Autotaxin Expression in Bladder and Renal Cancer

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Abstract

Autotaxin is an extracellular enzyme that generates lysophosphatidic acid (LPA). LPA binds up to six different cell surface G protein-coupled receptors to initiate signaling resulting in cell survival, invasion and angiogenesis. For this reason autotaxin has emerged as a therapeutic target in several different malignancies.

I have used immunohistochemistry to explore the expression of autotaxin and its correlation with clinico-pathological variables in bladder and renal cancer.

I show that in bladder cancer, tumours from patients with muscle invasive disease were significantly more likely to show strong autotaxin expression than were those tumours from patients without evidence of muscle involvement (p=0.009). This observation is not only consistent with the known functions of autotaxin/LPA in promoting tumour invasion, but suggests that the potential use autotaxin inhibitors in preventing bladder cancer progression warrants further investigation.

Although I failed to detect autotaxin expression in the tumour cells of patients with renal cancer, I did observe high-level expression of autotaxin on the tumour-associated vasculature, which in many cases was not apparent in blood vessels of matched normal renal tissues. This points to an important role for autotaxin in renal cancer-associated angiogenesis and suggests a potential role for autotaxin inhibition as an anti-angiogenesis therapy.

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Introduction

1.1 Bladder Cancer

Bladder cancer is the fourth most common malignancy in males and twelfth most common in females in the UK. It has an incidence of 10,000 new cases and 5,000 deaths per year (www.statistics.gov.uk, Cancer Research UK). Approximately 70% of patients present with disease that is confined to the mucosa (stage Ta, CIS), or submucosal (stage T1). These cases are described as non-muscle invasive bladder cancer (NMIBC) and have excellent long term survival but are characterized by frequent recurrences (Messing et al., 1995) The remaining 30% of cases are described as muscle-invasive bladder cancer (MIBC) (EAU 2012). This type of bladder cancer has a high mortality rate within 2 years (Prout and Marshall, 1956).

Rates of morbidity and mortality in the UK are similar to those in Europe and North America.

Bladder cancer surveillance can be life long and presents the healthcare system with a huge financial burden not to mention the psychological burden imposed on the patient. For example, Stenzl et al (2008) estimated that the cost per patient from diagnosis to death was $96,000 to $187,000 and that the total cost within the United States in 2001 was $3.7 billion.

Therefore there is a desperate need to identify causative factors, at a molecular level, that could be used as therapeutic targets to improve diagnosis, treatment and the follow-up of these patients.

1.1.1 Pathology of bladder cancer

Greater than 90% of all bladder cancers are transitional cell carcinoma (TCC) (Lopez-Beltran, 2008). This group can further be divided into papillary, sessile and carcinoma *in situ* (CIS). CIS is presumed to be a histologically recognizable precursor of invasive carcinoma.

Approximately 5% are squamous cell carcinoma and are associated with chronic irritation of the bladder epithelium. The majority of these cases are linked to chronic schistosomiasis infection but other associations are the presence of a long-term catheter and bladder calculi.

Less than 2% are adenocarcinoma of the bladder. There are 3 types including papillary, polypoid and nodular. They are associated with chronic infection; glandular cystitis is frequently associated with this lesion, or bladder extrophy.

1.1.2 Grade and Stage of bladder cancer

According to the 1973 WHO grading transitional call carcinoma is graded into well (G1), moderately (G2), and poorly (G3) differentiated tumours. However this has been superseded by the 2004 WHO grading which grades TCC into urothelial papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma and high-grade papillary urothelial carcinoma.

The papilloma is composed of a delicate fibrovascular core covered by normal urothelium. A PUNLMP is defined as a papillary fibrovascular growth covered by proliferated urothelium exceeding normal thickness. PUNLMP have a low risk of progression, but they are not completely benign and tend to recur. The low-grade papillary urothelial carcinoma group includes all former grade 1 (WHO 1973) cases and some grade 2 cases.

The 2004 WHO classification system is recommended as it stratifies according to risk potential. However the 1973 WHO classification system is still very much in use today as many find it a simpler system to use.

Table 1.1: WHO Grading in 1973 and in 2004 (Epstein et al. (1998) & Sauter et al. (2004))

1973 WHO Grading
Urothelial Papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading system (papillary lesions)
Urothelial papilloma (completely benign lesion)
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade (LG) papillary urothelial carcinoma
High-grade (HG) papillary urothelial carcinoma

For staging, TNM (tumour, lymph nodes and metastases) 2010 is used for bladder cancer as can be seen illustrated below. pTa includes tumours that have not invaded or breached the basement membrane. pT1 includes those tumours that have breached the basement membrane, pT2 includes those that have invaded the muscularis mucosa, pT3 includes those invading the perivesical tissue and pT4 includes those invading neighbouring organs (prostate, uterus, vagina, pelvic and abdominal wall).

Table 1.2: TNM Classification of Urinary Bladder Cancer (Sobin et al. 2009)
T- Primary Tumour
Tx Primary tumour cannot be assessed T0 No evidence of primary tumour Ta Non-invasive papillary carcinoma Tis Carcinoma in situ: "flat tumour" T1 Tumour invades subepithelial connective tissue or lamina propria T2 Tumour invades muscle: T2a Tumour invades superficial muscle (inner half) T2b Tumour invades deep muscle (outer half) T3 Tumour invades perivesical tissue: T3a Microscopically T3b Macroscopically T4 Tumour invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall T4a Tumour invades prostate stroma, seminal vesicles, uterus or vagina T4b Tumour invades pelvic wall or abdominal wall
N- Regional Lymph Nodes
Nx Regional lymph nodes cannot be assessed N0 No regional lymph nodes metastases N1 Metastases in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral) N2 Metastases in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral) N3 Metastases in common iliac lymph node(s)
M- Distant Metastases
M0 No distant metastasis

M1 Distant metastasis

1.1.3 Epidemiology of bladder cancer – Incidence and Mortality

In the United Kingdom (UK) bladder cancer is the seventh most common cancer (2011), accounting for 3% of all new cases. It is the fourth most common cancer in males (4% of male total), whilst it is the 12th most common cancer in females (2% of female total). In 2011, there were 10,399 new cases of bladder cancer in the UK, 7452 (72%) in men and 2947 (28%) in women, giving a male: female ratio of approximately 2.5:1 (Cancer Research UK, 2015).

Crude incidence rates show that there are 24 new bladder cancer cases for every 100,000 males in the UK, and 9 for every 100,000 females.

Bladder cancer incidence is strongly related to age with the highest incidence rates being in those above 60 years of age (www.cancerresearchuk.org).

In the UK, in 2010, the lifetime risk of developing bladder cancer was 1 in 40 for men and 1 in 100 for women.

In Europe, bladder cancer is the 5th most common cancer with more than 151,000 new cases diagnosed in 2012 (4% of the total). Worldwide, it is the 9th most common cancer, with around 429,800 new cases diagnosed in 2012 (3% of the total).

Age standardized mortality rates globally vary from 2-10 per 100,000 per year for men and 0.5-4 per 100,000 per year for women.

1.1.4 Bladder cancer risk factors

There are several well-established risk factors for bladder cancer.

Firstly, tobacco smoking is thought to be responsible for 50-65% of male cases and 20-30% of female cases (Lokeshwar et al. 2005). Incidence of bladder cancer has been found to be directly related to duration of smoking and the number of cigarettes smoked per day (Stenzl et al. 2010). The risk is also higher for those smoking from a young age and those exposed to tobacco during childhood (May et al. 2003). Furthermore, there was a significant

reduction in risk in those who stopped smoking, 40% reduction within 1-4 years of stopping and 60% reduction after 25 years (Stenzl et al. 2010).

The second most important risk factor is occupational exposure to chemicals. These cases account for 20-25% of bladder cancer cases (EAU, 2012). Individuals working in industries that use rubbers, paint, dyes and chemicals are most at risk.

There have been reports of increased rates of secondary bladder malignancies after external beam radiation therapy (ERBT) for gynaecological malignancies, with relative risks of 2 to 4 (Chrouser et al. 2006). Some believe there is an association between dietary factors and bladder cancer however no causal relationship has been reported (EAU Guidelines 2015). The EPIC study is a multi-centre cohort study examining the association between diet, environmental and lifestyle factors and cancer. So far this study has failed to report any links between red meat, fruit and vegetable consumption and fluid intake and bladder cancer. However, recently they have described a link between dietary intake of flavonols and ligans and risk of aggressive bladder cancer (Zamora-Ros et al. 2014)

The link between bladder schistosomiasis (bilharzia) and squamous cell carcinoma (SCC) of the bladder is well established. However, according to recent data from Egypt, there has been a shift from SCC to transitional cell carcinoma (TCC). This is thought to be due to a decline in the detection of bilharzia eggs in the urine samples due to better control of the disease in rural populations (Grim et al. 2003, Divrik et al. 2006).

Separate to schistosomiasis, chronic urinary tract infection has been linked to muscle invasive bladder cancer, particularly invasive squamous cell

carcinoma. Several case-control studies have demonstrated a direct association between bladder cancer and urinary tract infections, some reporting as much as a twofold increase in risk of bladder cancer in patients suffering from recurrent urinary tract infections (EAU Guidelines, 2012).

Several chemotherapy agents have been implicated in the development of muscle invasive bladder cancer, in particular cyclophosphamide, (Payne et al. 2013) an alkylating agent used in the treatment of non-neoplastic diseases. The metabolite of cyclophosphamide, acrolein, is thought to be responsible for the increased incidence of bladder cancer. This effect was found to be independent of the association of haemorrhagic cystitis with the same treatment (Kaldor et al. 1995, Travis et al. 1995).

An association between upper tract tumours and bladder cancer has been reported. A 1.7% - 26% incidence of non-muscle invasive bladder cancer has been reported after a diagnosis of upper urinary tract tumour (EAU, 2012). In a retrospective study of 1529 patients with primary non-muscle invasive bladder carcinoma, those who were found to have a tumour in the bladder trigone were almost 6 times more likely to develop a synchronous tumour in the upper urinary tract (Palou et al. 2005).

Vaidya et al. (2001) performed a retrospective study of patients who underwent radical cystectomy and demonstrated that women were more likely than men (85% vs. 51%) to be diagnosed with primary muscle invasive bladder cancer. They also suggested that women were more likely to be older at diagnosis and that delayed diagnosis was more likely in women after haematuria due to the many differential diagnoses such as urinary tract infections which are commoner in women than men (Cardenas-Turanzas et al. 2006).

Hormonal levels, oestrogen and androgen, have also been deemed responsible for some of the differences in gender prevalence of bladder cancer (McGrath et al. 2006, Scosyrev et al. 2009, Stenzl et al. 2010). Post-menopausal status was found to be associated with an increased risk of bladder cancer even after adjusting for smoking status.   A recent study of Egyptian women found that multiple pregnancies and use of the oral contraceptive pill was associated with decreased risk of developing bladder cancer while younger age at menopause (<45 years) was associated with increased risk (Wolpert et al. 2012).

The SEER (surveillance, epidemiology and end results) study based on 13,234 cancer cases (including female breast, colorectal, prostate, lung, bronchus, uterine cervix, ovarian, melanoma and urinary bladder) diagnosed from 1979-2003, showed that survival from diagnosis was associated with socioeconomic status (health income, education, income and poverty status) such that low socioeconomic status was associated with reduced survival from diagnosis compared with higher socioeconomic status (Abdollah et al. 2013).

They also found that when comparing black and white racial origin, black racial origin had unfavourable prognoses compared with white racial origin, even after adjustments for socioeconomic status were made.

1.1.5 Existing treatments for bladder cancer

Treatment of bladder cancer differs according to grade and stage of the tumour. Generally, non-muscle invasive bladder cancer is treated with an initial resection followed by intravesical chemotherapy (mitomycin C) and/or intravesical Bacillus Calmette-Guerin (BCG) immunotherapy. Muscle invasive bladder cancer however, is treated with initial resection to gain histology but is then followed by a combination of either surgery with or without adjuvant or neo-adjuvant chemotherapy or radiotherapy depending on the grade and stage. The aim of treatment for muscle invasive bladder

cancer is to remove all loco-regional disease including the primary tumour as well as regional lymph nodes. Neo-adjuvant or adjuvant chemotherapy may also be given with the intention of targeting occult regional nodes and visceral metastases when the risk is high. Radical cystectomy provides good control of the primary tumour as it involves removal of the entire bladder, however the surrounding structures such as perivesical tissue, prostate, and seminal vesicles in men and ovaries, uterus, cervix and anterior vagina in women are also removed.

Chemotherapy given before cystectomy, known as neo-adjuvant chemotherapy, is done with the intention of treating micro-metastatic disease present at diagnosis. It is normally considered in patients with stage T2-T4a muscle invasive disease.

Adjuvant chemotherapy has been advised for patients deemed at high risk of recurrence in an attempt to delay recurrence and prolong survival. The advantage of giving chemotherapy after surgery is that full pathological staging is available.

In patients wishing to preserve sexual function and therefore quality of life or those not fit for surgery can be offered a bladder preserving approach which consists of a combination of chemotherapy and radiotherapy. This would only be appropriate in patients with solitary T2 or early T2 tumours less than 6cm in size, no tumour-associated hydronephrosis, tumours allowing a visibly complete transurethral resection of the tumour, invasive tumours in the absence of carcinoma in situ, and renal function that will permit subsequent chemotherapy (Wein et al. 2012).

1.1.6 Molecular Biology of Bladder Cancer

Cancer is abnormal and unregulated cell growth that occurs after a triggering event that results in cell change. Two types of gene have been associated in

initiating these cancer-forming changes; proto-oncogenes and tumour suppressor genes.

Proto-oncogenes become drivers of cancer as a result of point mutations in the genetic code that result in unregulated growth, or gene amplification or translocation, both of which increase the level of protein. The resultant proto-oncogene becomes an oncogene that can promote cancer (Lengauer et al. 1998).

Tumour suppressor genes normally act to inhibit cancer formation, however deletions in the allele or frameshift mutations may have the opposite effect thereby contributing to the development of cancer.

There have been two pathways thought to be involved in bladder cancer formation. The first is from normal urothelium to low grade non-invasive and the second is from normal urothelium to CIS and then muscle invasive disease. A third pathway has now been proposed involving normal urothelium to hyperplasia/dysplasia to high-grade papillary carcinoma and then to muscle invasive disease (Wein et al. 2012).

Recognized genetic mutations in low-grade non-muscle invasive disease are alterations in the fibroblast growth factor receptor 3 (FGFR 3) and deletions of chromosome 9q. While mutations in p53 and RB loss are associated with high-grade aggressive disease.

The conversion of normal urothelium to dysplasia is associated with deletions in chromosome 9 in 75% of cases, abnormal p53 in 50% and increased cellular growth in all cases (Mallofre et al. 2003). The main genetic changes separating non-muscle invasive disease from muscle invasive are high FGFR-3 and low TP53 mutation rates and genetic instability (Wein et al. 2012).

1.1.7 Bladder Cancer Prognosis Programme

The Bladder Cancer Prognosis Programme (BCPP) is a large epidemiological prospective longitudinal cohort study of all patients with newly diagnosed bladder cancer within the West Midlands, UK. It was initiated in 2005 by the Cancer Research UK Bladder Cancer Group, based at the University of Birmingham (UK).

There were 3 clear objectives of the BCPP. First to assess what effect, if any, modifiable *lifestyle factors* such as dietary habits, smoking, fluid intake and environmental exposures, have on recurrence and progression of bladder cancer.
It also considered *quality of life variables* and possible associations with recurrence and progression of bladder cancer.
The role of *molecular biomarkers* in predicting bladder cancer recurrence and progression in addition to investigating whether or not selenium and vitamin E play any role in reducing the risk of bladder cancer recurrence and progression in patients with non-muscle invasive bladder cancer, are among some of the studies that have arisen out of the BCPP study.

The BCPP bio-bank is one of the world's largest and most detailed collections and comprises fresh frozen bladder cancer tissues, blood and urine. Each sample is linked to over a thousand variables, relating to clinical, epidemiological and pathological data as well as follow up data on disease recurrence and progression.

BCPP aimed to recruit patients within the West Midlands who had newly diagnosed bladder cancer over a 3-year period. Specifically, patients over the age of 18 years were recruited at haematuria clinics based on cystoscopic findings suggestive of bladder cancer. The cohort included patients with any

stage of the disease providing it was newly diagnosed. A history within the last decade of urethra, bladder, ureter or renal pelvis malignancy excluded patients from the cohort as did a diagnosis of HIV infection or any other condition that may have compromised patient safety.

Data collection was initiated at diagnosis and performed by research nurses trained to interview patients prior to surgery about socio-demographic information such as age, sex, ethnicity, education, marital status, etc. They also asked about lifestyle factors (including smoking history, exposure to industrial dyes, etc.), past medical history, drug history, social history including dietary intake (total fluid intake, alcohol, caffeine, sweeteners, vitamins), family history and social support networks. The European Organisation for Research and Treatment for Cancer (EORTS) questionnaire was used along with the general cancer questionnaire QLQ-C30.

In addition, patients were sent a self-completion questionnaire after transurethral resection of bladder tumour (TURBT) and prior to their first follow-up at around 3 months from initial diagnosis.

Biological samples including blood, urine, fresh and formalin-fixed paraffin-embedded tumour samples were also collected at diagnosis.

Following the initial TURBT but prior to the first follow- up appointment patients were asked to complete a 7 day food and fluid diary and give toenail samples to measure for selenium levels. At 3 months and then annually, life style factors, quality of life variables and patient surveillance preferences were recorded and questionnaires repeated.

The primary endpoints of BCPP were disease recurrence and progression. Recurrence being defined as new occurrence of a bladder tumour at the same or different site as the initial tumour. Recurrences identified at the first check cystoscopy at 3 months were excluded as this was considered to be incomplete resection of primary tumour at the initial resection. Progression was defined as recurrence with an increased tumour grade compared with

the initial tumour ranging from grade 1 or 2 to 3, or an increase in TNM stage, or new occurrence of carcinoma in situ (CIS), or new multiple tumours following resection of a solitary lesion, or refractory disease requiring a cystectomy.

All the information relating to the BCPP is held on an anonymised database. For the purposes of this study a subset of cases were selected from the BCPP cohort and immunohistochemistry was performed. Epidemiological data from the database was extracted and a new smaller database was established. This database was then updated with the results of the immunohistochemistry undertaken, investigating the association of autotaxin with bladder cancer. Statistical analysis was performed and the results are discussed in subsequent chapters. More information is available in the "Materials and Methods" chapter.

1.2 Renal Cancer

Renal cell carcinoma (RCC) accounts for 2-3% of all adult malignancies (Campbell-Walsh, 2012). It is the most deadly of the urological malignancies. Historically, there has been a 30-40% mortality rate associated with RCC, compared with a 20% mortality rate associated with bladder and prostate carcinomas (Landis et al, 1999; Pantuck et al, 2001b).

1.2.1 Pathology of renal cancer

Renal cell carcinoma (RCC) is the most common of the renal cancers accounting for 90% of all cases. Although RCC tends to grow as a single tumour within a kidney, there may be more than one tumour within any single kidney. The three commonest types of RCC are, clear cell, papillary and chromophobe types. Clear cell is the commonest form representing 80-90% of RCC. Microscopically clear cell type is composed of pale or clear cells as the name suggests. Papillary is the second most common type of RCC accounting for approximately 10% of all RCC. Papillary tumours form small finger-like projections and can also be known as chromophilic as the cells, in the presence of certain dyes, look pink under the microscope. Chromophobe RCC accounts for about 5% of all RCC. The cells of this type are also clear as in clear cell, however in chromophobe they tend to be larger in size.

Rare types of RCC include collecting duct, multilocular, medullary, mucinous and spindle cell.

Other types of renal tumours include transitional cell carcinoma, Wilm's tumour and renal sarcomas, as well as benign tumours such as oncocytomas and angiomyolipomas.

5-10% of all renal cancers are transitional cell carcinoma (TCC). This type of tumour begins in the lining of the renal pelvis, which is composed of transitional cells. Wilm's tumour almost always occurs in children. Renal

sarcoma is rare and begins in the blood vessels of the connective tissue of the kidney. This type accounts for <1% of all kidney cancers.

Table 1.3 describes the major subtypes of renal cell carcinoma.

Table 1.3: Major histological subtypes of RCC (EAU 2013)

Histological subtype	Percentage of RCC	Histological description	Associated genetic changes
Clear cell (cRCC)	80-90%	Most cRCC are composed predominantly of cells containing clear cytoplasm, although eosinophilic cytoplasm predominates in some cells. The growth pattern may be solid, tubular, and cystic.	Identified by the specific deletion of chromosome 3p and mutation of the VHL gene. Other changes are duplication of the chromosome band 5q22, deletion of chromosome 6q, 8p, 9p, and 14q.
Papillary (pRCC)	10-15%	Most pRCCs have small cells with scanty cytoplasm, but also basophilic, eosinophilic, or pale-staining characteristics. A papillary growth pattern predominates, although there may be tubular papillary and solid architectures. Necrotic areas are common. Papillary RCC can be divided into two different subtypes: type 1 with small cells and pale cytoplasm and type 2 with large cells and eosinophilic cytoplasm, the latter having a worse prognosis.	The most consistent genetic alterations are trisomies of chromosomes 3q, 7, 8, 12, 16, 17, and loss of the y chromosome.
Chromophobe (chRCC)	4-5%	The cells of chRCC may have pale or eosinophilic granular cytoplasm. Growth usually occurs in solid sheets.	The genetic characteristic are a combination of loss of chromosomes 1, 2, 6, 10, 13, and 17.

1.2.2 Grade and Stage of renal cancer

Fuhrman nuclear grade is the most commonly utilised grading system used to describe renal cancer. The most aggressive pattern observed defines the Fuhrman grade. 1 = well-differentiated, 2 = moderately differentiated, 3 and 4 = poorly differentiated. This is based on nuclear size, outline and nucleoli. The grading system is an independent prognostic factor.

Leibovich Score (LS) is another scoring system for cRCC. It is a histological scoring tool that takes into account 5 different clinical and pathological parameters and has been used in cases of localized cRCC to predict likely progression to metastatic cRCC (Leibovich et al. 2003). The score considers pathological stage, nodal status, tumour size, nuclear grade and histological tumour necrosis (Ravand et al. 2008). Using these parameters, tumours are assigned a score ranging from zero to 11 and categorised into low- (0–2), intermediate- (3–5) or high-risk (≥6) groups. The LS can accurately assess the risk of metastasis in patients after radical nephrectomy and therefore is used to guide the postoperative intensity of follow-up and possible entry into clinical trials for patients to receive adjuvant therapy (Vasdev et al. 2014).

Staging is based on the 2009 TNM system classification system and can be seen in table 1.4 below.

Table 1.4 : The 2009 TNM staging classification system for Renal Cell Carcinoma (Sobin et al. 2009) & (Wittekind et al. 2012) *Taken from EAU Guidelines 2015.*
T- Primary tumour

TX   Primary tumour cannot be assessed
 	
T0   No evidence of primary tumour
 	
T1   Tumour ≤7cm in greatest dimension, limited to the kidney
 	
      T1a Tumour ≤ 4cm in greatest dimension, limited to the kidney
 	
      T1b Tumour > 4cm but ≤ 7cm in greatest dimension
 	
T2   Tumour > 7cm in greatest dimension, limited to the kidney
 	
      T2a Tumour > 7cm but ≤ 10cm in greatest dimension
 	
      T2b Tumour > 10cm limited to the kidney
 	
T3   Tumour extends into major veins or directly invades adrenal gland or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota's fascia
 	
      T3a Tumour grossly extends into the renal vein or its segmental (muscle containing) branches or tumour invades perirenal and/or renal sinus (peripelvic0 fat but not beyond Gerota's fascia
 	
      T3b tumour grossly extends into the vena cava below the diaphragm
 	
      T3c tumour grossly extends into vena cava above the diaphragm or invades the wall of the vena cava
 	
T4   Tumour invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland)

N- Regional lymph nodes
NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis N1 Metastasis in a single regional lymph node N2 Metastasis in more than 1 regional lymph node				
M- Distant metastases				
M0 No distant metastasis M1 Distant metastasis				
TNM stage grouping				
Stage I	T1		N0	M0
Stage II	T2		N0	M0
Stage III	T3		N0	M0
	T1, T2, T3		N1	M0

1.2.3 Epidemiology of renal cancer- Incidence and Mortality

Approximately 12 new cases are diagnosed per 100,000 population per year. There is a male-to-female preponderance of 3:2 (Landis et al, 1999; Wallen at al, 2007; DeCastro and McKiernon, 2008; Woldrich et al, 2008; Carrizosa and Godley, 2009).

Typically, RCC presents in the sixth or seventh decade of life (Pantuck et al, 2001b; Wallen et al, 2007). Most cases of RCC are believed to be sporadic, with only 2-3% thought to be familial (Lipworth et al, 2006).

The incidence of RCC has increased in recent years due to the more prevalent use of ultrasonography and CT in the investigation of abdominal symptoms and therefore the incidental finding of RCC has risen greatly.

1.2.4 Renal cancer risk factors

There are three known risk factors for RCC; tobacco exposure, obesity and hypertension. The greatest risk factor is tobacco exposure with a relative risk ranging from 1.4 to 2.5 compared with controls (Wein et al., 2012). All forms of tobacco appear to be involved and risk increases with increasing dose or pack-years (Kantor, 1977). Relative risk is directly related to duration of smoking and falls gradually after cessation (La Vecchia et al, 1990).

Obesity is another important risk factor for RCC with a relative risk of 1.07 for each unit of rising body mass index (Chow et al. 2000). There is an increase in both obesity and RCC in Western countries with around 40% of the cases of RCC in the US have been linked to obesity (Calle and Kaaks, 2004). Potential mechanisms proposed to account for this link include lipid peroxidation resulting in DNA adducts, raised expression of insulin-like growth factor-1, raised oestrogen levels and raised arterionephroscellerosis and local inflammation (Kasiske et al., 1992, Huang et al., 1998, Gago-Dominguez et al., 2002)

Hypertension is the third recognized risk factor for RCC. There have been suggestions that it is the treatment for hypertension such as anti-hypertensives and diuretics that are responsible for the increased risk of RCC. However the weight of evidence now suggests that it is the underlying disorder rather than the treatment (McLaughlin et al. 1995, Yuan et al., 1998, Lipworth et al., 2006). Renal injury caused directly by hypertension as well as inflammation and metabolic and functional changes in the renal tubules are thought to increase the vulnerability to carcinogens (Lipworth et al, 2006).

1.2.5 Existing treatments for renal cancer

Treatment of localized disease involves surveillance of small renal masses, surgery, either radical or partial nephrectomy with or without adrenalectomy and lymph node dissection, or percutaneous approaches. These are

minimally invasive techniques and include percutaneous radiofrequency ablation (RFA), cryoablation (CAB), microwave ablation, laser ablation and high-intensity focused ultrasound ablation (HIFU).

Treatment for metastatic disease involves surgery usually in the form of a debulking cytoreductive nephrectomy; a tumour nephrectomy. For the majority of patients this is a palliative procedure and other treatments will ultimately be required, such as radiotherapy especially if bone metastases are present.

Systemic treatment for metastatic RCC involves a combination of chemotherapy, interleukin-2 and angiogenesis inhibitor drugs and more recently immune checkpoint inhibitors.

1.2.6 Molecular Biology of Renal Cancer

The histological subtypes of RCC have been outlines above. The molecular genetics underlying these subtypes has allowed the development of molecular targeted agents that have extended survival especially in patients with metastatic RCC.

An example of this is von Hippel-Lindau disease which is a familial form of clear cell RCC. It is a rare autosomal dominant disease affecting 1 in 36,000 live births. There are 3 major manifestations of the disease and these are clear cell RCC, haemangioblastoma of the retina and cerebellum and phaeochromocytoma. It is characterized by inheritance of a mutated VHL tumour suppressor gene on the short arm of chromosome 3 (3p25-26).

The main function of the VHL gene is to target the hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) for ubiquitin-mediated degradation, ensuring levels of HIFs are kept low under normal conditions. Inactivation or mutation of the VHL gene therefore results in inappropriate HIF 1 and 2 up-regulation and accumulation in cells under normoxic conditions. This subsequently causes

up-regulation of vascular endothial growth factor (VEGF), which is the primary growth factor in RCC (Wein et al. 2012).

The VHL pathway is illustrated in figure 1.1 below.

Knowledge of the molecular basis of VHL has enabled the development of Sunitinib, which is a tyrosine kinase inhibitor. Specifically sunitinib inhibits VEGF and platelet derived growth factor (PDGF). Motzer et al. 2006 found that in 106 patients who underwent radical nephrectomy and then treatment with Sunitinib there was an overall response rate of 33% with a 14 month median duration of response. Median survival progression free survival was 8.8 months and overall survival was 23.9 months.

Figure 1.1 The VHL and HIF Pathway under normal conditions and in RCC, showing the part of Sunitinib's place in the pathway.
(Taken from : Marston Linehan W & Srinivasan R (2013). Targeted therapies: treating advanced kidney cancer-miles to go before we sleep. Nature reviews Clinical Oncology 10, 614-615.
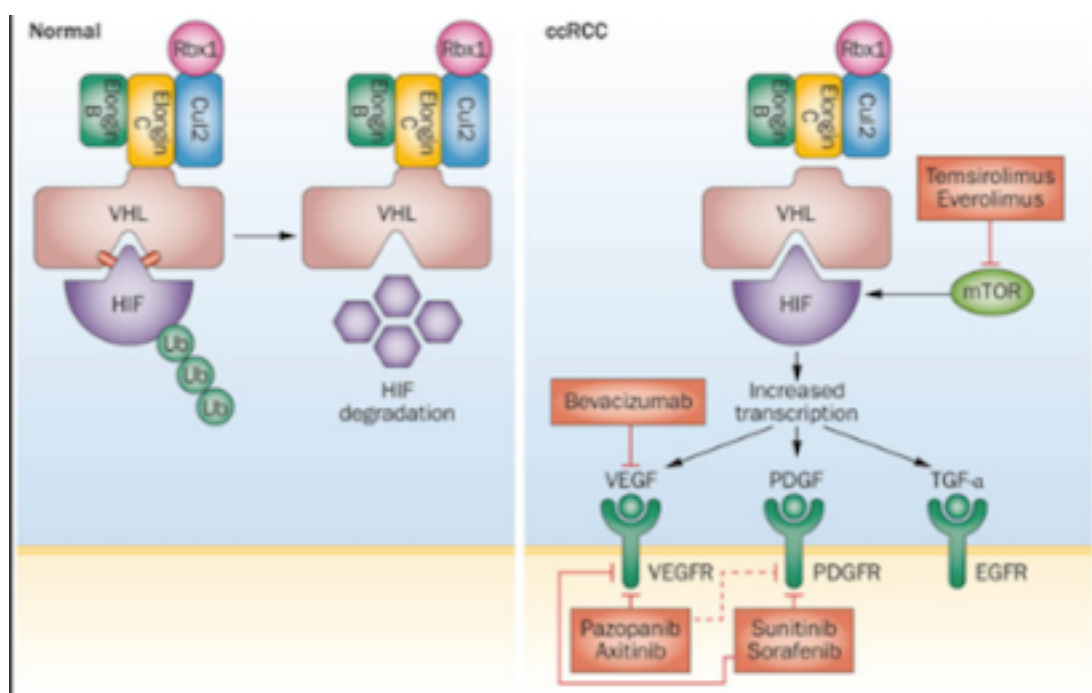
1.3 Biomarkers

There has been increasing interest in recent years around personalized medicine and management of diseases. Biomarkers have become an important tool in this process. There are different categories of markers that play a role in different stages of the disease process.

Diagnostic markers are parameters that can aid in the diagnosis of diseases. For example it has been proposed that sputum minichromosome maintenance (MCM) immunocytochemistry could be used in the diagnosis of lung cancer (Ramnath et al. 2001, Hashimoto et al. 2004).

Prognostic markers refer to a marker that provides information on the likely course of the cancer disease in the untreated individual. For example, a prognostic marker could identify those patients with cancers most likely to recur after initial surgical treatment for example and therefore those who would benefit from adjuvant systemic treatment.

Predictive biomarkers refer to markers that can be used to select the therapy with the highest likelihood of success in a given individual patient. They help identify subgroups of individuals who are most likely to respond to a particular therapy. Therefore predictive biomarkers form the basis for personalized or individualized therapy. For example, oestrogen and progesterone receptors are used to predict whether or not a patient will respond to endocrine therapy in breast cancer. Her2 status is used to predict if a patient will respond to Herceptin and KRAS mutation is used to predict whether a patient will demonstrate resistance to EGFr antibody therapy.

Autotaxin has the potential to be a valuable biomarker.

1.4 Autotaxin

Autotaxin (ATX), encoded by the ENNP2 gene, is a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase enzyme family (NNP). It was first reported in 1992 when it was identified as a tumour cell motility factor in a melanoma cell line (Stracke et al. 1992). It is a 125-kilodalton glycoprotein and is present in biological fluids such as cerebrospinal fluid (CSF), malignant ascites and blood (Fotopoulou et al. 2010). It is encoded by a single gene on human chromosome 8 and has 3 different isoforms; α, β, and γ (Tania et al. 2010). It is synthesized as a pro-enzyme and secreted into the extracellular space (Jansen et al. 2005). Autotaxin is then thought to communicate and influence target cells via integrins (Houben et al. 2011). It is found in higher concentrations in the blood of women (0.625-1.323mg/L) than in men (0.438-0.914mg/L) (Nakamura et al. 2008) and has been shown to play a role in several diseases and conditions including obesity (Ferry et al. 2003), rheumatoid arthritis (Bourgoin et al. 2010), chronic pain (Inoue et al. 2008a, Inoue et al. 2008b), and cancer (Okudaira et al. 2010). Recent studies have suggested that ATX expression in cancer cells has a direct role in promoting bone metastases and that by silencing ATX expression in these cells bone metastases are reduced without reduction in tumour volume (David et al. 2010).

Autotaxin is active in the lysophosphatidic acid (LPA) pathway (figure 1.2) and is responsible for the conversion of lysophosphatidyl-choline (LPC) to LPA.

LPA is a phospholipid derivative that acts as a signaling molecule. It was first identified in ovarian cancer by Sedlakova et al. in 2006. They reported plasma LPA levels to be higher in patients with ovarian cancer compared with controls with no ovarian pathology and patients with benign ovarian disease

(Sedlakova et al 2008). They also found that plasma LPA levels were associated with stage and histological type of ovarian cancer and concluded that LPA could be used as a useful marker for ovarian cancer especially the early stages of the disease.

LPA elicits a variety of responses by binding to one or more of 6 specific G protein-coupled receptors, (encoded by LPAR genes 1-6), which in turn initiate subsequent cell signaling pathways resulting in cell proliferation, survival, motility, invasion, and angiogenesis. As such, aberrant LPA signaling has been linked to various types of cancer.

The role of each of the LPA receptors is not clear, however loss of LPA_6 function has been implicated in the development of bladder cancer, suggesting that LPA_6 may be a tumour suppressor (Mills et al. 2003, Lee et al. 2007, Houben et al. 2011).

Similarly, LPA_1 mRNA expression was found to be significantly higher in muscle invasive bladder cancer specimens than in non-muscle invasive specimens (Kataoka et al. 2014) and strong LPA_1 expression was reported on the cell membranes in muscle invasive specimens.

Renal cancer is a highly vascular tumour thought to be dependent on aberrant angiogenesis (Su et al. 2013).   Su et al. showed that autotaxin was exclusively expressed in RCC tumour vessels but neither in tumour cells nor in normal renal capillaries. They also found that autotaxin, produced by RCC endothelium, acted through LPA signaling to promote tumourigenesis so they proposed a role for the autotaxin-LPA signaling pathway in renal cancer progression.

VEGF signalling pathway has also been associated with renal angiogenesis and is the target for Sunitinib, which is the standard treatment for advanced RCC. Su et al. (2013) looked at the development of acquired resistance against Sunitinib in RCC and the role of autotaxin in it. They found that

autotaxin expression was increased in the tumour vasculature of RCC but decreased with the treatment of Sunitinib. They proposed a role for the autotaxin-LPA signaling pathway in the acquired resistance of RCC to Sunitinib through targeting tumour cell and vascular interactions.

Therefore, it is clear that autotaxin is an essential component in the LPA pathway and that a major function of that pathway is tumour cell survival and invasion, and angiogenesis. The potential role of autotaxin as an attractive therapeutic target is obvious.

In this thesis the relation between autotaxin expression and the clinico-pathological variables in two cohorts, bladder and renal cancer, was investigated.
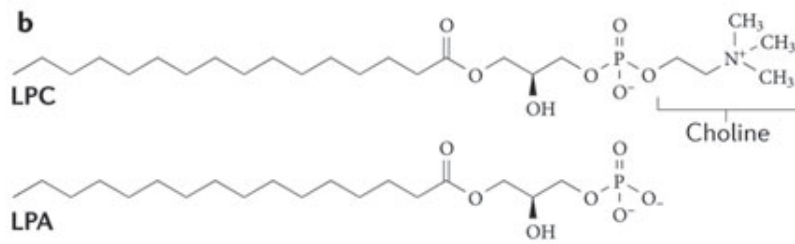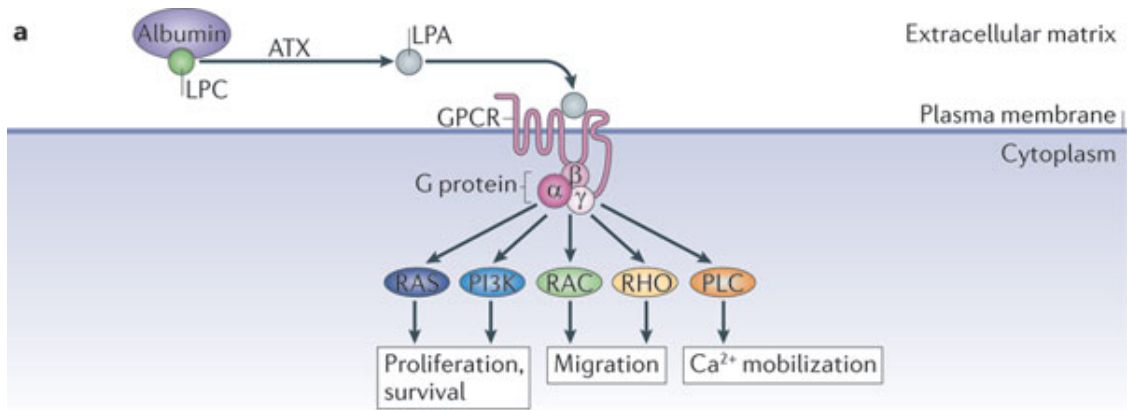
Figure 1.2 The LPA Pathway
Taken from Woolenaar WH & Perrakis A (2011).

Materials and Methods

This chapter describes the basic laboratory techniques used. Any modification to these materials and methods have been specified in the relevant chapter.

2.1 Materials

All tissue samples used in these studies were from archival, formalin fixed paraffin embedded tissue blocks, Department of Pathology, University Hospital Birmingham. Tissue sections were cut at 4µm on a rotatory microtome, placed onto charged slides (PFM, UK) and heated in a 60⁰C incubator for 1 hour prior to use.

2.2 Immunohistochemistry

Two related immunohistochemistry approaches were used. Both gave similar results.

2.2.1 Citrate Buffer Retrieval Method

Paraffin embedded sections of bladder or renal cancer tissue were baked in the oven at 60 degrees for 1 hour. The citrate buffer was made using 1.26g sodium citrate, 0.25g citric acid, and 1L H_2O. The pH was adjusted to pH6 with concentration of NaOH. The water bath was heated to a temperature of 96 degrees and the container of citrate buffer was placed in the water bath. The slides were immersed in Histoclear for 10 minutes then IMS for a further 10 minutes. They were then rinsed with water for 5-10 minutes. Slides were immersed in hydrogen peroxide 0.3% (1:100 dilution of 30% H_2O_2 stock in

dH$_2$O) for 10 minutes and then rinsed with water for a further 5-10 minutes. The slides were then placed in the hot citrate buffer for 20 minutes.

Following antigen retrieval slides were taken out and allowed to cool for 30 minutes -1 hour and then rinsed in water for 10 minutes.

Using a marker pen a circle was carefully drawn around the tissue. Slides were placed in Tris buffered saline pH 7.6 (TBS) for 5 minutes then the section of tissue was covered with horse serum 2.5% for 30-40 minutes. The liquid was removed from the slides and then the tissue was covered with primary, rat monoclonal antibody 2B4 (1:200) against autotaxin (1μL of abx: 200μL of PBS) on each slide (200μl per slide) and placed in the fridge overnight at 4 degrees. Following this the slides were rinsed in TBST for 20-30 minutes and then the secondary antibody (rabbit anti-rat (Vector, UK) at 1/200 dilution) was applied for 10 minutes. The slides were once again rinsed with TBS for 10 minutes and then the tertiary reagent was applied for 30 minutes, then rinsed with TBST for a further 30 minutes.

The immunostaining procedure was then visualized with 3,3-diaminobenzidine (DAB) (30μl in 1 ml dilutent, putting 150-200μl on each slide for 1 minute) according to the manufacturer's instruction. They were then rinsed with water for 5 mins, haemotoxylin for 1.5 mins, cold water for 2 mins, hot water for 2 mins, cold water again for 2 min, IMS for 10 mins, then histoclear for 10 mins. Following this the tissue was mounted using DPX mountant.

2.2.2 The ALTER Method

The second retrieval method involved de-paraffinising the sections and rehydrated to water as previously described (2.2.1). After a low temperature retrieval technique, ALTER (Reynolds *et al* 2002) immuno-staining was performed on a Dako Autostainer. In brief this comprised of a 10-minute endogenous peroxidise block (Dako, UK) followed by a 10-minute protein block in 2% casein (Vector Labs, UK). Sections were then incubated in optimally diluted autotaxin antibody for one hour, rabbit anti-rat (Vector Labs, UK) at 1/200 dilution for 10 minutes, Vector ImmPRESS rabbit secondary for 30 minutes and visualised in NovaRED chromagen (Vector Labs, UK) for 5 minutes. All buffer washes were performed with EnVision FLEX wash buffer (Dako, UK). Sections were counterstained with Meyers haematoxylin, dehydrated and mounted with a glass coverslip in DPX.

2.3 Scoring of Autotaxin Expression

After staining was completed, the cases were interpreted by two independent observers, a consultant histopathology and myself. They were scored according to whether autotaxin expression was 'strong', 'weak', or 'negative'. Examples of each category are shown in Figure 2.2.1a-d and 2.2.2a-c in the bladder and renal chapters respectively. None of the renal cancer tumours were positive for autotaxin, but the tumour endothelium was, so expression in figures 2.2.2a-2.2.2c refers to vessels only.

2.4 Databases

2.4.1 Bladder Database

The bladder database, as seen in Figure 2.3.1 below, was initially created as part of the West Midlands bladder cancer prognosis programme (BCPP). It includes extensive epidemiological information on over 1000 patients with bladder cancer within the West Midlands. In this study a smaller subset of the original database was used to populate a new database.

Among some of the variables included in the database were gender, date of birth, age at recruitment, disease status at death, cause of death, grade and stage of disease, marital status, ethnicity, smoking status, and other medical comorbidities such as depression, myocardial infarction, arrhythmias, pulmonary emboli, stroke and diabetes and date of recurrence of disease. Paraffin embedded tissue blocks were available for all patients in the database. Blocks were retrieved and fresh sections cut before performing immunohistochemistry.

The database was then updated and new information regarding autotaxin expression in this cohort was added.

Figure 2.3.1 – Screenshot of the bladder database

2.4.2 Renal Database

The renal database (seen in Figure 2.3.2 below) was initially created to establish epidemiological information of a cohort of metastatic renal cell carcinoma patients who were being treated with Sunitinib. It included 59 patients, 14 females and 45 males with a median age of 60 years. Renal biopsies were taken from each patient prior to commencing treatment with Sunitinib. Paraffin embedded tissue blocks were available for all patients. Blocks were retrieved and fresh sections cut in preparation for immunohistochemistry.

The database was updated as part of the current piece of work and new information was gathered. This involved retrieving information from hospital notes, letters and on-line databases.

Among some of the variables included in the database were gender, age, grade and stage of disease, date of starting Sunitinib treatment, vascular invasion of the tumour, fat necrosis, sarcomatoid component, heng score, date of last follow-up, VEGF-R status, SPHK1 status, and CA-9 status.

All patients had their histology reviewed by a consultant pathologist, date of last follow-up was updated as well as date of death, and autotaxin expression established by immunohistochemistry was added.

Histology date	type of sample	Date of starting sunitinib	histotype	Grading	vascular invasion	fat invasion	T	N	necrosis	sarcomatoid component
26/01/10	pancreas and spleen	30/06/06	0	2	x	na	na	na	2	0
31/03/05	retroperitoneal mass	30/06/06	0	3	x	na	na	na	2	0
13/06/06	left kidney	30/06/06	0	3	1	1	3	x	2	0
03/03/06	left kidney	30/06/06	5	3	1	1	3	1	1	0
13/02/02	right kidney	30/06/06	0	1	1	0	1	x	2	0
08/05/00	tumorectomy (left kidney)	30/06/06	0	1	1	1	x	0	2	0
08/05/06	right kidney	30/06/06	0	3	1	0	3	x	2	0
16/02/06	left kidney	04/07/06	4	3	1	1	3	x	1	0
18/10/04	right nephrectomy	04/07/06	1	3	1	1	3	2	1	0
04/05/01	right kidney	04/07/06	0	1	1	0	1	x	2	0
24/12/07	left kidney	04/07/06	0	3	1	1	3	0	1	0
28/12/05	right kidney	06/07/06	4	3	x	1	3	?	1	0
11/01/07	right kidney	06/07/06	0	4	0	0	1	x	2	50%
03/03/06	right kidney	06/07/06	0	3	1	1	3	x	1	0
16/05/00	left kidney	11/07/06	0	1	0	1	3	x	2	0
18/10/05	right kidney	13/07/06	0	2	0	0	1	x	2	0
16/01/06	left kidney	13/07/06	0	4	1	1	3	0	2	80%
03/04/06	left kidney	11/07/06	4	4	0	1	3	x	2	focal
05/12/05	left kidney	13/07/06	0	4	1	1	3	0	1	0
20/08/02	left kidney	18/07/06	4	x	x	x	x	x	x	0
03/04/06	right kidney	18/07/06	0	2	0	0	3	x	2	0
17/05/05	right kidney	17/08/06	1	3	1	1	3	1	2	0
25/07/05	bladder	01/08/06	0	2	na	na	na	na	2	0
07/06/05	left kidney	01/12/06	0	3	0	0	1	x	2	0
09/12/05	scalp	05/04/07	0	x	na	na	na	na	2	0
21/05/02	left kidney	15/05/07	1	x	0	0	2	0	2	0
26/07/04	left kidney	18/05/07	0	2	1	0	1	x	0	0
23/05/08	left kidney	29/11/07	0	2	0	1	3	x	0	0
24/02/03	tumorectomy (VHL snd)	24/08/07	0	1	0	na	na	na	2	0
24/04/06	left kidney	29/01/08	0	3	1	1	3	x	2	0

Figure 2.3.2 – Screenshot of the renal database

2.5 Statistical Analysis

All quantifiable data were subjected to statistical analysis in the IBM SPSS Statistics (version 21). The advice of an experienced statistician was sought to guide the appropriate use of tests. *P* values were taken to indicate a significant difference between the data sets when the *P* was below 0.05.

Pearson's Chi- Square test is used to decipher whether or not a significant relationship existed between two categorical variables. Essentially it measures how well the observed distribution of data fits with the distribution expected if the variables are independent. It is suitable for unpaired data and large samples.

Linear-by Linear Association test considers the trend of the data and indicates the strongest association between two variables. It represents any relationship between two variables that depict a straight line when plotted next to each other in a graph. It is used for ordinal categorical data sets.

When numbers were small *Fischer's Exact Test* was used rather than Pearson's Chi-Square. It assesses whether or not two classifications are associated. It is used with categorical data.

Kappa statistic and coefficient is a statistic that measures inter-rater agreement for qualitative, categorical items. It was used to assess the association between autotaxin expression in vessels compared with expression in tumour.

When considering the relationship between autotaxin expression and survival *log rank tests* were used. Log rank tests compare the survival times between two or more groups (non-parametric), such as strong and weak autotaxin expression. The null hypothesis for a log rank test is that the groups have the same survival.

Kaplan-Meiers estimate is used to measure the fraction of subjects living for a certain time after a treatment/event. The starting point in this piece of work was histology date and the terminating event was time to death. The resulting Kaplan-Meier product was plotted creating a survival curve.

Multivariate survival analysis was also undertaken. This technique is used to analyze data that may arise from more than one variable, for example, gender, disease grade and stage, lymph node status and number of metastatic sites. Multivariable analysis was used with the renal cohort but not with the bladder cohort as the number of positive stainers was too small. Univariate analysis on the bladder cohort did not show any significant effects for autotaxin and therefore multivariate analysis was considered unnecessary.

Expression of Autotaxin in Relation to Clinico-pathological Finding in a Cohort of Bladder Cancer Patients

3.1 Introduction

In the U.K. bladder cancer is the fourth most common malignancy in males and the 12th most common in females. It affects >10,000 individuals each year (www.statistics.gov.uk). It is the most common tumour occurring in the urinary tract and accounts for one in every 28 new cases of cancer every year (Zeegers et al. 2009). Bladder cancer is responsible for > 5,000 deaths each year in the UK (ONS). Both incidence and mortality rates increase with age and given the ageing population bladder cancer will remain an important health problem.

The majority of bladder cancers (70%) are either confined to the lamina propria or superficial to this i.e., non-muscle invasive. These patients undergo extensive life-long follow-up with cystoscopy. While many patients find on going surveillance reassuring others find it stressful. Schoever et al. (1987) reported adverse psychological impact caused by long-term cystoscopic follow-up and intravesical treatment.

In terms of healthcare expenditure, bladder cancer is the 5th most expensive cancer to treat in the U.S. (Botteman et al. 2003). Furthermore, among all cancers treated with in the Medicare system it has been found to have the highest cost per patient from diagnosis to death (Riley et al. 1995). Cost of patient management for bladder cancer in the UK has previously been found

to be more expensive than other cancers for example, prostrate cancer (Sangar et al. 2005).

However, it is not only the cost of treating bladder cancer that is the problem, some of the current treatments risk significant side effects. BCG or Bacillus Calmette-Guerin for example, was first used as a treatment for bladder cancer by Morales in 1976. Its role is in the treatment of intermediate and high-risk non-muscle invasive bladder cancer. It is live attenuated Mycobacterium bovis. Although not well understood, the mechanisms of action are thought to include the attachment of BCG to the urothelium via the fibronectin receptor which is then internalized within the bladder cancer cell. It is also thought to promote the secretion of cytokines and chemokines, and the presentation of BCG and/or cancer cell antigens to cells of the immune system (Redelman-Sidi et al. 2014).

Sylvester et al. (2002) meta-analysis of 24 randomised trials involving 4863 patients with non-muscle invasive bladder cancer found that an induction followed by maintenance of BCG following initial resection of bladder tumour resulted in a relative risk reduction of disease progression of 27%. Common side effects experienced when having BCG treatment for bladder cancer include urinary frequency, dysuria, malaise and mild fever for up to 24 hours after treatment. Less common but more serious side effects include arthralgia, rash, headache, and high fever for at least 48 hours. These symptoms suggest BCG sepsis and require urgent medical attention and anti-tuberculosis therapy as well as steroids.

A second treatment currently used for non-muscle invasive bladder cancer is mitomycin C (MMC). It is an anti-tumour antibiotic and its mechanism of action involves cross-linking complementary DNA strands and alkylates in bladder tumour cells.

Sylvester et al. (2004) found that a single post-operative dose of intravesical MMC within the first 24 hours following bladder tumour resection resulted in a 39% decrease in the relative risk of recurrence with adjuvant treatment. Side effects of MMC include irritation to the skin if it comes in contact, urinary tract infections with symptoms of frequency, dysuria and systemic upset, and if it is inadvertently given in the presence of a bladder perforation the MMC can then be absorbed into the blood stream and results in haematological abnormalities as well as pain and discomfort with in the peritoneal cavity.

Therefore, current treatment of bladder cancer not only poses a financial burden but also can lead to serious side effects to the patient.

One potential therapeutic target in bladder cancer is autotaxin. Autotaxin is an enzyme that converts LPC to LPA. Little is known about the expression of autotaxin and if it is involved in the pathogenesis of bladder cancer. Lee et al. (2007) found a link between familial bladder cancer and the LPA_6 receptor but literature search found no other report of either autotaxin expression or LPAR expression in bladder cancer.

Were autotaxin shown to be expressed then autotaxin inhibitors might be considered in the treatment of patients with bladder cancer.

The work set out in this chapter aimed to describe autotaxin expression in bladder cancer and its relationship to clinico-pathological observations.

3.2 Materials and Methods

New sections were made from paraffin embedded blocks available from the BCPP cohort. An antibody targeting autotaxin was validated using tonsil samples and was then used to stain the bladder sections for autotaxin expression. Immunohistochemistry was performed using the methods set out in the Materials and Methods chapter.

3.3 Results

I first performed a pilot study comprising 124 cases of bladder cancer to establish the prevalence of autotaxin expression and to determine if analysis of a larger cohort was likely to produce meaningful results.

3.3.1.1 Autotaxin Expression and Association with Stage of Bladder Cancer

32/124 cases expressed autotaxin within tumour cells. pT2 tumours were more likely to show strong staining (27% versus 7%) however the significance tests (both for association and for linear trend) showed a trend to statistical significance with p=0.060 and p=0.069 respectively.

Stage		ATX Staining NONE	ATX Staining WEAK	ATX Staining STRONG	Total
pTa	Count	53	11	5	69
	% within stage	76.8%	15.9%	7.2%	100%
pT1	Count	24	3	2	29
	% within stage	82.8%	10.3%	6.9%	100%
pT2	Count	15	4	7	26
	% within stage	57.7%	15.4%	26.9%	100%
Total	Count	92	18	14	124
	% within stage	74.2%	14.5%	11.3%	100%

Table 3.3.A – Autotaxin Staining vs. Tumour Stage

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	8.886	4	0.064	*0.060*
Linear-by-Linear Association	3.312	1	0.069	*0.069*
N of Valid Cases	124			

Table 3.3.B – Statistical Analysis of Autotaxin Staining vs. Tumour Stage

Next, the impact of autotaxin expression on presence or absence of tumour invasion into muscle was examined. Muscle invasion is an important prognostic factor with muscle invasion indicating a poorer prognosis.

77/92 (78.6%) patients with non-muscle invasive disease pTa/pT1 showed no autotaxin expression compared with 15/26 (57.7%) of patients with muscle invasive tumours pT2. 7/98 (7.1%) of non-muscle invasive pTa/pT1 showed strong autotaxin expression compared with 7/26 (26.9%) of muscle invasive pT2. When pT2 (invasive) were compared with pTa and pT1 combined, both an association and a trend were significant (p=0.015 and p=0.009). This suggests that autotaxin expression is associated with muscle invasion and as such autotaxin could be a marker of a poorer prognosis in bladder cancer.

		ATX staining NONE	ATX Staining WEAK	ATX Staining STRONG	Total
Non-Muscle Invasive (pta & pT1)	Count	77	14	7	98
	% within NMI	78.6%	14.3%	7.1%	100%
Muscle Invasive (pT2)	Count	15	4	7	26
	% within MI	57.7%	15.4%	26.9%	100%
Total	Count	92	18	14	124
	%	74.2%	14.5%	11.3%	100%

Table 3.3.C - Autotaxin Staining vs. Presence of Absence of Muscle Invasion

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	8.345	2	0.015	*0.015*
Linear-by-Linear Association	7.339	1	0.007	*0.009*
N of Valid Cases	124			

Table 3.3.D – Statistical Analysis of Autotaxin Staining vs. Presence or Absence of Muscle Invasion

Furthermore, when comparing the relationship between muscle invasion and autotaxin expression following re-grouping in to 'none' staining versus 'weak/strong' staining, 77/97 (78.6%) of non-muscle invasive group showed no autotaxin expression compared with 15/26 (57.7%) of the muscle invasive group. 21/98 (21.4%) of the non-muscle invasive group showed weak/strong autotaxin expression compared with 11/26 (42.4%) of the muscle invasive group. The association between muscle invasiveness and the weak/strong staining group was significant (p=0.031).

		ATX Staining NONE	ATX Staining WEAK/STRONG	Total
Non-Muscle Invasive	Count	77	21	98
	% within non-muscle invasive	78.6%	21.4%	100%
Muscle Invasive	Count	15	11	26
	% within muscle invasive	57.7%	42.4%	100%
Total	Count	92	32	124
	%	74.2%	25.8%	100%

Table 3.3.E - Autotaxin Staining vs. Dichotomized Staining (none vs. weak/strong)

	Value	df	Asymp. Sign. (2-sided)
Pearson Chi-Square	4.679	1	*0.031*
N of Valid Cases	124		

Tables 3.3.F – Statistical Analysis of Autotaxin Staining vs. Dichotomized Staining (none vs. weak/strong)

Further re-grouping in to 'none/weak' and 'strong' groups then showed that 91/98 (92.9%) of non-muscle invasive cohort showed no/weak expression compared with 19/26 (73.1%) of muscle invasive. 7/93 (7.1%) of non-muscle invasive cohort showed strong expression compared with 7/26 (26.9) of muscle invasive. This association was found to be significant (p=0.01). This result once again supports the contention that autotaxin could be a marker of muscle invasiveness in bladder cancer and therefore of poorer prognosis.

		ATX Staining NONE/WEAK	ATX Staining STRONG	Total
Non-Muscle Invasive	Count	91	7	98
	% within non -muscle invasive	92.9%	7.1%	100%
Muscle Invasive	Count	19	7	26
	% within muscle invasive	73.1%	26.9%	100%
Total	Count	110	14	124
	%	88.7%	11.3%	100%

Table 3.3.G - Autotaxin Staining vs. Dichotomized Staining (None/Weak vs. Strong)

	Value	df	Asymp. Sign. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Fischer's Exact Test				*0.010*	0.010
N of Valid Cases	124				

Table 3.3.H – Statistical Analysis of Autotaxin Staining vs. Dichotomized Staining (None/Weak vs. Strong)

3.3.1.2 Autotaxin Tumour Expression versus Grade of Tumour

Tumour grade, based on the 1973 WHO classification discussed above, describes how abnormal the tumour cells are compared with normal cells when viewed under the microscope. It is an indicator of how the tumour will behave, how quickly it will grow and spread. If the tumour cells morphologically resemble normal cells then the tumour is called 'well-differentiated'. If the tumour cells are very different to normal cells, the tumour is called 'poorly differentiated'. Based on this a numerical grade is given to the cancer.

Tumour grade was compared with autotaxin expression. Although the percentage of cases strongly staining increased with grade (7%, 11%, 14%), this linear association was not significant (p=0.532).

Grade		ATX Tumour Staining NONE	ATX Tumour Staining WEAK	ATX Tumour Staining STRONG	Total
1	Count	23	6	2	31
	% with in grade	74.2%	19.4%	6.5%	100%
2	Count	22	3	3	28
	% with in grade	78.6%	10.7%	10.7%	100%
3	Count	48	9	9	66
	% with in grade	72.7%	13.6%	13.6%	100%
Total	Count	93	18	14	125
	%	74.4%	14.4%	11.2%	100%

Table 3.3.I – Autotaxin Tumour Staining vs. Grade of Tumour

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Linear-by-Linear Association	0.423	1	0.515	*0.532*
N of Valid Cases	124			

Table 3.3.J- Statistical Analysis of Autotaxin Tumour Staining vs. Grade of Tumour Significance Values

3.3.1.3 Tumour Grade versus Autotaxin Expression (Dichotomized, None/Weak vs. Strong)

Tumour grade was considered in relation to autotaxin expression when groups were separated into 'none/weak' and 'strong' expression.

29/31 (93.5%) of G1 cases showed none/weak expression compared with 25/28 (89.3) of G2 and 57/66 (86.4%) of G3.

2/31 (6.5%) of G1 cases showed strong expression compared with 3/28 (10.7%) of G2 and 9/66 (13.6%) of G3.

No significant association or trend was observed (p=0.576, p=0.320 respectively).

Grade		ATX Staining NONE/WEAK	ATX Staining STRONG	Total
1	Count	29	2	31
	% with in grade	93.5%	6.5%	100%
2	Count	25	3	28
	% with in grade	89.3%	10.7%	100%
3	Count	57	9	66
	% with in grade	86.4%	13.6%	100%
Total	Count	111	14	125
	%	88.8%	12.2%	100%

Table 3.3.K- Grade vs. Autotaxin Staining (Dichotomized, None/Weak vs. Strong)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson's Chi-Square	1.103	2	0.576	*0.566*
Linear-by-Linear Association	1.085	1	0.298	*0.320*
N of Valid Cases	124			

Table 3.3.L- Statistical Analysis of Grade vs. Autotaxin Staining (Dichotomized, None/Weak vs. Strong) Significance Values

3.3.2 The Expanded Cohort

Having demonstrated an association between autotaxin expression and muscle invasiveness in the pilot cohort, an extended cohort was assessed. The pilot 124 cases were supplemented with a further 241 to achieve a cohort of 365 cases. 50 cases were excluded for technical reasons including high background and poor section quality. Because I had also observed variable staining of vessels in the initial cohort, vessel staining was scored in all cases. Autotaxin staining was available for 316 (vessels) and 315 (tumours). There were no CIS or T3-4 included in the cohort and therefore analysis only included Ta-T2.

In the pilot cohort of 124 cases autotaxin staining was observed in 79.4% (vessels) and 25.6% (tumour) of the study subjects.

When the cohort was expanded to 365 cases the prevalence of the positive staining fell to 71.2% (vessels) and 12.7% (tumour).

	Autotaxin - Vessels	Autotaxin -Tumour
None	28.8% (91)	87.3% (275)
Weak Staining	34.8% (110)	7.3% (23)
Strong Staining	36.4% (115)	5.4% (17)
Positive for Staining	71.2% (225)	12.7% (40)

Table 3.3.M – Summary of Staining Results for the Expanded Cohort

3.3.2.1 Autotaxin Expression in Tumour versus Stage

An association between autotaxin expression in tumour and stage was then evaluated. 128/146 (87.7%) of pTa showed no expression of autotaxin compared with 59/73 (80.0%) of pT2. While 4/146 (2.7%) of pTa showed strong expression compared with 9/73 (12.3%) of pT2. A p-value of 0.028 was achieved suggesting a significant association between autotaxin expression and stage of disease. However there was no significant trend towards stronger staining with higher stages (test for trend p=0.083) as one might expect and the effect disappeared when strong and weak staining are combined (p=0.165).

			ATX Tumour None	ATX Tumour Weak	ATX Tumour Strong	Total
Stage	pTa	Count	128	14	4	146
		% within stage	87.7%	9.6%	2.7%	100.0%
	pT1	Count	71	3	4	78
		% within stage	91.0%	3.8%	5.1%	100.0%
	pT2	Count	59	5	9	73
		% within stage	80.8%	6.8%	12.3%	100.0%
Total		Count	258	22	17	297
		%	86.9%	7.4%	5.7%	100.0%

Table 3.3.N Autotaxin Expression in Tumour vs. Stage.

I then tested for an association between autotaxin staining and grade of tumour however no significant association (p=0.487) or trend (p=0.577) between tumour grade and autotaxin expression was found.

When testing for an association between autotaxin expression and muscle invasion 199/244 (88.8%) of the NMI group showed no expression compared with 59/73 (80.8%) of MI tumours. 8/224 (3.6%) of NMI showed strong expression compared with 9/73 (12.3%) of MI.
The association (p=0.020) and trend (p=0.016) was found to be significant (Table 3.3.2.B). However it was no longer significant if strong and weak expression were combined (p=0.078) but was strengthened if weak and negative expression were combined (p=0.009), indicating that the degree of tumour expression is important. This shows that even within the expanded cohort, autotaxin may be an important prognostic marker of poor prognosis of bladder cancer.

		ATX Tumour None	ATX Tumour Weak	ATX Tumour Strong	Total
NMI	Count	199	17	8	224
	% within NMI	88.8%	7.6%	3.6%	100.0%
MI	Count	59	5	9	73
	% within MI	80.8%	6.8%	12.3%	100.0%
Total	Count	258	22	17	297
	%	86.9%	7.4%	5.7%	100.0%

Table 3.3.O Autotaxin Expression vs. Muscle Invasion.
p=0.020, test for trend p=0.016

		ATX Tumour None	ATX Tumour Weak/Strong	Total
NMI	Count	199	25	224
	% within NMI	88.8%	11.2%	100.0%
MI	Count	59	14	73
	% within MI	80.8%	19.2%	100.0%
Total	Count	258	39	297
	%	86.9%	13.1%	100.0%

Table 3.3.P Autotaxin Expression vs. Muscle Invasion. p=0.078

		ATX Tumour None/Weak	ATX Tumour Strong	Total
NMI	Count	216	8	224
	% within NMI	96.4%	3.6%	100.0%
MI	Count	64	9	73
	% within MI	87.7%	12.3%	100.0%
Total	Count	280	17	297
	%	94.3%	5.7%	100.0%

Table 3.3.Q Autotaxin Expression vs. Muscle Invasion. p=0.009

3.3.2.2 Association between Autotaxin Expression in Vessels, compared with Expression in Tumour

Whether or not there was a link between autotaxin staining of the vessels and of the tumour was also considered. However there was no evidence found to support such a correlation. Chi- square test (p=0.083) indicates no association, and the Kappa statistic of *0.024* indicates poor agreement between autotaxin expression in tumour and vessels.

			ATX vessels NONE	ATX vessels WEAK	ATX vessels STRONG	Total
ATX Tumour	None	Count	82	99	94	275
		% of total	26.0%	31.4%	29.8%	87.3%
	Weak	Count	3	8	12	23
		% of total	1.0%	2.5%	3.8%	7.3%
	Strong	Count	6	2	9	17
		% of total	1.9%	0.6%	2.9%	5.4%
Total		Count	91	109	115	315
		% of total	28.9%	34.6%	36.5%	100%

Table 3.3.R Association between Autotaxin Expression in Vessels, compared with Expression in Tumour

The tests previously performed for autotaxin tumour expression in the pilot cohort were then repeated on the expanded cohort for vessel expression and no association was found between autotaxin vessel staining and grade (p=0.351), stage (p=0.420) or muscle invasiveness (p=0.158).

			ATX Vessels NONE	ATX Vessels WEAK	ATX Vessels STRONG	Total
Stage	pTa	Count	43	48	55	146
		% within stage	29.5%	32.9%	37.7%	100.0%
	pT1	Count	19	26	33	78
		% within stage	24.4%	33.3%	42.3%	100.0%
	pT2	Count	22	31	21	74
		% within stage	29.7%	41.9%	28.4%	100.0%
Total		Count	84	105	109	298
		% within stage	28.2%	35.2%	36.6%	100.0%

Table 3.3.S Autotaxin Expression in Stage vs. Vessels. P=0.420

			ATX Vessels NONE	ATX Vessels WEAK	ATX Vessels STRONG	Total
Grade	G1	Count	21	19	29	69
		% within stage	30.4%	27.5%	42.0%	100.0%
	G2	Count	20	26	32	78
		% within stage	25.6%	33.3%	41.0%	100.0%
	G3	Count	44	59	47	150
		% within stage	29.3%	39.3%	31.3%	100.0%
Total		Count	85	104	108	297
		% within stage	28.6%	35.0%	36.4%	100.0%

Table 3.3.T Autotaxin Expression in Grade vs. Vessels. P=0.351

			ATX Vessels NONE	ATX Vessels WEAK	ATX Vessels STRONG	Total
Muscle invasion	NMI	Count	64	73	88	225
		% within NMI	28.4%	32.4%	39.1%	100.0%
	MI	Count	21	31	20	72
		% within MI	29.2%	43.1%	27.8%	100.0%
Total		Count	85	104	108	297
		%	28.6%	35.0%	36.4%	100.0%

Table 3.3.U Autotaxin Expression in Vessels vs. Muscle Invasiveness. P=0.158

3.3.2.3 The Association between Autotaxin Expression and Survival/Event-Free Survival

As the data demonstrated a possible association between expression of autotaxin and muscle invasiveness, I looked at whether this effect of autotaxin expression impacted on survival. However there was no significant effects of autotaxin tumour expression on survival.

No significant association was found between degree of staining, strong/weak compared with none, and overall survival in months (p=0.944) (Figure 3.1).
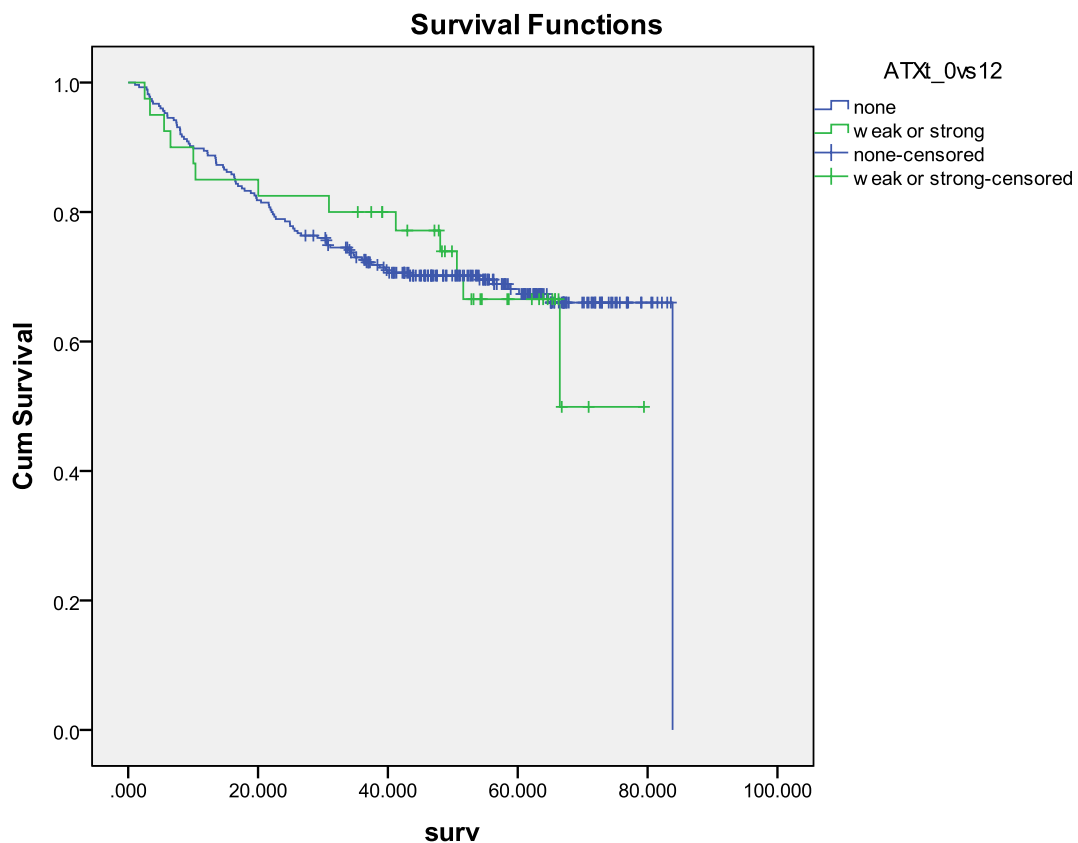

Figure 3.1 – Kaplan-Meier plot showing the association between autotaxin expression and survival/event-free survival (y axis). Overall survival in months (x axis). P=0.944

Staining	5-Year Survival	95% CI	No. Subjects	No. Deaths
Strong or Weak	66.5%	50.6% to 82.5%	40	13
None	68.1%	62.3% to 73.9%	275	87

Table 3.3.V– The Association between Autotaxin Expression and Survival/Event-Free Survival in Months.

Similarly, I found no significant association found between degree of staining and event free survival in months (p=0.724) (Figure 3.2).
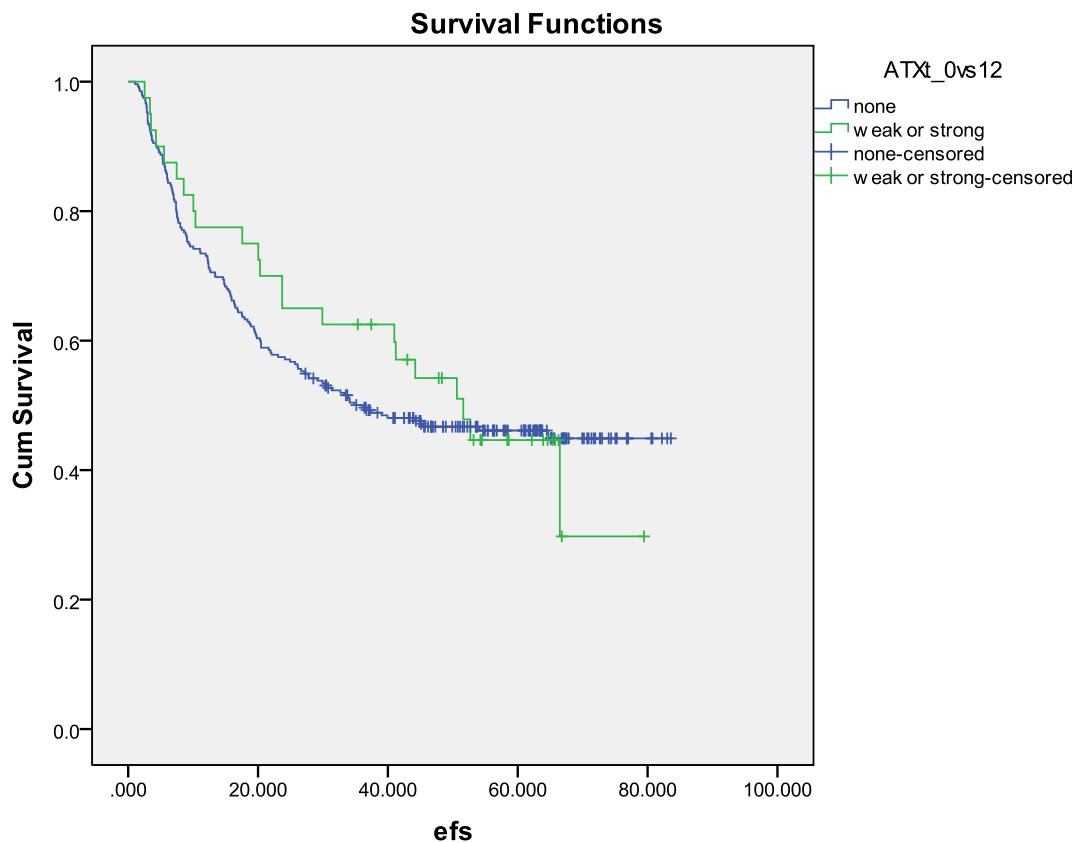
Figure 3.2 – Kaplan-Meier plot of event-free survival in months (x axis). Cumulative survival in % (y axis), P=0.724
(Events = first record of either disease recurrence, disease progression or death)

Staining	5-Year EFS	95% CI	No. Subjects	No. Events
Strong or Weak	44.6%	28.4% to 60.9%	40	22
None	46.1%	40.1% to 52.1%	275	147

Table 3.3.W – Table 3.3.2.5 – Strong or Weak Staining versus None; Event Free Survival in Months

Events	Strong or Weak Staining	No Staining
Disease Progression	4 (18%)	28 (19.0%)
Disease Recurrence	8 (36%)	60 (40.8%)
Death without Progression/Recurrence	10 (46%)	59 (40.1%)

Table 3.3.X – Disease Progression and Recurrence Free Survival

3.3.2.3.1 Strong Staining versus Weak or None

The association of autotaxin with survival was then considered after grouping staining into 'strong' versus 'weak or none' staining.

Despite the small numbers, there was a suggestion of poorer survival in strong stainers. This most probably resulted from the association of staining with higher disease stage.
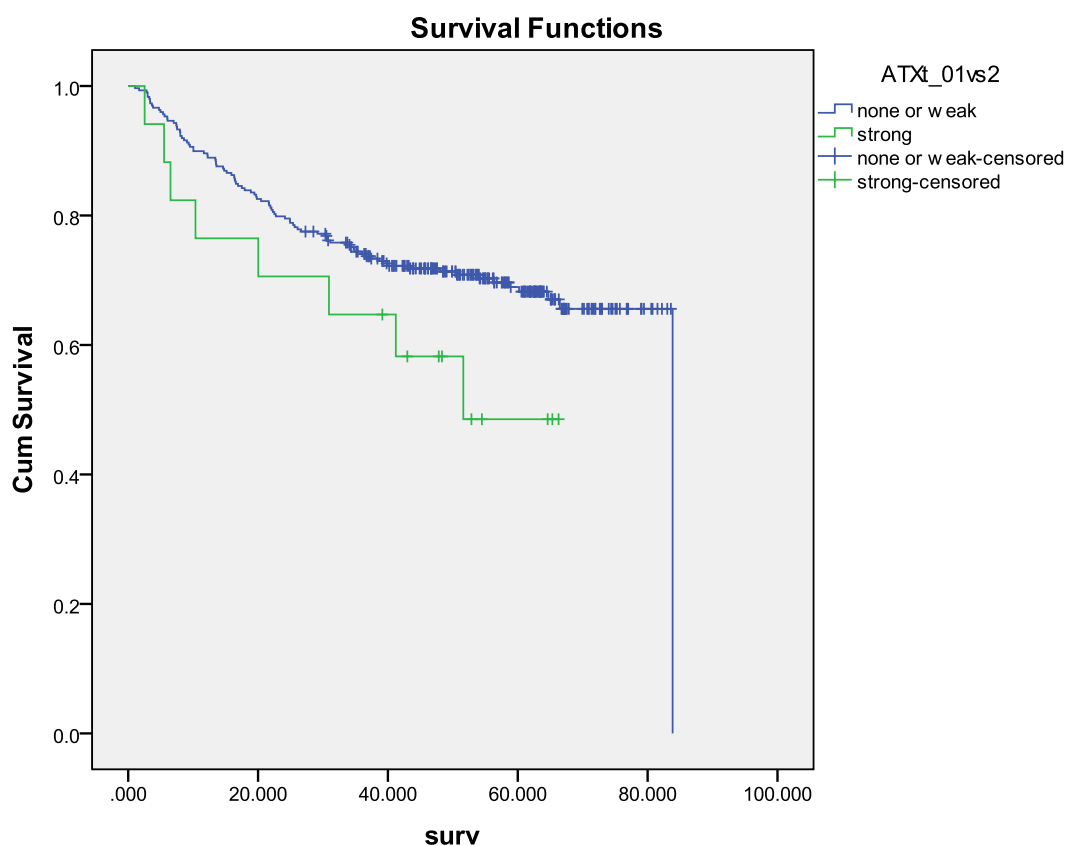
Figure 3.3 – Kaplan-Meier plot of overall survival in months (x axis). Cumulative survival as % (y axis). P=0.120
The sudden drop of the survival curve, seen in graph 3.3, after 80 months is an artifact caused by the death of the subject with the longest follow-up time.

Staining	5-Year Survival	95% CI	No. Subjects	No. Deaths
Strong	48.5%	22.2% to 74.8%	17	8
None/Weak	68.9%	63.4% to 74.5%	298	92

Table 3.3.Y – Strong Staining versus Weak/None; Overall Survival

The censor date was therefore re-set to 28/2/2013 rather than 31/12/2012, thereby increasing the follow-up time and eliminating the sudden drop of the survival curve as can be seen in figure 3.4.

It is worth noting that four further deaths were recorded in the extension period.

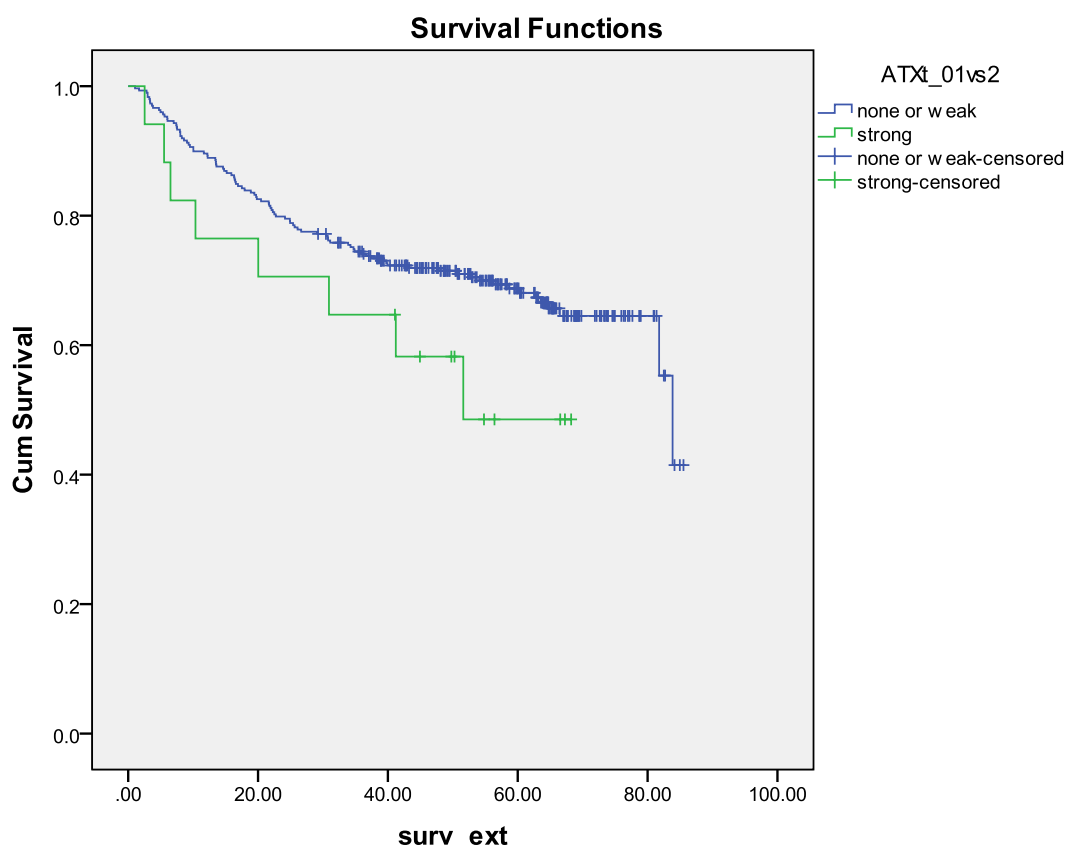Figure 3.4 – Kaplan-Meier plot of overall survival in months (x axis). Cumulative survival as % (y axis). p=0.145 –Log-rank Test (for equality of survival curves)

Staining	5-Year Survival	95% CI	No. Subjects	No. Deaths
Strong	48.5%	22.2% to 74.8%	17	8
Weak or None	68.8%	63.3% to 74.3%	298	96

Table 3.3.Z – Overall Survival in Months with Increased Follow-up data

The difference in survival is still not statistically significant, however some late deaths still occur in the 'none/weak' staining group.

Some survival times for the live cases, which are a measure of quality of follow-up, are shown below in table 3.3.3. Minimum survival in months for the 'strong' staining group, including 9 cases only, is 41.09 while maximum survival is 68.22 months. This is compared with the 'weak or none' staining group, including 202 cases, in which minimum survival in months is 29.24 and maximum survival is 85.53 months.

Staining	No. Cases	Minimum	Medium	Maximum
Strong	9	41.09	54.802	68.22
Weak or None	202	29.24	58.52	85.53

Table 3.3.1.A - Follow-Up Time in Months (Survival Time for Live Cases Only)

The "pattern of censoring" for the two groups is illustrated in graph 3.5 below. There appears to be no obvious difference in the quality of follow-up, but the non-staining group does appear to have a longer follow-up.
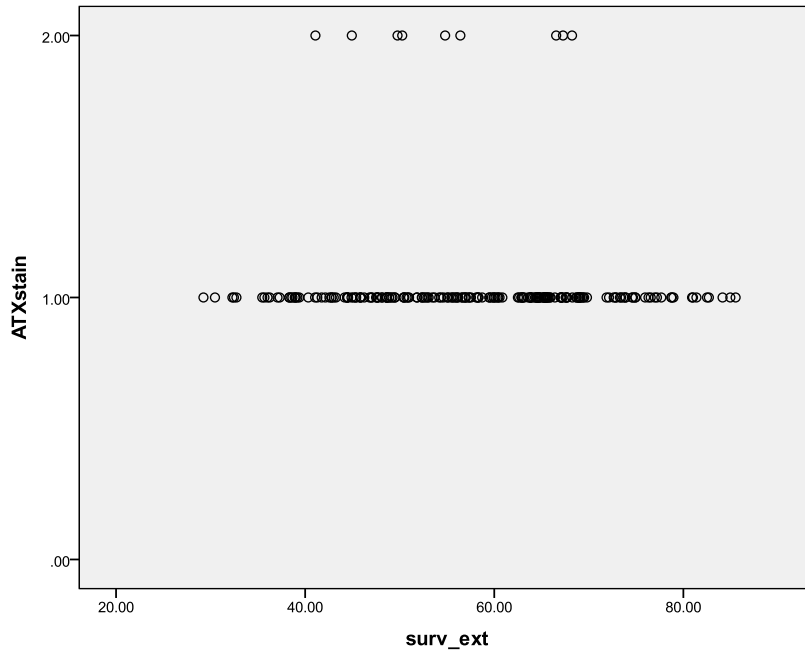
Figure 3.5 – Pattern of Censoring for the 2 groups of strong staining (2.00) vs weak/none (1.00), across survival in months.

To elucidate further whether or not longer follow-up time for the none/weak staining group has affected the results, follow-up time can be truncated at 69 months, i.e., any subjects with longer follow-up then 69 months are classed as "alive" at 69.00 months.

However, the p-value remains unchanged at p=0.145.

Considering event-free survival there was no significant association found between staining and event free survival, p=0.720, as illustrated in Figure 3.6 below.
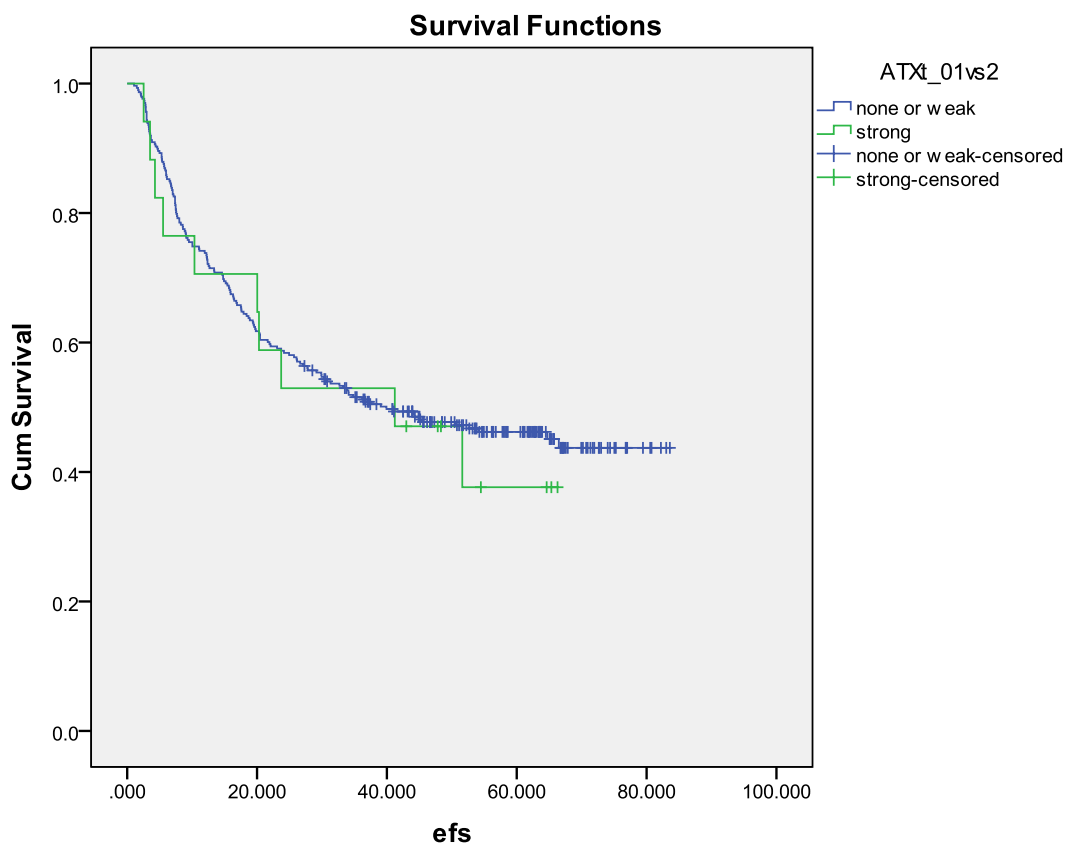
Figure 3.6 – Event-Free Survival in Months against cumulative survival as %, p=0.720

Staining	5-Year EFS	95% CI	No. Subjects	No. Events
Strong	37.6%	12.5% to 62.8%	17	10
Weak or None	46.2%	40.3% to 52.0%	298	159

Table 3.3.1.B– Increasing Follow-Up – Overall Survival in Months

Events	Strong Staining	Weak or None
Disease Progression	1 (10%)	31 (19.5%)
Disease Recurrence	3 (30%)	65 (40.9%)
Death without Progression/Recurrence	6 (60%)	63 (39.6%)

Table 3.3.1.C – Increasing Follow-Up – Disease Progression/Recurrence in Months

3.3.2.4. The Association of Disease Stage (Muscle Invasive or Non-Muscle Invasive) and Disease Progression.

BCPP defines "progression' as tumour recurring at a higher stage or grade than the original. Alternatively, the "standard" definition describes progression when a tumour becomes muscle-invasive, the original having been non-invasive (pTa and pT1)).

3.3.2.4.1 Progression-Free Survival versus Autotaxin Staining

Using the BCPP definition of progression, figure 3.7 shows progression-free survival in months in 280 none/weakly staining patients (who experienced 36 (13%) disease progression events) and 17 strongly staining patients (who experienced 1 (6%) disease progression events).

The strongly staining subjects appear to have fewer progressive events, but the log-rank test for equality of survival indicates no difference (p=0.455). Five-year progression-free survival is 93.8% (95% CI 81.9%-100%) for the 'strong' staining group and only 84.55% (95% CI 79.7 to 89.3%) for the 'none/weakly' staining group. The difference is particularly marked in the non-invasive subgroup.
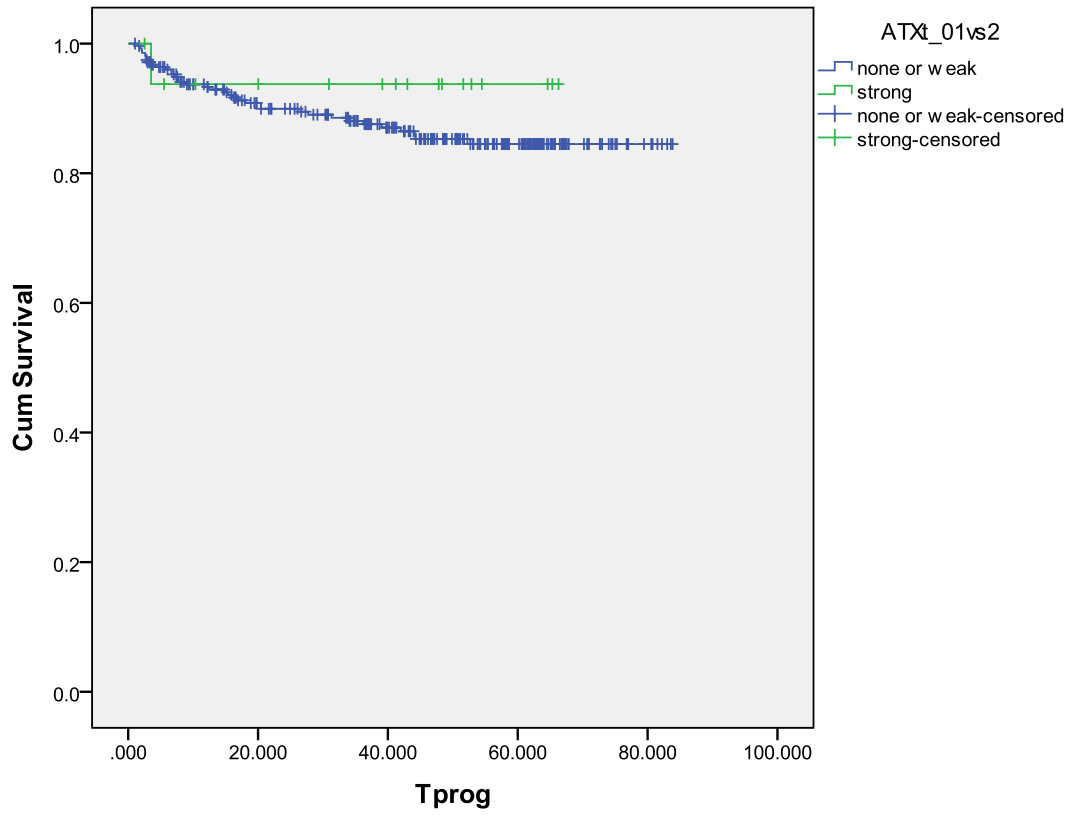
Figure 3.7 - Progression-Free Survival in months versus Autotaxin Staining

When considering only non-invasive tumours (pTa and pT1), which included 224 subjects and disease progression, the association was not significant with a p=0.280, as shown in Figure 3.8.
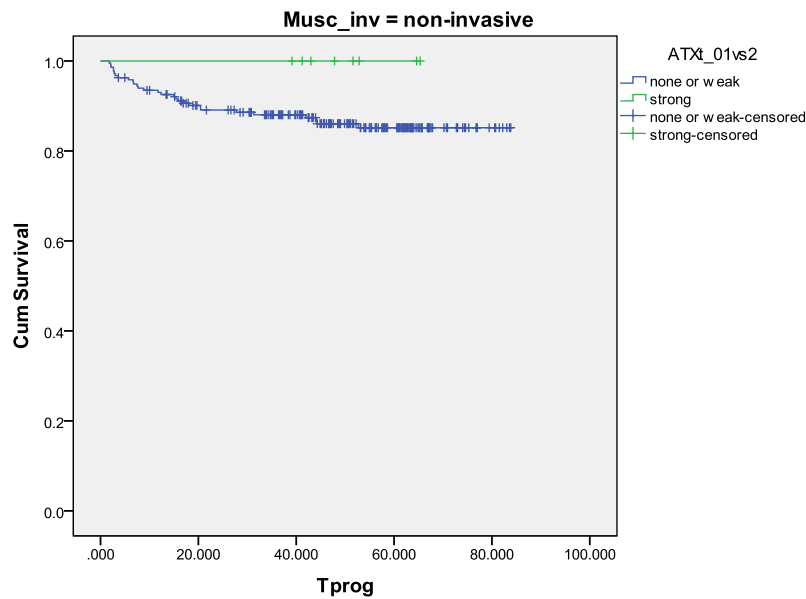

Figure 3.8 – Progression free survival in months in non-invasive tumours (pTa and pT1), p=0.280

When considering only muscle-invasive tumours (pT2) (73 subjects) and disease progression the association remained non significant (p=0.985, Figure 3.9).
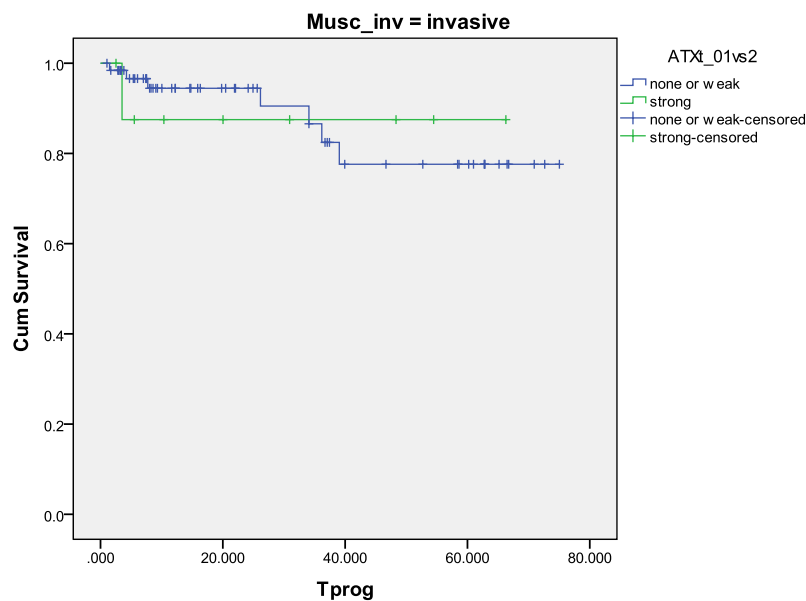
Figure 3.9 – Progrssion free survival in months in muscle-invasive tumours (pT2), p=0.985

Therefore, using the BCPP definition of progression, there was no association between autotaxin expression and progression-free survival although the strongly staining group does appears to have fewer progression events.

The effect is similar when considering the standard definition of progression.

Figure 4.0 shows a progression-free survival in months for 216 'none/weakly' staining patients who experienced 6 (2.8%) events (progressions) and 8 'strong' staining patients who had no events.

Again 'strong' autotaxin-staining in the tumour appears inversely linked to progression but the log-rank test for equality of survival indicated no difference (P=0.642).

Five year progression-free survival is 100% (95% CI not calculable) for the 'strong' staining group and 97.6% (95% CI 95.6 to 99.7%)) for the 'none/weakly' staining group.
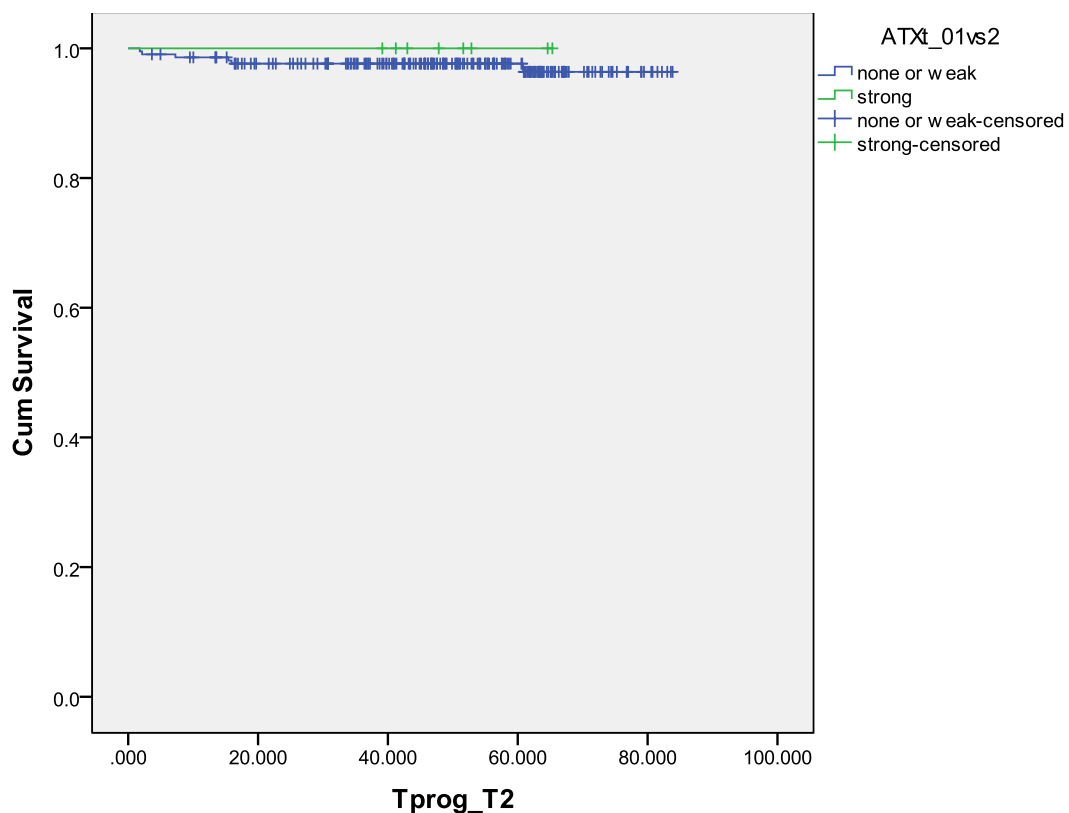
Figure 3.10 – Progression free survival in months in 224 pTa and pT1 Patients.

3.4 Illustration of Autotaxin Expression in Bladder Cancer
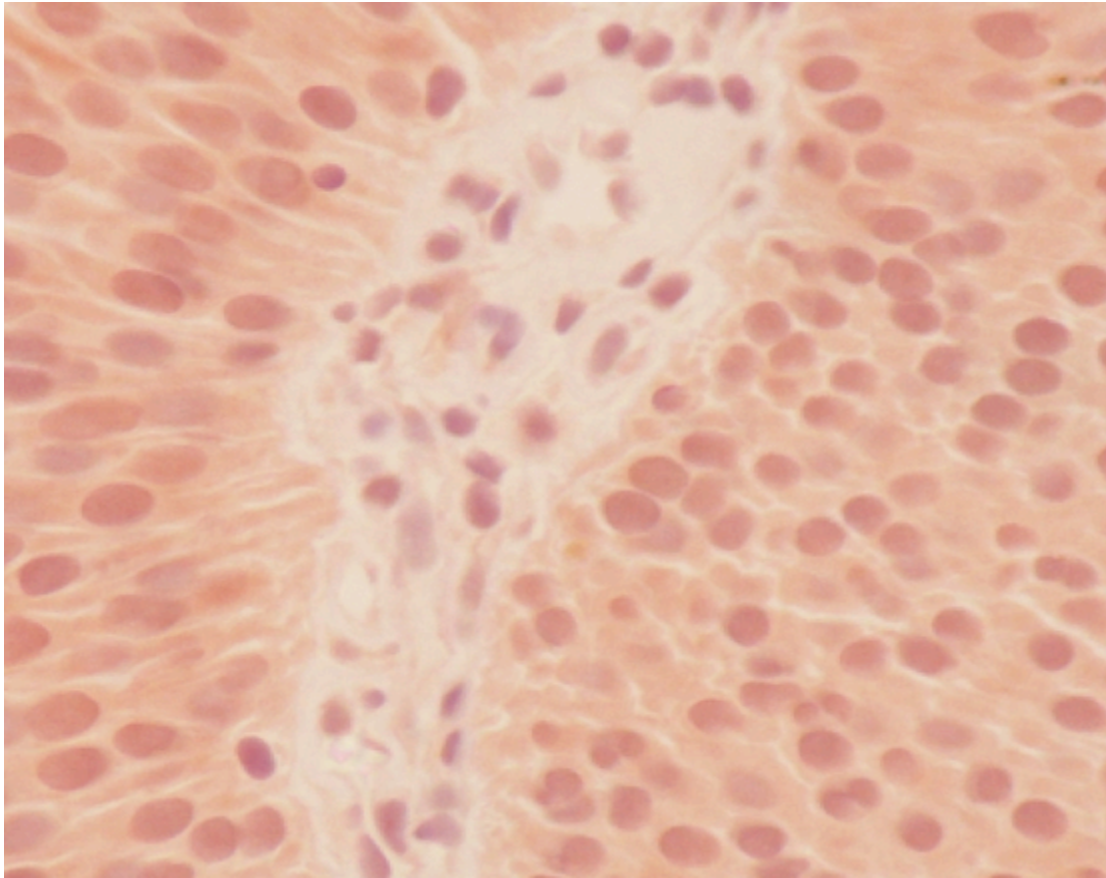
Figure 3.11. Tumour cells showing weak cytoplasmic expression of autotaxin in bladder cancer
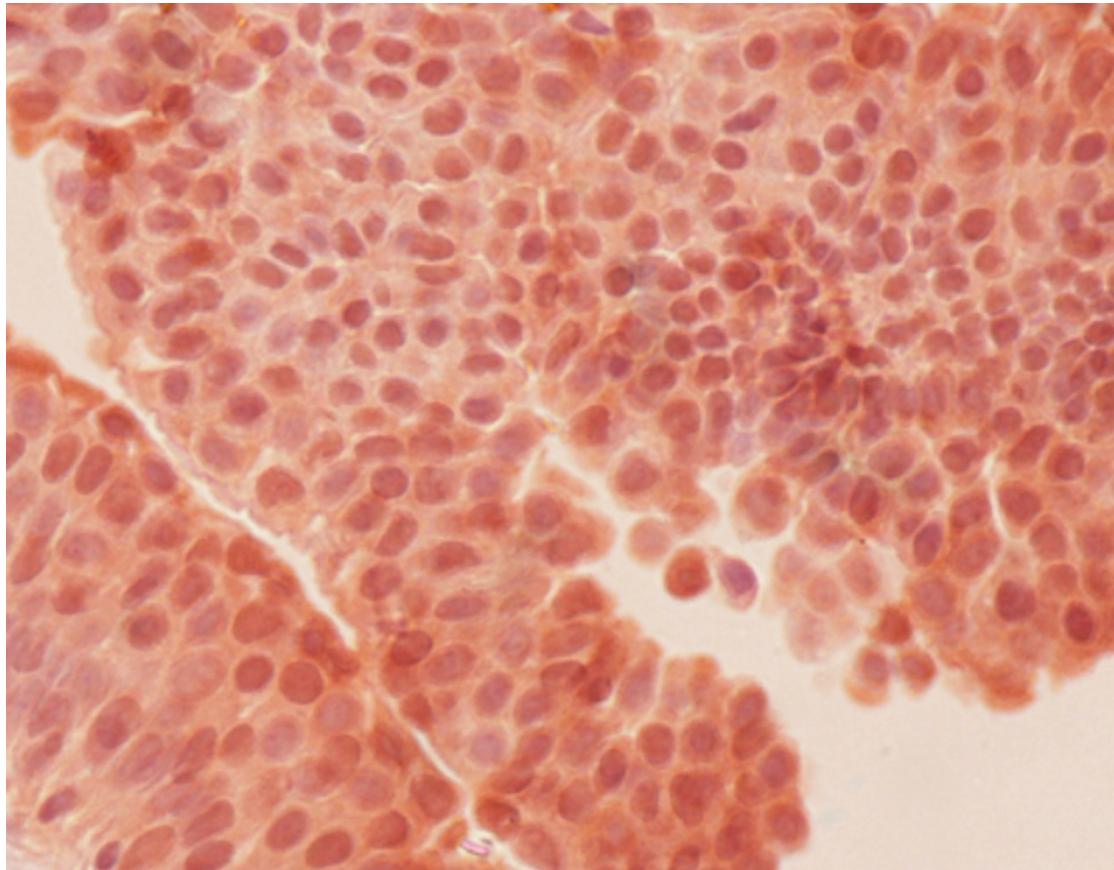
Figure 3.12. Tumour cells showing strong cytoplasmic expression of autotaxin in bladder cancer
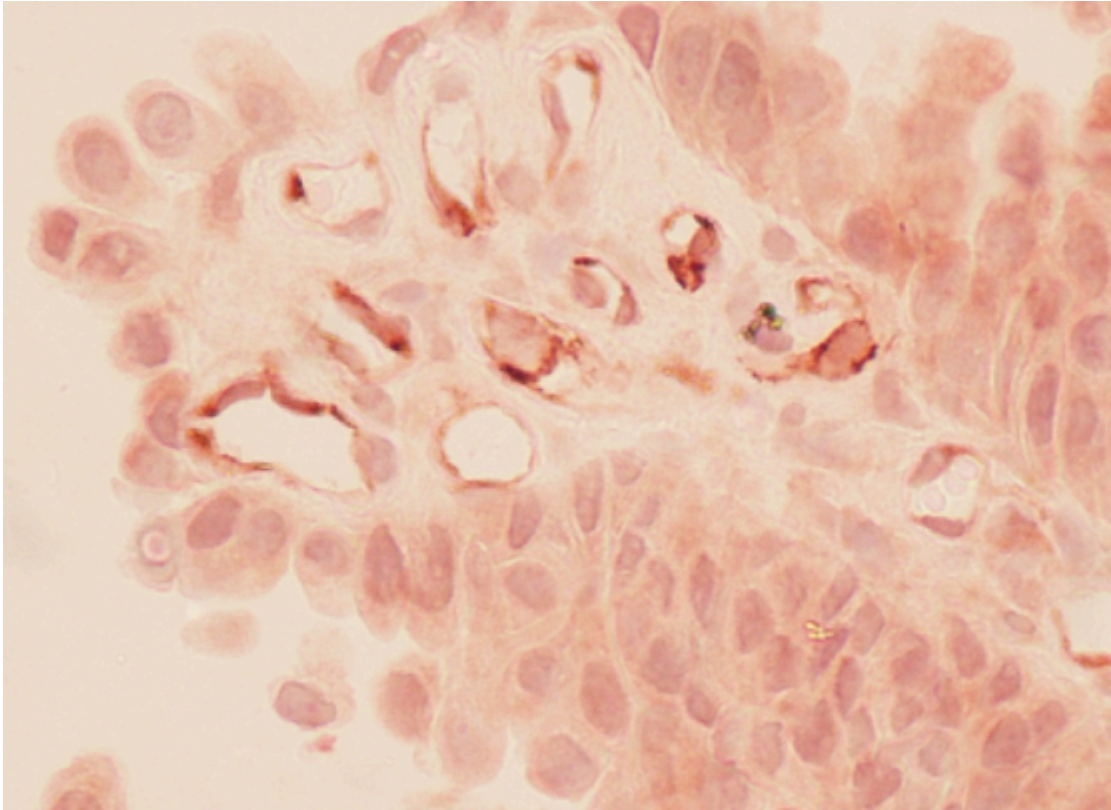
Figure 3.13. Vessels weak for autotaxin expression in bladder cancer
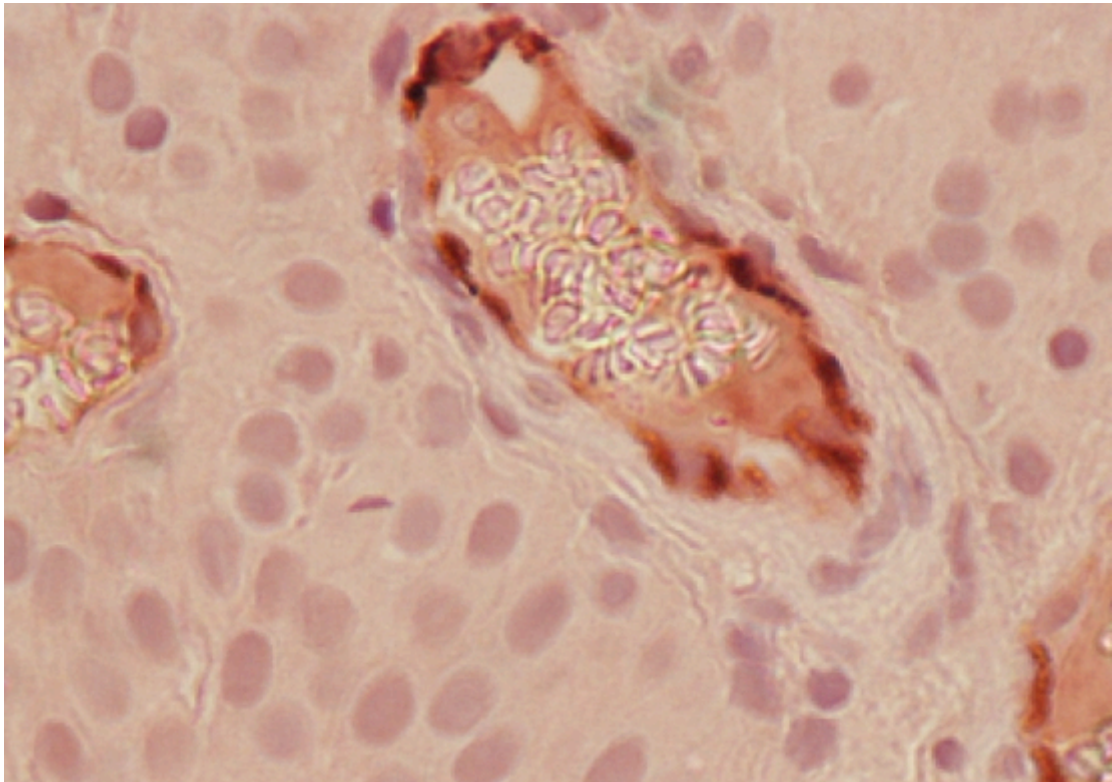
Figure 3.14. Vessels strong for autotaxin expression in bladder cancer

3.5 Comparisons between the Initial and Expanded Bladder Cohorts

Gender

Sex

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Female	33	26.6	26.6	26.6
	Male	91	73.4	73.4	100.0
	Total	124	100.0	100.0	

Table 3.5A- Gender of the initial cohort (124)

Sex

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Female	69	21.9	21.9	21.9
	Male	246	78.1	78.1	100.0
	Total	315	100.0	100.0	

Table 3.5B- Gender of the expanded cohort (15)

Gender distribution was similar for the two groups. In the initial cohort 26.6% were female compared with 73.4% male. In the expanded cohort 21.9% were female and 78.1% were male.

Marital Status

Married

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	not married	46	37.1	39.0	39.0
	married or cohabiting	72	58.1	61.0	100.0
	Total	118	95.2	100.0	
Missing	System	6	4.8		
Total		124	100.0		

Table 3.5C- Marital status in the initial cohort

Married

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	not married	115	36.5	37.6	37.6
	married or cohabiting	191	60.6	62.4	100.0
	Total	306	97.1	100.0	
Missing	System	9	2.9		
Total		315	100.0		

Table 3.5D- Marital status in the expanded cohort

37.1% of the initial cohort were not married and 58.1% were married or cohabiting. 36.5% of the expanded cohort were not married as compared with 60.6% who were cohabiting. Therefore both groups were similar in terms of marital status.

Ethnicity

Eth

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	White	114	91.9	96.6	96.6
	Non-White	4	3.2	3.4	100.0
	Total	118	95.2	100.0	
Missing	System	6	4.8		
Total		124	100.0		

Table 3.5E- Ethnicity of the initial cohort

Eth

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	White	300	95.2	98.0	98.0
	Non-White	6	1.9	2.0	100.0
	Total	306	97.1	100.0	
Missing	System	9	2.9		
Total		315	100.0		

Table 3.5F- Ethnicity of the expanded cohort

91.2% of the initial cohort were classed as white and 3.2% as non-white ethnicity.
95.2% of the expanded cohort were classed as white and 1.9% as non-white. Therefore both groups were similar in terms of ethnicity.

Smoking Status

smoke

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Never smoked	28	22.6	23.9	23.9
	ex-smoker	69	55.6	59.0	82.9
	smoker	20	16.1	17.1	100.0
	Total	117	94.4	100.0	
Missing	System	7	5.6		
Total		124	100.0		

Table 3.5G- Smoking status of the initial cohort

smoke

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Never smoked	62	19.7	20.4	20.4
	ex-smoker	171	54.3	56.3	76.6
	smoker	71	22.5	23.4	100.0
	Total	304	96.5	100.0	
Missing	System	11	3.5		
Total		315	100.0		

Table 3.5G- Smoking status of the expanded cohort

22.6% of the initial cohort had never smoked, 55.6% were ex-smokers and 16.1% were smokers.
19.7% of the expanded cohort had never smoked, 54.3% were ex-smokers and 22.5% were smokers.
There was a larger percentage of smokers in the expanded cohort but comparable figures of never smoked and ex-smokers.

Therefore the initial and expanded cohorts appear similar in terms of gender, ethnicity, marital status and smoking status.

3.6 Discussion

At the onset of this piece of work there had been little work done on the association between autotaxin expression and bladder cancer. A literature review revealed a study by Lee et al. (2007), in which an association of the LPA_6 receptor with familial bladder cancer was reported. LPA_6, previously known as P2RY5, couples to the $G\alpha_{13}$-RHoA signaling pathway which has a regulatory role with the actin cytoskeleton. The expression of the gene P2RY5 can be induced by T cell activation (Kaplan et al. 1993) as well as in LPA-stimulated fibroblasts (Stortlers et al. 2008)(Houben et al. 2011). Crawford et al. (2008) suggested that LPA_6/P2RY5 could represent a potential risk marker for bladder cancer.

However with the growing interest in the role of the autotaxin-LPA signaling pathway in tumour growth and invasion, and angiogenesis, other groups started focusing on this pathway.

More recently, Kataoka et al. (2014) found that LPA_1 mRNA expression was significantly increased in muscle invasive bladder cancer samples than non-muscle invasive samples, and strong LPA_1 expression was found on cell membranes in muscle invasive samples. They also reported that LPA treatment resulted in an increase in T24 cell invasion while treatment with LPA_1 siRNA or LPA_1 inhibitor decreased cell invasion. They concluded that LPA signaling and LPA_1 activation in particular promoted bladder cancer invasion and suggested that LPA_1 could be used to identify bladder cancers with high invasive potential and furthermore could itself be used as a therapeutic target in the treatment of invasive bladder cancer.

Work in this chapter has made two interesting observations.

First, a significant association between stage of bladder cancer and higher expression of autotaxin. Second, an association between autotaxin expression and disease progression.

As advancing stage of disease normally corresponds with poorer prognosis, identifying autotaxin expression in newly diagnosed bladder cancers could in the future be used as a marker of poor prognosis. Furthermore, inhibitors of autotaxin could play a role in modifying the disease. For example, Benesch et L. 2014, found that a new autotaxin inhibitor called ONO-8430506 decreased initial lung tumour growth and subsequent metastatic nodules both by 60% compared with control mice. If inhibitors of autotaxin were found to have a similar effect in bladder cancer then current treatments of bladder cancer could be avoided along with their side effects.

That said, monitoring the side effects of autotaxin inhibitors will also be crucial. Although no autotaxin/LPA-targeting cancer drugs are available for clinical use at present, development is progressing rapidly (Tabuchi et al. 2015). There is however a report of LPA_1 deficiency resulting in schizophrenia type pathology in mice (Harrison et al. 2003).

Weaknesses of the current study include small sample size as well as the absence of normal bladder mucosa with which to compare autotaxin expression. However obtaining samples of truly 'normal' bladder is extremely difficult. Even sections of normal bladder mucosa taken from a bladder which has cancer elsewhere would be expected to exhibit a degree of field change.

Future work would involve increasing the cohort number, assessing autotaxin expression in normal bladder mucosa and comparing it with that of expression in bladder cancer.

As muscle invasive bladder cancer is associated with a higher mortality than non muscle invasive bladder cancer it would be interesting to further tease out differences in expression between NMI and MI bladder cancer to ascertain if there is a potential role of autotaxin in MI bladder cancer.

Expression of Autotaxin in a Cohort of Metastatic Renal Cancer Patients Treated with Sunitinib

4.1 Introduction

Renal cell carcinoma (RCC) represents 2-3% of all cancers worldwide, with the western nations demonstrating the highest incidence (European Network of Cancer Registries). Incidence of renal cell carcinoma has increased by approximately 2% over the last 11 years both worldwide and in Europe, however rates in Sweden and Denmark have gradually fallen (Lindblad et al. 2004).

In Europe alone, there were estimated to have been 84,400 new cases of RCC and 43,700 kidney related deaths in 2012 (Ferlay et al. 2013).

There are different types of RCC, each with different histo-pathological and genetic features. There is a 1.5:1 male predominance and a peak incidence of between 60 and 70 years.

Risk factors include smoking, obesity, hypertension (Lipworth et al. 2006) as well as having a first-degree relative with RCC (Clague et al. 2009).

The evidence as yet does not support a strong link between dietary habits and exposure to specific carcinogens. Smoking cessation and preventing obesity have been found to be associated with reduced risk, as has moderate alcohol consumption (Bellocco et al. 2012, Song et al. 2012).

Over 50% of RCC are detected incidentally by ultrasound (US) or computed tomography (CT). However, these tumours tend to be smaller and of lower stage (Patard et al. 2002, Kato et al. 2004, Tsui et al. 2000).

Treatment options for RCC differ depending on stage. For localised disease, surgery is the only curative option. Alternatives to surgery include the

ablative therapies such as cryoablation and radiofrequency ablation. For locally advanced disease, i.e., lymph node involvement or venous thrombosis, surgery remains an option but often neo-adjuvant treatments are also considered however the evidence from randomized controlled trials has yet to be reported.

Treatment for advanced/metastatic RCC involves cytoreductive surgery, local therapy to metastases such as radiotherapy to bone metastases, or systemic therapies. Systemic therapies include chemotherapy, targeted therapies such as the tyrosine kinase inhibitor monoclonal antibody against VEGF or mTOR inhibitors such as temsirolimus or everolimus, and in selected cases immunotherapy such as interleukin-2.

Sunitinib is a multi-targeted tyrosine kinase inhibitor (TKI). It predominately targets the vascular endothelial growth factor receptor (VEGFR) and is used as the first line therapy for advanced and/or metastatic renal call carcinoma. Most patients develop resistance to Sunitinib after one year while some appear to have an innate resistance and do not respond at all (Motzer et al. 2009). Resistance is marked by renewed angiogenesis and disease progression.

Whether open or laparoscopic surgery, the risks posed are the same general risks as for any major surgery as well as risks specific to nephrectomies such as renal failure, pneumothorax, etc. The side effects associated with systemic treatment comprise nausea, vomiting, diarrhoea, hypertension and hand and foot syndrome, and clearly these can seriously affect an individual's quality of life.

There are many treatments available for RCC but none are satisfactory. Since one of the main functions of LPA is angiogenesis, and aberrant angiogenesis has been found to be a contributory factor in the development of RCC (Su et al. 2013), then autotaxin and the LPA signaling pathway represent a potential therapeutic target in the treatment of renal cancer. At present there is little known about the role of autotaxin and LPA in renal cancer. This chapter will describe the expression of autotaxin in the tumours of a cohort of patients with renal cancer.

4.2 Materials and Methods

New sections were made from paraffin embedded blocks available from the renal cohort. An antibody targeting autotaxin was validated using tonsil and was then used to study autotaxin expression in these tumours. Immunohistochemistry was performed using the methods set out in the materials and methods chapter.

4.3 Results

The sample size of this cohort was small and as a result it was not possible to analyze the data using the same statistical tests as had been used for the bladder work.

4.3.1 Cohort Characteristics

The subject characteristics of the cohort itself were considered and are illustrated below. By comparing characteristics of the present cohort with national figures published by Cancer Research UK for the time period 2009-2011 it can be determined whether or not the present cohort is a representative sample.

There were 59 patients in the cohort, 45 (76%) male and 14 (24%) female. Similarly, incidence rates nationally are higher for males than for females at age 35 and above (the gap is not significant in younger age groups), and this gap is widest at 85+, when the male: female incidence ratio of age-specific rates (to account for the different proportions of males to females in each age group) is around 2.1:1.

Comparing age distribution in the present cohort (figure 4.2) with national figures (figure 4.1) a similar distribution can be seen. Age-specific incidence rates rise sharply from around age 40- 50, peaking in the 60- 70 age group.
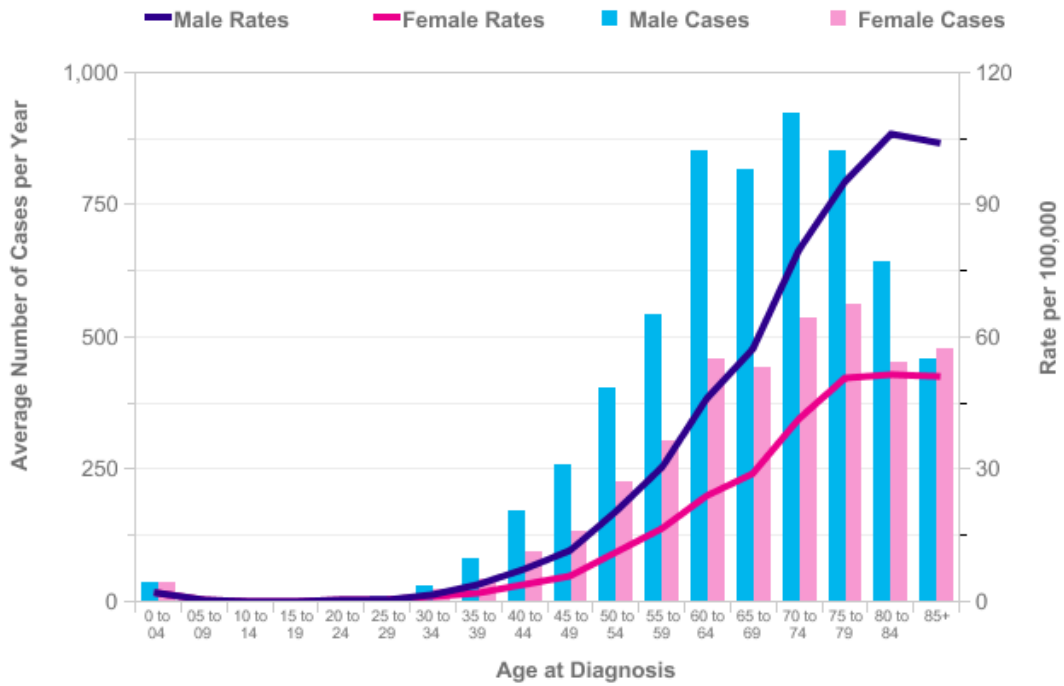
Figure 4.1 Kidney Cancer 2009-2011. Taken from Cancer Research UK. Average number of new case per year and age-specific incidence rates per 100,000 population, UK.
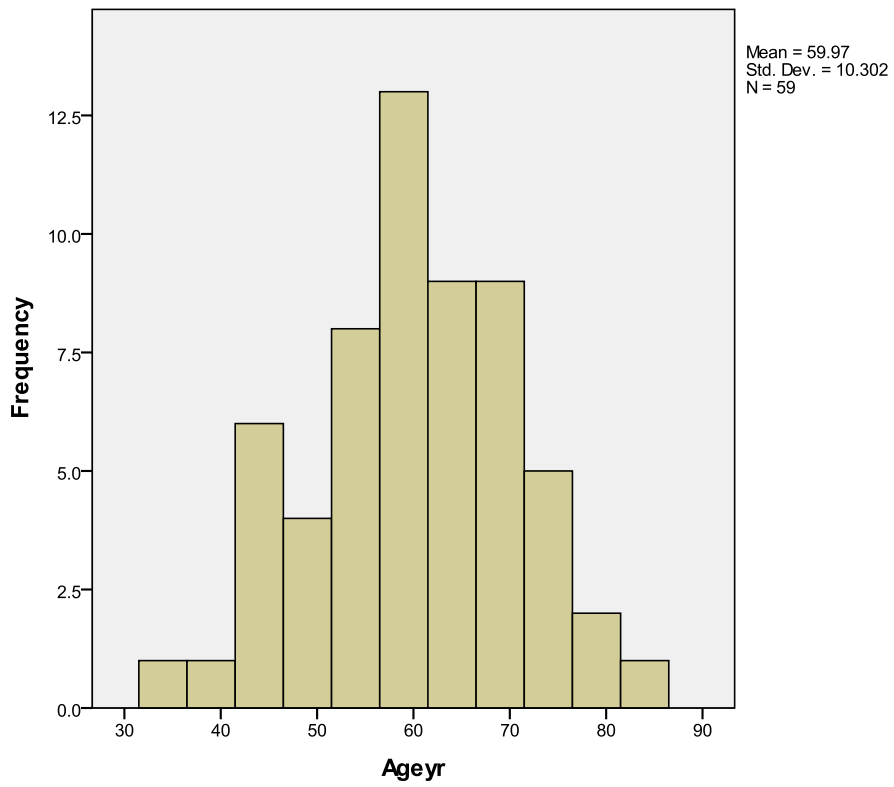
Figure 4.2 Age distribution of Renal Cohort

The median age of the cohort at diagnosis was 60 years (range 34-85 years).

Stage at diagnosis was similar between the cohort and national figures for stage I and II but differed for stage III, IV and cases where stage was not known (Table 4A). The present study cohort included only metastatic renal cancer and hence percentage of cases which were stage 3 (68.6%) is higher in the study cohort than that observed nationally.

Stage at Diagnosis	% of Cases – Present Study Cohort	% of Cases – England 2012
Stage I	17.6	15.7
Stage II	3.9	3.8
Stage III	68.6	8.4
Stage IV	5.9	15.3
Stage not known	3.9	56.8

Table 4A. Proportion of kidney cancer diagnosed at each stage, all ages, in the present cohort compared with figures in England in 2012 (Cancer Research UK, 2010)

Time from diagnosis to trial entry was considered and is illustrated in Figure 4.1A and 4.1B below. The mean time from diagnosis to trial entry was 22.6-31.9 months while the median time from diagnosis to trial entry was 11.7-14.1 months.
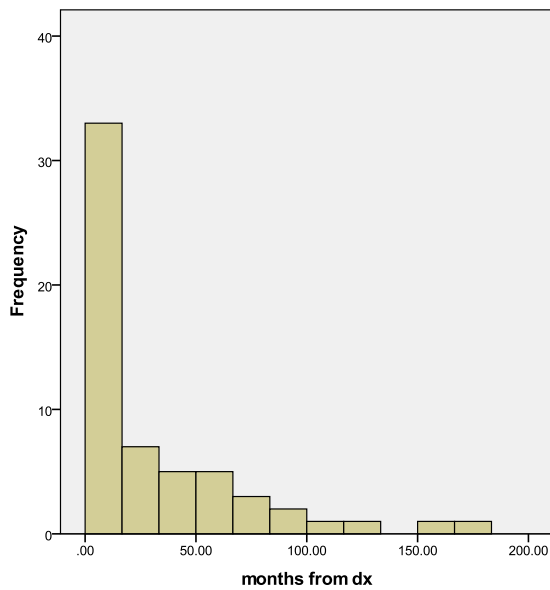
Figure 4.1A
Months as stated in the data file
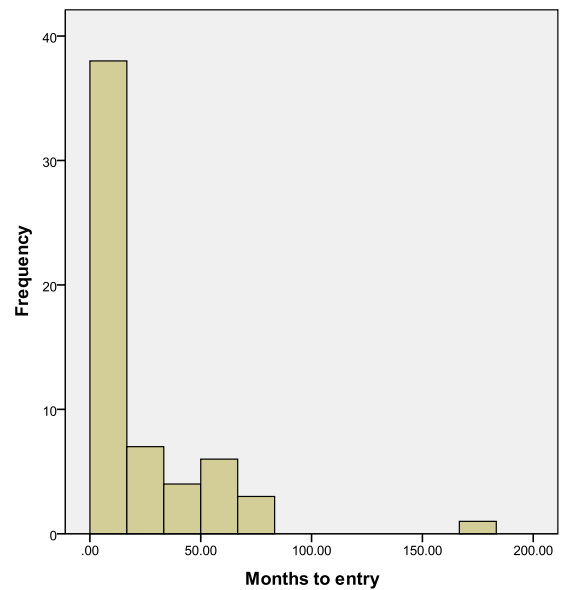
Figure 4.1B
Months as calculated from the dates

Survival was recorded from the date of trial entry to the date of death (43 patients) or the date last seen alive (16 patients). The survival curve is illustrated in Figure 4.1D below.
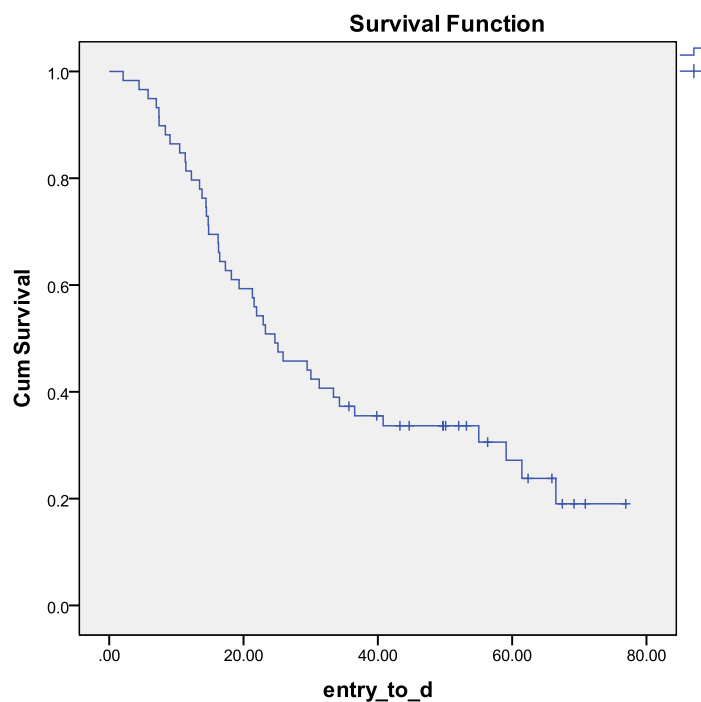
Figure 4.1D Kaplan Meier plot showing date of trial entry until death in months (x) vs. cumulative survival (%) (y)

1-year survival rate was 81.4% (95% CI 71.4 to 91.3),
3-year survival rate was 37.3% (95% CI 24.9 to 49.6) and 5-year survival rate was 27.2% (95% CI 14.5 to 39.9). Therefore, survival dropped substantially with time in this cohort. Median survival was 24.67 months (95% CI 16.19-33.15).

However, comparing these results with national statistics, 1-year survival is similar but 5-year survival nationally is much higher; 56.2% compared with 27.2%.

	1-year survival Cohort	1-year survival England	5-year survival Cohort	5-year survival England
Net Survival	81.4	72.4	27.2	56.2
95% LCI	71.4	72.3	14.5	56.0
95% UCI	91.3	72.4	39.9	56.4

Table 4B Comparing 1 and 5-year survival rates between the cohort and adults (age 15-99), England and Wales, 2010-2011.
LCI- lower confidence interval, UCI- upper confidence interval.

Disease progression can be defined as any change in the disease such that it becomes more severe. This may involve worsening grade or stage of the disease, increase in tumour volume or new diagnosis of metastases.

In the present study disease progression was seen in 48 cases. There were no patients who died without disease progression.
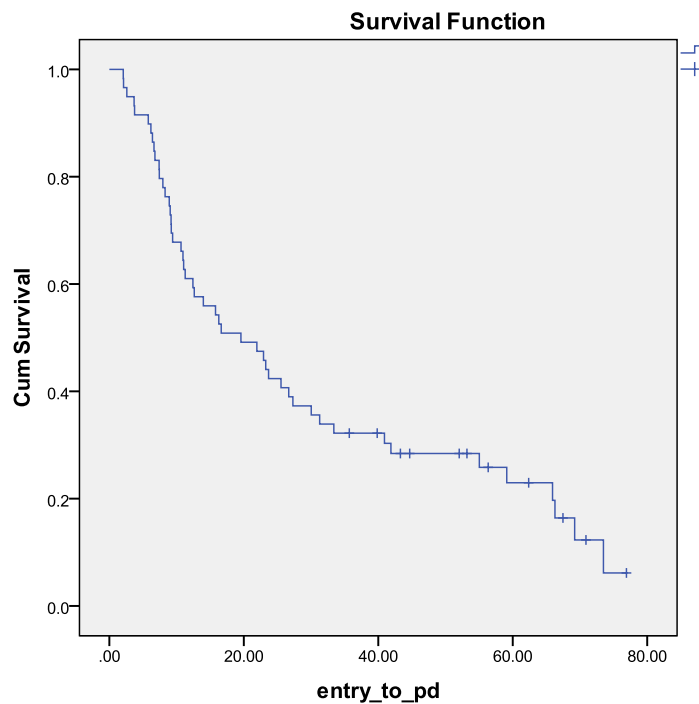
Figure 4.1E Progression-free survival in months vs. cumulative survival (%).

1 year progression-free survival rate was 61.0% (95% CI 48.6 to 73.5). 3 year progression-free survival rate was 32.2% (95% CI 20.3 to 44.1) and 5 year progression-free survival rate was 23.0% (95% CI 11.4 to 34.6).

Median progression free survival was 19.57 months (95% CI 9.59-29.55).

Sections from 45 subjects in the cohort were stained for autotaxin. Specimens were excluded from 14 subjects due to poor quality paraffin blocks. Expression of autotaxin in vessels was reported; all tumour tissues stained negative for autotaxin expression.

The effect of autotaxin expression on survival was considered initially.

Comparing autotaxin expression in all groups individually, none, weak and strong, no significant association with survival was found (p=0.689).
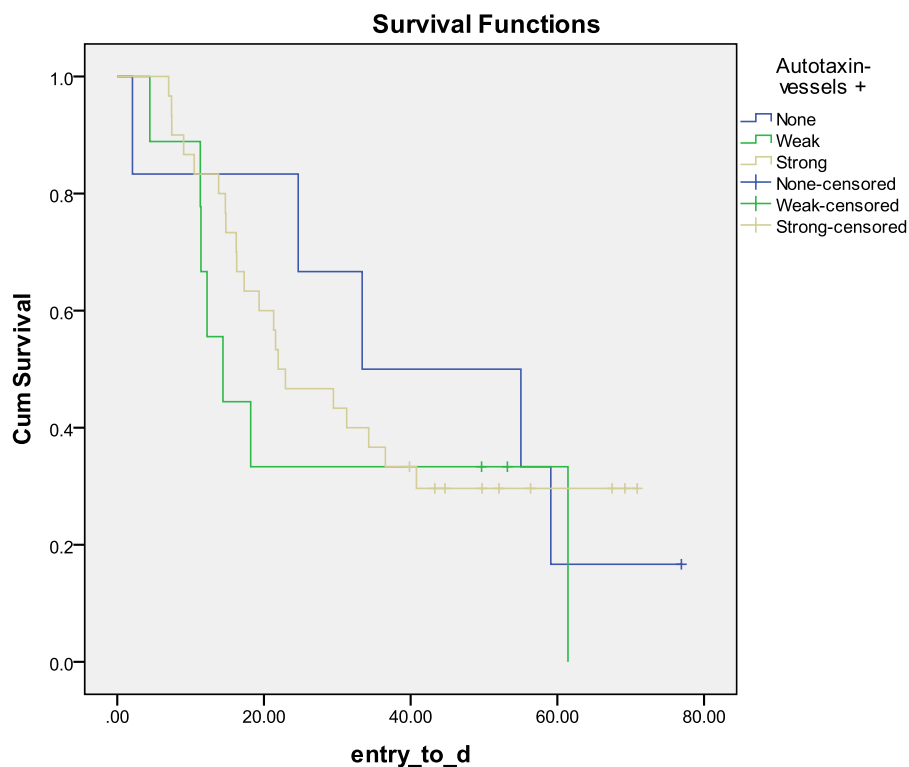
Figure 4.2A – Survival in months vs. cumulative survival (%) in all three groups; none (n=6) vs. weak (N=9) vs. strong (N=30).

p=0.689

Grouping the no autotaxin expression and weak groups together and comparing with strong, no significant association with survival was found (p=0.731).

Survival Functions

Figure 4.2B – Survival in months vs. cumulative survival (%) for none or weak (N=15) vs. strong (N=30).

p=0.731

Comparing the no autotaxin expression group with the weak and strong groups combined, no significant association with survival was found (p=0.704).

Figure 4.2C – Survival in months vs. cumulative survival (%) for none (n=6) vs. weak or strong (N=39).

p=0.704

Therefore the level of autotaxin expression (in vessels) was not found to influence survival, and grouping the weak staining cases with either the negative or the strongly staining cases did not alter the findings.

The effect of autotaxin expression on time to disease progression was then evaluated.

Survival Functions
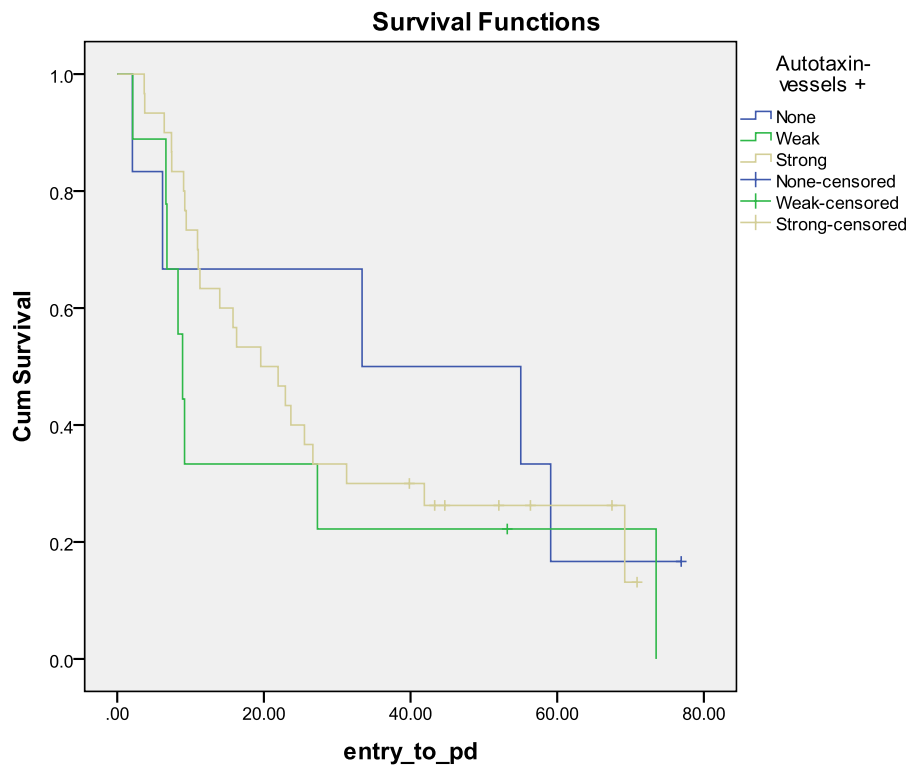
Figure 4.2D-Time to disease progression in months vs. cumulative survival (%) in groups none (N=6), weak (N=9) and strong (N=30).

p=0.566

Comparing autotaxin expression in all groups individually, negative, weak and strong, no significant association with time to disease progression was found (p=0.566).

Grouping the no autotaxin expression and weak groups together and comparing with strong, no significant association with time to disease progression was found (p=0.731).
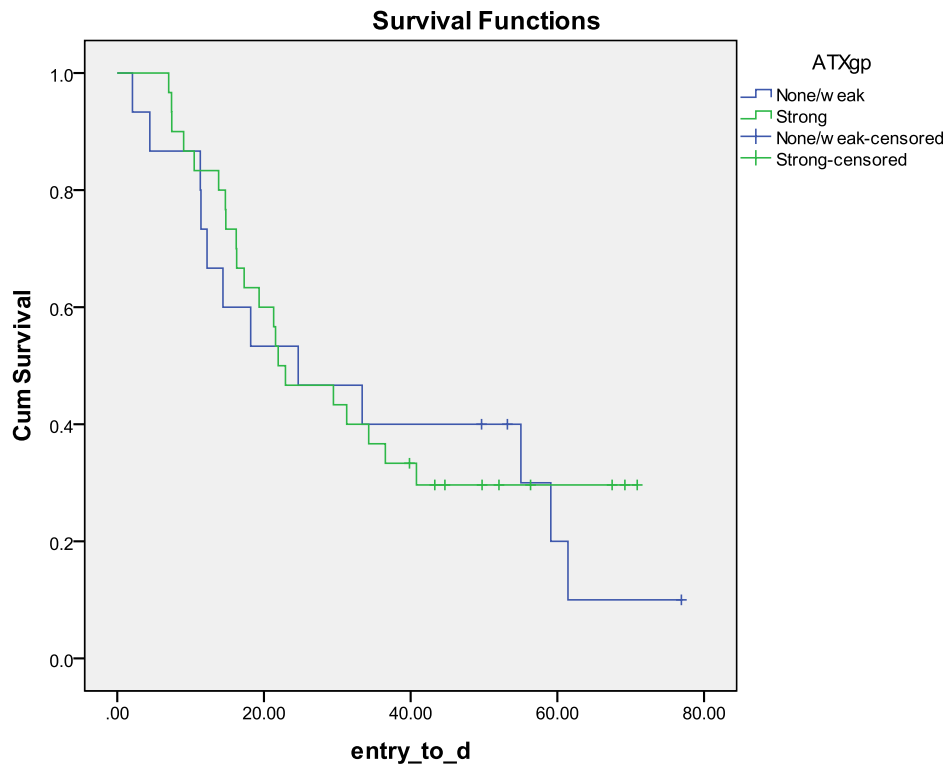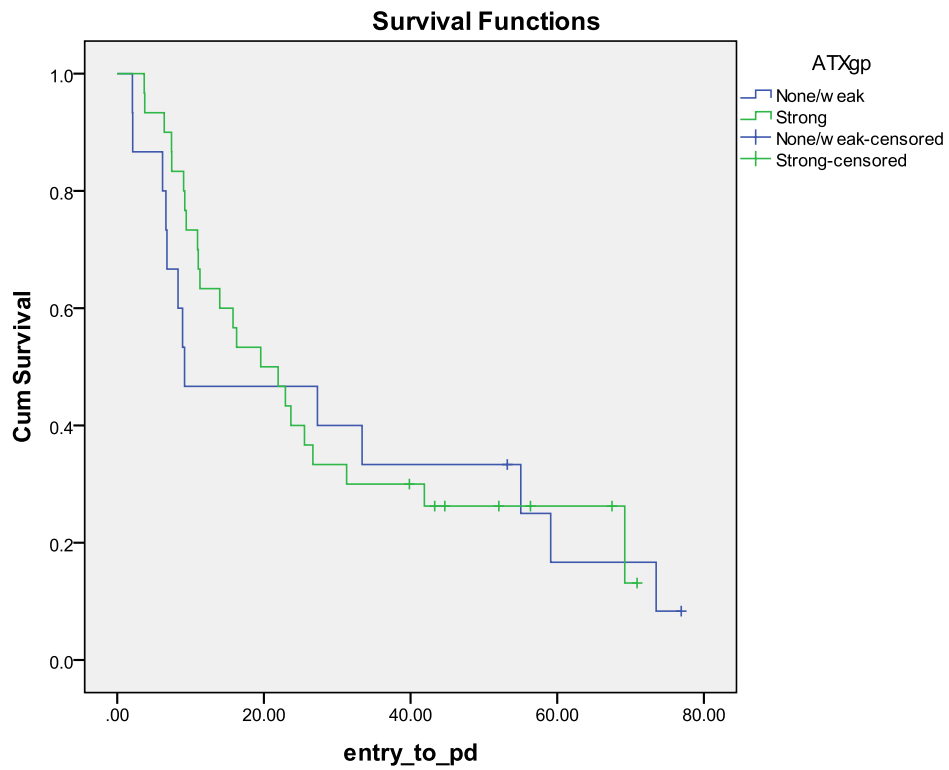

Figure 4.2E- Time to disease progression in months vs. cumulative survival (%) in groups none/weak (N=15) and strong (N=30).
p=0.785

Comparing the no autotaxin expression group with the weak and strong groups combined, no significant association with survival was found (p=0.531).
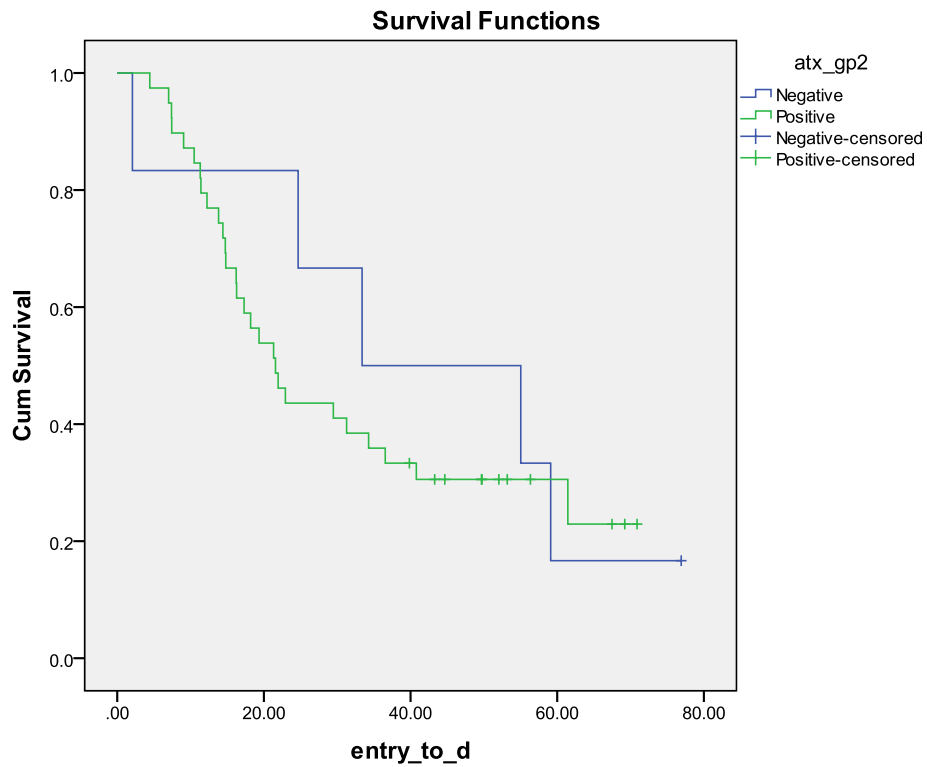

Survival Functions
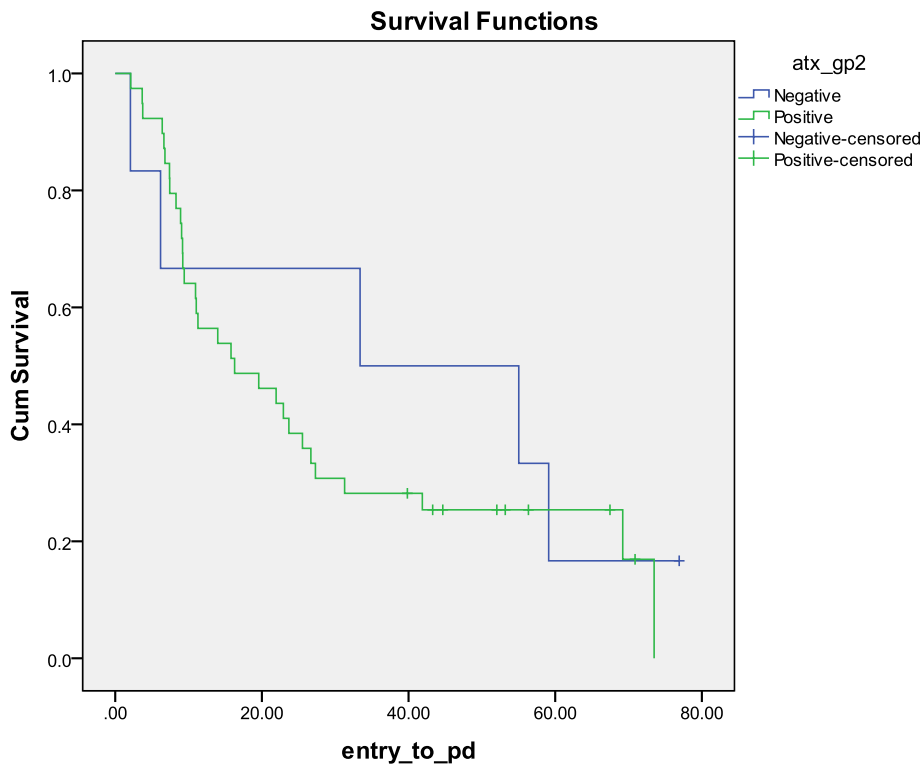
Figure 4.2F- Time to disease progression in months vs. cumulative survival (%) in groups none (N=6) and weak or strong (N=39).
p=0.531

Therefore, similarly, the level of autotaxin expression did not affect time to progression; grouping the weak staining cases with either the negative or the strongly staining cases did not alter the findings.

It is worth noting that, although not statistically significant, the high level expression of autotaxin found on the tumour associated vasculature was not apparent in the blood vessels of a small number of matched normal renal tissue that were tested.

4.3.3 Multivariate survival analysis (Time to death)

Multivariate survival analysis was performed using histology date as the fixed starting point and 'time to death' as the terminating event.

As the cohort in this study was small, only limited multivariate modeling could be employed.

Models with more than 2 variables are likely to be unreliable, so bivariate Cox proportional hazards regression was used to determine if autotaxin is of prognostic significance in the presence of these other factors.

Autotaxin was grouped as none or weak staining (n=15) vs. strong staining (n=30).

Factors used:	Age in years (continuous variable)
	Gender
	Disease grade (1 & 2 vs. 3 vs. 4)
	T-stage ((T1 & T2 vs. T3 & T4)
	N-stage (N0 vs. N1&N2 vs.Nx)
	Heng Score (Good vs. Intermediate vs. poor) (The Heng score is a prognostic indicator used in metastatic renal cell carcinoma. The factors used to derive the Heng score were not tested individually.)
	No. metastatic sites (1 vs. 2 vs. >2)
	Sarcomatoid factor (present/absent)
	CA9 (positive/negative) CA9 is over-expressed in VHL mutated clear cell renal cell carcinoma.

Marker	Co-factor	Marker HR*	95%CI for HR	Model fit (p)	No. cases
ATX	-	0.882	0.432 to 1.801	0.733	45
ATX	Age	0.934	0.454 to 1.922	0.541	45
ATX	Gender	0.883	0.432 to 1.802	0.942	45
ATX	Grade	1.059	0.475 to 2.358	0.696	41
ATX	T-stage	1.179	0.534 to 2.606	0.405	38
ATX	N -stage	1.217	0.554 to 2.671	0.925	39
ATX	Heng Score	0.740	0.341 to 1.606	0.158	45
ATX	Metastasis sites	0.776	0.350 to 1.719	0.730	45
ATX	Sarcomatoid factor	0.812	0.394 to 1.670	0.453	45
ATX	CA9 (Pos vs. Neg)	0.846	0.407 to 1.757	0.317	44

While strong autotaxin staining is associated with a slightly lower mortality risk (hazard ratio), its value (0.882) was not significantly different from 1.00 (as shown by the confidence interval of 0.432 to 1.801).

The testing of all other factors in bivariate models did not produce any significant effect for autotaxin – neither were any of the bivariate models significantly more predictive of outcome than the null model (i.e. one where no factors affect survival). Therefore there is no evidence that autotaxin has any prognostic significance in RCC.

4.3.4 Multivariate Survival analysis (Time to disease progression)

Multivariate analysis was then performed using histology date as the starting point again but this time using 'time to disease progression' as the terminating event.

Marker	Co-factor	Marker HR*	95%CI for HR	Model fit (p)	No. cases
ATX	-	0.906	0.447 to 1.837	0.786	45
ATX	Age	0.942	0.461 to 1.924	0.734	45
ATX	Gender	0.907	0.447 to 1.842	0.963	45
ATX	Grade	0.987	0.439 to 2.219	0.920	41
ATX	T-stage	1.054	0.481 to 2.309	0.358	38
ATX	N -stage	1.068	0.489 to 2.331	0.873	39
ATX	Heng Score	0.774	0.363 to 1.651	0.222	45
ATX	Metastasis sites	0.756	0.342 to 1.669	0.755	45
ATX	Sarcomatoid factor	0.843	0.410 to 1.735	0.666	45
ATX	CA9 (Pos vs. Neg)	0.926	0.452 to 1.897	0.809	44

Once again no significant association was found between autotaxin expression, and any of the co-factors and time to disease progression.

In summary, in renal cell carcinoma, autotaxin does not appear to be associated with time to disease progression, however, the sample size was small.

4.4 Illustration of Autotaxin Expression in Renal cancer

As none of the renal cancer tumours were positive for autotaxin, expression below refers to vessels only.
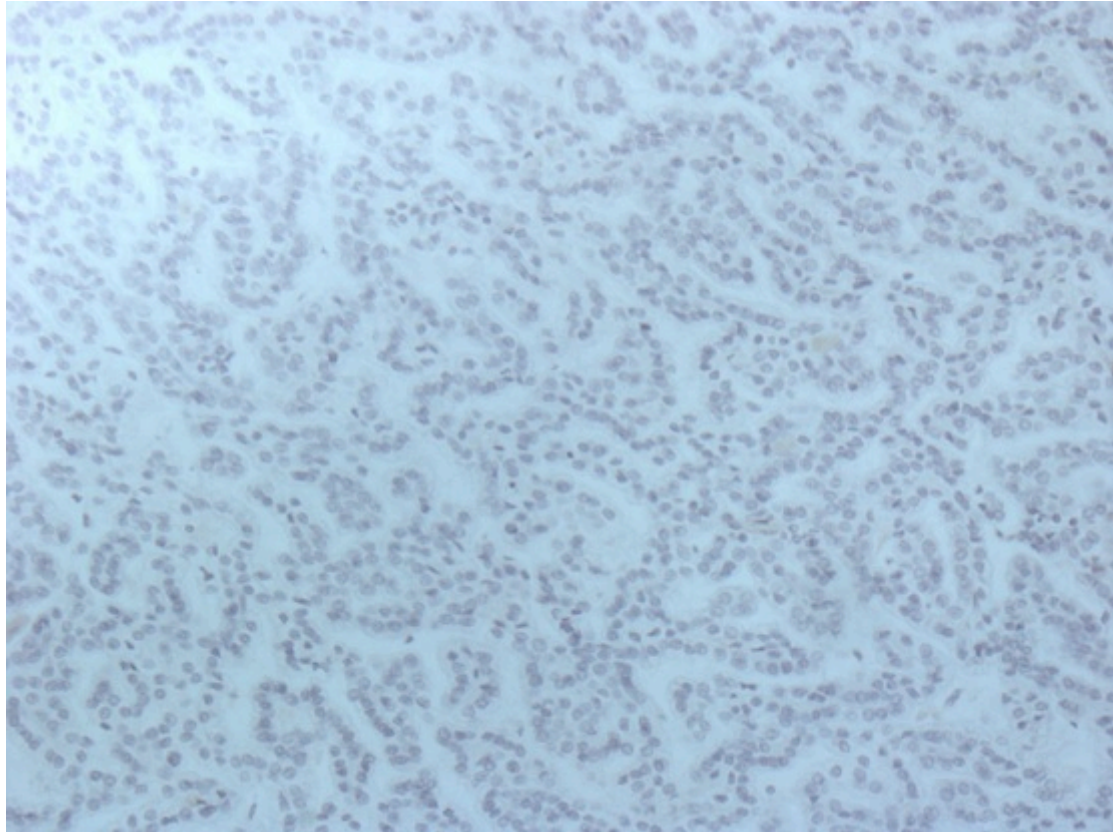

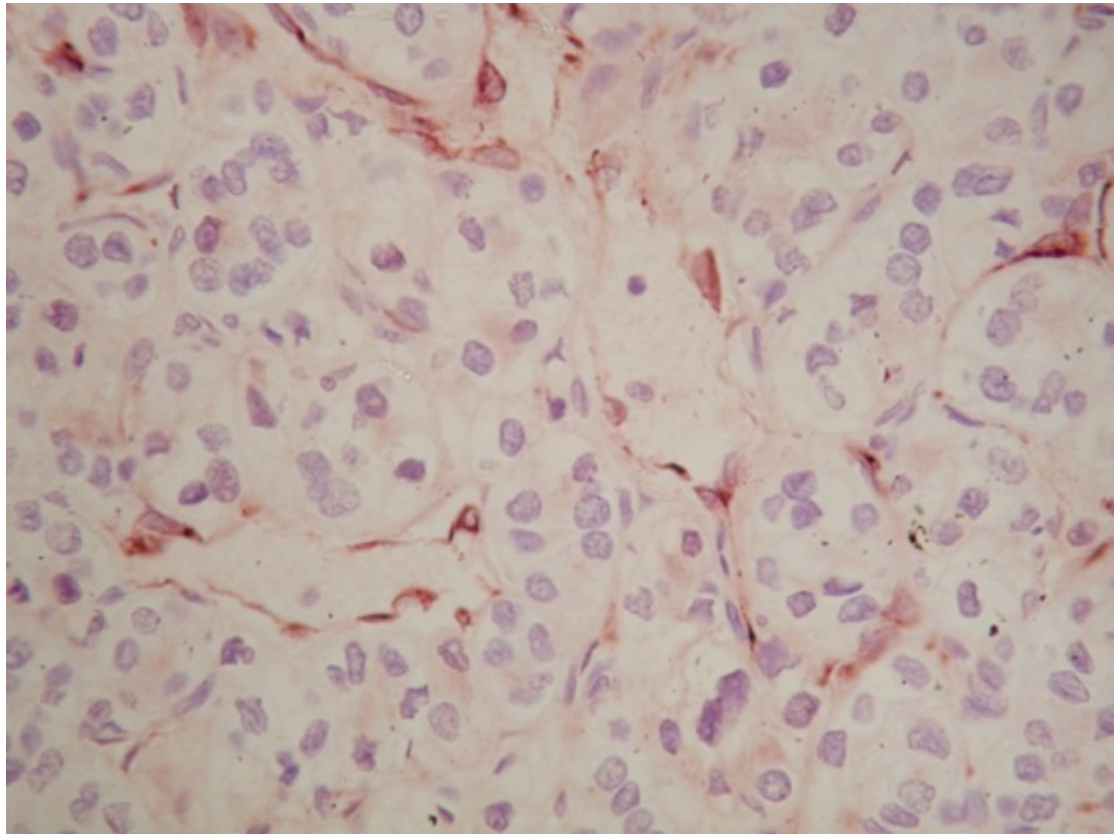Figure 4.4A. Vessels negative autotaxin expression in renal cancer

Figure 4.4B. Vessels weak autotaxin expression in renal cancer
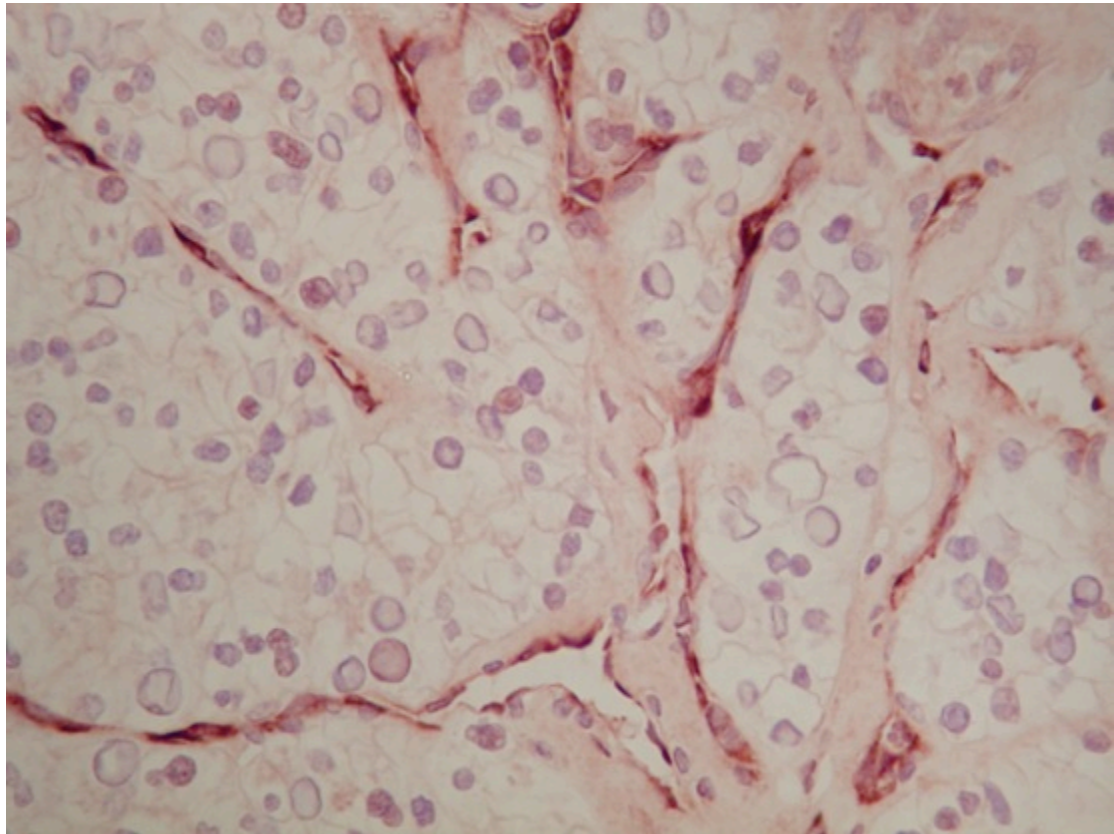
Figure 4.4C. Vessels strong autotaxin expression in renal cancer

4.5 Discussion

This study focused on a small cohort of 59 patients with metastatic renal cell carcinoma treated with Sunitinib. The advantages of a clinical cohort such as this one was the excellent follow up clinical data. Tissue from these subjects was stained for autotaxin expression.

Autotaxin expression in vessels only was reported as I did not observe any staining in tumour.

There was no significant association found between autotaxin expression and survival or time to disease progression.

Furthermore, multivariate analysis found that autotaxin had no prognostic significance in relation to renal cell carcinoma and no association with time to disease progression.

At the onset a literature review failed to identify any previous work on the association between autotaxin and renal cancer. However, in 2013 Su et al. reported that autotaxin was expressed in endothelial cells of tumour vessels but not in the tumour cells, confirming the findings of this study. They also found that autotaxin was not expressed in the endothelial cells of the normal renal capillaries, again similar to the findings of this study. Interestingly, they showed that endothelial autotaxin expression in renal cell carcinoma was down regulated after Sunitinib treatment.

Future work would involve expanding the current cohort as any significant associations may have been difficult to identify with such small numbers.

It would also be interesting to compare autotaxin expression in renal cell carcinoma with expression in pathologically normal renal tissue. However it is very difficult to obtain truly normal renal tissue. Specimens from

nephrectomies performed for benign disease are likely to still be abnormal histologically and normal tissue from a cancerous kidney will likely demonstrate field changes. It may be possible to identify specimens from renal biopsies performed at the request of nephrologists investigating nephrological conditions.

Comparing autotaxin expression in renal cell carcinoma tumours treated with and without Sunitinib would also be another interesting study to establish if Sunitinib itself affected autotaxin expression as the study by Su et al. (2013) suggests.

Discussion

In this thesis the expression of autotaxin in relation to the clinico-pathological findings of the two urological malignancies, bladder and renal cancer, was evaluated.

5.1 Bladder Cancer

5.1.1 Main Findings

I found that in bladder cancer, high-level expression of autotaxin was significantly associated with higher stage of disease, muscle invasion and disease progression.

Strong autotaxin expression was found in higher stages of the disease. Higher stage generally refers to T2 disease, the stage at which the tumour invades the muscle wall of the bladder, and therefore corresponds to muscle invasive disease.

This is a potentially important finding as advancing stage of disease/muscle invasion generally corresponds with poorer prognosis. Therefore, if autotaxin could be used as a reliable marker of higher stage disease then autotaxin inhibitors could have a potential role in early disease modification and preventing subsequent muscle invasive disease.

5.1.2 Previous Work

A literature review did not identify any previous work on the association between autotaxin and bladder cancer however studies focusing on the LPA signaling pathway, of which autotaxin is a part, and in particular LPA receptors and bladder cancer have been reported.

Lee et al. (2007) reported that the LPA_6 receptor was associated with familial bladder cancer. They used bladder cancer as the model to identify clonal

genetic factors associated with growth advantage. This then allowed them to track the evolution of bladder cancer from intra-urothelial precursor lesions. They found that the LPA_6 receptor, which is associated with the tumour suppressor gene RB1, was associated with loss of the normal variation with in a cell line and initial development of neoplasia in situ.

The G protein-receptor 87 (GPR87), which has been identified as a LPA receptor (Tabata et al. 2007), has been found to be associated with bladder cancer. Okazoe et al. (2013) found that GPR87 was expressed in five different human bladder cancer cell lines and that by silencing GPR87 gene expression cell viability was reduced. They reported GPR87 expression was positive in 38 (54%) of a total of 71 tumours. Furthermore they found that patients with GPR87 positive tumours had a shorter recurrence free survival than those with GPR87 negative tumours (p=0.010). Multivariate analysis showed that GPR87 staining status was an independent prognostic marker for intravesical recurrence (p=0.041). Progression from NMI bladder cancer to MI cancer was more frequently observed among the patients with GPR87 positive tumours although this was not statistically significant (p=0.056).

Kataoka et al. (2015) reported that LPA signaling via the LPA_1 receptor promoted bladder cancer invasion. They found LPA_1 mRNA was significantly higher in muscle invasive bladder cancer samples than in non-muscle invasive samples. They described strong LPA_1 expression on the cell membrane in muscle invasive samples. Furthermore they found that LPA treatment increased T24 cell invasion while treatment with a LPA_1 inhibitor lead to a decrease in invasiveness. They also reported that LPA treatment increased ROCK1 (a serinethreonine kinase that regulates cell differentiation and migration) expression and myosin light chain phosphorylation, both factors thought to affect cancer development.

5.1.3 Drawback of Present Work

Inadequacies of the present study considering the association between autotaxin expression and bladder cancer include that although the study number was reasonable the low prevalence of autotaxin expression generally under powered the ability to identify change in bladder cancer. If the study was to be repeated it would be worthwhile performing a power calculation to establish how many patient samples I would need to sufficiently power analysis.

The aim of this project was to consider the association between autotaxin and bladder cancer. Therefore, there was no intention to analyze the relation between autotaxin and normal bladder tissue however this would be an interesting area to look at in future work as normal bladder would serve as a good control group. A small number of normal bladder specimens were examined in this study but it is possible that that they did not represent truly 'normal' bladder mucosa. Both practically and ethically it is very difficult to collect histologically normal bladder mucosa. Even 'normal' tissue from a bladder with cancer is likely to demonstrate field changes so therefore cannot be considered to be completely normal.

A further inadequacy of the current study is that the analysis does not allow me to analyze if the effects that I have reported is an effect specific to neoplastic cancer or if it could for example be found in inflamed bladder mucosa also.

5.1.4 Future Work

Future work would involve performing a power calculation initially to determine the number of patients needed to power analysis as well as including normal bladder tissue to use as a control.

5.2 Renal cancer

5.2.1 Main Findings

I found that in renal cancer, that there was no significant association between autotaxin expression and survival or time to disease progression. Multivariate analysis also found that autotaxin had no prognostic significance in relation to renal cell carcinoma and no association with time to disease progression.

I did however show that the high level expression of autotaxin observed on the tumour-associated vasculature was not apparent in the blood vessels of matched normal renal tissues. Unfortunately, the number of matched normal samples was too small to allow statistical analysis.

5.2.2   Previous Work

At the start of this study there was no published work on autotaxin and renal cancer however since embarking on this project Su et al. (2013) reported that altered autotaxin expression was detected in sunitinib-treated tumour vasculature of human RCC and in the tumour endothelial cells of RCC xenograft models when adapting to Sunitinib.

In the current study autotaxin expression in vessels only was reported as expression was found to be negative in all tumour cases.

Su et al. (2013) also reported that autotaxin and LPA, which is the catalytic product of autotaxin, were both involved in the regulation of signaling pathways in RCC in vitro, as well as cell motility. They found that by using an LPA_1 antagonist to block the LPA receptor 1 or gene silencing of LPA_1 in RCC cells resulted in increased LPA-mediated intracellular signaling and invasion in vitro. The LPA_1 antagonist also reduced RCC tumourigenesis in vivo. Furthermore, LPA_1 antagonist given along with Sunitinib was found to prolong the sensitivity of RCC to sunitinib in xenograft models, which implies

that autotaxin-LPA signaling may play a role in acquired resistance against Sunitinib in RCC.

5.2.3 Drawback of Present Work

There were several inadequacies of this analysis. The cohort of 59 patients is too small to detect any significant associations between autotaxin expression and renal cancer with confidence.

The cohort comprised patients with metastatic renal cell carcinoma who were about to commence treatment with Sunitinib. Future analysis would include renal cell carcinomas samples of various stages taken before, as well as after, treatment had commenced, to establish what effect if any Sunitinib had on autotaxin expression.

There were also too few histologically normal renal tissue samples included to allow any firm conclusions to be drawn. Similarly to that of bladder cancer collecting normal renal tissue is problematic both practically and ethically.

5.2.4 Future Work

Future work would involve expanding the cohort number, patients with various stages of the disease, and having a cohort of normal renal tissue to act as the control.

5.3 Autotaxin inhibitors

Autotaxin is secreted by multiple different cancer cells types and this contributes to the cells invasive properties (Gotoh et al. 2012). Ovarian cancer cells produce LPA at high levels (Baker at al. 2002, Sutphen et al. 2004). However it is not clear whether elevated LPA levels that result from secretion of autotaxin by cancer cells could be used as a marker for cancer. Plasma autotaxin itself does not appear to be cancer specific as high levels have been found in benign conditions such as liver disease (Nakagawa et al. 2011, Waranabe et al. 2007), pregnancy (Iwasawa et al. 2009) and cardiac conditions (Kimura et al. 2010).

Autotaxin is one of 40 genes that have been found to be up-regulated in highly metastatic cancers (Euer et al. 2002). Expression of autotaxin in mice resulted in mammary intraepithelial neoplasia, invasive and metastatic tumours (Liu et al. 2009). LPA was found to result in the chemo-resistance of ovarian cancer cells to cisplatin and adriamycin (ES at al. 2009) and paclitaxel-induced apoptosis in cancer cells inhibited by autotaxin (Samadi et al. 2009). Overexpression of autotaxin was found in patients with recurrent disease after chemotherapy treatment (Jazaeri et al. 2005). Gotoh et al. (2012) found that autotaxin was a potential drug-resistance gene in ovarian cancer and Vidot et al. (2010) showed that by inhibiting autotaxin it was possible to increase the sensitivity of resistant cancer cells.

Benesch et al. (2014) described how inhibition of autotaxin delayed breast tumour growth and lung metastases in mice. They proposed that by reducing LPA production and signaling in tumours it could offer a new approach to halting the development of metastases and chemotherapy and radiotherapy resistance (Brindley et al. 2013).

Van Meeteren et al. (2005) describe LPA as having an inhibitory feedback effect on autotaxin. If this is the case then LPA analogues that inhibit

autotaxin without activating the LPA receptors could potentially prevent cancer proliferation and invasion.

There have been multiple publications describing various autotaxin inhibitors over the last few years and other therapeutics targeting the autotaxin-LPA-LPA receptor signaling pathway. These inhibitors come in a variety of structures including metal chelators such as Bithionol, lipid-based inhibitors such as HA155, and small molecule inhibitors such as H2L-7905958.

While pharmaceutical companies all over the world compete to find the next cancer treatment, autotaxin inhibitors will certainly be among the forerunners.

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