Title : Characterisation of the expression of tumour antigens and biomarkers in myeloid leukaemia and ovarian cancer

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Characterisation of the Expression of Tumour Antigens and Biomarkers in Myeloid Leukaemia and Ovarian Cancer

By

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PhD

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Abstract

Acute myeloid leukaemia (AML) and ovarian cancer (OVC) are two difficult to treat cancers. AML is often treatable however minimal residual disease (MRD) endures such that many patients who achieve remission eventually relapse and succumb to the disease. OVC affects approximately 7000 women in the U.K. every year. It can occur at any age but is most common after menopause. Diagnosis at an early stage of disease greatly improves the chances of survival however, patients tend to be diagnosed in the later stages of disease when treatment is often less effective. Immunotherapy has the potential to reduce MRD and delay or prevent relapse. In order for immunotherapy to work, tumour antigens need to be identified and characterised so they can be effectively targeted. Personalised treatments require the identification of biomarkers, for disease detection and confirmation, as well as to provide an indication of best treatment and the prediction of survival.

PASD1 has been found to be frequently expressed in haematological malignancies and I wanted to determine if there was a correlation between the presence of antigen-specific T cells in the periphery of patients with AML and PASD1 protein expression in the leukaemic cells. The expression of other leukaemia antigens were concurrently examined as comparators. I performed RT-PCR on nine antigens and immunocytochemistry on PASD1 in 18 samples from AML patients. I found a correlation between PASD1 expression in AML samples and the presence of PASD1-specific T cells as detected on the pMHC array.

OVC lacks suitable targets for immunotherapy with few CTAs having been identified. I examined the expression of SSX2IP and the CTAs PASD1 and SSX2 in OVC. I compared the protein expression of these known tumour antigens to the "gold standard" biomarker for the diagnosis of OVC, CA125 and two other proteins known to be promising in the diagnosis of OVC, HE4 and WT1. I analysed commercially available paraffin-embedded OVC multiple

tissue arrays (MTAs) containing 191 samples, predominantly stage I (n= 166), II (n= 15) and III (n= 6) OVC as well as healthy donor (n= 8) and normal adjacent tissues (n= 8). Scoring was performed in a single blinded fashion. I found SSX2A to be expressed at a score level of 3 with a frequency (37/191) that exceeded that of CA125 (14/191), HE4 (14/191), WT1 (1//191) or PASD1 (0/191). To confirm this expression I used two additional commercially-available antibodies that recognise the region common to SSX2A and B, and an antibody specific for SSX2A. Using SSX2 peptides, I blocked the immunolabelling of SSX2 in SSX2-positive cell lines showing that the immunolabelling of SSX2 and SSX2A was specific. I demonstrated that the expression of SSX2 and specifically SSX2A was reproducible and restricted to ovarian cancer with little or no expression in endometrial tissues, or diseased or inflamed endometrial tissue.

In summary, these studies demonstrated that PASD1 expression in leukaemia cells correlated with the presence of PASD1-specific T cells in the periphery of presentation AML patients. I have shown that PASD1 specific-T cells are present in AML patients at diagnosis and that immunotherapy targeting PASD1 could be used to break tolerance and clear residual leukaemia cells during first remission. Analysis of the expression of three antigens in OVC, identified the specific expression of SSX2, in particular SSX2A in OVC but not healthy or diseased endometrial tissues. The expression of SSX2A was more frequent and more specific to OVC, than HE4 and WT1, and more frequent at higher intensity, especially in early stage OVC, than CA125. SSX2 and explicitly SSX2A requires further investigation to determine whether the high level of background at score 2 can be reduced with better blocking of non-specific sites. This may require the use of different SSX2 antibodies or an improved staining protocol.

Author's Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire.

It has not been submitted before for any degree or examination in any other University.

Name of candidate: Ghazala Khan

Signature:

Date: 20 December 2016

Dedications

For my Dad.

For my parents who gave me the world. They allowed me to follow my dreams and encouraged me to be the best. I am who I am because of them.

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Abbreviations

ABC	ATP-binding cassette
ADIP	Afadin DIL domain-interacting protein
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
APL	Altered peptide ligand
BAGE	B melanoma antigen
BIRC5	Baculoviral IAP Repeat Containing 5
CA125	Cancer Antigen 125
CC	Cervical cancer
CEA	Carcinoembryonic antigen
CML	Chronic Myeloid Leukaemia
CR	Complete response
CRC	Colorectal adenocarcinoma
CT	Cancer-testis
CTAs	Cancer-testes antigens
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
DAB	3,3'-Diaminobenzidine
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EFS	Event-free survival
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
EOC	Epithelial ovarian cancer
ESC	Endometrial serous carcinoma
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCM	Flow cytometry analysis
FDA	Food and Drug Administration
FIGO	The International Federation of Gynecology and Obstetrics
FRET	fluorescence resonance energy transfer
GC	Gastric cancer
GRINL1A	N-Methyl D-Aspartate-Like 1A combined protein
HCC	Hepatocellular Carcinoma
HCT	Hematopoietic cell transplantation
HE4	Human epididymis protein 4
HGPIN	High-grade prostatic intraepithelial neoplasia
HL	Hodgkin’s lymphoma
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
ICC	Immunocytochemistry
IFNγ	Interferon-gamma
IgG	Immunoglobulins
IHC	Immunohistochemistry

IL-2	Interleukin-2
LAA	Leukaemia associated antigens
LAK	lymphokine-activated killer
LMP	Low malignant potential
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MRD	Minimal residual disease
NAT	Normal adjacent tissue
NHL	Non-Hodgkin's Lymphoma
NSCL	Non-small cell lung
NT	Normal tissue
OS	Overall survival
OSC	Ovarian serous carcinoma
OVC	Ovarian cancer
PASD1	Per ARNT SIM PAS domain containing 1
PBMNC	Peripheral blood mononuclear
PD-1	Programmed death-1
PFC	Polychromatic flow cytometry
PFS	Progression-free survival
pMHC	Peptide-MHC
PPC	Primary peritoneal carcinoma
PR	Partial response
PRAME	Preferentially expressed antigen in melanoma
RAYS	Recombinant antigen expression on yeast surface
RHAMM	Receptor for Hyaluronan Mediated Motility
RT-PCR	Reverse transcription polymerase chain reaction
RQ-PCR	Real-time PCR
SCID	Severe combined immunodeficiency disorder
SCT	Stem cell transplantation
SEREX	Serological identification of antigens by recombinant expression cloning
SERPA	Serological proteome analysis
SSX2	Synovial Sarcoma X breakpoint 2
SSX2IP	Synovial Sarcoma X breakpoint 2 Interacting Protein
STIC	Serous tubal intraepithelial carcinoma
TAAs	Tumour associated antigens
TAE	Tris-acetate-EDTA
TAP	Transporter associated with antigen transport
TBS	Tris-buffered saline
TBI	Total body irradiation
TCR	T cell receptor
TIL	Tumour-infiltrating T leucocyte
TMAs	Tumour Microarrays
Tregs	Regulatory T cells
TVU	Transvaginal ultrasound
VEGF	Vascular endothelial growth factor
WAP	Whey-acidic-protein
WFDC2	Four-disulfide core domain protein 2
WT1	Wilms Tumour Protein

CHAPTER 1: INTRODUCTION

1.1 Cancer and conventional therapy

Cancer affects 338,623 people in the UK and is most frequent in patients over the age of 65 peaking in incidence at the age of 85. The frequency of cancer in older individuals (≥ 65 years) is 2085.3/100,000 compared with those aged less than 65, which is 193.9 per 100,000 and more than half of all cancers diagnosed (58%) are found in the elderly (> 65) (Yancik & Ries, 1994). Overall cancer survival rates have been improving and have doubled in the last 40 years such that cancer survival is now measured in a 10-year time-frame, rather than 5 years, at around 50% (Cancer Research UK, 2016). The frequency of cancer in the population rises with age, due to environmental and genetic factors, mistakes by DNA polymerase during the division of cells and the copying of DNA leading to spontaneous mutations that can lead to cancer. There is also an increasingly obvious impact of diet on cancer incidence (25% of the British population is obese) and certain cancers occur more frequently and in more aggressive forms in overweight individuals (MacInnes et al, 2003; Panagopoulou et al, 2012). Breast cancer patients had an additional 45% of visceral fat, abdominal fat surrounding the internal organs, compared with healthy controls (Schapira et al, 1994) and a higher mortality rate was associated with body mass index ≥ 30 kg/m^2 (considered obese) when compared to <25 kg/m^2 (Dal Maso et al, 2008). As the waist circumference increases there seems to be a correlation with increased risk of colon cancer (Larsson and Wolk, 2007). There are more than 200 different cancers involving a range of cells from different body tissues, which behave in unique ways. However, the focus of many researchers and clinicians is on early diagnosis and preferably prior to symptoms asserting themselves, which would lead to higher survival rates. For example, in ovarian cancer (OVC) survival rates are 90% at 5-years post diagnosis for those patients with stage I disease compared with <50% when OVC is diagnosis at the later

stages (III and IV) as is most commonly the case. Relative survival rates at 10 years post diagnosis are 84% and 59% for stage I and II disease, respectively, which reduces quite dramatically to 23% and 8% for stages III and IV, respectively (Baldwin et al, 2012).

There are three types of conventional treatments for solid cancers; surgery, radiation and chemotherapy. Many solid tumours will eventually require the use of surgery in order to aid their removal. Surgery can be very effective in helping with the diagnosis via a biopsy and removing early stage tumours before metastatic cancers develop which become extremely difficult to treat. The main aim of surgical intervention is to resect the tumour and some of the surrounding healthy tissue along with some lymph nodes. Following surgery a combination of chemotherapeutic cytotoxic drugs are administered to kill any residual cancer cells. Cancerous cells are known to divide rapidly in comparison to normal cells which is the quality exploited by the drugs. However, some normal cells also divide constantly and these are also susceptible to damage by the chemotherapeutic drugs such as hair follicles, which is why hair loss is a common side effect of chemotherapy (Siegel et al, 2012).

Radiotherapy comprises the use of high-energy X-rays targeted at the tumour using a linear accelerator (external) or injecting radioactive molecules at the site of the tumour (internal). An exact dose of X-rays are directed at the tumour causing DNA damage that the cancer cells are unable to recover from due to gene mutations, contrary to affected healthy cells which can survive due to DNA repair mechanisms. Radiotherapy can be used in combinations with surgery or chemotherapy (Becker, 2012).

Treatments for liquid tumours and particularly leukaemia differ in that the cancer originates in the bone marrow, is diagnosed by biopsy and often is disseminated throughout the body via the blood stream. In addition, leukaemic cells are often perturbed precursors of adaptive or innate immune cells and so the immune response is uniquely affected by the disease.

1.2 Leukaemia

Leukaemia is a haematological malignancy arising from an accumulation of haematopoietic stem cells often referred to as blasts. The UK incidence is 1 in 100,000. Haematopoiesis describes the process which forms the myeloid and lymphoid cell lineages from stem cells which in turn produce blood cells such as granulocytes and lymphocytes. The classification of leukaemia is governed by the cells of the blood involved i.e. myeloid leukaemia if the initial transformed cell is a precursor to myeloid cells or if it is lymphoid then lymphocytic leukaemia is described. A genetic mutation causes a stem cell to undergo changes, which are passed on to its progeny and over the course of cell division the number of mutations accumulate leading to increasing numbers of leukemic cells in the bone marrow. The rapid increase in the numbers of these immature cells condenses the space available for healthy blood cells causing a reduction in immune cells produced to fight infections. Progression of leukaemia can vary with acute leukaemia occurring and advancing rapidly in weeks and requiring treatment promptly, whereas chronic leukaemia takes months to develop and often treatment can be delayed. The four main types of leukaemia are acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), acute lymphocytic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL) (Bain, 2010).

AML is rare in children but is commonly observed in adults over the age of 65. Typically at diagnoses, the bone marrow sample comprises of about 10^{12} blasts cells. Prognosis depends on the severity of the illness at the point of diagnosis. Patients with AML usually present because of complications of disordered haematopoiesis: bleeding, fatigue, refractory infections, or the clinical consequences of an extremely high white blood cell count: difficulty breathing, confusion, or other symptoms of organ failure (Showel and Levis, 2014). CML is commonly detected in older patients (50-60 years) and >95% of patients with CML have increased numbers of hematopoietic cells expressing the oncogenic BCR-ABL1 fusion gene. The

translocation produces the Philadelphia chromosome (Ph) (Nowell and Hungerford, 1960), which encodes the constitutively active BCR-ABL1 protein tyrosine kinase (Faderl et al, 1999; Sawyers, 1999). This constant activation of the oncogene causes unregulated proliferation of the transformed hematopoietic cells therefore reducing the numbers of normal white blood cells (Stein et al, 2013). More than half of the patients affected by ALL are children and treatment is very effective, leading to a 90% 5-year survival rate, however in adult ALL there are poor outcomes. A quarter of adults with ALL have the translocation t(9;22) while the most common translocation found in childhood ALL is t(12;21) which produces the TEL-AML1 gene. Common symptoms include anaemia and infection (Howard and Hamilton, 2008). CLL is most common in the elderly, the majority of patients are over 50 years of age and have poor prognosis. Chromosome deletions such as 11q, 13q and 17p are observed in CLL while CD38 or ZAP70 expression and mutations in IGHV are indicators of poor survival (Puiggros et al, 2014).

1.2.1 Conventional treatment of acute leukaemia - AML

Treatment of AML is usually comprised of two stages. induction therapy, which involves giving DNA-damaging agents such as cytarabine, a nucleoside analogue, in combination with an anthracycline (which among other actions, inhibits DNA and RNA synthesis) such as daunorubicin. These drugs aim to cause disease remission by inducing the abnormal cells to undergo apoptosis as they predominantly affect rapidly dividing cells, such as leukemic cells, and most patients achieve a remission.

Almost all AML patients will achieve first remission however many patients will relapse and require further treatment (Dores et al, 2012). Further treatment consists of additional chemotherapy or bone marrow stem cell transplantation (SCT). Around 70-80% of AML patients aged less than 65 achieve remission through chemotherapy treatment (Dohner et al, 2010), but around half relapse without SCT. Even with SCT, over one third will relapse

(Cornelissen et al, 2007). Currently the median survival for AML is around one year; however there has been a steady increase in the overall survival in younger patients (Maynadie et al, 2013). This can be explained, to some extent, as being due to improvements in palliative care and support (Showel and Levis, 2014).

1.2.2 Conventional treatment of acute leukaemia - ALL

Treatment of ALL usually includes chemotherapy and should be started as soon as possible after diagnosis. The first of the three phases of treatment is called remission induction. Once remission has been achieved consolidation therapy is given, this involves chemotherapy and sometimes a stem cell transplantation. The final stage is maintenance therapy, this involves low dose chemotherapy and steroids for up to 2 years. In contrast, Ph+ ALL is treated as detailed in Section 1.2.3.

1.2.3 Conventional treatment for CML

The tyrosine kinase inhibitor, imatinib (STI571, Gleevec; Novartis), is administered at 400 mg daily, and is the standard therapy for CML patients in chronic phase (Druker et al, 2001). 82% of patients have been found to achieve a complete cytogenetic response with imatinib (Jabbour et al, 2008) however some patients do not respond to imatinib, and even those that do, often develop resistance, therefore alternative treatment options are required. This has led to the development of second-generation tyrosine kinase inhibitors such as nilotinib, which is more potent than imatinib and is currently approved for the treatment of newly diagnosed, imatinib-resistant or imatinib-intolerant CML and Ph+ ALL (Kantarjian et al, 2006). Bone marrow or SCT are performed when patients become resistant to the drugs and maybe in combination with total body irradiation (TBI) (Thomas et al, 1976). Following chemotherapy, high energy rays (radiotherapy) are used on the whole body to kill off remaining bone marrow cells. Then the patient's own bone marrow or stem cells, or donor cells are administered via drip. TBI treatment is carried out twice a day for 3 or 4 days (Cancer Research UK, 2015).

1.3 OVC

Over 90% of OVC cases are malignant epithelial carcinomas (EOC). These can be further divided into five main types: (high-grade serous (70%), endometrioid (10%), clear cell (10%), mucinous (3%), and low grade serous carcinomas (<5%) according to histopathology and molecular genetic alterations (Prat, 2012).

Around 225,500 women worldwide are diagnosed with OVC every year and there are about 140,200 associated deaths (Jemal et al, 2011). OVC is the fifth most common cancer in the UK, even more prevalent than cervical cancer (CC). Gradually, over the past decade, the incidence has been increasing and currently the lifetime risk is 1.8% (Stack and Fishman, 2013). This may reflect an aging population caused by improved treatments for other diseases, improved standards of living and healthcare developments. Although OVC can occur at any age, more than 85% of women are over 50 years of age at diagnosis (Stack and Fishman, 2013). While the chances of developing OVC are increased in those with close relatives who have been affected, most individuals have no family history.

The different types of OVC are classified by the type of cell from which the cancer originates from and include epithelial, germ cell and stromal OVC, the most common type being EOC. As the early stages of the disease is generally asymptomatic, over 75% of patients are diagnosed in the later stages of the disease (stage III and IV) (Rosen et al, 2005) when patients present with pelvic or abdominal pain, urinary frequency or urgency, increased abdominal size or bloating. The 5-year survival rate at this stage is 12-20% (Stack and Fishman, 2013). However, if OVC is diagnosed in the early stages of disease (stage I) there is a 90% survival rate (Stack and Fishman, 2013). About 75% of patients with stage III cancer at presentation relapse after surgery and chemotherapy and 80-90% die of the disease (Prat, 2012). Currently the marker CA125 is used as an aid in the detection of OVC, however CA125 is found in 75-90% of stage III and IV tumours, and/or serous tumours, but has a false positive rate of 80% (Moss et al,

2005). Serous tumours are likely to develop from serous tubal intraepithelial carcinoma (STIC) precursor lesions in the fallopian tubes (Dietl, 2014; Salvador et al, 2008) which then coat the ovary and may fall into the abdominal cavity making it difficult to detect them at the earliest stages of disease. In contrast clear cell/endometroid tumours arise in the ovary (McMeekin et al, 1995) and can be detected in the early stages of disease (Ledermann et al, 2013), although this is still rarely the case (Maringe et al, 2012).

1.3.1 Types of OVC

There are three main types of OVC depending on the cells of origin:

1. EOC
2. Germ cell tumours
3. Sex cord stromal cell tumours

EOC is the most common type of OVC (**Figure 1.1**). The epithelial cells which cover the surface of the ovaries is where the malignant transformation of the stem cell occurs. About 90% of all OVCs are of epithelial derivation. These can be further sub-divided into the following types; serous, endometrioid, mucinous and clear cell tumours (George et al, 2016). EOC is associated with high mortality, due to its frequent diagnosis in the late stages of disease in 70% of women (George et al, 2016). Some EOC are called borderline tumours or tumours of low malignant potential (LMP). These tumours include those which cannot be clearly recognised as malignant cancer cells when viewed under the microscope because they appear similar to both aggressive and benign ovarian tumours (Fischerova et al, 2012)

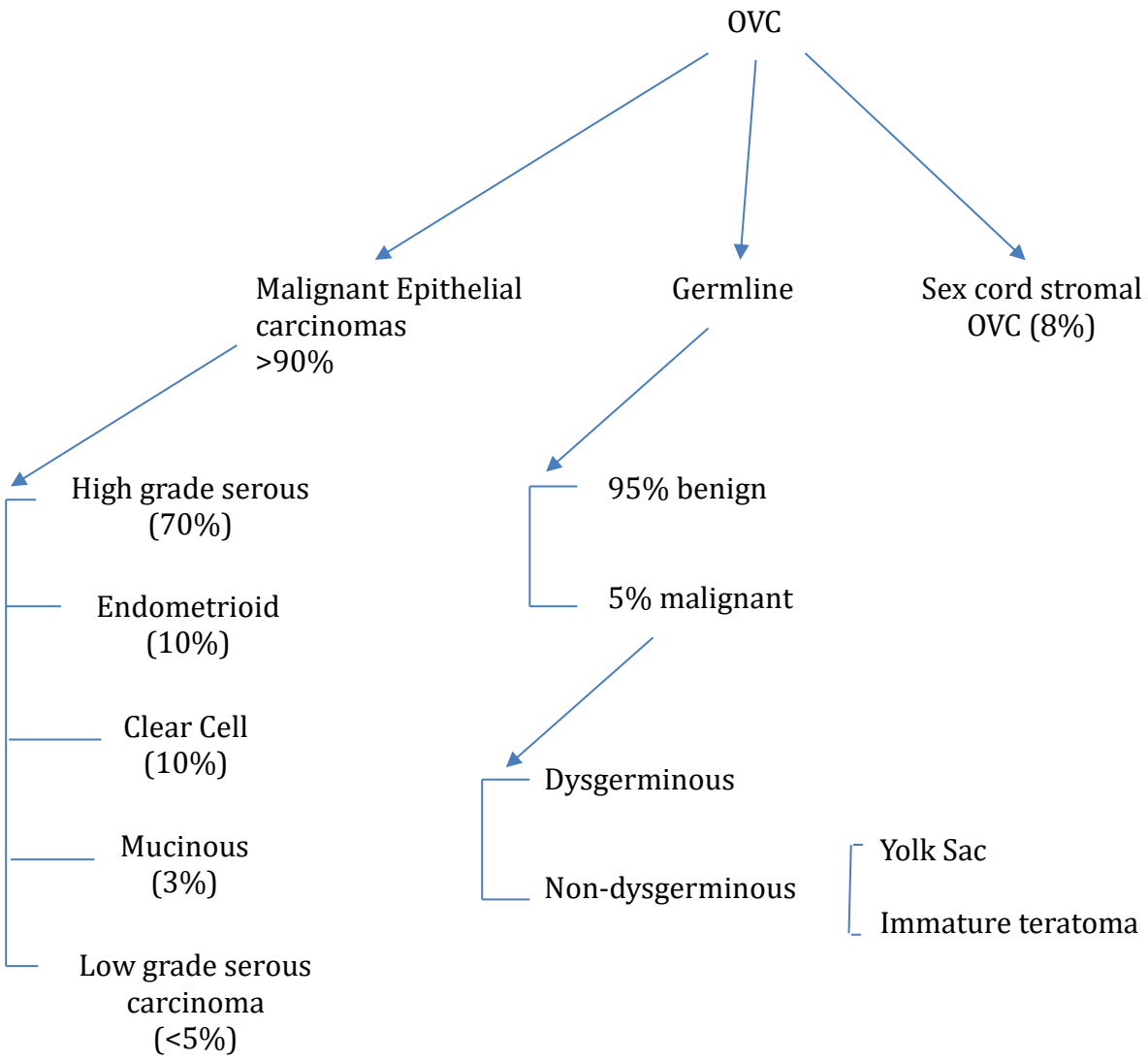

Figure 1.1 Breakdown of the sub-types of OVC. 90% of tumour that affect the ovary are epithelial and 70% of these are serous accounting for the majority of diagnoses. It is believed that high grade serous OVC originate at the far end of the fallopian tube, rather than the surface of the ovary, and then spread to the ovary. Despite the variety of OVC the majority are still treated with chemotherapy and surgery.

Germ cells in the ovaries are the cells which develop into ova. Many germ cell tumours are benign however approximately 5% of the cases are malignant (Low et al, 2000). Malignant tumours are subdivided into dysgerminomas and non-dysgerminomas. Dysgerminomas generally affects both ovaries whereas non-dysgerminomas tends to be restricted to only one.

Germ cell tumours of the malignant dysgerminous and non-dysgerminous type are both found in girls and young women. Types of non-dysgerminomas tumours include yolk sac tumours and immature teratoma. Dermoid cysts are benign tumours also known as mature teratomas. Many germ cell tumours (90%) are treatable even if detected at the later stages since they are mainly found in teenagers and young adults (Crowder, 2004).

Sex cord stromal cell tumours originate from the gonadal stroma and sex cord cells of the ovary which include granulosa cells, theca cells, fibroblasts, Leydig cells and Sertoli cells. About 8% of OVCs are sex cord stromal cell tumours and can affect women of all ages. The more common types are granulosa cell tumours, fibrothecomas and Sertoli-Leydig cell tumours. These tumours are relatively rare and are usually considered to be low-grade cancers, with around 70% of the cases at stage I at presentation.

Another uncommon cancer but very similar to EOC is known as primary peritoneal carcinoma (PPC) which develops from the abdominal lining. This is due to the fact that the cells constitute the lining of the abdomen as the ovary surface. PPC is similar to OVC in its characteristics and development.

1.3.2 Stages of OVC

The International Federation of Gynecology and Obstetrics (FIGO) have updated their definition of the staging system for OVC in 2014 (**Table 1.1**).

Table 1.1. A concise summary of the stages of OVC according to FIGO

Stage	Description
Stage 1	One or both ovaries affected
1a	Cancer contained within one ovary, no external cancer cells present
1b	Cancer contained within both ovaries, no external cancer cells present
1c	As 1a and 1b and on the surface of one or both ovary or burst capsule(s) or cancer cells present in abdomen
Stage 2	One or both ovaries affected and spread to further organs
2a	Spread to the uterus and/or fallopian tubes
2b	Further spreading in pelvic tissue
2c	As 2a and 2b and on the surface of one or both ovary or burst capsule(s) or cancer cells present in abdomen
Stage 3	One or both ovaries affected, or the peritoneum, and extended to the lining of the pelvis and abdomen and/or nearby lymph nodes
3a	Metastasis on the lining of abdomen beyond the pelvis microscopic only
3b	Visible peritoneal metastasis beyond the pelvis <2 cm
3c	Peritoneal metastasis beyond the pelvis >2 cm and/or regional lymph node metastasis
Stage 4	Distant metastasis to other organs

Stage I

The cancer is contained inside the ovaries with no spreading.

Stage IA; the cancer cells have developed in one ovary, and the tumour is limited to the inside of the ovary. Washings from the abdomen and pelvis (taken during surgery) have no cancer cells detected.

Stage IB; the cancer is in both ovaries but still contained within, with no cancer cells on the surface of the ovary. Washings from the abdomen and pelvis show no cancer cells present.

Stage IC; the cancer is present in one or both ovaries as well as any of the following:

i. The tissue surrounding the tumour, known as the capsule ruptures due to surgery leading to cancer cells leaking into the abdomen and pelvis (surgical spill). This is stage IC1.

ii. Cancer is on the outer surface of at least one of the ovaries or the capsule ruptures prior to surgery leading to cancer cells leaking into the abdomen and pelvis. This is stage IC2.

iii. Washings from the abdomen or pelvis show cancer cells present.

Stage II

The cancer is in one or both ovaries and has spread to other organs (such as the uterus, fallopian tubes, bladder, the sigmoid colon, or the rectum) within the pelvis. It has not spread to lymph nodes or distant sites.

Stage IIA: the cancer has spread to the uterus and/or fallopian tubes, but has not spread to the pelvic lymph nodes or distant organs

Stage IIB; the cancer has spread to more intraperitoneal tissues

Stage III

The tumour is in one or both ovaries, or the peritoneum, and has also extended to the lining of the pelvis and abdomen and/or nearby lymph nodes

Stage IIIA1; cancer found in retroperitoneal lymph nodes only by cytological or histological examination

Stage IIIA1(i); Positive retroperitoneal lymph nodes only, with metastasis up to 10 mm

Stage IIIA1(ii); As StageIIIA1(i) however with metastasis greater that 10mm

Stage IIIA2; tumours found external to the pelvis, with or without being present in retroperitoneal lymph nodes

Stage IIIB; peritoneal metastasis beyond the pelvis of up to 2 cm diameter with or without metastasis to the retroperitoneal lymph nodes

Stage IIIC; metastasis of more than 2cm, with or without metastasis to the retroperitoneal lymph nodes and metastasis to the capsule of the liver and the spleen

Stage IV

Distant metastases, excluding peritoneal metastases

Stage IVA; Distant metastases, excluding peritoneal metastases, with pleural effusion determined by cytology.

Stage IVB; Distant metastases, excluding peritoneal metastases, including parenchymal metastases and metastases to organs beyond the abdominal (including inguinal (groin) lymph nodes and lymph nodes outside of the abdominal cavity).

Currently in the UK there is no screening program for OVC therefore diagnosing it requires multiple platforms.

1.3.3 Diagnosis of OVC

In order to check for possible abnormalities in the ovaries, a pelvic examination is carried out by a doctor by inserting gloved fingers into the vagina while applying pressure on the abdomen to feel for any swellings and/or tenderness. Another test known as transvaginal sonography applies sound waves to abdominal tissues using an ultrasound probe inserted into the vagina. This affords clear pictures of the ovaries to detect any masses. However, it is not possible to tell if a particular mass observed is benign or cancerous which can result in healthy women undergoing unnecessary operations. The marker CA125 can be used to judge the efficacy of treatment but it not recommended as a method of screening since it can be increased in a variety of benign conditions such as menstruation or ovarian cysts (Koninckx et al, 1996). All the above methods are non-specific for early stage OVC and if any abnormalities are discovered, further tests are required for confirmation. A combination of CA125 and TVU (transvaginal

ultrasound) have been proven to be inefficient in detecting early stage OVC (Olivier et al, 2006; Woodward et al, 2007).

1.3.4 Conventional treatment of OVC

The highest survival rates for OVC patients are achieved by those who are diagnosed at the early stages of disease (**Figure 1.2**). There is a great impetus to develop early stage screening strategies which will increase the survival rates in patients with OVC.

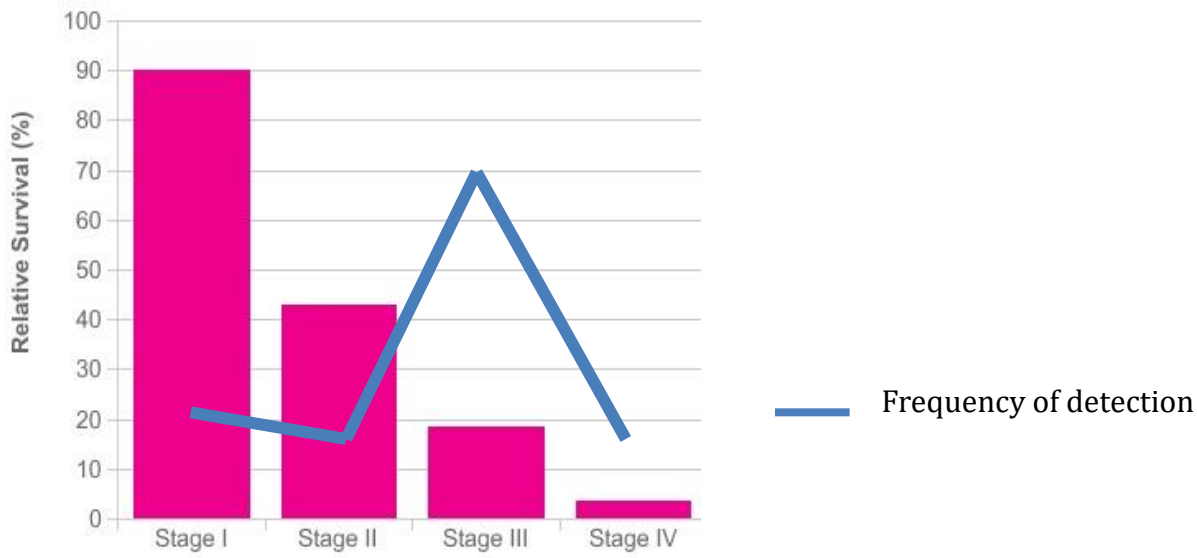

Figure 1.2. **The relative survival of OVC patients depending on the stage of disease at detection and the frequency of detection.** Graph modified from Cancer Research U.K. web site with data from (Berek and Hacker, 2010; Howlader et al, 2016).

Currently the most common treatments are surgery and chemotherapy depending on the stage of disease at diagnosis. At the earliest stage, it may be possible to have only the one ovary removed to effectively eliminate all the cancer cells present. However, it is very rare for OVC to be detected at stage I or II, accounting for 25% and 10% of all diagnoses, respectively (Berek and Hacker, 2010) for OVCs. Early stage diagnosis will usually lead to surgery to remove the

ovaries, the fallopian tubes and the womb in order to ensure that all the cancer is removed. Once the cancer has advanced and spread, surgical intervention can only attempt to reduce the bulk of the disease after which chemotherapy is used to destroy any remaining cancer cells. If the cancer load is too large then chemotherapeutic drugs will be used to reduce the tumour and then surgery is more effective at removing the cancer. Common chemotherapy drugs are carboplatin and paclitaxel (Bois et al, 2003). Olaparib, a poly(ADP-ribose) polymerase inhibitor, was approved by the FDA (Food and Drug Administration) for OVC patients harbouring BRCA mutations who have already undergone chemotherapeutic treatments (Bornstein and Jimeno, 2016). In a phase 3 trial cediranib, an angiogenesis inhibitor, was found to increase progression-free survival (PFS) in relapsed platinum-sensitive OVC, however a number of toxic effects were identified including diarrhoea and hypertension causing termination of the trial (Ledermann et al, 2016). A phase 2 trial studying the combination of olaparib and cediranib in recurrent platinum-sensitive OVC also found an improvement in PFS compared with olaparib alone however the combination was also found to have greater toxicity (Liu et al, 2014).

The monoclonal antibody bevacizumab can be used along with chemotherapy that targets the vascular endothelial growth factor therefore blocking angiogenesis and this lack of blood supply restricts tumour growth (Garcia and Singh, 2013).

To complement conventional therapies, cancer immunotherapy could be used to stimulate the body's own immune system to lyse residual cancer cells post-surgery.

1.4 Cancer and the immune system

The role of the immune system in humans is multifaceted providing a coordinated series of reactions, referred to as immunity, formulated against foreign elements found in the body such as viruses and bacteria, which would otherwise have the ability to cause life-limiting disease.

Immunity can be subdivided into two separate branches that cooperate with each other; innate and acquired immunity. Innate immunity is the first line of defence against all foreign pathogens that cross the skin barrier whereas acquired immunity is specifically targeted against antigens. The immune system is also able to eradicate cells of the human body, which can become cancerous due to genetic mutations or viral infections.

Class I and II MHC (major histocompatibility complex) are present on the surface of nucleated cells and present processed peptides from proteins inside the cell to T cells. MHC molecules are membrane glycoprotein complexes, which have the ability to bind with specific antigenic peptides. Class I is expressed on all nucleated cells whereas class II is only on certain immune cells such as macrophages. Multiple alleles exist for the MHC gene, which guarantees a diverse range of peptides are able to be presented to T cells. T cells can destroy infected cells if peptides in the context of "danger" are detected (Matzinger, 2002). MHC in humans is known as the human leukocyte antigen (HLA) system. MHC class I HLA molecules are highly polymorphic and generally stimulate T cells to provide the best defence against infections.

1.4.1 T cell responses

Mature T cells are able to travel in between the blood and lymphoid tissues prior to being activated and these are known as naïve T cells. The T cells are stimulated for action when they come into contact with an antigen they can recognise, presented on the MHC class I molecules. In relation to the MHC class I pathway, endogenous proteins are fragmented and one of the smaller peptides is bound to the peptide binding site of the MHC class I molecule. This MHC class I-peptide complex travels to the surface of the cell via the Golgi body. T cells expressing a specific TCR (T cell receptor) for the antigen recognises it and forms a TCR: MHC class I-peptide complex. CD8 is expressed on the surface of cytotoxic T lymphocytes (CTL), often referred to as CD8 T cells. The CD8 protein aids the pMHC:TCR interaction by binding to the MHCI molecule. Activation of T cells causes the T cell population to expand in order to mount

an immune response resulting in effector populations of $CD8^+$ CTL and the release of cytotoxic cytokines such as TNF-α and IFN-γ and the granules perforin and granzymes (Mak and Saunders, 2005).

1.4.2 B-cell responses

B cells are part of the adaptive immune response known as humoral immunity. The priming of B cells occurs when the B cell receptor binds a specific antigen on the surface of the B cell. The antigen is internalised by receptor-mediated endocytosis and is processed into smaller fragments of peptides. A fragment is then bound to MHC class II and displayed on the surface of the B cell. This peptide-MHC class II complex is recognised by helper T cells, which also express the CD4 molecule on their surface that is involved in the pMHC class II:TCR complex. Secondary binding of the CD40 receptor on the B cell with its ligand on the T helper cell additionally guides the amplitude of the response. This interaction causes the release of interleukin-2 (IL-2) thereby activating the B cells to mature into plasma cells and producing antibodies that are secreted from the cell against the antigen.

1.4.3 Immune surveillance and immune evasion

The link between immune responses and cancer is evident from findings such as patients with a compromised immune system having an increased tumour incidence (Penn et al, 1971) and cancer patient sera evidencing recognition of autologous cancer antigens (Sahin et al, 1995). Children who have previously been treated for cancer are 10-20 times more likely to develop a second tumour and patients suffering from autoimmune diseases have an increased risk of malignancy (Mueller and Pizzo, 1995). The risk of lymphomas for kidney transplant recipients were 11.8 times greater than healthy controls over a decade follow-up period (Opelz and Dohler, 2004) while HIV patients who have a low $CD4^+$ count have an increased risk of virus causing cancers such as Hodgkin's lymphoma (HL) caused by Epstein Barr Virus (EBV) (Corthay, 2014).

Immune surveillance describes the concept of the immune system being able to identify and eliminate foreign elements, which can cause infections such as bacteria, as well as being able to distinguish cancerous cells from normal cells. It is possible there are cancer cells developing in the body constantly but due to immune surveillance they are destroyed before a noticeable tumour develops.

The immune surveillance principle has been further described by the updated concept of tumour immunoediting (Dunn et al, 2002). Tumour immunoediting is divided into three stages known as elimination, equilibrium and escape. At the elimination stage the immune system identifies and eradicates any precancerous cells which develop in the body. This can lead to either all cancerous cells being destroyed or in some cases some residual cells remain evading the immune system by evolution, developing or having features that hide them from the immune system. Early genetic changes are not required to maintain the cancer phenotype but the tumour cell gathers more and more mutations, through genetic instability, as part of the tumourigenesis process (Roschke and Rozenblum, 2013). Residual tumour cells are involved in the second stage of immunoediting, where equilibrium is achieved because the number of cancer cells being destroyed by the immune system is equal to the number of new cancer cells being made. In this stage tumour cells continue to accrue additional genetic mutations which alters the gene expression profiles of these cells leading to some antigens being overexpressed compared to normal levels in the human body. These mutations can provide tumour-specific antigens that can be ultimately used as biomarkers of disease and/or targets for immunotherapy.

The equilibrium stage manages to control the growth of the tumour by still killing some tumour cells however the tumour will outgrow this stage as it acquires more mutations that help it circumvent the immune system. Immune editing describes the training of the immune system by the tumour, to recognise “were normal” cells as healthy and clonally delete immune cells that would otherwise kill the tumour cells (Chan et al, 2007). Eventually the acquisition of

mutations leads to multiple tumour cell populations (**Figure 1.3**) which can lead to multiple clones and eventually the immune system can no longer cope. Some of the cells can actively circumvent the immune system by suppression and in the final stage, escape is achieved and the tumour grows freely. Mechanisms of immune escape include absence of cancer antigens able to generate an immune response and/or decreased number of MHC class I molecules on the tumour cell surface by virtue of genetic mutation. Induction of T cell anergy, low tumour infiltrating lymphocyte (TIL) numbers and the release of immunosuppressive agents such as IL-10 (Kim and Chen, 2016) can also help tumour cells avoid immune surveillance.

1.5 Immunotherapy

Although conventional treatments can be successful for leukaemia and OVC, on the whole aggressive types and stages are still particularly challenging to diagnose and treat. The future of cancer treatment is currently directed towards immunotherapy, which is seen as the best opportunity for personalised and more effective treatments that could significantly increase survival rates (Schadendorf et al, 2015). Immunotherapy allows the body's own immune system to fight cancer cells and potentially protect against cancer development in the future (Ryan et al, 2016).

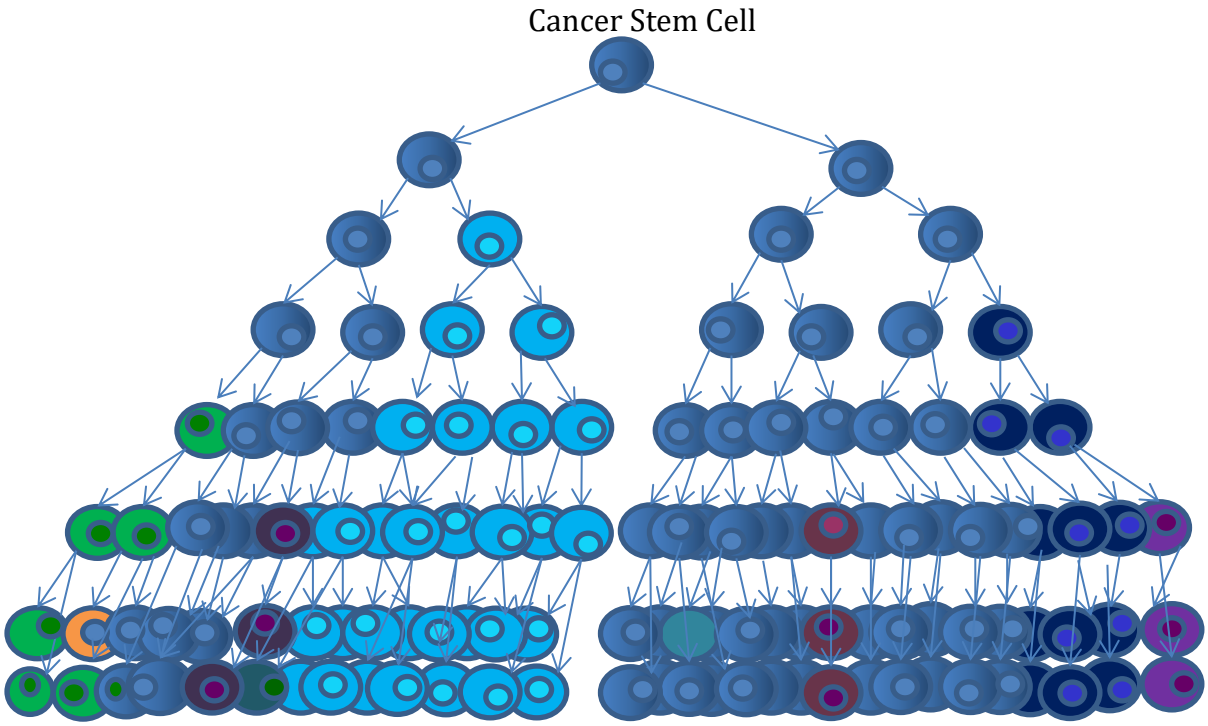

Figure 1.3. Accumulation of mutations generates multiple tumour stem cell derived populations. Different colours visually indicates the change a gene mutation (often caused by genomic instability during tumourigenesis) has on the clonality of the population. The figure shows how rapidly the population can expand within six generations from a single cancer stem cell. The tumour cells evade the immune system by virtue of their cloaking techniques (ie low MHC expression, low tumour antigen expression) and can overwhelm the immune system with their numbers once the tumour load is great enough.

The ideal immunotherapy targets should play a role in tumour progression (Zhang et al, 2009). To optimise the quest for tumour antigens and focus studies on a limited number of antigens, Cheever et al (2009) identified the nine characteristics of an ideal tumour antigen and listed them in order of importance (1 being the most important): (1) there should be some therapeutic benefit of the antigen compared to no antigen; (2) the antigen should be immunogenic i.e. be able to generate a response from immune cells; (3) the progression of the tumour should include a role for the antigen; (4) the antigen should be specific for the cancer and not be expressed in normal cells; (5) the antigen should have high expression levels in the positive cells; (6) be expressed in cancer stem cells; (7) tumours positive for the antigen should be in a significant number of patients; (8) availability of immunogenic epitopes and (9) intracellular location of antigen (Cheever et al, 2009). For example, p53 (Soussi, 2000) is one of the most desirable targets for immunotherapy – targeting p53 can kill both the evolving tumour cell population and any cancer "stem" cell which harbours this as an early tumourigenesis stage aberration. By targeting p53 you prevent its support of further tumour growth. In addition, a number of tumour antigens have been shown to be useful biomarkers for cancer diagnosis (Haralambieva et al, 2000), disease stage (Guinn et al, 2007) and survival (Guinn et al, 2009).

1.5.1 Strategies for immunotherapy

One of the biggest debates in cancer immunotherapy remains which approach will be the most effective. Although a great deal of work takes place in research labs which generates promising

preclinical data, these data do not generally emulate the results obtained in clinical trials. Despite their common pre-clinical use, mouse models are not always able to elucidate possible side effects of the agents being tested, since the variability of the patient's characteristics such as age and weight cannot all be mimicked in mice (Klevorn and Teague, 2016). In 1999 Jesse Gelsinger's death led to a halt in many immunotherapy clinical trials in the US and led to an extensive review of practices in immunotherapy clinical trials. In 2004 Rosenberg et al (Rosenberg et al, 2004) surveyed the data collected from clinical trials on 440 patients with metastatic cancer, all of whom had been treated in the Surgery Branch of the National Cancer Institute. The study showed that only 2.6% of immunotherapy clinical trials had worked and this reflected the experiences of other similar Research Institutions.

In 2005 Peng et al described the first immunotherapy clinical trial that used Ad (Adenovirus)-p53 in China and its success renewed interest in immunotherapy. In 1995 Ad-p53 a clinical trial involved 135 head and neck squamous cell carcinoma patients. 75% of the participants had late stage disease and previous treatment had not been successful. Split into two groups the test group received Ad-p53 and radiotherapy while the control group only received radiotherapy. The test group had CR and PR rates of 64% and 29% respectively while the control group had 19% CR and 60% PR ($p<0.01$). The data generated by this trial led to the approval of Ad-p53 under the commercial name of Gendicine by the China Food and Drug Administration for use in head and neck cancer patients (Peng, 2005; Roth, 2010). A number of reviews now chart the development and exciting prospects of cancer immunotherapy (Devaud et al, 2013; Rosenberg, 2014; Schumacher and Schreiber, 2015; Yang, 2015) and in 2013 Science named Cancer Immunotherapy the Breakthrough of the Year (Couzin-Frankel, 2013).

When T cells were found to be able to recognise and kill cancer cells (Wolfel et al, 1989), it was thought that T cell therapies would be the most effective form of immunotherapy. This is

due to our belief that T cells have an exquisite specificity for epitopes within tumour antigens and are able to effectively kill cancer cells in a controlled manner. CTLs can be stimulated in a number of ways such as through the use of dendritic cells (DCs) (Zizzari et al, 2011), peptide vaccines (Bae et al, 2012), DNA vaccines (Nguyen-Hoai et al, 2012) and natural killer cells (Anderson et al, 2012).

DCs are APCs that are able to cross present by ingesting and processing extracellular antigens and presenting them on MHC class I molecules (Nierkens et al, 2013), therefore they have received a lot of attention for their potential use in cancer immunotherapy. DC therapy involves extracting the patient's own monocytes and activating them to DCs, a process of maturation that requires cytokine stimulation and the feeding of cancer antigens to the DCs. The DCs are then injected back into the body in order to stimulate the immune system to eliminate the antigen expressing cancer cells (Sabado and Bhardwaj, 2013). A patient who relapsed twice, was given DC therapy between January (when she relapsed for second time) and the following August during which time the disease remained stable (for 9 months) until further progression and death. An advantage of DC therapy was that there were no adverse effects observed locally or generally (Massumoto et al, 2008). Alternatively, DCs pulsed by peptide and injected into the skin led to a response rate of 28% in patients. This percentage increased to 35.7% when immature DCs are injected straight into the tumour and even higher to 40% for advanced pancreatic cancer (Nakamura et al, 2012).

When a tumour antigen is secreted into the circulation in high levels, immune tolerance can be induced in the thymus. $CD8\alpha^{-}Sirp\alpha^{+}$, a subset of DCs, are able to capture tumour antigens in the blood, which can induce tolerance through a direct interaction with Tregs or negative selection. Tregs are cells which are part of the tolerance system which prevents autoimmunity (Pacholczyk and Kern, 2008; Baba et al, 2012b). Simultaneous Treg depletions (using anti-

CD25 antibodies for instance) may aid the effectiveness of immunotherapy in some cancer types where Treg infiltration into the tumour is rife (Jing et al, 2011; Baba et al, 2012a).

Monoclonal antibodies are used to treat a number of cancers including low-grade or follicular non-Hodgkin's lymphoma (NHL) and CLL through treatment with rituximab, which is a CD20 specific antibody. Rituximab targets CD20 present on the surface of the B cells including the malignant NHL and CLL cells (Yang et al, 1999). The VEGF inhibitor Bevacizumab is a humanised IgG1 antibody which blocks angiogenesis therefore restricting a tumours ability to gain a blood supply. It has been shown to be effective in a number of cancers including OVC, where 16–21% of patients with relapsed OVC responded (Garcia and Singh, 2013) while efficacy was shown in colorectal cancer (CRC) (McCormack and Keam, 2008) and glioblastoma (Carter et al, 2016).

It is likely that the best strategy for the effective treatment of cancer, where antibody therapies are not the answer, may include a combination of conventional and immunotherapy techniques (Peng, 2005) or even a combination of immunotherapy techniques as demonstrated in increasing numbers of mouse models (Bose et al, 2012) and clinical trials (Karan and Van Veldhuizen, 2012; Ciccarese et al, 2016; Head et al, 2016). Subsequently adoptive T cell therapy has been shown to be very promising with the number of cells being returned to patients (Gattinoni et al, 2005) and their status – activated but not matured (Klebanoff et al, 2011), being the main considerations. TIL therapy has been used to treat patients with stage IV melanoma. TILs are obtained from the blood, lymph nodes or from a tumour tissue biopsy. TILs are isolated, activated and expanded using IL-2 *in vitro*. The patient undergoes lympho-depleting chemotherapy prior to the T cells being injected back in to the blood (Kvistborg et al, 2012).

A recent and promising therapy approach has been in the area of checkpoint inhibitors. The immune checkpoints cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed

death 1 (PD-1) are on the surface of activated T cells and are negatively regulating receptors. CTLA4 diminishes the role of the receptor CD28 in the activation of T cells by competing for its ligands (Rudd et al, 2009). PD-1 surface receptor moderates T cells in the periphery during an immune response and prevents response to self antigens. When it interacts with its ligand, PD-L1, it prevents T cell proliferation and cytokine release (Freeman et al, 2000). A significant rise in $CD8^+$ TILs was observed when PD-1 was blocked by antibody with improved IFN-γ secretion (Kodumudi et al, 2016).

There are a number of excellent reviews in this area of research that aim to identify and discuss effective immunotherapy strategies for the future. These include cellular immunotherapy (Smits et al, 2011), whole cell vaccines (Keenan and Jaffee, 2012), multidrug resistance (Curiel, 2012), DCs (Palucka and Banchereau, 2012), oncolytic viruses (Guo et al, 2015) and nanotechnology (Goldberg, 2015). Targeted therapeutic strategies along with ever improving designs in clinical trials pave the way for further success (Mellman et al, 2011).

A combination therapeutic approach has for a long time been seen as the best line of attack against cancer, using conventional therapy to reduce the tumour load and immunotherapy to removal residual and at times, dissipated disease.

In addition, combinations of immunotherapy could further enhance survival, reducing residual disease where there are escape variants and where the cancer is heterogeneous in its targets. Combining the antibodies anti-CTLA-4 and anti-4-1BB revealed $CD8^+$ immune responses against advanced MC38 tumours as well as establishment of memory T cells. Combination treatments reduced autoimmunity in comparison to a single antibody therapy (Kocak et al, 2006) and often offer an opportunity to eliminate escape variants. Combination therapy could be the answer for drug resistant tumours as the resistance mechanisms of the tumour can be identified and targeted alongside. Two cell lines (breast and gastric cancer) resistant to

sacituzumab govitecan became susceptible to it through the use of an ATP-binding cassette (ABC) transporter inhibitor used in combination with antibody treatment (Chang et al, 2016). ABC transporters can cause drug resistance by efflux-removal of the drug from the cell (Borges-Walmsley et al, 2003).

Promising combination therapies utilising antibodies include Lapatinib with trastuzumab in Her2 positive breast cancer (Baselga et al, 2012), Dabrafenib and Trametinib in relapsed OVC (Robert et al, 2015), carboplatin and pemetrexed in advanced non-small cell lung (NSCL) cancer (Zukin et al, 2013), pidilizumab and rituximab in follicular lymphoma (Westin et al, 2014), albumin-bound paclitaxel and gemcitabine in pancreatic cancer (Von Hoff et al, 2013), nivolumab and ipilimumab in untreated metastatic melanoma (Larkin et al, 2015), cisplatin and topotecan or cisplatin and gemcitabine in advanced CC (Leath et al, 2013) and bevacizumab plus oral capecitabine plus irinotecan in metastatic colon cancer (Ducreux et al, 2013).

1.5.2 The role of immunotherapy to remove MRD from leukaemia patients in remission

Minimal residual disease (MRD) refers to the number of cancer cells present in a patient's body, particularly in leukaemia patients where MRD tends to be dissipated rather than discretely located. Treatment for leukaemia is often successful however, recurrence is seen in about 50% of younger patients and 90% of older AML patients (Schlenk and Döhner, 2013) and MRD provides a way to predict relapse 2-3 months prior to the development of symptoms (San Miguel et al, 1997). AML patients with cytogenetics abnormalities such as t(8;21) and t(15;17) are 80% more likely to relapse than those with normal karyotypes in their blasts (Savani, 2010) and patients with the translocation t(8;21) were found to relapse more than once while patients with the t(15;17) translocation showed only one relapse (Garson et al, 1989). Death of patients with leukaemia are generally due to disease relapse and patients in first complete remission who are positive for MRD prior to hematopoietic cell transplantation

(HCT) were more likely to die (2.61 times) or relapse (4.9 times) a second time than patients who were MRD negative (Walter et al, 2013).

Immunotherapy, the stimulation of a patient's immune system to kill diseased cells, provides a possible method to remove MRD from cancer patients in first remission, when the burden of disease is low. In addition, immunotherapy should be specific to the diseased cells unlike other conventional treatment options (Liu and Kline, 2013).

1.5.2.1 Importance of T cell responses in controlling cancer

In the 1890's Dr William B Coley, the "Father of Cancer Immunotherapy", realised that cancer patients who had contracted acute bacterial infections could concurrently have a reduction in their tumour size. Dr Coley went on to successfully treat a patient with an inoperable malignant tumour by injecting live bacteria into the mass thus establishing the field of cancer immunotherapy (Nauts et al, 1946). It has been known that the immune system is able to fight against cancer with evidence for this from patients who are immunocompromised by transplantation, HIV and severe combined immunodeficiency syndrome, all of whom have an increased incidence of developing cancer (Penn, 1988). Expansion of tumour-specific T cells can be introduced into cancer patients in the form of vaccines targeting TAAs or by adoptive T cell therapy, thereby improving the eradication of cancer cells by the immune system (Dougan and Dranoff, 2009). Therefore, it is important to understand which TAAs are being expressed by tumour cells, and the TAA-specific T cells available within patients to respond to them, in order to determine if a particular immunotherapy treatment could reasonably be expected to be effective. Cancer immunology involves understanding the mechanisms of actions of the pathways that are important for immune surveillance and tumour rejection to get a better insight into how they can fail. To this end, immunotherapy, which boosts the patient's own immune system to recognize and eradicate cancer cells, is believed to hold the most potential for a life-long cure (Finn, 2012). A number of leukaemia associated antigens (LAAs)

have been found in recent years including B melanoma antigen (BAGE) (Boel et al, 1995), Preferentially Expressed Antigen in Melanoma (PRAME) (Ikeda et al, 1997), Receptor for Hyaluronan Mediated Motility (RHAMM) (Greiner et al, 2002) and WT1 (Call et al, 1990). Some LAAs have been found to be recognised by antibodies in leukaemia patients at disease presentation such as PASD1, SSX2IP and Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate-Like 1A Combined Protein (GRINL1A) (Guinn et al, 2005) and RHAMM (Greiner et al, 2006). LAAs have been shown to be associated with clinical outcome in AML patients e.g. DNA microarray analysis of 116 AML patients showed that increased expression of G250 mRNA was linked with longer overall survival (P = 0.022). LAAs such RHAMM and Survivin have been shown, *in vitro*, to be associated with an increase in the cellular proliferation of leukemic blasts. In contrast to this *in vitro* function, elevated expression of LAAs such as SSX2IP and PRAME were found to be associated with an improved clinical outcome in AML (Greiner et al, 2008).

1.5.2.2 Antigen-specific T cells

When a healthy cell starts to undergo the process of tumourigenesis it will express antigens, at levels or with mutations, which were not expressed previously (van Bruggen et al, 1991). These are known as TAAs. TAAs can be used as targets for immunotherapy, allowing scientists to stimulate the immune system to kill tumour cells (Khodadoust and Alizadeh, 2014). As conventional treatments can induce remission in many AML patients, then immunotherapy has the potential to kill residual tumour cells, MRD, and prevent or delay relapse (Barrett and Le Blanc, 2010).

Since TAAs are self-antigens which are expressed abnormally in tumour cells they are targeted by the adaptive branch of the immune system. The idea that cancer cells express antigens that are specific to them has been known for many years starting with the work of the Boon group who identified the cancer-testis antigen (CTA) MAGE-1, a melanoma antigen (van Bruggen et

al, 1991). Studies showed that T cells were able kill cancer cells. They showed that they could expand TILs using IL-2, inject the TILs into mice with metastatic tumours and they demonstrated that these TILs were up to 100 times more effective at killing cancer cells than lymphokine-activated killer (LAK) cells (Rosenberg et al, 1986). Indeed melanoma TILs were better at killing autologous tumour cells when compared to allogeneic TILs (Muul et al, 1987) and 6/13 melanoma patients who received TILs specific for the TAA MART-1 along with high dose of IL-2 were observed to achieve tumour regression of MART-1 positive tumours (Dudley et al, 2002).

Tumour antigens provide a promising target for immunotherapy, as a strategy in the quest to fight cancer, and require further investigation since few antigens have been identified in some of the most difficult to treat cancers such as adult leukaemia and OVC. To date immunotherapy clinical trials have had a range of efficacy showing both potential and a need for further investigation to optimise treatments especially in combination with conventional therapies (Dudley et al, 2002; Khodadoust and Alizadeh, 2014; Sterman et al, 2015) and each other (recently reviewed in acute leukaemia by Ishii and Barett, 2016 and in OVC by Coukos et al, 2016).

Our interest is in identifying the tumour antigens targeted by T cells in acute leukaemia patients and novel antigenic targets for OVC where few immunotherapeutic targets have been found to date.

1.5.3 The identification of tumour antigens as targets for immunotherapy

SEREX (Sahin et al, 1995), SERPA (Klade et al, 2001) and peptide elution from MHC for mass spectrometry analysis (Castelli et al, 1995) are commonly used to identify antigenic targets in a cancer type. There are of course pros and cons to each including cost and labour intensity. Most of the focus concerns tumour types which lack suitable targets for

immunotherapy and for which conventional or current therapies still do not overcome poor survival rates (≤50% over 10-years post-diagnosis). An additional major benefit of new antigen identification is that such antigens can provide invaluable insight into disease mechanisms (Guinn et al, 2008), act as biomarkers of disease stage (Guinn et al, 2006) and predict survival rates at disease presentation (Greiner et al, 2006; Guinn et al, 2007b; 2009; Liberante et al, 2013).

The quest to identify novel antigens has been the focus of a number of studies over the last four decades (reviewed in Vigneron et al, 2013; Lu & Robbins, 2016). Autologous typing had been used to identify antigens including alpha fetoprotein in hepatoma and germ cell tumours, carcinoembryonic antigen (CEA) in gastrointestinal cancers, prostate-specific antigen in prostate cancer, cancer antigen 125 (CA-125) in OVC and AU in melanomas (reviewed in (Old, 1981; Thomas and Sweep, 2001). However this technique has limitations, including a requirement that the tumour cells under examination can be cultured *ex vivo*. The recognition of antigens by low titre antibodies in patients often prevents their further characterisation.

1.5.4 The quest to identify CTAs

Tumour antigens are classified into the following categories: CT, mutational, differentiation, amplified/overexpressed, splice variant and viral antigens (Tureci et al, 1999). CTAs show restrictive expression, their presence only in tumours and in testis rendering them very attractive therapeutic targets. The testis is an immunologically protected site i.e. lacking in MHC class I expression (Chen et al, 1997), therefore targeting CTAs should not lead to catastrophic auto-immune responses against healthy tissue (Scanlan et al, 2004). Few CTA have been identified in OVC, however I have reviewed the most notable relevant to OVC and acute leukaemia, and therefore this thesis, in **Section 1.7**.

The Boon group developed representational difference analysis and were successful in identifying a number CTAs predominantly in melanoma patient samples, including the MAGE family of CTAs, and one antigen from renal cancer, RAGE (Martelange et al, 2000; Tyson et al, 2002). In 1995 Sahin et al. (Sahin et al, 1995) described the use of the SEREX which could identify antigens in a range of cancer tissues (Sahin et al, 1995; Tureci et al, 1999). This technique uses patient immunoglobulins (IgG) from peripheral blood sera to immunoscreen cDNA from tumours, cell lines or normal testis tissues in the form of polypeptides on the capsid surface of the phage. There are over 2,000 tumour antigens detailed in the Cancer Immunome database (http://ludwig-sun5.unil.ch/CancerImmunomeDB) each identified using the SEREX technique (Tureci et al, 2005). SEREX was validated by the identification of known antigens such as synovial Sarcoma X2 (SSX2) (Tureci et al, 1996), mutated p53 (Scanlan et al, 1998) and the AKT oncogene (Obata et al, 2000).

To maximise the likelihood of finding CTAs, cDNA libraries made from healthy testis cDNA, which also benefit from a wide range of gene expression due to global promoter hypomethylation and their rapid proliferation (compared to many other tissues), have been immunoscreened with patient sera. This has led to the identification of a number of CTAs including cTAGE-1, NY-ESO-1, SSX2 (Jager et al, 1999; Eichmuller et al, 2001) and PASD1 (Liggins et al, 2004b; Guinn et al, 2005). One-third of all antigens identified by SEREX were found to be novel and many have progressed from bench-to-bedside as the focus of clinical trials, most notably those targeting NY-ESO-1 positive tumour cells (von Boehmer et al, 2013; Sonpavde et al, 2014).

Other methods for the identification of tumour antigens, with relevance to this thesis include:-

1.5.4.1 The in silico identification of TAAs and the verification of their expression using reverse transcription – polymerase chain reaction (RT-PCR) and real time PCR (RQ-PCR)

The identification of tumour antigens through the mining of online data has provided a rich resource of known antigens. Their verification through RT-PCR and RQ-PCR has confirmed the expression of a number of previously identified TAAs in a range of solid and haematological malignancies (Adams et al, 2002; Forgber et al, 2009; Krackhardt et al, 2002; Qian et al, 2007; Wang et al, 2009). Although this has provided important antigen expression information and a good starting point to identify potential antigenic targets in a range of cancers, these studies are entirely limited to tumour antigens that had already been identified.

1.5.4.2 cDNA microarrays

The differential expression of tumour antigens and/or protein biomarkers between cell and disease subtypes have been directly compared on cDNA microarrays and has allowed our improved understanding of lymphomas (Nishiu et al, 2002) and aided our development of personalised therapies (Brennan et al, 2007). Microarray technology is able to distinguish between different subtypes of a particular cancer as well as identify the expression of novel antigens (De Pitta et al, 2005). Minimal residual disease is a very important tool in the detection of impending relapse in patients who have had some form of treatment. Markers for minimal residual disease in acute lymphocytic leukaemia were identified by gene profiling (Chen et al, 2001b). cDNA microarray has been used to identify the frequency of elevated tumour antigen expression in AML (Guinn et al, 2005) and also associations between specific cytogenetic abnormalities and relative levels of tumour antigen expression (Guinn et al, 2008). Microarrays have also been used to elucidate the possible function of tumour antigens such as SSX breakpoint 2 Interacting Protein (SSX2IP) in a sub-group patients harbouring cytogenetic abnormalities such as t(8;21) associated with mitotic spindle failure and the association between the elevated expression of some tumour antigens (SSX2IP, RHAMM and

SURVIVIN) at disease presentation and patient survival (Guinn et al, 2009) in AML.

1.5.4.3 Mass Spectrometry

Mass spectrometry involves the analysis of peptides eluted from the MHC of antigen presenting cells (Dutoit et al, 2012; Knights et al, 2006; Stickel et al, 2009) or proteins in serum (Mohamedali et al, 2009). This area is reviewed more completely by Hillen and Stevanovic, 2006 and Stern, 2007. Mass spectrometry has demonstrated that as many as 10,000 different peptide species are presented by individual class I MHC alleles (Zarling et al, 2000). The technique, its strengths and limitations are extensively reviewed by Yates et al, 2009.

1.5.4.4 Protein microarrays

Protein microarrays involve the immunoscreening of protein arrays (approximately 9,000 full length proteins and functional domains) which may be purchased from companies such as Invitrogen, Functional Genomics or Cambridge Protein Arrays. Antibodies in sera from patients (Gunawardana and Diamandis, 2007; Chen and Snyder, 2010; Life Technologies, 2014) can be detected using generic secondary antibodies (fluorescently conjugated anti-human IgG) and visualised on microarray scanners.

1.5.5 Have we identified enough tumour antigens?

The debate now focusses on whether we have found enough tumour antigens. Supported by the National Cancer Institute, Cheever et al. (Cheever et al, 2009) reported a short-list of antigens which demonstrated properties and should be funded by agencies to ensure a limited number of the most promising targets were realised in immunotherapy clinical trials (detailed in **Section 1.5**). However in some cancers, and most notably haematological malignancies, few of the antigens on this short-list were expressed with a frequency that would justify targeting them in clinical trials (reviewed by Guinn, 2015). For some cancers, effective immunotherapy targets have not been identified (i.e. OVC and adult acute lymphocytic leukaemia) and better

targets that may also help us better understand the biology of the disease and provide biomarkers. The antigens that have already been identified in acute leukaemia and OVC and relevant to my studies are reviewed in **Sections 1.6 and 1.7**.

1.6 Tumour associated antigens

By definition tumour associated antigens are recognised by the immune system by virtue of their differential expression in cancer cells compared with healthy cells. They differ from CTAs in that CTAs have a unique property of expression, which is restricted to cancer cells and immunologically protected MHC class I negative tissues, such as the testes and placenta. Other healthy tissues show no or very little expression. This section describes the main tumour antigens I have investigated in the studies described in this thesis and provides background information as to why they were chosen for my studies.

1.6.1 Proteinase 3

Proteinase 3 (PR3) is a serine proteinase present in the primary granules of neutrophils and monocytes. It has been found to be involved in Granulomatosis with polyangiitis, formerly called Wegener's granulomatosis, a vasculitis disease that causes inflammation of the blood vessels mainly affecting lungs, kidneys and sinuses (Niles et al, 1989). Using immunocytochemistry (ICC) and flow cytometry PR3 was detected in AML and CML patient's bone marrow but not in ALL or CML patient samples (Dengler et al, 1995). A PR3 HLA-A2-restricted peptide was recognized by CTL that killed leukaemia cells from HLA-A2 patients (Molldrem et al, 1996) and a novel peptide derived from PR3, restricted to HLA-B*1510 positive patients, was found in isolated leukocytes from a CML patient (Knights et al, 2006).

1.6.2 SSX2IP

SSX2IP was discovered using a yeast two-hybrid screening to identify proteins that may react with SSX2. Using SSX2 as a probe, SSX2IP was revealed as a partner (de Bruijn et al, 2002).

It is thought that SSX2IP regulates the function of SSX2 in the testes and malignant cells (de Bruijn et al, 2002).

The SSX2IP gene is located on chromosome 1p22.3 (Maglott et al, 2007) and includes over 46 kb and consists of 14 exons however the first one is not translated (de Bruijn et al, 2002). The gene contains 33 introns. 18 different mRNAs are produced; 17 spliced and 1 un-spliced form (Thierry-Mieg and Thierry-Mieg, 2006). SSX2IP in rodents is known as the afadin DIL domain-interacting protein (ADIP) (Asada et al, 2003) and in chickens is named light-inducible and clock-controlled gene (LCG) (Hatori et al, 2006).

SSX2IP was identified as a leukaemia associated antigen through SEREX immunoscreening of a testes cDNA library and was shown to be preferentially recognized by sera from AML patients when compared to normal donor sera. RT-PCR showed that SSX2IP was expressed in 33% of presentation AML patient samples, with no expression in normal donor haematopoietic samples (Breslin et al, 2007; Guinn et al, 2005). In mice, ADIP has been shown to interact with afadin (Asada et al, 2003), the human equivalent of AF-6. AF-6 may be involved in signal transduction at special cell–cell junctions (Prasad et al, 1993). Microarray analysis indicated that SSX2IP was expressed at lower levels in AML patients harbouring a t(8;21) translocation. This translocation is linked with neutralizing the spindle checkpoint leading to the higher levels of aneuploidy seen in this sub-group of AML (M2) patients. SSX2IP expression levels, and those of a number of genes involved in the cell cycle, were found to be concurrently elevated in acute promyelocytic leukaemia harbouring the t(15;17) translocation (Denniss et al, 2007).

Dennis et al (2007) investigated whether the low frequency of SSX2IP-positive cells observed by ICC and ≤16% expression detected by surface staining in flow cytometry was due to a cell cycle-related expression. Human myeloid cells were blocked at G0 phase of the cell cycle and SSX2IP expression peaked at 24-26 hours after release. Blocking the same cells at the G1/S

interface showed expression peaked at 14-17 hours on the cell surface. This peak expression of SSX2IP during mitosis was confirmed by confocal microscopy (Denniss et al, 2007).

1.6.3 Survivin

Survivin is a member of inhibitor of apoptosis (IAP) family and is encoded by the Baculoviral IAP Repeat Containing 5 (*BIRC5)* gene. The human survivin gene is located on chromosome 17q25 and is 14.7 kb (Ambrosini et al, 1997). It has been found to be mainly expressed during fetal development in undifferentiated tissues, regulating the cell cycle by blocking apoptosis via caspase 9, which is activated in both extrinsic and intrinsic pathways (LaCasse et al, 1998). This implies survivin is erroneously activated in tumour cells. Survivin has also been observed in G2/M in a cell cycle-dependent manner and is capable of binding to mitotic spindle microtubules suggesting checkpoint regulation (Li et al, 1998).

Survivin has been identified in a wide variety of cancers including lung adenocarcinoma, squamous lung cancer, breast, prostate, pancreatic and colon carcinomas (Ambrosini et al, 1997), soft tissue sarcomas (Kappler et al, 1997) and malignant glioma (Chakravarti et al, 2002). Analysis of NHL revealed 70-90% expression of survivin in 55% of high grade lymphomas while no expression was detected in low grade lymphomas (Ambrosini et al, 1997).

Recent studies have suggested that survivin may not be specific to cancer cells but some normal cells may also express it at lower levels. Studies have detected survivin in normal adult cells, such as basal keratinocytes (Dallaglio et al, 2014), human cord blood cells $CD34^{+}$, T cells (Fukuda et al, 2002), vascular endothelial cells (Mesri et al, 2001) and erythroid cells (Gurbuxani et al, 2005). Any disturbance in the expression of survivin could cause antagonistic effects on all or any of these cells.

1.6.4 Tyrosinase

Tyrosinase was detected and identified in melanoma cells when CTLs that had been isolated from HLA-A2 patients were shown to recognise and lyse tyrosinase positive tumour cells (Brichard et al, 1993). Tyrosinase is expressed in normal melanocytes such as skin and mucous membrane but not in other normal tissue (Jim'enze et al, 1988). $CD4^+$ T cells obtained from the peripheral blood of a melanoma patient were found to be reactive to synthetic peptides derived from tyrosinase. Clones were able to recognize the tyrosinase peptide p386-406 when bound to the HLA-DR15 (DRB1*1501) molecule (Kobayashi et al, 1998). However tyrosinase expression was found to be lacking in melanoma metastases in comparison to gp100 and MART. This absence seems to be related to the amount of infiltrating $CD8^+$ and $CD4^+$ T cells implying that the immune cells mount a response against cells expressing tyrosinase (Bartlett et al, 2014).

1.6.5 Wilms tumour 1 (WT1)

A mutation in the WT1 tumour suppressor gene inactivates it, leading to the development of Wilms' tumour of the kidney (Haber et al, 1990). It encodes a protein with four C-terminal Zinc-fingers characteristically found in transcription factors (Hohenstein and Hastie, 2006). WT1 genetic mutations are found in many syndromes: Denys-Drash syndrome (Pelletier et al, 1991), Frasier syndrome (Klamt et al, 1998) and WAGR (Wilms' tumour, aniridia, genitourinary malformations, mental retardation) syndrome (Gessler et al, 1990). In some malignancies the detection of minimal residual disease can be based on WT1 transcript levels, these include acute leukaemia (Inoue et al, 1994), desmoplastic small round cell tumours (Lae et al, 2002), breast cancer (Silberstein et al, 1997), de novo lung cancers (Oji et al, 2002), and the differentiation of retinoblastoma cells (Wagner et al, 2002). Due to post-transcriptional modifications there are 24 different isoforms of WT1 mRNA known, each with distinct functions (Wagner et al, 2003b).

Of a cohort of 73 patients, immunoglobulins IgM and IgG WT1 antibodies have been found in 55% of haematological cancers and together were detected in 33%. While out of 43 healthy volunteers 16% had IgM, 5% IgG and none had both (Elisseeva et al, 2002). Patients who achieve greater overall survival and longer constant remission are observed to have a higher number of WT1-specific T cells compared to relapsed patients (Casalegno-Garduño et al, 2016). T helper type 1 WT1 antibodies of the IgG1, IgG2, and IgG3 sub-classes were significantly higher in leukaemia and myelodysplastic syndrome patients' blood than in healthy volunteers (Wu et al, 2005).

1.7 CTAs

The restricted expression of CT antigens to healthy MHC class I-deficient germline cells makes them appealing targets for immunotherapeutic strategies because they provide tumour-specific antigens for MHC class I-restricted $CD8^+$ T cells (Smith and McNeel, 2010). Developing immunogenic cancer vaccines that target these antigens has become a priority in how cancer is diagnosed and treated. Boon and colleagues were the first to clone a human tumour antigen named melanoma antigen-1 or MAGE-1 (van der Bruggen et al, 1991). Subsequently other CT antigens were discovered by the group namely BAGE and GAGE gene families. Common characteristics to CT antigens include mostly being encoded by multigene families, often mapping to the X chromosome, having their expression level epigenetically regulated with drugs such as 5-aza-2-deoxycytidine and although the functions of many are still unidentified they are known to be involved in tumourigenesis (Smith and McNeel, 2010).

1.7.1 HAGE

In 2002 Adams et al investigated the expression of 10 CT antigens in presentation 26 AML and 42 CML. They found little or no expression of MAGE-A1, -A3, -A6, -A12, BAGE, GAGE, LAGE-1, NY-ESO-1 and RAGE. However, in contrast to previous studies (Martelange et al, 2000) they found that HAGE was expressed in 57% of the CML patient samples examined and

23% of AML patient samples by RT-PCR, and confirmed by Q-PCR. HAGE was found to be induced in a dose dependent manner by 5-aza-2′-deoxycytidine (Stankovic et al, 2008) and detectable by qPCR in 14.8% (11/74) AML patients (Chen et al, 2011).

TMAs with 16 different tumours on them exhibited expression of HAGE protein in 75% of cancerous tissues including liver, kidney and stomach, whereas no or very little expression was found in healthy tissues (Mathieu et al, 2010). Silencing the HAGE gene in melanoma decreases the RAS protein expression, which in turn leads to a reduction in the activation of the AKT and ERK signalling pathways, resulting in inhibition of tumour growth (Linley et al, 2012). HAGE appears to be a marker for poor prognosis in breast cancer since high expression of HAGE was linked to aggressive disease ($p<0.01$) and poor survival ($p<0.001$) (Abdel-Fatah et al, 2014). HAGE is part of the DEAD-box RNA helicases which implies that its function may include RNA metabolism in malignant cells (Riley et al, 2009).

1.7.2 MAGE family

MAGE-A4 expression was studied in 74 patients with ovarian tumour, including 10 with serous cystadenomas, 11 with serous tumours of borderline malignancy and 53 with serous carcinomas. Fourteen patients were stage I or II and 39 were stages III or IV. Using immunohistochemistry (IHC) MAGE-A4 expression was found to be present in 30/53 (57 %) of the serous carcinomas and in 1/11 (9 %) of the serous tumours of borderline malignancy but no staining was detected in the normal ovary. Kaplan-Meier survival curves were constructed, followed by the log rank test to determine MAGE-A4 expression and survival. A significant inverse correlation was found between MAGE-A4 expression and patient survival. Advanced stage cases (stages III and IV) expressing MAGE-A4 exhibited the poorest prognosis (Yakirevich et al, 2003). MAGE-1 was found, by RT-PCR, to be expressed in 15/27 (56 %) malignant ovarian tissue specimens (Gillespie et al, 1998). Through enzyme-linked immunosorbent assay (ELISA), serum MAGE-4 protein was considered positive in 13/60 (22

%) of primary OVC patients and a predictor of poor survival following surgery (Kawagoe et al, 2000). RT-PCR was used to test 44 ascites specimens. BAGE mRNA was detected in 15/27 samples (56 %), MAGE-1 mRNA was detectable in 2/27 samples (7 %), 8/27 samples (30 %) had detectable MAGE-3 mRNA, and 8/27 samples (30 %) had detectable GAGE1/2 mRNA (Hofmann and Ruschenburg, 2002; Zhang et al, 2010). Cox regression model showed the expression of MAGE-A3 as a marker of poor prognosis in non-small cell lung carcinoma (NSCLC) (Gure et al, 2005) and in pancreatic ductal adenocarcinoma by quantitative real-time RT-PCR assay (Kim et al, 2006).

1.7.2.1 MAGE immune responses

MAGE-A3 antigen was the first human TAA shown to be recognized by $CD8^+$ T cells (Gaugler et al, 1994). Out of 122 patients with non-small cell lung carcinoma (NSCLC), 35 % developed recurrent disease following treatment with a recombinant MAGE-A3-based vaccine while in the placebo group numbering 60, 43 % of the patients relapsed (Brichard and Godechal, 2013). MAGE-A3 in combination with the immunostimulant AS15, produced a better antigen-specific response in NSCLC patients (Brichard and Godechal, 2013). T cells were observed to react against epitopes from MAGE-A1, MAGE-A2 and MAGE-A3 in multiple myeloma patients (Goodyear et al, 2005). Multiple myeloma patients with advanced disease produce an immune response against MAGE-C1/CT7 (Fontecedro et al, 2007).

1.7.3 NY-ESO-1

NY-ESO-1 is a 22 kDa protein located at Xq28 identified by SEREX in oesophageal squamous cell carcinoma patient serum (Chen et al, 1997) and mRNA was detected in 41/123 (33 %) oesophageal cancer patients (Fujita et al, 2004). mRNA has also been detected in a variety of other cancers; melanoma 23/67, breast cancer 10/33, prostate cancer 4/16 and bladder cancer 4/5 (Chen et al, 1997). NY-ESO-1 protein was found by IHC in 4/11 metastatic melanoma, 2/14 breast cancers, 2/9 bladder cancers and 2/3 synovial sarcomas but not in colon or renal

cancers (Jungbluth et al, 2001). Increased expression of NY-ESO-1 in melanoma samples was shown to lead to a decrease in the number of $CD3^+$ tumour infiltrating lymphocytes (Giavina-Bianchi et al, 2015).

1.7.3.1 NY-ESO-1 in immunotherapy

In clinical trials T cell responses were detected in 10/11 patients expressing NY-ESO-1 antibody while no immune response was observed in NY-ESO-1 negative patients (Jager et al, 2000). Using tetramers to HLA-A*0201/NY-ESO-1157-165, investigators found specific T cells in multiple myeloma patient samples, which when expanded were able to lyse primary tumour cells (Rhee et al, 2005).

Clinical trial responses to NY-ESO-1 targeted therapy have been described by a number of studies in the last 15 years. These include CD4 responses to peptide vaccines administered with ISCOMATRIX to patients with advanced melanoma (Klein et all, 2015), antibody and T cell response following injection of overlapping long NY-ESO-1 peptides with adjuvants in patients with OVC (Sabbatini et al, 2012), and remissions in patients who have received adoptive therapy for melanoma and synovial cell sarcoma using lymphocytes with a modified TCR that recognises NY-ESO-1 (Robbins et al, 2011 and 2015). NY-ESO-1, including its targeting in clinical trials, has been recently reviewed by Esfandiary and Ghoafouri, 2015.

1.7.4 PASD1

The CTA per ARNT SIM (PAS) domain containing 1 (PASD1) gene was identified through the immunoscreening of testes cDNA libraries (Liggins et al, 2004b; Guinn et al, 2005) using the SEREX technique (Sahin et al, 1995). A full-length cDNA clone encoding the novel antigen OX-TES-1 was identified through the immunoscreening of a testis cDNA library with diffuse large B-cell lymphoma (DLBCL) sera (Liggins et al, 2004b). It was initially named OX-TES-1 and subsequently given the HUGO approved name PASD1. Guinn et al, 2005 also identified

PASD1 through the immunoscreening of a testis cDNA library with presentation AML sera. The sequence was initially named GKT-AML-20. Further investigation of the structure of the PASD1 transcripts identified by the two groups indicated that OX-TES-1 encoded a different variant of PASD1 (named PASD1_v1) than GKT-AML20 (subsequently named PASD1_v2). Liggins et al, 2004a revealed that the longer PASD1_v2 sequence had retained intron 14 during alternative splicing such that the stop codon in intron 14 was read and led to the production of a shorter PASD1b protein (Figure 1.4).

1.7.4.1 Expression of PASD1 mRNA variants in cell lines/tissues

PASD1_v1 mRNA was detected by RT–PCR in seven DLBCL-derived cell lines. PASD1_v2 mRNA appears to be preferentially expressed in cell lines derived from non-germinal centre DLBCL. A number of investigations have demonstrated PASD1 expression in haematological malignancies including 4/12 (33 %) AML samples and 1/6 (17 %) CML (Guinn et al, 2005) **(Table 1.2)**. In addition, PASD1 expression was found at contrasting frequencies of 14/16 and <5 % in multiple myeloma (Sahota et al, 2006; van Duin et al, 2011) respectively. These data may reflect the differing techniques used for detection of PASD1 gene and protein expression and the site that probe sets bind in the 3' region of the cDNAs.

1.7.4.2 Expression of PASD1 protein variants in different cell lines and tissues

Markers were used to determine the prognostic value of PASD1. Germinal markers CD10 and BCL-6 can indicate good prognosis whereas the non-germinal centre marker MUM1 is indicative of poor survival. The two de novo DLBCL patients' who generated immune response to PASD1 were of a poor prognosis non-germinal centre subtype (Liggins et al, 2004a).

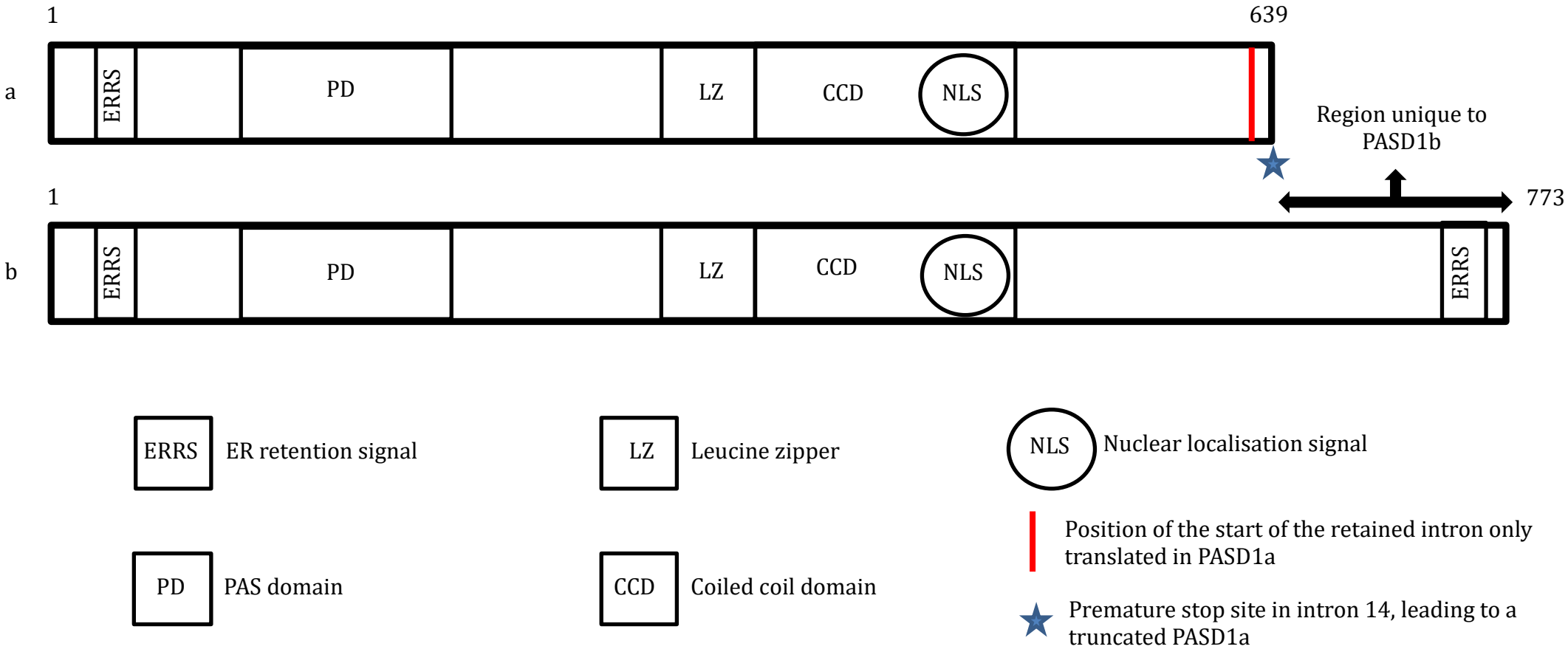

Figure 1.4 Diagrammatic representation of the two known variants of PASD1. The longer PASD1_v1 transcript has a retained intron 14 which has a premature stop site. This is translated into a shorter PASD1a protein (LIggins et al, 2004a). The shorter PASD1_v2 transcript does not have a retained intron and is transcribed into the longer PASD1b protein. PASD1b is 134mino acids longer than PASD1b.

Table 1.2 PASD1 expression in human tissues (taken from Khan et al, 2014)

Tissue	Technique	Reference
Expression in 25 of 68 solid tumours	Probing Northern blot arrays	Liggins et al, 2004a
A range of normal tissues including brain, liver, kidney, placenta, breast, uterus or ovary	RT-PCR and ICC	Cooper et al, 2006; Guinn et al, 2005
4 of 12 AML patients, and 1 of 6 CML patients	RT-PCR and RQ-PCR	Guinn et al, 2005
Normal testicular tissues expression was only found in the nuclei of a subpopulation of spermatogonia. Labelling intensity decreased with maturity of the spermatogoa.	Immunostaining	Cooper et al, 2006
PASD1-1 expression in 21 of 51 DLBCL, 4 of 9 mantle cell lymphoma, 4 of 15 follicular lymphomas, 4 of 12 Burkitt's lymphoma. PASD1-2: 11 of 52 DLBCL, 2 of 4 MM, 4/10 peripheral T cell lymphoma and a range of other tumour cells from patients with haematological malignancies. 41 % overall. PASD1-1 was mostly cytoplasmic and weak nuclear staining in DLBCL and MM. Stronger labelling towards the periphery of the tumour. PASD1-2 was nuclear staining.	Immunostaining	Cooper et al, 2006
14 of 16 primary MM samples including 9 presentation and 7 previously treated cases. Two of four primary MM tumour samples.	RQ-PCR ICC	Sahota et al, 2006
PASD1 (22/25) cell lines derived from 21 B- and 4 T-cell malignancies	RT-PCR	Liggins et al, 2010
Not found in 78 basal cell carcinoma	RQ-PCR	Ghafouri-Fard et al, 2010
3.4 % of 320 newly diagnosed and 264 relapse cases of MM	Microarray using Affymetrix GeneChips	van Duin et al, 2011

In a cohort of haematological malignancy derived cell lines, the sub-cellular localisation of PASD1, as determined by immunostaining with monoclonal antibodies, was variable (Cooper et al, 2006). Cooper et al. showed that in the non-germinal centre DLBCL-derived cells OCI-Ly3 (Yee et al, 1989), PASD1-1 strongly labelled the cell membrane and cytoplasm while there was no staining of these cells with PASD1-2. Expression of PASD1 was also demonstrated in the FEDP (ALK-negative anaplastic large-cell lymphoma) cell line in the cytoplasm. Strong

cytoplasmic labelling with PASD1-2 was also observed in a subpopulation of Granta519 cells. KM-H2, established from the pleural effusion of a patient with Hodgkin's disease of mixed cellular type (Kamesaki et al, 1986) and the mantle cell lymphoma (MCL)-derived cell line Granta519 showed nuclear staining with PASD1-1 and PASD1-2 antibodies. K562 cells derived from a patient with myeloid leukaemia (Klein et al, 1976) and the Thiel multiple myeloma (MM) cell line all exhibited nuclear staining. The detection of nuclear staining was not unexpected and likely reflects the presence of a nuclear localisation signal in the common region of the PASD1-1 and PASD1-2 proteins and the role of PASD1 as a transcription factor (Xu et al, 2016) like many other SEREX-defined antigens (Chen, 2004).

PASD1 has been shown to suppress circadian rhythms. The circadian clock regulates and responds to the physiological and environmental changes by regulating transcription in a roughly 24 h cycle. PASD1 through its interaction with CLOCK:BMAL1 reduces transcription regulation leading to transformation of cells (Michael et al, 2015).

However, published studies to date have predominantly examined haematological malignancies with few studies indicating the finding of PASD1 in solid tumours (**Table 1.2**). This may reflect the fact that many studies do not publish negative data (discussed in Guinn, 2014) or that the expression of PASD1 has not been examined in many solid tumours except basal cell carcinoma (Ghafouri-Fard et al, 2010).

1.7.4.3 PASD1 in immune responses

Humoral responses to PASD1 have been demonstrated through the use of SEREX (Guinn et al, 2005; Liggins et al, 2004b) that showed that at disease presentation 4 out of 10 patients with DLBCL, 6 out of 17 AML and 1 of 6 CML patients had already mounted humoral immune responses releasing antibodies into the peripheral blood volume of these patients. Similar antibody responses were not found in the periphery of 20 and 10 healthy donors in these studies,

respectively. SEREX is based on the existence of humoral immune responses but SEREX-defined antigen are often also recognised by T cells, probably reflecting the dual role of $CD4^+$ helper T cells in the elucidation of $CD8^+$ and B cell responses (Lederman et al, 1992a; 1992b).

The question remains why these humoral, and cellular, immune responses were insufficient to kill PASD1 positive tumour cells. One theory is that tumour cells may induce some immune response in the mid-stages of the cancers' development, when there are enough tumour cells to be seen by the immune response, but as these tumour cells proliferate they downregulate the immune system of the patient and escape effective killing (Dunn et al, 2004). Many of the antigens recognised by SEREX are nuclear transcription factors with low immunogenicity. It is proposed that those antigens with high enough immunogenicity would have already induced tumour cell killing. Only antigens with poor immunogenicity would have escaped immune surveillance. Immunotherapy, therefore, offers a way to enhance immune recognition of CT and tumour-associated antigens by the immune system and induce effective residual tumour cell killing. Indeed the elevated expression of some tumour antigens at disease presentation has been shown to be associated with enhanced patient survival (Greiner et al, 2006; Guinn et al, 2009; Liberante et al, 2013). It is proposed that during conventional therapy, the resulting tumour lysis leads to inflammation and provide the requisite "danger signals" (Matzinger, 1994) which could cause the induction of effective anti-tumour immune responses. Cancer cells with elevated antigen expression would be better able to induce immune responses, when killed by chemo/radiotherapy, antigen would spill out, be mopped up by APCs, presented to the cellular immune system, that would lead to the killing of antigen positive tumour cells and subsequent epitope spreading (Chen et al, 2005; Dai et al, 2005). To support this there is increasing evidence that chemotherapy may have a synergistic effect with the immune response to support this possibility (Szczepanski et al, 2013).

T-cell immunogenic epitopes within PASD1a and PASD1b (Ait-Tahar et al, 2009; Hardwick et al, 2013) were identified using the TEPITOPE (Sturniolo et al, 1999), SYFPEITHI (Rammensee et al, 1999) and Bioinformatics and Molecular Analysis Section (BIMAS) (Parker et al, 1994) prediction programmes. The capacity of the MHC class I epitopes to bind HLA-A2 were confirmed using T2 assays (assay detailed in (Alexander et al, 1989)) which can predict A2-peptide off-rates. MHC class I antigen presentation and cell surface expression depends on the peptide travelling into the endoplasmic reticulum or Golgi by the MHC encoded transporters TAP-1 and TAP-2. T2 cells are TAP-deficient but do express MHC class I on their surface which is only stabilised when binding a peptide. The T2 binding assay can be used to determine how well a peptide can stabilise HLA-A*0201 MHC allele (Wang et al, 2002). The avidity and half-life of peptide binding to MHC were detected by fluorescent activated cells (FACs) analysis using anti-HLA-A2. In each case the criteria used required that the epitopes under investigation should not be similar to epitopes from other known human proteins. Hardwick et al, 2013 utilised a cut-off of similarity to known proteins across the 9 a.a. peptides examined was ≥ 40 % when compared to other known human proteins. In addition, the group favoured the utilisation of SYFPEITHI predicted epitopes in favour of BIMAS ones (Hardwick et al, 2013). None of the seven MHC class I binding nonamers (named sequentially Pw4 through to Pw10) identified using predictive programmes were able to stabilise HLA-A*0201 in T2 assays and all had poor SYFPEITHI scores (Hardwick et al, 2013). However, modification of one of the anchor residues at a.a. 2 or 9 (to a lysine, valine or isoleucine) did lead to epitopes with improved SYFPEITHI scores and enhanced and/or extended periods of binding to HLA-A*0201 in T2 assays. One of these modified peptides, named Pa14, was shown to be able to stimulate patient T cells. This caused a very limited expansion in $CD8^+$ T cell numbers from two of three HLA-A*0201 positive, PASD1-positive AML patient samples. This corresponds with the findings of others (Rezvani et al, 2009) who also found limitations in the

expansion that can be achieved with AML T cells *ex vivo*. A 2-3 week limited expansion is the maximum that has been achieved prior to AML T cell death. Reasons for the limited responses may be due to the presence of myeloid suppressor cells in mixed lymphocyte assays (Mougiakakos et al, 2013), interleukin-6 (IL-6) secretion by myeloid leukaemia cells (Buggins et al, 2008) and/or defects in T cell populations in myeloid leukaemia patients (Wendelbo et al, 2004). However, stimulation of T cells from a single colon cancer patient, in this study, led to a substantial increase in the number of Pa14-specific T cells to 13.6 % of the $CD8^+$ cell population after four rounds of weekly Pa14 stimulation, with Pa14- specific IFNγ responses being evidenced (Hardwick et al, 2013).

CTL responses to PASD1 were also detected in DLBCL patients (Ait-Tahar et al, 2009). IFNγ release was detected in 21 out of 29 HLA-A*0201-positive DLBCL patients following short-term culture of their peripheral blood mononuclear cells stimulated with five HLA-A*0201-restricted PASD1 peptides. However, there was no response in the 21 patients who were HLA-A*0201-negative. IFNγ is a cytokine released mainly by natural killer and natural killer T cells and is a component of innate immunity. It can also be released by Th1 CD4 and CD8 T cells subsequent to acquired immunity to a specific antigen. IFNγ can activate macrophages and induces class II MHC molecule expression (Schoenborn and Wilson, 2007).

CD4 T helper cells are known to promote immunity in a number of ways such as stimulating the production of antibodies. CD4 responses against PASD1 epitopes were investigated by Ait-Tahar et al. (Ait-Tahar et al, 2011) in patients with DLBCL. They showed that immunogenic PASD1 epitopes predicted to bind several class II DR beta 1 alleles were able to induce $CD4^+$ T helper responses to PASD1-positive cells from patients with DLBCL. Two of the five peptides (PASD1(6) and PASD1(7)) were shown to be immunogenic in 14 of the 32 patients tested and T-helper cell lines generated from two patients were able to lyse PASD1 positive cell lines derived from haematological malignancies. $CD4^+$ T helper cell lines raised from two

patients were able to lyse PASD1-positive tumour cell lines corroborating that these T cells recognized intracellular expressed PASD1. The PASD1-negative cell line was not lysed.

A new PASD1 immune response has been described in a melanoma patient as they achieved complete remission after initial detection of autoantibodies against melanoma antigen A3 (MAGEA3) (Stamell et al, 2013).

1.7.5 SSX2

SSX2 was identified due to the translocation t(X;18) found to be common in synovial sarcoma tumours (Smith et al, 1987). Synovial sarcoma is a soft tissue tumour predominantly affecting children and young adults. It was found that as a result of this translocation the SYT gene on chromosome 18 is fused together with SSX genes, SSX1 or SSX2, on the X chromosome leading to the production of a SYT-SSX fusion protein (Clark et al, 1994). The gene was first identified using SEREX on melanoma patient samples as the CTA, HOM-MEL-40 (Tureci et al, 1996). Using SEREX it was found that melanoma patients exhibited immune responses to SSX2, however healthy controls showed no response. SSX2 belongs to a family of SSX genes (**Figure 1.5**), all five members share strong sequence homology, act as transcriptional repressors (Lim et al, 1998) and are associated with several Polycomb group proteins (Soulez et al, 1999). Dos Santos et al (dos Santos et al, 2000) demonstrated that SSX nuclear expression in the testis was found to be restricted to spermatogenic cells, mainly spermatogonia with some expression in the healthy thyroid (Tureci et al, 1996). Treatment of an SSX-negative cell line with 5-aza-2'-deoxycytidine, a demethylating agent, led to SSX RNA and protein expression, indicating a role for methylation in transcription regulation. SSX2 transcripts have been identified in a number of cell lines most notably leukaemia (Hoffman et al, 2014), myeloma (Atanackovic et al, 2007) and melanoma (dos Santos et al, 2000). In addition, transcripts were detected by RT-PCR in a significant proportion of human melanomas (50 %), hepatocarcinomas (50 %), thyroid cancer (50 %), colon cancer (26 %), prostate cancer (20 %)

and breast carcinoma (19 %) as well as in haematological cancers (11 %), brain tumour (9 %) and stomach cancer (8 %) (Tureci et al, 1996). SSX2 expression has been demonstrated by RT-PCR in 13% of endometrial cancers (Tureci et al, 1998) and 3-10 % of OVCs (Hasegawa et al, 2004; Valmori et al, 2006), but 26 % of OVCs if you score SSX1, 2 and 4 together (Valmori et al, 2006). The presence of SSX2 expression in so many tumour types suggested that this antigen is upregulated in cancer independently from fusion events (Smith and McNeel, 2010).

Although examining mRNA indicates the expression of a gene and can be a very useful and sensitive tool, it does not always predict protein abundance nor does it give insight into the sub-cellular localisation of a protein which can indicate a normal or altered function (Guo et al, 2008). Taylor et al (Taylor et al, 2005) found that SSX1, SSX2, SSX4, and SSX5 were all expressed in 20 % of patients with multiple myeloma (MM), and this expression was found to correlate with adverse prognosis and reduced survival. Analysis of heterogeneous SSX protein expression in patient samples have indicated expression in 34 % of primary and metastatic melanoma patients (dos Santos et al, 2000), 26 % of high-grade prostatic intraepithelial neoplasia (HGPIN) (Smith et al, 2011), 23 % of prostate metastatic lesions but not primary lesions. In malignant bone and soft tissue tumours there was significantly higher expression than in benign tumours ($P<0.0001$), expression in stage III tumours was significantly higher than that in stage I or II tumours ($P<0.005$) (Naka et al, 2005) indicating that expression of SSX2 protein appeared to increase with disease grade at least in these cancers.

```
SSX1  mngddtfakr prddakasek rskafddiat yfskkewkkm kysekisyvy mkrnykamtk
SSX2A mngddafarr ptvgaqipek iqkafddiak yfskeewekm kasekifyvy mkrkyeamtk
SSX2B mngddafarr ptvgaqipek iqkafddiak yfskeewekm kasekifyvy mkrkyeamtk
SSX3  mngddtfarr ptvgaqipek iqkafddiak yfskeewekm kvsekivyvy mkrkyeamtk
SSX4  mngddafarr prddaqisek lrkafddiak yfskkewekm kssekivyvy mklnyevmtk
SSX5  mngddafvrr prvgsqipqk mqkafddiak yfsekewekm kasekiiyvy mkrkyeamtk
SSX6  mngddafakr prddakasek rskafddiak yfskeewekm kfsekiscvh mkrkyeamtk

SSX1  lgfkvtlppf mcnkqatdfq gndfdndhnr riqvehpqmt fgrlhriipk impkkpaede
SSX2A lgfkatlppf mcnkraedfq gndldndpnr gnqverpqmt fgrlqgispk impkkpaeeg
SSX2B lgfkatlppf mcnkraedfq gndldndpnr gnqverpqmt fgrlqgispk impkkpaeeg
SSX3  lgfkailpsf mrnkrvtdfq gndfdndpnr gnqvqrpqmt fgrlqgifpk impkkpaeeg
SSX4  lgfkvtlppf mrskraadfh gndfgndrnh rnqverpqmt fgslqrifpk impkkpaeee
SSX5  lgfkatlppf mrnkrvadfq gndfdndpnr gnqvehpqmt fgrlqgifpk itpekpaeeg
SSX6  lgfnvtlslf mrnkratdsq rndsdndrnr gneverpqmt fgrlqriipk impekpaeeg

SSX1  ndskgvseas gpqndgkqlh ppgkanisek inkrsgpkrg khawthrlre rkqlviyeei
SSX2A ndseevpeas gpqndgkelc ppgkpttsek ihersgnrea qekeerrgta hrwssqnthn
SSX2B ndseevpeas gpqndgkelc ppgkpttsek ihersgpkrg ehawthrlre rkqlviyeei
SSX3  nvskevpeas gpqndgkqlc ppgkpttsek inmisgpkrg ehawthrlre rkqlviyeei
SSX4  nglkevpeas gpqndgkqlc ppgnpstlek inktsgpkrg khawthrlre rkqlvvyeei
SSX5  ndskgvpeas gpqnngkqlr psgklntsek vnktsgpkrg khawthrvre rkqlviyeei
SSX6  sdskgvpeas gpqndgkklc ppgkasssek ihersgpkrg khawthrlre rkqlviyeei

SSX1  sdpeedde
SSX2A igrfslstsm gavhgtpkti thnrdpkggn mpgptdcvre nsw
SSX2B sdpeedde
SSX3  sdpeedde
SSX4  sdpeedde
SSX5  sdppedde
SSX6  sdpeeddk
```

Figure 1.5 SSX family members showing the C terminal region unique to SSX2A. The differences in the amino acid sequence between the six SSX family members compared to SSX1 are shown in red font.

1.7.5.1 SSX2 immune responses

Anti-SSX2 antibodies have also been found in 11 % of melanoma patients (Tureci et al, 1996) and 3 % of colon cancer patients (Scanlan et al, 2002) but rarely in other cancers. Similarly studies of OVC patients have demonstrated the absence of detectable anti-SSX2 in plasma in OVC patients (Lu et al, 2011). However Taylor et al found that reactivities with nucelophosmin, cathepsin D, p53 and SSX common antigen were significantly higher in patients with all stages of OVC compared with controls and women with benign ovarian disease (Taylor et al, 2009). Sera from a subgroup of the patients were tested for SSX2 and SSX4 antibody by ELISA and recombinant antigen expression on yeast surface (RAYS).

Aberrant expression of these antigens was found in 31/120 (26 %) of ovarian tumours. Antibodies to SSX2 and SSX4 were detectable in two patients (2 %) (Valmori et al, 2006).

There has been little demonstration of SSX2 antibodies in patient plasma. In 194 multiple myeloma (MM) plasma samples, 3.1 % of patients were found to have SSX2 specific antibodies. SSX2 antibodies were able to activate complement and increase CTA uptake by antigen presenting cells. SSX2 antibodies were restricted to IgG3 (Luetkens et al, 2014).

1.7.5.2 SSX2 as a target for immunotherapy – evidence of naturally occurring T cell responses in patients

In metastatic melanoma the peptide SSX2 (41-49) was identified as an HLA-A2-restricted epitope (**Table 1.3**). $CD8^+$ T cells specific for SSX2(41-49) were present in the tumour-infiltrated lymph node population by multimer staining, and isolated CTL clones were able to lyse HLA-$A2^+$ tumour cells expressing SSX2 (Ayyoub et al, 2002). SSX2–derived T cell epitope, mapping to the 37–58 region and surrounding the SSX2 41–49 epitope, was recognized by $CD4^+$ T cells from melanoma patients (Abate-Daga et al, 2014; Ayyoub et al, 2004).

In a hepatocellular carcinoma (HCC) patient, SSX2-specific $CD8^+$ T cells were detected in tumour infiltrating lymphocytes but not in the normal lymphocytes of patients or in the peripheral blood mononuclear cell samples taken on the day of surgery (Bricard et al, 2005). In two of six HLA-$A2^+$ HCC patients, the group found that MAGE-A10 and/or SSX2-specific $CD8^+$ T cells naturally responded to the disease, because they were enriched in tumour lesions but not in non-tumoural liver (Bricard et al, 2005). Isolated T cells specifically and efficiently killed tumour cells *in vitro*, providing evidence that these CTL were selected *in vivo* for high avidity Ag recognition. Therefore, besides melanoma, HCC is the second solid human tumour

with clear evidence for *in vivo* tumour recognition by T cells, providing the rational for specific immunotherapy, based on immunization with CT Ags such as MAGE-A10 and SSX2.

Table 1.3 SSX2 epitopes identified to date

SSX2 epitopes	Cancer and detectable immune response	Publication
$SSX2_{p37–58}$	specific CD4+ T cells were found in 11 of 19 melanoma patients	Ayyoub et al, 2004
$SSX2_{p41-49}$	Identified in metastatic melanoma	Ayyoub et al, 2002
$SSX2_{p45-59}$	3/6 breast cancer patients, 1/5 healthy controls	Neumann et al, 2004
$SSX2_{p103-111}$	5/7 breast cancers samples showed immune response	Wagner et al, 2003a

SSX4-specific $CD4^+$ T cells were found to recognise two novel SSX4-derived T-cell epitopes in association with HLA-DR (human leukocyte antigen) (Valmori et al, 2006). In 2011, Smith et al (Smith et al, 2011) described a single HLA-A2–restricted epitope, SSX2 p103-111 RLQGISPKI with a SYFPEITHI score of 23 and a BIMAS score of 10.433.

Using the SYFPEITHI algorithm Wagner et al (Wagner et al, 2003a) identified a HOM-MEL-40/SSX2-derived epitope with high binding affinity for HLA-A*0201. Stimulation with p103-111 induced HOM-MEL-40-specific CTLs in five out of seven patients with HOM-MEL-40/SSX2 positive breast cancers and in six of eleven healthy controls. HLA-A*0201 specificity for p103-111 was shown by blocking with specific antibodies. Prestimulated p103-111 specific $CD8^+$ T cells reacted with SSX2-transfected COS7/A2 cells as well as with the HLA-A*0201 positive cell line SK-MEL-37 that is known to express HOM-MEL-40/SSX2. The same $CD8^+$ cells did not lyse negative controls. p103-111 peptide vaccine could be applied to a large number of cancers which are HOM-MEL-40/SSX2 (Wagner et al, 2003a).

Side population (SP) cells are progenitor cells from normal and malignant tissues which have increased resistance to chemotherapy and radiotherapy. HL SP cells express higher levels of the TAAs MAGEA4, SSX2, survivin, and NY-ESO-1, which allowed them to be specifically recognized and killed by TAA-specific CTLs (Shafer et al, 2010). The expression of SSX2 in SP cells suggests that SSX2 may be expressed in tumour stem cells and that this should be explored further in OVC.

A DNA vaccine encoding altered peptide ligand (APL) was developed in which the anchor residues of the p41-49 and p103-111 epitopes were changed. Investigations of how these changes affected epitope binding, generated increased numbers of $CD8^+$ T cells specific for SSX2 and led to the production of epitope-specific Th1 cytokines (Smith et al, 2014).

1.8 Measuring the immune responses to tumour antigens – with a focus on pMHC arrays

Once a tumour antigen has been identified it is important to investigate how capable it is of stimulating an immune response compared with other antigens. Therefore assays are used to determine T cell responses against the antigen of interest. These assays usually involve the measurement of lymphocyte proliferation in response to exposure to antigen such as carboxyfluorescein diacetate succinimidyl ester dye, lymphocyte proliferation assays and [3H]-thymidine incorporation assay; assays that measure cytokine production such as enzyme-linked immunosorbent spot assay (ELIspot) and flow cytometry (intracellular and secreted cytokine) or killing assays such as chromium-release CTL assays. These will not be discussed in detail here but can be found in Immunology Methods Manual, 1997. More recently methods have been developed that can simultaneously detect the specificity of T cells for epitopes within antigens. These include flow cytometry using pMHCs, including the “combinatorial encoding” approach (Hadrup et al, 2009; Newell et al, 2009) reviewed in Hadrup and Schumacher, 2010.

1.8.1 Peptide-MHC (pMHCs)

pMHC based assays circumvent the issues caused by measuring T cell proliferation. T cell proliferation assays can provide information on whether an immune response can be generated but will not determine which T cells, if they are indeed T cells, have been activated, unless a single population has been purified. pMHCs, often referred to as tetramers, can be used to identify antigen specific T cells. They are produced through the refolding of β2-microglobulin and heavy chains in MHC molecules with the appropriate epitope of interest. The pMHC is then labelled with biotin using BirA enzyme. A streptavidin molecule conjugated to a fluorescent detector binds to four pMHCs to make tetramers or other multimers of pMHC (for example dimers, pentamers or dextramers) courtesy of the biotin-avidin interface (Sims et al, 2010). T cell populations are added to this mixture and T cells with the specific receptor for the epitope of interest will bind and be measureable by flow cytometry (Shen et al, 2010). Shen et al (Shen et al, 2010) have found that cross-reactive T cells i.e. T cells that recognise two different antigens can be identified providing an extra tool in vaccine development. In some cases, antigen specific T cells may not bind tetramers due to being undifferentiated and unable to accumulate TCR molecules close to the antigen. Another reason could be low affinity between TCR and MHC (Khan et al, 2010). Techniques based on the use of pMHCs include pMHC arrays (Chen et al, 2005), NACS (Kwong et al, 2009) and the combinatorial approach (Hadrup and Schumacher, 2010; Newell et al, 2009). Each provide high throughput analysis of multiple T cell populations with a variety of pros and cons to each technique including issues with background, specificity/binding capacity of individual pMHC complexes, activated induced cell death of pMHC bound T cells, internalisation of pMHCs following T cell binding (Whelan et al, 1999), cost and labour intensity. Sequencing of TCRs (2-3 million every 2-3 days) by companies such as TRON gGmbH (Johannes Gutenberg University Mainz, Germany) and Adaptive Biotechnologies (Seattle, USA) will provide a new way of analysing

T cell populations which will be informative with regards to which TCRs are present but not necessarily whether they are present on mature, anergic, activated or functional T cells nor which sub-group of T cells are harbouring them [helper T cells, CTLs, Th17 cells or indeed regulatory T cells (Tregs)]. This technology allows the first opportunity to examine an extremely large number of TCRs in a very short time and will revolutionise how we examine T cell responses in patients in the future.

1.8.2 pMHC arrays

pMHC arrays were first described by (Soen et al, 2003), to examine which specific T cell populations were present in the periphery of patients with cancer. Similar tests are often performed with flow cytometry (Jung et al, 1993; Picker et al, 1995) which is time limited, expensive and can be difficult to perform and exclude debris and cell clumps. To examine multiple specific T cell populations by flow cytometry, groups have used methods such as polychromatic flow cytometry (PFC) (Estes et al, 2010) and multiparametric flow cytometry analysis (FCM) (Camisaschi et al, 2014). Flow cytometry has many advantages such as enabling the analysis of multiple parameters at the single cell level thus identifying immunoreactive subset populations and antigens to many diseases including cancer. Much data can be generated with relatively small cell numbers. However interpreting such a large amount of data can become a very complex and laborious task (Lugli et al, 2009).

pMHC arrays (Chen et al, 2005; Soen et al, 2003) (**Figure 1.6**) provide a strategy to determine which specific $CD8^+$ T cell populations are present in the peripheral blood of patients without a pre-stimulation/expansion step. Antigens identified by the techniques described already can be used to help expand the pMHC array for future studies.

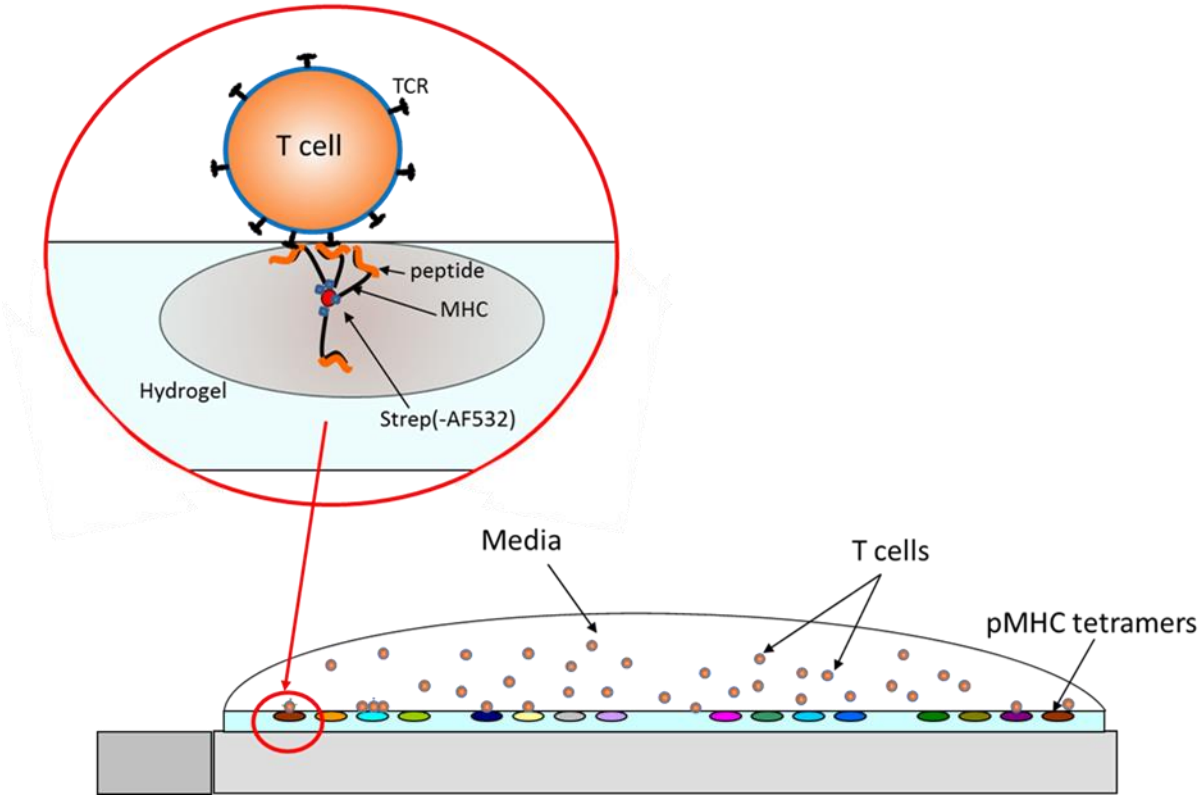

Figure 1.6 Diagrammatic representation of the pMHC array. Lipophilically dyed CD8+ T cells (0.8-1.2 x 10^6 per microarray) were incubated at 37°C in warm colourless X-VIVO 15 media with the pMHC array. Each pMHC spot contains 1ng of tetramer and each slide can hold up to 1,000 spots of pMHC. Taken from Brooks et al, 2015. PASD1 epitopes are frequently recognised by "untouched" CD8+ T cells from presentation myeloid leukaemia patients.

The pMHC array provides a means to investigate epitope spreading and changes in T cell specificities with disease progression. The technique benefits from the low number of purified $CD8^+$ T cells required for each array (0.5-2 x 106/array), which can be purified from 20 ml of patient peripheral blood using StemCell $CD8^+$ negative isolation beads providing "untouched" T cells (Bonney et al, 2015). The purified $CD8^+$ T cells are then lipophyllically dyed with DiD (Molecular Probes), washed and incubated with the pMHC array. The pMHC array has a detection limit of 0.02 % matching the sensitivity we can reproducibly achieve with flow cytometry when analysing patient samples. Where sample availability permits pMHC array data should be validated by flow cytometry (Dittmann et al, 2005) using the same pMHC

tetramers as used in the pMHC array. The pMHC array has the added advantage that it can be used for the initial screening of a relatively small number of $CD8^+$ T cells against a large number of pMHCs on the array, and a short-list of T cell populations which are shown to exist on the pMHC array can then be quantitated by flow cytometry (limiting the amount of sample required in subsequent studies). The pMHC array can be used to analyse patient samples at a number of disease time-points (presentation, post- treatment (surgery and/or radiotherapy) and with disease progression) to examine how T cell responses to tumour antigens change with treatment, to examine epitope spreading (where those epitopes are present on the array) and to correlate changing immune responses with clinical responses.

1.9 Biomarkers in cancer

The National Cancer Institute described biomarkers as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease." Biomarkers are self-proteins or metabolites whose levels of expression or mutation can suggest the presence of a tumour or tumour progression as well as a marker that enables the monitoring of tumour destruction in response to treatment. Ideally biomarkers should be obtained from blood and/or urine making access easier and any analysis cost effective. They can include transcription factors, cell surface markers or secreted proteins. Good biomarkers have the ability to allow the diagnosis of cancer in its earliest stage thereby reducing the number of cancer deaths by helping to enable accurate diagnoses and early appropriate treatment. Early treatment occurs at a stage when it is most effective and enables the effective utilisation of individualised treatments – saving patients unnecessary side effects and the health care system the unnecessary cost of treatments that will not help treat patients. The ongoing improvements in our understanding of tumourigenesis has aided the identification of better biomarkers and along with more sensitive methods, it is increasingly likely that in the future biomarkers will be used more effectively.

In cancer research, biomarkers are employed in a number of ways. A biomarker can be used to identify the type of cancer present (diagnostic), a prognostic biomarker can determine the probable progression of disease if left untreated and whether recurrence of the cancer is likely and a predictive biomarker assess how a cancer patient may respond to a particular treatment (**Figure 1.7**).

1.9.1 Biomarkers in OVC

The effectiveness of a particular therapeutic vaccine depends on the TAA targeted. In an ideal model the best antigen should have the following characteristics: non-self or differentially expressed in a disease state, be specific to the tumour cells, detectable in a large number of patients, involved in tumour progression, immunogenic and preferably be on the cell surface so it is easily accessible by an antibody (Hung et al, 2008).

In OVC serous carcinomas are uncommon at FIGO stage I and are usually part of progressive disease, while 95 % of FIGO stage III and IV patients have the serous type of OVC (Colombo et al, 2010). Even though early stage OVC tends to have a less aggressive histotype, it is still difficult to diagnose. It may be possible to treat aggressive (later stage) OVC following surgery and chemotherapy which is used to reduce the disease load. Then immunotherapy can be used to target minimal residual disease and deliver a more effective overall treatment (Hung et al, 2008).

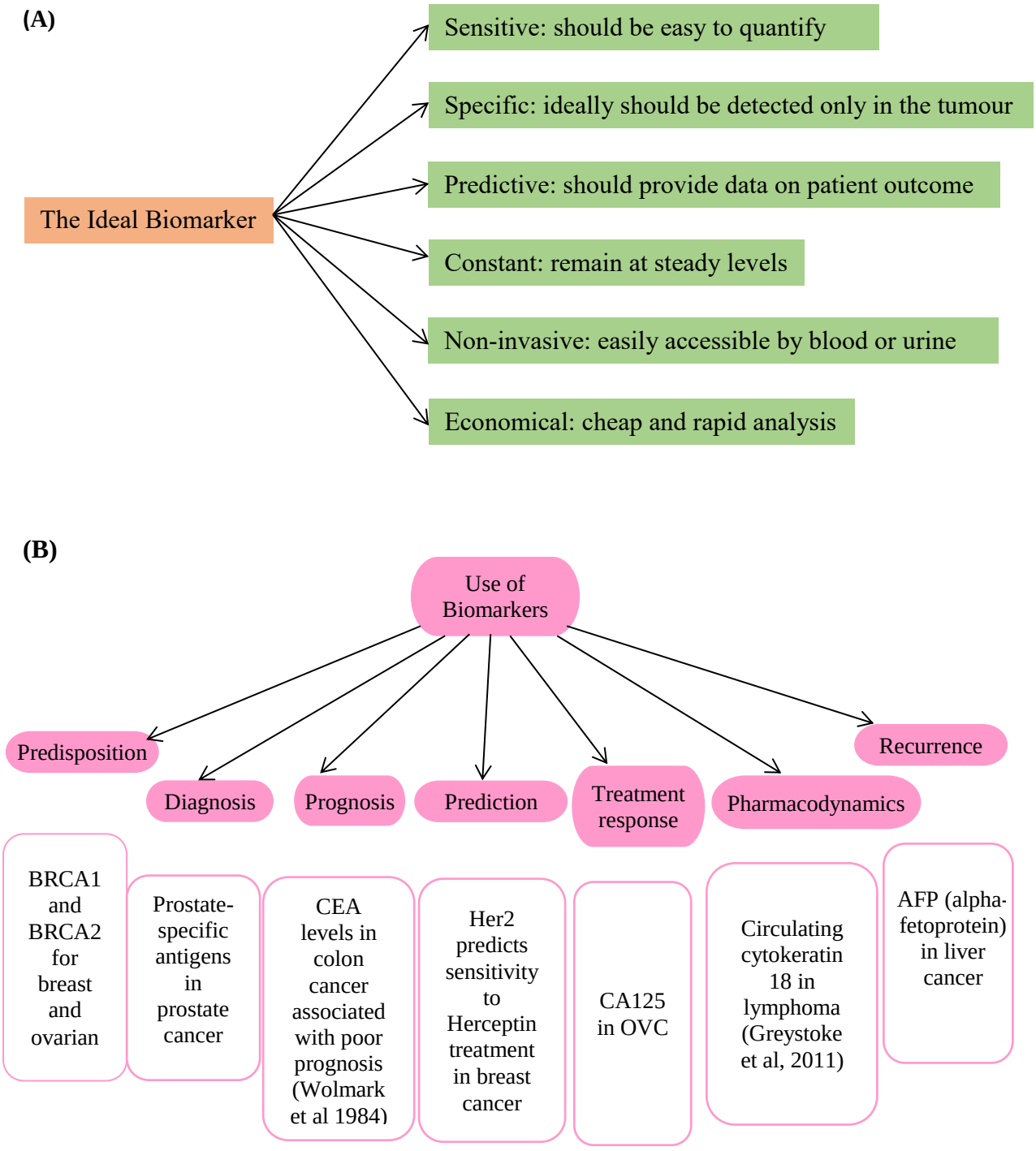

Figure 1.7. The characteristics of an ideal biomarker and their uses in cancer therapy and diagnosis. Few biomarkers have all of the ideal characteristics indicated in (**A**) but the more they have the better they usually are. The uses of biomarkers shown in (**B**) demonstrate their scope for improving cancer detection and cancer care, impacting on outcomes and minimising unnecessary treatments and side effects.

1.9.1.1 CA125

CA125 was first identified by the monoclonal antibody OC125 that was detected in mice in response to being vaccinated with EOC cell lines (Bast et al, 1981). An assay was developed which could detect CA125 in patient sera where 82 % (83/101) of OVC patients were found to have higher levels of CA125. Levels were also correlated with progression or regression of disease by rising or falling. Notably only 1 % in a cohort of 888 healthy people and 6 % (of 143) with non-malignant disease had high CA125 levels (Bast et al, 1983). However, CA125 is unsuitable in the early stages of EOC when it is known to be elevated in a number of benign conditions such as pregnancy and tuberculosis (Meden and Fattahi-Meibodi, 1998) with variable expression between patients [recently reviewed in (Su et al, 2013)]. CA125 is used to investigate a wide spectrum of symptoms attributed to a number of benign and malignant gynaecological conditions and not only for OVC (Moss et al, 2004). It also appears to work better as part of a panel to improve specificity, sensitivity (100 %) and differentiation of OVC from endometriosis (Bandiera et al, 2013; Jiang et al, 2013). In one study, CA125 demonstrated better sensitivity and specificity when used with three other biomarkers (apolipoprotein, a truncated form of transthyretin and a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4) than alone in early stage OVC (Zhang et al, 2004). A combination of CA125 with TVU only managed to detect OVC at an advanced stage, and 3/4 early stage tumour patients screened had normal results preceding the diagnosis (Olivier et al, 2006). Due to its poor sensitivity and specificity, CA125 was not recommended for use in screening asymptomatic women by the National Academy of Clinical Biochemistry (NACB) Panel (Sturgeon et al, 2008). There still remains a need to identify singularly good biomarkers which could positively influence disease outcome by enabling the early detection and therefore treatment of patients with OVC.

1.9.1.2 Human epididymis protein 4 (HE4)

HE4, also called whey-acidic-protein (WAP) four-disulfide core domain protein 2 (WFDC2) was originally demonstrated to have tissue specific expression in the epididymis (Kirchhoff et al, 1991) and is expressed in normal tissues of the reproductive and respiratory tract in multiple isoforms (Bingle et al, 2002), where it may play a role in the innate immunity (Bingle et al, 2006). Using comparative hybridization of cDNA arrays, the gene human epididymis secretory protein 4 (HE4) was found to be overexpressed in OVC (Schummer et al, 1999). Further studies exhibited a possible role in OVC cell line adhesion and motility (Lu et al, 2012). HE4 was also up-regulated in renal disease in a mouse model with fibrosis of the kidney due to a halt in the degradation of type I collagen and subsequent neutralising of HE4 inhibited fibrosis and resumed collagen degradation (LeBleu et al, 2013). In lung cancer HE4 may also be abnormally expressed (Molina et al, 2011). In human OVC cells HE4 is produced as a protein with a size of about 13 kDa and converted to a 25 kDa secreted glycosylated protein (Moore et al, 2014). It was found to be overexpressed in EOC in comparison to normal ovarian epithelium (Hellstrom et al, 2003), especially in serous and endometrioid histotype (Drapkin et al, 2005) and the serum HE4 levels in women with EOC were shown to be clinically relevant (Moore et al, 2014). However, HE4 does not seem to be specific for OVC as expression has also been found in other cancers such as pulmonary and endometrial adenocarcinomas (Moore et al, 2008a). Although HE4 may have better specificity than CA125, the performance of both improves when they are used in combination and not as singular detection markers (Escudero et al, 2011; Moore et al, 2008b).

1.9.1.3 NY-ESO-1

LAGE-1 and NY-ESO-1 are 94 % homologous and therefore often investigated together. Odunsi et al, (Odunsi et al, 2003) evaluated a total of 190 patients by RT-PCR and/or IHC, 21/190 (11 %) were at tumour stages IA-IIC and 169/190 (89 %) were at stages IIIA-IV. NY-

ESO-1 expression was detected in 82/190 samples (43 %) and out of 107 tumour samples investigated for LAGE-1 mRNA expression 22 (21 %) were positive. NY-ESO-1 and LAGE-1 mRNA coexpression was seen in 12/107 (11 %) specimens. The expression of either NY-ESO-1 or LAGE-1 mRNA was present in 42/107 (40 %) EOC specimens. NY-ESO-1/LAGE-1 antibodies were present in 11/37 (30 %) of patients with NY-ESO-1- and LAGE-1-positive tumours, and only one patient with an NY-ESO-1- and LAGE-1-negative tumour had NY-ESO-1 antibody. NY-ESO-1 expression also correlated with advanced stage EOC (Odunsi et al, 2003). Yakirevich et al (Yakirevich et al, 2003), found that 10/53 (19 %) of serous carcinoma patient samples were positive for NY-ESO-1. Levels of antibodies to NY-ESO-1 were studied in type I and II OVC patients. Antibodies to NY-ESO-1 were detected in high levels in the plasma of patients with type II tumour however this was significance in only one of the two statistical tests performed. NY-ESO-1 antibodies were not observed in the plasma of type I OVC patients (Lu et al, 2011). NY-ESO-1 mRNA was detected in 2/8 OVC samples however transcripts were also found in normal ovary (Chen et al, 1997). NY-ESO-1 was found to be able to distinguish between early stage disease and Stage III/IV (late stage) OVC as higher expression was found in later stages (Taylor et al, 2009).

1.9.1.4 WT1

WT1 is commonly expressed in ovarian serous carcinomas and is considered to be a diagnostic marker of these tumours, however 34/77 (44 %) of endometrial serous carcinoma that expressed WT1 also had a decreased disease-free survival rate (Hedley et al, 2014). WT1 can be used in a panel with oestrogen receptor and progesterone receptor to distinguish between endometrial serous carcinoma (ESC) and ovarian serous carcinoma (OSC) but alone neither of these antigens were capable of doing so. WT1 was detected in 81 % OSC and 36 % of ESC (Fadare et al, 2013) however the OSC samples examined were all late stage.

Low levels of WT1 antibodies in the plasma of patients diagnosed at stages III-IV and grade 3 carcinomas were related to improved survival in patients and patients with high WT1 antibodies in plasma as well as positive staining in cancer tissues had shorter survival (Andersson et al, 2014). It is interesting that WT1, as with a number of other tumour antigens, is also a biomarker for cancer.

1.9.2 The dual role of tumour antigens as biomarkers for cancer

Although tumour antigens were often investigated for their potential as targets for immunotherapy first, a growing body of work suggests that some can also act as biomarkers of disease (Schumacher & Schreiber 2015). Elevated expression of the CTA PRAME has been an indicator for poor survival and a reduced disease-free survival rate in solid tumours (Yao et al, 2014). In myelodysplastic syndrome very high and very low levels of PRAME were found to be related to poor survival (Liberante et al 2013). In acute promyelocytic leukaemia patients who harbour the t(15;17) translocation, decreased expression of PRAME correlated with shortened overall survival (Santamaria et al, 2008) whereas the typically favourable t(8;21) translocation was associated with a higher level of PRAME in AML M2 patients (van Baren et al, 1998).

The LAA SSX2IP has been found to be a marker of improved survival rates in AML patients who have no cytogenetic aberrations (Guinn et al, 2009). While high transcripts of G250/CA9 and RHAMM are associated with longer overall survival (Greiner et al, 2006). Humoral responses against NY-ESO-1 may correlate with poor survival in hormone refractory prostate cancer (Fosså et al, 2004).

1.9.3 Validation of biomarkers

Validation of biomarkers involves the development of assays that have to be optimised for testing the expression of the biomarker robustly. Firstly an analytical validation step determines

the ability of the assay to accurately measure the biomarker in patient samples and a second clinical validation step ensures that the data generated is reliable as an indicator of the desired outcome. The assay used for validation can include RT-PCR and qPCR (genetic analysis), IHC (protein detection and locale in tissues), fluorescent in situ hybridization (cytogenetic analysis) (Marchiò et al, 2011;Goossens et al, 2015). Clinical evaluations should show clear advantages of using the biomarker in comparison to current best practise, be economically viable and have high bioavailability (Goossens et al, 2015).

1.10 Hypothesis and Aims of study

1.10.1 Hypothesis

I hypothesised that the cancer-testis antigens, PASD1 and SSX2, and the SSX2 interacting protein SSX2IP, would make promising targets for the immunotherapy of acute myeloid leukaemia and ovarian cancer in the early stages of disease. To test this hypothesis I examined the expression of the antigens in these two forms of cancer and compared their expression to the existence of PASD1-specific T cell responses in AML and to other proteins (immunotherapeutic targets and biomarkers) known to be expressed in OVC.

1.10.2 Aims of the study

1. To investigate whether there is a correlation between the expression of the tumour antigen, PASD1, in leukaemia cells and antigen-specific T cell responses in the periphery;

2. To examine the expression of known tumour associated and cancer-testis antigens in OVC;

3. To determine whether a cancer-testis antigen expressed in OVC can also act as a biomarker for the disease.

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue Culture

2.1.1 Cell lines

All cell lines were obtained from ATCC except for A2780 (Sigma-Aldrich Co. Ltd), KM-H2 and KYO-1 (The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH). Cells are grown in RPMI 1640 media (Sigma-Aldrich Company Ltd., Dorset, U.K.) containing 10 % foetal bovine serum (FBS)(Thermo Fisher Scientific, Leicestershire, UK) and 1 % penicillin and streptomycin (Thermo Fisher Scientific), in a humidified incubator at 37°C with 5 % CO_2 (**Table 2.1**).

Table 2.1 Cell lines, disease and original source details.

Cell lines	Cancer type	Species	Adherence	Original reference
A2780	Ovarian adenocarcinoma (OVC)	Human	Adherent	Hamilton et al, 1984
HCT116	Colorectal carcinoma	Human	Adherent	Brattain et al, 1981
HeLa	CC	Human	Adherent	Scherer et al, 1953
HL60	AML	Human	Non-adherent	Collins et al, 1977
Jurkats	Acute T cell leukaemia	Human	Non-adherent	Schneider et al, 1977
K562	CML	Human	Non-adherent	Lozzio and Lozzio, 1975
KM- H2	HL	Human	Non-adherent	Kamesaki et al, 1986
KYO-1	CML	Human	Non-adherent	Ohkubo et al, 1985
OVCAR3	Ovarian adenocarcinoma (OVC)	Human	Adherent	Hamilton et al, 1983
SK-MEL 28	Melanoma	Human	Adherent	Carey et al, 1976
SKOV3	Ovarian adenocarcinoma (OVC)	Human	Adherent	Fogh et al, 1977
SW480	CRC	Human	Adherent	Leibovitz et al., 1976
THIEL	Multiple myeloma (MM)	Human	Adherent	Chi et al, 2011

2.1.2 Varying CO_2 levels

To determine whether tumour antigen protein expression was affected by differing levels of CO_2 (covering a range from 3.5 - 6 %), K562 cells were incubated in one CO_2 level for 7 days prior to being harvested.

2.2 Human samples

2.2.1 Colon cancer patient samples

The TMAs were a kind gift from Dr Alex Mirnezami, University of Southampton. There were between 80-100 colon cancer samples on each array but no further clinical information was available.

2.2.2 Leukaemia patient samples

All patient samples (Appendix I) were collected following informed consent in accordance with the Declaration of Helsinki following Local Research Ethics Committee approval (NREC No. 06/H0606/88) and University Research Ethics Committee Approval.

2.2.3 OVC patient samples

All Tumour Microarrays (TMAs) were prepared by US Biomax, Inc. (Rockwille, United States). Patient information for these samples is available in Appendix II.

2.2.4 Healthy donor samples

Healthy donor blood samples were obtained from the Department of Haematology, Southampton General Hospital following informed consent (Local Research Ethics Committee, Southampton University Hospitals NHS Trust, Southampton U.K., LREC submission number 228/02/T).

2.2.5 Processing leukaemia patient samples for analyses

2.2.5.1 Health and safety considerations around the use of patient samples

Processing of patients samples was carried out in consideration of health and safety requirements. All samples were processed within containment level 2 laminar flow hoods. Personal safety equipment, lab coat and gloves were worn at all times. All waste was disposed of in containers with 5 % fresh Virkon for 4-16 h and then autoclaved.

2.2.5.2 Bone marrow and peripheral blood

The bone marrow or peripheral blood samples were aliquoted at 5 ml per 50 ml universal container (UC) (Fisher Scientific) and 45 ml of 1 X red blood cell lysis buffer (155 mM (ammonium chloride (NH_4Cl), 12 mM sodium bicarbonate ($NaHCO_3$) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (all from Sigma) added. The samples were placed at room temperature (RT) for 30 min and subsequently centrifuged at 530 x g for 5 min. The supernatant was discarded and where multiple tubes of blood from the same individual had been lysed the white cell pellets were pooled together. 1 ml of X-VIVO-15 media (Lonza, Slough, UK) was added and the cells were counted using a disposable haemacytometer (Immune Systems Ltd, Devon, UK). Cells were diluted to 4-8 x 10^6/ml in X-VIVO-15, 1 % human AB sera (Sigma) and 10 % DMSO (Thermo Fisher Scientific), 1 ml aliquoted per cryovial (Thermo Fisher Scientific) and placed in the -80 °C freezer for a minimum of 4 h, then transferred to a liquid nitrogen tank (Locator® 4 Cryo Biological Storage System, Barnstead/ThermoLyne®, Sigman-Aldrich Co. Ltd.) for longer term storage.

2.2.5.3 Isolation of serum from clotted blood

Blood collected in the absence of anti-coagulant contained a solid clot that was removed using tweezers and discarded in an autoclavable bin bag, and following autoclaving was finally disposed of by incineration. The remaining blood was transferred to 15 ml UC and centrifuged at 800 x g for 8 min. The straw coloured yellow serum was harvested, mixed in a 15 ml UC and split equally between ten 1.5 ml eppendorfs for long term storage in the -80 °C (Thermo FisherFisher Revco® ExF -86C ULT Upright Freezer).

2.3 Counting cells

To count cells, 10 µl of the cell suspension was mixed with 10 µl of Trypan blue solution (Sigma). 10 µl of this mixture was applied to the edge of the coverslip of the disposable haemocytometer (FastRead 102 Disposable Counting Slides, Immune Systems Ltd, UK) and moved into the void by capillary action. The haemocytometer has grid lines which form different sized squares when viewed under the microscope at x 10 objective **(Figure 2.1**). Sixteen of the smallest squares make up one large square.

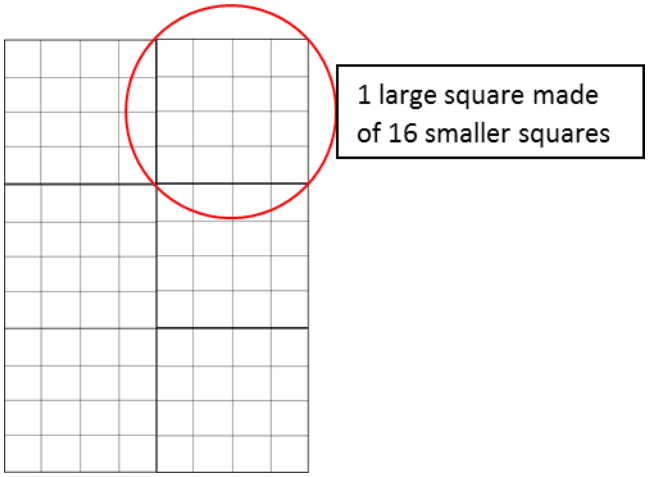

Figure 2.1 Visual of the grids on the disposable haemacytometer viewed under the microscope. The haemacytometer used contained six large squares each made up of 16 smaller squares. Cells in one of the larger squares, in a known volume are counted to determine the number of cells per ml.

Cells were counted in one large square (as indicated by a red circle in **Figure 2.1**). Each of the large squares were 1 mm x 1 mm, and the area between the slide and the coverslip was 0.1 mm, therefore the volume was 0.1 mm^3. To determine the total cells per ml the number of cells counted was multiplied by the dilution factor (X 2) and the resulting number was further multiplied by 10^4/ ml.

2.4 Freezing cell lines and patient samples in liquid nitrogen

Adherent cells were washed with sterile 1 x PBS (bought as 10 x PBS tablets from Sigma Aldrich Co Ltd, Gillingham, UK) and detached using 1 ml of trypsin and placing the flask in the incubator at 37 °C for 1 min. Then 9 ml of RPMI media including 10 % FBS was added to achieve a final volume of 10 ml total. For non-adherent cells the previous step was missed.

10 µl of the cell suspension was used for the counting of cells as described in **section 2.3** and the remaining cell suspension was centrifuged at 530 x g for 5 min. The cells were resuspended in RPMI media to achieve a cell concentration of 8-16 $x10^6$ cells/ml and an equal volume of freezing media (80 % FBS and 20 % Dimethyl sulfoxide (DMSO) (both from Thermo Fisher Scientific) was added to achieve a final cell concentration of 4-8 x 10^6/ml. A 1 ml volume of cells were placed in cryovials and into Mr Frosty^{TM} Freezing Containers with isopropanol (both Thermo Fisher Scientific) at -80 °C for 4-16 h prior to transfer to liquid nitrogen storage.

2.5 Defrosting cell lines and patient samples stored in liquid nitrogen

Cell lines were taken from liquid nitrogen and placed in a preheated water bath at 37 °C for 1 min and gently agitated. Once partially (approximately 40 %) defrosted, the cells were added into 20 ml of RPMI media and allowed to recover overnight in a tissue culture incubator before passaging was considered.

2.6 RT-PCR

2.6.1 RNA extraction

RNeasy mini Kit (Bioline) was used to extract mRNA and the manufacturer's instructions were followed. In brief, 350 µl buffer RW1 was added to RNeasy column and centrifuged for 15 sec at 8000 x g, and the flow-through discarded. 10 µl DNase 1 stock solution was added to 70 µl of RDD Buffer and gently mixed. 80 µl of DNase 1 incubation mix was added to the RNeasy column membrane and placed at room temp for 15 min. 350 µl of Buffer RW1 was added to

the RNeasy column and the column centrifuged for 15 sec at 8000 x g. The flow-through was discarded and 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 sec at 8000 x g to wash the membrane. The tube was left open for up to 2 min to dry. 35 µl of DEPC water, pre-warmed to 37 ºC was added to the column membrane and incubated for 60 sec, then the mRNA eluted by centrifugation for 1 min at 8000 x g. The mRNA was stored at 4 ºC for immediate use or aliquoted and stored at -20 ºC for up to 6 months or -80 ºC if storage for a longer period was required.

2.6.2 1st strand synthesis

RevertAid 1st strand synthesis on RNA (Thermo Fisher Scientific U.K) was used to prepare cDNA. The protocol was as manufacturer's instructions as follows: 11 µl of total RNA (from **Section 2.6.1**) was added at a concentration of 300 ng to 1 µl of random hexamer primer. Diethylpyrocarbonate (DEPC) treated water was added to increase the volume to 12 µl and the mixture is incubated at 70 ºC for 1 h in a heating block. The following components were added in the order given; 5X Reaction buffer, RiboLock RNase Inhibitor, 10 mM dNTP Mix and RevertAid M-MuLV Reverse Transcriptase (all Thermo Fisher Scientific). The tube of reactants were vortexed and then microfuged to bring the reactants to the bottom of the tube. The reaction was then incubated for 5 min at 25 ºC followed by 60 min at 42 ºC. The reaction was terminated by incubation for 5 min at 70 ºC, aliquoted and stored at 4 ºC for use within 6 months, or stored at -20 ºC for the longer term. 1st strand cDNA products were used directly in polymerase chain reactions (PCR) (**Section 2.6.3**).

2.6.3 RT-PCR

The following components were added to PCR tubes in the following order; 12.5 µl of ready mix (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002 % gelatin, 0.4 mM dNTP mix (dATP, dCTP, dGTP, TTP), stabilizers, 0.06 units Taq DNA Polymerase/ml), 1 µl of each primer (4 µM) (Sigma) **(Table 2.2)**, 1 µl of cDNA (300 ng/ul) and sterile water to a final

volume of 25 µl. The tubes were placed into a Thermo Fisher™ Applied Biosystems™ 2720 Thermal Cycler and a programme called GAPDH.cyc provided the following PCR conditions; 94 °C for 2 min, 30 cycles of [94 °C for 30 sec, 50 °C for 40 sec, and 72 °C for 40 sec], followed by 72 °C for 7 min and 4 °C overnight. The reactions were analysed on a 1 % agarose gel (**Section 2.6.4**).

2.6.3.1 Optimisation of the RT-PCR technique for each antigen

All primers used were from previous publications (**Table 2.2**), except p68 and SSX2, which I designed using a primer designing tool from NCBI called Primer-BLAST: http://www.ncbi.nlm.nih.gov/tools/primer-blast/. I chose primers based on experience of use in the lab by my colleagues or recent publications. I mapped every primer onto genomic and transcribed sequences using the NCBI database to check that primers mapped solely to the gDNA of interest and that they flanked an intron. The latter ensured I would see a different PCR product size if the amplicon was primed from cDNA or contaminating gDNA (**Table 2.2**). I used GAPDH to demonstrate my capacity to amplify PCR products from the cDNA and the integrity of the cDNA for PCR amplification. All mRNA was treated with DNase before transcription to cDNA as part of the RNA extraction kit. PCR amplification of mRNA (50 µl), in which PCR reactions were set up replacing cDNA as template with mRNA, demonstrated the absence of gDNA contamination prior to synthesis to produce cDNA. In every experiment, one tube (the "blank") contained all of the reagents (ReadyMix, primers and water) except the template which was volume to volume replaced with sterile water. Using the NanoDrop 2000, mRNA and cDNA used in all experiments had 260/280 of ~2 and 1.7-1.9 respectively.

Table 2.2 Primers used for PCR analysis of cell lines and patient samples.

Primers	Sequence	Tm (ºC)	Reference	Expected band size (bp)	Band size from gDNA (bp)
GAPDH	Forward: 5'-ACCCACTCCTCCACCTTTG-3'	64.0	Dotzlaw et al., 1997	178	648
	Reverse: 5'-CTCTTGTGCTCTTGCTGGG-3'	63.8			
SSX2	Forward: 5'-AGTGTATATGAGGCAGGCCG-3'	60.7	Khan et al, unpublished	954*	44,374
	Reverse: 5'-TGTACGGACCCTTTTTGGGG-3'	70			
SSX2IP	Forward: 5' TGAATGAGCTGCTTGTGCTT 3'	63.7	Guinn et al., 2005b	207	3,615
	Reverse: 5' GCTGATGCAAATTCCTGTTCT 3'	63.1			
MelanA	Forward: 5' ACTGCTCATCGGCTGTTG 3'	62.8	Coulie et al., 1994	268	11,145
	Reverse: 5' TCAGCCATGTCTCAGGTG 3'	61.1			
G250	Forward: 5' GTCTCGCTTGGAAGAAATCG 3'	63.8	Liu et al., 2012a	200	2,038
	Reverse: 5' AGAGGGTGTGGAGCTGCTTA 3'	63.9			
CEACAM5	Forward: 5' CGCATACAGTGGTCGAGAGA 3'	64.1	Liebig et al., 2005	362	5,292
	Reverse: 5' TGTAGCTTGCTGTGTCATTT 3'	58.5			
MUC1	Forward: 5' CGTCGTGGACATTGATGGTACC 3'	68.4	Brossart et al., 2001	287	1,093
	Reverse: 5' GGTACCTCCTCTCACCTCCTCCAA3'	69.5			
NY-ESO-1	Forward: 5' CCCCACCGCTTCCCGTG 3'	72.6	Ries et al, 2009	275	513
	Reverse: 5' CTGGCCACTCGTGCTGGGA 3'	72.4			
PASD1	Forward: 5'-AGCCACCTCTGTGCTGACTT-3'	64.1	Guinn et al., 2005b	233	25,699
	Reverse: 5'-GGTTCAACGTACACGGCTTT-3'	63.8			
p68	Forward: 5'-CTCAGGGCCCATAGTGCAA-3'	65.0	Khan et al unpublished	655	1,144
	Reverse: 5'-ACCCGCGTGTCTGATAATCC-3'	66.0			
Survivin	Forward: 5-GATGACGACCCCATAGAGGAAC-3'	65.6	Johnen et al, 2012	85	1,958
	Reverse: 5'- GGGTTAATTCTTCAAACTGCTTCT-3'	62.5			
Tyrosinase	Forward: 5'-ACAACAGCCATCAGTCT-3'	53.3	Abrahamsen et al., 2005	291	36,694
	Reverse: 5'-CCTGTACCTGGGACATT-3'	55.4			
WT1	Forward: 5'-GGCATCTGAGACCAGTGAGAA-3'	64.4	Cilloni et al., 2002	483	7,376
	Reverse: 5'-GAGAGTCAGACTTGAAAGCAGT-3'	59.3			

*I did try Tureci et al, 1998 SSX2 primers but they did not work in my hands and so I used new primers I designed.

To demonstrate that the RT-PCR experiments worked as expected I extracted mRNA from cell lines that were known to express the antigen of interest (**Table 2.3**) based on data from previous studies and showed transcription of the genes of interest in the cell lines I had.

Table 2.3 Known transcript expression of antigens in human cell lines

Cell line	Antigens expressed	Method	References
KG1	SSX2IP	IHC	Denniss et al, 2007
K562†	SSX2IP	IHC	Denniss et al, 2007
	SSX	RT-PCR	dos Santos et al, 2000
	PASD1	RT-PCR, qPCR, IHC	Hardwick et al, 2013
	WT1	RT-PCR	Inoue et al, 1994
	MUC1	Immunoblotting	Kawano et al, 2007
	P68	Western Blot	Yang et al, 2005
	Survivin	RT-PCR & Western Blot	Schmidt et al, 2003
HeLa	PASD1	MTAs	Liggins et al, 2004a
Sk-mel-28	MelanA	RT-PCR	Chen et al, 1996
SW480	G250	Real-time PCR	Lal et al, 2001
Jurkats	PASD1	RT-PCR	Guinn et al, 2005

†CEACAM5, NY-ESO-1 and Tyrosinase, expression was found in K562 cells but had not been previously reported.

2.6.4 Agarose gel electrophoresis

To make a 1 % agarose gel, 1 g of agarose powder was dissolved in 100 ml of 1 x Tris-acetate-EDTA (TAE) (40Tris, 20 mM acetic acid, 1 mM EDTA) buffer by placing it in the microwave. Once dissolved the agarose solution is cooled by running under the cold tap. Once sufficiently cooled, ethidium bromide was added and the whole mixture was poured into a gel mould with a comb to make wells. After 15 min the gel had solidified and was placed into an electrophoresis tank with TAE buffer. 3 µl of loading buffer was mixed with 12 µl of the PCR reaction and added to the wells. 5 µl of the DNA HyperLadder™ 50 bp (Bioline U.K) was used as a marker.

2.7 IHC

2.7.1 Preparation of samples for IHC

Cells were counted and resuspended at 5 x 10^6/ ml in sterile PBS. Glass slides (Fisher Scientific) were cleaned with pure methanol and 10 µl of cell solution was spotted on each microscope slide at two independent sites. The slides are air dried 4-6 h, double wrapped in saran wrap and stored at -20 °C.

2.7.2 Antibodies

Antibodies were used are described in **Table 2.4**.

We used two PASD1 mouse anti-human monoclonal antibodies which were a kind gift from Professor Alison Banham, (John Radcliffe Hospital, University of Oxford, UK). Both variants of the PASD1 protein, PASD1a and PASD1b, are detected by the antibody PASD1-1 which binds between a.a. 195-474, while PASD1-2 binds the PASD1b protein in a region between a.a. 540-773 (Cooper et al, 2006; **Figure 2.2**). PASD1 has previously been shown to be expressed in primary spermatagonia with expression decreasing with sperm maturation.

I used three independently derived antibodies (all Abcam) to identify SSX2 protein expression during my studies (**Figure 2.3**). A pSSX2 antibody that bound to SSX2 in the N' terminus between a.a. 21-70, was renamed pSSX2 (N) for clarity. I also used two antibodies that were specific for SSX2A. These were named mSSX2A (clone 4D10) a mouse monoclonal antibody, and pSSX2A, a rabbit polyclonal that each recognise SSX2A between a.a. 176-223.

Table 2.4 Antibodies used for ICC

Antibodies	Antibody clone name	Type (Isotype)	Source (product number)	Species	Dilution	Stock [conc] (µg/ml)
Actin	ACTN05(C4)	Monoclonal ($IgG_{1)}$	Abcam (ab3280)	Mouse anti-human	1/100	2
mSSX2A	4D10	Monoclonal (IgG_1)	Abcam (ab117972)	Mouse anti-human	1/100	6.7
pSSX2(N)	N/A	Polyclonal (IgG)	Abcam ab182361	Rabbit anti-human	1/100	10
pSSX2A	N/A	Polyclonal, IgG	Abcam ab48571	Rabbit anti-human	1.100	10
CA125	OC125	Monoclonal, IgG_1	AbD Serotec MCA1914H	Mouse anti-human	1/100	90
Anti-HE4	EPR4743	Monoclonal, IgG	Abcam ab109298	Rabbit anti-human	1/100	17.18
Anti-WT1	1E9	Monoclonal, IgG_{2a}	Abcam ab118873	Mouse anti-human	1/100	5
SSX2IP	N/A	Polyclonal, IgG	Abcam ab10256	Goat anti-human	1/100	5
PASD1-1: reacts with PASD1a and PASD1b	2ALCC136	N/A	University of Oxford	Mouse anti-human	1/250	NK
PASD1-2: recognises PASD1b	2ALCC128	N/A	University of Oxford	Mouse anti-human	1/50	NK
Isotype control for actin, mSSX2A and PASD1 Abs	MOPC-21	Monoclonal, IgG_1	Abcam ab18443	Mouse anti-human	1/100	5
Isotype control for pSSX2(N), pSSX2A and HE4 Abs	N/A	Polyclonal, IgG	Abcam ab37416	Mouse anti-human	1/100	50
Isotype control for SSX2IP Ab		Polyclonal, IgG	Abcam ab79108	Goat anti-human	1/100	5
Secondary Ab for actin, mSSX2A, CA125, WT1 and PASD1	N/A	N/A	Envision kit, Dako	HRP anti-mouse	N/A	N/A
Secondary Ab for pSSX2(N), pSSX2A and HE4	N/A	Polyclonal, IgG	Abcam ab6721	Goat anti-rabbit	1/100	20
Interlinking Ab for SSX2IP	GT175	N/A	University of Oxford	Mouse anti-goat	1/100	NK

Ab: antibody; HRP: Horseradish peroxidase; **M: monoclonal; NK: not known; N/A: not applicable; N: N' terminal; p: polyclonal**

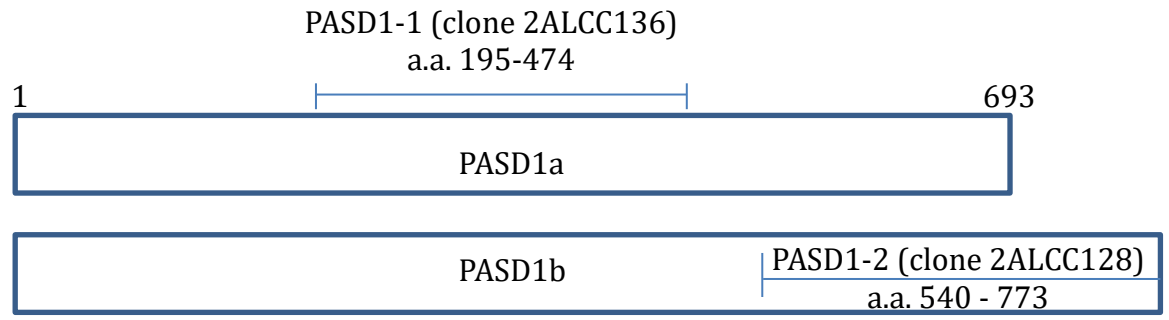

Figure 2.2 Schematic diagram indicating the location that each of the PASD1 monoclonal antibodies bind to on the PASD1 variant. It has been suggested that there are a number of PASD1 variants but only the two indicated have been characterised (Cooper et al, 2006). Although binding of PASD1-2 indicates the expression of PASD1b in cells, the PASD1-1 antibody does not distinguish between PASD1a and b expression, but when used in combination with PASD1-2, PASD1b expression can be discounted.

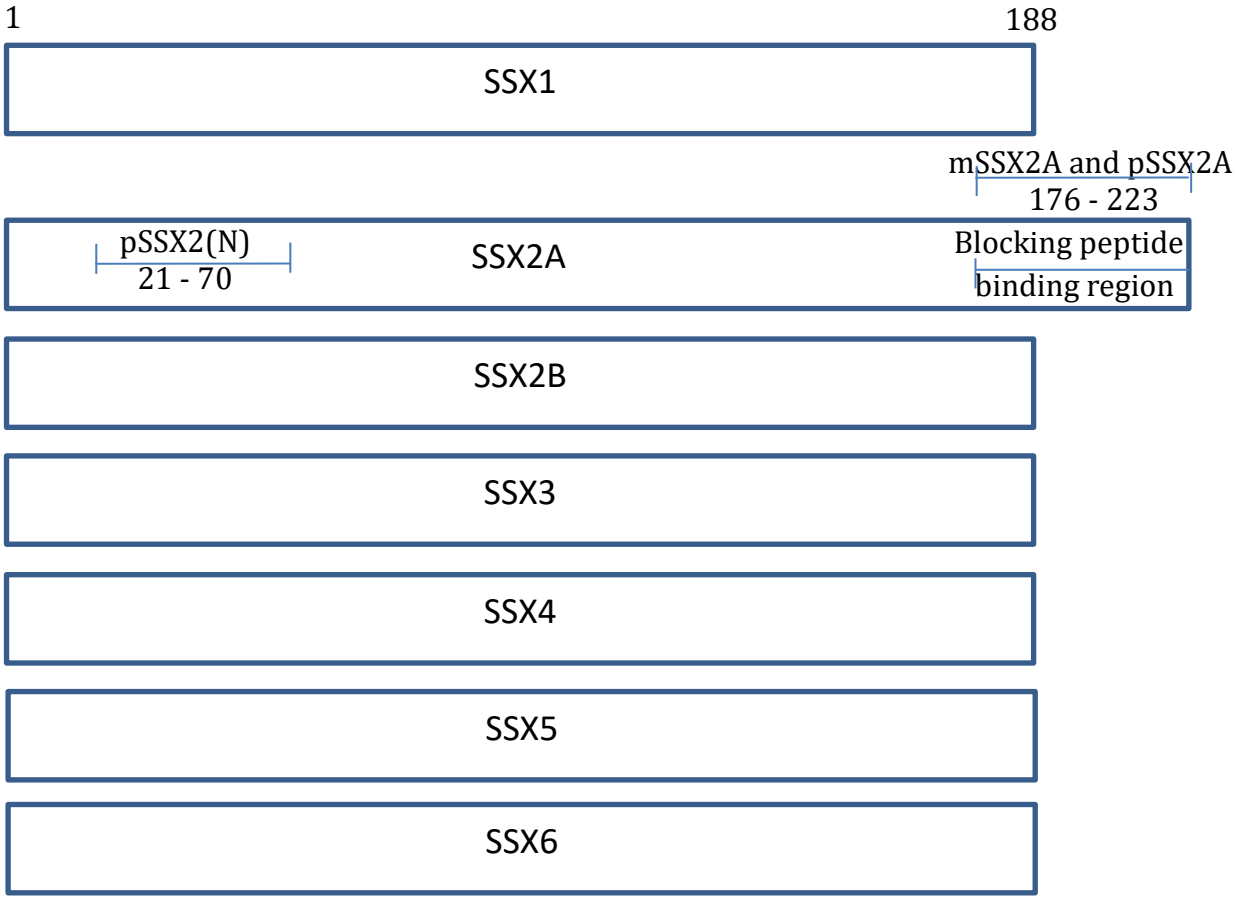

Figure 2.3 Schematic of the SSX family of variants and the location of antibody binding. SSX2A has a unique carboxy region only bound by the mSSX2A and pSSX2A antibodies. The third antibody used in these studies bound to a region common to both SSX2A and SSX2B referred to as pSSX2(N).

2.7.3 ICC on frozen samples

Slides were taken from storage at -20 ºC, and defrosted for 20 min at RT before removing the saran wrap. A diamond pen (Fisher Scientific) was used to mark around the area of the slide to be stained (the 'button'). The cells were fixed by inserting the slides into a coplin jar containing 50 ml of 100 % cold methanol (Fisher Scientific) on ice for 15 min. When analysing leukaemia samples an additional 1 ml of H_20_2 was added to the methanol. The slides were removed from the fixative and placed horizontally into a moisture chamber face up. They were washed three times in Tris-buffered saline (TBS) (0.15 M sodium chloride; 0.05 M TRIS-HCl buffer; pH 7.6) (Sigma) and the excess TBS was carefully removed from the periphery of each of the buttons using dry white tissue. A peroxidase block from the Envision Kit (Dako) was added to the cells for 5 min. The appropriate primary antibodies were diluted in TBS (**Table 2.4**), and 40 µl of the antibody solution was added to each button. Anti-actin was used as a positive control. The slides were incubated with primary antibody for 1 h at RT then washed three times with TBS. Only SSX2IP antibody was produced in goat, therefore an intermediary antibody called GT175 (kind gift from Professor Alison Banham, University of Oxford) was added after the primary for 1 h at RT as a linker to the secondary antibody. The secondary antibody was supplied as part of the Envision+ System which is horseradish peroxidase (HRP) based and includes 3,3'-diaminobenzidine (DAB) as the detector reagent in the kit (Dako). HRP conjugated anti-mouse IgG antibody which was added and incubated for 30 min at RT. Following washes with TBS, 20-30 µl of DAB substrate from the Envision kit was added to each cell button, incubated for 5 min then washed gently with water. 40 µl of 1:5 dilution Mayer's haematoxylin: Lillie's modification (Dako) was added to each button as a counterstain, and washed with copious amounts of tap water to remove all excess stain. Slides were mounted in DPX mountant (VWR) and imaged using the virtual microscopy system

Olympus Dotslide (University of Southampton) at 40 X magnification at the University of Southampton.

2.7.4 IHC on TMAs

Using a method shared by Linden Lyne in the laboratory of Professor Alison Banham (Liggins et al, 2004a) paraffin-embedded ovarian TMAs containing 208 samples (product no.OV2084) (Biomax U.S) and endometrial TMAs with 80 samples (product no.UT801) (Biomax) were de waxed in citroclear (TCS Biosciences U.K) twice for 5 min. The slides were then placed in 100 % ethanol twice for 5 min (air drying in the interval for a few seconds) and then once in 50 % ethanol for 5 min. The arrays were washed with tap water and antigen retrieval was performed in Tris/EDTA buffer pH9 (Sigma) in the microwave (800W) for 11 min of full power. Following cooling, the arrays were washed in TBS. Peroxide block (Dako) was added for 5 min and the tissue arrays washed again in TBS. From this point staining was carried out as per ICC on frozen samples (**Section 2.7.2**). Scoring was performed in a single blinded fashion by Dr Barbara Guinn. Patient information and tumour characteristics are available in appendix II.

2.7.5 Blocking protocol

To establish the specificity of the SSX2 antibodies being used, antibody blocking was performed. Blocking peptides for all SSX2 antibodies tested were purchased from Abcam **(Table 2.5)**. All three antibodies which recognised SSX2 named mSSX2A, pSSX2(N) and pSSX2A, were diluted in TBS along with the blocking peptide. The peptide was 20 X the concentration of the antibodies (1 µM Ab:20 µM peptide) which had been previously used successfully to block antigens (Professor JoAnne McLaurin, University of Toronto, Canada, personal communication). The mixture was placed at 4 °C overnight in a rotator and ICC was performed as described in **section 2.7.2** using the blocked antibodies in direct comparison to the same antibodies incubated overnight with an equal volume of TBS to show the specificity of the antibodies for the target peptide.

Table 2.5 Sequences of peptides used to block antibodies in ICC

Peptide	Source & catalogue number	Antibodies the peptide blocks	Sequence	a.a.
N' terminal SSX2	Abcam Ab192644	pSSX2(N)	**IQKAFDDIAKYFSKEEWEKMKASEKI FYVYMKRKYEAMTKLGFKATLPPF**	**21-70**
C' terminal SSX2	Abcam Ab182921	mSSX2A and pSSX2A (both C-terminal)	**QNTHNIGRFSLSTSMGAVHGTPKTIT HNRDPKGGNMPGPTDCVRENSW**	**176-223**

2.7.6 Scoring of samples following ICC/IHC

Staining intensity was classified on a scale of 0 to 3 (0: no staining; 1: background; 2: weak staining; 3: moderate staining; 4: strong staining) based on the system described by Biesterfeld et al, 1996. The percentage of positively stained cells in cell lines and leukaemia patient samples were scored by cell count on a scale of 0 to 4 (0: 0 %; 1: 1-10 %; 2: 11-50 %; 3: 51-80 %; 4: > 80 %) as well as the number of stained cells (% in 200 cells where available) and sub-location (nuclear, surface, cytoplasmic). The immunoreactivity score was achieved by the multiplication of the intensity score with the percentage of positive cells (% in counts of 200 cells) (Deng et al, 2014). Immunoreactivity scores of 0= negative, 1-29= weak, 30-143= moderate, 144-228= high and >228= very high staining.

2.8 Statistical Analyses

The statistical analysis was performed by Professor Ken Mills at the CCRB. It was carried out using Partek Genomic Suite that has statistical analysis capability. A 2-tailed paired t-test was used to see if there was a difference in both directions. A significance level of 0.05 allows 0.25 in each tail of the distribution. A paired T-test compares the same samples in different conditions to see whether the differences between them are significant. In this project, the same samples were subject to different antigens and the subsequent labelling was analysed.

CHAPTER 3: EXPRESSION OF TUMOUR ANTIGENS IN HUMAN LEUKAEMIA PATIENT SAMPLES AND CORRELATION WITH ANTIGEN RECOGNITION BY SPECIFIC CD8$^+$ T CELLS

3.1 Introduction

Dr Guinn's group had previously shown that the CTA, PASD1 was recognised by antibodies in the sera of presentation AML patients (Guinn et al, 2005) and that it was the most frequently expressed CT antigen in presentation AML samples. Subsequently Brooks et al, (2015) used the pMHC array to determine the frequency with which "untouched" CD8$^+$ T cells from presentation AML patients recognised different antigens and the epitopes therein that were relevant to AML. I played a small part in this study (that was funded by a Cancer Research U.K. Discovery Committee Award; C1510/A11926). The primary aim of the study was to prioritise the PASD1 epitopes identified by Ait-Tahar et al, 2010 called PASD1(1), PASD1(2) and PASD1(5) in comparison to an analogue peptide, Pa14, identified by Hardwick et al, 2013, using the pMHC array. My role was to determine whether patients' whose CD8$^+$ T cells recognised PASD1 epitopes also had PASD1 transcripts and/or protein expression in their leukaemia cells. The T cell specificities examined by the pMHC array included antigens and epitopes therein that had been identified, predominantly in myeloid leukaemia, by other investigators (**Table 3.1**). These additional LAAs provided comparators for T cell recognition by CD8$^+$ cells from leukaemia patients with T-cell specificity for the PASD1 epitopes.

Table 3.1 Frequency of expression of the antigens on the pMHC array in haematological malignancies, with a focus on myeloid leukaemias.

LAA	Frequency of expression in AML	References
G250	60 % of myeloid leukaemia patients showed T cell responses	Greiner et al, 2006
Gp100	80/853 leukaemia patients were positive	Greaves et al, 1983
HAGE	57 % of CML and 23 % of AML patients samples	Adams et al, 2002
MelanA	3/26 AML patients had specific T cells	Brooks et al, 2015
MUC1	67 % of AML and 92 % of myeloma patients	Brossart et al, 2001
PASD1	33 % of AML and 17 % of CML patient samples	Guinn et al, 2005
Proteinase 3	Antibody detection 2-5 times greater in AML and CML than in healthy samples	Dengler et al, 1995
p68 RNA-helicase	Detected in 2/3 myeloid leukaemia cell lines K562 and KG1 but not in HL60	Lin et al, 2012
Survivin	detected in 75/125 of *de novo* AML cases	Adida et al, 2000
Tyrosinase	3/26 AML patients had specific T cells	Brooks et al, 2015
WT1	Transcripts detected in 73 % of patients	Schmid et al, 1997
P53	Mutations found in 1/39 de novo AML patients	Fenaux et al, 1991

In order to correlate the T cell recognition of HLA-A2 restricted PASD1 epitopes identified by previous investigators (Ait-Tahar et al, 2009; Hardwick et al, 2013) with the expression of PASD1 in the leukaemia cells, the same patient samples were used for both analyses where possible. The leukaemia samples I chose to study for PASD1 expression were HLA-A2 positive, while some were positive for the presence of PASD1-specific T cells and some were not. Some of the HLA-A2 patients had T cells that bound to the HLA-A2 positive pMHCs which may have been due to the promiscuity of the TCR which only recognises one or two amino acids in an epitope and as such one TCR can recognize numerous pMHC. As the pMHC array analyses had included other leukaemia associated antigens and their epitopes, I also chose to study these antigens alongside PASD1. The pMHCs on the array were determined by availability from Professor Hans-Georg Rammensee's group at the University of Tubingen and relevance, where possible, to AML. All had been tested by the investigators who had requested they were made as part of collaborations with the Rammensee group prior to our studies. The exception to this were five HLA-A2 restricted PASD1 epitopes, made for the pMHC array study as part of a collaboration with Dr Barbara Guinn (**Table 3.2**).

Table 3.2. pMHCs used on the array to detect virus and LAA-specific T cell populations within the peripheral blood CD8$^+$ population of leukaemia patients.

Epitope	HLA type	Amino acid sequence	Ref[a]
ALK-SLA human	HLA-A*0201	SLAMLDLLHV	Passoni et al, 2002
CMV pp65	HLA-A*0201	NLVPMVATV	Wills et al, 1996
Flu M1	HLA-A*0201	GILGFVFTL	Bodmer et al, 1989
G250	HLA-A*0201	HLSTAFARV	Vissers et al, 1999
Gp100	HLA-A*0201	KTWGQYWQV	Skipper et al, 1999
HAGE	HLA-A*0201	DLILGNISV	Mathieu et al, 2007
HBV	HLA-A*0201	FLLTRILTI	Rehermann et al, 1996
HPV16 E7	HLA-A*0201	YMLDLQPETT	Kast et al, 1994
HPV16 L1	HLA-A*0201	ICWGNQLFV	Voss et al, 1997
Library	HLA-A*0201	[c]	Arnold et al, 1997
MelanA mod	HLA-A*0201	ELAGIGILTV	Valmori et al, 1998
MUC1	HLA-A*0201	NLTISDVSV	Carmon et al, 2000
Muc1 mod	HLA-A*0201	KLLLTVLTV	Hoff et al, 2010
MUC-1 tandem repeat	HLA-A*0201	STAPPVHNV	Brossart et al, 1999
MUC1_HUMAN mod	HLA-A*0201	SLAPPVHNV	Rodulf, 2008
PASD1(1)	HLA-A*0201	QLLDGFMITL	Cooper et al, 2006
PASD1(2)	HLA-A*0201	YLVGNVCIL	Cooper et al, 2006
PASD1 (Pa14)	HLA-A*0201	RLWQELSDI	Hardwick et al, 2013
PASD1(5)	HLA-A*0201	ELSDSLGPV	Cooper et al, 2006
Proteinase 3	HLA-A*0201	VLQELNVTV	Molldrem et al, 1996
p68 RNA-helicase	HLA-A*0201	YLLPAIVHI	Verma et al, 2010
$Survivin_{5-11}$	HLA-A*0201	TLPPAWQPL	Schmitz et al, 2000
$Survivin_{96-104}$	HLA-A*0201	LTTLGEFLKL	Schmitz et al, 2000
Tyrosinase	HLA-A*0201	YMDGTMSQV	Skipper et al, 1999
VMSA_HPBV	HLA-A*0201	WLSLLVPFV	Nayersina et al, 1993
$WT1_{37-45}$	HLA-A*0201	VLDFAPPGA	Smithgall et al, 2001
$WT1_{126-134}$	HLA-A*0201	RMFPNAPYL	Goa et al, 2000
$EBV\_BMRF1_{105-114}$	HLA-A*0101 mut	AVEQASLQFY	NK
$EBV\_BZLF_{340-348}$	HLA-A*0101 mut	VVETLSSSY	NK
EBV BZLF	HLA-A*0101 245V[b]	DSELEIKRY	NK
$LMP2\_EBV_{410-420}$	HLA-A*0101 mut	LTEWGSGNRTY	NK
$MAGE\ 1_{161-169}$	HLA-A*0101	EADPTGHSY	Mukherji et al, 1995
$EBV\ BMLF1_{298-306}$	HLA A*0301 mut	SLSKVILTLK	NK
$EBV\ BRLF1_{148-156}$	HLA-A*0301 mut	RVRAYTYSK	Benninger-Doring, et al, 1999
$EBV\ EBNA3_{471-479}$	HLA-A*0301	RLRAEAQVK	Hill et al, 1995
LMP1_EBV mod.	HLA-A*0301	ALFLGIVLK	NK
p53	HLA-A*0301	RVRAMAIYK	Zhang et al, 1996
$p53_{321-330}$	HLA-B*0702	KPLDGEYFTL	Gnjatic et al, 1995
EBV BZLF1	HLA-B*0801	RAKFKQLL	Steven et al, 1997
$EBV\ EBNA3_{325-333}$	HLA-B*0801 wt	FLRGRAYGL	Misko et al, 1990

[a]Original reference for the epitope, [b]245V mutation of MHC class I as described by (Goa et al, 2000); [c]a random selection of 6,000 peptides, generated as described in reference (Arnold et al, 1997).
HLA: human leukocyte antigen; Mod: modified; Mut: mutated; NK: not known.

I examined PASD1 expression in samples from the leukaemia patients whose T cells were examined on the pMHC array by immunolabelling and for completeness RT-PCR.

As positive controls for the effective amplification of cDNA by each primer pair, a number of cells lines were used; K562, KG1, Jurkats, HeLa, SW480 and SK Mel 28. These cell lines were chosen on the basis that they had either previously been shown to express the antigen under investigation or when I tested them acted as suitable controls for the amplification of the gene transcripts in question (**Table 2.3**). The leukaemia cell line K562 provided a suitable positive control for many of the LAAs but where it wasn't suitable other cell lines were found.

To optimise ICC for the PASD1 antibodies, a number of leukaemia cell lines including K562 and solid tumour cell lines including SW480 were used. K562 and SW480 had been shown to express the PASD1 protein previously (Cooper et al, 2006; Hardwick et al, 2013). An embryonic kidney cell line (HEK 293) was used as a definitive negative control as PASD1 has been shown to be absent from kidney tissue (Cooper et al, 2006) previously.

For the pMHC array study $CD8^+$ T cells were negatively isolated from the peripheral blood of patients with leukaemia or from healthy donors, and were referred to as "untouched" as they were not expanded or stimulated with peptide *ex vivo* prior to their analyses on the array. These "untouched" T cells were dyed with the lipophilic tracer, DiD, and incubated with polyacrylamide arrays printed with pMHCs from more than 50 tumour-associated antigen and viral epitopes (including HLA-A*0201/ CMV and Flu controls) (**Table 3.2**). The pMHC array study does not detect the functionality of the T cells and although some groups have shown the pMHC array can be modified to detect cytokines (Chen et al, 2005), Hans Vergauwen while studying with the Guinn group did not find this to be possible (Vergauwen, 2011, MSc by Research thesis).

I investigated a cohort of leukaemia patient samples (some of whom had T cells that bound to pMHCs on the array and some that did not), depending on the samples available in storage and the previous data obtained, which numbered 18 and three normal donor controls (PBMNCs) to

determine whether there was a correlation between the presence of LAA-specific T cells in the peripheral blood of these patients (**Table 3.3**) and expression of the genes (transcripts and protein) in the leucocytes from the same patients.

The pMHC array study showed that PASD1 epitopes were the most frequently recognised. Of the 26 AML patients whose $CD8^+$ T cells were analysed eight had T cells that recognised one or more of the LAA epitopes and while four of these patients had T cells that recognised PASD1(2), three patients had T cells that recognised PASD1 Pa14 and one patient had T cells that recognised the PASD1(5) epitope. The cut-off point for a positive result was that at least 50 % of the six spots on the pMHC in each of the two areas had to have a detectable number of T cells attached (approximately 40). For this reason ALL003 and AML001 were not identified as scoring positive for T cell recognition. Patient ALL003 was positive in two of six spots in total and AML001 was positive for two of six spots in one area.

I then examined a small subset of AML patients to determine whether their leukaemia cells expressed PASD1 protein and whether this correlated with the presence of PASD1-specific $CD8^+$ T cells in their periphery. For completeness I also examined whether PASD1 transcripts were detectable in the patients' leukaemia cells along with the other LAAs used in the pMHC study.

Table 3.3 Binding of virus and LAA-specific pMHCs by "untouched" CD8$^+$ T cells purified from AML, ALL and CML patients (data taken from Brooks et al, 2015). The table shows the results from patient samples that were analysed on the pMHC array and were subsequently analysed by RT-PCR for tumour antigen expression and for PASD1 expression by ICC.

Patient ID	HLA-A2/CMV status	HLA-A2 - pMHC Molecules													
		CMV IE1	CMV pp65	Flu M1	CEAM 5	G250	MelanA	MUC1 950-958	PASD1 Pa14	PASD1(1)	PASD1(2)	PASD1(5)	p68 RNA Helicase	Tyrosin-ase	WT1 126-134
AML001	+/-														
AML003	+/+														
AML004	+/+	+	+						+					+	+
AML006	-[a]/+														
AML008	+/+		+						+						
AML009	-/nk														
AML013	+/nk														
AML014	+/nk										+				
AML015	+/nk														
AML018	-/nk														
AML019	+[a]/nk														
AML021	-[a]/nk				+										
AML023	+/nk														
AML024	Nk/nk														
ALL001	+/+	+	+												
ALL002	+/nk														
CML001	+/nk														
CML002	-/nk														

nk: not known

3.2 Aims

- To correlate the detection of PASD1 specific T cells on the pMHC array with PASD1 gene transcripts and protein expression in leukaemia cells from the same patient samples.
- To investigate whether patients with T cells that bound to pMHCs encoding epitopes expressed the same antigen (by virtue of gene transcripts) in their tumour cells.

3.3 Results

3.3.1 Detection of TAA transcription in cell lines

I wanted to determine which of the leukaemia patient samples expressed transcripts of the tumour antigens being studied on the pMHC array, and investigate whether patients that had LAA-specific T cells, as detected by the pMHC array, also expressed transcripts for the same antigens being expressed in their malignant cells. I limited my study to the tumour antigens that were also investigated on the pMHC array and that were recognised by patient T cells with some samples that were HLA-A2 negative or $CD8^+$ T cell negative to act as negative controls. I optimised RT-PCR analysis using a panel of cell lines, at least one of which was known to express the antigen of interest (**Table 2.3**). The primers used for RT-PCR are detailed and referenced in **Table 2.2**. I found expression of each LAA in at least one of the cell lines, in agreement with already published data, where available (**Table 3.4; Figure 3.1**). K562 provided a positive control for the expression of most of the LAAs under test except G250, MelanA and SSX2.

Table 3.4 Expression of PCR transcripts in human cancer cell lines.

	CEACAM5	G250	GAPDH*	MelanA	MUC1	NY-ESO-1	P68	PASD1a+b	SSX2#	SSX2IP#	Survivin	Tyrosinase	WT1
K562	+	-	+	-	+	+	+	+	-	+	+	+	+
KG1			+					-	-	+			-
HeLa			+						+	+			
SW480		+	+	-					-	+			-
Jurkats			+										
SKMel28			+	+									

*GAPDH was used as a positive control for the competency of the cDNA for amplification.
#SSX2 and SSX2IP pMHCs were not available for array analysis but were examined by RT-PCR in the human cancer cell lines due to our groups prior interest in these antigens
Cells that are empty on the table indicate that this PCR analysis was not done. Data is representative of at least two independent experiments in which mRNA was extracted from cells, used to produce mRNA, transcribed into cDNA and used for PCR analysis.

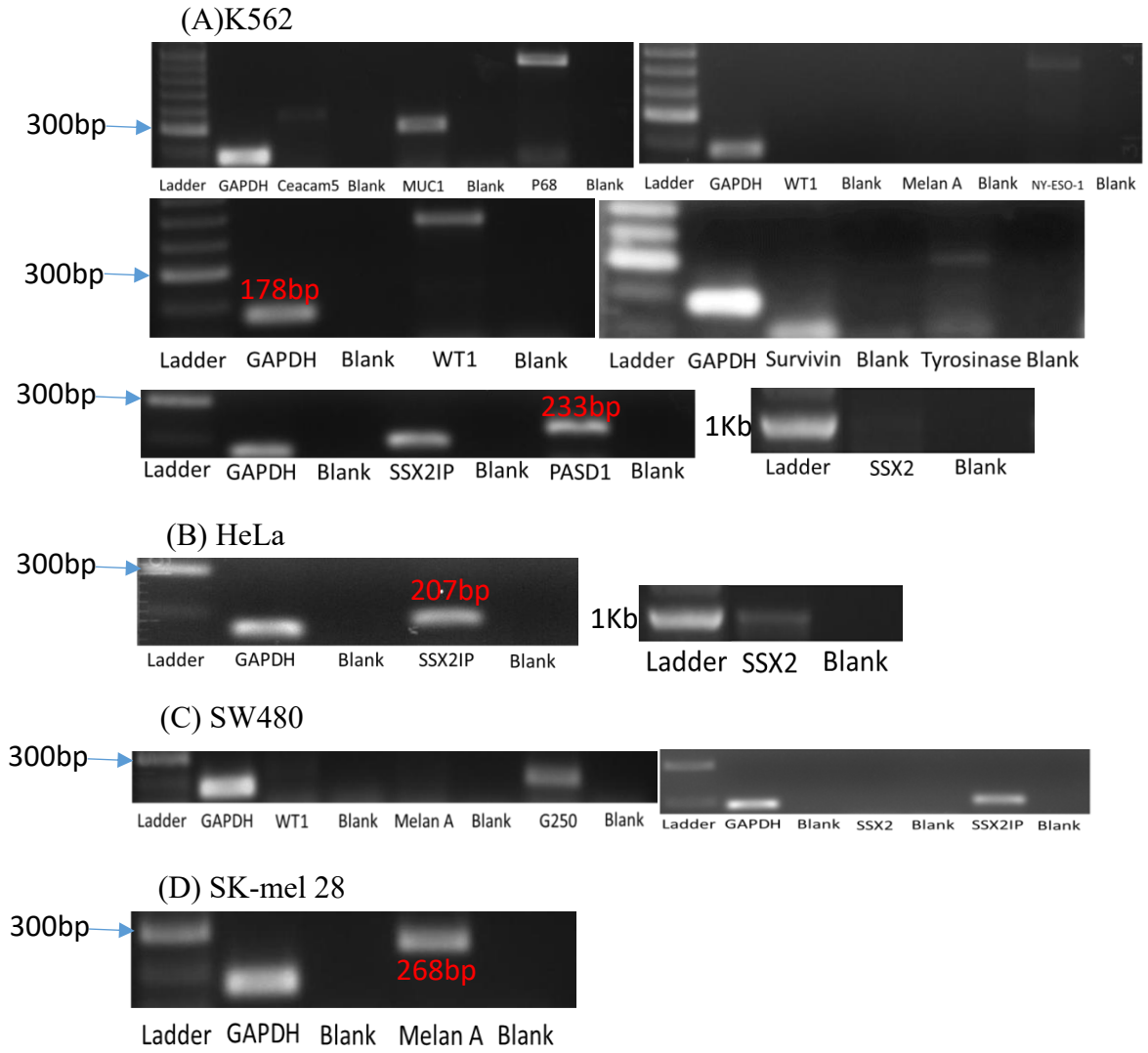

Figure 3.1 Optimisation of the RT-PCR analysis of tumour antigen expression in cell lines. PCR conditions (primers, $MgCl_2$ concentration and annealing temperature) were optimised to ensure products were made from cDNA from at least one of the cell lines under test for each antigen. 1 % agarose gel electrophoresis was used to separate PCR products for analysis. Ladder is HyperLadder II with the brightest band indicated as being 300bp, sizes of bands are shown in red in bp.

3.3.2 Transcription of LAAs in leukaemia patient samples

Transcriptional expression was carried out as an extra comparator as these LAAs were used in detecting T cells on the pMHC array beside PASD1. Eight LAAs (CEACAM5, G250, MelanA, MUC1, P68, PASD1, Tyrosinase and WT1) were investigated by RT-PCR in 18 leukaemia patient samples (14 AML, 2 CML, 2 ALL) (**Figure 3.2),** all of whom had LAA-specific-T cells for these antigens when examined on the pMHC array. In addition I examined three samples of PBMNCs from healthy donors. Surprisingly healthy donor PBMNCs were found to express

six of the eight antigens examined (CEACAM5, G250, MUC1, P68, Tyrosinase and WT1), the exceptions being PASD1 or MelanA. Each experiment was repeated at least twice and included a no template control, colloquially referred to as a "blank" and a positive control for PCR amplification, GAPDH.

I found that only four of the eighteen leukaemia samples analysed on the pMHC array had LAA-specific T cells that recognised an epitope from an LAA. However all 18 samples produced transcripts detectable for at least one of the LAAs as determined by RT-PCR. G250, MUC1, p68, tyrosinase and WT1 transcripts were found in samples that did not have detectable LAA-specific T cells in their periphery, against these antigens however in one patient sample (AML004), had WT1 transcripts in the tumour cells and WT1-specific T cells were detected on the pMHC array **(Table 3.5)**.

A PASD1 amplification product was detected in the following patient samples; AML006, AML013, AML015, AML018 and AML026. This equates to 28 % (5/18) of patient samples, similar to previous findings by Guinn et al, 2005. However, with the exception of AML006, the PASD1 amplicon detected in the patient samples was not the 233bp band expected based on the findings from previous publications (Guinn et al, 2005b)(**Table 2.4**; **Figure 3.3).** Cooper et al, (Cooper et al, 2006) also described the finding of additional PASD1 variants in their studies as determined by expected protein weights when Western blotting DLBCL cell lines.

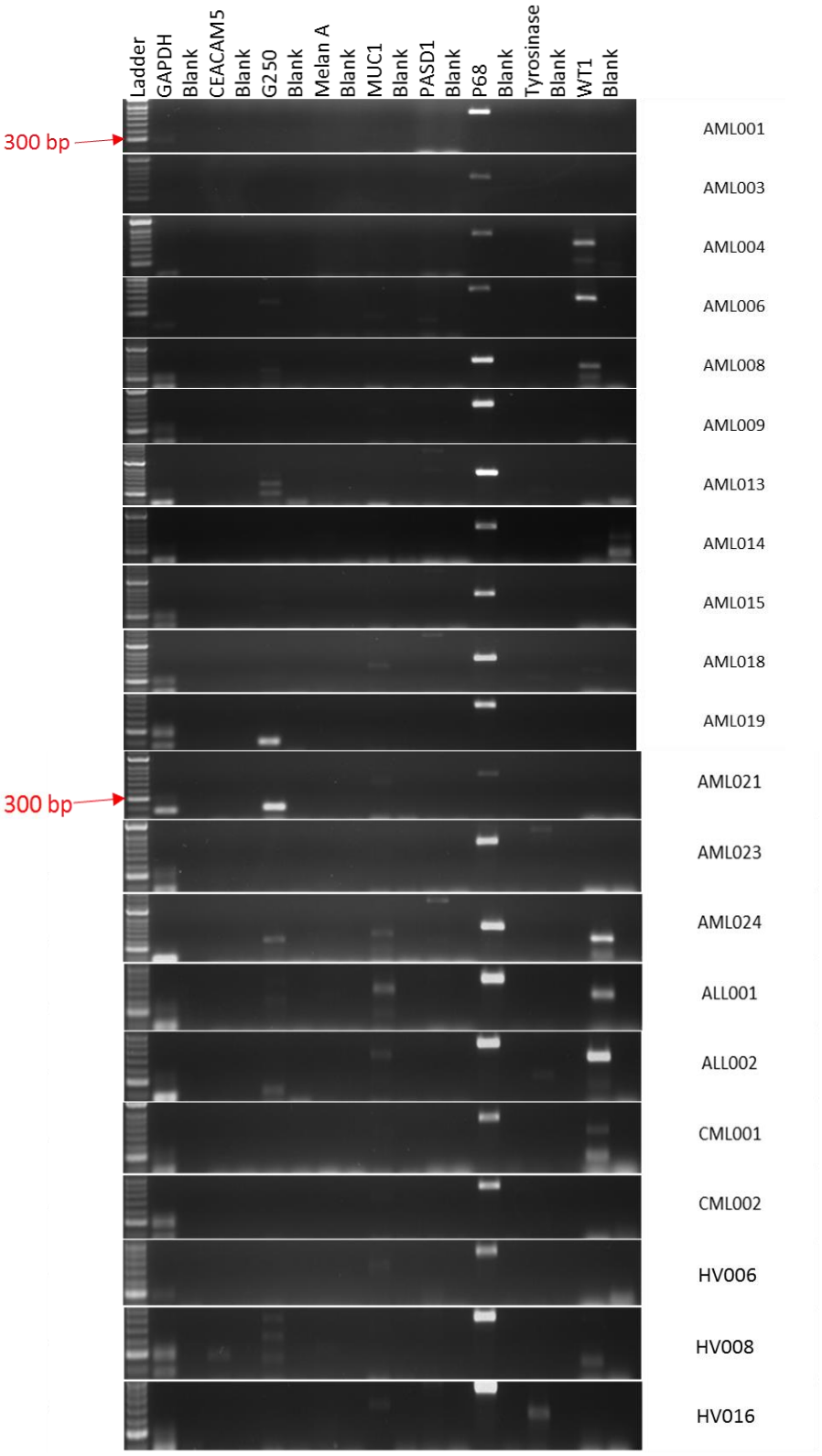

Figure 3.2 RT-PCR analysis of antigen expression in leukaemia patient samples. The LAAs tested were variably expressed by patient samples as indicated in **Table 3.4**. GAPDH was used as a positive control for the competency of the PCR amplification process. Healthy volunteer (PBMNCs) were used as controls, the ladder was HyperLadder II. Data is representative of at least two independent experiments.

Table 3.5 Detection of LAA transcription in patient samples and normal donors analysed on the pMHC array.

	GAPDH	CEACAM5	G250	MelanA	MUC1	PASD1	P68	Tyrosinase	WT1
AML001	+	-	-	-	-	-	+	-	-
AML003	-	-	-	-	-	-	+	-	-
AML004	+	-	-	-	-	-	+	-	+
AML006	+	-	+	-	+	+	+	-	+
AML008	+	-	+	-	-	-	+	-	+
AML009	+	-	-	-	-	-	+	-	-
AML013	+	-	+	-	-	+	+	+	-
AML014	+	-	-	-	-	-	+	-	-
AML015	+	-	-	-	-	+	+	-	-
AML018	+	-	-	-	+	+	+	+	+
AML019	+	-	+	-	-	-	+	-	-
AML021	+	-	+	-	+	-	+	+	-
AML023	+	-	-	-	-	-	+	-	-
AML024	+	-	+	-	+	+	+	-	+
ALL001	+	-	+	-	+	-	+	-	+
ALL002	+	-	+	-	+	-	+	+	+
CML001	+	-	-	-	-	-	+	-	+
CML002	+	-	-	-	-	-	+	-	-
ND1	+	-	-	-	+	-	+	-	-
ND2	+	+	+	-	+	-	+	-	+
ND3	+	-	-	-	-	-	+	+	-

†: Housekeeping gene used to show that the cDNA template was intact and could be amplified; +: band of the expected size; +: band not of the expected size for amplification from cDNA or gDNA. ND: normal donor PBMNC

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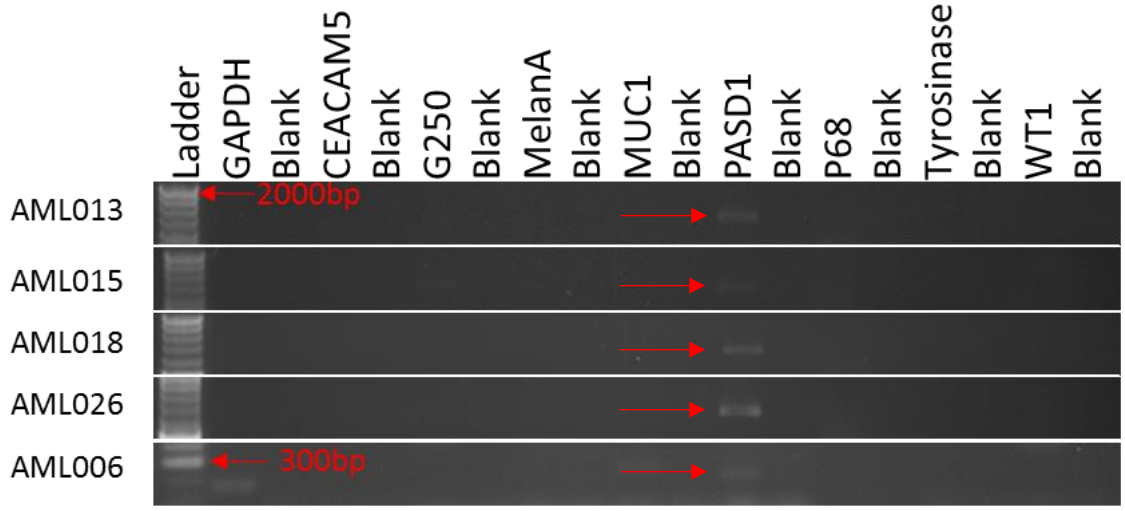


Figure 3.3 Detection of PASD1 transcripts in leukaemia patient samples. The amplification of a PASD1 PCR product was detected in samples AML006, AML013, AML015, AML018 and AML026, however only AML006 cells produced a transcript of the expected size (233bp). The other PASD1 PCR products were transcripts each of the same greater size (approximately 1600bp).

3.3.3 Detection of PASD1 protein expression in cell lines

PASD1 was the most frequently recognised tumour antigen by T cells from AML patients on the pMHC array. To achieve my aim of confirming whether PASD1 protein was being translated, the expression of PASD1 protein in AML patient samples was investigated using ICC. I optimised the immunolabelling assay using cancer cell lines that were known to be positive such as K562, or as one of the controls, negative (HEK 293), for PASD1 protein expression (**Figure 3.4**). Two anti-PASD1 antibodies were used as described previously (**Section 2.7.2**). PASD1a and PASD1b, were detectable using the PASD1-1 antibody which bound between a.a. 195-474, PASD1b alone was detected through the use of the PASD1-2 antibody that binds the PASD1b protein in a region between a.a. 540-773 (Cooper et al, 2006). Using PASD1-1 antibody, PASD1a and b were detected in K562, THIEL and SW480 while PASD1-2 also immunolabelled PASD1b in the same cell lines (**Table 3.6**). This confirms previous data that demonstrated PASD1 expression in K562, SW480 and G361 cell lines, with the highest expression being in SW480 and G361 (Liggins et al, 2004a; summarised in Table 2.1). The staining, with both PASD1-1 and PASD1-2 antibodies was seen to be cytoplasmic and nuclear, as described previously (Cooper et al, 2006). Immunolabelling of PASD1 protein in SK-Mel-28 was only successful when using the PASD1-2 antibody that recognises PASD1b only, and indicating that this cell line only produces the shorter PASD1_v2 transcript that is translated into the longer PASD1b protein. There was no detectable PASD1 protein in KM-H2.

Table 3.6 PASD1 protein expression in human cancer cell lines.

Cell line	Cells only	Actin	Isotype control	PASD1 a & b	PASD1 b
K562	-	+	-	+	+
KM-H2	-	+	-	-	-
Thiel	-	+	-	+	+
SW480	-	+	-	+	+
HEK 293	-	+	-	-	-
Sk-Mel-28	-	+	-	-	+

3.3.4 Detection of PASD1 protein expression in AML patient samples

As my aim was to correlate the PASD1 T cells detection with PASD1 protein expression, AML samples were labelled with PASD1 antibodies by ICC. PASD1a + b protein expression were detected in the following three patient samples: AML004, AML008 and AML014 (**Figure 3.5; Table 3.7**). The other 12 samples tested for PASD1 expression were found to be negative. In the patients who were positive for PASD1 protein expression, the expression was in a mixture of all possible sub-cellular localisations in each patient (**Table 3.8**).

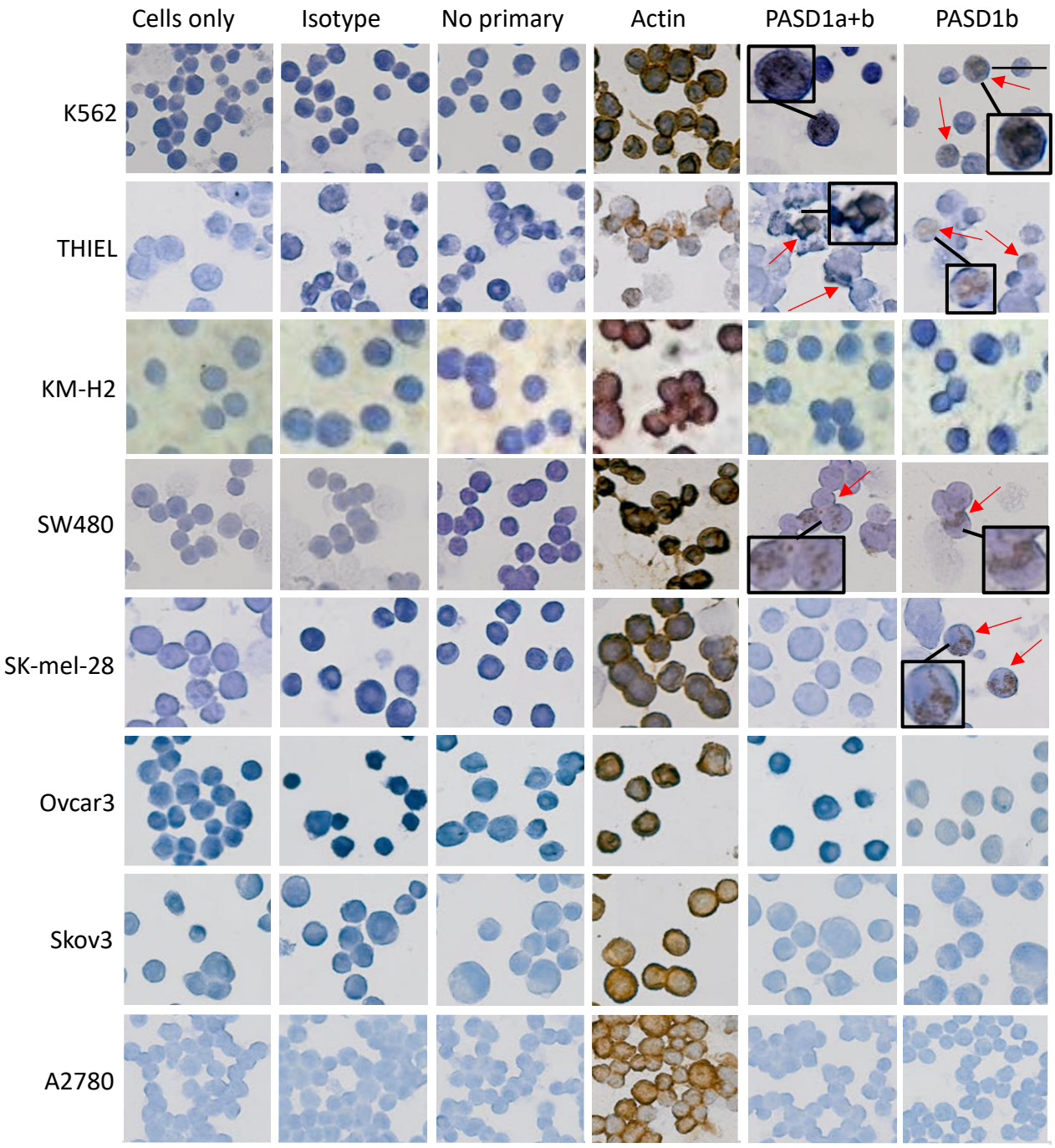


Figure 3.4 Optimisation for ICC showing expression of PASD1 protein in human cell lines. PASD1 was found to be expressed in the following cell lines: K562, THIEL, SW480, Sk-Mel-28 but not in KM-H2. Black boxes contain enlarged images of positively staining cells. Cells only and isotype antibodies were used as negative controls while no primary (replaced by TBS alone) was used to determine background staining. Actin was used as a positive control to demonstrate the ICC was working as it should be. All magnification is x400 and images are representative of at least two independent experiments.

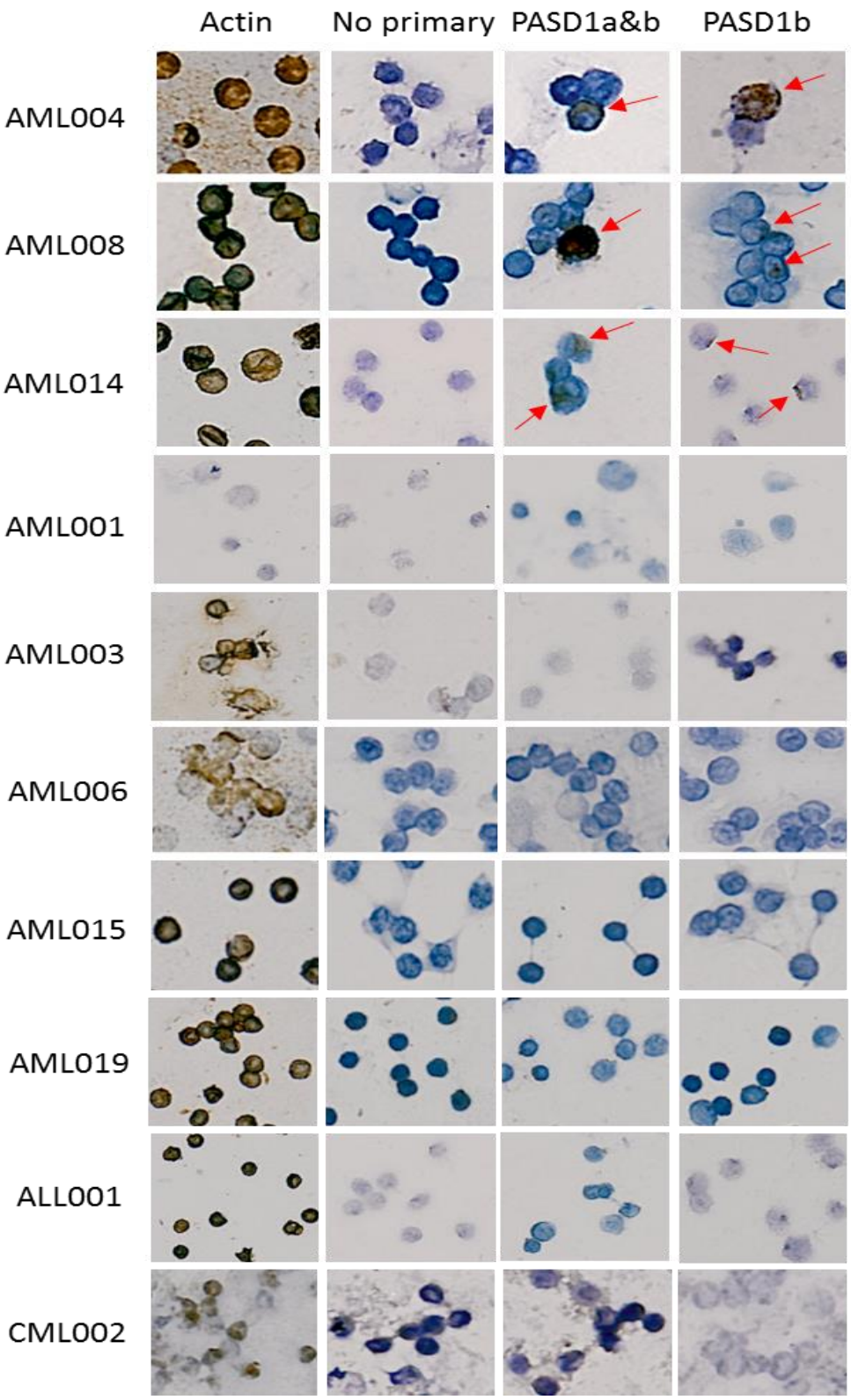


Figure 3.5. Expression of PASD1 in AML patient samples. Both variants of PASD1 were expressed in the cells from AML004, AML008 and AML014. All other samples were negative. No primary (replaced by TBS) was used as a negative control and actin was used as a positive control. X400 magnification with at least two independent experiments.

Table 3.7 PASD1 protein expression as detected by immunolabelling in leukaemia patient samples. Representative of at least two independent experiments.

Samples	Actin	No primary	PASD1 136 (PASD1a + b)	PASD1 128 (PASD1b)
AML001	+	-	-	-
AML003	+	-	-	-
AML004	+	-	+	+
AML006	+	-	-	-
AML008	+	-	+	+
AML009	+	-	-	-
AML014	+	-	+	+
AML018	+	-	-	-
AML019	+	-	-	-
AML021	+	-	-	-
AML023	+	-	-	-
AML026	+	-	-	-
ALL001	+	-	-	-
ALL002	+	-	-	-
CML002	+	-	-	-
Total	**15/15**	**0/15**	**3/15**	**3/15**

Table 3.8 Frequency of cells that are positive for PASD1a and b, intensity and sub-cellular localisation of staining. Data is representative of at least two independent experiments.

	PASD1a + b				PASD1b			
	Frequency of expression /200 cells (%)	**Intensity of staining**	**Subcellular localisation**	**Immunoreactivity score**	**Frequency of expression /200 cells (%)**	**Intensity of staining**	**Subcellular localisation**	**Immunoreactivity score**
AML004	8/200 (4)	1	Cytoplasmic	4	79/200 (40)	2	Surface, nuclear, cytoplasmic	80
AML008	48/200 (24)	3	Nuclear, cytoplasmic	72	26/200 (13)	1	Nuclear	13
AML014	85/200 (43)	2	Nuclear, cytoplasmic	86	34/200 (17)	2	Surface	34

PASD1-specific $CD8^+$ T cells were identified in the peripheral blood of AML004, AML008 and AML014 through the use of pMHC arrays (**Table 3.9;** Brooks et al, 2015). My studies found that every patient who had $CD8^+$ T cells that recognised PASD1 also had PASD1 expression as detected by ICC. PASD1 protein expression was found in AML004, AML008 and AML014 while data for AML013, AML015 and CML001 was discarded due to non-specific immunolabelling of the cells in these samples with isotype control antibody.

Table 3.9 Direct comparison of the antigens detected by RT-PCR, ICC and the LAA-specific CD8+ T cells detected on the pMHC array, from each patient.

Sample id	RT-PCR (antigens detected)	ICC for PASD1	LAA-specific T cells detected on the pMHC array
AML001	P68	Negative	Non detected
AML003	P68	Negative	Non detected
AML004	P68 & WT1	**Positive**	**PASD1,** Tyrosinase & WT1
AML006	G250, MUC1, p68, **PASD1** & WT1	Negative	Non detected
AML008	G250, p68 & WT1	**Positive**	**PASD1**
AML009	P68	Negative	Non detected
AML013	P68, G250, **PASD1** & tyrosinase	-	Non detected
AML014	P68	**Positive**	**PASD1**
AML015	P68 & **PASD1**	-	Non detected
AML018	MUC1, p68, **PASD1**, tyrosinase & WT1	Negative	Non detected
AML019	G250 & p68	Negative	Non detected
AML021	G250, MUC1, p68 & tyrosinase	Negative	CEACAM5
AML023	P68	Negative	Non detected
AML026	G250, MUC1, p68, **PASD1** & WT1	Negative	Non detected
ALL001	G250, MUC1, p68 & WT1	Negative	Non detected
ALL002	G250, MUC1, p68, tyrosinase & WT1	Negative	Non detected
CML001	P68 & WT1	-	Non detected
CML002	P68	Negative	Non detected
HV1	MUC1, p68	-	Non detected
HV2	CEACAM5, G250, MUC1, p68, WT1	-	Non detected
HV3	P68, tyrosinase	-	Non detected

-: not done.

None of the samples with detectable PASD1 transcripts had detectable PASD1 protein or PASD1-specific T cells in their periphery, although the number of PASD1-positive samples by any of the three techniques was low.

3.3.5 Correlation between PASD1 transcription, protein expression and survival in AML patients.

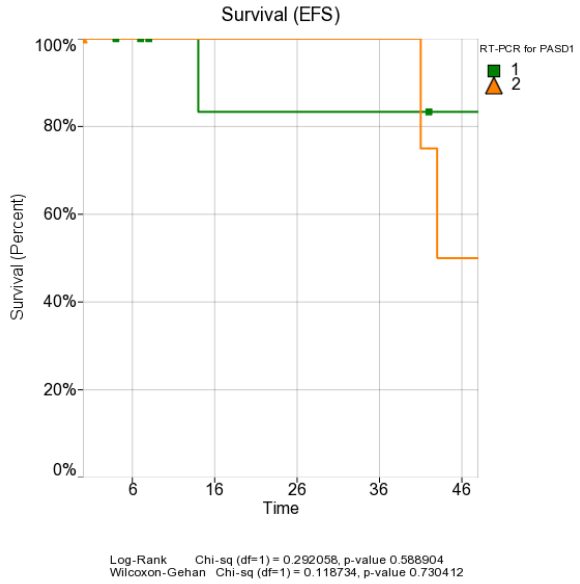
Professor Ken Mills, Centre for Cancer Research and Cell Biology, Queen's University Belfast kindly performed statistical analysis to determine whether the expression of PASD1 as determined by RT-PCR and/or the presence of PASD1 specific T cells correlated with survival in the AML patients analysed (**Table 3.10**). A 2-tailed paired T test was carried out using the Partek Genomic Suite with the significance level of 0.05. Professor Mills did not examine whether the expression of PASD1 as detected by ICC correlated with survival as the number of patients positive for PASD1 protein expression were so small (n=3). In addition there was no significant correlation between PASD1 detected by RT-PCR (n=5) and overall survival (OS) (**Figure 3.6A**) and event free survival (EFS) (**Figure 3.6B**) (p values were 0.589 and 0.357, respectively). The presence of PASD1 specific T cells detected on the pMHC array (n=12) and OS (**Figure 3.6C**) and EFS (**Figure 3.6D**) had p values of 0.836 and 0.605, respectively, indicating there was no significant correlation. It was noted that the number of patients who were positive for PASD1 by RT-PCR were very small and that statistical analyses may be worth revisiting when I have a larger number of patients who are positive for PASD1 by RT-PCR, and/or were positive for PASD1-specific T cells on the pMHC array.

Table 3.10 Summary of the data for PASD1 expression detected with each of the three techniques, pMHC arrays, ICC and RT-PCR. Each of the samples were given a score of 1 (negative) or 2 (positive) for each of the techniques used.

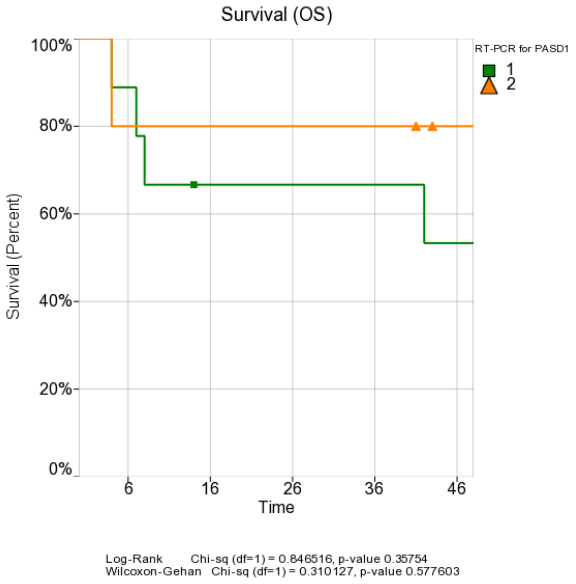
Patient ID	Age[a]	Disease[b]	Survival in months (status)	Presence of PASD1-specific T cells	ICC positivity	RT-PCR positivity
AML001	59	AML (M2)	59 (D)	1	1	1
AML002	65	AML	29 (D)	2	-	-
AML003	48	AML	49 (A)	1	1	1
AML004	46	AML	14 (A)	2	2	1
AML005	62	AML	4 (D)	1	-	-
AML006	68	AML (M4)	2 (D)	1	1	2
AML007	64	AML	2 (D)	1	-	-
AML008	50	AML	42 (D)	2	2	1
AML009	30	AML (M4)	66 (A)	1	1	1
AML010	30	AML	83 (A)	2	-	-
AML011	63	AML	20 (A)	1	-	-
AML012	71	AML	50 (A)	2	-	-
AML013	45	AML	43 (A)	2	-	2
AML014	57	MDS/AML	8 (D)	2	2	1
AML015	19	AML	51 (A)	1	-	2
AML016	26	AML	25 (D)	2	-	-
AML017	59	AML	50 (A)	2	-	-
AML018	NK	AML	64 (A)	1	1	2
AML019	65	AML	49 (A)	1	1	1
AML020	54	AML	23 (D)	2	-	-
AML021	82	AML	4 (D)	1	1	1
AML022	64	AML	7 (D)	2	-	-
AML023	54	MDS	7 (D)	1	1	1
AML024	62	AML/MDS	6 (D)	1	-	-
AML025	77	AML	34 (D)	1	-	-
AML026	62	AML	41 (A)	1	1	2
ALL001	22	ALL	38 (A)	1	1	1
ALL002	65	ALL	3 (D)	1	1	1
ALL003	NK	ALL	NK	2	-	-
ALL004	NK	ALL	NK	1	-	-
ALL005	22	T-ALL	2 (A)	1	-	-
ALL006	50	cALL	17 (A)	1	-	-
ALL007	26	cALL	16 (A)	1	-	-
CML001	67	CML	41 (A)	1	-	1
CML002	21	CML	16 (A)	1	1	1
CML003	63	CML	23 (A)	1	-	-
CML004	32	CML	27 (A)	1	-	-
CML005	61	CML-CD2[d]	48 (A)	1		-
Total				**12/38**	**3/15**	**5/18**

A= alive; D = dead; NK: not known; -: not done.

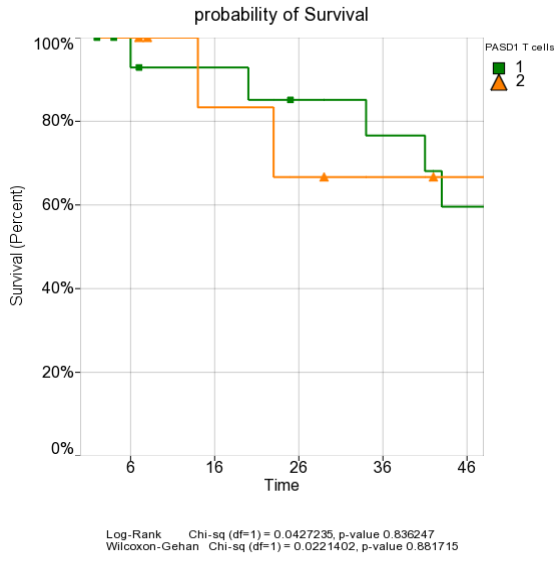
(a) RT-PCR positivity for PASD1 and EFS (p=0.59)



(b) RT-PCR positivity for PASD1 and OS (p=0.36)



(c) pMHC array positivity and EFS (p=0.84)



(d) pMHC array positivity and OS (p=0.6)

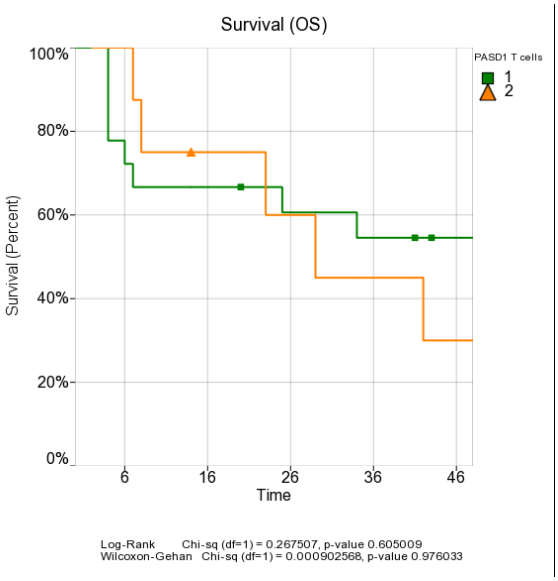


Figure 3.6 Kaplan-Meier survival curves based on the (a+b) expression of PASD1 as determined by RT-PCR or (c+d) the binding of PASD1-specific T cells to pMHC spots on an array. The relationship between PASD1 expression by RT-PCR (n=5, brown line) or the presence of PASD1-specific T cells (n=12, brown line), or not (blue line) and (a+c) EFS or (b+d) OS was not found to be statistically significant (shown in parenthesis following the graph title).

3.4 Discussion

To help complete a study for publication (Brooks et al, 2015) which investigated the presence of LAA-specific CD8+ T cells in the periphery of leukaemia patients, I examined the expression of LAAs that were available on the pMHC array and were recognised by $CD8^+$ T cells from some leukaemia patients for their expression in leukaemia cells. To do this I used RT-PCR to investigate whether LAA transcripts were present in the leukemic cells and ICC to examine protein levels in the cells. I was particularly keen to determine whether LAA transcripts and specifically PASD1 protein in the leukaemia cells correlated with the presence of PASD1-specific $CD8^+$ specific T cells in the periphery.

RT-PCR transcripts for p68 (18/18), G250 (8/18) and WT1 (7/18) were frequently detected in the leukaemia samples examined. This did not correlate with the findings of LAA-specific T cells in these patients. For some patients this may reflect the fact that the HLA-restriction of the pMHCs did not match the patients' haplotype. Many of the pMHCs on the array were HLA-A*0201 restricted reflecting the high frequency of this gene in Caucasian populations (Browning and Krausa, 1996). When researchers in Western Europe examine T cell responses they often chose HLA-A*0201 restricted epitopes because of the predominance of HLA-A2 as a haplotype in approximately 40% of the Caucasian population. Thus the pMHCs on the array, were chosen based of their availability in Professor Hans-Georg Rammensee's group, and their relevance to leukaemia. HLA-A2 negative patients were used as controls. The pMHC array could have been less haplotype restricted but reflected the pMHCs that have been requested by Professor Rammensee's collaborators and our interest in LAAs. Some LAAs fail to elicit a T cell response in patients due to immune editing by the tumour (discussed in **section 1.4.3**) and some T cells may be anergic, at the very least contributing to poor responses to antigen and their infrequent presence in individuals. In addition the pMHC array is likely to underestimate the number of patients with LAA-specific T cell responses (Brooks et al, 2015) as the scoring

criteria was very strict. For a positive score LAA-specific T cells had to bind to four of six pMHC spots at two independent sites on the array.

The pMHC only harboured one HLA-restricted epitope for p68 and G250, and two WT1 epitopes. G250 and WT1 have previously been shown to be highly expressed in AML patients where 67 % (40/60) and 51 % (18/35) patient samples were shown to express WT1 and G250 transcripts respectively (Greiner et al, 2004). AML is dependent on p68, inhibiting p68 expression inhibits AML cell proliferation in vitro and AML progression in vivo. However inhibiting p68 expression is not toxic to healthy bone marrow cells (Mazurek et al, 2014).

The most frequent LAA-specific pMHCs on the array were the five epitopes belonging to PASD1. This may have skewed the data towards the detection of PASD1-specific T cells more than those from other LAAs. However the frequency of detection of PASD1-specific T cells was close to the expected frequency of PASD1 expression in diagnosis AML patients (33 %; Guinn et al, 2005) especially when considering the frequency of HLA-A2 restricted individuals in the Caucasian population.

My data showed 5/18 (28 %) patient samples were positive for PASD1 by RT-PCR which correlates with previous data (Guinn et al, 2005). However the five AML patient samples (AML006, AML013, AML015, AML018 and AML026) that had detectable PASD1 transcripts by RT-PCR were negative for the presence of PASD1-specific T cells on the pMHC array. AML006 and AML018 were HLA-A2 negative and AML026 had a unknown haplotype. The presence of PASD1 transcripts in the tumour but the failure of detection of PASD1-specific T cells may reflect the absence of the correct haplotype in the patient, T cell anergy, under-detection of T cell responses due to the high criteria for the scoring of positivity when using the pMHC array, the falling off of T cells from the pMHC array that was shown by electron-microscopy to cause pitting in the gel (Hardwick et al, 2013) or the fact that PASD1 may have

already led to the tumour editing the immune system and the thymic deletion of any PASD1-specific T cells (reviewed in Chan et al, 2006) as part of the early development of the tumour. To date PASD1 expression has been anecdotally related to later stage and more aggressive disease in DLBCL (Liggins et al, 2004b) and diagnosis AML (Guinn et al, 2005). The tumour may have mutated antigen (relatively rarely detected in tumour antigens, to date and not yet found with PASD1) or the APC may not have been presenting the LAA and its epitopes in the context of "danger" (Matzinger, 1998) leading to anergy and/or clonal deletion. In addition I may have detected PASD1 transcription less frequently in AML patients as I used a one stage PCR amplification protocol (Guinn et al, 2005) in contrast to Liggins et al, 2004a who used two stage PCR (where PCR products from the first stage are used to provide template for a second round of PCR amplifications) to detect PASD1 transcripts in DLBCL patient samples. Although two stage PCR increases sensitivity it can also increase the rate of false positives and with my primers and reagents was not found to be required.

AML006 was used as a negative control for the pMHC study for PASD1-specific CD8+ T cells. However AML006 was the only patient sample that expressed a PASD1 transcript of the expected size of 233bp (as detected by RT-PCR analysis). Four of the five patient samples (AML013, AML015, AML018 and AML026) who had PASD1 transcripts detectable by RT-PCR had transcripts that were approximately 1600bp in length. As the PASD1 primers used were exon-exon, and intron junction spanning, it is likely that the large transcripts were not the products of genomic contamination. This was confirmed by the fact that the predicted genomic DNA transcript should have been many thousands of bp in length, if it could have been amplified by the PCR program. In addition I cleaned all extracted mRNA to remove any contaminating gDNA using ISOLATE II RNA Mini kit (Bioline BIO-52072). I performed PCR on an aliquot of mRNA (which had not been reverse transcribed to cDNA) to check that there was no contaminating gDNA in the template. Amplification products were only obtained from

cDNA, and not from mRNA when it was used as a template, and the 1600bp product sizes were too small to be amplified from the gDNA **(see Table 2.2)**. Hence the longer PCR product obtained from the AML patients may have been a transcript variant. Liggins et al have also described the presence of multiple transcripts from PASD1 including PASD1_v1 originally identified through the immunoscreening of a testes cDNA library with DLBCL sera (Liggins et al, 2004a) and PASD1_v2 that is a shorter transcript than PASD1_v1 and was identified through the immunoscreening of a testes cDNA library with AML sera (Guinn et al, 2005). PASD1_v1 has a retained intron between exons 14 and 15 and produces a shorter PASD1a protein due to the premature stop site retained in the intron (Liggins et al, 2004a). Cooper et al (2006) have also described the possible existence of multiple variants of PASD1 by the ICC technique. However I did not sequence the larger amplicons I obtained by RT-PCR due to the low copy number of the template and difficulties in repeating the PCR amplification due to template decline over time. Real time qPCR would have been an alternative method to utilise as it is able quantitate the actual DNA copy number which unfortunately was not possible onsite.

Of the patients who had an amplifiable PASD1 PCR product (AML006, AML013, AML015, AML018 and AML026) at diagnosis, four of the five patients (AML013, AML015, AML018 and AML026) were in complete remission for up to 2 years after diagnosis. There are some antigens which are observed to be markers for survival but PASD1 has not been found to fall into this category to date (Guinn & Mills, unpublished data). However there has been one indication that PASD1 may play a role in patient survival. Three DLBCL patients who were found to respond to PASD1 at a year post diagnosis were still in remission at the conclusion of the study (Ait-Tahar et al, 2009). In addition, ten of the fifteen patients who elicited a CD4 Th response to a PASD1 peptide (DGFMITLSTDGVIICVAENI) continued to be in complete remission for the duration of another study by the same group (Ait-Tahar et al, 2011).

The presence of PASD1-specific T cells did not prevent the cancer from developing or successfully help the immune system to destroy the leukaemia cells in the patients I studied. It was recently established that PASD1 blocks circadian rhythms (Michael et al, 2015) and therefore would not be expressed in all cells all of the time therefore allowing some cells to evade the immune system and develop into cancer. This may show that although the patient's cancer cells are expressing PASD1, and PASD1-specific T cells are present in the periphery, they may be anergic. It is also possible that while there are transcripts of PASD1 in patient's samples, they may not necessarily be being translated into protein. T cell anergy can lead to immunologic self-tolerance where T cells are functionally inactivated. Other studies have described how immunotherapy can break tolerance (Rice et al, 2008) and it may be that patients who express tumour antigens respond better to chemotherapy (by achieving a more durable remission) because of the "danger" signals that the chemotherapy-induced cell death of cancer cells causes (Matzinger et al, 1994). It is proposed that tumour antigen in dead or dying targeted tumour cells are mopped up by the immune system in the presence of inflammation (danger) stimulating immune "effector" cells to kill more tumour.

The pMHC array was not a functional study and so the existence of T cells would not indicate their capacity to recognise and kill tumour cells that express that antigen. Brooks et al, 2015 found that the T cells that bound to the pMHC array died quickly (within 20 minutes) perhaps reflecting the toxic environment of the polyacrylamide gel that provided a bed for pMHCs and/or the high concentration of pMHCs presented to T cells on a flat surface leading to the induction of activation induced cell death. Previous investigators have also shown that T cells internalise pMHCs (Whelan et al, 1999) and pits were found on the pMHC array suggesting we are underestimating the amount of T cells that can recognise pMHCs on the array (Brooks et al, 2015).

In addition to PASD1, another seven LAAs were investigated by RT-PCR in order to compare LAA-specific T cell detection on the pMHC array with LAA gene transcript expression by RT-PCR. However even in the patients samples in which LAA transcripts were seen, no corresponding LAA-specific T cells were detected. This could be due to having T cell deletion as part of the education of the immune system by the tumour cells or insufficient T cell responses against the auto-antigens in the tumour so there is too low a frequency of LAA specific-T cells for the artificially high detection limits of the pMHC array.

RT-PCR has many advantages such as being expeditious, it has relatively fewer steps compared to other techniques such as Northern Blot, and it remains the most effective technique in detecting rare or low abundance mRNAs (Bustin and Nolan, 2004). However there are also limitations to the RT-PCR method since its success depends a lot on the extraction method, the number of tumour cells in a given samples and the stability of the RNA extracted (Bustin and Nolan, 2004; Ko et al, 1998). However it's most notable limitation is that unlike RQ-PCR (Muller et al, 2002) it is not quantitative. There are a number of mechanisms that leukaemia cells can exploit in order to evade an anti-tumour immune response. For a successful immune response to occur the TCR must bind a specific antigen peptide presented on the MHC molecule, on an APC, and for leukaemia patients this is often the leukaemia cell itself. For effective T cell stimulation, co-stimulatory CD28 receptors on the T cells must bind B7 ligands (CD80/CD86) expressed on the leukaemia cell or anergy is induced (Leung and Suh, 2014). AML cells show a number of features that can allow them to avoid an immune attack. CD80 is rarely expressed on AML blasts and where it is the level of expression is very low (Whiteway et al, 2003). Patients who express both CD80 and CD86 ligands remain in remission for a longer period of time (Whiteway et al, 2003). CD80 and CD86 can also bind to CTLA-4, a member of the CD28 family, and generate inhibitory signals (Parry et al, 2005) limiting the extent of an immune response. Programmed death-1 (PD-1) is the specific receptor for B7-H1

and B7-DC, members of the B7 family, and is involved in terminating immune responses and inducing tolerance by triggering the production of the anti-inflammatory IL-10 (Dong et al, 1999). However AML cells can shed ligands for co-stimulatory molecules such as the 4-1BB ligand, which may allow the leukaemia to circumvent T cell attack by the binding of soluble ligand to the T cell. LAAs may also be expressed in normal tissues, including the thymus, consequently the T cells detect the antigen with lower potency, the response generated will be weaker (Teague and Kline, 2013) and thus the T cells are potentially clonally deleted.

As PASD1 is being investigated as a potential target for immunotherapy, it is important to correlate the detection of CD8+ T cells with protein expression in cancer cells to determine if this is viable option (Ait-Tahar et al, 2009; Brooks et al, 2015). Two antibodies (**Table 2.4**) were used by ICC to detect expression of PASD1 in cell lines and patient samples that had been spotted on to glass slides. As I was interested in the determine sub-cellular localisation of PASD1, which may be indicative of normal or abnormal function, the cells were air dried to ensure I don't cause artefacts due to cytospinning the cell contents to the wall of the cell. PASD1 is a transcription factor, related closely to the CLOCK gene in mice (Michael et al, 2015) and is generally undetectable in healthy cells except the most immature spermatogonia (Cooper et al, 2006).

The cell lines K562, THIEL, SW480, KM-H2, Sk-Mel-28 were used to optimise the ICC protocol and HEK293 cell line was used as negative control. K562, THIEL, SW480 were found to be positive with both PASD1 variants (PASD1a and b) as found previously (Liggins et al, 2004a; Guinn et al, 2005; Sahota et al, 2006; Ait-Tahar et al, 2011). Sk-Mel-28 was positive with only PASD1b antibody which is a novel finding but melanoma cells have been found to express PASD1 previously, for example G361 (Liggins et al, 2004a). KM-H2 did not have detectable PASD1 expression but protein expression had been described previously by Cooper et al (2006). This may reflect a different source for the KM-H2 cells, mine were from a lab at

the University of Wales, College of Medicine in Cardiff in the early 1990s. There have been some previous descriptions of cell lines being contaminated by other cell lines, such as HeLa (Coriell et al, 1958) or being mis-labelled over time, or being grown in ways that lead to competition and change, especially with many passages. The best way to investigate the lack of PASD1 expression in our KM-H2 cells may have been to perform two rounds of PCR using the same primers as used by Cooper et al (2006), to have my KM-H2 cells haplotyped to check they appear to be the same haplotype as described in the original manuscript describing their derivation (Kamesaki et al, 1986) or to ask Professor Banham for some of their KM-H2 cells and directly compare the PASD1 expression to determine whether the issue was my performance of the technique.

For ICC actin was used as a positive control and no primary was used as a negative control – to detect the background levels of staining by non-specific sources. In three samples (AML013, AML015 and CML001) the no primary was positive due to the non-specific staining seen on primary tissue samples which can be "sticky" and provide false positives (Fritschy, 2008), therefore these samples and the data obtained from them was rejected. In order to avoid background staining a number of approaches were used; peroxidase block was used as part of the Envision kit, the DAB incubation time was reduced from 5 min to 1-2 min and aggressive washing (increased volume and frequency) was applied to remove unbound reagents.

The pMHC array may allow us to ascertain whether a patient's immune system is likely to respond well to an immunotherapy treatment i.e. to a vaccine which stimulates a certain population of T cells. It is known leukaemia patients with elevated LAA levels (such as SSX2IP) at diagnosis have a better prognosis (Guinn et al, 2009) and I think that this is because when tumour cells are lysed by conventional therapy the LAAs spill out, causes inflammation and attracts an effective immune response. pMHC array data may indicate whether specific

CD8+ populations are present in the periphery and whether they influence the response patients have to chemotherapy and OS and EFS.

In this study I found that when PASD1 specific T cells were detected, PASD1 protein was also present in the patients' leukaemia cells. Sub-cellular localisation can indicate function and so changes in sub-cellular localisation can indicate when mutations that have occurred (Hung and Link, 2011) or novel isoforms created (Cooper et al, 2006). Expression as detected by both PASD1 antibodies was found to be nuclear, cytoplasmic and surface which correlates with the findings of Cooper et al (2006). Although ICC is able to show localisation of proteins it can be difficult to ascertain surface expression and this could have been achieved with other methods such as fluorescent staining or in situ hybridisation. Two antibodies were used (Cooper et al, 2006), ACCL 136 which detects both PASD1a and PASD1b isoforms and ACCL 128 detects just the PASD1b isoform as far as I am aware, and using these antibodies it was possible to determine which isoform (PASD1a and b) were being expressed in patients samples. In the patient's samples that were positive for PASD1 expression (AML004, AML008 and AML014) stained with both anti-PASD1 antibodies, so we are unable to determine whether PASD1a is expressed as well as PASD1b. This result correlates with the pMHC array data where in the same AML samples PASD1-specific T cells were detected to the analogue peptides Pa14 which are located at 691-699 a.a (Hardwick et al., 2013). As Pa14 is located in the carboxy end of PASD1 it confirms not only the expression of PASD1b by ICC but the recognition of the part of the PASD1 protein unique to PASD1b. PASD1 activates the transcription factor STAT3 in the nucleus leading to cell proliferation and migration evidencing PASD1 localisation and function (Xu et al, 2016).

The circadian clock is the internal biological clock which oversees the cellular activity of all cells in a 24 h cycle regulating processes such as gene transcription, translation and cell division. The CLOCK:BMAL1 complex ensures that the relevant clock factors (Period and

Cryptochrome) are activated at the right time which in turn deactivate CLOCK:BMAL1 when they are no longer needed in a negative feedback loop safeguarding normalcy. PASD1 is able to disrupt this complex due to its similarity to CLOCK, thereby effectively switching off the circadian rhythms leading to diseases such as diabetes and cancer (Michael et al, 2015).

The data obtained by ICC did not correlate with the RT-PCR data. Inconsistencies in PASD1 expression have been found in earlier studies within and between tumour cells (Cooper et al, 2006; Sahota et al, 2006) signifying further novel isoforms and its role in circadian rhythms. This observation is common with other CTAs for example NY-ESO-1 (Jungbluth et al, 2005) and MAGE (Dhodapkar et al, 2003). The detection of a transcript does not always lead to the detection of the protein. There are multiple processes which regulate transcription and translation that effects gene and protein expression; post-transcriptional modifications such as splicing, mRNA degradation and half-life, protein regulation and stability and different characteristics that can typify each isoform (Vogel and Marcotte, 2012). My data indicated the percentage expression of PASD1 in cells ranged between 4-43 % and this is similar range to previous studies showing expression of PASD1 in up to 40 % but not in all cells (Sahota et al, 2006; Hardwick et al, 2015) possibly do due its role in circadian rhythms which are individual to each cell and independent of surrounding cells (Nagoshi et al, 2004).

Statistical analysis was performed by calculating the paired T test p value for 0.05 significance and returned non-significant p values for EFS and OS in relation to RT-PCR positivity but no real conclusions can be made due to the low number of patients. When looking at pMHC positivity the trend showed that PASD1 positive patients had worse EFS and OS however again the p values were not significant. Looking at cytogenetics all five leukaemia patients who were positive for PASD1 expression by RT-PCR had normal genetics, while in the cohort of twelve patients examined only six had normal cytogenetics. Patient AML008 for whom the results of their karyotyping was not available is not included. Possibly the fact that these five patients

had no additional chromosomal abnormalities meant they also had a longer OS but not all cytogenetic abberrations found in AML are detrimental with regards to survival (Grimwade et al, 1998), including t(8;12), t(15;17) and inv(16). Previously 75 % of AML patients who were positive for PASD1 gene expression by RT-PCR, also had a normal karyotype, while 58 % of patients who were negative for PASD1 had a normal karyotype, however this was not statistically significant (Baghdady et al, 2013).

Two of the three patients who were ICC positive for PASD1 expression, had cytogenetics abnormalities; these were AML004 (del7q) and AML014 (trisomy 8, abnormal 13) and the third patient's cytogenetics were not known (AML008). Four of the twelve patients who were PASD1 negative by immunolabelling had an abnormal karyotype. However the numbers of patients in any one group are too small to draw any conclusions and no correlations between PASD1 expression and the presence or absence of cytogenetic abnormalities have been described previously probably due to the relatively small number of AML patients with PASD1 positivity that have been studied to date.

There are a number of strategies to target PASD1 for immunotherapy. Joseph-Pietras et al. (Joseph-Pietras et al, 2010) used the pDOM-epitope DNA vaccine design (Rice et al, 2008) to compare the efficacy of the whole PASD1_v1 cDNA (FL) in vaccination studies compared with the CTL PASD1(1)38–47 and PASD1(2)167-175 epitopes (Ait-Tahar et al, 2009). The group found a greater T-cell response in HHD mice (Firat et al, 1999) to PASD1(1) than PASD1(2) in IFNγ ELISpot assays and significant CTL killing of loaded and endogenously PASD1 positive myeloma cell lines. Vaccines containing the FL PASD1 induced greater anti-PASD1(1) responses compared with anti-PASD1(2) suggesting immunodominance. In addition the FL PASD1 vaccine could induce CTL that were capable of killing MM cells. The DNA fusion gene vaccine has also been used to assess PASD1 analogue peptides in HHD humanized mice (Hardwick et al, 2013). One of the epitopes, Pa14, is an analogue of the wild

type Pw8 peptide. The group showed that immunising mice with Pa14, induced immune responses against the modified (Pa14) and wild type (Pw8) peptides in studies using mixed lymphocyte reaction (MLR), ELISpot and CTL assays. Splenocytes from vaccinated mice demonstrated *in vitro* cytotoxicity against myeloid leukaemia tumour cells, which were either exogenously loaded with the corresponding wild type peptide (Pw8) or presented endogenously processed PASD1 peptides. Of note mice immunised with a pDOM-Pw8 DNA vaccine were unable to mount a significant immune response but mice immunised with the modified peptide pDOM-Pa14 killed Pw8 loaded and endogenously PASD1 expressing targets. Further epitopes could be identified through the immunoscreening of short overlapping peptide libraries (Komatsu et al, 2013; Lewinsohn et al, 2013) although some of the already predicted PASD1 epitopes (Ait-Tahar et al, 2011; Ait-Tahar et al, 2009; Hardwick et al, 2013) remain to be more thoroughly studied *in vivo*.

The expression of PASD1 in a range of tumour types, especially haematological malignancies, suggests that PASD1 specifically has potential as a target for the immunotherapy of these difficult to treat haematological malignancies that do not respond well to conventional therapy and frequently lack a more suitable and targeted therapy for the removal of minimal residual disease when their immune systems are recovering from conventional treatment.

CHAPTER 4: TUMOUR ANTIGEN EXPRESSION IN CANCER CELLS

4.1 Introduction

Finding antigens for immunotherapy in OVC and indeed cancer in general, I believe to be the most vital direction to take in order to combat the disease and since early detection is seen as the best chance a patient has of successful treatment antigens that can also act as biomarkers would provide much improvement on current practice. Therefore, I looked at antigens which had not been investigated thoroughly in OVC previously. Initially I examined antigens that my supervisor had found to be important in AML. The reason being that the expression of SSX2IP had been shown to be on the surface of leukaemia cells during mitosis (Denniss et al, 2007). I wanted to determine whether this was the case in other cancers as this would make SSX2IP a promising target for antibody therapies in other tumour types. I also wanted to determine whether SSX2, a CT antigen, co-localised with SSX2IP in its expression on the surface of cancer cells.

The expression of PASD1 has not previously been examined OVC and my supervisor had found PASD1 expression was limited to a sub-population of cells in the K562 cell line (Hardwick et al, 2013) but it's expression was not found to be cell cycle related (Denniss & Guinn, unpublished data). When I couldn't replicate the expression of SSX2IP in K562 I wanted to determine whether it was due to a difference in CO_2 levels between the incubators and whether hypoxia, known to play a role in tumour growth and resistance (Wilson and Hay, 2011), was playing a role in the expression of the tumour antigens in my hands.

As controls for my study I also investigated the presence of antigens known to be expressed in OVC (HE4, CA125 and WT1) (Bast et al, 1981; Kirchhoff et al, 1991; Hwang et al, 2004) to see how PASD1, SSX2IP and SSX2 compared to the current "gold standard" and popular biomarkers for OVC.

To investigate antigens in OVC, TMAs were purchased from Biomax, US which contained mostly early stage OVC samples with a few late stage as well as normal tissue (NT) and normal adjacent ovarian tissue (NAT).

4.2 Aims

- To determine whether PASD1, SSX2 and SSX2IP expression suggests they would make promising targets for the immunotherapy of OVC.
- To determine whether SSX2IP, and its partner protein SSX2, are expressed on the surface of solid tumour cancer cells
- To investigate the impact of different CO_2 levels on PASD1, SSX2 and SSX2IP expression in tumour cells

Antibodies to the antigens of interest (PASD1, SSX2IP and SSX2) were optimised on the OVC cell lines (Ovcar3, Skov3 and A2780) as well as on other haematological cell lines where their expression had been shown previously or to provide negative controls had not. As SSX2IP is an LAA, K562 (leukaemia cell line) was used as a positive control and HL60 was used as a negative control (Denniss et al, 2007). Other solid tumour cell lines (HeLa and SW480) were also used for SSX2IP as well as the OVC cell lines Ovcar3, Skov3 and A2780. A second solid tumour control was used in HCT116 (colon cancer). PASD1 and SSX2 antibodies were tested on OVC cell lines Ovcar3, Skov3 and A2780.

Initially SSX2IP, which was known to be positive in K562 (Denniss et al, 2007), could not be immunolabelled with the same antibody clone from Abcam. I used K562 cells that had been grown at various levels of CO_2 (3.5-6 %) in an incubator in Southampton to see whether a difference in CO_2 levels was causing the lack of reproducibility.

Following optimisation of antibodies on cell lines, FFPE colon cancer TMAs were used to optimise IHC protocol. These were provided by Dr Alex Mirnezami and were used to immunolabel the samples for PASD1, SSX2IP and SSX2 expression.

OVC TMAs were purchased to compare the expression of PASD1, SSX2IP and SSX2 with the current "gold" standard for OVC diagnosis, CA125 (Bast et al, 1981), as well as the most promising other antigens for the detection of OVC, HE4 and WT1 (Hellstrom et al, 2003; Fadare et al, 2013). Cells only and isotype were used as negative controls and actin as a positive control.

4.3 Results

4.3.1 Expression of tumour antigens in cell lines (ICC)

Before staining TMAs to test the labelling in OVC samples each antigen was tested and optimised on cells lines. Each ICC experiment was performed at least twice and the results were reproducible. Immunolabelling of actin was used as a positive control to demonstrate that all of the components for ICC were working properly.

4.3.1.1 Expression of SSX2IP in cancer cell lines

To determine whether I could detect SSX2IP antigen expression in OVC samples and to investigate if SSX2IP is found on the surface of cancer cells, I initially examined the expression of SSX2IP in a number of human cancer cell lines (**Figure 4.1; Table 4.1**). I showed in reproducible experiments that the antibodies were all working using the cell lines K562, KM-H2, HeLa, SW480, HL60, Ovcar3, Skov3 and A2780 (**Section 3.3.3**). K562 was previously shown to express SSX2IP (Denniss et al, 2007). Prior to the studies described in this chapter, SSX2IP had been shown to be a leukaemia antigen and had not investigated in solid tumours. It has also not been looked at in KM-H2, HeLa and SW480 previously, although HL60 has been shown to be negative for SSX2IP by Denniss et al (2007).

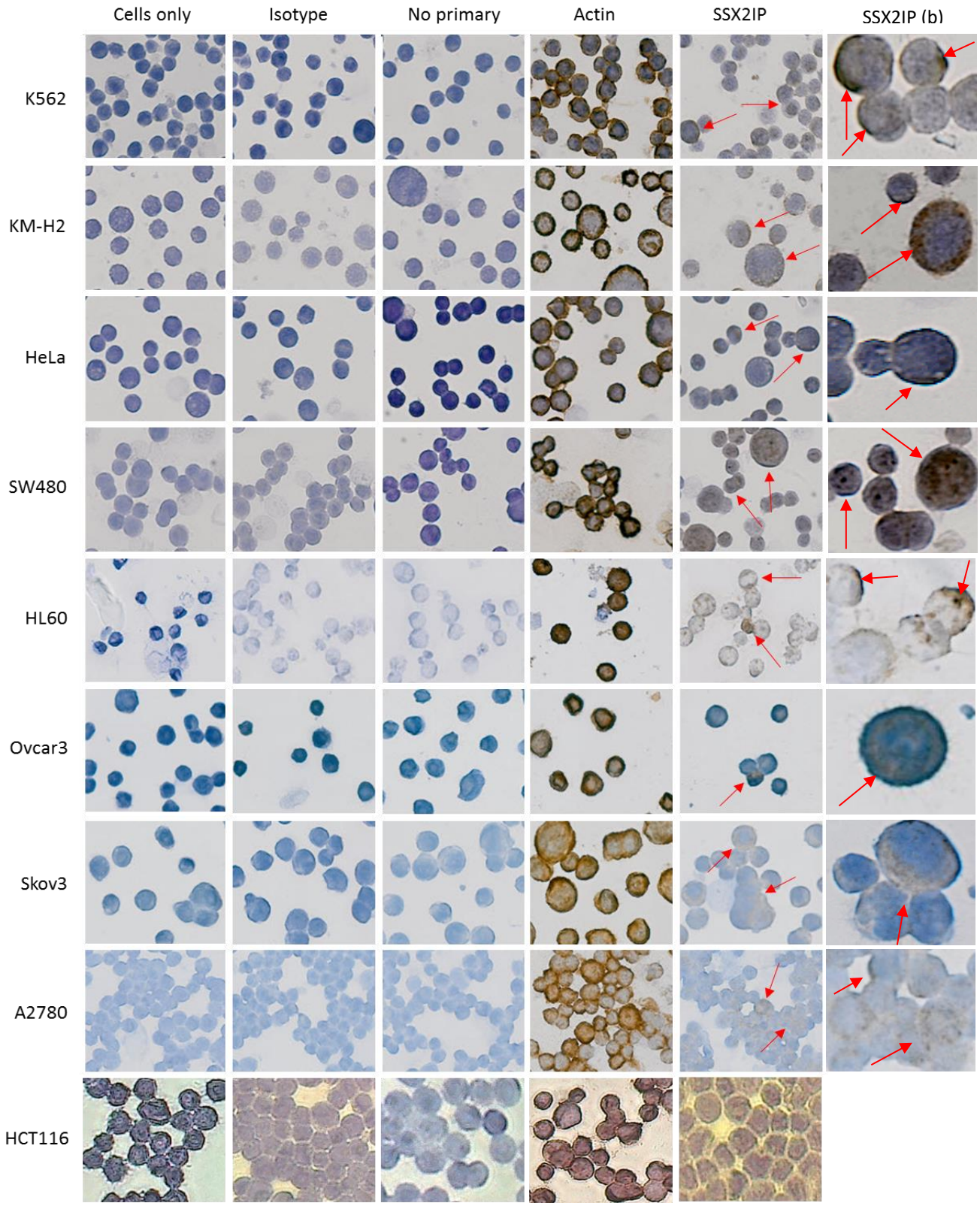


Figure 4.1 Expression of SSX2IP in solid tumour and leukaemia cell lines. SSX2IP was detected in all cell lines tested: K562, KM-H2, HeLa SW480, HL60, including the ovarian cell lines Skov3, Ovcar3 and A2780. HCT116 was found to be negative for SSX2IP. Cells only, isotype and no primary were used as negative controls and β-actin was used as a positive control. Magnification was x400 except column SSX2IP (b) where a separate images have been enlarged to try to show detail.

Table 4.1 Expression and sub-cellular localisation of SSX2IP in human cancer cell lines

	Cell line	Cells only	Actin	Isotype control	SSX2IP	Percentage expression	Sub-cellular localisation	Staining intensity¶	Immunoreactivity score
AML	K562	-	+	-	+	81	Surface	3	243
	HL60	-	+	-	+	47	Surface, Nuclear	2	94
MM	Thiel	-	+	-	-	-	-	0	0
HL	KM-H2	-	+	-	+	77	Surface	2	144
CRC	SW480	-	+	-	+	92	Surface, Cytoplasm	3	276
	HCT116 wt	-	+	-	-	-	-	0	0
CC	HeLa	-	+	-	+	24	Surface, Nuclear	3	72
OVC	Ovcar3	-	+	-	+	11	Cytoplasm	1	11
	A2780	-	+	-	+	23	Surface, Nuclear	1	23
	Skov3	-	+	-	+	20	Cytoplasm, Nuclear	1	20

¶staining intensity for SSX2IP

4.3.1.2a CTA expression in OVC cell lines - PASD1

PASD1 expression was examined in several human cancer cell lines to perfect the ICC protocol prior to labelling OVC patients samples, as detailed in **Table 3.6**. In addition I examined its expression in a further three OVC cell lines as part of this study. By ICC PASD1a and b, and PASD1b alone, were not detected in Ovcar3, Skov3 and A2780 (**Figure 4.2; Table 4.2**). PASD1a and b proteins were not detected in HeLa, while PASD1b alone as detected by the PASD1-128 antibody was detectable in HeLa.

4.3.1.2b CT antigen expression in OVC cell lines - SSX2A

SSX2A protein was found in a number of solid and haematological malignancies cell lines which included OVC cell lines. In haematological cell lines the mSSX2A antibody detected SSX2A expression in the following cells lines; K562, KYO-1, KM-H2 and Thiel **(Table 4.3)**. SSX2A immunolabelling by mSSX2A was found in THIEL but not HL60. SSX2A was also detected in the three OVC cell lines. Sk-Mel-28 (melanoma) was positively stained with mSSX2A **(Figure 4.3)** which correlates with a previous study (dos Santos et al, 2000).

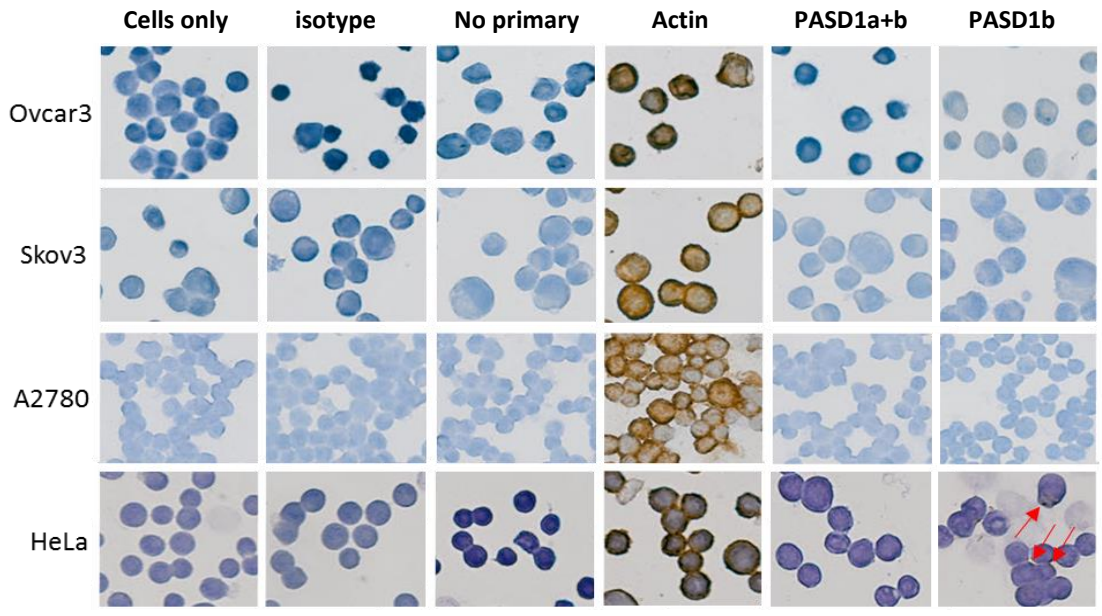


Figure 4.2 PASD1a and b expression in OVC cell lines. PASD1b was found to be expressed in the CC cell line, HeLa, but not in the three OVC cell lines. All pictures taken as magnification x400.

Table 4.2 PASD1 expression in OVC cell lines.

Cell line	Cells only	Actin	Isotype control	PASD1 a & b	PASD1 b	Percentage expression	Sub-cellular localisation	Immunoreactivity score
Ovcar3	-	+	-	-	-	-	-	0
Skov3	-	+	-	-	-	-	-	0
A2780	-	+	-	-	-	-	-	0
HeLa	-	+	-	-	+	5 %	Surface	5

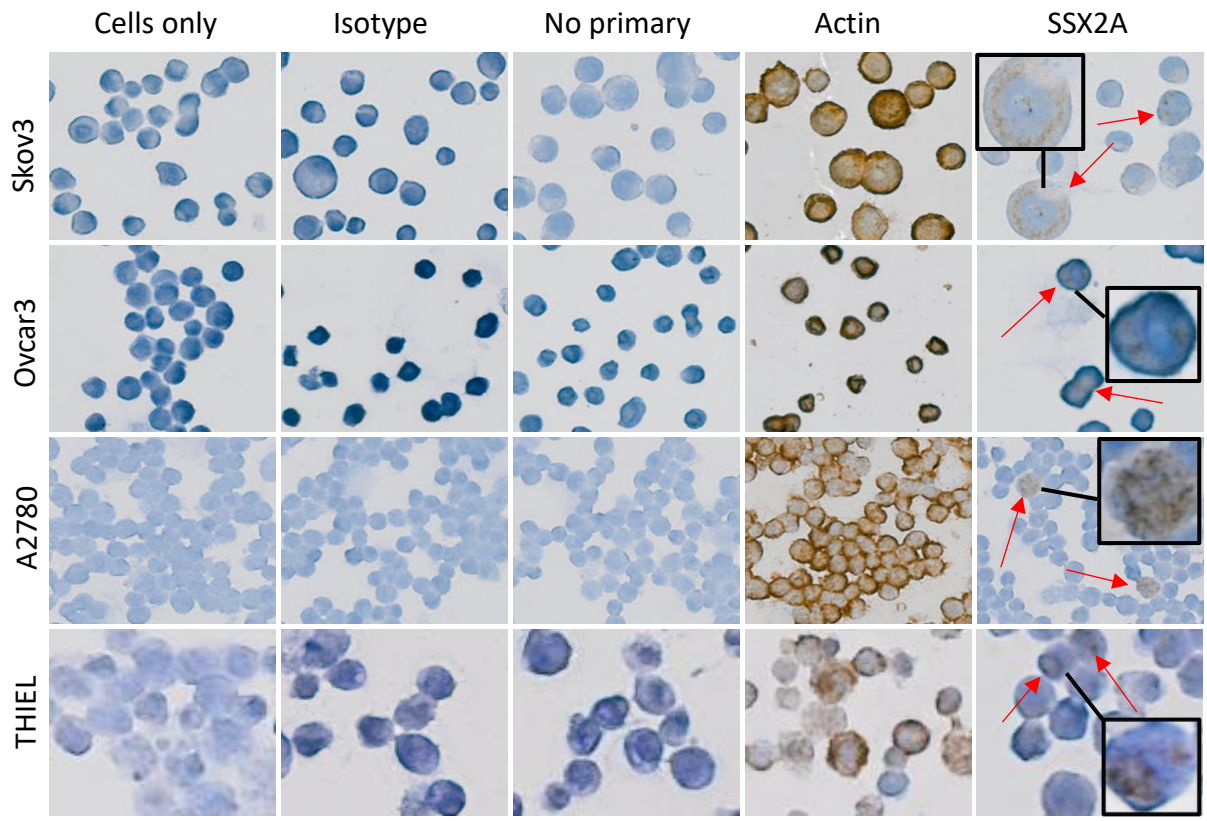


Figure 4.3 Expression of SSX2A in OVC cell lines. SSX2A was detected in the cytoplasm of all three OVC cell lines tested (red arrows). Black boxes contain enlarged images of positively staining cells. A2780 and Skov3 cell lines had higher intensity staining than Ovcar3. Cells only, isotype and no primary were all negative and actin was used as a positive control. Magnification is x400 and data is representative of at least two replicates. The black boxes contain an enlarged image of cells to show more detail.

Table 4.3. ICC analysis of SSX2A expression in the human cancer cell lines.

	Cell line	Cells only	Actin	Isotype control	SSX2A	Percentage expression	Staining intensity	Sub-cellular localisation	Immunoreactivity score
AML	K562	-	+	-	+	85	3	Surface, cytoplasm	255
	HL60	-	+	-	-	-	0	-	0
Multiple myeloma	Thiel	-	+	-	+	10	2	Nuclear, Cytoplasm	20
Hodgkin's Lymphoma	KM-H2	-	+	-	+	60	3	Nuclear, surface	180
CRC	SW480	-	+	-	-	-	0	-	0
	HCT116 wt	-	+	-	-	-	0	-	0
Cervical cancer	HeLa	-	+	-	-	-	0	-	0
OVC	Ovcar3	-	+	-	+	6	1	Cytoplasm	6
	A2780	-	+	-	+	30	1	Surface, Cytoplasm	30
	Skov3	-	+	-	+	5	1	Surface, Cytoplasm	5

4.3.1.3 Impact of CO_2 levels on PASD1 and SSX2A expression

I was concerned as to why I could not duplicate previous findings with regards to SSX2IP expression on K562. One suggestion was a difference in CO_2 in the humid chambers of the incubators despite them being calibrated at both sites (Southampton and King's College London) and so I investigated the effect of varying CO_2 levels on the expression of the antigens. I examined a range of CO_2 levels from 3.5 - 6 % including 5 % the normal level in a humidified chamber. I found both forms of PASD1, SSX2A and SSX2IP were each expressed at 5 % and 5.5 % CO_2 levels (**Table 4.4; Figure 4.4**) but these antigens were not expressed in CO_2 levels above or below this. The percentage expression of each antigen (SSX2A and PASD1a and PASD1a+b) was conistent at 5% and 5.5% CO_2 levels. However it was interesting to note that PASD1 expression was only found on the surface of K562 cells at 5.5% CO_2 levels. Unfortunately I did not get SSX2IP expression on the cell lines in the varied CO_2 levels, even at 5% CO_2, but I now believe that was caused by a defunct, but in-date, SSX2IP antibody that was subsequently replaced with a new vial and staining of SSX2IP again worked on K562 as expected in all subsequent studies shown in this thesis.

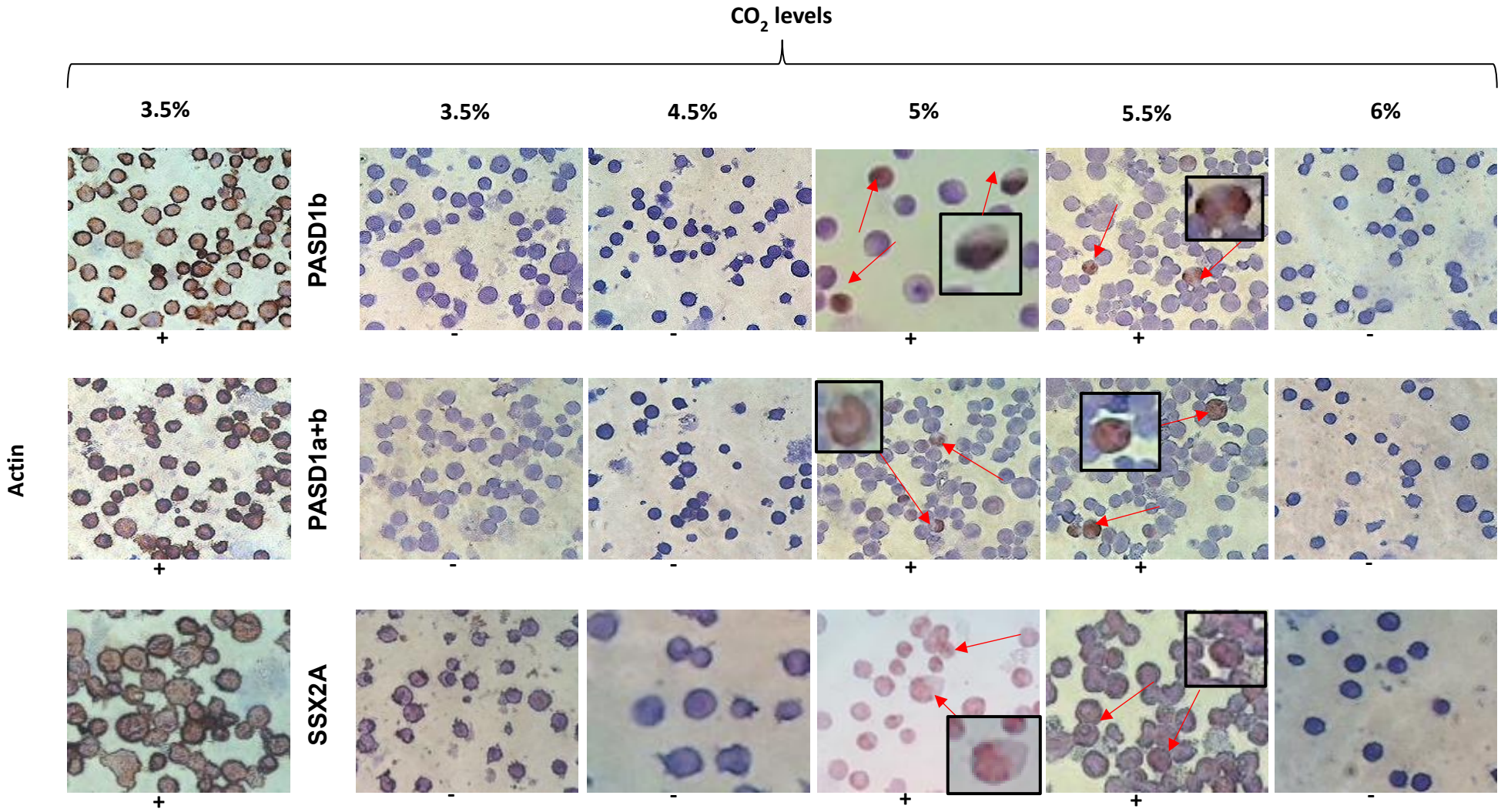


Figure 4.4 The effect of varying CO_2 levels on the expression of PASD1 and SSX2A in K562 cells. The expression of both antigens was restricted to 5 and 5.5 % CO_2 but no expression was seen at 3.5, 4.5 and 6 % CO_2 following a 2-week incubation. All images were taken at a magnification of x400. The image within the black box is an enlarged section to show more detail.

Table 4.4 Effect of CO_2 levels on (a) PASD1 and (b) SSX2A expression in K562 cells.

CO_2 level (%)	Cells only	Isotype control	Actin *	PASD1 a+b	PASD1b	Percentage expression (%)		Subcellular localisation		Immunoreactivity score	
						a+b	b	a+b	b	a+b	b
3.5	-	-	+	-	-	-	-	-	-	0	0
4.5	-	-	+	-	-	-	-	-	-	0	0
5	-	-	+	3	3	21	18	Nuclear, Cytoplasm	Nuclear, Cytoplasm	63	54
5.5	-	-	+	3	3	17	20	Nuclear, cytoplasm, surface	Nuclear, Surface	51	60
6	-	-	+	-	-	-	-	-	-	0	0

CO_2 level (%)	Cells only	Isotype control	Actin*	SSX2A	Percentage expression	Subcellular localisation	Immunoreactivity score
3.5	-	-	+	-	-	-	0
4.5	-	-	+	-	-	-	0
5	-	-	+	3	95	Nuclear, cytoplasm	285
5.5	-	-	+	3	80	Cytoplasm, surface	240
6	-	-	+	-	-	-	0

+: positive result following immunolabelling of protein with antibody; -: negative result, no immunolabelling

4.3.2 Expression of tumour antigens in CRC TMAs – optimisation of immunolabelling on paraffin-embedded tissue

I stained seven TMAs of CRC patient material, which were a gift from Dr Alex Mirnezami (Southampton University Hospital Trust) (**Table 4.5; Figure 4.5**) to ensure the immunolabelling method I was planning to use worked in my hands. It had been hoped this would form part of a study of TAA expression in CRC to complement work performed by Viktoriya Boncheva as part of her MPhil studies (V. Boncheva, MPhil, 2014). Although no further slides were received, this work did provide an opportunity to practise my IHC technique on paraffin-embedded TMAs using my antigens of interest: SSX2, SSX2IP and PASD1. Two separate TMAs were analysed and were designated 1A (seven slides) and 1B (six slides). PASD1 was not significantly expressed, no samples were positive for PASD1b and 5/88 were positive for PASD1a+b however these were not above the background levels. Sample 1A had no expression of SSX2IP above background however sample 1B had SSX2IP expression in 15 % of the CRC tissues above the background level of staining. SSX2A was found in 21 % of the samples on slide 1A and 10 % of the sample on slide 1B, both above background levels.

The commercially available isotype control led to unsatisfactorily high levels of background staining. Colleagues at the LRF monoclonal antibody facility, University of Oxford recommended I use TBS as our control for background staining. However the low levels of immunolabelling achieved with PASD1 led me to choose this as a monoclonal antibody control for non-specific antibody labelling on the tissue arrays.

4.3.3 Scoring of samples

Antigen expression was examined in NT, NAT and stage I, Ia, Ib, Ic, II, III and IV OVC and skin cancer tissues. CA125 was used as an industry standard comparator. Staining intensity was indicated by the immunolabelling intensity on the cells as follows:- 0 and 1: negative and background staining, respectively; 2–4 was considered to be positive with 2 being moderate, 3

high and 4 very high levels of immunolabelling by antibody (**Figure 4.6**). Actin was used as the positive control to confirm the immunostaining protocol was working and provide a staining intensity comparator and cells only provided a control for background staining with or without haematoxylin as a differential stain. A small number of tissue cores were missing from the TMAs following immunolabelling and so data on these samples are absent from the figures. Melanoma (skin cancer) tissue on each TMA was used as a positive control for PASD1 immunolabelling which was often negative otherwise.

4.3.4 Expression of TAA/CTAs in OVC

SSX2, SSX2IP and PASD1 staining in OVC was compared to the well-known CA125. CA125 expression was used as the standard marker for OVC although it is widely regarded as poor detector of OVC. Only scores of ≥2 were considered positive and I found many of the scores for CA125 to be 0 and 1. At stage I positivity was 14 % (23/165, 1 missing), stage II it was 7 % (1/14, 1 missing), with no samples scoring 2 or above at stage III and IV (**Figure 4.7**). Although immunolabelling that scored 2 and 3 was considered higher than the negative and background levels, it occurred infrequently within the tissues.

Table 4.5 Analysis of tumour antigen expression in CRC TMAs.

Antigens	Cells only		Actin		Isotype Control		SSX2A		SSX2IP		PASD1a+b		PASD1b	
Slide id	1A	1B	1A	1B	1A	1B	1A	1B	1A	1B	1A	1B	1A	1B
No. of positive patient samples	0/81	0/89	82/82	90/90	35/81	38/91	54/84	52/100	20/81	52/92	5/88	-	0/80	0/92
Frequency of TAA expression (%)	0	0	100	100	43	42	64	52	25	57	6	-	0	0
Percentage of positive cells above the staining achieved with isotype control (%)	-	-	57	58	-	-	21	10	0	15	-	-	-	-

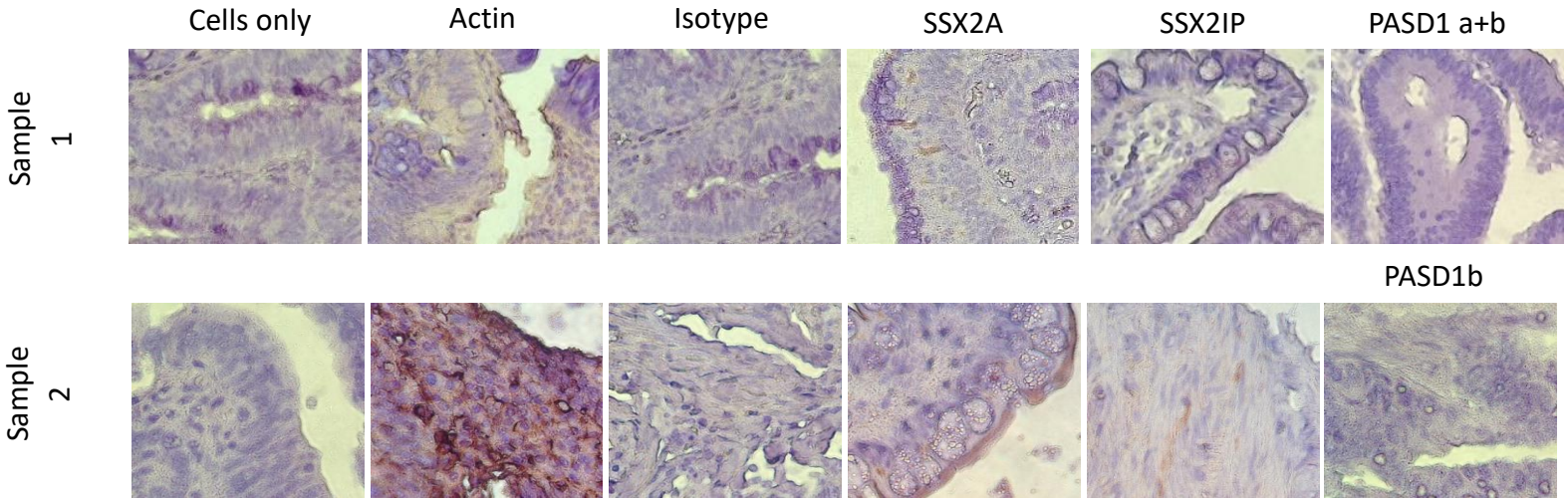


Figure 4.5 Expression of SSX2A, SSX2IP and PASD1 in FFPE-CRC samples as detected by immunolabelling. Image shows the immunolabelling of two samples, labelled Sample 1 and 2 for ease, as representative of the staining observed. Neither PASD1a and b, nor PASD1b, were expressed above background levels in the samples assessed while SSX2A and SSX2IP were expressed in up to 21 % of the samples examined. These experiments were not repeated but within each slide (1A and 1B) there was a range of 81-92 independent tissues. Magnification: x400

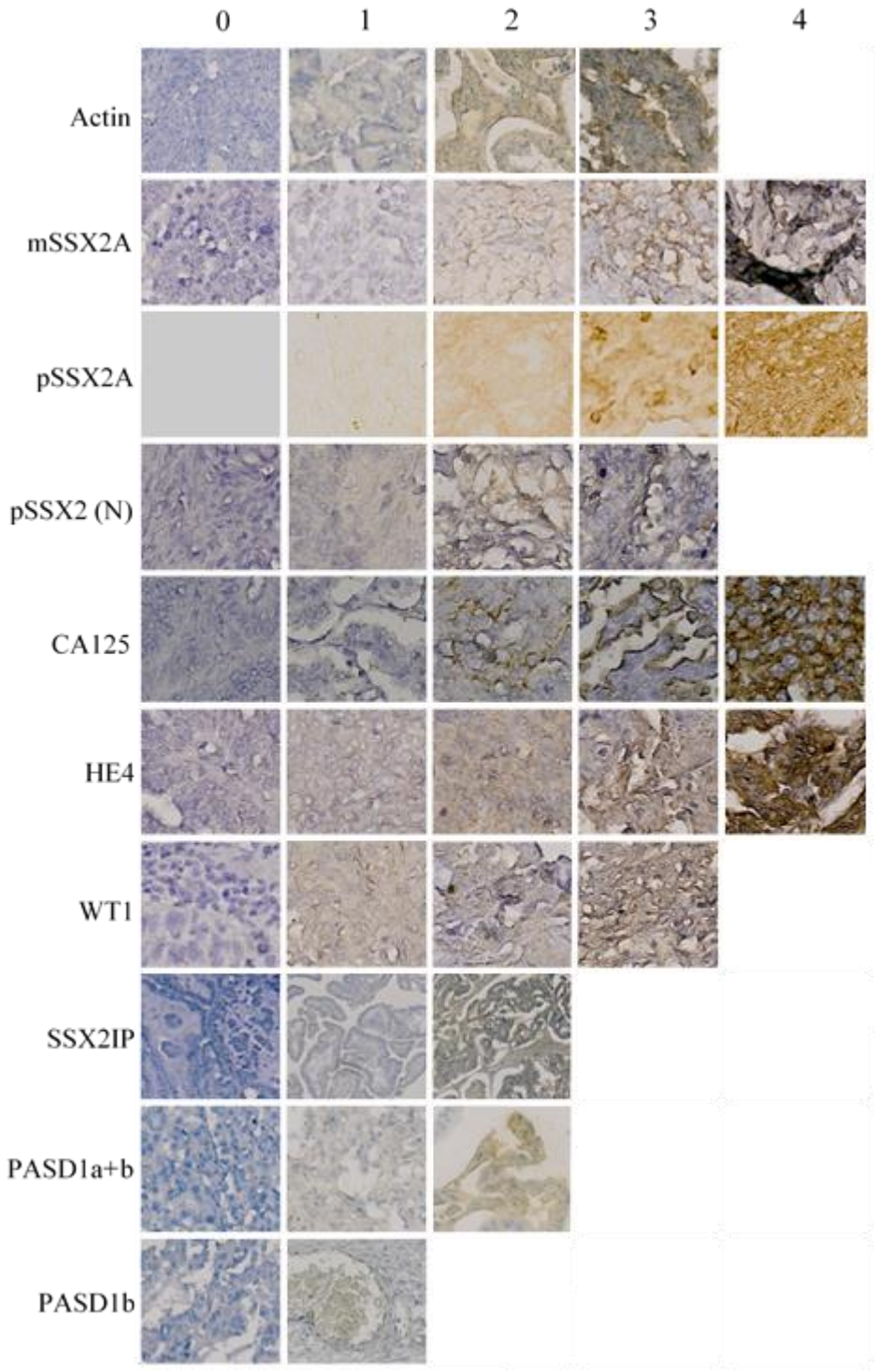


Figure 4.6. Representative scoring of the immunolabelled tumour antigens in OVC TMAs. Blank areas of the figure are due to a lack of immunolabelling at this level. Scores of 0–1 was considered to be negative or background staining respectively, 2–4 was considered to be positive with 2 being moderate, 3 high and 4 very high levels of immunolabelling. Scoring was performed with no knowledge of which tissue section were in which positions on the TMA. Magnification was x400.

4.3.4.1 PASD1

PASD1 is a CTA in blood cancer and therefore I wanted study it in OVC. When examining the immunolabelling of PASD1 in OVC tissue samples, the skin cancer tissue acted as a positive control for successful immunolabelling within those slides. Actin demonstrated that all tissues could be stained while PASD1 immunolabelling had also been demonstrated on human cancer cell lines (**Section 3.3.3**). Both of the PASD1 variants, identified by the PASD1-1 and PASD1-2 antibodies, scored as negative and at back ground levels, 0 and 1 respectively, in 188/189 OVC tissues and only one sample achieved a score of 2 for both. There was very little background staining following immunolabelling with antibody to either PASD1 variant (PASD1-1 or PASD1-2). PASD1a+b scored only 0 in NAT and 0-1 in NT, whereas PASD1b had scores ranging 0-1 for NT and only 1/8 sample scored 2 with the remaining 7 scoring 0-1 for NAT **(Table 4.6; Figure 4.7)**.

4.3.4.2 SSX2IP

I found no significant expression of SSX2IP in the OVC tissue samples **(Table 4.6; Figure 4.8)**. 181 of the 188 OVC samples achieved a background score of 0 or 1, and only 7/188 scored 2 with no samples scoring any higher. All eight of the NAT scored 0 and 1 for SSX2IP while two of eight NT scored 2 and the rest scored 0 or 1.

4.3.4.3 SSX2A

SSX2 has not been extensively looked at in OVC and so it was used to label OVC samples to determine if it is detectable. Expression of SSX2A was detected in a large number of patient samples at all stages of OVC including stage I and II. SSX2A was detected in 39 % (64/165, 1 missing) of stage I and 21 % (3/14, 1 missing) of stage II, which is higher than PASD1, SSX2IP and CA125 in the same stages (**Figure 4.8**). SSX2A was also found in a number of later stage tissues (stage III and IV) on the TMAs. SSX2A was found in 33 % (2/6) of stage III and 60 % (3/4) of stage IV samples and this was a higher frequency than PASD1, SSX2IP or CA125.

CA125 has been described in later stages of OVC but not consistently (Moss et al, 2005). There was staining seen in NT (50 %) and in NAT (13 %) (**Figure 4.9**) which has been seen previously with PASD1 in pre-malignant cells (Cooper et al, 2006)**.** This frequency of expression in NT exceeded the frequency of SSX2A immunolabelling in OVC stage I, II and III but may be explained by the fact that DAB precipitate is not chemically bound to the substrate at the site of its' oxidation (**Figure 4.10**) which means that it may have washed over the site of the NT and become ensnared. On further analysis it appeared that the staining of NT by DAB was non-specific, when compared to the cell specific immunolabelling seen within the ovarian tissues (**Figure 4.11**). However this does not explain why the NAT was not also stained non-specifically at the level the NT were. TMAs from Biomax US used by collaborators previously were found to be inaccurate where healthy tissues on the slides were found to be in fact premalignant (Banham group, University of Oxford, UK personal communication). The other option would be to have a pathologist determine whether the SSX2 staining was specific in health tissues and NAT compared with OVC tissues.

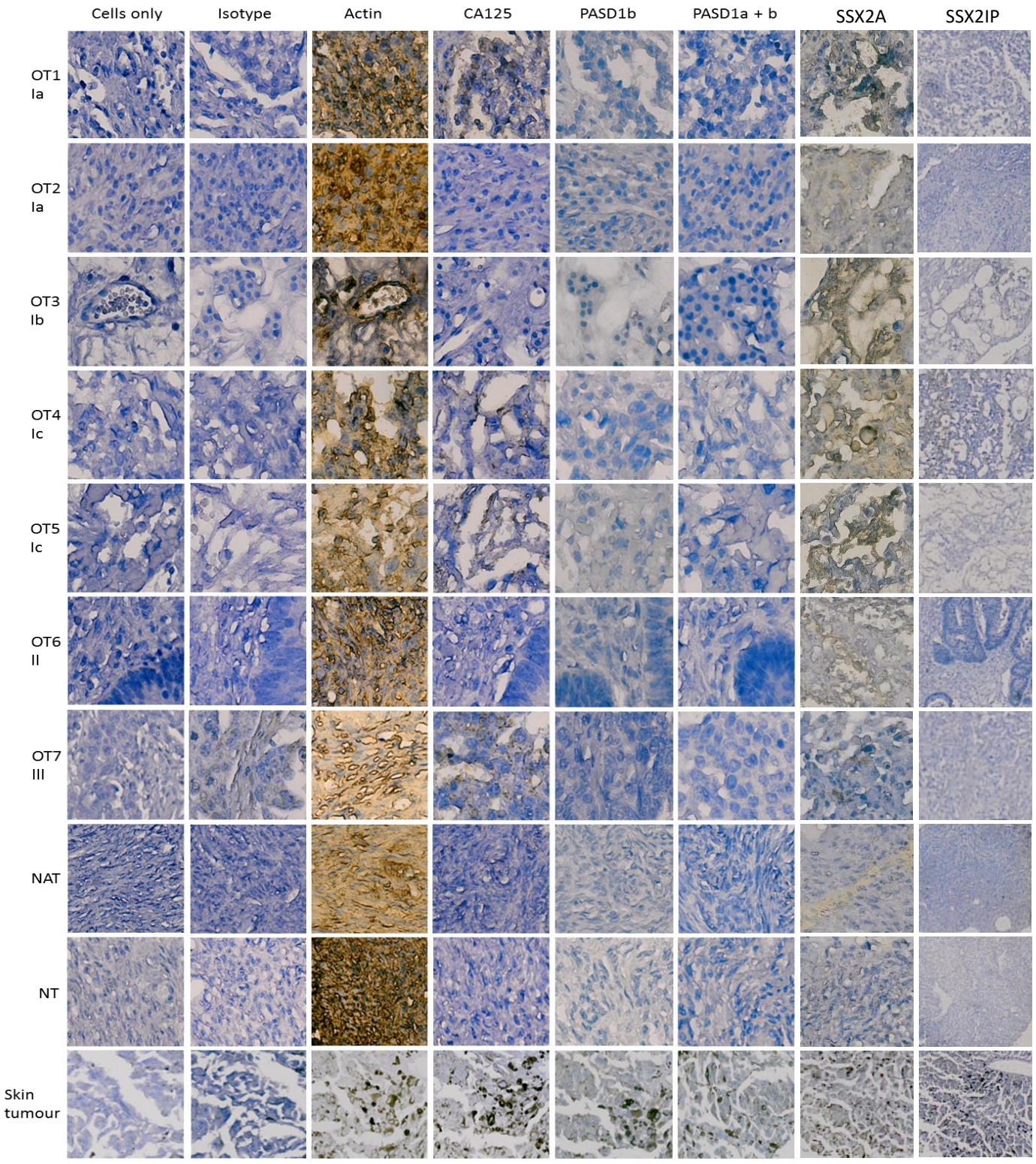

Cells only
Isotype
Actin
CA125
PASD1b
PASD1a + b
SSX2A
SSX2IP
OT1 Ia
OT2 Ia
OT3 Ib
OT4 Ic
OT5 Ic
OT6 II
OT7 III
NAT
NT
Skin tumour

Figure 4.7 Expression of TAA/CTAs in OVC TMAs. Images show the tumour antigen immunolabelling of OVC at stages Ia-III. PASD1 and SSX2IP were predominantly negative in OVC with very few samples scoring above 1 - background. SSX2A was frequently positive for immunolabelling, scoring 2 and above. CA125 was used as a comparator as it is one of the currently used proteins to confirm a diagnosis of OVC. Cells only and isotype were used as negative controls and actin as a positive control. Skin tumour tissue, already on the TMAs, was used as a further control for the test antibodies. Skin tumour tissue showed high expression of actin, CA125, SSX2IP, SSX2A and PASD1 (3 and above) but no immunolabelling with the negative controls (cells only and isotype). NAT and NT were also tested and mostly achieved a score of 2 or above when immunolabelled with actin with only two NT scoring 1.

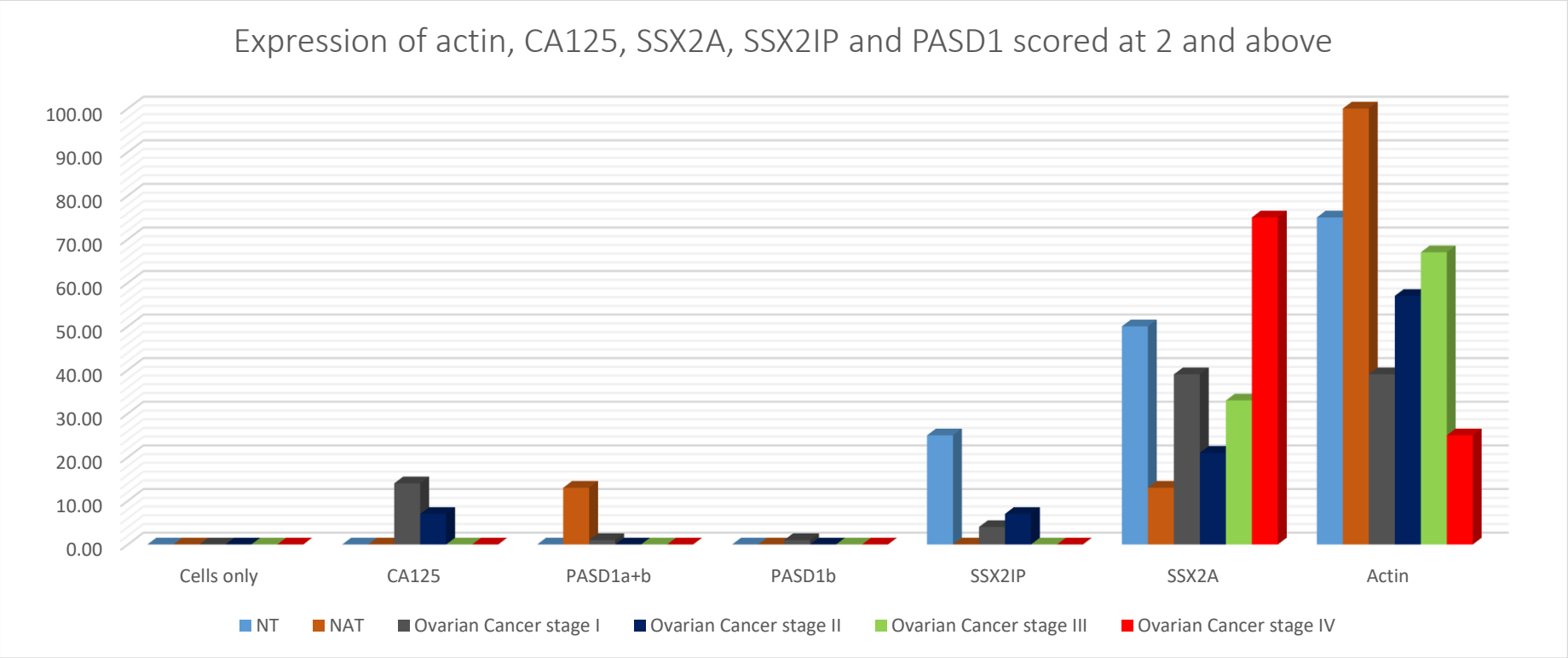


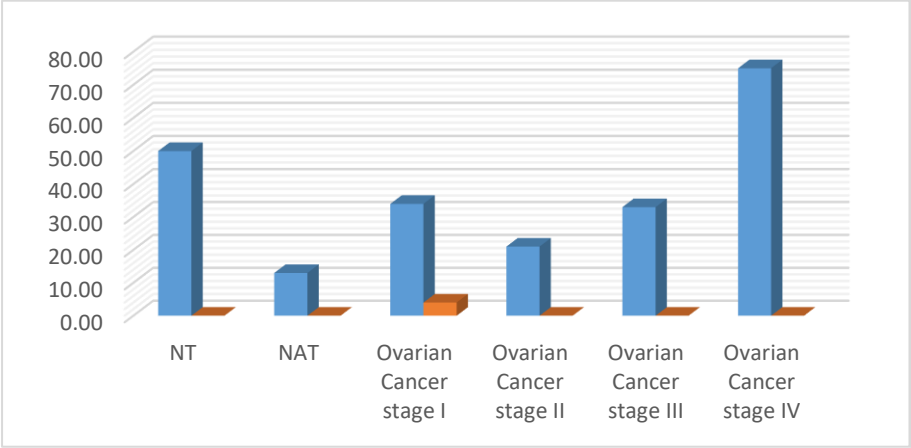
Figure 4.8. Frequency of tumour antigen immunolabelling of OVC tissues on the TMA. CA125 was used as a comparator to the other TAAs as it is currently used to confirm the diagnosis of OVC in clinical practice (Moss et al, 2005). Actin was used as the positive control to confirm the immunostaining protocol was working and cells only provided a control for "non-specific" background staining. Very little antigen expression was observed with CA125, PASD1 and SSX2IP but promising levels of SSX2A were detected at all OVC disease stages. The levels of staining shown here encompasses scores of 2 and above. Although there was staining of NT and NAT tissue this appeared to be non-specific compared to tumour tissue.

Table 4.6 Frequency of immunolabelling of antigens in OVC tissues.

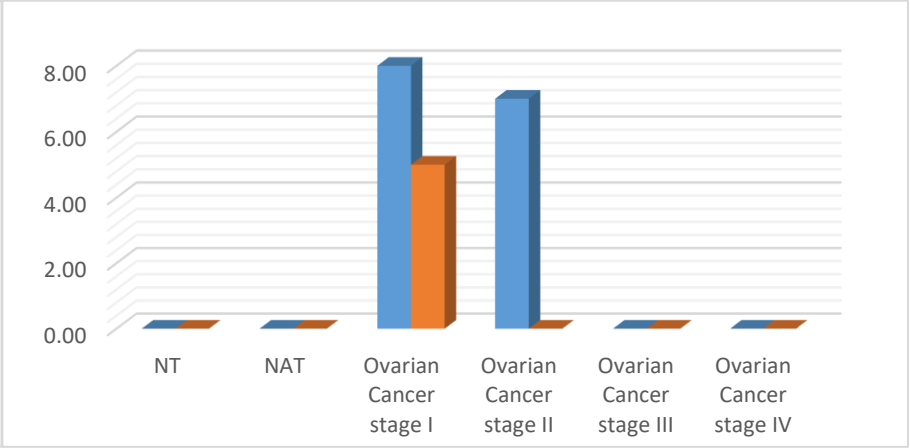
	Frequency of antigen expression in tissues* (%)						
	Cells only	CA125	PASD1a+b	PASD1b	SSX2IP	SSX2A	Actin
NT	0.00	0.00	0.00	0.00	25.00	50.00	75.00
NAT	0.00	0.00	13.00	13.00	0.00	13.00	100.00
OVC stage I	0.00	14.00	1.00	1.00	4.00	39.00	39.00
OVC stage II	0.00	7.00	0.00	0.00	7.00	21.00	57.00
OVC stage III	0.00	0.00	0.00	0.00	0.00	33.00	67.00
OVC stage IV	0.00	0.00	0.00	0.00	0.00	75.00	25.00

*The scores included in this study were those of 2 and above.

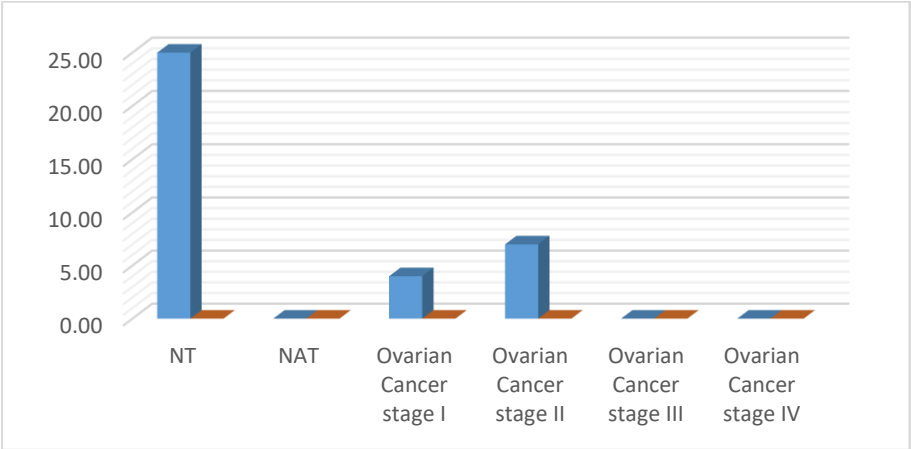
(a) SSX2A



(b) CA125



(c) SSX2IP



(d) SSX2A, CA125 and SSX2IP immunolabelling at level 2 and above

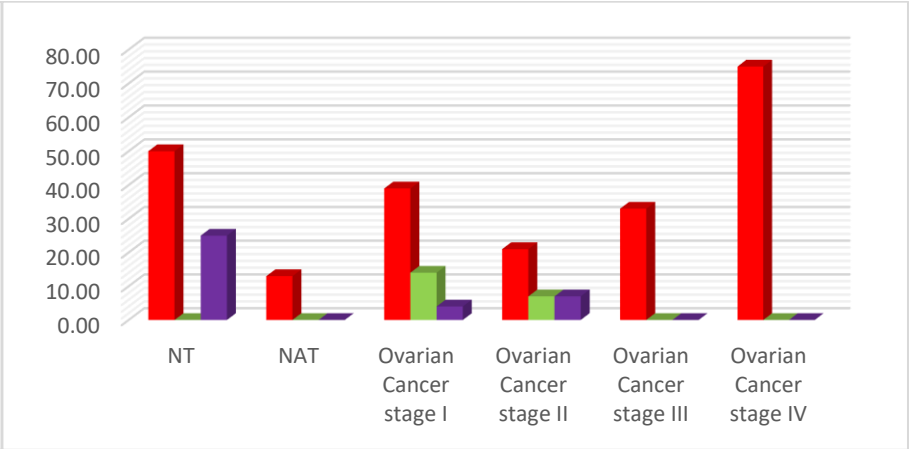


Figure 4.9. Immunolabelling of antigens at score level 2 and above in OVC and normal tissues. I have examined the intensity of staining, at level 2 and above (a) SSX2, (b) CA125, and (c) SSX2. Blue bars are indicative of level 2 immunolabelling and orange of level 3. In addition I directly compared (d) staining at level 2 and above for each antigen with SSX2A in red, CA125 in green and SSX2IP in purple. Y-axis shows percentage of tissue cores immunolabelled.

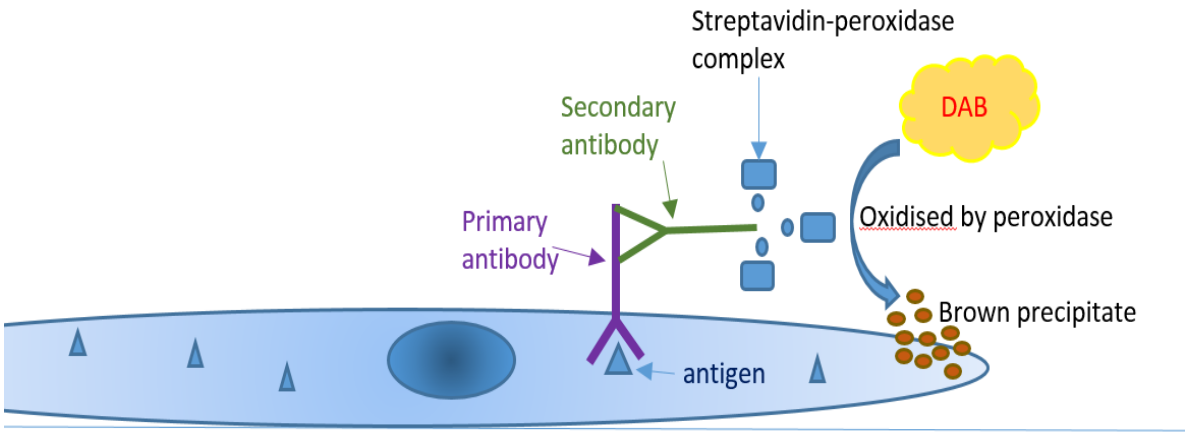


Figure 4.10. Diagrammatical representation of the immunohistochemistry technique indicating the deposition of DAB near the site of the peroxidase conjugated secondary antibody. Figure is based on a similar image depicted by BiteSizeBio.

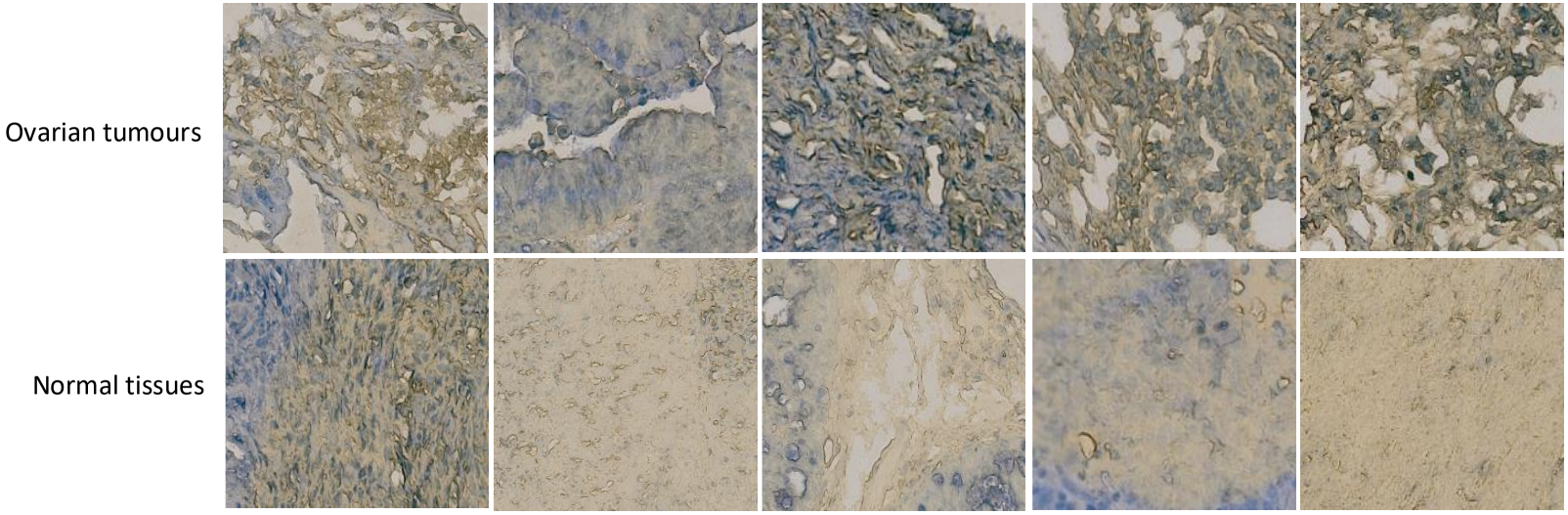


Figure 4.11. The comparison of staining observed with SSX2A in tumour tissues and normal tissues. It shows that in tumour tissue the immunolabelling adhered to individual cells, these tissues were each scored 3, whereas in NT and NAT the staining was general and although it received the score of 2-3, it did not appear to reflect the staining of individual cells. The staining of NT and NAT appeared similar in appearance to the background staining we had observed with isotype control but at higher levels.

4.4 Discussion

The aim of this project was to identify antigens for immunotherapy for early stage OVC which could subsequently be potential biomarkers of disease (SSX2, SSX2IP and PASD1). A variety of cell lines, haematological and solid cancers including OVC, were used since both SSX2IP and PASD1 are known as leukaemia antigens. All antigens were expressed in a number of the cells lines used, but SSX2A and SSX2IP only were expressed in OVC cell lines, PASD1 was negative. Of note, only PASD1b was detected in HeLa cells and appeared to be on the surface suggesting a possible surface antigen in cervical cancer.

PASD1 expression has previously been demonstrated in some more advanced tumour stages, and in some solid tumour cell lines such as Hn5 (head and neck)(Guinn et al, 2005b), SW480 (Liggins et al, 2004a) and H1299 (lung cancer)(Hardwick et al, 2013). 25 of 68 solid tumour tissue expressed PASD1 (Liggins et al, 2004a). PASD1 has also been found to be expressed in 22/25 cell lines derived from 21 B- and 4 T-cell malignancies by RT-PCR (Liggins et al, 2010). I now add to this knowledge showing that PASD1 is not frequently expressed in OVC particularly at the early stages of disease (which I recently published in Khan et al, 2015). This adds to a growing list of solid tumours (including bladder and basal cell carcinoma (Ghafouri-Fard et al, 2010)) which do not appear to express PASD1 at notable levels. Perhaps the biggest indication of the lack of PASD1 expression in solid tumours comes from the lack of publications. This is disappointing as PASD1 has one of the most restricted expressions of any CTA in healthy tissue having been found to be expressed in only the most immature spermatogonia at very high levels (Chen, G-Y. 2011, Cancer Research Institute Annual Meeting, personal communication). If found its expression in solid tumours would make it a very attractive CTA for immunotherapy targeting.

Since one of my initial projects was looking at SSX2IP in solid tumours I started by trying to repeat the studies of my predecessors (Denniss et al, 2007). I tried immunolabelling the cell

line K562 which was known to express SSX2IP on its surface. However I was unable to immunolabel this cell line and get the same results. Knowing that incubators can vary in their levels of CO_2 and that this can impact antigen expression (**Section 1.8**), I examined antigen expression in a range of different CO_2 levels. Unfortunately this did not yield any SSX2IP immunolabelling in K562 cells. Subsequently a new batch of SSX2IP antibody was purchased and I could detect SSX2IP on K562, and other cell lines, replicating work that had been described previously.

I also examined whether PASD1 and SSX2A expression was affected by CO_2 levels as at that time I was hoping to perform studies to examine SSX2IP expression and its' sub-cellular localisation in other cancer cells. Despite numerous attempts I found little positive impact of increased or decreased CO_2 levels on PASD1 or SSX2A expression in K562. Both PASD1-1 and PASD1-2 antibodies stained K562 at the 5% and 5.5% CO_2 levels but not 3.5 %, 4.5 % or 6 %. Since PASD1 seems to be a predominately a haematological antigen its' expression may not be subverted by CO_2 levels in the same way as antigens associated with solid tumour might be. A solid tumour is a mass of cells with, at the later stages, a hollow centre whereas liquid cancers diffuse throughout the body therefore they will not be subject to the same stressors provided by altered O_2 levels. However what was interesting was that at 5.5% CO_2 there was surface expression of PASD1 on the K562 cells suggesting that increased CO_2 levels may impact on the sub-cellular localisation of this antigens' expression and this observation requires further investigation outside the scope of the studies described here. SSX2A was also only expressed at 5 and 5.5 % CO_2, but with an impact on its' sub-cellular localisation at 5.5% CO_2. There appeared to be surface expression at 5.5 % but not 5 % suggesting some process in work at higher CO_2 however this cannot be confirmed just by visualising but would require another technique such as fluorescence microscopy. I only used 6 % CO_2 as the highest levels of CO_2

and may have seen greater effects from actually altering O_2 levels to induce create hypoxic and anoxic conditions rather than just altering CO_2 levels in a tissue culture incubator.

Liggins et al (Liggins et al, 2004a) had previous analysed the expression of PASD1 in three OVC tissues samples by dot blotting (based on the Western blotting technique). The PASD1 expression was negative to extremely weak and required a long exposure time in order to be detectable when compared to the other solid tumour tissues tested such as kidney and prostate. It is not clear what stages the OVC tissue samples they analysed were but it matches my findings of very low level of expression of PASD1 in OVC.

I was gifted seven TMAs labelled 1A and six TMAs labelled 1B, harbouring ≤88 and ≤100 tissue cores respectively. These tissues were paraffin-embedded unlike the cell lines and leukaemia cells I had analysed by ICC previously. I used these FFPE tissues as an opportunity to optimise the IHC protocol for subsequent immunolabelling of PASD1, SSX2IP and SSX2 in OVC TMAs. SSX2A and SSX2IP were both detected in colon cancer samples. SSX2 has been previously found in 25 % of colon cancer samples (Tureci et al, 1996). This was further verified by my colleague (Payalben Savaliya, personal communication) who found SSX2 to be an important indicator for survival in colon cancer using microarray datasets. This study is now being prepared for publication (Boncheva, Mills and Guinn, in preparation).

One NAT tissue achieved a score of 2 for PASD1b (1/8) but there is some evidence that PASD1 mRNA may be present in histologically normal tissues signalling the potential of the cells to become cancerous (Liggins et al, 2004a; Ait-Tahar et al, 2009). The immunolabelling of PASD1 was seen to fade over time probably due to the dissolution of DAB precipitate into the faramount aqueous mounting media (Espada et al, 2005; Soini et al, 2002). When I realised this was the cause of the loss of DAB precipitate I changed practise to use DPX, a synthetic resin mounting media, instead. Indifferently, and on repeat experiments, PASD1 was not found

to be frequently or highly expressed - only one OVC stage 2 sample had expression at a score of 2. PASD1 expression has previously been shown to be associated with the more aggressive forms of lymphoma (Liggins et al, 2004b) and it may be that if I had analysed more later stage III and IV OVC samples we may have found more samples immunolabelling at a score of 2 or above for PASD1 expression. The TMA had only six OVC stage III and four stage IV samples. In cell lines investigators have found a range of frequencies of PASD1 expression from 17.6±3.6 % in K562 to >99% in H1299 (Hardwick et al, 2013) and recently PASD1 function has been linked with circadian rhythms (Michael et al, 2015). PASD1 was found to suppress the 24 h biological clock which controls the physiological and chemical processes of all cells in a 24 h period. This implies that PASD1 would not be expressed in all cells at all times and therefore would not make a suitable biomarker as it would need to be expressed in a large number of tumour cells and be visible at any time the test is performed.

CA125 and HE4 were expressed more frequently than PASD1 in my study of OVC samples however CA125 has been proven to have low specificity and sensitivity, and inconsistent expression patterns amongst OVC patients. Therefore CA125 is mainly used as a marker to detect disease relapse and for monitoring treatment efficacy rather than confirming the diagnosis of primary OVC (Zhen et al, 2014). There are also suggestions that CA125 may be more effective as a marker for predicting advanced stage disease (Kim et al, 2015). CA125 and HE4 would not make optimal tumour antigens for immunotherapy because they are expressed in normal cells and benign conditions, making it difficult to break tolerance against them and maintain specificity for cancerous cells and not healthy or inflamed/damaged tissues. In fact using irradiated immune cells from patient themselves, a donor or tumour lysates were found to be ineffective against CA125 expression (Dranoff et al, 1993).

In a literature review, serum CA125 levels have been found to be a good prognostic indicator for patients with OVC (Gupta and Lis, 2009) while three months post-surgery serum CA125

levels can provide an independent prognostic marker, identifying high risk patients for whom further treatment may be beneficial (Sevelda et al, 1989). In contrast CA125 has also been found in EOC where raised CA125 correlated with a poorer overall disease-specific survival (66.1 vs 87.8 months, p = 0.021) (Myriokefalitaki et al, 2015). These manuscripts demonstrate both the value of CA125 as a marker of late stage OVC and the confusing picture around its value in early stage OVC.

SSX2IP is an LAA which was identified as an interacting partner to SSX2 (**Section 1.6.2**). It has been investigated in a number of haematological malignancies (Breslin et al, 2007) but infrequently in solid tumours, with the exception of cell lines. I wanted to look at its expression in OVC due its interesting expression on the surface of AML cells during mitosis (Denniss et al, 2007). Although both SSX2A and SSX2IP were visually appeared to be labelled on the surface of cells, further studies would be required to find if they co-localise such as fluorescence resonance energy transfer (FRET). Elevated expression of SSX2IP has been shown to lead to an increase in tumour size and thrombus ($P < 0.05$) and significantly shorter survival time ($P = 0.004$) in patients with hepatocellular carcinoma (HCC). SSX2IP was also found to promote peritoneal spreading and liver metastasis of HCC cells in nude mouse model and appears to augment drug resistance (Li et al, 2013b). In gastric cancer (GC), 43/66 (65 %) of tumour tissues demonstrated elevated expression of SSX2IP protein in comparison to matched healthy tissues. Tumour suppressor miR-338-3p (micro-RNA) functions by inhibiting cell proliferation, migration, invasion and apoptosis in GC and raised levels of SSX2IP to some extent reverses the inhibitory effect of miR-338-3p in GC cells without altering its expression. This suggests SSX2IP is a functional target of miR-338-3p in GC (Li et al, 2013a) and plays a functional role in a number of cancer types.

Guinn et al, 2008 showed an association between mitotic spindle failure in patients with AML M2, associated with a t(8;21) translocation and SSX2IP expression. Subsequently other

investigators have shown that SSX2IP accrues at spindle poles and interacts with the γ-tubulin ring complex (γ-TuRC) and the centriolar satellite protein PCM-1. Reduction in SSX2IP levels hampered the γ-TuRC loading onto centrosomes leading to failure of spindle formation (Barenz et al, 2013) and the human mitotic spindle disanchored 1 (hMsd1)/SSX2IP is thought to be a novel microtubule-anchoring factor (Hori et al, 2014). I found SSX2IP to be expressed in a very small number of OVC samples, showing some expression in NT (25% of eight) and 4 % of 166 stage I and 7 % of 15 stage II OVC. Thus SSX2IP offers no advantage over existing biomarkers for OVC. I believe the search for its expression on the surface of other malignant cells may determine its' value as an immunotherapeutic target for antibody therapies.

SSX2 has been found to be expressed in a number of cancers including breast and hepatocarcarcinoma (Tureci et al, 1996)(**reviewed in Section 1.7.4**). SSX24 expression as detected by immunostaining showed that five of 143 early-stage non-small cell lung cancers (NSCLCs) (Greve et al, 2014) and seven of eight prostate cancer cell lines transcribed SSX2 at varying levels (Smith et al, 2011). In contrast early studies of the expression of the SSX2 gene indicated it was not found OVC (none of three) by RT-PCR (Tureci et al, 1996) however SSX4, by the same method was detected in 6/12 (50 %) OVC tissues (Tureci et al, 1998). Subsequent studies using RT-PCR examined the expression of the SSX genes in gynaecological cancers. Hasegawa et al (2004a) showed that 2/50 (4 %) of endometrial and 1/25 (4 %) of CC (Hasegawa et al, 2004a) expressed SSX2 while Valmori et al, (2006) demonstrated SSX2 expression in 10 % of EOC patient samples (Valmori et al, 2006). Of all the positive tumours identified, one of two SSX1 and all four SSX2 mRNA positive tumours were found to co-express SSX4 mRNA (Hasegawa et al, 2004a**).** SSX4 mRNA expression has been observed in 5/40 (13 %) OVCs as well as in endometrial cancers in 12/50 (24 %), and in 5/25 (20 %) CC (Hasegawa et al, 2004a). Other members of the SSX family have been detected in various cancers. Expression of SSX1 (3/118; 2.5 %) and SSX4 (19/120; 16 %) was detected

in EOC specimens using RT-PCR (Valmori et al, 2006). This contradicts the findings of another study where no expression of SSX1 was found in OVC but was found in a very small number of endometrial cancer (1/50; 2 %) and CC samples (1/25;4 %)(Hasegawa et al, 2004a).

Actin was found to be expressed in almost all of the samples analysed and showed that the IHC assay and all of its composite reagents were working appropriately. Isotype control were predominantly negative but can score positive at low levels (1 and rarely 2) on primary tissues due to non-specific binding. Other investigators have shown that IgGs can bind with low affinity to many tissue components producing false positives (Fritschy, 2008).

SSX2 has been found to have very restricted expression in previous studies (Tureci et al, 1996), being found in tumour cells and immunologically protected sites such as the testes with very weak expression if the thyroid (Tureci et al, 1998; Lim et al, 2011). As such SSX2 would make a very specific target for cancer immunotherapies (Tureci et al, 1996). However I did detect a lot of staining in NT (50% of eight tissues at level 2) and (13% of eight at level 2) NAT. The staining pattern appeared to be non-specific in the NT and NAT. All of the NT and NAT samples were located along one edge of the TMA slide and I noticed that samples at the edge of the arrays tended to capture more background staining. However this does not explain why the NAT did not stain non-specifically as frequently as the NT did. Further analysis by a pathologist would have provided a more definitive answer regarding the specificity of the staining, which unfortunately I was not able to arrange. This reflects the time constraints on pathologists and our need to collaborate with clinicians to facilitate professional scoring of primary samples. A pathologist could also pinpoint accurately the location of the antigens within the tissue bed and help determine whether there was specific staining of certain types of cells within OVC samples.

This part of my studies had aimed to determine whether PASD1, SSX2 and SSX2IP could make promising targets for the immunotherapy of OVC. PASD1 and SSX2IP had low expression in OVC suggesting they would be poor targets for the immunotherapy of OVC. Initially I was unable to replicate the work of my predecessors and show that SSX2IP was expressed on the surface of K562 cells, but experiments that involved altering CO_2 levels during cell growth led to my finding that PASD1 is expressed on the surface of K562 when CO_2 levels are slightly elevated (5.5%). This suggests that further investigations, on whether the sub-cellular localisation of the expression of TAAs, such as SSX2IP and PASD1, are changed by slight changes in CO_2 level are worthy of future consideration. Analysis of SSX2A in OVC samples showed consistent expression at all stages including stage 1 and II suggesting that it required further investigation.

CHAPTER 5: VALIDATION OF SSX2 AS A BIOMARKER FOR EARLY STAGE OVC

5.1 Introduction

SSX2 has been shown to be a CTA with restricted expression in healthy tissues (reviewed in **Section 1.7.4**). This does not rule out the possibility that SSX2 is expressed in NT although CTAs are often found to have low level expression in few healthy tissues, usually the pancreas or brain, and high levels of expression in immunologically protected sites such as the testes and placenta. However my analyses of NT had shown high levels of, what appeared to be non-specific staining, with only OVC stage IV exceeding the frequency of staining of tissues in NT (Chapter 4). Few investigations have examined SSX2 expression in solid tumours and so I extended my investigations to determine whether the expression of SSX2 was specific to OVC and whether the staining of ovarian healthy tissues could be differentiated. I also wanted to determine whether SSX2 was expressed in related inflammatory diseases and healthy tissues. There are two variants of SSX2, although they are rarely discerned in the scientific literature and so I investigated their expression independently to see whether I could see which were being expressed in OVC.

I chose two additional, commercially available SSX2 antibodies for further analysis to determine whether the issue with background could be reduced by the use of alternative antibodies and whether I could differentiate between SSX2 and SSX2A immunolabelling. One of the commercial antibodies bound solely to the C' terminal of SSX2, and this was specific for SSX2A and referred to as pSSX2A. This overlapped with the recognition of SSX2A achieved by the monoclonal antibody mSSX2A (Chapter 4) and should have generated similar results. The third antibody I chose bound to the region of SSX2 common to both SSX2A and SSX2B, referred to as pSSX2(N)(discussed in detail in **Section 2.7.2**). Each SSX2 antibody was tested on the following cell lines, K562, HL60, HeLa and SW480, to

optimise the protocol for ICC for each new antibody to include haematological and solid tumour cells lines. Cell lines that were positive for SSX2 (K562 and Skov3) were used to test the specificity of the antibodies for SSX2 through the use of peptide blocking.

All three SSX2 antibodies were used to immunolabel SSX2A (pSSX2A and mSSX2A), and SSX2 (pSSX2(N)), in patient samples on MTAs and directly compared to HE4 and WT1 immunolabelling which have recently been investigated for their usefulness as OVC biomarkers (described in **Section 1.9.1.2 and 1.9.1.4** respectively). HE4 was chosen in place of CA125 for the experiment where pSSX2A was analysed. This was to determine whether HE4 was a better biomarker in my hands than CA125.

5.2 Aims

- To confirm the frequency of expression of SSX2 and specifically SSX2A in OVC using three independent antibodies
- To determine whether SSX2 and specifically SSX2A are expressed in OVC, healthy and diseased related tissues

5.3 Results

5.3.1 Optimisation of SSX2 and SSX2A immunolabelling using commercially available antibodies

Both SSX2 and specifically SSX2A were found to be expressed in the K562, KM-H2, KY0-1, HL60, HeLa and SW480 **(Figures 5.1 & 5.2)** cell lines. This confirms the data from previous studies where each of these cell lines including K562 (dos Santos et al, 2000), KM-H2 (Colleoni et al, 2002), HL60 (Hoffman et al, 2014) and HeLa (The Human Protein Atlas) were shown to express SSX2. The exception was SW480 where we found SSX2 and SSX2A expression although it had not previously been reported.

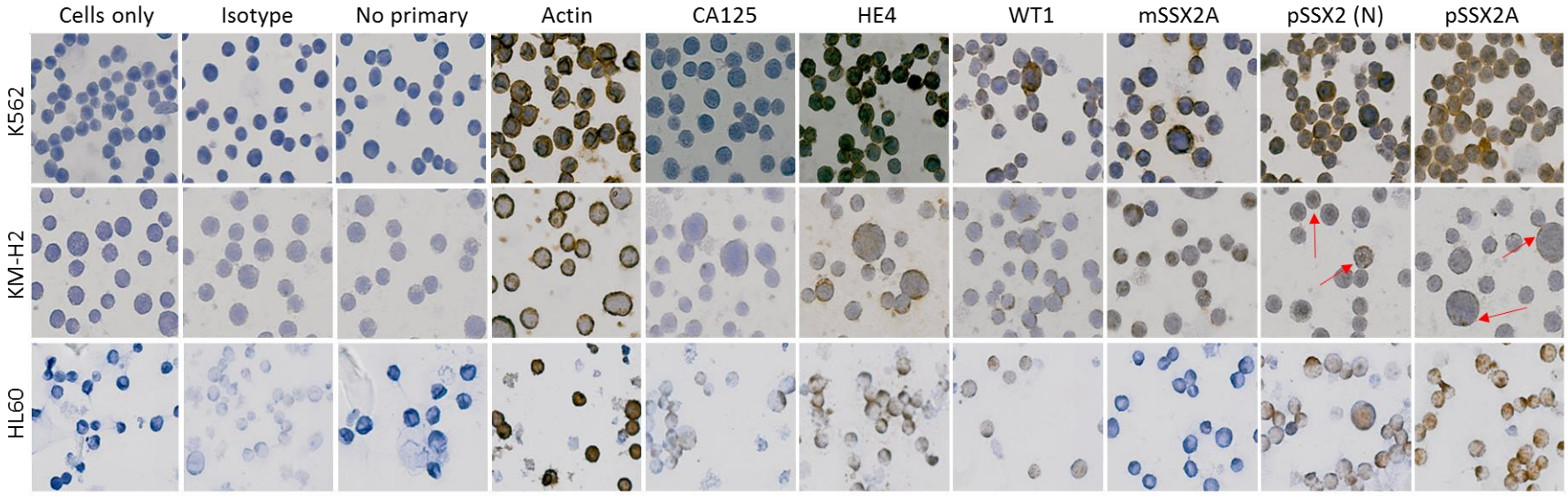


Figure 5.1 Expression of SSX2 and SSX2A, as detected by three commercially available antibodies, in AML and HL cell lines. SSX2 expression was detected in K562 (CML), HL60 (AML) and KM-H2 (HL) cell lines but SSX2A was not detected in HL60. HE4 and WT1 were detected in all three cell lines and CA125 in KM-H2 and HL60. Actin was used a positive control and cells only, isotype control and no primary antibody all acted as negative controls.

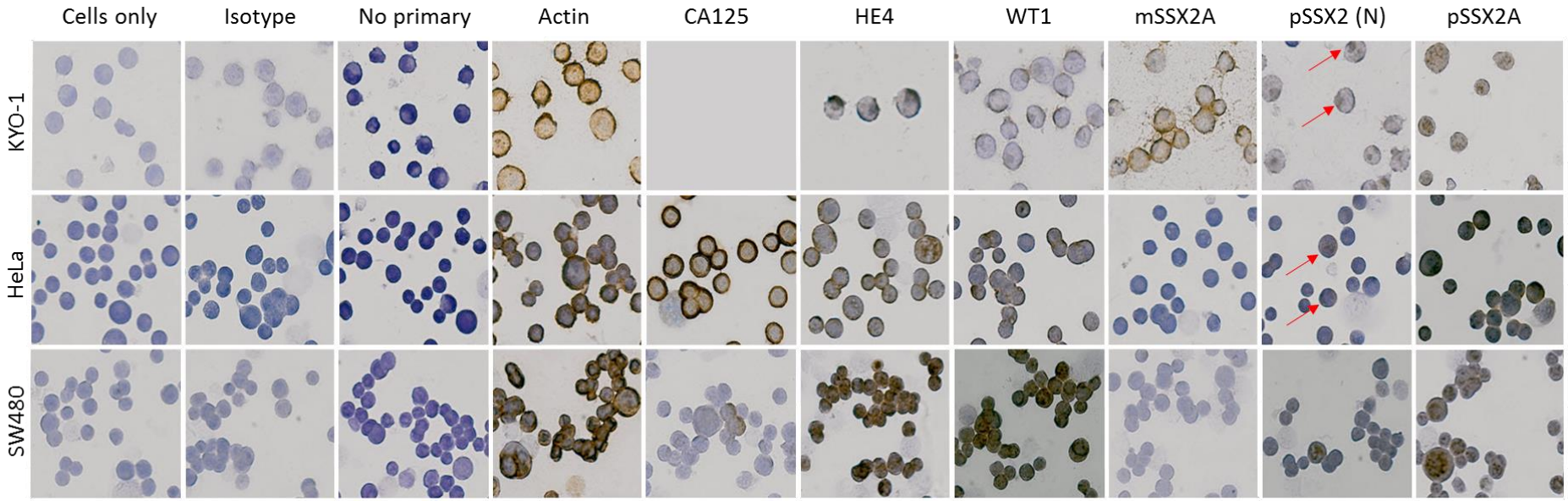


Figure 5.2 Expression of SSX2 in CML, CC and CRC cell lines. pSSX2(N) and pSSX2A were found in KYO-1 (CML), HeLa (CC) and SW480 (CRC) cell lines however mSSX2A was found only in KYO-1. HE4 and WTI were detected in all cell lines tested whereas CA125 was also found in HeLa and SW480, non-OVC cell lines.

5.3.2 Blocking SSX2 and SSX2A antibodies using specific peptides

To show that the commercial antibodies were specific for SSX2, and SSX2A, I incubated the antibodies with their respective SSX2 peptides prior to immunolabelling (**Section 2.7.4)**. Two cell lines (K562 and Skov3) were found to immunolabel with all three antibodies, and these were used to demonstrate whether we could block SSX2 and SSX2A immunolabelling with the SSX2 and SSX2A specific peptides (**Section 2.7.5**). All three antibodies were successfully blocked by their respective peptides, suggesting that these antibodies were specific for SSX2 and SSX2A as described by the manufacturer **(Figure 5.3).**

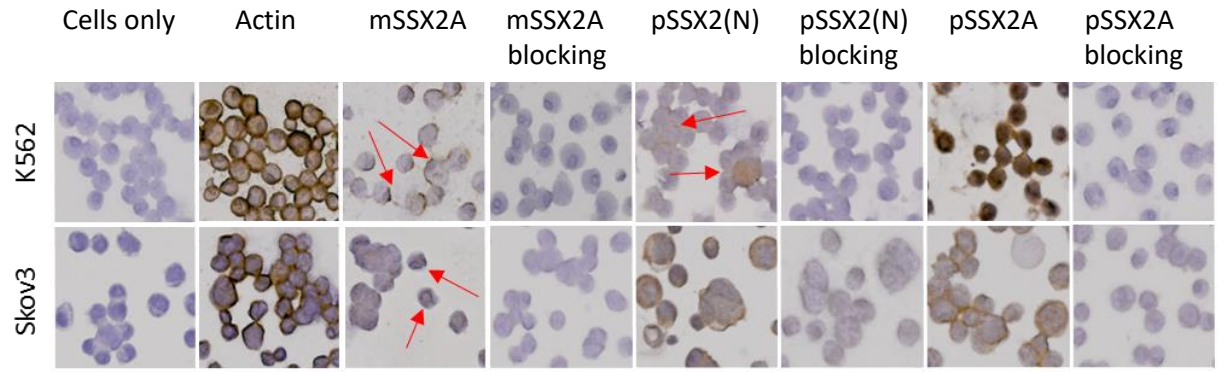


Figure 5.3 Demonstrable blocking of SSX2 antibodies by SSX2 peptides. To examine the specificity of the SSX2 antibodies, blocking peptides were used. K562 and Skov3 cell lines were used. All three commercially obtained SSX2 antibodies were successfully blocked from immunolabellng SSX2 in the human cancer cell lines. Magnification x 400. Images are representative of 3 repeats of this experiment.

5.3.3 Analysis of SSX2A and HE4 expression in OVC tumour samples

A new SSX2A antibody was used to further analyse if SSX2A is consistent in labelling OVC. Expression of SSX2A was detected through the use of the pSSX2A antibody on OVC TMAs containing samples from various stages of disease. Using the pSSX2A antibody I found that SSX2A protein was expressed in all stages of OVC; 21 % (34/165, 1 missing) in stage I, 40 % (6/15) in stage II, 17 % (1/6) in stage III and 50 % (2/4) in stage IV **(Figure 5.4).** There was no immunolabelling of SSX2A in NT or NAT when using the pSSX2A antibody. Due to

the poor results I obtained with CA125 (Section 4.XX) I compared SSX2 staining to the relatively recently investigated biomarker, HE4 (**Section 1.9.1.2**). SSX2A immunolabelling with pSSX2A antibody exceeded the level and frequency of expression of HE4, in the same patient samples. HE4 was positive in 8 % (13/165, 1 missing) stage I, 13 % (2/15) of stage II OVC while no expression was found in stage III and IV OVC samples. There was also no immunolabelling of HE4 in the NT or NAT and there was no background staining of the tissues obtained when using the isotype control.

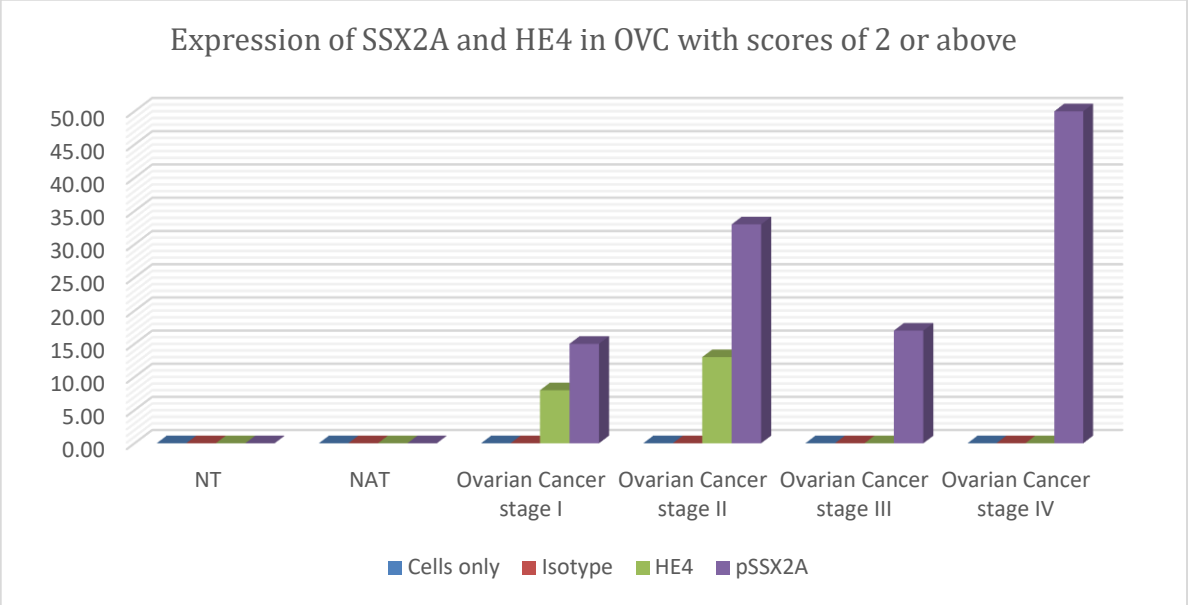


Figure 5.4 Frequency of expression of SSX2A in comparison to HE4. HE4 was also used as a positive control as it is known to be expressed in OVC. In this study there was minimal expression found of HE4 in the early stage samples and none in the later stages. SSX2A was detected in 20 % of stage I, 40 % stage II, 20 % stage III and 50 % stage IV OVC patient samples. Both SSX2A and HE4 were not found in NAT or NT. This experiment was performed only once for each antibody although each slide tested had 208 samples on it, acting as 208 independent experiments.

5.3.4 Specificity of the expression of tumour antigens in endometrial tissue

A potential biomarker should ideally be specific to the disease state it identifies to avoid unnecessary procedures. To determine whether the SSX2A expression I had detected using pSSX2A and mSSX2A was specific to OVC or could also be found in healthy and diseased endometrial tissue, as is the case for HE4 (**Section 1.9.1.2**), I examined the expression of SSX2A on endometrial disease spectrum TMAs. The TMAs included normal endometrial tissue, malignancy of the uterus, endometrial hyperplasia, as well as metastasis – endometrial adenocarcinoma. The highest expression in malignant endometrial tumours with scores of 2 and above was observed with HE4 with 27/30 (90 %) samples while immunolabelling SSX2A with pSSX2A occurred in 15/30 samples (50 %) (**Figures 5.5 and 5.6**). CA125 was immunolabelled to a lesser extent in 9/30 (30 %) samples. However immunolabelling with the mSSX2A antibody indicated there was very little expression of SSX2A in endometrial cancer samples where no samples had a score of 2 or above. HE4 and pSSX2A were found to immunolabel normal endometrial tissues with the majority of samples, 9/16 scoring 3-4 for HE4 and 13/16 scoring 2-3 with pSSX2A. CA125 was positive in 6/16 NT scoring 2-3. However SSX2A, when immunolabelled with mSSX2A, was only positive in 2 out of 16 malignant uterus samples each achieving a score of 2. Cells only and isotype were negative as expected. Scores for each sample are provided in appendix II. The endometrial TMAs contained endometrial cancer which had metastasised (n=6). These included single samples of fibrofatty tissue, lymph node, pelvic cavity, ovary and two samples of abdominal cavity. mSSX2 had a score of 1 on the single fibrofatty tissues sample, pSSX2A achieved a score of 2 on all metastatic tissues apart from in one of the abdominal samples where the score was 1. CA125 had score of 1 in the ovary, 2 in fibrofatty tissue, pelvic tissue and 1 out of 2 of the abdominal cavity and 3 in the lymph node while CA125 was negative in the remaining abdominal cavity tissue. HE4 had a score of 1 in 3 tissues (1/2 abdominal cavity, fibrofatty

tissue, lymph node) and 2 in 3 tissue (1/2 abdominal cavity, pelvic cavity, ovary) **(Figure 5.7)**.

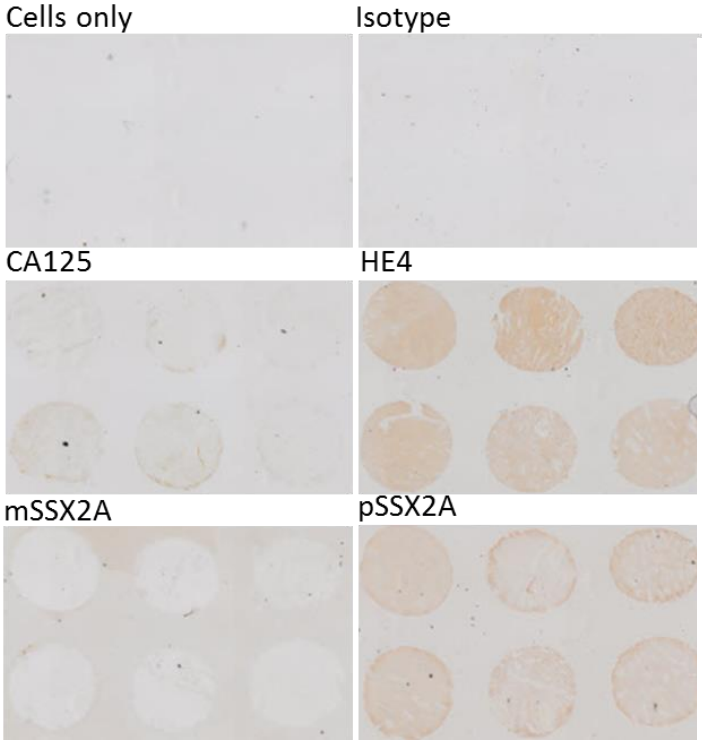
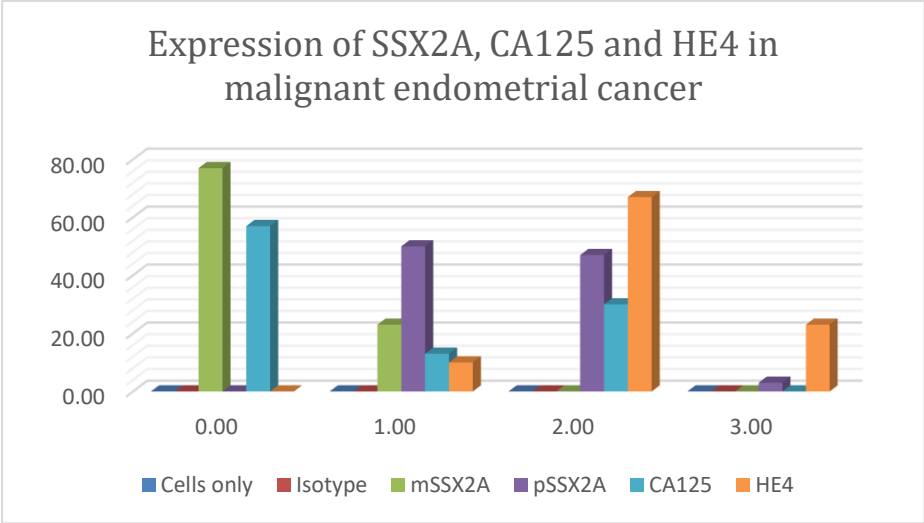
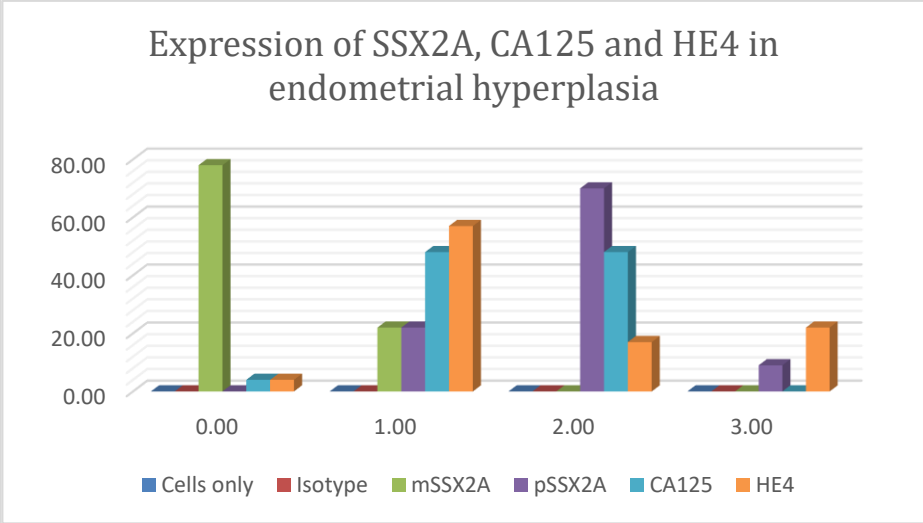


Figure 5.5 TMAs of endometrial cancer enabled the analysis of CA125, HE4, and SSX2A expression. Immunolabelling of HE4 and pSSX2A was found with a score of 2 in most samples, while CA125 immunolabelling achieved a score of 1 in most samples. Very little staining was observed with mSSX2A (seen with a score of zero in the image). Cells only and isotype control were also negative. In order to clearly observe the absence of immunolabelling no counterstain was used.

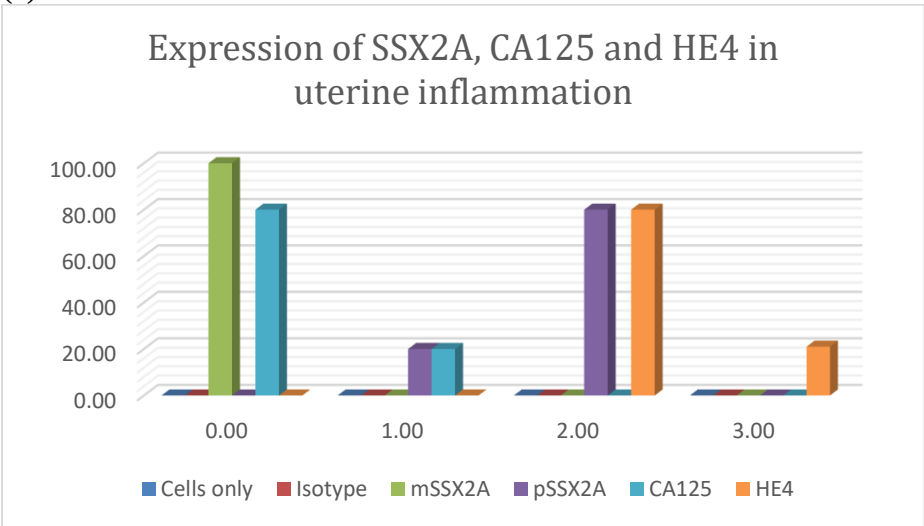
(a)


Expression of SSX2A, CA125 and HE4 in malignant endometrial cancer
80.00
60.00
40.00
20.00
0.00
0.00
1.00
2.00
3.00
Cells only
Isotype
mSSX2A
pSSX2A
CA125
HE4

(b)


Expression of SSX2A, CA125 and HE4 in endometrial hyperplasia
80.00
60.00
40.00
20.00
0.00
0.00
1.00
2.00
3.00
Cells only
Isotype
mSSX2A
pSSX2A
CA125
HE4

(c)


Expression of SSX2A, CA125 and HE4 in uterine inflammation
100.00
80.00
60.00
40.00
20.00
0.00
0.00
1.00
2.00
3.00
Cells only
Isotype
mSSX2A
pSSX2A
CA125
HE4

(d)

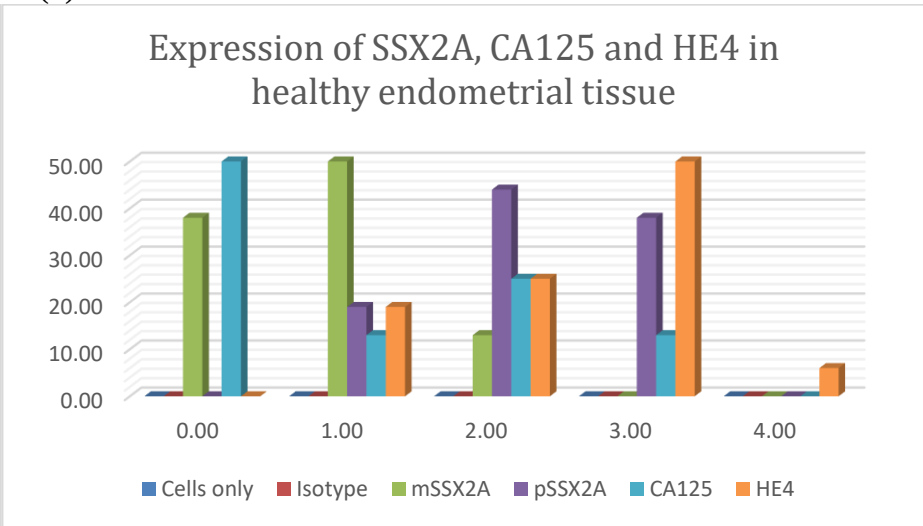

Expression of SSX2A, CA125 and HE4 in healthy endometrial tissue
50.00
40.00
30.00
20.00
10.00
0.00
0.00
1.00
2.00
3.00
4.00
Cells only
Isotype
mSSX2A
pSSX2A
CA125
HE4

Figure 5.6 Immunolabelling of various endometrial tissues on TMAs. (a) Malignant endometrial cancer: only labelling with pSSX2A, CA125 and HE4 succeeded in scoring 2 and above while labelling with mSSX2A was found to be the background level of 0-1, (b) endometrial hyperplasia: mSSX2A staining scored mainly background of 0-1 while pSSX2A, CA125 and HE4 all scored 2 and above with the highest labelling being by pSSX2A, (c) in inflammation tissues mSSX2A staining was found to be negative only scoring 0 and similarly CA125 only scored 0-1 while pSSX2A and HE4 both labelled with scores of 2 and above, and (d) weak expression was seen with mSSX2A in normal endometrial tissue (2/16) whereas pSSX2A, CA125 and HE4 show much higher labelling with HE4 reaching up to 50%.

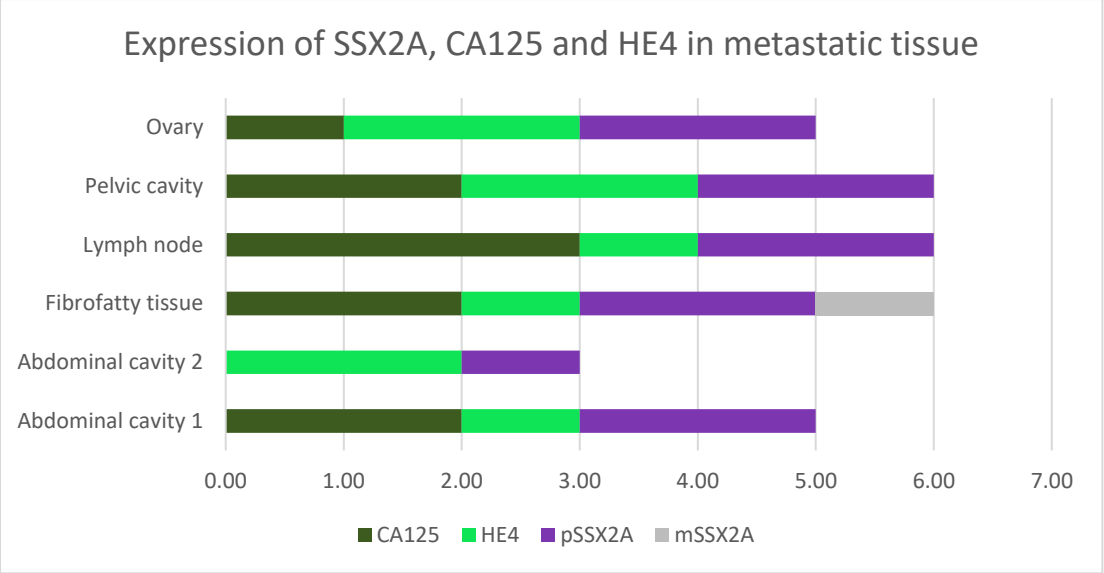


Figure 5.7: Labelling of endometrial metastatic tissue on TMAs. Endometrial metastatic tissue at a variety of secondary locations appeared to label with pSSX2A and HE4 highly, with CA125 having lower expression while mSSX2A shows virtually no staining apart from in the fibrofatty sample with a score of 1.

5.3.5 Analysis of SSX2 and SSX2A expression in OVC

To discern whether SSX2 and SSX2A are both expressed in OVC and to repeat the most promising of my findings I examined the expression of SSX2A in OVC using mSSX2A (used to immunolabel SSX2A in Chapter 4) and the pSSX2 (N) antibody. I didn't used the pSSX2A antibody as it was found to label endometrial tissue. Scores of 2 and above were considered positive. I found expression of SSX2 in OVC patient samples at almost every stage of OVC (Figures **5.8, 5.9 and 5.10**). mSSX2A immunolabelled SSX2A in 54 % (88/162, 4 missing) of samples from stage I, 50 % (7/14, 1 missing) from stage II, 17 % (1/6) of samples from stage III and 25 % (1/4) from stage IV patients, whereas the pSSX2 (N) immunolabelled SSX2 in 33 % (54/162, 4 missing) of samples from stage I, 50 % (7/14, 1 missing) from stage II and 25 % (1/4) from stage IV while no SSX2 protein was detected with this antibody in any stage III samples. In comparison, CA125 was found in 32 % (52/164, 2 missing) of stage I, 36 % (5/14, 1 missing) of stage II and 20 % (1/5, 1 missing) stage III and 75 % (3/4, 1 missing) of stage IV patient samples. HE4 was expressed in 32 %

(52/164, 2 missing) of samples from stage I, 50 % (7/14, 1 missing) of stage II, 20 % (1/5, 1 missing) of stage III and 75 % (3/4) of stage IV patient samples. WT1 was found in 22 % (35/162, 4 missing) of stage I, 29 % (4/14, 1 missing) of stage II with no detection in stage III and stage IV OVC samples. HE4, WT1 and both the SSX2 (mSSX2A and pSSX2 (N)) antibodies led to positive scoring in NT and NAT although SSX2 was found at slightly lower levels in NT (29 % (2/7, 1 missing) and 50 % (4/8) with mSSX2A and 20% (1/5, 3 missing) and 38 % (3/8) with pSSX2 (N)) compared with HE4 (50 % (4/8) and 38 % (3/8) and WT1 (38 % (3/8) and 25 % 2/8), respectively. CA125 was not immunolabelled in the NT or NAT (**Figure 5.10c**). Positive samples were taken as 2 and above however when scores 3 and above are analysed it appears that mSSX2A is labelled exceedingly better at the higher intensity than pSSX2A, CA125, HE4 and WT1 **(Figure 5.11a and b).** CA125 has been previously known to have little or weak expression in normal ovarian epithelium (Rosen et al, 2005) although some studies do show that CA125 may be positive as 4 out of 11 epithelial cell samples obtained from normal ovaries and fallopian tubes had CA125 expression (Neunteufel & Breitenecker, 1989). While HE4 has not been found in normal ovaries, it is highly expressed in benign OVC cancers and other ovarian disease such as ovarian surface cysts (Georgakopoulos et al, 2012).

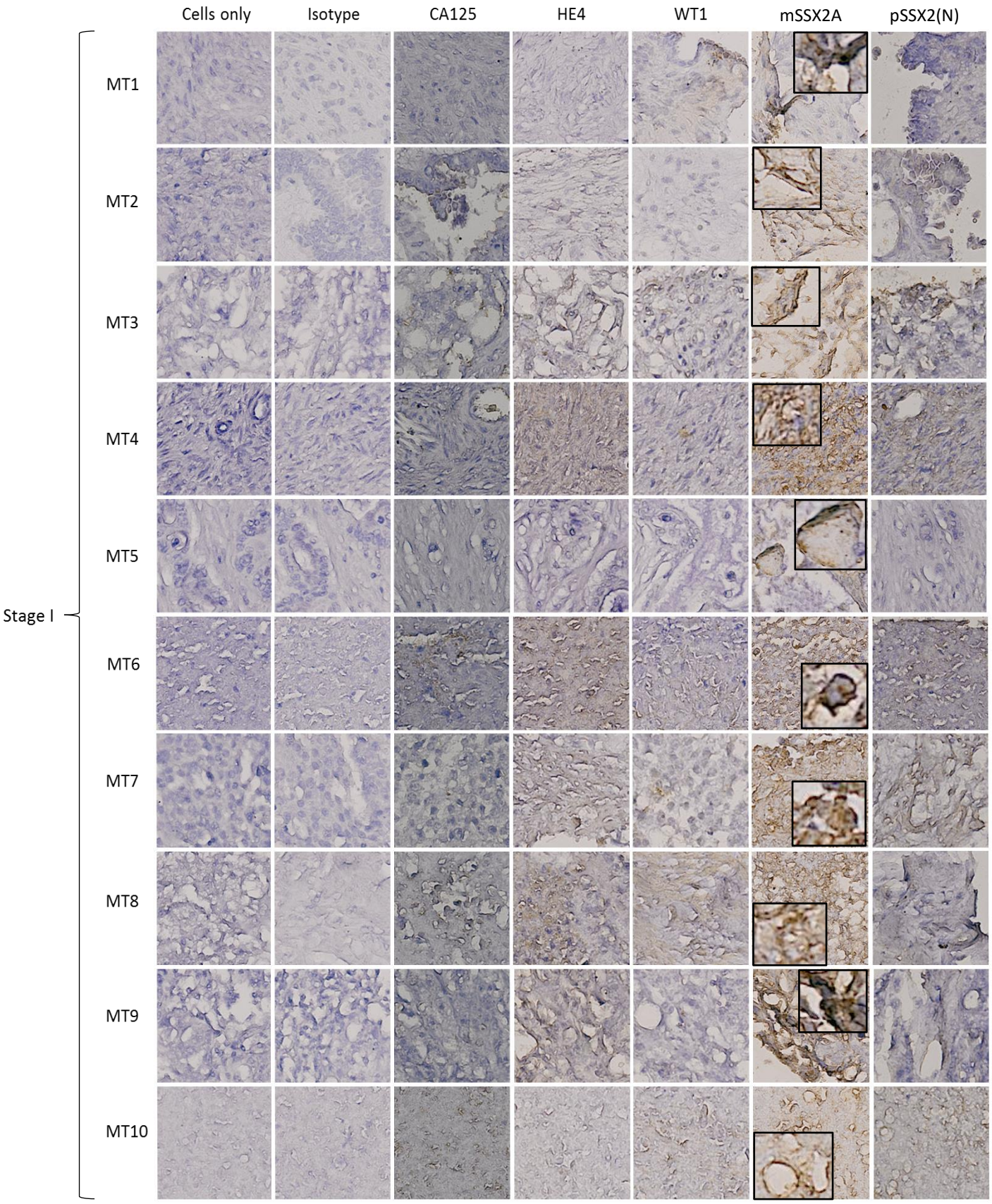

Cells only
Isotype
CA125
HE4
WT1
mSSX2A
pSSX2(N)
MT1
MT2
MT3
MT4
MT5
MT6
MT7
MT8
MT9
MT10
Stage I

Figure 5.8 Immunolabelling of SSX2A and SSX2 in stage I OVC tissues with mSSX2A and pSSX2 (N) antibodies, respectively. SSX2 antigen immunolabelling was visible by virtue of a brown deposition. Actin was used as a positive control to demonstrate that IHC staining was working properly and cells only and isotype were used as negative controls to indicate the background staining that occurred in the absence of specific antibody-antigen binding. CA125 was used as a comparator to SSX2A and SSX2 immunolabelling as it is the current NHS "gold standard" indicator for the presence of OVC cells. Expression of SSX2A is seen in the early stages of OVC and is more convincing than CA125. WTI, HE4 and SSX2 (labelled by pSSX2(N) antibody) are also expressed but not as intensely as SSX2A. Magnification was 400x except for immunolabelling of small areas of tissue with the SSX2A shown in black boxes and enlarged to aid visualisation of the immunolabelling. Images represent results from a single experiment in which MTAs which included XX stage I OVC samples, immunolabelled with each antibody or control.

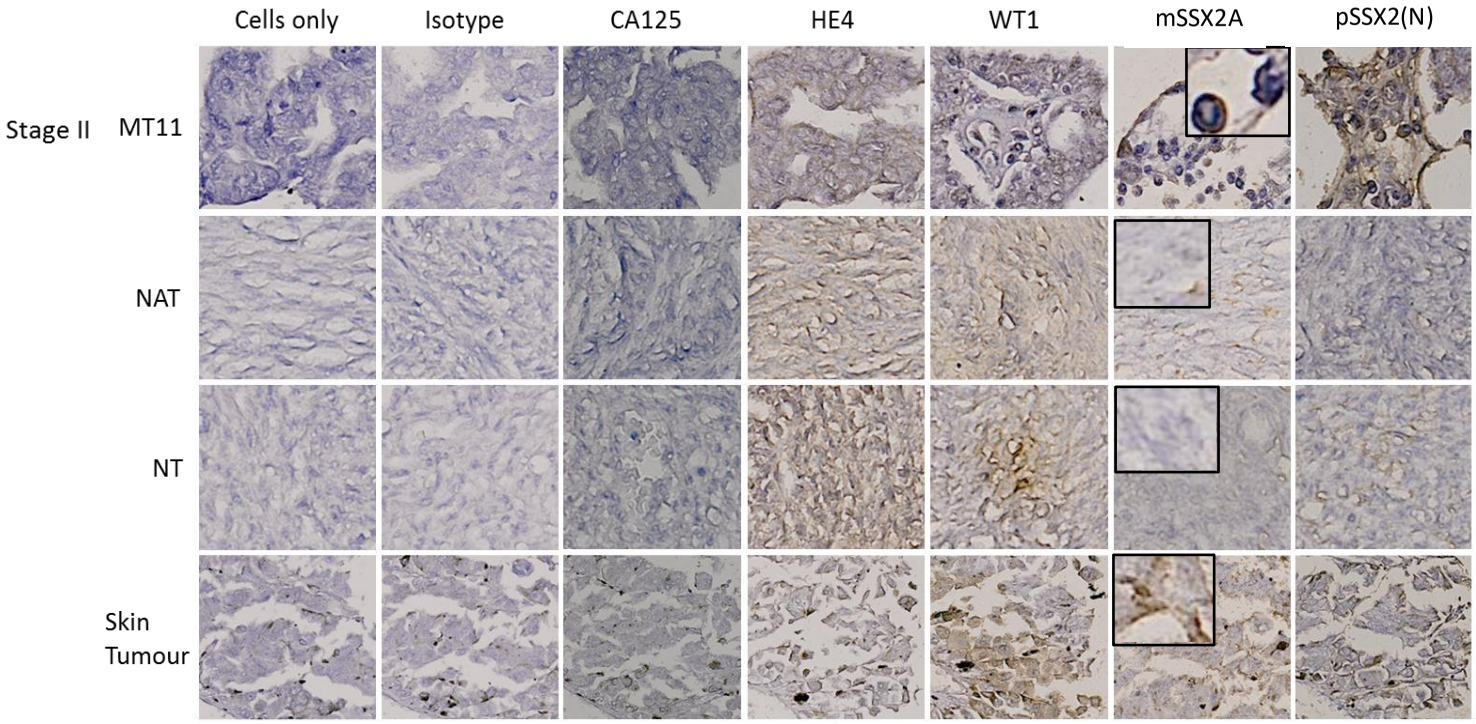
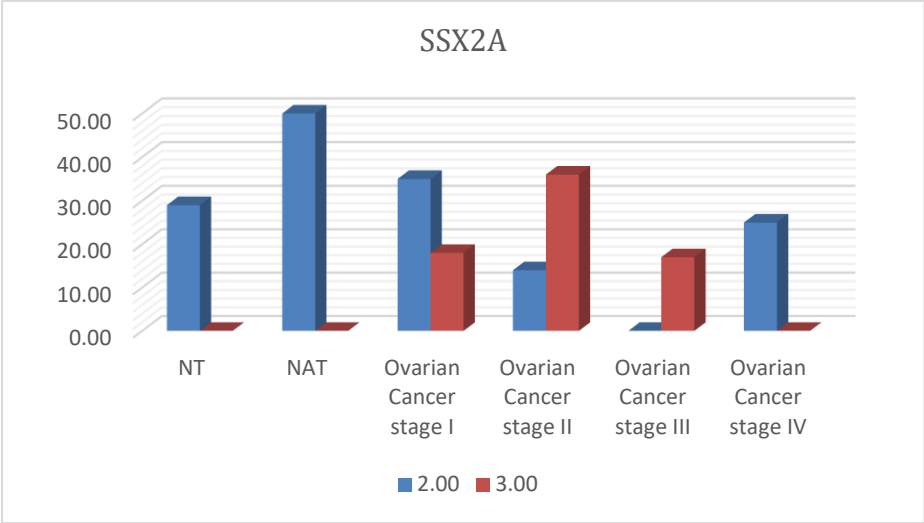
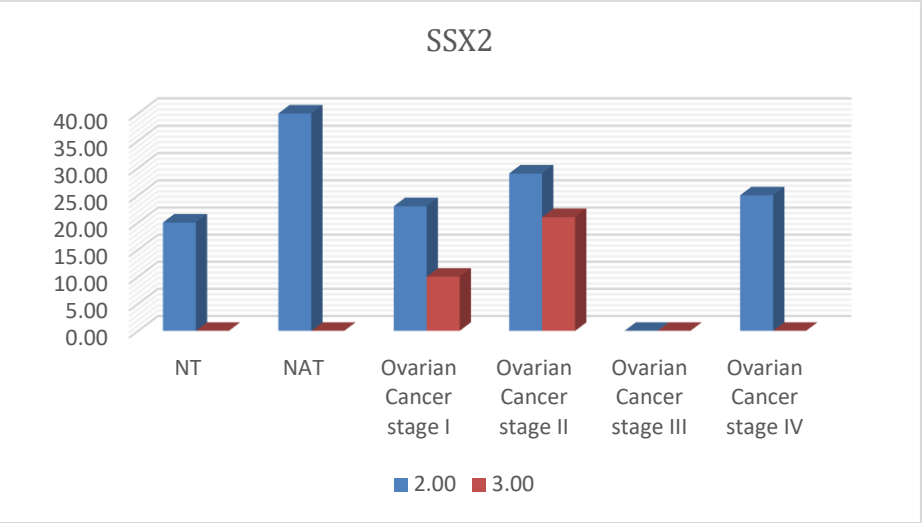


Figure 5.9 Images of the IHC staining of stage II OVC tissues, NAT and NT samples. SSX2 was shown to have superior expression at stage II of OVC than CA125, HE4 and WT1. There was some staining seen in NAT and NT samples for all tested antigens except CA125. A skin tumour samples was included on the TMAs and was used as a positive control for the expression of a number of TAAs including HE4, SSX2, SSX2A and WT1. The data is representative of one experiment on 14 tissues from stage II, NAT (n=8) and NT (n=7). Magnification was x400.

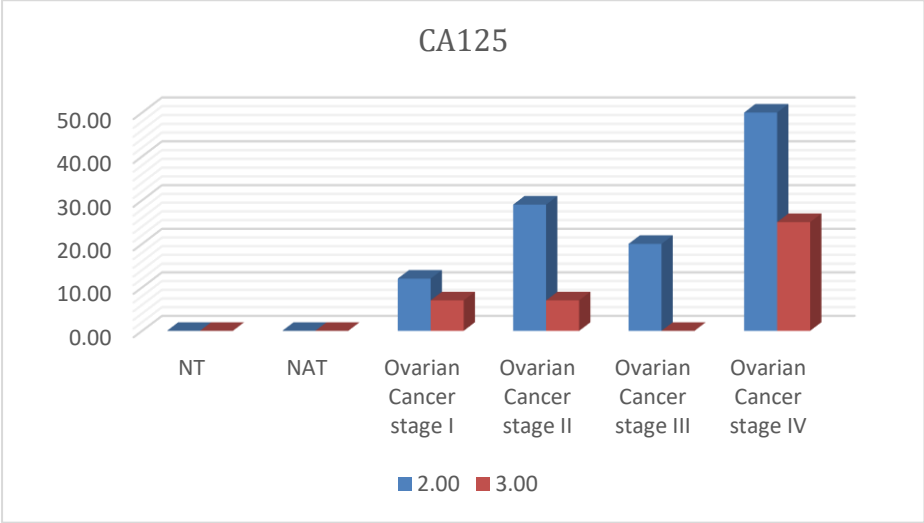
(a)


SSX2A
50.00
40.00
30.00
20.00
10.00
0.00
NT
NAT
Ovarian Cancer stage I
Ovarian Cancer stage II
Ovarian Cancer stage III
Ovarian Cancer stage IV
2.00
3.00

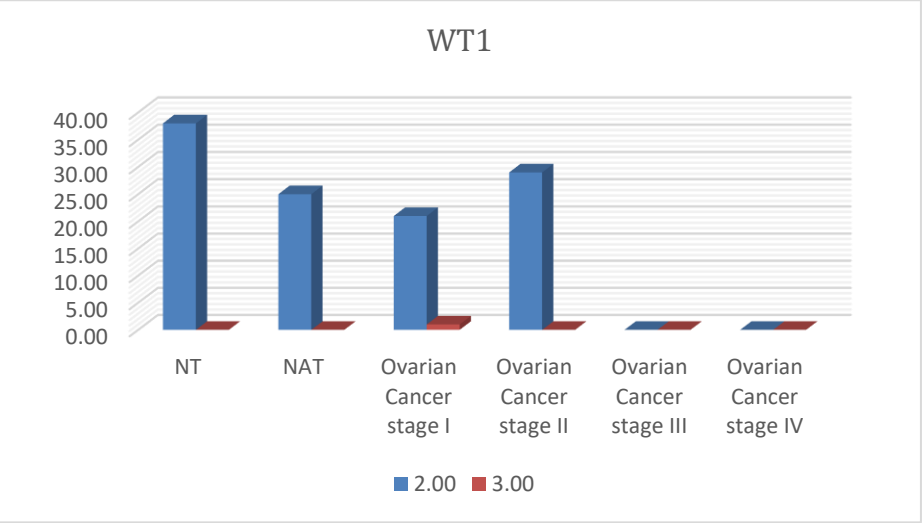
(b)


SSX2
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
NT
NAT
Ovarian Cancer stage I
Ovarian Cancer stage II
Ovarian Cancer stage III
Ovarian Cancer stage IV
2.00
3.00

(c)


CA125
50.00
40.00
30.00
20.00
10.00
0.00
NT
NAT
Ovarian Cancer stage I
Ovarian Cancer stage II
Ovarian Cancer stage III
Ovarian Cancer stage IV
2.00
3.00

(d)


WT1
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
NT
NAT
Ovarian Cancer stage I
Ovarian Cancer stage II
Ovarian Cancer stage III
Ovarian Cancer stage IV
2.00
3.00

(e)

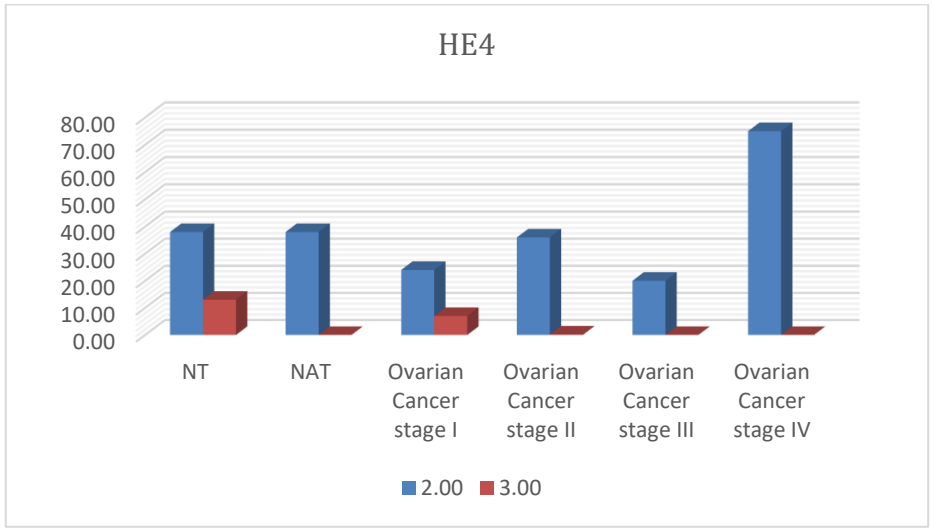
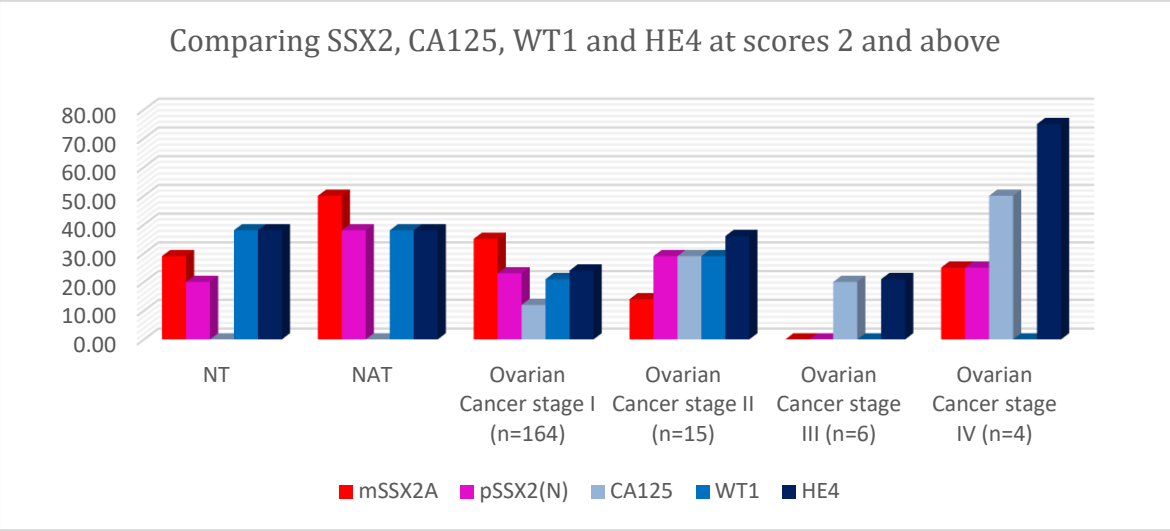


Figure 5.10 Percentage of samples which scored 2 or 3 for each biomarkers being investigated. (a) SSX2A, staining is seen at score 2 in every stage of OVC except for stage III. Immunolabelling is seen in normal tissues at the score of 2, while SSX2A is the only biomarker labelling at the higher intensity score of 3 solely in OVC at the earlier stages, with no normal tissue staining, (b) SSX2 staining is also observed in all stages including NT and NAT at score 2 however at score 3 it is only expressed in stages I and II with no expression in normal tissue, (c) CA125 is not found to be in normal tissues but is found at all stages of OVC however at the higher intensity score of 3 it has very weak expression in stages I and II with a slight improvement at stage IV, (d) WT1 was labelled highly in normal tissues and at lower levels at stages I and II but very little expression is found at score 3 and (e) HE4 has similar expression in normal tissues, stage I, II and III but is seen highly labelled at stage IV. Very little expression is found at score 3.

(a)



(b)

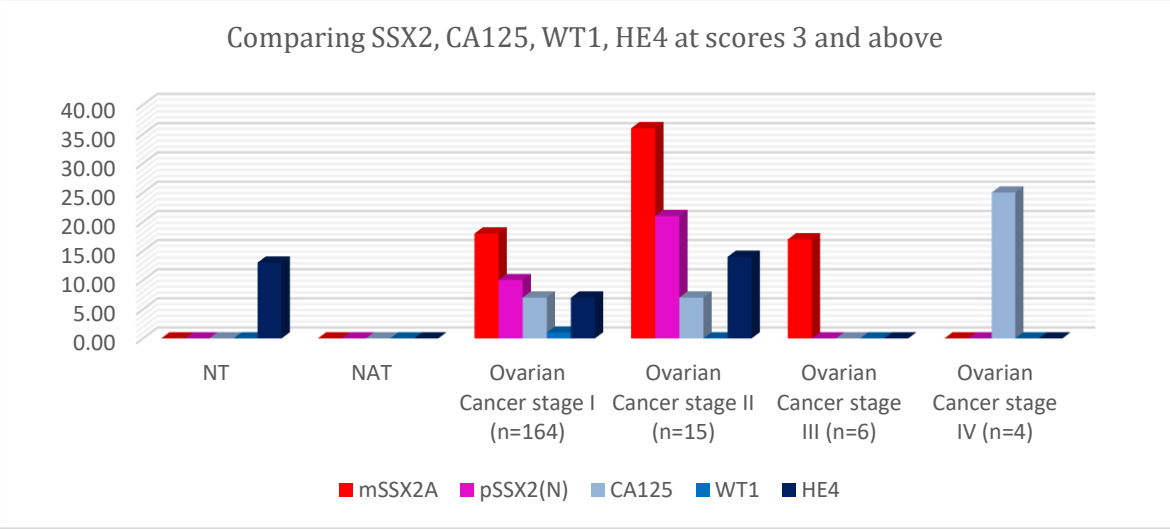


Figure 5.11 Percentage expression of SSX2, SSX2A, CA125, WT1 and HE4 at scores of (a) 2 and above and (b) 3 and above. Higher expression is seen with SSX2A than the other biomarkers in stage I OVC samples at scores of 2 and above. However there is labelling of NT and NAT by all biomarkers except CA125. At scores of 3 and above SSX2A labelling exceeds that of others at stages I and II with NT and NAT showing no staining apart for HE4.

5.3.6 Comparison of SSX2A expression between serous and non-serous OVC

All subtypes of ovarian tumours can be classified into serous and non-serous subtypes. More of the non-serous subtypes are early stage. SSX2A was seen to be expressed uniformly in all types of early stage OVC (76 - 77 %) (**Table 5.1**) whereas CA125, HE4 and WT1 were slightly more likely to be expressed in serous subtypes of OVC, at frequencies of 56 %, 57 % and 58 % respectively, when compared to non-serous subtypes (42 %, 49 % and 49 %, respectively).

Table 5.1 Number of positively scoring OVC samples differentiated into serous and non-serous tumour types.

Total positive OVC samples by immunolabelling			
	Non-serous	Serous	P value*
CA125	23/55 (42 %)	76/136 (56 %)	0.0782
HE4	27/55 (49 %)	78/136 (57 %)	0.299
WT1	27/55 (49 %)	79/136 (58 %)	0.257
SSX2A	42/55 (76 %)	105/136 (77 %)	0.9

*P value is the result of a paired t-test.

5.3.7 Statistical analysis to determine whether SSX2A immunolabelling occurs significantly more frequently than labelling with other known biomarkers

A paired T-test was used to analyse the results from the patient samples analysed in Section 5.3.2. Early stage samples (stage I) which scored 2 and above were compared for each antigen **(Table 5.2)** however the data was not found to be statistically significant as p values were all higher than 0.05. Samples that scored 2 or above when immunolabelled with each antigen were also compared between patients with serous cancer (n=136) with those with non-serous cancer (n=55). There was a significant difference between the expression of SSX2A and all other markers (CA125, HE4 and WT1) in serous and non-serous OVC **(Table**

5.3). In contrast, no difference was observed when comparing antigen expression between CA125, HE4 and WT1 in serous or non-serous samples.

Table 5.2 Statistical analysis showing the difference (p values) between the scores of SSX2, SSX2A, CA125, WT1 and HE4 in early stage OVC samples (stage I). No data extended to a significant level of $p<0.05$. P values were generated by a paired t-test.

	SSX2A	SSX2	CA125	WT1	HE4
SSX2A	-	0.095254	0.122898	0.531248	0.115526
SSX2	-	-	0.5	0.152234	0.5
CA125	-	-	-	0.318755	0.5
WT1	-	-	-	-	0.28258
HE4	-	-	-	-	-

Table 5.3 Comparisons of the expression of CA125, HE4, WT1 and SSX2A in (a) non-serous and b) serous OVC samples. P values are achieved through the use of a paired t-test.

a

Non-serous	CA125	HE4	WT1	SSX2A
CA125	-	0.444	0.444	0.000023
HE4		-	1	0.003098
WT1			-	0.003098
SSX2A				-

b

Serous	CA125	HE4	WT1	SSX2A
CA125	-	0.807	0.713	0.00019
HE4		-	0.902	0.000484
WT1			-	0.000752
SSX2A				-

Data highlighted in yellow has reached statistical significance.

5.4 Discussion

The biomarkers which have been identified to date (**Section 1.10.1**) show limitations in their usefulness caused by imperfect specificity or reproducibility with regards to the immunolabelling of OVC samples. Most biomarkers work best in combination, such as HE4 and CA125, and therefore I had hoped to identify one marker that could indicate the presence of OVC cells in tissues. This would be economical, require less controls and be potentially more sensitive as there is less risk of false negative results through background/non-specific staining. Treatment is known to be most effective when OVC is diagnosed early, and so the ideal biomarker would also be one that recognised OVC in the early stages, especially as this correlates very significantly with early interventions and long term survival.

It is widely accepted that CA125 is not a very good biomarker for OVC (**Section 1.9.1.1**) therefore for an antigen to be better than CA125 does not equate with it being satisfactory and I wanted to find a biomarker that is an improvement compared to current best practise for the confirmation of an OVC diagnosis, such as HE4 and WT1. If I was able to identify a more robust biomarker (with regards to sensitivity and specificity) there is an increased likelihood it would reach the clinic and impact on patient care. However it is acknowledged that in the absence of a better biomarker CA125 needs to be a reference point.

My data suggested that SSX2 may have potential as a single early stage biomarker for OVC. SSX2 immunolabelling was found at the highest frequency with high intensity (score 3 and above) in early stage OVC patient samples. This contrasted starkly with the current “gold standard” biomarker used to confirm a diagnosis of OVC, CA125. pSSX2 (N) antibody was able to widely label OVC, however it was also detected at low levels in endometrial cancer. As a polyclonal antibody it is prone to non-specific binding especially on tissues and so some level of non-specific/background staining was expected. The specificity of the immunolabelling by pSSX2A will need to be interpreted by a pathologist to determine

whether the staining in endometrial tissue was specific or non-specific. However mSSX2A was found to immunolabel almost as many samples as the pSSX2A antibody, with little immunolabelling in endometrial tissues, showing it to be a specific and sensitive detector of OVC. Since both of these antibodies were blocked by the SSX2 peptide on cell lines, mSSX2A was taken as the most appropriate for further studies. In NAT and NT samples some expression was seen with all antibodies (HE4, WT1, pSSX2 (N), mSSX2A and pSSX2A) with the exception of CA125. CA125 for the main part seems undetectable in normal ovarian epithelium (Kobayashi et al, 1993; De los Frailes et al, 1993) however due to the inconsistent nature of CA125 some studies have found expression (reviewed in Felder et al, 2014). Out of the three SSX2 antibodies, the highest scores in terms of immunolabelling OVC samples were observed with the two polyclonal antibodies (pSSX2A and pSSX2 (N)). As previously described, the staining seen in normal tissue did not appear to be specific. There was no detailed information about how the normal donor samples were collected so it is possible some of these samples could have in fact been pre-cancerous. All staining does require a pathologists critical and highly trained skills to establish true positivity but as a preliminary dataset the data suggests that immunolabelling SSX2 may provide a more specific and sensitive label for early stage OVC then current best practise. At the higher intensities (3 and above) mSSX2A labelling was found to be elevated compared to the other biomarkers at OVC stages I-III. This indicates that SSX2A could detect early stage OVC cells more accurately. There is some evidence that CTAs may be detectable in pre-cancerous cells (Liggins et al., 2004a) which may otherwise seem healthy, therefore the TMAs should be examined by a pathologist to determine whether the tissues labelled normal adjacent and normal tissues were indeed healthy and not in fact early stage pre-cancerous. CA125 is already known to have a variable expression in OVC samples and alters with the stage of EOC. It can be detected in 80 - 90 % of late stage OVC but only about 50 % of early stage

EOC may express CA125 (Jacobs and Menon, 2004). It has previously been shown that the presentation of advanced stage disease is more likely to be of the serous subtype as 82 % of stage III patients are serous while non-serous subtype are more likely to occur in early stage (over 90 % of cases) (Gilks et al, 2008) thus taking subtypes into consideration when looking at biomarker expression is very important. SSX2A expression was found to be consistent in both non-serous and serous OVC. However CA125, HE4 and WT1 were found to be more frequently expressed in serous concurring with previous data where 75 % of serous carcinoma cases were positive for CA125 and WT1 (Drapkin et al, 2005; Kobel et al, 2008). This implies that SSX2A may be an important biomarker for both the serous and non-serous types of early stage OVC, and SSX2 has previously been suggested to play a role in the development of tumours (Tureci et al, 1996) making it a particularly appealing antigen to act as a target for immunotherapy (Cheever et al, 2009). HE4 showed no positive labelling in NT when used alongside pSSX2A antibody but higher staining was observed when used with all other possible biomarkers, suggesting that more aggressive washing could be one of the potential solutions as only four TMAs were used earlier while seven were used at once the second time around. Ovarian cancer tissues however were labelled similarly both times.

To further establish whether SSX2A would be specific to OVC as a diagnostic biomarker, immunolabelling of SSX2A was carried out on endometrial cancer TMAs. Endometrial tumours showed expression of CA125, HE4 and SSX2A (when immunolabelled with pSSX2A) but very little immunolabelling of SSX2A with mSSX2A. I believe that the expression seen with pSSX2A was due to it being polyclonal and more likely to bind tissues non-specifically. SSX2, also referred to as HOM-MEL-40 (Tureci et al, 1996), has already been examined in a range of healthy tissues including the ovary, colon and breast. No expression of SSX2 has been found except in the testis with weak expression in the thyroid gland (Tureci et al, 1996) making this a particularly promising biomarker if background

levels can be shown to be low in NT and NAT, and a very specific target for immunotherapy. Although SSX2 expression in thyroid tissue is present, albeit at low levels, autoimmunity that could destroy the thyroid following immunotherapy targeting SSX2, can be controlled for, and would be balanced out by the removal of OVC cells that could lead to patient death.

Despite its use as a biomarker for the diagnosis of OVC, CA125 is not very specific to OVC and can be detected in a number of benign and malignant conditions such as menstruation, pregnancy, benign pelvic tumours, pelvic inflammatory diseases and peritonitis (Daoud and Bodor, 1991). However recent studies in which the levels of 14 currently promising OVC-related biomarkers, including CA125 and SSX protein, were measured through detectable antibodies in the plasma of 151 OVC patients, 23 with borderline ovarian tumours, 55 with benign tumours and 75 healthy controls and showed that CA125 exhibited the greatest power to discriminate the plasma samples of type II cancer patients from normal volunteers. The potential of CA125 to act as a biomarker for stage II OVC is improved when it is used in combination with the detection of auto-antibodies to p53 (Lu et al, 2011). HE4 and WT1 have more recently been identified as possible biomarkers in OVC however they also work best as part of panels (Van Gorp et al, 2011) and are more frequently expressed in the advanced stage disease (Olivier et al, 2006). Median CA125 and HE4 levels were elevated in stage III and IV endometrial tumours ($p<0.001$) (Brennan et al, 2014). WT1 has been known to be an oncogene and highly expressed in a number of leukaemia types; it was expressed in 7/16 cases of ALL, 15/22 of AML and 8/10 of blast crisis CML (Miwa et al, 1992). CA125 has been shown to be sensitive in the detection of more than 90% of cases of OVC recurrence post-chemotherapy leading the US Food and Drug Administration (FDA) to endorse its use to determine residual disease (Simmons et al, 2013).

Statistical analysis showed a difference in the expression of SSX2A and other antigens tested between serous and non-serous OVC. When looking at non-serous OVC samples, there was a

statistically significant different between SSX2A and CA125 (p= 0.000023), HE4 (p= 0.003098) and WT1 (p= 0.003098). Similarly in serous samples the same trend is observed between SSX2A (when immunolabelled with mSSX2A) and CA125 (p= 0.00019), HE4 p= 0.000484) and WT1 (p= 0.000752). This implies that SSX2A offers an improvement on the current biomarkers, could be used to confirm an OVC diagnosis in the early stages of OVC and is not limited to serous or non-serous types.

SSX2A expression was higher when considering scores of highest intensity in OVC samples at every stage compared with CA125 and PASD1. The expression of SSX2A was not more predominant in serous compared with non-serous OVC. This contrasts with CA125, PASD1 and SSX2IP both in expression frequency, levels (2 and above) and the predominant expression in early stage disease. SSX2 has great potential as a target for cancer immunotherapy due to its previous findings of restricted expression in healthy tissues and frequent expression in early stage OVC. SSX2 epitopes have already been identified and investigated in melanoma, HCC, and breast cancer (reviewed in **Section 1.7.4.1**) but its' possible role as a biomarker for OVC has yet to be explored however that may be change.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The aim of this study was to identify and characterise antigens that were frequently expressed in two cancers that the research team I worked with and I were interested in (myeloid leukaemia and OVC). Frequently expressed antigens may provide novel targets for immunotherapy, if their expression is limited to cancer cells and immunologically protected sites. By virtue of their restricted expression some antigens have also been found to have a dual role as biomarkers for disease and in this regard I identified a CTA that was frequently expressed in the early stages of OVC. My studies have successfully identified targets for immunotherapy in leukaemia and OVC (Chapter 3 and 4) and an antigen that may act as a diagnostic biomarker for OVC (Chapter 5).

PASD1 was found to be present in leukaemia cells which correlated with the presence of PASD1-specific T cells being detected in the periphery of the same patients using the pMHC array.

With the help of Professor Ken Mills, I investigated whether patients who had PASD1 transcripts as detected by RT-PCR and PASD1-specific T cells as detected by pMHC array had longer survival rates however there was no significant correlation although the number of samples analysed was very low. To progress this study I would investigate a much larger population of AML patients in order to determine if there was a correlation between PASD1 transcripts and/or the presence of PASD1-specific T cells and survival. Further statistical analysis does not provide any more information since the number of patients was so low and contradictory conclusions are obtained, for example Kaplan Mieier curves show that having detectable PASD1 transcripts improves EFS but OS is found to be greatly reduced. This could be because the PASD1 transcipts detected were not the correct size and would therefore require sequencing which could not be carried out due to the small amount of product detected. This could also mean that PASD1 may play a dual role where it prevents or deters cancer early on but then encourages cancer progression at a late stage such as TGFβ (Lebrun, 2012).

I found some data which suggested that the presence of PASD1 is linked with normal karyotype which is supported by previous studies (Baghdady et al, 2013). Again a larger number of patient's samples will be required to investigate this further. This could be achieved through the analysis of data from studies by Guinn et al, 2015, Baghdady et al, 2013 and chapter 4 in this thesis. Microarray data which examined a correlation between the levels of PASD1 expression (above and below median) with survival and/or cytogenetic abnormalities (or not) could provide insight into the role of PASD1 in AML. Similar studies with SSX2IP elucidated its role in mitosis (Denniss et al, 2007).

The transcripts obtained for PASD1 were of a much larger product size than expected from the primers and our knowledge of the intron-inton boundaries that these span. There is evidence that more that the two PASD1 transcripts described to date (Liggins et al, 2004a) do exist (Cooper et al, 2006). As the product initially did not seem correct it was not considered for sequencing but using newly acquired AML patient samples would allow the same RT-PCR analysis to be performed and the PASD1 products sequenced to determine whether these are the products of novel PASD1 transcripts could be informative.

The pMHC array technique (Soen et al, 2003b) enables analyses of specific T cell populations by virtue of their recognition of pMHCs. This analysis allows the simultaneous analysis of many T cell populations thereby showing if a patient's immune system has the correct armoury to target an expressed TAA. Bonney et al demonstrated that pMHC arrays are capable of detecting around 40 independent but TAA specific-$CD8^+$ T cells and could identify up to 40 specific T cell populations in a small sample size without haplotype restriction. pMHC arrays can be used to look for longer and overlapping epitopes and other antigens important in solid tumours as well. This information can lead to targeted immunotherapeutic treatment which may be more effective at breaking tolerance and instigating an effective T cell response. In addition my group hope to examine whether the presence of specific T cell populations can predict response to chemotherapy in terms of survival and achievement of first remission. In addition the pMHC array can be used to follow

specific T cell populations and determine which immunotherapy strategies should be used (i.e. which antigens/epitopes therein should be targeted). The group would like to extend the pMHC array used in the studies described in chapter 4 to encompass a wider range of class I and potentially class II epitopes, and develop solid tumour specific pMHC arrays in the future. The pMHC array is restricted only by the pMHCs available already.

The pMHC array (Soen et al, 2003) only measures presence of antigen-specific T cells and does not measure T cell functionality. However the pMHC array does allow the analysis of a small number of negatively purified $CD8^+$ T cells (from 20 ml of peripheral blood) that are "untouched". This enables the short-listing of which antigen-specific T cell populations are present in patients and enable their further analysis through sampling of an additional volume of blood. Such experiments could then include intracellular cytokine assays, ELISAs, ELISpot assays and CTL assays to further assess T cell functionality in a limited number of relevant T cell populations. Previously Vergauwen (2011) tried to detect which cytokines were being secreted by T cells bound to pMHCs on the array, however unlike other groups (Chen et al, 2005), Vergauwen did not find this to be possible. The Davis group are now focussing on using flow cytomtery based methods, rather than pMHC arrays, with a number of investigators describing difficulties with the background sticking of T cells to the pMHC array.

Assays such as the combinatorial approach described by Hadrup et al, 2009, again detects specific T cell populations but not functionality. However flow cytometry based methods can be adapted to examine T cell responses and cytokine secretion, indicating the functionality of the T cells. To prevent the cytokine from exiting the cell a transport inhibitor is added e.g. brefeldin A. The cells are then fixed in paraformaldehyde and permeabilized to allow the anti-cytokine antibody to bind. The use of an intracellular cytokine staining assay to detect the cytokine IFNγ shows high reproducibility and linearity with little background (Flesch et al, 2012). Duration of culture prior to antigen stimulation, as well as the cytokine accumulation period, are the critical parameters of these

methods. In both murine and cattle models, following 2-6 hours in culture, T cells are shown to produce a mixture of cytokines IFNγ, IL-2 and tumour necrosis factor-α, however following 6-16 hours of culture only IFN-γ cytokine was found (Kaveh et al, 2012).

Other assays that can detected T cell responses to antigen include ELISpot assays where T cells are plated with the antigen and the production of cytokine is measured through cytokine production.

When considering the best method for immunotherapy the use of multiple peptides from distinct TAAs to stimulate immune cells have been shown to be very effective. A cocktail of four multiple myeloma antigen peptides were used to stimulate T lymphocytes from HLA-A2 positive people induced IFNγ production, cell proliferation and cytotoxicity against HLA-A2 positive multiple myeloma patients' cells (Bae et al, 2012). Indeed long peptides may offer the advantage of allowing the immune system to choose the epitope(s) it can best process and present from a peptide sequence and induce an effective cytotoxic T cell response in the presence of longer $CD4^+$ helper motifs (Zwaveling et al, 2002). Conversely sometimes longer proteins can inhibit $CD8^+$ T cells responses (Rice et al, 2002) but this may vary depending on the constituents of individual protein sequences.

PASD1 remains a promising target for immunotherapy approaches especially in haematological malignancies. The work in chapter 4 supports previous evidence that patient leukaemia cells express PASD1 and our colleagues have shown that AML patients have detectable $CD8^+$ T cells specific to PASD1 epitopes. In some cases there may not be PASD1-specific T cells present even though PASD1 seems to be expressed by RT-PCR, however it is possible that there are more epitopes of PASD1 yet to be identified, that a different isoform of PASD1 is playing a role in tumour development or that the PASD1 mRNA may not be translated into protein in these patients. I found PASD1 protein expression varied from 4 – 43 % of the cells in each of the AML patients tested and this is consistent with previous studies (Hardwick et al, 2013) and likely reflects PASD1 function in circadian rhythms (Michael et al, 2015). The frequency of PASD1 expression in OVC was found to be varied. Variation in frequencies within the same cancer may also occur due to

differences in primers, PCR cycles and machines or patient selection (i.e. patient numbers, geography, age, disease stage, underlying factors). For example SSX2 expression frequencies have been found to range from 10-46.7% in HCC (Chen et al, 2001a; Luo et al, 2002; Peng et al, 2005; Tureci et al., 1996) and 2-25% in colon cancer (Mashino et al, 2001; Tureci et al, 1998; Tureci et al, 1996). Screening peptide libraries would likely identify additional PASD1 targets for immunotherapy.

Recently PASD1 been found to suppress circadian rhythms (Michael et al, 2015) implying it will not be expressed in all cells at all times. It would be of interest to investigate this further. Investigating PASD1 at various time points would give a better understanding of when PASD1 is detected in the cell cycle and antigen specific treatments can be applied at the optimal time in the future to be more effective.

My failure to find PASD1 in OVC adds to a growing body of evidence which suggests that unlike many other CT antigens, PASD1 is found predominantly in haematological malignancies. Further studies which also find PASD1 is not expressed in solid tumours may not be published as null data is invariably not. However to date PASD1 expression has not been found in bladder cancer (G-Y Chen, CRI annual meeting, 2011), basal cell carcinoma (Ghafouri-Fard et al, 2010) and now in OVC (Khan et al, 2015).

SSX2IP expression previously shown to interact with SSX2 but this does not seem to be the case on OVC. This may reflect the fact that when first identified a YAC system was used and only part of SSX to identify its interacting partner. SSX2IP expression has been described in leukaemia (Guinn et al, 2005) but was not found to be expressed in OVC in my study.

OVC effects increasing number of people every year, however it is very difficult to diagnose. Early diagnosis significantly increases the chance of survival, but the majority of cases are diagnosed at a late stage. Therefore identifying novel biomarkers is imperative to improve survival rates. To that end, using OVC TMAs, I have identified a potential early stage biomarker for OVC named SSX2,

more specifically the variant SSX2A. Further work needs to be carried out in order to establish the specificity of the SSX2 antibodies for early stage OVC and why there was so much background staining of NT and NAT. Although I did my best to analyse the staining patterns, the stained TMAs should be analysed by a qualified pathologist to determine why there was such high levels of background staining of the NT and NAT. I believe the staining of normal tissue was not as specific as the immunolabelling of tumour tissue and a pathologist would be able to confirm this belief. Also the ICC staining was only analysed by looking down a light microscope to see where the antigen location appeared to be i.e surface, nuclear etc but further techniques can be applied such as fluorescence microscopy for endorsement. Further studies on samples from patients attending local hospitals would provide tissues from gynaecological cancers, normal/healthy tissues and disease (endometriosis and inflammation) allowing the optimisation of the immunolabelling techniques on locally handled frozen tissues aiding our interpretation of results through help from clinical colleagues. This may also help by allowing the reduction in background staining of healthy tissues as the samples would be processed and prepared by the team rather than a company in the US.

Further work should be carried out on SSX2 such as plasmid constructs can be used to further investigate the effect of SSX2, SSX2A and other isoforms expression on transformed cells. Clones can be isolated with varying levels of mRNA expression and investigated to determine the effect of SSX2 protein expression on transformed cells. Similar studies have been performed to investigate the function of PASD1 (Liggins et al, 2004a) SSX2 (Abate-Daga et al, 2014). Differing levels of SSX2 and knockdown studies would show us how the cells are affected and the possible function of the antigen that can be exploited in the future. Knock out of SSX2 with siRNA can show impact on cell behaviour. Mass spec quantitative analyses can be used to detect SSX2 in OVC patient's samples.

There is some evidence that SSX2 protein may be secreted into urine, due to its small size, and if this is the case it would make an easily detected biomarker for early OVC diagnosis, using lateral

flow assays in well-woman clinics. SSX2 is expressed in Pichia pastoris as a means to produce a delayed-type hypersensitivity skin test reagent for monitoring SSX2-specific anti-cancer immune responses. SSX2 was detected intracellularly in P. pastoris despite the addition of the Saccharomyces cerevisiae alpha-mating factor secretion signal. Increasing the SSX2 gene copy number did not improve its secretion but did enhance intracellular SSX2 levels. SSX2 with its C-terminal nuclear localization signal (NLS) deleted (SSX2NORD), however, was secreted. Indirect immunofluorescence indicated that SSX2 containing the NLS did not translocate to the nucleus but accumulated in the endoplasmic reticulum (ER). Experimental results further suggested that SSX2 containing the NLS was misfolded in the ER, while deletion of the NLS facilitated correct folding of SSX2 inside the ER and improved its secretion. Production of SSX2NORD was scaled-up to a 2 L fermentor using a fed-batch protocol to maintain methanol at a concentration of 1 g/L. Decreasing the cultivation temperature from 25 °C to 16 °C improved protein stability in the culture supernatant. In this process, after 120 h cultivation, the wet cell weight of P. pastoris reached 280 mg/ml, and the yield of SSX2NORD was 21.6 mg/l (Huang et al, 2010).

To determine whether I could detect SSX2 and SSX2A in urine I could perform a sandwich ELISA. This would allow me to quantitate the amount of SSX2 in the urine. I would need to analyse the presence of SSX2 secretion in age and sex-matched healthy donor urine to ascertain baseline levels and how they are affected by time of day, sample collection and storage. To this end we have already obtained UREC approval and collected 48 healthy donor urine samples (Andrew Mead, personal communication), predominantly from women over 40 years of age, which were aliquoted and stored in -80 ^{o}C. Dr Guinn has recently started a collaboration with Professor Anthony Maraveyas, Hull and East Yorkshire Hospitals NHS Trust, which would enable the collection of OVC patient samples for the detection and characterisation of TAAs in body fluids and tissues. Recent studies have demonstrated that HE4 is secreted into the urine of OVC patients (Liao et al, 2015) and it would be interesting to determine whether this form of detection has circumvented

issues with HE4 and its detection in other diseases such as endometriosis which makes it less specific for OVC. I would be interested in developing a lateral flow assay that could be used to detect SSX2 in patient urine as a screening assay, ideally for us in GP-led well woman clinics for people aged over 50.

Three SSX2 antibodies were used to investigate SSX2 and SSX2A expression and its' potential as a biomarker in OVC. All were able to immunolabel OVC patient samples in MTAs and provide us with a better understanding as to whether SSX2A could be a biomarker. Data also showed some staining in NT and NAT with mSSX2A (Chapter 4 and 5) and pSSX2(N) (Chapter 5). This contrasts with what I had expected from the existing published literature but could be explained as follows. It has been previously reported that some CTAs have been found to be present in normal adjacent tissue, such as PASD1, which has been detected in premalignant surrounding cells which may at first appear normal (Cooper et al, 2006). In previous studies SSX2 has been notably absent from healthy tissues except in the testes (Tureci et al, 1996) which is why it has been named a CTA. However Tureci et al, looked for transcripts whereas I was looking for protein since that would be more important to its role as a biomarker. It is also possible the healthy tissues were not as healthy as hoped and in fact were premalignant, depending on how and from where they were collected. mSSX2A antibody did not label the endometrial cancer samples while pSSX2A did and this could be because polyclonal antibodies tend to bind multiple epitopes and are not generally as specific as monoclonal antibodies which only bind to one epitope leading to a "dirtier" staining pattern.

An alternative option, to get away from the issues around using IHC in PPFE tissue arrays would be to identify novel urine proteins that can differentiate early stage OVC samples from healthy donor sera using protein arrays or 2D gel electrophoresis. These proteins would need to be validated for their potential as biomarkers as described already (**Section 1.10.3**).

Usually investigators look for antibodies against the antigen of interest in patient sera. SSX2-specific antibodies have been shown to activate complement and increase the CTA uptake by

antigen presenting cells. SSX2-specific antibodies were mainly confined to the subclass IgG3 (Luetkens et al, 2014). Sahin et al (Sahin et al, 1995) identified the HOM-MEL-40 antigen by SEREX immunoscreening of a melanoma cDNA library with autologous sera. In 194 MM plasma samples, 3.1% of patients were determined to have SSX2 specific antibodies. Anti-SSX2 antibodies have also been found in 11% of melanoma patients (Tureci et al, 1996) and 3% of colon cancer patients (Scanlan et al, 2002). Aberrant expression of the combined SSX antigens (SSX1, SSX2, and SSX4) was found in 31/120 (26%) of ovarian tumours (Valmori et al, 2006). Sera from a subgroup of the patients were tested for SSX2 and SSX4 antibody by ELISA and recombinant antigen expression on yeast surface (RAYS). Antibodies to SSX2 and SSX4 were detectable in two patients (2%) (Valmori et al, 2006). Another study of 151 OVC patients plasma showed that there were no common SSX antibodies but this may reflect the use of 293T cells to produce tagged-SSX2 protein for the study (Lu et al, 2011). However Taylor et al found immune reactions against nucelophosmin, cathepsin D, p53 and SSX common antigen at all stages of OVC which were higher than benign disease and healthy controls (Taylor et al, 2009).

SSX2 epitopes have been identified as part of a strategy for targeting cancer cells that present SSX2, using immunotherapy. In metastatic melanoma the peptide SSX2 (41-49) was identified as an HLA-A2-restricted epitope. $CD8^+$ T cells specific for SSX2 (41-49) were present in the tumour-infiltrated lymph node population by multimer staining, and isolated CTL clones were able to lyse HLA-$A2^+$ tumour cells expressing SSX2 (Ayyoub et al, 2002). SSX2–derived T cell epitope, mapping to the 37–58 region and surrounding the SSX2 41–49 epitope, was recognized by $CD4^+$ T cells from melanoma patients (Ayyoub et al, 2004; Abate-Daga et al, 2014).

In a HCC patient, SSX2-specific $CD8^+$ T cells were detected in tumour infiltrating lymphocytes but not in normal lymphocytes of patient and in peripheral blood mononuclear cell samples taken on the day of surgery (Bricard et al, 2005). In two of six HLA-$A2^+$ HCC patients, it was found that MAGE-A10- and/or SSX2-specific $CD8^+$ T cells naturally responded to the disease, because they

were enriched in tumour lesions but not in non-tumoral liver (Bricard et al, 2005). Isolated T cells specifically and highly killed tumour cells *in vitro*, providing evidence that these CTL were selected *in vivo* for high avidity Ag recognition. Therefore, besides melanoma, HCC is the second solid human tumour with clear evidence for *in vivo* tumour recognition by T cells, providing the rational for specific immunotherapy, based on immunization with CT Ags such as MAGE-A10 and SSX2.

SSX4-specific $CD4^+$ T cells which were found to recognise two novel SSX4-derived T-cell epitopes in association with HLA-DR (human leukocyte antigen) were identified (Valmori et al, 2006). Smith et al (Smith et al, 2011) analysed a single HLA-A2–restricted epitope, SSX2 p103-111 RLQGISPKI (Gure et al, 2002) with a SYFPEITHI score of 23 and a BIMAS score of 10.433.

Using the SYFPEITHI algorithm Wagner et al (Wagner et al, 2003a) identified a HOM-MEL-40/SSX2-derived epitope with high binding affinity for HLA-A*0201. Stimulation with p103-111 induced HOM-MEL-40-specific CTLs in 5/7 patients with HOM-MEL-40/SSX2 positive breast cancers and in 6/11 healthy controls. HLA-A*0201 specificity for p103-111 was shown by blocking with specific antibodies. Prestimulated p103-111 specific $CD8^+$ T cells reacted with SSX2-transfected COS7/A2 cells as well as with the HLA-A*0201 positive cell line SK-MEL-37 that is known to express HOM-MEL-40/SSX2 but not with the negative controls. p103-111 peptide vaccine could be applied to a large number of cancers which are HOM-MEL-40/SSX2 (Wagner et al, 2003a).

Side population (SP) cells are progenitor cells from normal and malignant tissues which have increased resistance to chemotherapy and radiotherapy. Hodgkin lymphoma (HL) SP cells expressed higher levels of the TAAs MAGEA4, SSX2, survivin, and NY-ESO-1, which allowed them to be specifically recognized and killed by TAA-specific cytotoxic T lymphocytes (Shafer et al, 2010). The expression of SSX2 in SP cells suggests it may be expressed in tumour stem cells and this should be explored further in OVC.

A DNA vaccine encoding altered peptide ligand (APL) in which the anchor residues of the p41-49 and p103-111 epitopes were changed in order to investigate how these changes effected epitope binding, generated increased numbers of $CD8^+$ T cells specific for SSX2 and producing epitope-specific Th1 cytokines (Smith et al, 2014).

Alternatively, if SSX2 and SSX2A does not provide a good target for immunotherapy I could immunoscreen testes cDNA library with sera from OVC patients, although other groups have performed SEREX on OVC patient samples (Ishida et al, 2008; Kim et al, 2012). These groups did not immunoscreen OVC sera on a healthy donor testes cDNA library which would maximise their ability to find CT antigens.

In summary, I investigated whether there is a correlation between the expression of the tumour antigen, PASD1, in leukaemia cells and antigen-specific T cell responses in the periphery (**Aim 1; Chapter 3**) and found that the presence of PASD1-specific T cells in the periphery closely correlated with PASD1 protein expression in leukaemic cells. I wanted to investigate the expression of a number of known tumour associated and cancer-testis antigens in OVC (**Aim 2; Chapter 4**) and to this end I found that PASD1 and SSX2IP were infrequently expressed in OVC but SSX2A was expressed. At a score of 2 I found expression of SSX2A expression in NT and NAT at a frequency that was almost as high as in OVC stage IV and exceded OVC stage I, II and III. However the staining of SSX2A in NT and NAT did not look specific, unlike the immunolabelling of patient samples with mSSX2A anibody and required further investigation. I obtained two additional commercially available antibodies that could bind SSX2A (pSSX2A) and the core region of SSX2 (pSSX2(N)) and used them to immunolabel OVC tissues. It was clear that the antibodies that labelled SSX2 (pSSX2(N)) and SSX2A (mSSX2A) were specific for OVC at score levels of 3 and above. To determine whether the cancer-testis antigen, SSX2 and/or SSX2A could also act as a biomarker for OVC (**Aim 3; Chapter 5**) I examined their expression in healthy and diseased endometrial tissue. I found that SSX2 and SSX2A were only expressed in OVC and hope that in the

future the expression of SSX2 in OVC can be discerned from its perceived expression in NT and NAT.

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Appendix I. Leukaemia patients information

Patient information from leukaemia patient samples used in Chapter 3.

Patient ID	Age[a]	Disease[b]	Comments	Full Haplotype	WBC count[c]	Cytogenetics	FLT3 and NPM status	Survival in months (status)	Positive for TAA-specific T cells on the pMHC array (& detection of PASD1 by ICC)
AML001	59	AML (M2)	initial chem, failed to achieve CR with induction VUD allo; ATRA chemotherapy initially, as suspicion of APML; Admitted for ADE chemotherapy x 1 cycle; Admitted for Salvage Chemotherapy : FLA-IDA Allo tx	**A*02,** B*08;40, Cw*03,07 DRB1*0401, 0301, DQB1*0301, 0201/02	30.4	Normal	FLT3 and NPM1 wt	59 (D)	-(-)
AML002	65	AML	chemotherapy x? CR; Cycle 1 & 2 of DA chemotherapy; 4 cycles of MIDAC chemotherapy; Relapsed. Treated with DA 3+10. Followed by TaNK trial at Royal Free Hospital. Further relapse.	**A2**	12.0	Not tested	NK	29 (D)	+ (ND)
AML003	48	AML	chemotherapy x4 CR; AML15 trial patient randomized to: 2 x DA cycle ; High dose Ara-C & Mylotarg: HDAC cycle 4	**A2**	2.4	46, XX, many cells showed chromosome damage, ?artefact	FLT3 wt	49 (A)	-(-)
AML004	46	AML	chemotherapy x4 CR; AML15 trial patient randomized to: 2 x DA cycle:; MACE cycle 3: MIDAC cycle 4.	**A2**	6.0	Del 7q	FLT3 wt	14 (A)	+(+)
AML005	62	AML	chemotherapy x4 CR; AML 16 patient; DA 3+10 Cycle 1; DA 3+8 Cycle 2; MIDAC (modified) Cycle 3; MIDAC (modified) cycle 4; Completed.	A3	19.2	At diagnosis: 12p minus	NK	4 (D)	-(ND)
AML006	68	AML (M4)	chemotherapy x1 ; PR ; DA 1 x cycle	ND	176.1	Normal	ND	2 (D)	-(-)
AML007	64	AML	preceding MDS (CMML); chemotherapy x1 PR; DA & Cytarabine; Palliative chemotherapy : FLA	A3	31.8	ND	NK	2 (D)	-(ND)
AML008	50	AML	HDAC chemotherapy	**A02**,02;B*07,37;Cw*06,07 DRB1*0802,1501;DQB1*0302,0602	0.6	ND	NK	42 (D)	+(+)
AML009	30	AML (M4 FAB)	3xcycles ; DA & Ara-C 2 cycles ; cycle 3 : FLA-IDA Sib-allo	A1,24;B*08,15;C*03,07 DRB1*0301;DQB10603,0201	5.8	Other CG normal	FLT3 ITD Positive	66(A)	-(-)
AML010	30	AML	Idarubicin & ATRA ATRA maintenance chemotherapy only	A*24,*26;B*13,*38; Cw*06,*12 DRB1*04,*07;DQB1*02,*03	2.9	t(15;17)	AML M3 with PML-RARA translocation	83 (A)	+(ND)
AML011	63	AML	AML 16 (2 cycles ofDA+ATRA)	NK	NK	Trisomy 13	NK	20 (A)	-(ND)

AML012	71	AML	Low dose cytarabine	NK	NK	NK	NK	50 (A)	+(ND)
AML013	45	AML	AML 17 (2 cycles of ADE, 2 cycles of HDAC)	A*02, 03	NK	Normal	NK	43 (A)	+(-)
AML014	57	MDS/ AML	ADE & mylotarg MUD allo (Flu/Bu/Campath) GvHD	**A*02,**03, B*38, 40, Cw*03,12, DRB1*03;01, 13:01, DQB1*02:01, 06:03	NK	Trisomy 8 Abnomal 13	NK	8 (D)	+(+)
AML015	19	AML	AML 17	A*01,02	NK	Normal	FLT3 wt	51 (A)	-(-)
AML016	26	AML	Chemotherapy and then sibling allograft	A*11;30	NK	NK	MLL rearrange ment	25 (D)	+(ND)
AML017	59	AML	AML17 (2 cycles of Dax, MiDAC)	A*01,24	NK	NK	FLT3 wt	50 (A)	+(ND)
AML018	NK	AML	VR 2 x allotransplants, alive 100d after the 2nd transplant	A*11, B*07, 35, Cw*04,07, DRB1*01:01, 15:01; DQB1*05:01, 06;02	NK	Normal	NK	64 (A)	-(-)
AML019	65	AML	AML 16 (3 cycles DA)	NK	NK	Normal	NPM1	49 (A)	-(-)
AML020	54	AML	Dax2, HDAC, MiDAC	A*01:01; B*08:01:01; C*07:01, DRB1*03:01, DQB1*02:01	NK	Normal	FLT3 + NPM1+	23 (D)	+(ND)
AML021	82	AML	Low dose cytarabine	NK	NK	Normal		4 (D)	-(-)
AML022	64	AML	DA x2	A*03;11	NK	Normal	FLT3 ITD +	7 (D)	+(ND)
AML023	54	MDS	VUD allo	**A*02;**03	NK	Normal	NK	7 (D)	+(-)
AML024	62	AML/ MDS	AML 16 trial (3 cycles DA,& 1 cycle Mylotarg)	NK	NK	46, XX	NK	6 (D)	-(ND)
AML025	77	AML	LDAC	NK	NK	Normal	NK	34 (D)	-(ND)
AML026	62	AML	AML 16 (3 cycles DA)	NK	NK	46 XX	FLT3 wt NPM1+	41 (A)	-(-)
ALL001	22	ALL	Chemotherapy (UKALL 2003)	**A2**	50.4	46XY	(SET/CA N fusion transcript)	38 (A)	-(-)
ALL002	65	ALL	Chemotherapy	**A2**	230.0	t(4;11)	NK	3 (D)	-(-)
ALL003	NK	ALL	NK	NK	NK	ND	NK	NK	+(ND)
ALL004	NK	ALL	NK	NK	NK	ND	NK	NK	-(ND)
ALL005	22	T-ALL	UKALL 2003	**A*02,**03; B*35, 44; C*04,05; DRB1*14,15, DQB1*05, 06	NK	t(1;7)	NK	2 (A)	-(ND)
ALL006	50	cALL	UKALL XII; VUD allo	A*02 B*15, 57 Cw*03, 06: DRB1*01:01, 14:01, DQB1*05:01, 0503	NK	Normal	None	17 (A)	-(ND)

ALL007	26	cALL	UKALL XII	A*01,02; B*07, 13;Cw*06, 07 DRB1*15:01, 07:01; DQB1*06:02, 02;02	NK	t(1;19)	NK	16 (A)	-(ND)
CML001	67	CML	CP on Glivec, MMR	**A2 (by FACs)**	150.0	t(9;22)	NK	41 (A)	-(-)
CML002	21	CML	Imatinib, MMR	NK	NK	t(9;22)	NK	16 (A)	-(-)
CML003	63	CML	VUD allograft (poorly responsive to TKIs)	A*24, 31 B*40, 57 Cw*03, 06 DRB1*04:04, 07:01 DQB1*03:03, 03:02	NK	t(9;22)	NK	23 (A)	-(ND)
CML004	32	CML	Imatinib, MMR	NK	NK	t(9;22)	NK	27 (A)	-(ND)
CML005	61	CML – CP2[d]	Glivec ; Myeloid transformation; chemotherapy x 2 followed by an allo-transplant	A*03,11;B*15,44;Cw*03,05DRB1*0101,0401;DQB10302,0501	210.0	t(9;22)	NK	48 (A)	-(ND)

[a]Age at diagnosis

[b]Diagnosis at time of sampling

[c]White blood cell count at time of sampling x 10^9/L

[d]Myeloid blast transformation

ADE: induction therapy consisting of cytarabine, daunorubicin and etoposide; ALL: acute lymphocytic leukaemia; allo: allograft; AML: acute myeloid leukaemia; APML: acute promyelocytic leukaemia; Ara-C: Arabinosylcytosine; ATRA: all trans retinoic acid; CML: chronic myeloid leukaemia, CMML: chronic myelomonocytic leukaemia; CP: chronic phase; CR: complete remission; DA: daunorubicin and Arabinosylcytosine; FLA: chemotherapy using fludarabine and cytarabine; HDAC: histone deacetylase inhibitors; IDA: chemotherapy protocol using idarubicin; FLT3-ITD: FLT3 – internal tandem repeat; Glivec: contains imatinib and inhibits tyrosine kinases which contribute to disease, in this case inhibiting BCR-ABL activity in CML; LDAC: low dose cytarabine; MACE: chemotherapy consisting of amsacrine, cytarabine and etoposide; MDS: myelodysplastic syndrome; MIDAC: chemotherapy consisting of mitoxantrone and cytarabine; MUD: matched unrelated donor; ND: not done; NK: not known; NPM1: Nucleophosmin gene; PR: partial remission; TKI: tyrosine kinase inhibitors; transl: translocation; VUD: Volunteer unrelated donor; wt: wild type.

Appendix II. Scoring for each OVC and endometrial sample on the TMAs

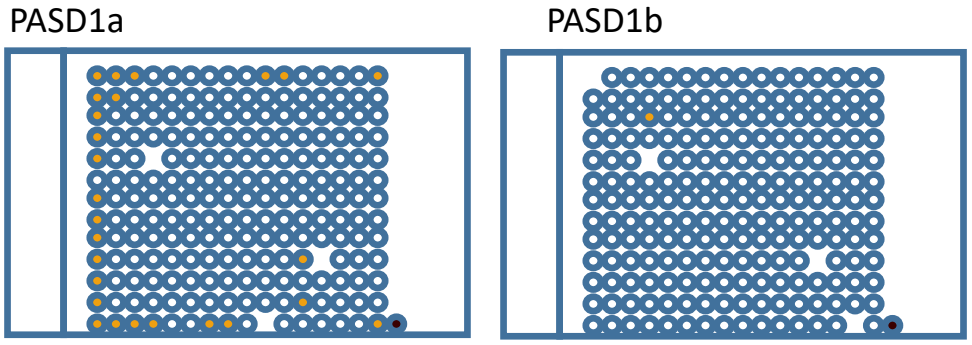
Scoring for each endometrial cancer sample on the TMAs

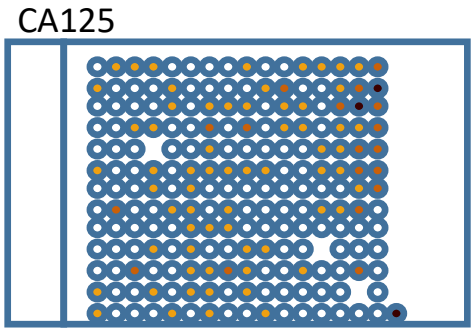
Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade	Type †	Cells only	Isotype	CA125	HE4	pSSX2	mSSX2
A1	1	F	60	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	2	2	1	0
A2	2	F	55	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	0	3	1	0
A3	3	F	62	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	1	2	2	1
A4	4	F	58	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	1	2	1	0
A5	5	F	61	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	0	2	2	0
A6	6	F	73	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	0	3	3	1
A7	7	F	51	Uterus	Endometrial adenocarcinoma	1 – 2	Malignant	0	0	0	3	2	0
A8	8	F	44	Uterus	Endometrial adenocarcinoma	1 – 2	Malignant	0	0	0	2	2	0
A9	9	F	48	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	2	3	3	0
A10	10	F	58	Uterus	Endometrial adenocarcinoma	1 – 2	Malignant	0	0	0	2	2	0
B1	11	F	60	Uterus	Endometrial adenocarcinoma (sparse)	1	Malignant	0	0	0	1	1	0
B2	12	F	49	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	0	2	1	1
B3	13	F	43	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	1	2	1	0
B4	14	F	51	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	1	2	1	0
B5	15	F	62	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	2	2	1	1
B6	16	F	62	Uterus	Endometrial adenocarcinoma	2	Malignant	0	0	2	2	1	0
B7	17	F	54	Uterus	Endometrial adenocarcinoma	1	Malignant	0	0	2	2	1	0
B8	18	F	52	Uterus	Endometrial adenocarcinoma	2	Malignant	0	0	2	2	2	0
B9	19	F	70	Uterus	Endometrial adenocarcinoma	2	Malignant	0	0	2	2	2	0
B10	20	F	56	Uterus	Endometrial adenocarcinoma	2 – 3	Malignant	0	0	0	3	1	1
C1	21	F	63	Uterus	Endometrial adenocarcinoma	3	Malignant	0	0	0	1	2	0
C2	22	F	58	Uterus	Endometrial adenocarcinoma	3	Malignant	0	0	0	2	2	1
C3	23	F	54	Uterus	Endometrial adenocarcinoma	3	Malignant	0	0	0	3	2	1
C4	24	F	63	Uterus	Endometrial adenocarcinoma	3	Malignant	0	0	0	2	1	0
C5	25	F	44	Uterus	Endometrial adenocarcinoma	3	Malignant	0	0	0	3	1	0
C6	26	F	53	Uterus	Squamous cell carcinoma	1	Malignant	0	0	0	2	1	0
C7	27	F	60	Uterus	Squamous cell carcinoma	2	Malignant	0	0	0	2	2	0
C8	28	F	45	Uterus	Squamous cell carcinoma	2 – 3	Malignant	0	0	2	2	2	0
C9	29	F	39	Uterus	Squamous cell carcinoma	3	Malignant	0	0	0	1	2	0
C10	30	F	44	Uterus	Squamous cell carcinoma	2	Malignant	0	0	2	2	1	0

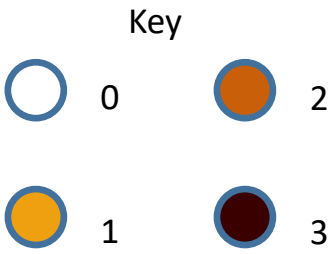
D1	31	F	39	Abdominal cavity	Metastatic endometrial adenocarcinoma	2	Metastasis	0	0	2	1	2	0
D2	32	F	63	Fibrofatty tissue	Metastatic endometrial adenocarcinoma (fibrofatty tissue)	–	Metastasis	0	0	2	1	2	1
D3	33	F	70	Lymph node	Metastatic endometrial adenocarcinoma	2	Metastasis	0	0	3	1	2	0
D4	34	F	69	Pelvic cavity	Metastatic endometrial adenocarcinoma	3	Metastasis	0	0	2	2	2	0
D5	35	F	51	Ovary	Metastatic endometrial adenocarcinoma	3	Metastasis	0	0	1	2	2	0
D6	36	F	40	Abdominal cavity	Metastatic dedifferentiated endometrial adenosquamous carcinoma	–	Metastasis	0	0	0	2	1	0
D7	37	F	37	Uterus	Endometrial polyp	–	Hyperplasia	0	0	2	1	2	0
D8	38	F	57	Uterus	Endometrial polyp	–	Hyperplasia	0	0	2	0	2	0
D9	39	F	69	Uterus	Endometrial polyp	–	Hyperplasia	0	0	1	1	2	0
D10	40	F	47	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	2	1	2	0
E1	41	F	51	Uterus	Hyperplasia of endometrium (sparse)	–	Hyperplasia	0	0	2	2	1	0
E2	42	F	52	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	2	2	2	1
E3	43	F	65	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	2	1	1	0
E4	44	F	40	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	1	1	2	0
E5	45	F	43	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	2	1	2	0
E6	46	F	37	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	1	1	2	0
E7	47	F	49	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	1	1	1	0
E8	48	F	70	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	1	1	2	1
E9	49	F	51	Uterus	Hyperplasia (smooth muscle tissue)	–	Hyperplasia	0	0	1	2	1	1
E10	50	F	43	Uterus	Hyperplasia (smooth muscle tissue)	–	Hyperplasia	0	0	2	1	2	0
F1	51	F	50	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	0	1	1	1
F2	52	F	52	Uterus	Endometrial glandular cystic hyperplasia	–	Hyperplasia	0	0	2	2	2	0
F3	53	F	64	Uterus	Endometrial simple hyperplasia	–	Hyperplasia	0	0	1	1	2	1
F4	54	F	69	Uterus	Endometrial glandular cystic hyperplasia	–	Hyperplasia	0	0	1	1	2	0
F5	55	F	41	Uterus	Endometrial glandular cystic hyperplasia	–	Hyperplasia	0	0	2	4	2	0
F6	56	F	50	Uterus	Endometrial adenomatous hyperplasia	–	Hyperplasia	0	0	2	4	3	0
F7	57	F	69	Uterus	Moderate atypical hyperplasia of endometrium	–	Hyperplasia	0	0	1	4	2	0
F8	58	F	44	Uterus	Severe atypical hyperplasia of endometrium, focal canceration	–	Hyperplasia	0	0	1	3	2	0
F9	59	F	48	Uterus	Endometrial glandular cystic hyperplasia	–	Hyperplasia	0	0	1	3	3	0
F10	60	F	48	Uterus	Chronic endometritis	–	Inflammation	0	0	1	3	2	0

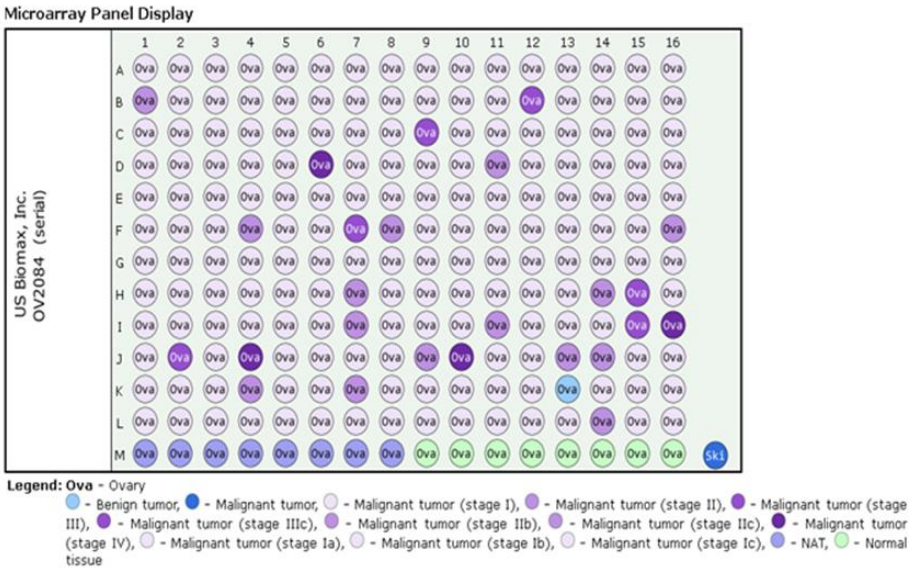
G1	61	F	63	Uterus	Chronic endometritis	–	Inflammation	0	0	0	2	2	0
G2	62	F	63	Uterus	Chronic endometritis (sparse)	–	Inflammation	0	0	0	2	2	0
G3	63	F	65	Uterus	Chronic endometritis	–	Inflammation	0	0	0	2	2	0
G4	64	F	51	Uterus	Acute endometritis	–	Inflammation	0	0	0	2	1	0
G5	65	F	46	Uterus	Cancer adjacent normal late proliferative endometrium tissue	–	Normal	0	0	2	2	2	0
G6	66	F	43	Uterus	Cancer adjacent normal endometrial tissue (smooth muscle)	–	Normal	0	0	2	3	3	0
G7	67	F	37	Uterus	Cancer adjacent normal proliferative endometrium tissue	–	Normal	0	0	1	3	2	0
G8	68	F	40	Uterus	Cancer adjacent normal endometrial tissue	–	Normal	0	0	3	3	3	0
G9	69	F	49	Uterus	Cancer adjacent normal proliferative endometrium tissue	–	Normal	0	0	2	2	3	0
G10	70	F	39	Uterus	Cancer adjacent normal endometrial tissue	–	Normal	0	0	2	3	3	0
H1	71	F	36	Uterus	Cancer adjacent normal proliferative endometrium tissue	–	Normal	0	0	0	1	2	2
H2	72	F	43	Uterus	Cancer adjacent normal proliferative endometrium tissue	–	Normal	0	0	0	2	1	2
H3	73	F	46	Uterus	Cancer adjacent normal proliferative endometrium tissue	–	Normal	0	0	0	1	2	1
H4	74	F	34	Uterus	Cancer adjacent normal endometrial tissue	–	Normal	0	0	0	1	1	1
H5	75	F	40	Uterus	Normal proliferative endometrium tissue	–	Normal	0	0	0	3	2	1
H6	76	F	21	Uterus	Normal proliferative endometrium tissue	–	Normal	0	0	0	4	2	1
H7	77	F	15	Uterus	Normal secretory endometrium tissue	–	Normal	0	0	0	3	1	1
H8	78	F	18	Uterus	Normal secretory endometrium tissue	–	Normal	0	0	1	3	2	1
H9	79	F	21	Uterus	Normal proliferative endometrium tissue	–	Normal	0	0	0	3	3	1
H10	80	F	21	Uterus	Normal secretory endometrium tissue	–	Normal	0	0	3	2	3	1
–	–	M	58	Skin	Malignant melanoma (tissue marker)	–	Malignant	3	3	3	3	3	3

Heatmap to show the scoring for PASD1 in OVC



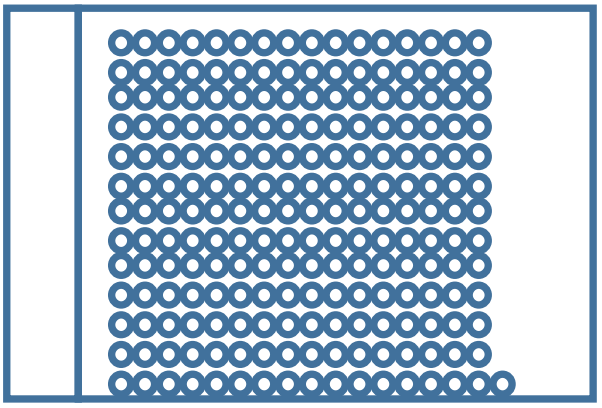




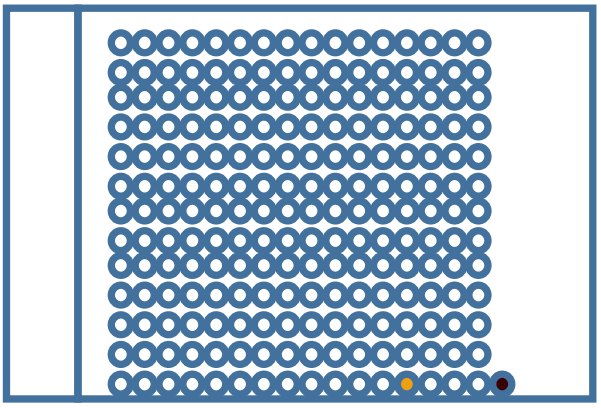


Heatmap showing the scoring for SSX2 in OVC

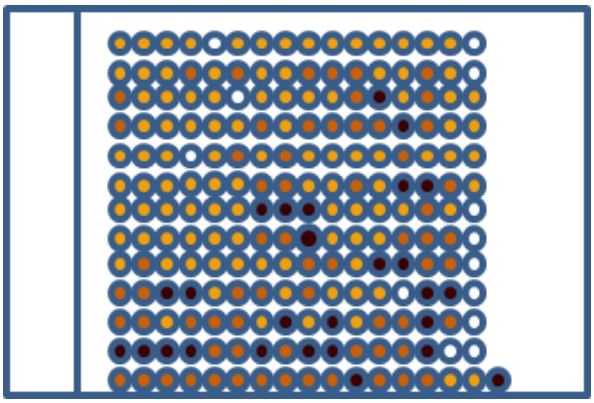
Cells only



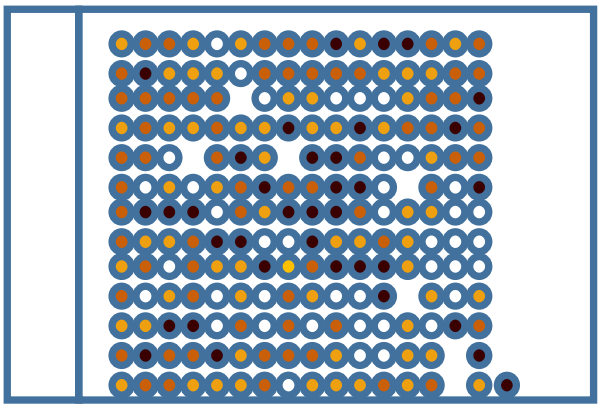
Isotype



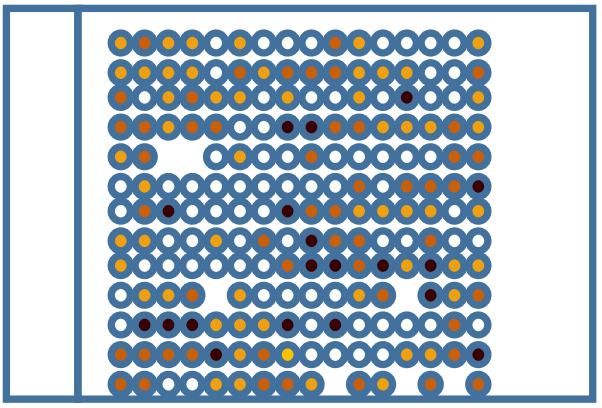
Actin



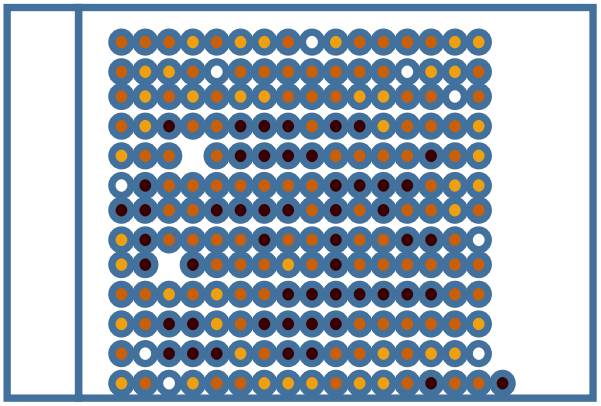
mSSX2



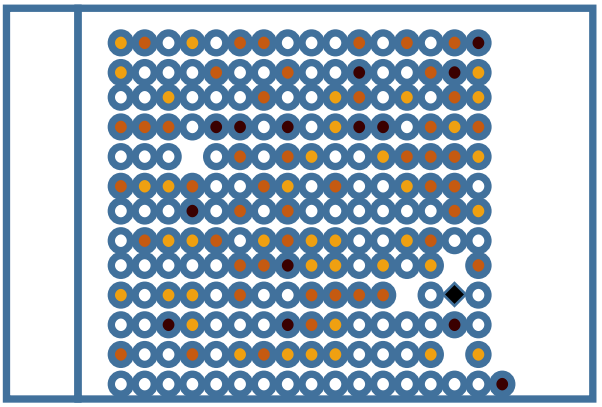
pSSX2 (N)

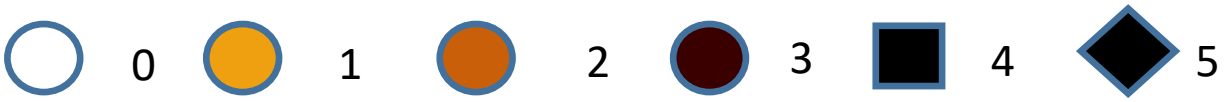


pSSX2

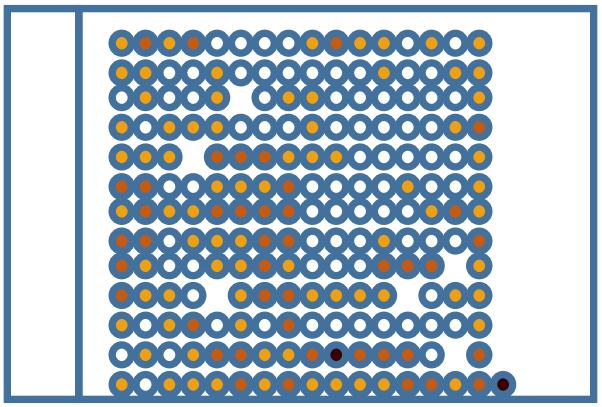


CA125

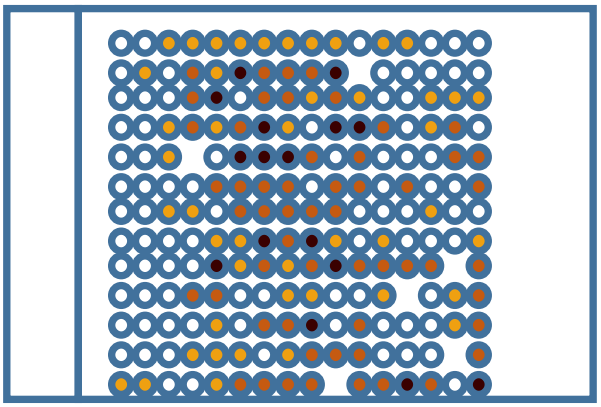




WT1

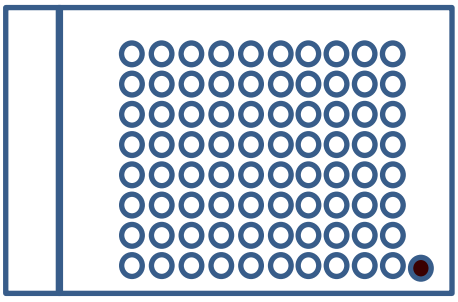


HE4

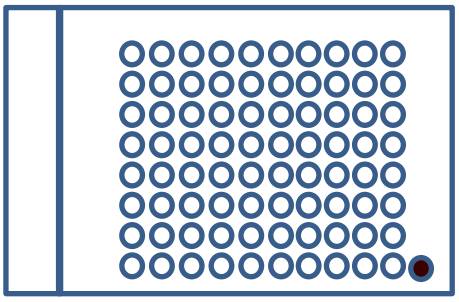


Heatmap showing scoring for SSX2 in endometrial cancer

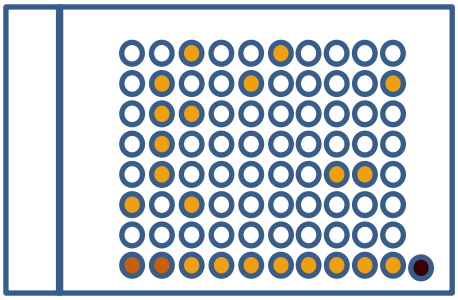
Cells only



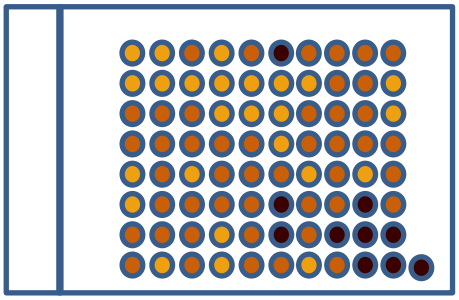
Isotype



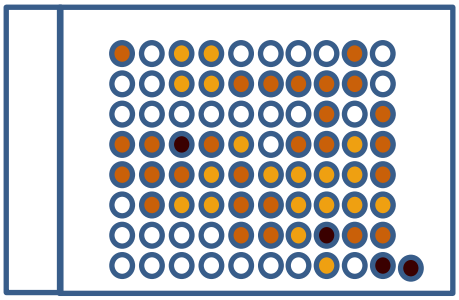
mSSX2



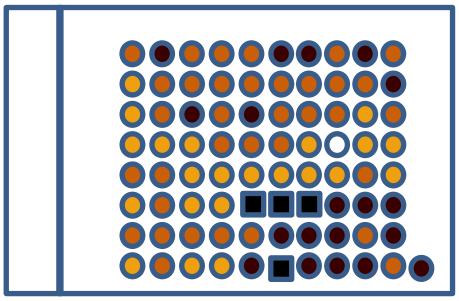
pSSX2



CA125



HE4



Key

Microarray Panel Display

US Biomax, Inc.
UT801 (serial)

	1	2	3	4	5	6	7	8	9	10	
A	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
B	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
C	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
D	Abd	Fib	Lym	Pel	Ova	Abd	Ute	Ute	Ute	Ute	
E	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
F	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
G	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	
H	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ute	Ski

Legend: Abd - Abdominal cavity, **Fib** - Fibrofatty tissue, **Lym** - Lymph node, **Ova** - Ovary, **Pel** - Pelvic cavity, **Ute** - Uterus
- Hyperplasia, - Inflammation, - Malignant tumor, - Metastasis, - Normal tissue