study metastasis of cancer cells in the chick
embryos [18]. Matrigel is one of the most suitable scaffolds used for implantation and grafting of
cancer cells onto the CAM. In our model, ovarian cancer cells and matrigel were mixed with or
without neutralizing antibodies before grafting onto the CAM of the chick embryos to assess ovarian
cancer cell invasion. The grafting of the matrigel in the CAM model allows continuous visualization of
the test site. Moreover, other studies have reported visible and solid tumors on the CAM of chick
embryos a few days after cancer cell inoculation [11,19]. We used histological assessment by means of
a pan-cytokeratin antibody, to allow the visualization of cancer cells invading into the mesoderm.

The CAM model has been previously employed to assess cancer metastasis [3,29]. In some studies
quantitative alu PCR was used to assess the presence of metastatic human cancer cells in chick embryo
organs [30]. Several studies have compared both CAM assays and mouse models to assess tumor
growth and metastasis. Colorectal cancer cells were reported to colonize the CAM similarly to
the mouse model [10]. Strojnik et al. conducted a study to compare the histological and
immunohistochemical characteristics of glioma tumor protein expression in the CAM and an
established rat model. They reported a similar profile of proteins expressed in both models [12].
In addition, the CAM model has also been used concurrently with the nude mice model to assess tumor
growth of fibrosarcoma (HT1080 cells) and human squamous carcinoma (Hep3 cells) cells [31].
Lyu et al. also showed that over expression of urokinase-type plasminogen activator receptor (u-PAR)
in Hep3 cells leads to an increase in cancer cell invasion in the CAM model as well as accelerated
tumor growth in the SCID mice model [32]. These studies demonstrate the validity of the CAM model
for in vivo analysis of cancer cell invasion and metastasis.

The CAM model has many advantages. It is cost effective, allows large scale screening and is an
easily reproducible in vivo model [4,7]. A comparison of the advantages and limitations of the CAM
against the mouse model are summarized in Table 1.
Table 1. Comparison of the advantages and limitations of the chick chorioallantoic membrane (CAM) and mouse model.

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<td>CAM</td>
<td>Short experimental assay (days)</td>
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<td>Availability of in vivo imaging</td>
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The CAM model has also been used in pre-clinical screening to assess the efficacy of drugs and inhibitors on tumor growth. Hagedorn et al. reported that treatment of human glioma cells with receptor tyrosine kinase inhibitors inhibited tumor growth in a CAM model [14]. Bekes et al., demonstrated that treatment of prostate cancer (PC3 cells) with u-PA activation blocking antibody mAb-112 significantly inhibited cancer cell invasion in the CAM model [35]. Additionally, the CAM model has been used to test the efficacy of chemotherapy agents (such as doxorubicin) in human leukemia cell lines and has been shown to reduce cancer cell growth in the CAM [18]. An important limitation of the CAM model is the inability to assess cancer–immune cell interactions. Examination of cancer–immune cell interactions requires the use of transgenic ovarian cancer models [36], however, these models are not widely available, are not suitable for high throughput screening, and cannot be used with primary ovarian cancer cells derived from clinical samples. The CAM assay is therefore an attractive model to rapidly assess the effectiveness of novel candidate therapeutic drugs and the in vivo inhibition of specific tumor types and subtypes in one consistent model.

We have shown that OV-90 ovarian cancer cells invade into the mesoderm of the CAM within three days of implantation, therefore making the OV-90 CAM model ideal for studying ovarian cancer invasion and metastasis. In contrast, the OVCAR-3 cells showed limited invasion in the CAM over the
three days of our assays, and would therefore be suitable for studying the role of molecules that promote ovarian cancer invasion. In conclusion, the CAM model provides a high throughput in vivo model for the assessment and evaluation of candidate pro-invasive molecules as well as potential therapeutic targets for ovarian cancer.

4. Materials and Methods

4.1. Cell Culture

The human ovarian cancer cell lines OVCAR-3, SKOV-3 and OV-90 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All ovarian cancer cell lines were maintained in RPMI 1640 medium supplemented with 4 mM L-glutamine, antibiotics (100 U penicillin G, 100 µg/mL streptomycin sulfate and 0.25 µg/mL amphotericin B), (Sigma-Aldrich, St. Louis, MO, USA). OVCAR-3 and SKOV-3 cells were supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich) and OV-90 cells were supplemented with 10% FBS. All cell lines were maintained at 37 °C in an environment of 5% CO₂.

4.2. Cell Motility and Invasion Assays

Cell motility and invasion assays were performed as previously described [37]. Briefly, the ovarian cancer cells (OVCAR-3, SKOV-3 and OV-90) were diluted to a cell concentration of $1 \times 10^6$ cells/mL and labeled with calcein-AM (1 µg/mL, Life Technologies, VIC, Australia). Ovarian cancer cells were mixed at room temperature for 1 hour in the dark on an oscillating platform. Ovarian cancer cells (50 µL, 40,000 cells) were loaded on top of uncoated 12 µm filter inserts (Disposable, 96-well plate, ChemoTx, Neuro Probe Inc, Gaitherburg, MD, USA) for motility assays or 12 µm filters coated with Geltrex (0.6 µL/well, 9 mg/mL, Life Technologies) for invasion assays. 10% FBS RPMI media was used as a chemo attractant. The cells were allowed to migrate and invade to the lower chamber for 6 hours. Non-migratory cells on the top of the filter were removed and the fluorescence was measured at 485–520 nm.

4.3. Chick Chorioallantoic Membrane Assay (CAM Assay)

Fertilized white leghorn chicken eggs were obtained from Hi-Chick, South Australia, Australia. Eggs were incubated in a MultiQuip Incubator (E2) at 37 °C with 60% humidity. Ethics approval was obtained by the University of Adelaide Animal Ethics Committee. A small window was made in the shell on day 3 of chick embryo development under aseptic conditions. The window was resealed with adhesive tape and eggs were returned to the incubator until day 11 of chick embryo development. On day 11, OVCAR-3, SKOV-3 and OV-90 cell suspensions ($9 \times 10^5$) were mixed with growth factor reduced matrigel (8.9 mg/mL, BD Biosciences, NSW, Australia) in a total volume of 30 µL. Control anti-mouse IgG (20 µg/mL) (Sigma Aldrich) and neutralizing antibody to protein A (20 µg/mL) (BD Biosciences) were mixed together with the OV-90 cancer cells and matrigel. Matrigel grafts were placed on top of the CAM and eggs were resealed and returned to the incubator for 72 hours until day 14 ($n = 6$ chicken embryos per cell line). Matrigel grafts with surrounding CAM were harvested from each embryo and fixed with 4% paraformaldehyde for 24 hours and embedded in paraffin. Serial
sections (6 µm) were stained with hematoxylin and eosin. Slides were digitally scanned using the NanoZoomer (Hamamatsu Photonics K.K., Japan).

4.4. Immunohistochemistry

CAM paraffin sections (6 µm) were incubated on a heat plate at 60 °C for 2 hours. Tissue sections were dewaxed with xylene and ethanol, followed by PBS washes. Antigen retrieval was performed by using 1% protease (Sigma-Aldrich) for 10 minutes on a heat plate at 37 °C. The endogenous peroxidase activity of the sections was quenched with 0.3% H₂O₂. Each tissue sample was blocked with 5% goat serum for 30 minutes before incubation with monoclonal mouse anti-human cytokeratin clone AE1/AE3 (1:50, Dako, VIC, Australia) at 4 °C overnight. Subsequently, the tissue sections were incubated sequentially with biotinylated goat anti-mouse (1:400, Dako), followed by streptavidin-HRP conjugated (1:500, Dako) for 1 hour at room temperature. Immunoreactivity was detected using diaminobenzidine/H₂O₂ substrate (Sigma-Aldrich). The sections were counterstained with 10% haematoxylin (Sigma-Aldrich), dehydrated and mounted in Pertex (Medite Medizintechnik, Germany). Slides were digitally scanned using the NanoZoomer (Hamamatsu Photonics K.K.). For quantitative analysis of ovarian cancer cell invasion into the mesoderm layer, 8 to 12 CAM images from each embryo were assessed by two independent researchers [16].

4.5. Statistical Analysis

All analyses were performed using SPSS 15.0 for Windows software (SPSS, Chicago, IL, USA). The student’s t-test and one-way ANOVA were used to determine statistical significance of ovarian cancer cell motility and invasion in vitro, and ovarian cancer cell invasion in the CAM model. Statistical significance was accepted at \(p < 0.05\).

5. Conclusions

The CAM assay is a robust technique that can be used to monitor invasion of ovarian cancer cell lines and to assess the role of novel molecules and potential therapeutic targets. It is a valuable alternative to murine in vivo models for the study of ovarian cancer invasion and metastasis.

Acknowledgments

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Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis

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ABSTRACT:

Our recent research identified the protein annexin A2 to be regulated by ovarian cancer-peritoneal cell interactions. This study investigated the role of annexin A2 in ovarian cancer metastasis and its potential utility as a novel therapeutic target, using in vitro and in vivo ovarian cancer models. Annexin A2 expression was examined by qRT-PCR and western blotting in ovarian cancer cell lines and immunohistochemistry in serous ovarian carcinoma tissues. Annexin A2 siRNAs were used to evaluate the effects of annexin A2 suppression on ovarian cancer cell adhesion, motility, and invasion. Furthermore, annexin A2 neutralizing antibodies were used to examine the role of annexin A2 in tumor invasion and metastasis in vivo using a chick chorioallantoic membrane assay and an intraperitoneal xenograft mouse model. Strong annexin A2 immunostaining was observed in 90% (38/42) of the serous ovarian cancer cells and was significantly increased in the cancer-associated stroma compared to non-malignant ovarian tissues. Annexin A2 siRNA significantly inhibited the motility and invasion of serous ovarian cancer cells and adhesion to the peritoneal cells. Annexin A2 neutralizing antibodies significantly inhibited OV-90 cell motility and invasion in vitro and in vivo using the chick chorioallantoic membrane assay. The growth of SKOV-3 cells and their peritoneal dissemination in nude mice was significantly inhibited by annexin A2 neutralizing antibodies. Annexin A2 plays a critical role in ovarian cancer metastasis and is therefore a potential novel therapeutic target against ovarian cancer.

INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer and ranks as the fifth most common cause of cancer-related death in women in the western world. It has been estimated that there will be 22,240 new cases of ovarian cancer and 14,030 deaths due to ovarian cancer in the United States in 2013 [1]. Despite improvements in the surgical treatment and the development of new chemotherapeutic agents over the last 10 years, ovarian cancer survival rates have not changed significantly. An increase of the ovarian cancer survival rate will require the successful development of more effective molecularly targeted therapies.

Ovarian cancer has a distinct predisposition for metastasizing via shedding of cancerous cells from the ovary into the peritoneal cavity and implanting onto the peritoneum that lines the pelvic organs. Once ovarian cancer cells adhere to the peritoneal cells, they migrate through the peritoneal layer and invade local organs. The
local invasion of organs, such as the bowel, eventually results in the death of the patient.

Our group has recently explored the interactions between ovarian cancer-peritoneal cells using an in vitro co-culture system [2]. One of the proteins identified by 2D gel electrophoresis and mass spectrometry to be regulated by ovarian cancer-peritoneal cell interactions was annexin A2 [3]. Annexin A2 is a multifunctional calcium phospholipid binding protein which binds to collagen I, cathespin B and tenasin-C [4], assists in maintaining the plasticity and rearrangement of the actin cytoskeleton [5] and a cellular redox regulatory protein [6]. Annexin A2 also plays an important role in the plasminogen activation system and acts as a tissue plasminogen activator (t-PA) receptor on the cell surface of endothelial and cancer cells, which mediates the conversion of plasminogen into plasmin [7, 8]. Various studies have found increased annexin A2 tissue levels in malignancies of the breast, pancreas, oropharynx, liver, kidney, and bowel (reviewed by [3]). Annexin A2 has been shown to promote cell invasion in malignancies of the breast, brain, liver, and pancreas [9-12] and enhances cell motility and cell adhesion of prostate and hepatocellular carcinoma cells [12, 13]. However, the knowledge on the role of annexin A2 in ovarian cancer is very limited. It was identified to be upregulated in ovarian cancer cell lines with high invasive capacity compared to those with low invasive properties [14]. Moreover, a large scale proteomic study identified annexin A2 to be upregulated in ovarian cancers when compared with normal ovarian tissue and benign lesions [15]. This study investigated annexin A2 expression in serous ovarian cancer tissues and cell lines and performed functional in vitro and in vivo studies to examine its role in ovarian cancer cell adhesion, motility, invasion and metastasis.

RESULTS

Expression of annexin A2 in human ovarian cancer and peritoneal cell lines

Real-time PCR results showed annexin A2 was expressed in human serous ovarian cancer cell lines (OVCAR-3, OVCAR-5, SKOV-3 and OV-90) and the peritoneal cell line, LP-9 (Fig. 2A). This was supported by 1D-western immunoblotting in the cell lysates as an annexin A2 band at 37 kDa was observed in all cell lines (Fig. 2B). These findings confirm that annexin A2 is produced by both ovarian cancer cells and peritoneal cells.

Annexin A2 is cleaved in ovarian cancer and peritoneal cells co-culture

Annexin A2 was found to be increased in the secretome of co-cultured OVCAR-5 and LP-9 cells using 2D gel electrophoresis and mass spectrometry [3]. Annexin A2 expression was further examined by western immunoblotting in the conditioned media (CM) from OVCAR-3, OVCAR-5, and SKOV-3 cells either cultured alone or in co-culture experiments with LP-9 cells after 48 h. A 37 kDa annexin A2 band at low abundance was observed in the CM from OVCAR-3, OVCAR-5, and SKOV-3 cells. In the LP-9 CM, two distinct bands corresponding to annexin A2 at approximately 37 and 36 kDa were observed. A shift in the 37 kDa band to a 35 kDa form was observed when LP-9 cells were co-cultured with OVCAR-3, OVCAR-5 or SKOV3 cells (Fig. 2C). A similar finding was observed with OV-90 and LP-9 cells (data not shown). Mass spectrometry of annexin A2 from the CM of the co-cultured OVCAR-5 and LP-9 (n=4) did not identify any annexin A2 peptides in the N-terminal domain (Supplementary Fig. 1). 2D-western immunoblotting was performed to confirm the presence...
of processed or cleaved annexin A2 in the co-cultured LP-9 and OVCAR-5 CM compared with LP-9 CM cultured alone (Fig. 2D). Multiple annexin A2 spots at 37 kDa and 35 kDa and pl values ranging from 6 to 8 in the co-cultured OVCAR-5 and LP-9 CM compared with the CM of LP-9 cells alone were observed. This result is consistent with the annexin A2 processing observed in the 1D-western immunoblotting.

**Annexin A2 is cleaved by protease plasmin**

Plasmin digestion experiment demonstrated that recombinant annexin A2 is cleaved by plasmin and annexin A2 cleavage was partially inhibited following treatment with α2-antiplasmin (Fig. 2E). To evaluate whether annexin A2 processing was mediated by other proteases, co-cultured OVCAR-5 and LP-9 cells were treated with MMP inhibitor, GM-6001, a broad spectrum protease inhibitor cocktail as well as plasmin inhibitors, ε-ACA and α2-antiplasmin. In the co-culture experiment, only α2-antiplasmin was able to partially block annexin A2 cleavage (Fig. 2F). The other inhibitors used in the co-culture experiment had no effect on annexin A2 processing.

Annexin A2 promotes ovarian cancer motility and invasion and aids peritoneal adhesion of ovarian cancer cells

To determine whether annexin A2 promotes ovarian cancer metastasis, we examined the effects of knocking down annexin A2 expression on ovarian cancer cell adhesion to the peritoneal cells, motility, and invasion.

![Figure 1](image-url)

**Figure 1: Annexin A2 immunostaining in human ovarian tissues and omental tissues.** Annexin A2 immunostaining is present in the epithelial cells of normal surface epithelium (A), serous cystadenomas (B) and serous borderline ovarian tumors (C). Annexin A2 immunostaining is present in membrane and cytoplasm of ovarian cancer cells and cancer associated stroma of stage 3 serous ovarian cancer tissues (D). Annexin A2 immunostaining is observed in peritoneal cells of the omentum (E) and serous ovarian cancer cells located adjacent to the peritoneal cells in omental implants (F). Insert in (A) is the negative control with no primary antibody. Magnification bar = 100 µm for all images.
Real-time PCR results showed annexin A2 siRNAs effectively knocked down annexin A2 expression up to 70% compared with negative control siRNA and non-treated OVCAR-5 cells (Supplementary Fig. 2A). Moreover, the knockdown of annexin A2 expression with siRNA A into OVCAR-3, OVCAR-5, SKOV-3, and OV-90 cells were confirmed by western immunoblotting (Supplementary Fig. 2B). Annexin A2 siRNA treated OVCAR-5, OV-90, SKOV-3, and OVCAR-3 cells showed significantly decreased motility (OVCAR-5 \( P = 0.0008 \), OV-90 \( P < 0.0001 \), SKOV-3 \( P < 0.0001 \) and OVCAR-3 \( P = 0.0069 \)) and invasion (OVCAR-5 \( P = 0.0047 \), OV-90 \( P = 0.0047 \), SKOV-3 \( P < 0.0001 \) and OVCAR-3 \( P = 0.0002 \)), compared with cells treated with the negative control siRNA (Fig. 3A-D). Furthermore, annexin A2 siRNA treated SKOV-3, OVCAR-5, and OV-90 cells had significantly decreased adhesion to LP-9 cells when compared with cells treated with negative control siRNA (Fig. 3A-C, OVCAR-5 \( P = 0.0005 \), OV-90 \( P < 0.0001 \), SKOV-3 \( P < 0.0001 \)). However, there was no difference in the adhesion to the peritoneal cells in annexin A2 siRNA treated OVCAR-3 cells (Fig. 3D, \( P = 0.9057 \)). We also observed a significant decrease in OV-90 cell motility \( (P = 0.0016) \) and invasion \( (P = 0.0031) \) after treatment with annexin A2 neutralizing antibody, compared with the mouse IgG antibody (Fig. 4A). Treatment of OVCAR-5 cells with annexin A2 neutralizing antibody also showed a significant decrease in cell motility and invasion compared with the mouse IgG antibody (data not shown).

**Annexin A2 promotes ovarian cancer cell invasion in the chick chorioallantoic membrane model**

Ovarian cancer cell invasion was assessed using the CAM (chick chorioallantoic membrane) assay. OV-90 cells were mixed together with matrigel and placed onto the CAM of the chick embryos (Fig. 4B). To evaluate the effects of annexin A2 on ovarian cancer cell invasion \textit{in vivo}, OV-90 cells were treated with the mouse IgG antibody and annexin A2 neutralizing antibody. Haematoxylin and eosin staining and pan-cytokeratin immunostaining of OV-90 cells treated with the mouse IgG antibody showed invasion of OV-90 cancer cells through the ectoderm into the mesoderm of the CAM and a
destruction of the ectoderm layer (Fig. 4C,D). In contrast, OV-90 cells treated with annexin A2 neutralizing antibody exhibited minimal invasion through the ectoderm and mesoderm of the CAM (Fig. 4E,F). Quantitative analysis showed that annexin A2 antibody significantly inhibited OV-90 cancer cell invasion into the CAM mesoderm. Treatment with annexin A2 antibody resulted in a 3.6 fold reduction in cancer cell invasion, compared with OV-90 cancer cells treated with mouse IgG antibody (Fig. 4G, \( P = 0.004 \), Mann-Whitney U test).

**Annexin A2 promotes ovarian cancer growth and metastasis**

We utilized an intraperitoneal xenograft mouse model to assess the role of annexin A2 in ovarian cancer metastasis in vivo. The extensive tumor development and peritoneal metastasis observed with mouse IgG treatment (Fig. 5A) was significantly reduced in mice treated with annexin A2 neutralizing antibody (Fig. 5B). Tumor burden measured by bioluminescence was significantly reduced by annexin A2 neutralizing antibody treatment compared with mouse IgG antibody treatment over a 36 day period (Fig. 5C, Supplementary Fig. 3 and \( P < 0.05 \), Mann-Whitney U test).

We investigated the possible mechanisms for the reduced tumor burden in the annexin A2 antibody treatment group by analysing tumor cell proliferation, cell apoptosis and angiogenesis. Neutralizing annexin A2 antibody had no effect on SKOV-3 cell proliferation (Ki67, Fig. 6A), but significantly increased SKOV-3 cell apoptosis (active caspase 3, Fig. 6B). No difference in vascular density was observed between the two treatment groups (CD34, Fig. 6C). Our findings suggest the reduced tumor burden and metastatic spread in the annexin A2 antibody treatment group is a result of reduced cell survival.

**DISCUSSION**

To our knowledge, this is the first study to investigate the role of annexin A2 in ovarian cancer invasion and metastasis. Here we show i) high annexin A2 expression in 90% of serous ovarian cells and increased annexin A2 levels in the cancer-associated stroma, ii) suppression of annexin A2 expression significantly reduces migration and invasion of 4 ovarian cancer cell lines, iii) down regulation of annexin A2 reduces adhesion of ovarian cancer cells
to the peritoneal cells, and iv) annexin A2 neutralizing antibodies blocks migration and invasion of ovarian cancer cells, both in vitro and in vivo. Collectively these results provide strong evidence that annexin A2 plays a pivotal role in ovarian cancer progression and metastasis.

Ovarian cancer cell implantation onto the peritoneal lining is a vital step in ovarian cancer metastasis. However, the mechanisms involved in this process are poorly

Figure 4: Annexin A2 promotes OV-90 cell motility and invasion in vitro and in vivo. (A) Cell motility and invasion of OV-90 cells treated with mouse IgG antibody or annexin A2 neutralizing antibody in vitro. Data are expressed as a percentage of control mouse IgG, mean ± SEM of quadruplicates from 3 independent experiments (n=12). *, significantly different from control (P < 0.05, Student t-test). (B) Chick chorioallantoic membrane (CAM) implanted with matrigel and OV-90 cancer cells (CMI) at day 14 of chick embryo development. The CAM layers ectoderm (ECT), mesoderm (MES) and endoderm (END) are evident. Haematoxylin and eosin and pan-cytokeratin immunohistochemistry of the CAM implanted with OV-90 cells in the presence of mouse IgG antibody (C,D) and annexin A2 neutralizing antibody (E,F). (G) Quantitative analysis of OV-90 cells invasion into the CAM. Data represents mean ± SEM from 72-80 images (n=12 chick embryos per treatment group from 2 independent experiments). *, significantly different from control (P < 0.05, Mann-Whitney U test). Magnification bar = 100 µm for all images.
characterized. In this study, annexin A2 was regulated as a result of ovarian cancer and peritoneal cell interactions. Although annexin A2 overexpression has been observed in various cancer types such as those of the pancreas [16], colorectal [17], breast [11, 18], prostate [13], liver [19] and brain [9, 20], to date, no information on the expression of annexin A2 in human ovarian cancer tissues and its functional role in ovarian cancer have been published.

Our immunohistochemical studies showed annexin A2 expression in both the membrane and cytoplasm of ovarian cancer cells, but high annexin A2 levels were also observed in the cancer associated stroma. Strong annexin A2 expression in stromal cells was observed for all clinical stages of human ovarian cancer (stage I to IV) compared with normal ovaries, serous cystadenomas and borderline ovarian tumors. Increased levels of annexin A2 were also present in ovarian cancer cells located adjacent to the peritoneal cells in the omental implants tissues.

**Figure 5: Annexin A2 promotes growth and metastasis of SKOV-3 cells.** Tumor burden of mice treated with mouse IgG antibody (A) or anti-annexin A2 antibody (B) on day 36 using IVIS imaging and at autopsy, with haematoxylin and eosin staining showing the tumor morphology. Images represent 1 sec acquisition time, and the photon emission transmitted from mice was captured and quantitated in photons/s/cm²/sr. (C) Tumor growth and metastasis measured by bioluminescence. Arrow indicates the time point of weekly antibody administration until day 36 (*P < 0.05, Mann-Whitney U test). Data represents mean ±SEM (n=5 mice per treatment group). Magnification bar = 100 µm for all images.
Annexin A2 was shown to be expressed in human ovarian cancer and peritoneal cell lines. We observed a shift in the full length 37 kDa annexin A2 band to a 35 kDa isoform in the CM of co-cultured ovarian cancer and peritoneal cells. Furthermore, peptide mass fingerprinting of the annexin A2 protein in the co-cultured CM failed to detect any peptides containing the first 30 amino acids of the N-terminal domain of annexin A2. These findings suggest that annexin A2 is cleaved at the N-terminal domain as a result of the ovarian cancer-peritoneal cell co-culture environment.

We investigated whether annexin A2 cleavage in the ovarian cancer peritoneal cell co-culture could be inhibited by specific proteases. Plasmin is one of the proteases at the cell surface involved in remodeling the tumor microenvironment [21]. We previously showed that plasmin was upregulated in the CM of co-cultured ovarian cancer and peritoneal cells [2]. Previous studies reported annexin A2 cleavage by plasmin at lysine 27 in the N-terminal domain in monocytes [22], and between lysine 307 and arginine 308 in the C-terminal domain in endothelial cells [23]. Annexin A2 is also cleaved by proteases such as glycogen synthase-3 [24] and MMP-7 [25]. We confirmed that plasmin cleaved recombinant annexin A2 and we demonstrated that annexin A2 cleavage in co-cultured ovarian cancer and peritoneal cells could be partially blocked by α2-antiplasmin.

Our findings suggest that the extracellular form of annexin A2 found in the cancer associated stroma in the ovarian cancer tissues may represent a cleaved secreted form of annexin A2, which may assist ovarian cancer progression and metastasis. Since annexin A2 lacks a signal peptide and is not secreted via the endoplasmic reticulum pathway, the mechanism that regulates annexin A2 secretion remains unknown. It has been reported previously that cleavage of annexin A2 at lysine 10 by MMP-7 can assist tumor invasion and metastasis of colorectal and breast cancer cell lines [25]. Moreover, annexin A2 isoforms and cleaved annexin A2 were observed in normal and squamous cell carcinoma tissues, confirmed by 2D-western immunoblotting [26]. However, further studies are required to investigate annexin A2 cleavage mechanisms, the functional role of cleaved annexin A2 and post-translational modifications of annexin A2.

**Figure 6: Annexin A2 neutralizing antibodies increase apoptosis.** Tumor sections of mice treated with mouse IgG and anti-annexin A2 antibodies were immunostained with (A) Ki67, (B) cleaved caspase 3 and (C) CD34. Data represents the median from 10 images per mice (n=6 for mouse IgG and n=5 for anti-annexin A2 treatment groups). *, significantly different from control (P < 0.05, Mann-Whitney U test). Magnification bar = 100 µm for all images.
In this study, we have demonstrated that suppression of annexin A2 using siRNA decreased ovarian cancer cell adhesion to the peritoneal cells, cell motility, and invasion in vitro. These observations concur with previous studies demonstrating decreased cell motility and invasion in cancers of the pancreas [10, 27], breast [28], brain [9], liver [12] and prostate [29] following treatment with annexin A2 siRNA. Moreover, our study demonstrated annexin A2 plays a role in ovarian cancer cell adhesion to the peritoneal cells. Shiozawa et al. reported that annexin A2 has a role in regulating prostate cancer cell adhesion to osteoblasts and endothelial cells using annexin A2 siRNA [13]. Silencing of annexin A2 expression significantly reduced cell adhesion of hepatocellular carcinoma cells [12] and cell adhesion of myeloid cells to human and murine osteoblasts cells [30]. Braden et al. also reported that down-regulation of annexin A2 expression using polymeric nanoparticles in prostate cancer cell line inhibits tumor growth in nude mice [29]. Therefore, annexin A2 contributes to cancer progression by enhancing cancer cell motility, invasion and adhesion.

The functional role of annexin A2 in ovarian cancer cell motility and invasion was also assessed using annexin A2 neutralizing antibody. We showed OV-90 cells treated with neutralizing annexin A2 antibody significantly decreased motility and invasion in vitro and invasion in vivo in the chick embryo CAM model. Annexin A2 neutralizing antibodies also significantly inhibited growth and metastasis of SKOV-3 cells in nude mice. Our finding are consistent with previous reports that have shown annexin A2 neutralizing antibodies to inhibit cell migration, invasion, and to block plasminogen activation of breast cancer cells [11] and monocytes [31] in vitro. Moreover, anti-annexin A2 antibodies have been shown to inhibit pancreatic cancer metastasis in a mouse model of pancreatic ductal adenocarcinoma [10] and to inhibit tumor growth in breast cancer and Lewis Lung Carcinoma xenograft mouse models [32, 33]. A significant reduction of glioma tumor growth and progression in the annexin A2 knockout mice model associated with a decrease in cancer cell invasion, angiogenesis and proliferation has also been reported [9]. Tumor growth of fibrosarcoma (HT1080) and lung cancer (A459) cell lines in NOD-SCID mice has also been shown to be inhibited following annexin A2 depletion [6]. Furthermore, Zhai et al. showed a decrease in glioma tumor burden as a result of an increase in apoptosis in tumors of the annexin A2 knockout mice [9]. Similarly, we observed an increase in cell apoptosis, but no difference in the cell proliferation or vascular density in the tumor sections of mice with SKOV-3/GFP-Luc cells treated with anti-annexin A2 antibody. The in vivo findings from our CAM model and intraperitoneal xenograft mouse model were consistent with our in vitro observations demonstrating annexin A2 promotes ovarian cancer cell motility, invasion and tumor growth.

In conclusion, our findings demonstrate that annexin A2 plays an important role in ovarian cancer metastasis. Anti-annexin A2 antibodies significantly blocked ovarian cancer cell invasion in the CAM model and cancer cell peritoneal dissemination in the intraperitoneal xenograft mouse model. Annexin A2 is therefore a promising novel therapeutic target against ovarian cancer.

MATERIALS AND METHODS

Patient’s tissue samples

Archived formalin fixed paraffin tissue blocks from 20 matching primary tumors and their metastatic implants, 10 cases of each of early stage serous ovarian cancer (stage I and II), advanced stage serous ovarian cancer (stage III and IV) and borderline ovarian tumors, 16 normal ovaries, 11 serous cystadenomas and 9 omental implants were obtained from the Institute of Medical Veterinary Science (SA Pathology), Adelaide, South Australia, Australia. Tissue microarrays (TMAs) were constructed from formalin-fixed, paraffin embedded tumor material with the approval from the Royal Adelaide Hospital ethics committee and each tissue block was represented by triplicate 1.0 mm diameter tissue cores.

Cell culture

The human serous ovarian cancer cell lines OVCAR-3, SKOV-3 and OV-90 were purchased from American Type Culture Collection (ATCC, VA, USA). OVCAR-5 cells were obtained from Dr Thomas Hamilton (Fox Chase Cancer Center, PA, USA) and the peritoneal cells, LP-9 were purchased from Coriell Cell Repositories (NJ, USA). All cell lines were maintained as previously described [2]. The SKOV-3/GFP-Luc cells (Cell Biolabs Inc., CA, USA) were maintained in RPMI 1640 medium supplemented with 4mM L-glutamine, antibiotics (100 U penicillin G, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B) and supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, MO, USA). All cell lines were maintained at 37°C in an environment of 5% CO2.

Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, NSW, Australia) and each RNA sample was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen), as per manufacturer’s instructions. Real-time PCR was performed in triplicates using human annexin A2 validated primers (QIAGEN, Australia) and SYBR Green PCR master mix (7900HT Fast Real-Time
PCR System, Applied Biosystems, NSW, Australia). The cycling parameters were: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and then 60°C for 1 min. The CT values were normalised relative to the housekeeping gene, L19 and were calibrated to the OVCAR-3 cells using the \(2^{-\Delta\Delta CT}\) quantitation method.

**Immunohistochemistry**

For the annexin A2 immunohistochemistry, tissue sections (5 µm) underwent microwave antigen retrieval (5 min 750 W, 15 min 350 W) in 10 mM citric acid buffer (pH 6.5), and were incubated overnight with mouse monoclonal antibody to annexin A2 (1/500, BD Biosciences, CA, USA) in blocking buffer (5% normal goat serum) at 4°C. Visualization of immunoreactivity was achieved using biotinylated anti-mouse immunoglobulins (1/400, Dako, NSW, Australia), streptavidin-peroxidase conjugate (1/500, Dako) and diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich), as previously described [2]. Normal prostate tissue was used as a positive control [34] and negative controls included no primary antibody and mouse IgG controls. For the xenograft mouse model experiment, mouse tumor tissue sections were immunostained with active caspase-3 (rabbit polyclonal, 1/200, Cell Signaling Technology, MA, USA), Ki67 (rabbit monoclonal, 1/400, Epitomics, CA, USA) and CD34 (rat monoclonal, 1/100, clone MEC14.7, Abcam, MA, USA), as previously described [35].

**Co-culture of ovarian cancer and peritoneal cells**

LP-9 cells were cultured in 6 well plates until they reached confluency. OVCAR-5, SKOV-3, OV-90 and OVCAR-3 cells were added (2 x 10^5 cells/well) to the LP-9 monolayer and co-cultured in direct contact for 48 h before the collection of the cell lysates and CM, as previously described [2]. OVCAR-5 and LP-9 cells were also co-cultured in the presence or absence of protease inhibitor cocktail (1/200, Sigma-Aldrich), MMP inhibitor, GM-6001 (20 µM, Calbiochem), ε-ACA (150 mM, Sigma-Aldrich) and α2-antiplasmin (0.2 µM, Calbiochem).

**1D-western immunoblotting**

Western immunoblotting was conducted as previously described [2] with a mouse monoclonal antibody to annexin A2 (1/2000, BD Biosciences) or β-actin antibody (1/2000, Abcam) and anti-mouse IgG peroxidase-conjugated secondary antibodies (1/2000, Dako). For the plasmin digestion, recombinant annexin A2 protein with a GST-tag (0.5 µg, Abnova, Taiwan) was incubated with plasmin (0.2 U/ml, Sigma-Aldrich) at 37°C in the presence and absence of α2-antiplasmin (0.2 µM, Calbiochem).

**2D-western immunoblotting**

The total protein concentration of CM from LP-9 alone and co-cultured OVCAR-5 and LP-9 was determined using EZQ protein assay (Invitrogen). The immobilized pH gradient (IPG) strips (11 cm, GE Healthcare, NJ, USA) were rehydrated in rehydration buffer containing 1.2% DeStreak, 0.5% C.A, 1% bromophenol blue and 4% thiourea-CHAPS (TUC) buffer overnight in IPGPhorII (GE Healthcare). A total of 50 µg protein sample was reduced with 1 M DTT, 0.8% IPG buffer, TUC buffer and bromophenol blue and applied to the IPG strip and isoelectric focusing was carried out using IPGPhorII at 20°C as previously described [2]. Western immunoblotting was performed as per 1D-western immunoblotting.

**Motility and invasion assays**

Cell motility and invasion assays were performed as previously described [36]. For the annexin A2 knockdown experiments, ovarian cancer cells were transfected with annexin A2 siRNAs (siRNA ID: s1384 (A) and s1385 (B), 10 nM ) or negative siRNA (Ambion, TX, USA) with Oligofectamine (Invitrogen) for 48 h. Ovarian cancer cells were also pre-treated with annexin A2 neutralizing antibody (20 µg/ml, BD Biosciences) or mouse IgG antibody (20 µg/ml, Sigma-Aldrich) for 2 h. Cells were labelled with calcein-AM (1 µg/ml, Invitrogen) and were loaded onto uncoated 12 µm filter inserts (96-well plate, ChemoTx, Neuro Probe, MD, USA) for migration assays or 12 µm filters coated with Geltrex (0.6 µl/well, Invitrogen) for invasion assays. The cells were allowed to migrate and invade to the lower chamber for 6 h and the bottom fluorescence of migratory cells was measured at 485-520 nm using the Triad series multimode detector (Dynex Technologies, VA, USA).

**Adhesion assay**

Adhesion assay were performed as previously described [2]. Briefly, ovarian cancer cells were transfected with either annexin A2 siRNA A or negative siRNA for 48 h and labelled with calcein-AM. Ovarian cancer cells were added to the LP-9 cell monolayer and were allowed to adhere for 8 min. The fluorescence of adhered cells was measured at 485-520 nm using the Triad series multimode detector (Dynex Technologies).
Chick chorioallantoic membrane assay

Fertilized white leghorn chicken eggs (Hi Chick, SA, Australia) were maintained at 37°C in 60% relative humidity in a Multiquip Incubator E2 (Multiquip Pty. Ltd., NSW Australia). Approval was obtained by the University of Adelaide Animal Ethics Committee. On day 3 of chick embryo development, a small opening was made under aseptic conditions in the egg shell. To investigate the effects of annexin A2 on ovarian cancer cell invasion in the CAM model, OV-90 cells (9x10⁶ cells) were mixed with matrigel (8.9 mg/mL, BD Biosciences) with either annexin A2 neutralizing antibody (20 μg/mL, BD Biosciences) or mouse IgG antibody (20 μg/mL, Sigma-Aldrich) in a total volume of 30 μl and placed on the CAM of day 11 chick embryos (n=6 chick embryos per treatment group). The invasion of the cancer cells through the ectoderm into the mesoderm was assessed on day 14 of chick embryo development in paraffin-embedded CAM sections stained with haematoxylin, eosin and pan-cytokeratin immunohistochemistry, as previously described [37].

Intraperitoneal SKOV-3 xenograft mouse model

An intraperitoneal SKOV-3 xenograft non-invasive and whole-body bioluminescent imaging model was used with the approval obtained by the University of Adelaide Animal Ethics Committee. Nude mice were injected intraperitoneally with SKOV-3/GFP-Luc cells (1x10⁸ cells/0.5ml) and were treated weekly with either 100 μg mouse IgG antibody or 100 μg anti-annexin A2 antibodies (BD Biosciences). Mice were injected intraperitoneally with D-luciferin (Caliper Life Sciences, MA, USA) solution at 150 mg luciferin/kg body weight and then gas-anesthetized with isoflurane (Bomac Pty. Ltd., NSW, Australia). Precisely, 10, 15 and 20 min following D-luciferin injection, images were acquired for 0.5 to 10 sec and the photon emission transmitted from mice was captured and quantitated in photons/s/cm²/sr using the IVIS Imaging System 100 (Xenogen Imaging Technology, CA, USA) with living image software (Igor Pro version 2.5). At autopsy, tumor tissues were fixed with 4% paraformaldehyde (Sigma-Aldrich) and embedded into paraffin blocks.

Immunohistochemical assessment

Slides were digitally scanned using the NanoZoomer (Hamamatsu Photonics, Japan) and images were captured using NDP view imaging software (Hamamatsu Photonics). The immunostaining intensity of annexin A2 in the epithelial and stromal compartments was assessed using a manual scoring method: strong (3+), moderate (2+), weak (1+), or negative (0). A score of 0 or 1+ was defined as low annexin A2 immunostaining and a score or 2+ or 3+ was defined as high annexin A2 immunostaining. The percentage of annexin A2 positive cancer cells were independently assessed in the ovarian cancer tissues. For the CAM assay, qualitative analysis to assess OV-90 cancer cell invasion was performed on 8 to 12 CAM images for each embryo as previously described [37]. For the mouse tumor tissue sections, ten random images of each tissue were captured at 40x magnification for Ki67 and activated caspase-3 and at 20x magnification for CD34. Colour threshold detection by the AnalySIS-Pro™ software (Soft Imaging System, Germany) was used to determine positive (brown pixels) and negative (purple pixels) stained cells. Data was expressed as percentage of positive pixels (positive brown stained divided by negative purple stained area) for Ki67 and percentage of positive CD34 pixels of the total tumor area. For active caspase 3, the number of positive cells in ten fields were counted manually and expressed as a percentage of the total tumor area. The mouse treatment groups were blinded until completion of all analyses.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 for Windows (SPSS Inc., IL, USA). The Chi-Square test was used to determine statistical significance between annexin A2 immunostaining in the ovarian and omental tissue groups. The Student’s t-test and one-way ANOVA with Dunnett C or Dunnett T post-hoc tests were used to determine statistical significance between control and treatment groups. For the CAM assay and xenograft mouse model experiments, the Mann-Whitney U test was used to determine the significance between control and treatment groups. Statistical significance was accepted at P < 0.05.

ACKNOWLEDGEMENTS

We thank Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA) for kindly providing the OVCAR-5 cell line. The authors thank Helen Hughes for collection of archival tissue blocks, Dr. Shalini Jindal for pathological assessment and Dr. Aleksandra Ochnick for the TMA construction. We thank Mrs Wendy Bonner for her help with tissue sectioning, Mr Adrian Kaczmarek for his technical assistance with the xenograft mouse model and Ms Izza Tan for her help with the image analysis. We also thank Professor Simon Koblar for his assistance in setting up the CAM assay. This research was supported by the Ovarian Cancer Research Foundation (OCRF) Australia, Cancer Council SA and the South Australian Health and Medical Research Institute (SAHMRI).
REFERENCES


Supplementary Figure 1: Ion-trap mass spectrometry LC-MS/MS analysis of annexin A2 spots from the 2D gel electrophoresis. No annexin A2 peptides (red peptides: positive identified sequence via Mascot search) were observed in the N-terminal domain of annexin A2 (amino acid 1-30) in the protein spots of the 2D gel electrophoresis-silver stained gel from OVCAR-5 and LP-9 co-cultured cells conditioned media [3].

Supplementary Figure 2: Annexin A2 expression after knockdown with annexin A2 siRNAs. (A) Annexin A2 real-time PCR expression of OVCAR-5 cells after treatment with annexin A2 siRNA A and siRNA B compared with the negative control siRNA and non-treated cells, assessed using $2^{\Delta\Delta CT}$ quantitation method. Data represents triplicate determinations ± SEM from 2 independent experiments. (B) Annexin A2 expression of OVCAR-5, SKOV-3, OVCAR-3 and OV-90 cells after knockdown with annexin A2 siRNA A and negative control siRNA confirmed by 1D-western immunoblotting. β-actin was used as a loading control.
Supplementary Figure 3: IVIS imaging of mice. Mice were treated once weekly for three weeks with either mouse IgG (top row), or anti-annexin A2 antibody (bottom row). Images represent tumor growth on days 5, 12, 19, 26, 30 and 36, assessed using the IVIS Imaging. Images represent 1 sec acquisition time, and the photon emission transmitted from mice was captured and quantitated in photons/s/cm²/sr.
**Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis – Lokman et al**

Supplementary Table 1: Annexin A2 epithelial and stromal immunostaining intensity in the normal ovaries, serous cystadenomas, serous borderline tumors and serous ovarian carcinomas (stage I-IV) tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Patient age (years) Median (range)</th>
<th>n</th>
<th>Annexin A2 epithelial staining</th>
<th>Annexin A2 stromal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intensity</td>
<td>% of positive cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low&lt;sup&gt;a&lt;/sup&gt;</td>
<td>High&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal ovaries</td>
<td>16</td>
<td></td>
<td>2/16 (12.5%)</td>
<td>14/16 (87.5%)</td>
</tr>
<tr>
<td>Serous cystadenomas</td>
<td>11</td>
<td></td>
<td>4/11 (36.3%)</td>
<td>7/11 (63.6%)</td>
</tr>
<tr>
<td>Serous borderline tumors</td>
<td>10</td>
<td></td>
<td>0/10 (0%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Serous ovarian carcinomas (Stage I-IV)</td>
<td>42</td>
<td></td>
<td>4/42 (10%)</td>
<td>38/42 (90%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td></td>
<td></td>
<td>P = 0.510</td>
<td>P = 0.248</td>
</tr>
</tbody>
</table>

<sup>a</sup> Annexin A2 low intensity score; no staining (0) and weak staining (1+)

<sup>b</sup> Annexin A2 high intensity score; moderate staining (2+) and strong staining (3+)
Supplementary Table 2: Annexin A2 epithelial and stromal immunostaining intensity in the primary tumor and matching omental metastasis tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Patient age (years) Median (range)</th>
<th>Annexin A2 epithelial staining</th>
<th>Annexin A2 stromal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intensity</td>
<td>Intensity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% of positive cells</td>
<td>% of positive cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤10</td>
<td>≥10</td>
</tr>
<tr>
<td>Primary tumor</td>
<td>21</td>
<td>68 (49-87)</td>
<td>0/21 (0%)</td>
<td>21/21 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7/21 (33.3%)</td>
<td>14/21 (66.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9/21 (42.9%)</td>
<td>12/21 (57.2%)</td>
</tr>
<tr>
<td>Matching omental metastasis</td>
<td>18</td>
<td></td>
<td>3/18 (16.7%)</td>
<td>15/18 (83.3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10/18 (55.6%)</td>
<td>8/18 (44.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4/18 (22.2%)</td>
<td>14/18 (77.8%)</td>
</tr>
<tr>
<td>Fisher’s exact test</td>
<td></td>
<td></td>
<td>P = 0.089</td>
<td>P = 0.206</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P = 0.307</td>
<td></td>
</tr>
</tbody>
</table>

*a* Annexin A2 low intensity score; no staining (0) and weak staining (1+)

*b* Annexin A2 high intensity score; moderate staining (2+) and strong staining (3+)
**Supplementary Table 3: Annexin A2 epithelial immunostaining intensity in the omental tissues.**

<table>
<thead>
<tr>
<th>Omental Tissue</th>
<th>n</th>
<th>Patient age (years)</th>
<th>Median (range)</th>
<th>Annexin A2 epithelial staining</th>
<th>% of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intensity</td>
<td>≤10</td>
</tr>
<tr>
<td>Cancer cells adjacent to the peritoneum</td>
<td>9</td>
<td>58</td>
<td>(40-70)</td>
<td>Low&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/9 (11.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8/9 (88.9%)</td>
</tr>
<tr>
<td>Cancer cells distant from the peritoneum</td>
<td>9</td>
<td>58</td>
<td>(40-70)</td>
<td>Low&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4/9 (44.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5/9 (55.5%)</td>
</tr>
<tr>
<td>Chi-squared test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = 0.114</td>
</tr>
</tbody>
</table>

<sup>a</sup> Annexin A2 low intensity score; no staining (0) and weak staining (1+)

<sup>b</sup> Annexin A2 high intensity score; moderate staining (2+) and strong staining (3+)