IDENTIFICATION AND EVALUATION OF BIOMARKERS FOR THE DETECTION OF BLADDER CANCER

By

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Abstract

BACKGROUND

Urinary Bladder Cancer (UBC) is the 5th most common cancer in the West (Cancer Research UK 2014). Continuous efforts have been made to develop non-invasive urine-based biomarkers with high sensitivity and specificity that would improve patients' quality of life, costs and lower the number of cystoscopies (Bryan et al. 2010). This can then be utilized as part of a multi-biomarker panel to develop a urine test for UBC diagnosis as a single marker is unable to replace current diagnostic invasive tools (Brentnall et al. 2012).

METHODS

Using a (LC-MS/MS) proteomic approach, 8 UBC and 1 normal bladder cell secretomes were analysed to identify secreted proteins that can be potential candidate biomarkers. phorbol 12-myristate 13-acetate (PMA) a PKC activator was used to treat whole secretomes and investigate all proteins released by cells whereas secretomes that were treated with the broad-spectrum inhibitor, Marimastat underwent ultracentrifugation to focus on proteins being shedded. Protein quantification was based upon stable isotope labelling by peptide demethylation (Chen et al. 2003). Two methods were utilized to select candidate biomarkers: Human Protein Atlas and Ingenuity Pathway Analysis. This project was interlinked to investigate Epithelial cell adhesion molecule (EpCAM) biology, a type I membrane protein that is cleaved by ADAM-17. This was done by examining the effects of PMA and Marimastat on its ectodomain shedding. The expression of its extracellular domain in cell lysates was determined by western blotting. This was followed by subsequent experiments to investigate cell phenotype and the effect of knocking down ADAM-17. This was concluded by measuring matrix metalloproteinase MMP-1 in urine of 323 UBC patients to investigate its potential use as a biomarker.

RESULTS:

Findings revealed 83 potential biomarkers in PMA-treated secretomes have been discovered by HPA analysis. Using the Biomarker Filter tab on IPA software four potential biomarkers were elucidated: GSTM1, AMARC, LAMC2 and APEG. IPA core analysis revealed many proteins were related to carcinogenic events such as invasion, proliferation, progression, metastasis and hyperplasia. PMA stimulates EpCAM release and increases cell viability and migration. Alternatively, Marimastat inhibits EpCAM release and has no noticeable effect on cell viability and migration. MMP-1 was elevated in a proportion of patients. In relation to stage, Mann-Whitney test revealed there was no significant difference found in Ta, T1 but there was a significant difference found in T2 whereas for grade, there was no significant difference for G1 and G2 but there was for G3. The AUC score for grade and stage was 0.653 and 0.605 respectively.

CONCLUSION:

PMA increases secretion and shedding of proteins and has an effect on the cell phenotype. Despite Marimastat inhibits shedding of EpCAM, it does not have an effect on cell phenotype. Urinary MMP1 is not a useful neither prognostic nor diagnostic biomarker of UBC. Elevated MMP-1 levels are only apparent in high stages and grade.

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1.0 Introduction

Urinary Bladder cancer (UBC) is the most common malignancy of the urinary tract (Dunn et al. 2013). It has a global incidence of more than 356,000 and prevalence approximately 2.7 million that continues to increase annually (Chen et al. 2012; Aben 2009). It mainly affects elderly people and the average age at the time of diagnosis is 73 (American Cancer Society 2014). Higher incidence rates are observed in North America, Europe and Australia compared to Far Eastern countries (Bartonová 2012). In the UK alone there are approximately 10,400 new cases attributed to bladder cancer yearly (7,500 men and 2,900 women) (Cancer Research UK 2014). In the USA the number of cases is estimated to be 74,690 new cases (about 56,390 in men and 18,300 in women) whereas in the EU there are 123,135 cases (approximately 96442 in men and 26693 in women) (American Cancer Society 2014, International Agency for Research on Cancer 2014). Possible explanations of the higher incidence in men than women are attributed to different historical cigarette smoking patterns; although it is not known whether hormonal differences play a role as well (Lotan et al. 2013; National Health Service, 2014). Mortality rates have risen; there are 5,000, 15,200 and 40,252 deaths in the UK, USA and EU respectively per annum (American Cancer Society 2014; Cancer Research UK 2014; International Agency for Research on Cancer 2014). However, reports have predicted that the burden of UBC will increase in developing areas. This is due to a combination of aging and growth of global population and exposure to cigarette smoking (Aben 2009).

1.1 Types of Bladder Cancer

1.1.1 *Urothelial carcinoma of the bladder*

Urothelial carcinoma of the bladder (UCB) accounts for 90% of UBC cases (Feldman 2009; Steinberg 2014). Table 1 shows the Tumour, Node, Metastases (TNM) 2002 classification that is used to identify stages and is approved by the Union International Centre in Cancer (Algaba et al. 2006). Stages Ta/T1 (confined to the mucosa/submucosa) are non-muscle invasive cancer (NMIBC) which was previously known as 'superficial' bladder cancer presents 70% of UCB cases (Bartonova 2012, Feldman 2009, Dunn et al. 2013, Netto 2012, Adab et al. 2002). It has a tendency to recur but is not immediately life-threatening (Bartonova 2012, Feldman 2009). Muscle-invasive cancers (MIBC) account for the remainder of UCB cases and represent life –threatening disease requiring radical therapy (Bartonova 2012, Feldman 2009, National Health Service 2014, Adab et al. 2002).

Table **1: The pathology of bladder cancer TNM classification of UBC (Algaba et al. 2006).**

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
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 (extravesical mass)
T4	Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumour invades prostate, uterus or vagina
T4b	Tumour invades pelvic wall or abdominal wall
N (Lymph nodes)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node 2 cm or less in greatest dimension
N2	Metastasis in a single lymph node more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension
M (Distant metastasis)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

1.1.2 *Other types of UBC*

There are other cancers besides UCB that initiate in the bladder but are rare. For instance, squamous cell bladder cancer that account for 5% of UBC cases (Cancer Research 2014). Under microscopic examination, squamous cells are flat cells that form the moist, skin-like tissues that line the body (American Cancer Society 2014, Cancer Research UK 2014). Majority of squamous cell carcinomas are invasive (American Cancer Society 2014). They are commonly found in developing countries that have a high incidence of bilharzias; a worm infection (Cancer Research UK 2014).

Adenocarcinoma accounts for 1-2% of cases and is a rare type of UBC. It develops from the mucus-secreting cells in the bladder lining (American Cancer Society 2014, Cancer Research UK 2014). Majority of adenocarcinomas are also invasive (American Cancer Society 2014).

Some bladder cancers are even rarer and are not formed from the urothelial lining. For instance, sarcomas that initiate in the muscle cells of the bladder (American Cancer Society 2014). Another example is Small cell carcinomas of the bladder (SCCB) that account for 1% of cases. They initiate as neuroendocrine cells which are nerve-like cells that develop quickly (American Cancer Society 2014). SCCB patients are treated with chemotherapeutic regimens used for small cell carcinoma of the lungs (SCLC) due to similarities found between both types of cancer (American Cancer Society 2014, Ismaili 2011).

1.2 Causal factors of UBC

Many studies have been conducted to investigate the aetiology of UBC; however the exact cause has not been elucidated (Chu 2013). Cigarette smoking and occupational exposure to chemicals are the two main factors that have been confirmed to increase UBC risk (Burger et al. 2013, Chu 2013). They account for 50% and 20% of cases respectively (Burger et al. 2013).

There are other factors that have been associated with UBC such as dietary factors, family history, race and socioeconomic status (Lotan 2013). It is important to note that some individuals with many risk factors do not actually have it whereas there are other individuals with bladder cancer that have several or no known risk factors (American Cancer Society 2014). This implies that having a risk factor or a combination of them does not indicate that one will develop bladder cancer (American Cancer Society 2014).

1.3 Symptoms

1.3.1 *Haematuria*

Haematuria (blood in urine) is the cardinal symptom of UBC and is normally painless (Bryan 2013b, National Health Service 2014). Statistics have shown that 85% of patients with bladder cancer have gross or microscopic haematuria (Feldman 2009, Budman 2008). However, a variety of benign inflammatory conditions that has no relation to UBC can also cause haematuria (Budman 2008, Feldman 2009).

1.3.2 *Other symptoms*

There are other symptoms such as frequent or sudden passing of urine and pain whilst urinating. However these symptoms are also associated with urine infection especially if there is no blood

present. Another explanation of these symptoms would be if the patient was male, this may be due to an enlarged prostate gland (Cancer Research UK 2014c). On the contrary, if it is cancer; the earlier it is diagnosed the easier it is to treat (Cancer Research UK 2014c).

1.4 Diagnosis

Early detection of UBC is necessary to improve prognosis and increase survival rates (Diamandis 2010a, Budman 2008). There are multiple diagnostic procedures for further investigations of patients suspected of having UBC. Generally, this compromises of imaging, biopsy and invasive evaluation with cystoscopy (Dawam 2012). Cystoscopy involves examining inside the bladder for tumours (The Patient Education Institute 2011). In some cases; the tumour can be removed during the procedure (The Patient Education Institute 2011).

1.5 Treatments

1.5.1. *Treatments for NMIBC*

Transurethral resection of bladder tumour (TURBT) is the first-line treatment for patients with NMIBC (Dunn et al. 2013). TURBT is a surgical procedure that removes the bladder tumour preventing it from metastasising to other organs of the body. A biopsy can also be taken at the same time and sent for examination (Guy's and St Thomas'NHS Foundation Trust n.d.). Chemotherapy following TURBT can reduce the recurrence rate by 39% in patients with low risk of recurrence and progression (Dunn et al. 2013).

Adjuvant treatments are often used due to risk of recurrence and progression to MIBC such as Bacillus Calmette–Guérin (BCG). BCG is a vaccine for tuberculosis and it promotes immune cells to become active in the bladder lining (Cancer Research UK 2014b). It can also work as an

immunotherapeutic agent to eliminate cancer cells that grow back or have remained behind in the bladder lining (Cancer Research UK 2014b). BCG is inserted directly to the bladder and this is known as intravesical treatment (Cancer Research UK 2014b). If the cancer re-occurs and progresses; cystectomy takes place which is a major operation to remove the tumour and not everyone is suitable for this surgical procedure (Cancer Research UK 2014b, UCLA 2014). Thus additional BCG treatment or chemotherapy is inserted which sometimes does not work well (Cancer Research UK 2013).

However, recent advances such as BCG in combination with instillation of hyperthermic mitomycin-C have proven to be effective (Cancer Research UK 2013, Compérat et al. 2011). Hypothermia is a treatment in which the bladder is heated to approximately 42^{o}C (Cancer Research UK 2013). Mitomycin C is a chemotherapy and is inserted through the bladder via a catheter; a tube (Cancer Research UK 2014a). The type of instillation is dependent on several factors such as stage, grade and multifocality of the tumour (Dunn et al. 2013).

1.5.2 *High-risk recurrent NMIBC and MIBC*

High-risk recurrent NMIBC and MIBC can be treated with curative intent by radical therapies such as radiotherapy, chemotherapy, and radical cystectomy (Diefenbach et al. 2012, Dunn et al. 2013, Choueriri 2008).

Recent findings suggested that the risk of recurrence after cystectomy has reduced from 40% to 6-13% and is dependent on tumour grade, stage and node status at the time of cystectomy (Diefenbach et al. 2012). In addition, studies have portrayed that radiotherapy and cystectomy have similiar long-term survival rates and there is no apparent changes linked to mode of treatment (Bryan 2010).

Therefore, long-term surveillance and treatment is necessary and current protocol states that cystoscopy adjunct with cytology (microscopic examination of cells present in the urine) should be implemented every 3 months for the first initial 1-2 years followed by every 6 months for 1-5 years and then annually as long as no recurrence has occurred (Budman 2008).

1.6 Disadvantages of current diagnostic procedures

The current protocol has significantly high cumulative costs of healthcare resources and patient time especially when patients' risk of recurrence is unequal (Bryan 2011, Budman 2008).

Furthermore, despite cystoscopy is a highly sensitive fundamental technique for most tumours, detection of Carcinoma in situ (CIS) or small papillary tumours that account for early recurrence can be suboptimal and can lead to incomplete resection (Chen et al. 2012, Bryan 2010, Parker 2011, Budman 2008).

However, there have been technological advances to improve the quality of TURBT and cystoscopy by limiting the number of cystoscopies or improving its sensitivity (Dunn et al. 2013). Amongst these is photodynamic diagnosis (PDD) and narrow-band imaging (NBI). They improve and increases detection rates of urothelial lesions compared to white-light cystoscopies (WLC) (Burger et al. 2008, Geavlete et al. 2012, Kausch et al. 2010). PDD is also known as fluorescence cystoscopy and utilizes 5-aminolevulinic acid (ALA) or its hexyl ester, hexaminolevulinate 5-ALA (Douglass 2007). NBI cystoscopy is a technique with high resolution that does not involve use of dyes (Geavlete et al. 2012). This leads to more patients having a complete resection and longer survival rates without recurrence (known as Recurrence-free survival), costs and better patient management (Burger et al. 2008, Kausch et al. 2010).

Nevertheless, cystoscopy is still as invasive and is a source of patient anxiety (Budman 2008).

Moreover, urine cytology as opposed to WLC is non-invasive. It has poor sensitivity but high specificity for low-grade, well-differentiated lesions. On the other hand, it has good sensitivity and specificity to detect high grade tumours (American Cancer Society 2014, Chen et al. 2012, Budman 2008). In addition, cytology can be challenging as it is dependent on the competency of the cytopathologist (Steinberg 2008).

1.7 Solutions

There is a need for the development of a non-invasive and inexpensive diagnostic tool such as urinary-based biomarkers with high sensitivity and specificity to detect UBC and/or differentiate between NMIBC and MIBC as alternative tools for the detection and surveillance of bladder cancer (Bryan 2010, Boustead et al. 2001). This would significantly improve diagnosis; lower healthcare costs, reduce morbidity and improve patient quality of life (Chen et al. 2012, Bryan 2010). Another potential utility of urinary biomarkers is that current evidence suggests that they have high sensitivity and specificity (>90%) for diagnosis of MIBC; this will lower the risk of delayed treatment (Bryan et al. 2013b).

1.8 Introduction to Biomarkers

Biomarkers are defined as biological molecules that can be objectively detected and measured in bodily fluids, tissues and cell lines (Chan 2013, National Institute of Health 2014). They can be mRNA, proteins, metabolites, DNA or mechanisms for instance proliferation, apoptosis and angiogenesis (Diamandis 2007). There are two types of biomarkers that offer distinct and obvious advantages: biomarkers of exposure, which are used in risk prediction, and biomarkers of disease, which are used in screening, diagnosis, recurrence, monitoring disease progression and response to

therapy (Diamandis 2007, Mayeux 2004). However, due to the minimal cancer prevalence, a biomarker has not yet been developed that meets all of these criteria (Diamandis 2007).

1.8.1 *Classes of Biomarkers*

There are several distinct types of cancer biomarkers based on different areas: genetics, proteomics, epigenetics, metabolomics, imaging technology and general physical techniques (Geng 2009).

1.8.1.1 DNA/cell-free DNA

Elevated levels of serum DNA concentrations are linked with cancer. Examples of substances that can serve as DNA biomarkers are alterations in DNA copy number and chromosomal aberrations (Weinstein 2014). Reports have shown that certain genes are hypemethylated in a range of cancers and some have been reported as biomarkers for UBC diagnosis. Bondaruk et al. revealed 6 potential methylation markers for UBC: SOX11, DBC1, MYO3A, PENK, CA10 and NKX6-2. Their sensitivity and specificity in urine elevate when measured as a panel. This further signifies how a panel of biomarkers increases both sensitivity and specificity.

1.8.1.2 RNA/microRNA

MicroRNAs (miRNAs) are small (22 nucleotides) non-coding RNAs that regulate the stability and translation of mRNA transcripts and play vital roles in various biological processes (Barik 2011, Choi et al. 2012). Choi et al. discovered that cell-free miRNAs were both present and stable in urine; specifically miR-145 levels were assessed and were able to distinguish between UBC patients and non-cancer controls. A higher recurrence risk was observed amongst NMIBC patients with lower miR-200a level compared to patients with higher miR-200a. This emphasises how miRNAs have shown for urine biomarker discovery.

1.8.1.3 Metabolic biomarkers

Metabolomics is the latest of the omics technologies and plays a critical role in early UBC diagnosis (Kim et al. 2014). Recent studies revealed potential candidate biomarkers via mass spectrometry-based metabolomics of urine of UBC patients that are useful for diagnosis and prognosis (Albericea et al. 2013). Examples of metabolites include tyrosine, histidine, phenylalanine and tryptophan that has been previously revealed but their role with cancer progression has only been established (Albericea et al. 2013).

1.8.1.4 Proteins

Proteins are another strategy for biomarker development. The proteome is the group of proteins that are encoded by the genome whereas the large-scale analysis of the structure and function of proteins is known as proteomics (Hudler 2014, Conrads et al. 2003). Proteins are attractive as biomarkers due to point of care testing, cost, reproducibility and great diversity (Brentnall et al. 2012). They are the end-point of many biological mechanisms and therefore they can precisely reveal the pathogenic phenotype (Hudler 2014).

1.8.2 Current biomarkers

The Food and Drug Administration (FDA) have approved several urine-based biomarkers (Cancer Research UK 2014, Bryan et al. 2011).

1.8.2.1 Nuclear Matrix Protein-22 (NMP22)

Nuclear matrix is a non-chromatin structure that normally provides support to the nucleus, gene expression and RNA transcription (Budman 2008). NMP-22 resides in the nuclear matrix whose role is to distribute chromatin to daughter cells during cell division (Steinberg 2013). It is released

from nuclei of tumour cells after they undergo apoptosis and are detected in urine (Steinberg 2013). Levels are higher in malignant than normal urothelial cells (Cancer Research UK 2014). The NMP-22 immunoassay test has many advantages than cytology. Amongst these advantages are it does not require prolonged times for examination nor need intact cells. Thus results can be obtained within half an hour, less costly and more sensitive than cytological analysis (Steinberg 2013).

1.8.2.2 Bladder Tumour Antigen (BTA)

BTA is another urine biomarker that can be released by normal and tumour cells where in the former as a response to autoimmune attack whereas in the latter to aid in evading the host's immune response (Cancer Research UK 2014, Budman 2008). BTA tests measures complement factor H–related protein (CFHrp) and complement factor H in urine that is picked up by two monoclonal antibodies (Cancer Research UK 2014, Steinberg 2013). The role of both factors is to inhibit the complement cascade which in turn inhibits cell lysis (Steinberg 2013). Dahse et al. revealed that CFHrp blocks C5 convertase activity and intervenes with C5b surface deposition and Membrane Attack Complex (MAC) synthesis. This mechanism is different to CFH and both proteins manage the complement pathway in an orderly manner. They bind to the same cellular surface sites and CFHrp activity increases as CFH prevents C3 convertase. Both factors reside in bladder cell lines and if detected there are a high risk of recurrence (Cancer Research UK 2014, Steinberg 2013). Many studies have been conducted where there is variability in terms of sensitivity and specificity (Steinberg 2013).

1.8.2.3 UroVysion

Chromosomal mutations have been linked with UCB. UroVysion is a multi-target interphase Fluorescence *in situ* hybridization (FISH) assay that can be utilized to identify urinary cells with

aneuploidy in chromosomes 17, 3, 7 and deletion of 9p21. Aneuploidy is an abnormal number of chromosomes (Gelbart 2000). This technique contains probes for centromeres of chromosomes mentioned and have been linked with high sensitivity and specificity. Studies have discovered that FISH is more sensitive and specific than urine cytology (Diefenbach et al. 2012). It is used to aid in initial diagnosis of UBC patients with haematuria and monitor tumour recurrence in patients that were diagnosed previously with UBC (Steinberg 2013).

1.8.2.4 Mcm5

Mcm5 test is a new test in which trials have discovered that its reliability and accuracy complements NMP22 test and a combination of both may aid in diagnosis (Cancer Research UK 2014). Mcm5 is a cell cycle biomarker of dysregulated growth (Burling et al. 2012). Studies have shown that the dual test detects 95% of significant diseases (Burling et al. 2012).

1.8.2.5 Disadvantages of current biomarkers

There are issues with current available biomarkers, they are less specific but have high sensitivity than cytology and some markers are unable to distinguish between urothelial malignancy and inflammation and other benign urological conditions (Budman 2008). FISH results may be used mainly when urine cytology results are inconclusive (Dunn et al. 2013).

Therefore, these biomarkers can act as adjunct to cystoscopy but is insufficient to replace it (Bryan 2010). There is an increase consensus by researchers that a panel of biomarkers is needed to elevate specificity and sensitivity for accurate diagnostic purposes and replace cystoscopies that a sole biomarker cannot fulfil (Hudler 2014).

1.9 Introduction to proteomics

The advent of proteomics play crucial roles in the identification, quantification and characterization of proteins that are involved in characterizing disease states, investigating cell signaling pathways, discovery of biomarkers and drug targets (Diamandis 2010b, Wehr 2006). Mass spectrometry (MS) is utilized with diverse separation ways and is the fundamental methodology of proteomics (Wehr 2006). There are two types of proteomics: top-down and bottom-up/shot-gun proteomics (Hudler 2014). The latter is usually used for such pursuits whereas the former can also be applied to many of these cases. Bottom-up proteomics consists of complex protein mixtures, purified mixtures that undergo proteolytic cleavage/enzymatic digestion into peptides. The peptides are then analysed by MS to identify the proteins (Hudler 2014, Wehr 2006).

Alternatively, Top-down proteomics consists of large protein fragments or intact proteins that undergo gas-phase fragmentation for analysis via MS without previous digestion into peptides (Wehr 2006). For characterization, proteins are extracted from the sample which is followed by fractionation. The fractions are then analysed by MS/MS (Wehr 2006). This is typically done by electrospray ionisation where the multiple charging increases dissociation and identifies intact protein by comparing the masses of the original precursor and product ion with the protein sequence databases (Wehr 2006).

1.9.1 *Mass Spectrometer*

Amongst the MS used are Liquid Chromatography – Mass Spectrometry (LC/MS) and Matrix Assisted Laser Desorption/Ionization (MALDI).

1.9.2.1 LC/MS

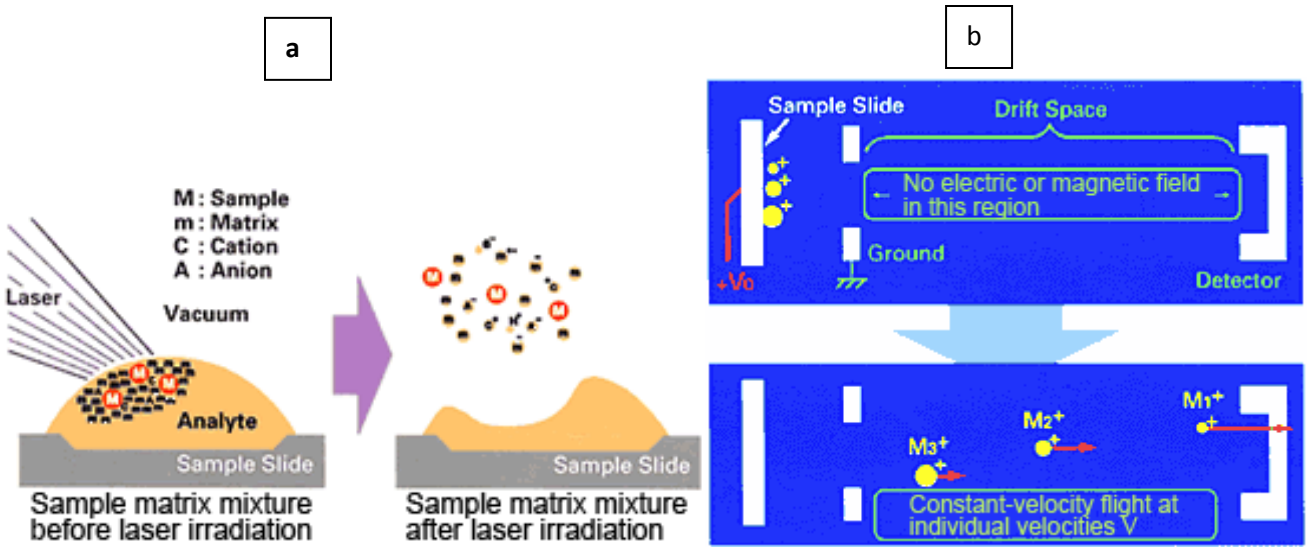
This technique consists of HPLC with MS. An interface is a particle beam that divides sample from solvent. It is needed to remove solvent and create gas phase ions. They are then transferred to optics of MS (Waters European Marketing n.d.). Majority of instruments have atmospheric pressure ionization (API) method in which removing the solvent and ionization are united in the source at atmospheric pressure (Waters European Marketing n.d.). There is also another type in which the step of solvent elimination and ionization are not together. This is known as electron impact ionization (EI). EI is useful in two major respects: when molecules do not undergo ionization with API and when electron impact spectrum is needed (Waters European Marketing n.d.).

1.9.2.2 MALDI-TOF MS

The sample is mixed with matrix; this is followed by the matrix absorbing the ultraviolet light. This results in conversion to heat energy. Some of the matrix is then heated rapidly and undergoes vapourisation with the sample as shown in Figure 1a overleaf. The next step is Time of Flight Mass Spectrometry (TOF MS) as shown in Figure 1b overleaf. There are different sizes of charged ions on sample slide. The variation in velocity between sample slide and ground will determine and attract the direction in which ions will travel. Lighter ions i.e those with smaller mass to charge ratios and those with highly charged ions shifts rapidly via the drift space until they reach detector (Shimadzu Corporation 2014).

Figure 1: An illustrated diagram of the principles of MALDI-TOF

In (a) It illustrates the MALDI principle where the sample and the matrix is missed before laser irradiation. Some of the matrix undergo vaporization with the sample. In (b) It illustrates the TOF principle whereby the direction of where the irons will travel is dependent on the charge size. Lighter ions (small m/z ratio) and those with highly charged ions move quicker than heavy ions (big m/z ratio). (Shimadzu Corporation 2014).



1.9.3 *Advantages of proteomics*

Proteomic technologies accelerates biomarker discovery and will aid in the understanding of gene function as well as identifying peptides/proteins in their post-translational modification such as phosphorylation or glycosylation, protein-protein interactions and comparing protein levels with potential application in growth and disease (Adams 2008, Gauthier et al. 2013). It will also have a dramatic change in cancer research and care (Srinivas 2002).

In particularly for bottom-up approaches, reversed-phase HPLC allows separation of peptides with solvents at high resolution. It has also allowed identification of proteins in complex mixtures such as cell lysates using capillary HPLC-MS-MS. In expression proteomics, staple isotope labeling and affinity tags have been developed in order to distinguish down-regulated and up-regulated proteins (Wehr 2006).

Stable isotope labeling is used in quantitative proteomics. Oxygen-18, deuterium, nitrogen-15 and carbon-13 isotopes are commonly used. They are incorporated via two methods: post-metabolically in peptides and proteins or metabolically in cells. When labeled peptide ions segregate in mass spectrometer, their intensity values are used for quantification (Impens et al. 2008).

In addition, top-down proteomics has advantages: enzymatic digestion of proteins is a time-consuming process and is not needed in this approach. The complete protein sequence can be accessed (Wehr 2006). Thus, degradation products, sequence variants and a mixture of post-translational modifications can be discovered (Doucette 2008, Flugge 1984).

1.9.4 Limitations of proteomics

There are limitations to the bottom-up approach such as partial characterization of different splice forms, endogenous protein cleavages and alternative modifications such as methylation and acetylation. Combinations of these forms can complex patterns of intact proteins isoforms (Kelleher et al. 2014). Another issue is high abundant peptides cause inadequate information about low abundant proteins (Wehr 2006).

Furthermore, top-down approaches is a relatively new field compared to bottom-up method. Amongst the limitations are: they do not work effectively with intact proteins that are larger than 50 kDa. Bioinformatic tools are primitive in contrast to bottom-up proteomics (Wehr 2006). Single-nucleotide polymorphisms, post-translational modifications, disulphide bonds and cleavages can alter the precursor mass and influence the fragment ion spectra. In addition, multiple protein fragments and co-isolation are identified in a single spectrum (Kim 2003).

1.9.5 *Sources of biomarkers for proteomics*

Previous attempts have been conducted to discover potential biomarkers using different sources (Bryan 2010). Amongst these are tissue samples which are not easily accessible and they having a heterogenous nature of cells (Hudler 2014). Thus for clinical diagnosis, it is better to use specimens that are minimally invasive, easily obtained, inexpensive and reproducible such as bodily fluids (Andersson et al. 2011). However, one of the limitations for their use is their complexity (Hudler 2014). For instance, blood has some highly abundant proteins such as albumin or globulin that can influence the detection of less abundant proteins (Andersson et al. 2011). Alternatively, urine has posed as a solution as abundant proteins presence is less than in serum and has direct contact with tumour (Bryan 2010). Cancer secretomes have shown to be a potential source for oncoproteomic use where cell lines and proximal fluids can be utilized that is easily accessible (Andersson et al. 2011, Diamandis 2010b, Makridakis 2010).

1.9.5.1 Secretomes

Secretomes consists of secreted proteins that compromise 10% of human genome (Diamandis 2010b). It mirrors the roles of cancer cells and consists of proteins that are secreted from cell surface and intracellular proteins that are released into supernatant due to cell lysis and apoptosis (Makridakis 2010). They also include proteins shed from the cell membrane due to proteolytic cleavage such as growth factors and cytokines (Makridakis 2010). They play key roles in cell signalling, communication and migration (Makridakis 2010). They may represent putative biomarkers or therapeutic targets for diverse range of cancers particularly UBC and can be detected in urine (Diamandis 2010b). There are two prominent pathways in which proteins are released

extracellularly (Chung et al. 2013). An overview of protein secretion pathways need to be addressed to increase understanding of secretome analysis.

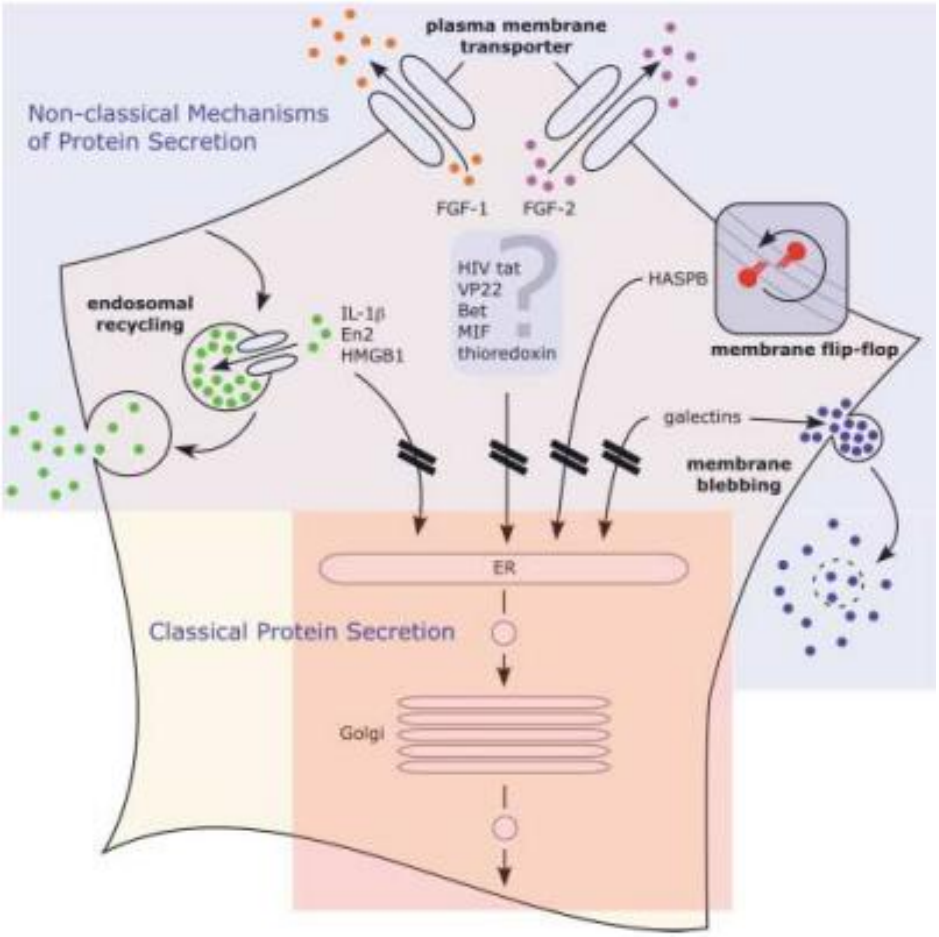
1.9.5.1.1 Protein secretion pathways

The classical secretory mechanism of protein synthesis and sorting compromises proteins that are synthesized by ribosomes contain an (endoplasmic reticulum) ER-signal peptide that associates with rough ER (Baltimore et al. 2000). Translation is completed in ER where new proteins are either integrated into transport vesicles that combine together to create *cis*-Golgi vesicles or they remain in the ER such as structural proteins or enzymes (Baltimore et al. 2000). The *cis*-Golgi cisterna converts to *trans*. Membrane-bound and luminal proteins undergo post-translation modifications such as associating with oligosaccharide chains (Baltimore et al. 2000). This is subsequently followed by another protein movement where some proteins move to the cell surface via small vesicles and undergo consistent secretion (Baltimore et al. 2000). Vesicles that bound to plasma membrane cause luminal protein release into the extracellular space (Nickel 2003). Other proteins travel to lysosomes and some remain in *trans*-Golgi cisternae (Baltimore et al. 2000).

The absence of ER/Golgi system in exporting proteins is known as the non-classical secretory pathways. There are a number of different types illustrated in Figure 2. For instance, export can take place via integrating into vesicles such as Engrailed 2 (En2) and High-mobility group box 1 (HMGB1). Another way is to transport proteins to extracellular space via translocation across plasma membrane as seen by Fibroblast growth factors (FGF) (Nickel 2003). In addition, it can also involve exosome synthesis which are labile vesicles formed at the cell surface via membrane blebbing. Proteins secreted through exosomes are released into extracellular space (Nickel 2003).

Tumour cells are in direct contact with the extracellular space. This allows them to progress via other hallmarks of cancer such as angiogenesis and metastasis where in order to facilitate the latter, extracellular matrix is degraded (Chung et al. 2013). These carcinogenic events are induced by key proteins secreted by tumour cells such as proteases, cytokines, chemokines, receptors and adhesion molecules (Chung et al. 2013). There is evidence that exosomes may play crucial roles in cancer development and progression (Blonder et al. 2009). In addition, tumour cells recruit stromal cells that also release secreted proteins. Thus, cancer secretomes consist of proteins that are secreted from secretory pathways in addition to proteins released by stromal cells and/or shedded from the cell surface that are potential candidate biomarkers (Chung et al. 2013)

Figure 2 Classical and Non-classical protein secretion pathway (Nickel 2003)



1.9.5.1.2 Limitations of cancer secretome analysis

Many studies conducted with secretomes have utilized conditioned medium (CM) of cultured cancer cell lines due to limitations of analysis of biological fluids (Chung et al. 2013). It is less complex which improves detection of less abundant proteins despite not representing the whole tumour microenvironment. However, secretome analysis also has its limitations. For instance, Fetal Bovine Serum (FBS) is added to the media and secreted proteins are covered by serum proteins. Secreted proteins are at low concentrations due to volumes of media utilized. Another challenge is contamination of intracellular proteins that occurs frequently during lysis (Chung et al. 2013). However, recent advances in proteomic technologies have led to improvements in resolution, sensitivity, and accuracy of mass spectrometry (Goodlett 2010).

1.9.5.2 Disadvantages of biomarkers

Many researchers have discovered potential candidate biomarkers to be used in routine clinical practice (Chan et al. 2013). Reasons why biomarkers fail to reach clinic is summarised in Figure 3. One of the confounding issues are growing costs of equipment, limited knowledge as to what is required for a clinical assay to be successful (Chan et al. 2013, Brentnall et al. 2012, Hudler 2014). Better bioinformatics is needed to process the large amount of data (Brentnall et al. 2012).

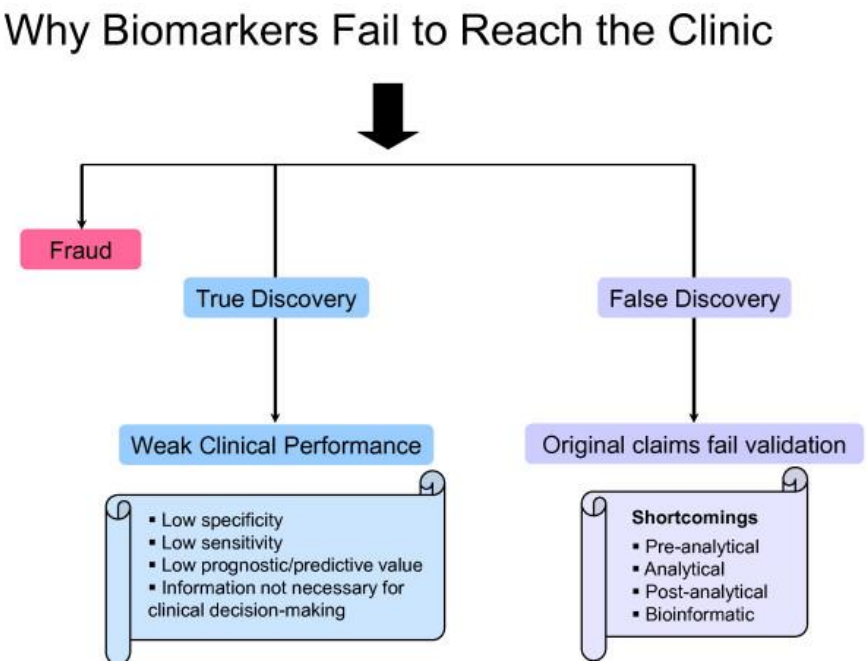


Figure 3: Summary of reasons for biomarker failure to reach the clinic. Apparent failures in diagnostics can be classified into three distinct categories as shown:Fraud, True Discovery and False Discovery (Diamandis 2012).

1.9.6 *Potential biomarkers from previous studies*

1.9.6.1 HAI and Midkine

Our group has previously published data on potential candidate biomarkers. Bryan et al. utilized shotgun proteomic approach combined with transcriptome analysis and discovered 10 biomarker candidates. This was based on the following approach: two microarray datasets compared 12-14 normal bladders with 15 Ta UCBs. A 2.5 fold change and $P<0.001$ criteria was used to select 44 genes upregulated in both data sets (Bryan et al. 2013a). This was then compared with protein list identified by two or more peptides in at least two of cell line secretomes. Two secreted proteins were then assessed for their potential in urine: Hepatocyte growth factor activator inhibitor type 1 (HAI-1) and midkine as they were secreted. The membrane-linked ligand for NKG2D receptor, ULBP-2, was used as a control as it was identified in secretomes but not substantially elevated in microarray data. ULBP-2 levels are higher in malignant than normal cells. It is also shed by proteases (Bryan et al. 2013a).

HAI-1 is a membrane-bound serine proteinase inhibitor that resides in epithelial cells. An example of proteinases it inhibits is matriptase (Hamasuna et al. 2000). It is shed into the ECM and is involved in cancer progression (Bryan et al. 2013a).

Midkine is an angiogenic growth factor and previous studies have shown it is up-regulated in UCB and is linked with late stages (Bryan et al. 2013a).

Findings suggest that levels were higher in invasive disease and were too high to be explained by haematuria and were likely due to tumour secretion (Bryan et al. 2013a).

1.9.6.2 Calreticulin

Arai et al. identified calreticulin, an endoplasmic reticulum chaperone as well as other candidate biomarkers via proteomic analysis. They discovered it to be elevated in UBC and their reported sensitivity and specificity were 73% and 86% respectively. The same group later discovered a soluble isoform of catechol-o-methyltransferase (s-COMT) snf gamma -synuclein (SNCG) (Arai et al. 2004). They then combined all three biomarkers and measured it in urine. The overall sensitivity and specificity was 76.8% and 77.4% respectively which was higher than when calreticulin was measured alone and had 71.4% and 77.8% respectively. This further signifies how a panel of biomarkers will improve UBC diagnosis.

The differences between our group study and Arai et al. is that our study utilized transciptome data combined with the proteomic data, whereas the latter utilized only the proteomic data. Amongst the advantages of using transcriptome data is it is publically available, lots of genome is included and the secreted proteins that are up-regulated do not incorporate as proteins in tissue and may have high mRNA levels instead (Bryan et al. 2013a).

In addition, Arai et al. evaluated the diagnostic capacity of a combination of biomarkers in a panel, whereas Bryan et al. were investigating potential biomarkers to be part of a panel of biomarkers.

1.9.6.3 EpCAM

Our group found several plasma membrane proteins via proteomic analysis whose extracellular domains have shown their potential as biomarkers. Extracellular domain of EpCAM that is cleaved by ADAM 17 kinase is being shed and also EGFR by UBC and can be potential prognostic biomarkers (Bryan et al. 2014). They discovered after measuring EpCAM in urine of UBC patients that levels increased with grade and stage. Thus, an increase of shedding of EpCAM and other cancer-associated proteins may be a source for biomarker discovery and may promote cell proliferation (Bryan et al. 2014).

1.9.6.4 Engrailed-2

Engrailed-2 (EN2) transcription factor protein is another potential biomarker that has been discovered by our group. It has an overall sensitivity of 82% and specificity of 75% in patients with NMIBC (Bryan et al. 2013c). Thus EN2 is a potential biomarker with higher sensitivity and specificity than current tests.

Ultimately, the proteomic approach, metabolomics, next-generation sequencing or a combination can will improve urine biomarker discovery and provide multi-marker urine test to detect bladder cancer and aid in therapeutic strategies (Goodlett 2010). Nevertheless, compared to other malignancies, bladder cancer research is significantly underfunded; hindering progress in improving diagnosis and therapeutics for patients with the disease (Bryan 2010).

2.0 Aims

- To use proteomic analysis of UBC cell lines secretomes to discover candidate urinary biomarkers for UBC. This can be achieved by identifying and focusing on proteins shedded from the surface of in vitro cultured UBC cells:

A) 'Whole' secretomes ± PMA.

Hypothesis: phorbol 12-myristate 13-acetate (PMA) is a PKC activator that stimulates secretion and shedding. It was used to treat whole secretomes and investigate all proteins released by cells (secreted and shedded proteins). Up-regulated proteins emphasises regulated release.

B) Ultracentrifugation of ± PMA secretomes

Hypothesis: After ultracentrifugation, vesicles/membranes are removed and any integral membrane proteins left must be shed extracellular domains. The proteolytic cleavage of the extracellular domains of membranes is known as ectodomain shedding. The focus will be on shedded proteins.

C) *Ultracentrifuged secretomes ± Marimastat.*

Hypothesis: Proteases that induce shedding are known as 'sheddases'. There are two types: a distegrin and metalloproteinase (ADAMs) and matrix metalloproteases (MMPs) that has potency against majority of MMPs. Marimastat, a broad spectrum metalloprotease inhibitor was utilized that covalently associates with the zinc atom at the active site of MMPs and inhibits these sheddases. Therefore, Marimastat can be used to confirm protease-based shedding.

D) ADAM17 siRNA to specifically inhibit this 'sheddase'

Hypothesis: There is evidence that ADAM17 induces proteolytic cleavage via its extracellular site.

- To measure one of the potential candidate biomarkers in bladder cancer patients' urine samples to make initial estimates of sensitivity and specificity.

3.0-Materials and Methods

3.1 Cell lines

A summary of bladder cell lines 8 UBC + one normal urothelium cell line were utilized in this study and are summarised in Table 2. HB-CLS-2 and 5637 were acquired from Cell Lines Service (CLS) GmbH (Eppelheim Germany). UROTsa was given as a kind gift from one of the members in the BCPP programme. The remaining cell lines were from Leeds from Professor Margaret Knowles.

Table 2: A Range of bladder cell lines used in the project representing different stages of Bladder cancer presenting a range of different grades profiles.

Name of bladder cell line	Description
RT4	**Transitional papillary tumour clinical stage T2, histological grade G1.**
UROtsa	**Isolated from normal human urothelium through immortalization with a construct containing the SV40 large T antigen.**
5637	**Derived from primary bladder carcinoma grade II.**
RTII2M	**Transitional cell carcinoma (histological grade G2)**
VMCUB	**Transitional cell carcinoma.**
T24	**Transitional cell carcinoma; grade III**
MGH-U3	**Transitional cell carcinoma grade I**
HB-CLS-2	**Derived from primary bladder carcinoma grading III**
SW780	**Derived from grade I papillary transitional cell carcinoma**

3.2 Chemicals and Inhibitors

To stimulate cells in culture, PMA was diluted to a final concentration of 2mM. Marimastat was diluted to a concentration of 5µM. Both are from Sigma-Aldrich (Dorset UK).

3.3 Tissue Culture

All cell lines were maintained according to the distributors' instructions. HB-CLS-2, 5637, RT112M and UROTsa were grown in RPMI-1640 media and 10% Fetal Calf Serum (FCS). SW780, T24 and VMCUB were grown in Dulbecco's Modified Eagle Medium (DMEM) and 10% FCS. RT4 was grown in McCoy's 5A Modified Medium and 10% FCS. MGH-U3 was grown in DMEM supplemenented with10% FCS and 1% amino acids. The mediums excluding McCoy's 5A medium were purchased from Sigma Aldrich (Dorset, UK). McCoy's 5A medium was purchased from (Life Technologies, Paisley, UK).

3.4 Harvesting of secretomes from cancer cell lines

Cells were grown to 80% confluence in T75cm^2 Corning Cell Culture vessels (Sigma Aldrich, Dorset, UK) in a 37^{0}C/5% CO2 *RS Biotech Galaxy R* incubator (C & M Scientific, West Lothian, *UK*). They were then washed four times with 10ml of relevant serum-free media per flask and incubated with for 18 hours with 6mL of relevant serum-free media per flask under several conditions (control, PMA, Marimastat, both PMA and Marimastat). The conditioned media were then spun down at 3.300 x *g* for 20 minutes utilizing Megafuges 2.0 R Universal Centrifuges (Heraeus, Hanau, Germany). They were then collected and concentrated using a 3kDa MWCO Millipore Amicon Ultra (Millipore, Darmstadt, Germany), spinning down at 3.300 x g until the final volume was 1ml.

For membrane fragment removal, 500µl of secretome was centrifuged at 136,000 x *g* for 90 minutes in a Beckman Optima Max instrument with TLASS rotor (Beckman Coulter, High Wycombe, UK). The supernatant were transferred to new tubes.

3.5 Trypsin Digestion

Proteins were prepared by the Filter-aided sample preparation (FASP) method based upon previous studies performed by our group (Wisniewski et al. 2009). The disulfide bridges in the proteins were reduced by 50mm DTT (Sigma-Aldrich, Dorset, UK). 2% CHAPs detergent (Melford, Suffolk, UK) was also added. Protein-detergent complexes and micelles were separated in 8M Urea presence (Sigma-Aldrich, Dorset, UK) (Wisniewski et al. 2009). They were left for an hour before being alkylated with 100mM iodoacetamide (Sigma-Aldrich, Dorset, UK) at room temperature for 30 min.

30kDa MWCO Centrifugal filter units (Sigma Aldrich, Dorset, UK) were subsequently washed several times in 100mM Tetraethylammonium bromide (TEAB) buffer before use (Flaker analytical #17902). After reduction and alkylation, proteins were added to filters and had several washings with TEAB. Utrafiltration was assisted by centrifugation to remove the DTT, CHAPs, iodacetamide and low molecular weight proteins and other components (Wisniewski et al. 2009). The protein suspension is digested with sequencing grade modified porcine trypsin (20 mg/mL; Promega, Madison, USA). The subsequent peptides are assembled as a filtrate and transferred to eppendorf tubes whilst molecules with high molecular weight including trypsin remain on the filter.

3.6 Protein Quantification

Protein quantification was based upon stable isotope labelling by peptide demethylation was conducted using formaladehyde reagent that labels the N-terminus and ε-amino group of the essential α-amino acid Lysine via reductive amination. The control secretome (no PMA) was

labelled 'light' $^{12}CH_2$-formaldehyde (light) whereas the experimental sample (PMA-treated secretome) underwent a 'heavy' labelling. This was a similar case to Marimastat to identify regulated proteins. Peptides were extracted with ACN containing 0.1% TFA v/v via Discovery HPLC and SPE C18 cartridges (Sigma Aldrich, Dorset, UK).

3.7 MS analysis

Digests were checked by MALDI-TOF for any presence of peptides (Bruker Daltonics II). This is followed with drying under ScanVac SpeedVac vacuum (LaboGenes) followed by adding 110µl of Mix mode A into the HPLC tubes and then seperated into 30 fractions by mixed mode anion exchange/reverse-phase HPLC (Ultimate 3000) with a temperature of 25^0C, 100ul min and inject volume of 100µl. The HPLC spectra programme was called Hystar software Version 3.2. They were then spotted on MALDI plate polished steel, 1µl of sample followed by 1µl of matrix α-Cyano-4-hydroxycinnamic acid (CHCA). They were then dried and dissolved in 0.1% formic acid and analysed by LC-MS/MS using a 90 min 0-40% acetonitrile gradient (75µm x 25cm C18 Pepmap column, Dionex) and Maxis impact mass spectrometer (Bruker Daltonic, Bremen, Germany).

3.8 Peptide Identification.

Peptides were identified using MASCOT 2.3 to investigate a custom database containing SWISSPROT human and bovine sequences and randomised versions thereof (Bovine sequences were included as the secretomes will inevitably contain traces of FCS) (Bryan et al. 2013). Mass tolerances for parent and fragment ions were 20 ppm and 0.05 Da respectively and the minimum peptide Mowse score was 5. Protein identifications were filtered using both a 1% false discovery threshold and a requirement for two or more peptides using Proteinscape 2.1 software.

3.9 Bioinformatics and Proteomic analysis

Subcellular localizations and categorization of protein function were determined by gene ontology tools String software (www.stringdp.org) and Panther software (www.pantherdp.org).

To select candidate biomarkers, proteomic data were compared with human protein atlas IHC (www.proteinatlas.org) data and Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA www.ingenuity.com) analysis. Both methods were conducted based upon previous studies.

3.9.1 *IPA analysis*

Karsani et al. determined cancer-associated proteins by comparing the proteome oral cancer cell lines with normal cell lines via two-dimensional gel electrophoresis. MALDI TOF/TOF was then utilized to reveal the identity of these proteins that were then classified based on function. IPA core analysis was used to determine which proteins were related to cancer development and progression. Another study was conducted on lung cancer using IPA analysis (Chen et al. 2012). Atri et al. compared renal cell carcinoma with normal renal tissues using label free proteomics. They conducted a pathway analysis using IPA software and discovered many cancer-associated processes such as glycolysis and oxidative phosphorylation.

Thus this selection method will be used to look at the cancer associated proteins and use IPA-Biomarker in which filters was used where it can provide potential biomarker candidates.

3.9.2 *HPA analysis*

Previous studies such as Chen et al. used a scoring method to discover candidate biomarkers by quantifying cancer/normal cells and cancer/normal tissues.

Thus, I will be looking at proteins that have been up-regulated by PMA by two-fold and down-regulated by Marimastat by half-fold. Further investigations will be conducted by looking at

proteins that are observed at high grade (T24, HB-CLS-2, SW780, RT112M and 5637) and not low-grade (RT4, MGH-U3 and VMCUB).

3.10 Western blot analysis

This was used to confirm the expression of EpCAM and ADAM17. For EpCAM, an EpEX antibody was used to present the extracellular domain of EpCAM. The protein concentration of cell lysates was determined by Pierce BCA Protein Assay (Thermo Fisher Scientific, Rockford, USA). Equal amounts of protein were then boiled in SDS-PAGE sample loading buffer containing 50 mM DTT, loaded and run on 4-12% NuPAGE gels (Thermo Fisher Scientific, Paiseley, UK). After gel electrophoresis, the resolved proteins were blotted transferred onto PVDF membranes (Millipore, Darmstadt, Germany). These were blocked with 1% dry milk in TBS. Membranes were then incubated overnight at 4 °C with the following primary antibody: anti-EpCAM antibody (BAF960; R&D Systems Europe Ltd). Blots were washed three times with TBST before the horseradish peroxidase-conjugated secondary antibodies. After three supplementary washes, blots were developed using ECL reagents (GE Healthcare). The blot was visualized with SRX-101A photon imaging systems (Konica Minolta).

For detection of ADAM17 in UBC cell lysates, we used Human TACE/ADAM17 Ectodomain Antibody (R&D Systems).

3.11 Knockdown of ADAM proteases by siRNA

ADAM17 were knocked down by RNA interference. Three different concentrations were utilized 0, 25, 50nM of siRNA in Darmafect transfection reagent. Three cell lines were utilized RT112M, HBCLS2, and 5637. Detection of ADAM17 protein was performed by Western blotting and its

effect on EpCAM shedding was investigated by ELISA. siRNA used was Human Adam17 (Thermo Scientific, #M-003453-01)

3.12 ELISA

All ELISAs of EpCAM, MMP1 were performed according to the manufacturer's instructions. Calibration curves were prepared using purified standards for the protein assessed using 2-fold dilutions of 1ng ml^{-1} standard for borh. Calibration curves were then plotted.

For MMP1 ELISA (DY901; R&D Systems Europe Ltd, Abingdon, UK), 20µl of PMA-treated secretomes was added whilst secretomes in other conditions 100µl was added. Absorbances at 450 nm of each aliquot were determined using the microplate reader.

Alternatively, for EpCAM ELISA utilised human EpCAM (DY960; R&D Systems Europe Ltd, Abingdon, UK). To each well of the assay were added 100µl of sample and 50 ml of PBS+ 1% BSA. The absorbance at 595 nm of each aliquot was determined using the microplate reader. This was performed to measure the levels of EpCAM in bladder cell secretomes and to investigate the effect of PMA and Marimastat on its release.

3.13 Wound healing assays

1ml of cell suspension with trypsin was added per well of 12 well plate. They were left to grow overnight and checked in morning under microscope for confluency. A black marker pen was placed to visualised the scratch of a 20µl pipette tip creating a wound. Q imaging (Rolera-XR Nikon eclipse) camera was used photographing images at 0, 5 and 20 hours. Wound closure was determined as the percentage of wound closure compared to respective negative control (regarded as 100%). Each experiment was performed in triplicate.

3.14 MTT assays

Cells were incubated in complete medium on 96 well plate for 24 hours. Once confluent, conditions were added where they undergo incubation overnight and then the subsequent MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent (Sigma Aldrich, Dorset, UK) is added to each well where a 3 hour incubation before reading it on plate reader. The absorbance at 595 nm of each aliquot was determined using the microplate reader.

The principle of this method is that MTT is converted into formazan crystals by living cells (Cloos 2011). This determines the mitochondrial activity that is linked to the quantity of viable cells. The MTT assay measures in vitro cytotoxic effects of drugs on cell lines (Cloos 2011). In relation to this study, this is performed to see how PMA and Marimastat influences influences cell viability.

3.15 Patient population and clinical specimens

Urine samples was collected as part of the Bladder Cancer Prognosis Programme (BCPP) between 2006 and 2009 (Billingham et al. 2009). Subjects were enrolled based upon initial findings suggestive of primary UCB. Before use, urine samples were defrosted at room temperature and vortexed using Fisons Whirli mixer. There were also non-cancer controls that consists of patients with non-UCB conditions. Information on the patients are summarised in Table 3. A total of 348 patient samples were investigated that vary in age, gender and grade (323 UBC, 25 non-cancer controls).

3.16 Biomarker validation

50µl of urine samples was added with 50µl of PBS+1%BSA for MMP-1 ELISA (DY901; R&D Systems Europe Ltd, Abingdon, UK). The absorbance at 450 nm of each aliquot was determined using the microplate reader.

3.17 Data analysis:

Averages were calculated for all data conducted. For MTT assays, student T-tests were calculated and a p-value < 0.05 was considered significant. Error bars were calculated based on standard deviation. Mann-Whitney test was performed to compare the different grades and stages. ROC analysis was performed on MMP1 concentration for specificity and sensitivity. The area under the ROC curve; (AUC) was calculated as a single measure for the discriminative efficacy of candidate marker.

4.0 Results

4.1 Comprehensive proteomic analysis of whole bladder cell line secretomes

One of the ways to investigate and identify proteins shedded from the surface of in vitro cultured bladder cancer cells was via Phorbol 12-myristate 13-acetate (PMA) that stimulates shedding. Whole secretomes was utilized to investigate all proteins (shedded and secreted) released by the cells.

In total, 2242 human proteins were identified in one or more of the 9 PMA-treated bladder cell line secretomes (UROTsa, HB-CLS-2, T24, RT4, VMCUB, SW780, RT112M, 5637, MGUH-3) where 2075 human proteins had two or more peptides. There was considerable overlap between the secretomes with 1122 proteins being identified in at least two out 9 secretomes and 137 were detected in all 9 secretomes. The 137 proteins are shown in Appendix A where their cellular location, molecular functions and cellular function is summarised using Panther database (http://www.pantherdb.org/).

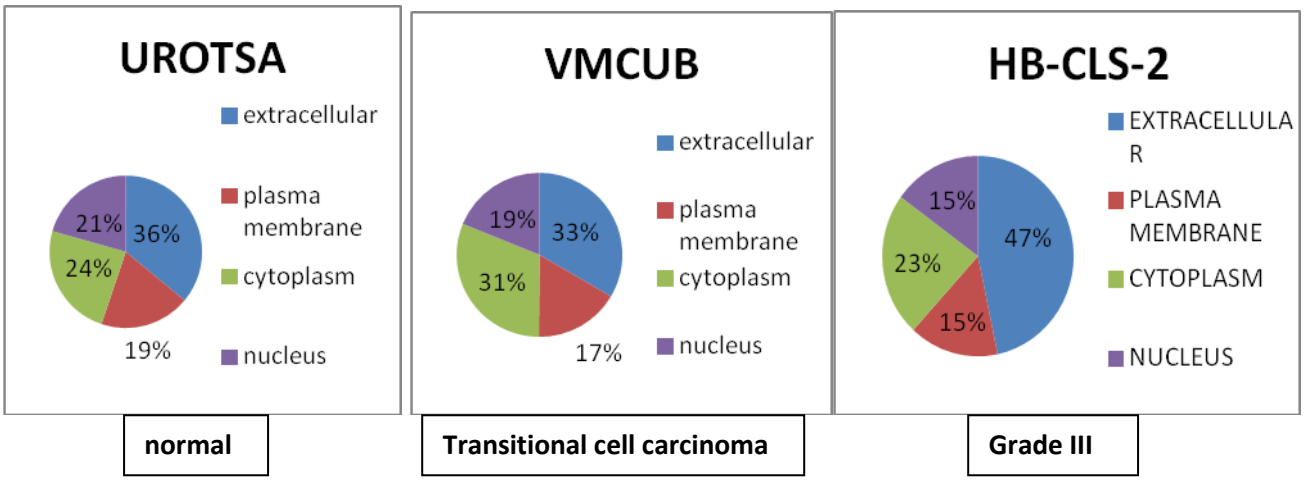
4.1.1 *The effect of PMA on whole secretomes' localisation*

Proteomic analysis has been conducted to determine what types of secreted proteins have been released and identified by mass-spectrometry and whether the locations of these proteins vary across the grades and the normal urothelium. Identified proteins were divided into categories based upon cellular location using the GO Cellular Components function in STRING 9.0 (www.string-db.org). They were distributed in the nucleus, plasma membrane, cytoplasm and extracellular space.

The secretomes were substantially enriched with extracellular proteins compared with the corresponding other cell compartments (nucleus, cytoplasm, plasma membrane). This was

consistent throughout the 9 whole secretomes regardless of normal urothelium and different grades. Therefore, due to this regular pattern, only three of the 9 secretomes have been presented at Figure 4: UROTsa; VMCUB (TCC) and HB-CLS-2 (Grade III) to provide an example of the proportion of proteins in repartition of each subcellular location. UROTsa (36%), VMCUB (33%) and HB-CLS-2 (47%) accounts for extracellular proteins. There were more plasma membrane-bound proteins in UROTsa than UBC secretomes. In addition, more cytoplasmic proteins are present in VMCUB than the higher grade HB-CLS-2 and normal UROTSA.

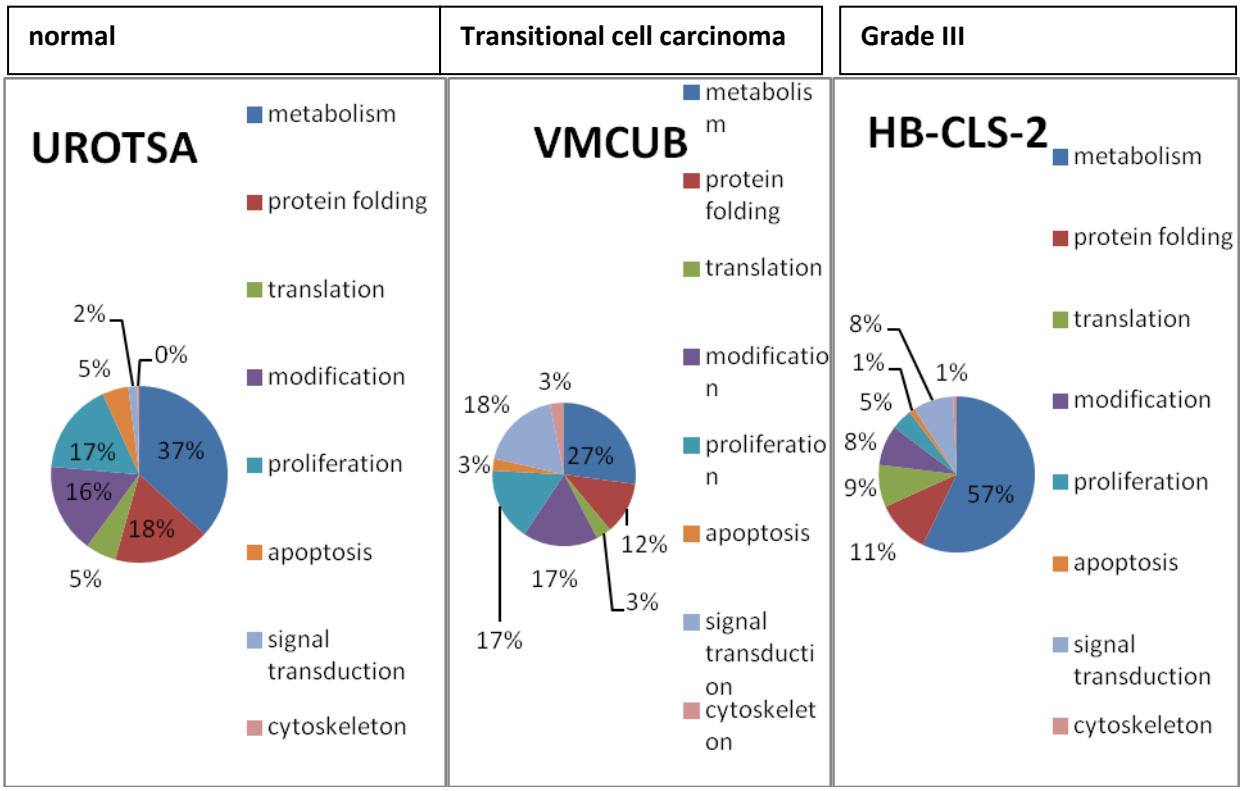
Figure 4: Cellular locations of the differentially expressed proteins in UROTsa (normal), VMCUB (TCC) and HB-CLS-2 (Grade III) PMA-treated whole secretomes identified by the proteomics approach. For UROTSA (extracellular =379 proteins, plasma membrane=202 proteins, cytoplasm=255 proteins, nucleus=217 proteins). For VMCUB (extracellular=363 proteins, plasma membrane=182 proteins, cytoplasm=337 proteins, nucleus=205 proteins). For HB-CLS-2 (extracellular=320 proteins, plasma membrane=103 proteins, cytoplasm=160 proteins, nucleus=102 proteins)



4.1.2 *The effect of PMA on whole secretomes' biological processes.*

The biological function was conducted to see whether it varies across the normal and UBC secretomes. They were determined using STRING 9.0 (www.string-db.org). They correspond to eight cellular protein groups, including metabolism-associated proteins, cytoskeleton-associated proteins, protein-folding, and translation, modification, apoptosis, proliferation and signal transduction. The results were consistent throughout the secretomes were the predominant function was metabolism. Thus, like Figure 4, only three secretomes were presented to show how regardless of normal/cancer and different grades, the predominant biological function was metabolism. HB-CLS-2 (Grade 3) had more proteins associated with the role than VMCUB (TCC) and UROTsa (normal) as shown in Figure 3B. However, there were a small proportion of proteins found in HB-CLS-2 (Grade 3) that are involved in proliferation compared to other secretomes. More proteins had an apoptotic and cytoskeletal functions in UROTsa (normal) than UBC cell lines. Thus differentially expressed proteins mainly located in extracellular and have a role in metabolism regardless of grade.

Figure 5: Biological processes of the differentially expressed proteins in UROTsa (normal), VMCUB (TCC) and HB-CLS-2 (Grade III) PMA-treated whole secretomes identified by the proteomics For UROTSA (metabolism=0 proteins, protein folding =92 proteins, translation = 44 proteins, Modification=14 proteins, proliferation=41 proteins,apoptosis=42 proteins,signal transduction=12 proteins, cytoskeleton=4 proteins). For VMCUB proteins' (metabolism=189 proteins, protein folding=82 proteins, translation=24 proteins, modification =118 proteins, proliferation=115 proteins, apoptosis=19 proteins, signal transduction=127 proteins, cytoskeleton=22 proteins). For HB-CLS-2 proteins'(metabolism=144 proteins, protein folding=28 proteins, translation=22 proteins,modification=21 proteins, proliferation=11 proteins, apoptosis=3 proteins,signal transduction=21 proteins, cytoskeleton=2 proteins).



4.1.3 *Cluster analysis of PMA data*

A cluster analysis was conducted on the PMA data to investigate how similar the data is from one cell line to the other. The most similar ones are clustered together and are shown as a 'heatmap' [data not shown]. It shows that the high grade and low grade were clustered separately. 118 proteins $p<0.05$ were between low and high grade. UROTsa (normal) clustered with high grade cell lines. This suggests that the expression of these proteins changes as the disease progresses. In addition, UROTsa may not be a reliable model of normal urothelium.

4.1.4 *Proteins up-regulated by PMA*

Up-regulated proteins emphasises regulated release. Those up-regulated by PMA are shown in Appendix B by two-fold. Up-regulated proteins were enriched for plasma membrane and extracellular secreted proteins. To identify proteins that have been up-regulated, quantitative proteomics was utilized where stable heavy isotope labelling using formaladehyde reagent labels the N-terminus and ε-amino group of the essential α-amino acid Lysine via reductive amination (Chen et al. 2003). The control secretome (no PMA) was labelled 'light' $^{12}CH_2$-formaldehyde (light) whereas the experimental sample (PMA-treated secretome) underwent a 'heavy' labelling. They were then vortexed together followed by an addition of sodium cyanoborohydride. Labelling was performed post-tryptic digestion and to ensure labelling has worked, it is detected on analysis of MALDI and LC/ESI-MS/MS (Chen et al. 2003). This labelling strategy is not influenced by peptide traits rather it focuses on mass (Demmers 2012). At the same retention time, the 'heavy' and 'light' peptides elute together from LC column and heavy leads to mass shift in mass spectrum the m/z value allowing peak isotopic pairs to be observed. Each peaks vary by 4 mass units that consists of one 'heavy' and one 'light' (Demmers 2012).

Table 3. A summary of the number of proteins that have been up-regulated and down-regulated by PMA across the 9 bladder cell lines Up-regulated proteins were identified by having a normalised median (H/L) ratio of >2 (increased by two-fold) whereas those that have been down-regulated were identified by having half fold ≤0.5. All appear to have more up-regulated proteins than down-regulated with the exception of HB-CLS-2 (Grade 3).

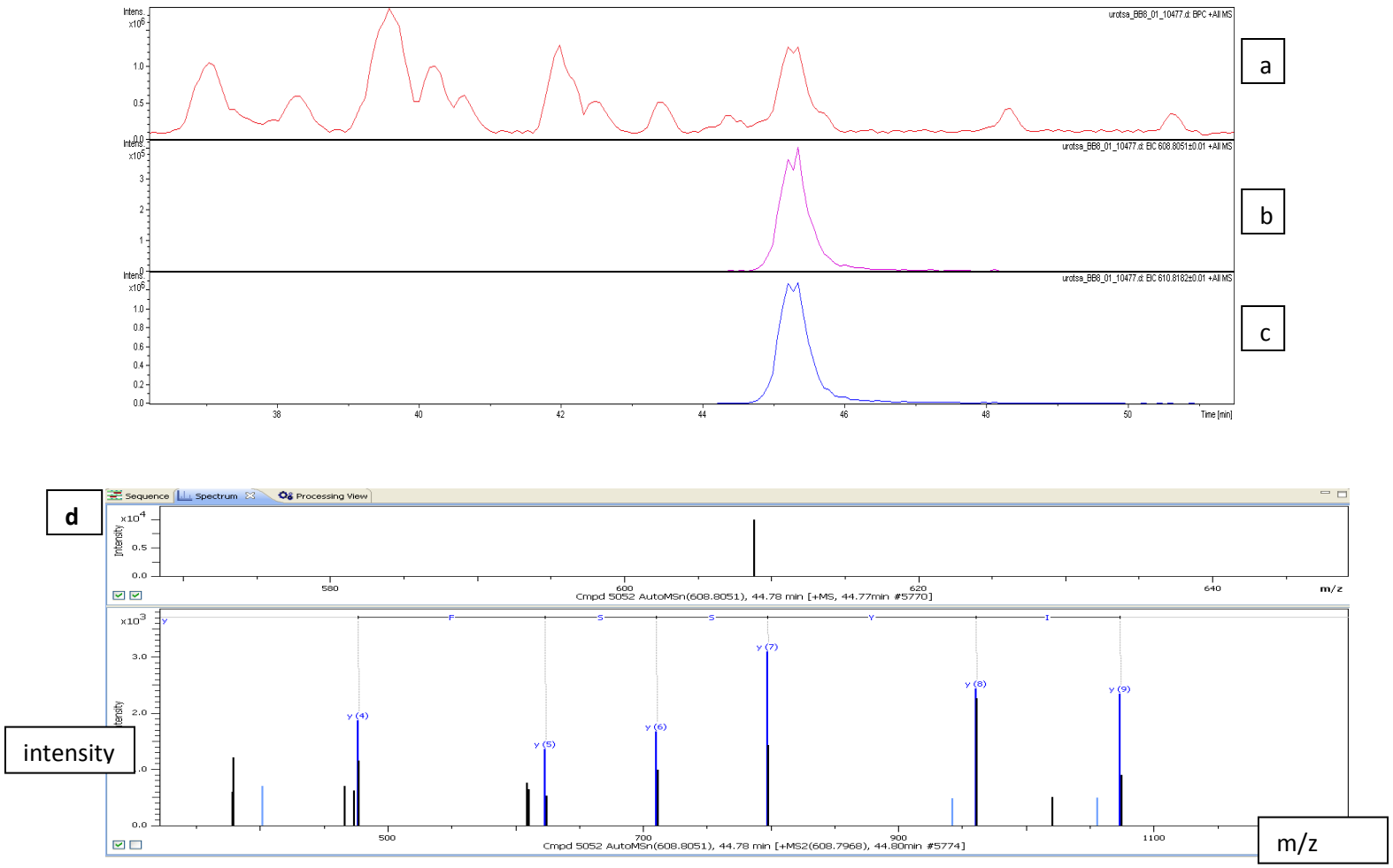
Name of cell line	**Total number of identified proteins**	**Total number of proteins upregulated by PMA**	**Total number of proteins downregulated by PMA**
UROTsa (normal)	**1008**	**173**	**69**
HB-CLS-2 (Grade 3)	**581**	**39**	**59**
VMCUB (TCC)	**712**	**98**	**53**
5637 (Grade 2)	**1062**	**102**	**32**
RT112M (Grade 2)	**671**	**177**	**152**
SW780 (Grade 1)	**1280**	**100**	**59**
T24 (Grade 3)	**654**	**57**	**35**
RT4 (Grade 1)	**1161**	**101**	**37**
MGUH-3 (Grade 1)	**814**	**51**	**43**

4.1.5 *Up-regulation of MMP-1 by PMA*

MMP-1 is amongst the proteins upregulated by PMA. It is an interstitial collagenease that targets type I, II and III (National Center for Biotechnology Information 2014). Figure 6 illustrates the MS and MS/MS spectra of MMP-. It is up-regulated by PMA in 7 out of 9 cell line secretomes this includes normal and UBC secretomes as shown in Table 4.

Figure 6. The identification of MMP-1 by MS/MS in dimethyl-labelled UROTsa whole secretome.

(a) The chromatogram of one HPLC fraction of demethyl-labelled UROTsa (normal) PMA secretome where retention time (minutes) plotted against intensity (%), (b) a light peptide of MMP-1 that has a relative intensity of 10^{-5}, (c) a heavy peptide of MMP-1 that has a relative intensity of 10^{-6}. Fractions are then analysed by MS where peptides undergo ionization and the mass-to-charge (m/z) ratio is measured to yield a precursor ion spectrum shown in (d) Precursor ion spectrum. Selected ions are then fragmented by collision-induced dissociation (CID) and the individual fragment ions measured by MS. (e) fragment ion spectra (MS/MS) of MMP-1 light and (f) fragment ion spectra (MS/MS) of MMP-1 heavy. (e) and (f) are continued overleaf.



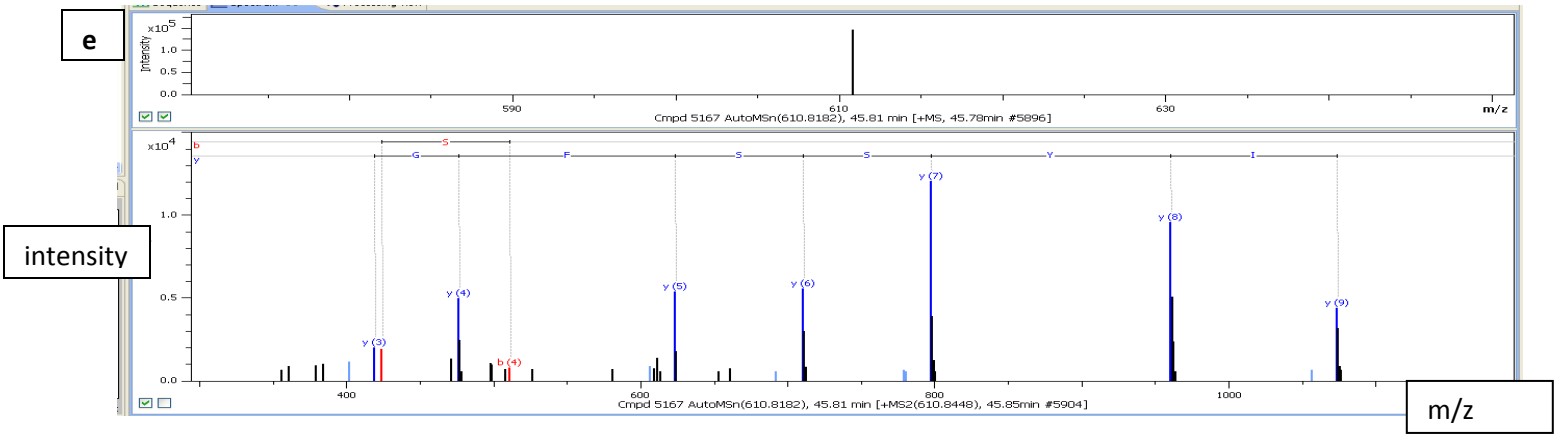


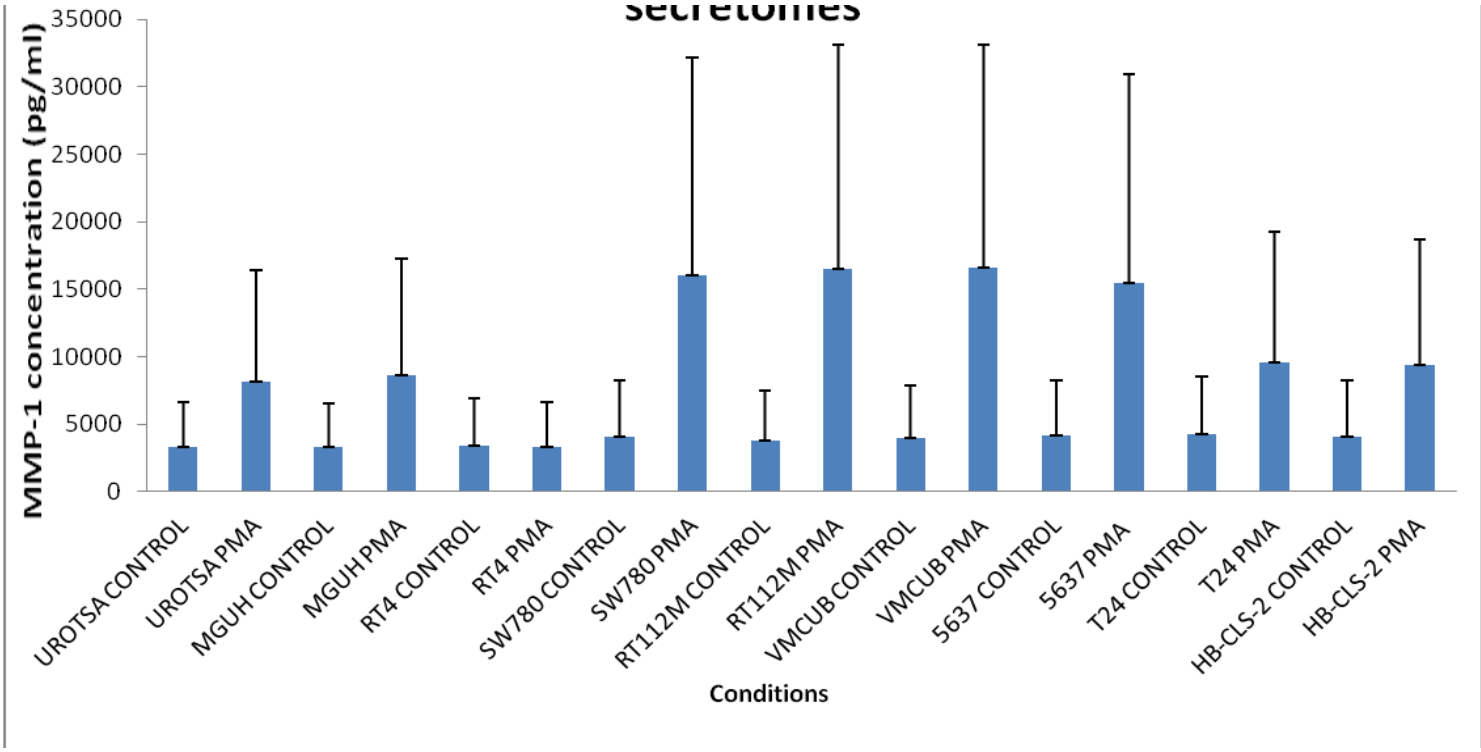
Table 4. The heavy-light (H/L) peptide ratios of MMP-1 in bladder cell secretomes measured by LC/MS/MS. UROTsa is a normal urothelium secretome; the rest are UBC secretomes. MMP-1 is upregulated in normal and UBC secretomes except for MGH-U3 (Grade 1) and HB-CLS-2 (Grade 3).

Name of PMA-treated secretome	UROTsa (normal)	MGH-U3 (Grade 1)	RT4 (Grade 1	SW780 (Grade 1)	RT112M (Grade 2	VMCUB (TCC)	5637 (Grade 2)	T24 (Grade 3)	HB-CLS-2 (Grade 3)
(H/L) ratio	19.6 ↑	Absent	5.4 ↑	21.8 ↑	12.8 ↑	5.47 ↑	11.9 ↑	25.4 ↑	Absent

4.1.5.1 PMA stimulate MMP-1 secretion in bladder cell secretomes

In response to the MS finding, an ELISA experiment was performed to investigate whether PMA stimulates MMP-1 secretion in bladder cell secretomes via PKC activation. The outcomes of the ELISA are illustrated in Figure 7. Results portrayed that MMP-1 concentration in whole secretomes is significantly elevated in presence of PMA than alone. This confirms what is already known that MMP-1 is secreted membrane-associated zinc endopeptidases localized in the ECM rather than membrane-bound similar to that of MMP-8 and MMP-13 that cleave interstitial fibrillar collagen (Acharya et al. 2006, Weizmann Institute of Science 2014).

Figure 7: Protein levels of MMP-1 in whole bladder cell secretomes. 20µl of PMA-treated secretomes whilst for control 100µl was added. Absorbances at 450 nm of each aliquot were determined using the microplate reader. PMA induces MMP-1 secretion and this is apparent across all 9 bladder cell secretomes. Error bars were plotted based with standard deviation of the data obtained.



4.2.The effect of ultracentrifugation on plasma membrane protein release in PMA-treated secretomes.

In order to understand the role of plasma membrane in biological processes, the plasma membrane proteome needs to be investigated. A change in membrane proteins is amongst the initial events that occur during pathological conditions (Hixson 2011). Thus, plasma membrane proteins are a useful source to discover potential biomarkers for diagnosis and prognosis. They are also useful as they can be target for drugs and monoclonal antibodies to inhibit enzymes and/or evade receptors necessary for cancer progression (Hixon 2011). Previous studies looked at locations and the types of membrane proteins present. In addition, our group have discovered that extracellular domains of EpCAM are shed into the conditioned media whereby membrane-bound EpCAM is released into cytoplasm.

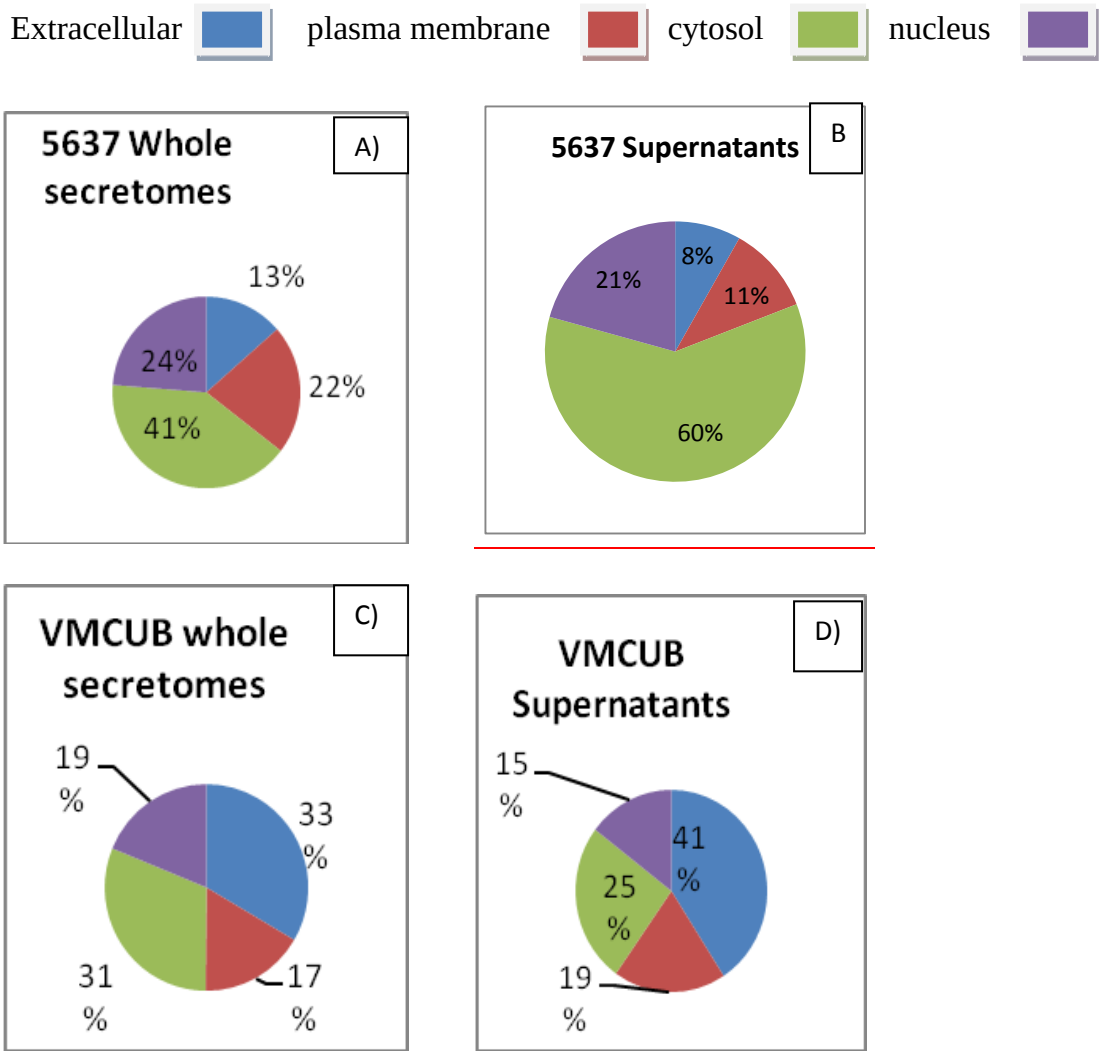
To test this hypothesis, ultracentrifugation took place on VMCUB (TCC) and 5637 (Grade II) PMA-treated secretomes to identify potential target proteins, those proteins that are still membrane-bound after ultracentrifugation could be potential biomarkers. There were 712 and 1062 proteins identified in VMCUB (TCC) and 5637 (Grade II) PMA-treated secretomes respectively whereas for supernatants more proteins were identified where 920 and 1251 proteins were identified in VMCUB (TCC) and 5637 (Grade II) respectively.

4.2.1 *The presence of plasma membrane bound proteins after ultracentrifugation*

The cellular locations of the proteins in VMCUB (TCC) and 5637 (Grade II) were determined using STRING 9.0 (www.string-db.org) and are shown in Figure 8. Findings suggest that there were still plasma membrane bound proteins released into the cytoplasm after ultra-centrifugation. There is also a slight reduction in the number of proteins located in extracellular space and nucleus. 173

proteins were assigned as membrane proteins in VMCUB (TCC) supernatants whereas 141 proteins were assigned as membrane proteins in 5637 (Grade II) supernatants.

Figure 8: Sorting of the identified proteins according to their subcellular localization.
A) 5637 Whole secretomes (extracellular=157,plasma membrane=251,cytosol=471,nucleus=274)
B) 5637 supernatants (extracellular=105,plasma membrane=114,cytoplasm=775,cytoplasm=775,nucleus=268)
C) VMCUB Whole secretomes (extracellular=363, plasma membrane=182,cytosol=337,nucleus=205)
D) VMCUB Supernatants (extracellular=369, plasma membrane=173, cytosol=230,nucleus=133)



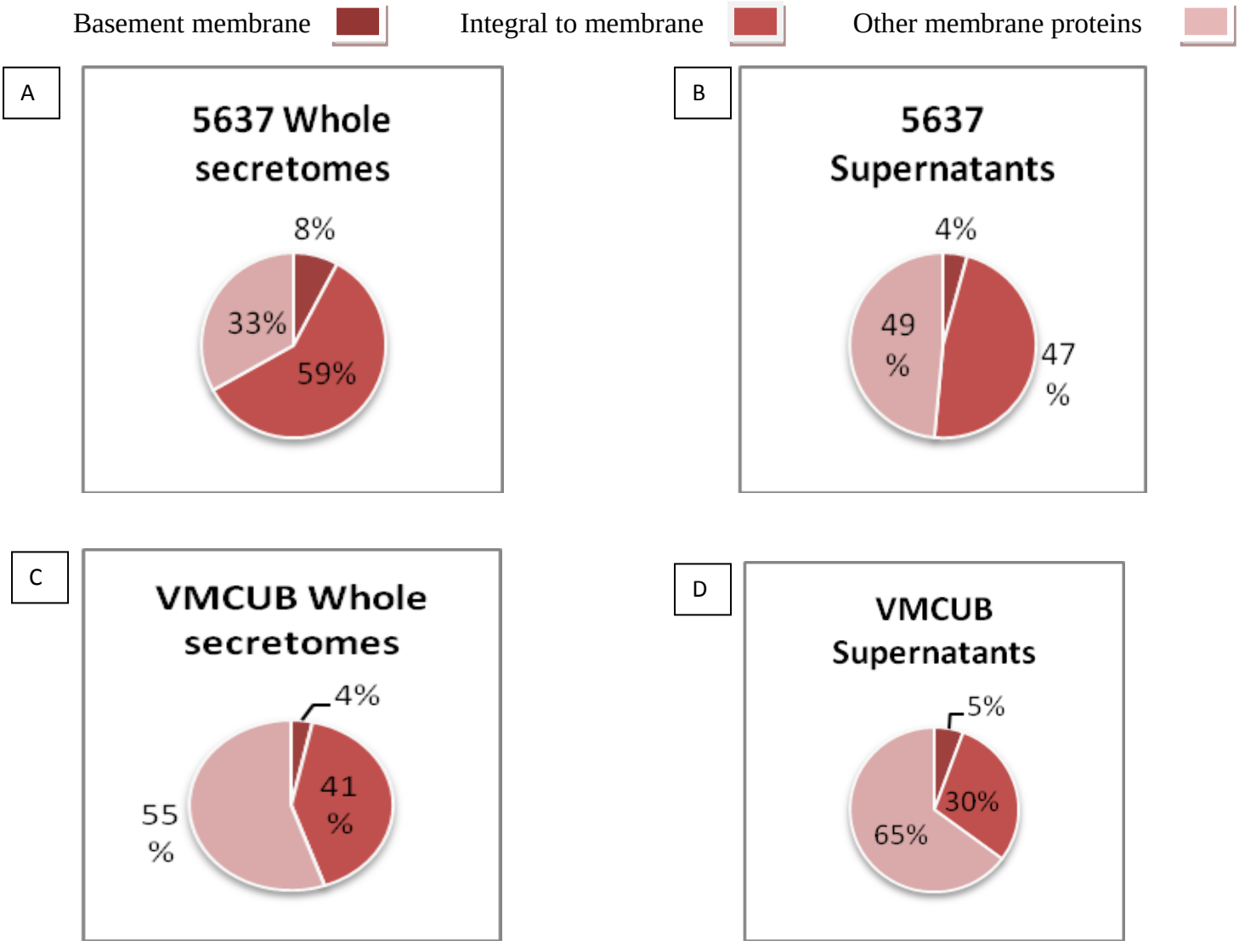
4.2.2 *Types of Membrane Proteins*

There are different types of membrane proteins that can be divided into basement membrane, integral membrane proteins and membrane-associated proteins as presented in Figure 9. Basement membrane proteoglycans are not only involved as structural components but also have a role in regulating several signalling pathways (Iozzo 2005). They engage in angiogenesis thus play a role in cancer progression (Iozzo 2005). Integral membrane proteins have two regions: hydrophilic and hydrophobic. It also has two domains: intracellular and extracellular (Srivastava n.d.). Integral membrane proteins have roles in many biological processes that include transport, pathogen invasion and cell signalling (Baldwin et al. 2010).

The different types of membrane proteins found in 5637 (Grade II) and VMCUB (TCC) with and without ultracentrifugation are shown in Figure 9.They were determined using STRING 9.0 (www.string-db.org). Findings have identified many membrane-associated proteins such as extracellular proteins. Large amount of 'integral to membrane' were discovered prior ultra centrifugation. However, after ultracentrifugation, there was a reduction in the 'integral to membrane' proteins and an increase in other membrane-associated proteins. Amongst the integral membranes found in both secretomes are: DSG2, EXT2, FAT1, GOLM1 and ILF3. In addition, there was a small amount of basement membrane proteins covered in both UBC secretomes.

Figure 9: Sorting of the identified proteins according to types of membrane proteins found in 5637 (Grade II) and VMCUB (TCC) whole secretomes and supernatants.

A) 5637 whole secretomes (basement membrane=18 proteins, integral to membrane=136 proteins, other membrane proteins=76 proteins)
B) 5637 Supernatant (basement membrane=10 proteins, integral to membrane=112 proteins, other membrane proteins=115 proteins)
C) VMCUB whole secretomes (basement membrane=6 proteins, integral to membrane=74 proteins, other membrane proteins=99 proteins)
D) VMCUB Supernatants (basement membrane=3 proteins, integral to membrane=16 proteins, other membrane proteins=35 proteins)



4.3 Ectodomain shedding of bladder cell lines.

4.3.1 Comprehensive proteomic analysis of ultracentrifuged bladder cell line secretomes

The 9 bladder cell secretomes underwent ultra-centrifugation to focus on shedded proteins. Marimastat; a broad spectrum MMP inhibitor inhibits the sheddases responsible for the shedding and was used to examine whether plasma membrane protein ectodomain were released by proteolytic cleavage. 1879 proteins were identified. 775 proteins identified in at least 3 UBC cell lines including 63 integral membrane proteins. There were no presence of ADAMs and MMPs except for MMP-10 that was down regulated. This was due to inhibitory effect of Marimastat. Similar to PMA proteomic analysis results; proteins were found predominately in the extracellular location and of a metabolic role and was consistent across all bladder cell secretomes. Appendix C summarises the proteins that have been down-regulated by Marimastat.

Furthermore, there was a biphasic effect on transmembrane proteins. Release of 40% was inhibited by Marimastat and 60% were not affected. This suggests that only some of the proteins are released by proteases that are inhibited by Marimastat.

4.3.2 Cluster analysis of Marimastat proteomic data

Results show several significant differences between low and high grade. Samples did not cluster, fewer proteins were identified and the data was more variable. Possible reasons for this include spanning of some proteins down that is partly due to Marimastat and partly due to lack of PMA, and also ultracentrifugation. Other reasons include experiments (digestions, mass spectrometry) that did not seem to be working as well later. Therefore, if there is less data and it is more variable then differences between the cell lines may not be detected.

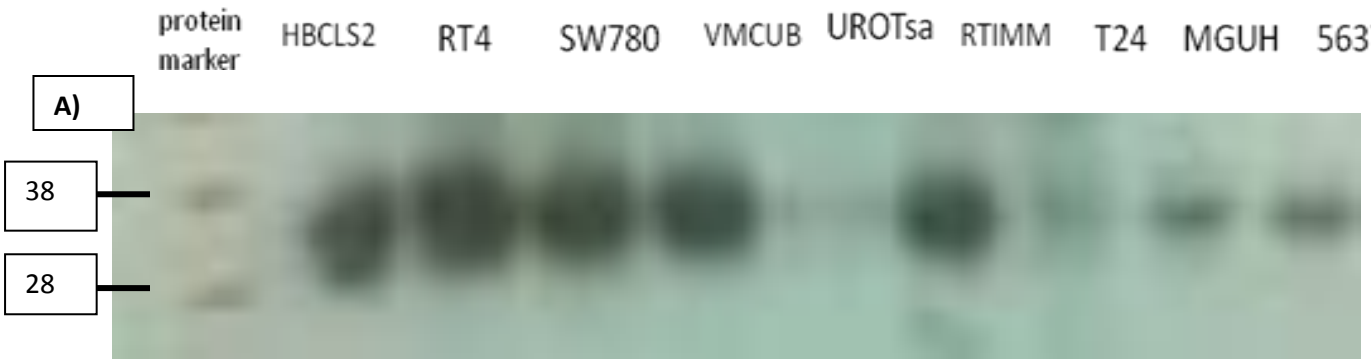
4.4 EpCAM Biology

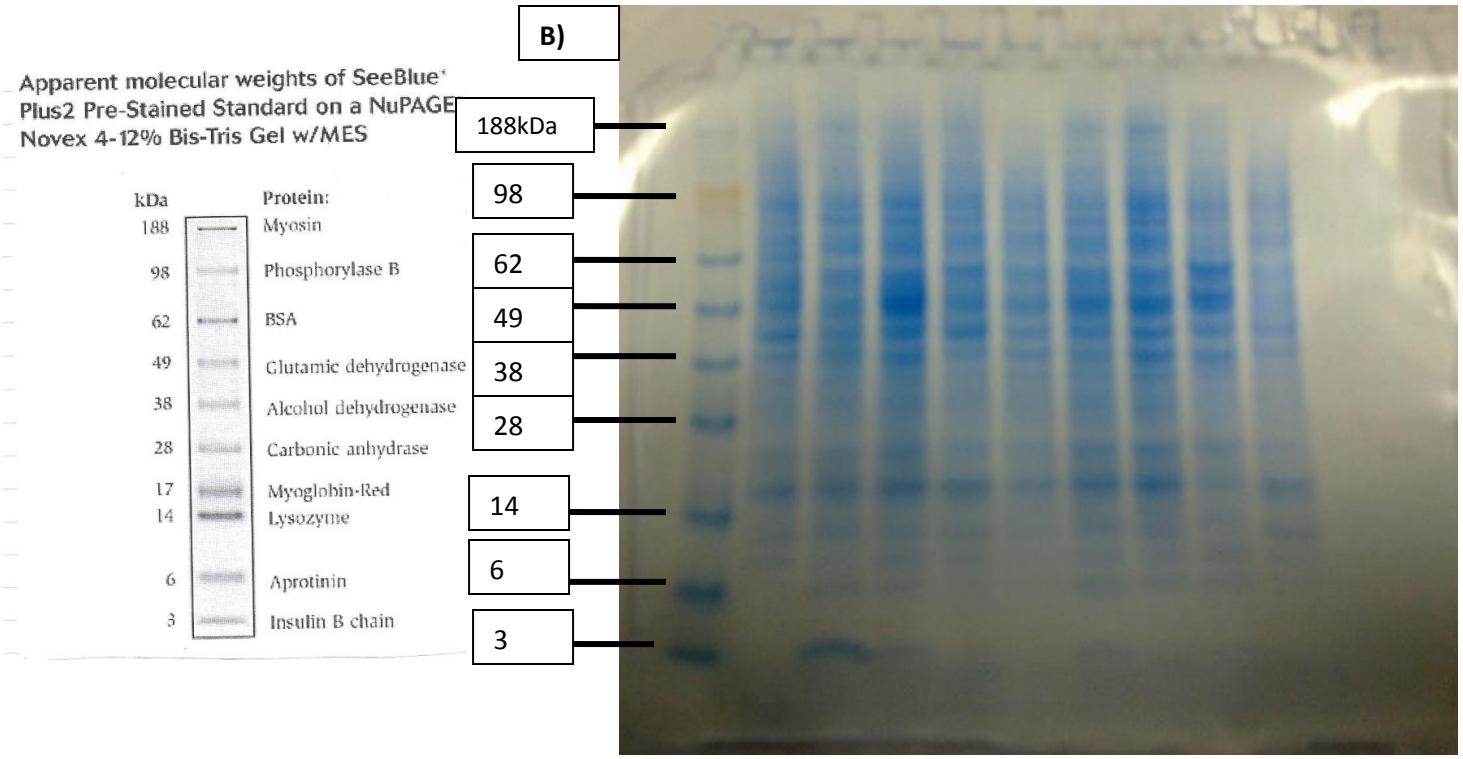
Previous published data from our group portrayed that EpCAM is one of the potential biomarkers of UBC. As its ectodomain is released, it can be used to confirm the effect of Marimastat as previous reports have implied that Marimastat inhibits 'sheddases' called ADAMs which are a family of metalloproteases that is responsible for proteolytic cleavage of ErbB ligands (Vaddi et al. 2007). At the same time, to investigate the effect of PMA on EpCAM release and what occurs in a combination of both PMA and Marimastat.

4.4.1 *Expression of EpCAM in cell lysates*

The abundance of EpCAM in bladder cell lines was investigated using cell lysates via Western blots as shown in Figure 11. An attempt has been done using secretomes [data not shown] but the results suggested that Western Blots was not sensitive enough to detect EpCAM in the secretomes which is why cell lysates were utilized. An EpEX antibody was used to present the extracellular domain of EpCAM. Results are consistent with studies that observed EpCAM between 30-40 kDa. UROTsa and T24 are EpCAM-negative. There is more abundance of EpCAM in HB-CLS-2, RT4, SW780, VMCUB, RT112M. There is lower abundance of EpCAM apparent in MGUH and 5637.

Figure 10: EpCAM presence in cell lysates a) Western blot on cell lysates (b) coomassie blue gel. 6µl protein marker and 15µl sample buffer and sample. EpECD runs about 30kDa and ICD runs about 5kDa. EpCAM can be seen between bands 28 and 38kDa. If EpCAM is glycosylated, it moves forward to the gel (33kDa). Cell lysates were loaded in the following order in both cases.

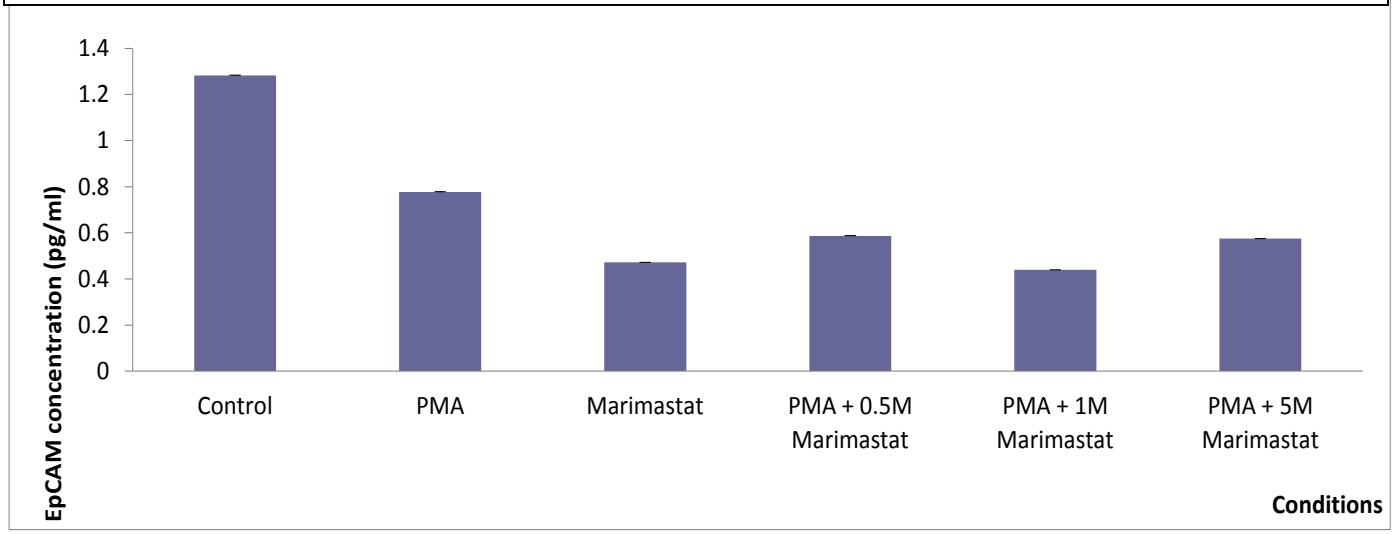




4.4.2 *The effect of Marimastat on EpCAM shedding*

Although EpCAM was not identified by enough peptides to make it into the proteomic dataset., the ELISA data showed that Marimastat inhibits EpCAM release – this can be used to justify 5µM as the working concentration. Initially, a range of different concentrations of Marimastat was investigated to determine the concentration that shows the most inhibitory effect on EpCAM release using SW780 ultracentrifuged secretomes as shown in Figure 11. Findings suggests that 5µM is shows more inhibitory effect than other concentrations. Thus 5µM Marimastat was conducted for the rest of the secretomes.

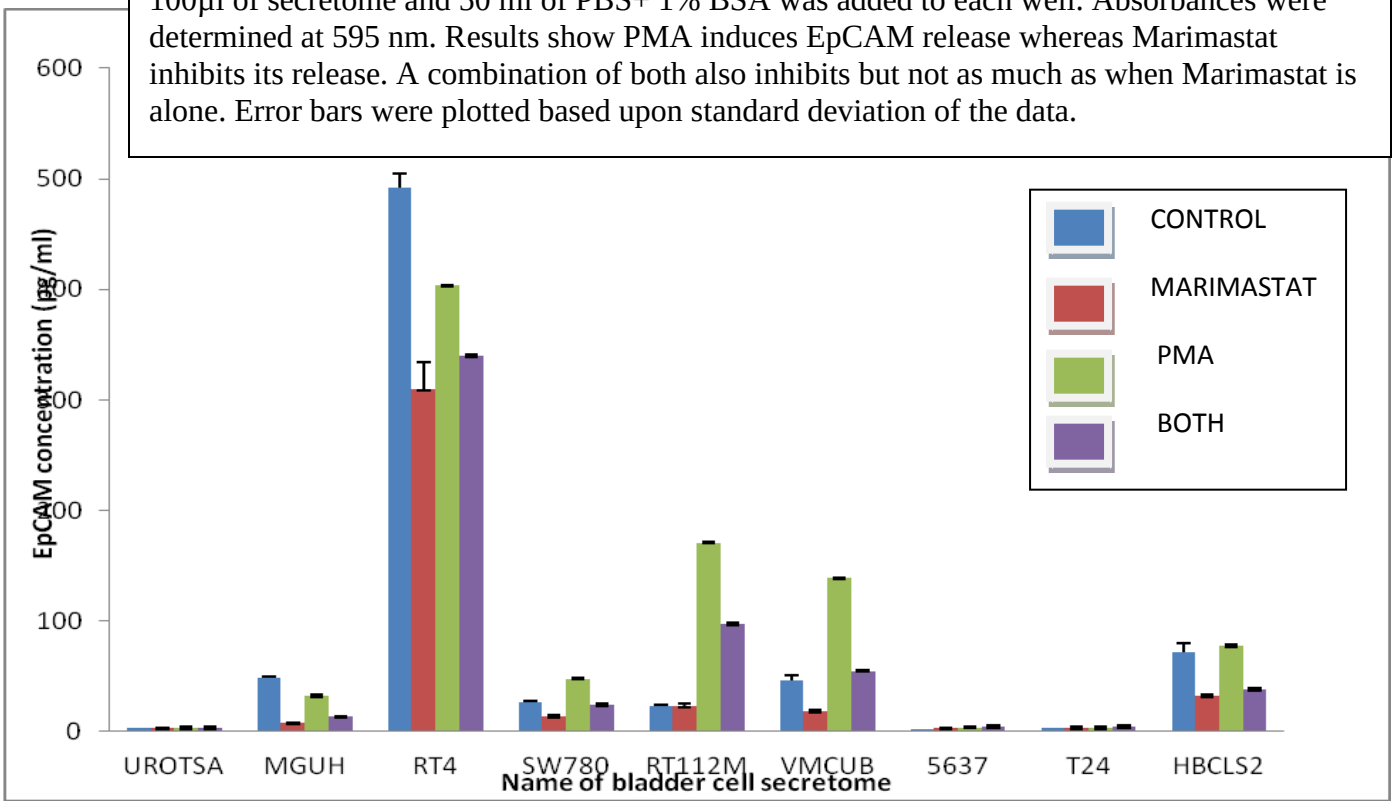
Figure 11: The effect of PMA and different concentrations of Marimastat on EpCAM shedding of SW780 supernatants. Sw780 is a Grade 1 bladder cell line. A combination of PMA and Marimastat shows some inhibitory release of EpCAM compared to PMA. Marimastat shows more inhibitory effect alone at 5µM than combined. PMA does not seem to induce EpCAM release much here. Error bars were plotted based upon standard deviation.



4.4.2.1 Marimastat inhibits EpCAM shedding in all bladder cell secretomes

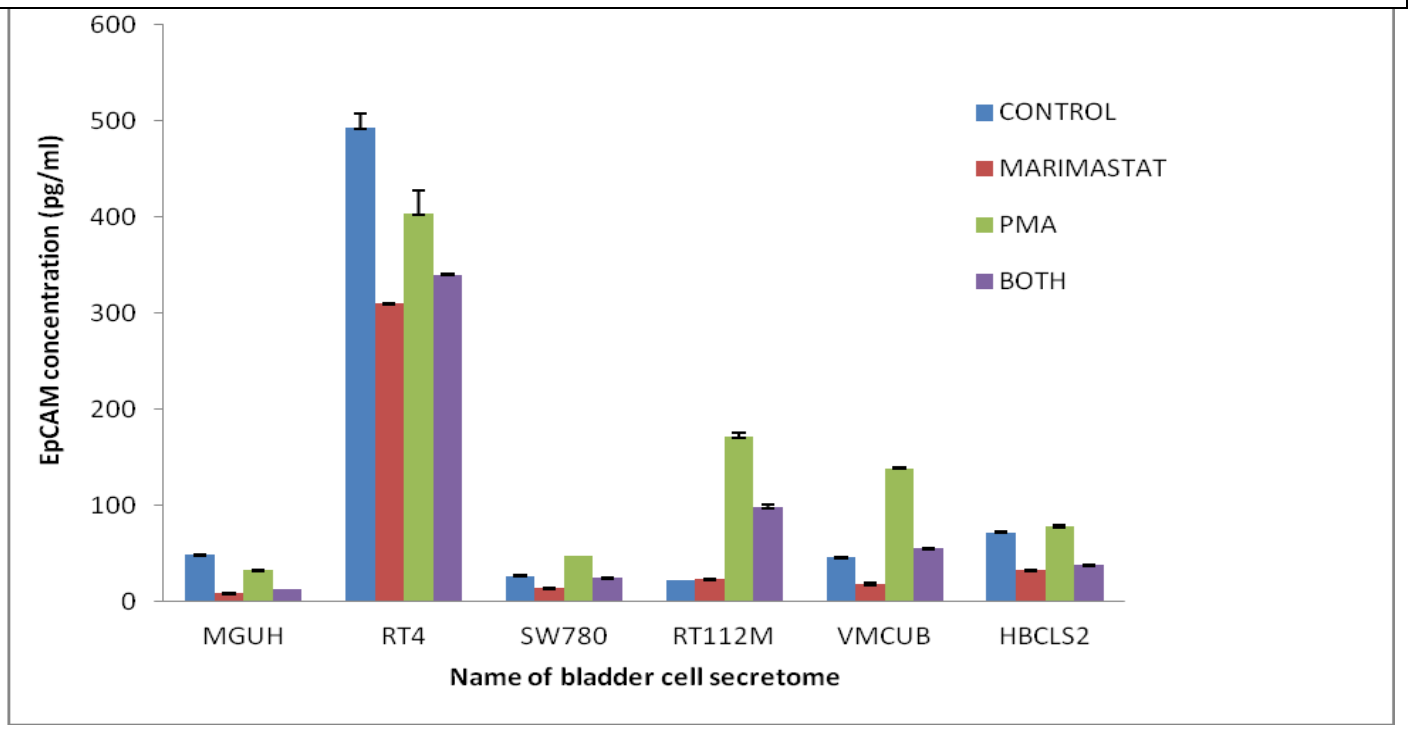
According to the ELISA results shown in Figure 12, there is higher EpCAM levels detected in RT4. This is consistent with results of western blot. There are also higher levels of RT112M, VMCUB and HBCLS2 similar to that of western blots. There appears to be a low level of EpCAM in UROTsa, T24 which opposes findings found in Western blot as there was no EpCAM detected. Marimastat reduces shedding of EpCAM. This occurs throughout different grades of bladder cancer cell lines and the normal urothelium UROTsa. PMA increases EpCAM shedding and there is a reduction in EpCAM release in combination of PMA and Marimastat. However, the reduction in EpCAM release is more in Marimastat alone than both as PMA increases its release.

Figure 12: The effect of Marimastat and PMA on EpCAM release using whole secretomes. 100µl of secretome and 50 ml of PBS+ 1% BSA was added to each well. Absorbances were determined at 595 nm. Results show PMA induces EpCAM release whereas Marimastat inhibits its release. A combination of both also inhibits but not as much as when Marimastat is alone. Error bars were plotted based upon standard deviation of the data.



A repeat was done but in this case with supernatants of secretomes as shown in Figure 13. The results complement of that of whole secretomes but at a lower concentration.

Figure 13: The effect of Marimastat and PMA on EpCAM release using ultracentrifuged secretomes. 100µl of secretome and 50 ml of PBS+ 1% BSA was added to each well. Absorbances were determined at 595 nm. Results show PMA induces EpCAM release whereas Marimastat inhibits its release. A combination of both also inhibits but not as much as when Marimastat is alone. Error bars were plotted based upon standard deviation of the data.



4.4.3 *The role of ADAM17 in EpCAM sheddase*

An attempt has been made to determine ADAM 17 abundance in cell lysates of UBC secretomes and to see its effect when knocked down on EpCAM release as previous studies revealed it to be responsible for its shedding. For siRNA knock down of EpCAM, it does not show much reduction on EpCAM concentrations on whole as well as ultracentrifugation [data not shown]. This may be due to the conditions it was grown in and may emphasise that the siRNA did not knock down ADAM 17.

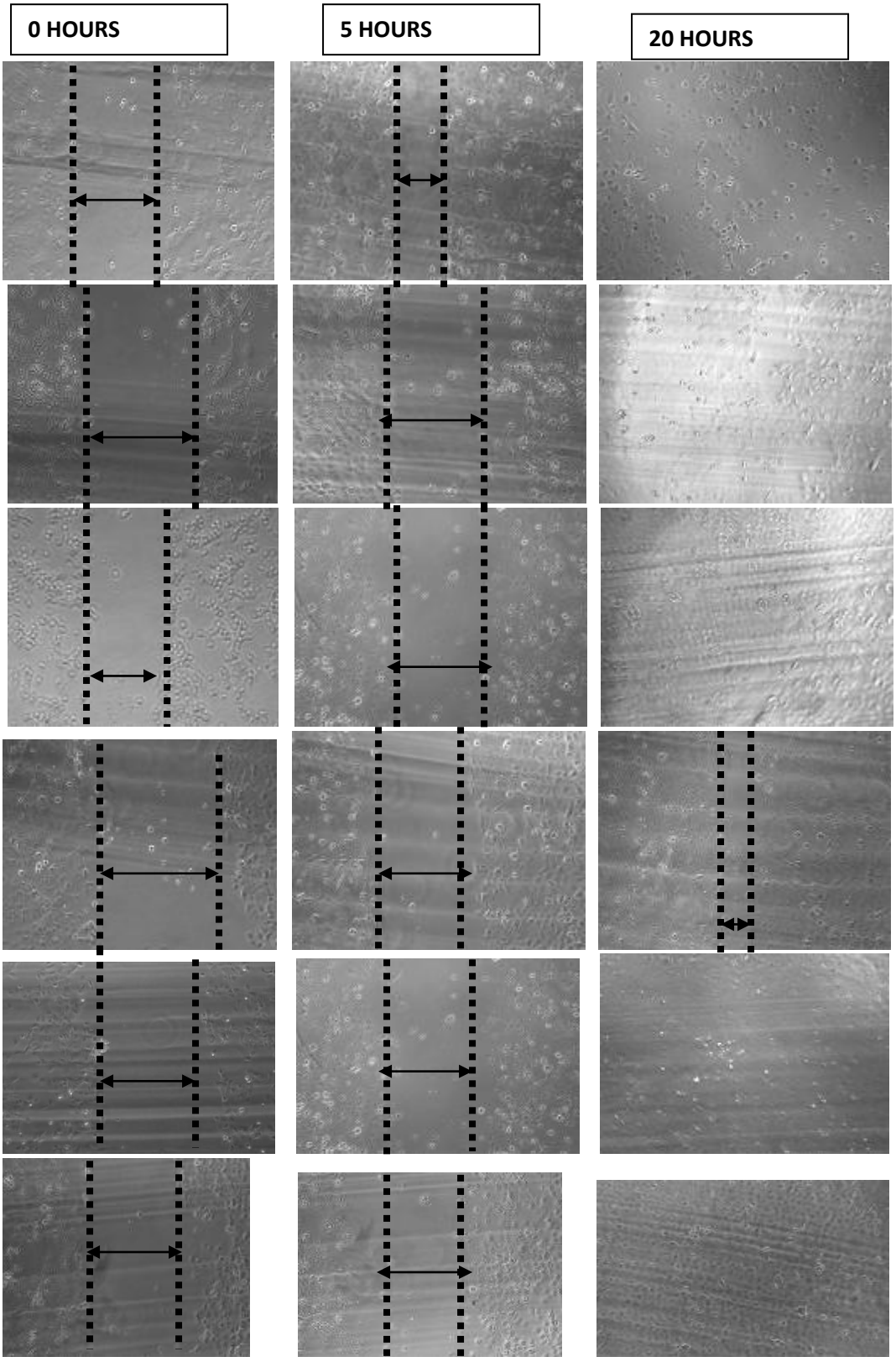
4.5 The effect of Marimastat and PMA on cell phenotype.

Because Marimastat prevented EpCAM cleavage, the next hypothesis, was that if it may inhibit proteolytic cleavage, it can also affect cell behaviour. This was investigated through wound-healing and MTT assays to see its affect on cell migration and cell viability respectively. At the same time, a hypothesis has been created whether PMA stimulates cell migration and viability, like how it stimulates shedding of EpCAM.

4.5.1 *Wound Healing*

This was performed to see if PMA and Marimastat affect cell migration. Photographs were taken at three time intervals: 0, 5 and 20 hours. The length of the wound was measured using a ruler via naked eye. Three measurements then work out the average. Figure 14 presents wound healing images at the three time intervals. Qualitative analysis suggests that PMA induces cell migration as time passes causing the wound to become narrow at 5 hours and then confluent at 20 hours. Alternatively, Marimastat did not seem to inhibit cell migration. There was more closure observed in Fetal Calf Serum (FCS) than serum-free (SF).

Figure 14: Wound Healing images of HB-CLS-2 (Grade III) This was done under different conditions at three intervals (0, 5 and 20 hours). It was repeated three times for fair testing and averages were calculated.



4.5.1.1 Marimastat does not inhibit cell migration

No significant inhibitory effect of 5µM Marimastat on cell-migration was found in both serum-free and in presence of nutritionally-filled media FCS conditions. Figure 15 presents how Marimastat affects percentage wound closure across different cell lines at 5 hours whereas Figure 16 presents how Marimastat affects percentage wound closure across different cell lines at 20 hours.

Figure 15: The effect of Marimastat on cell migration at 5 hours Photographs were taken at three time intervals: 0, 5 and 20 hours thrice. The length of the wound was measured using a ruler via naked eye. Three measurements then the average and standard deviation were calculated. Error bars were based upon standard deviations of the data obtained. Below is the average closure at 5 hours. It does not appear that Marimastat inhibits cells migration across the bladder cell lines. The data was plotted based upon grade; starting from left to right, it initiates from normal urothelium to HB-CLS-2 Grade III UBC cell lines.

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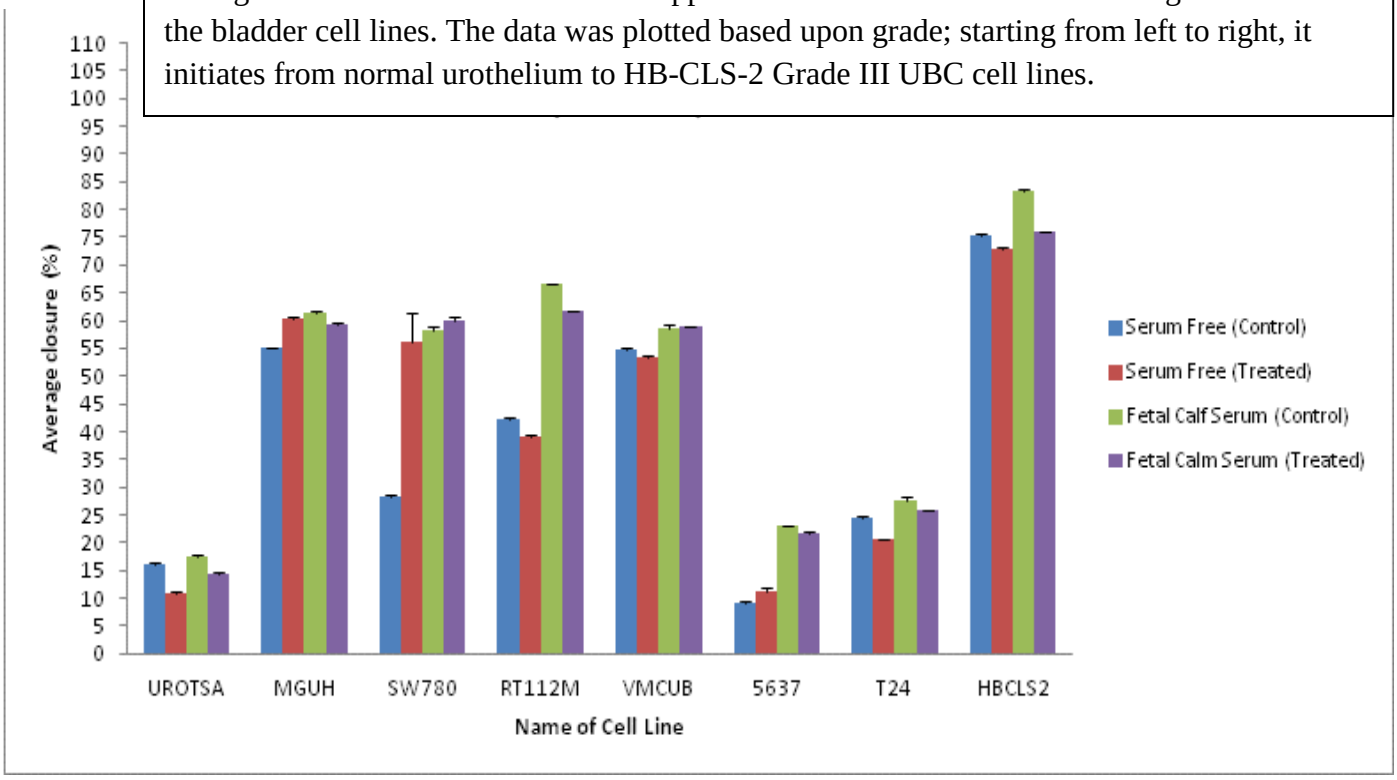
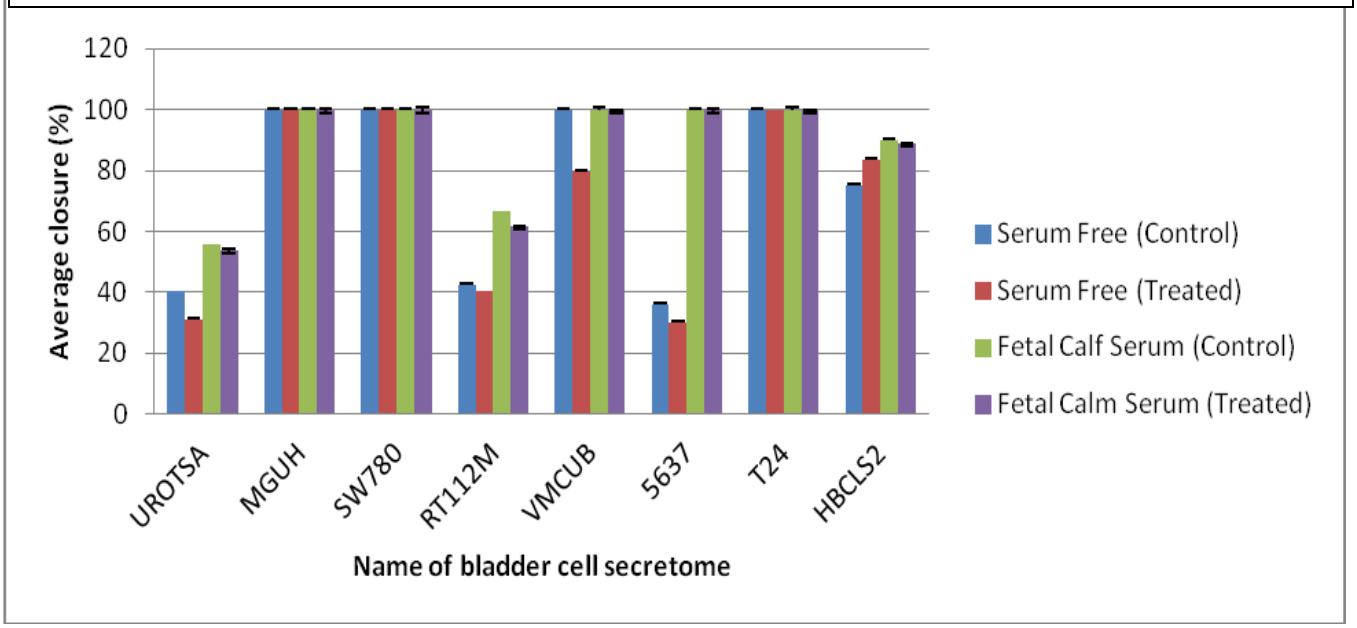


Figure 16: The effect of Marimastat on cell migration at 20 hours

Photographs were taken at three time intervals: 0, 5 and 20 hours thrice. The length of the wound was measured using a ruler via naked eye. Three measurements then the average and standard deviation were calculated. Error bars were based upon standard deviations of the data obtained. Below is the average closure at 20 hours. It does not appear that Marimastat inhibits cells migration across the bladder cell lines especially in Fetal Calf Serum. The data was plotted based upon grade; starting from left to right, it initiates from normal urothelium to HB-CLS-2 Grade III UBC cell lines. However, analysing the serum-free data, in some UBC cell lines such as RT112M (TCC) VMCUB (Grade 2) and 5637(Grade 2), there appears to be some inhibitory effect of cell migration by Marimastat compared to the control. This also occurs in normal urothelium serum-free data.



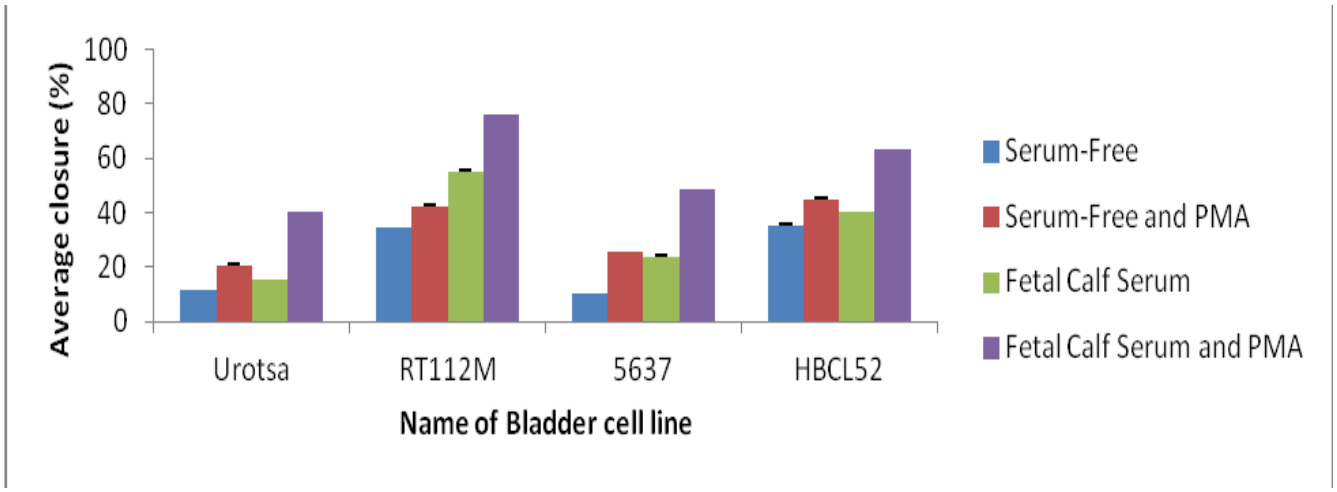
4.5.1.2 PMA stimulates cell migration

Cells have been treated with PMA, to express its effect on different grades of cancer cell lines. It was performed using the same method as the Marimastat investigation. Figure 17 presents how PMA affects percentage wound closure across different cell lines.

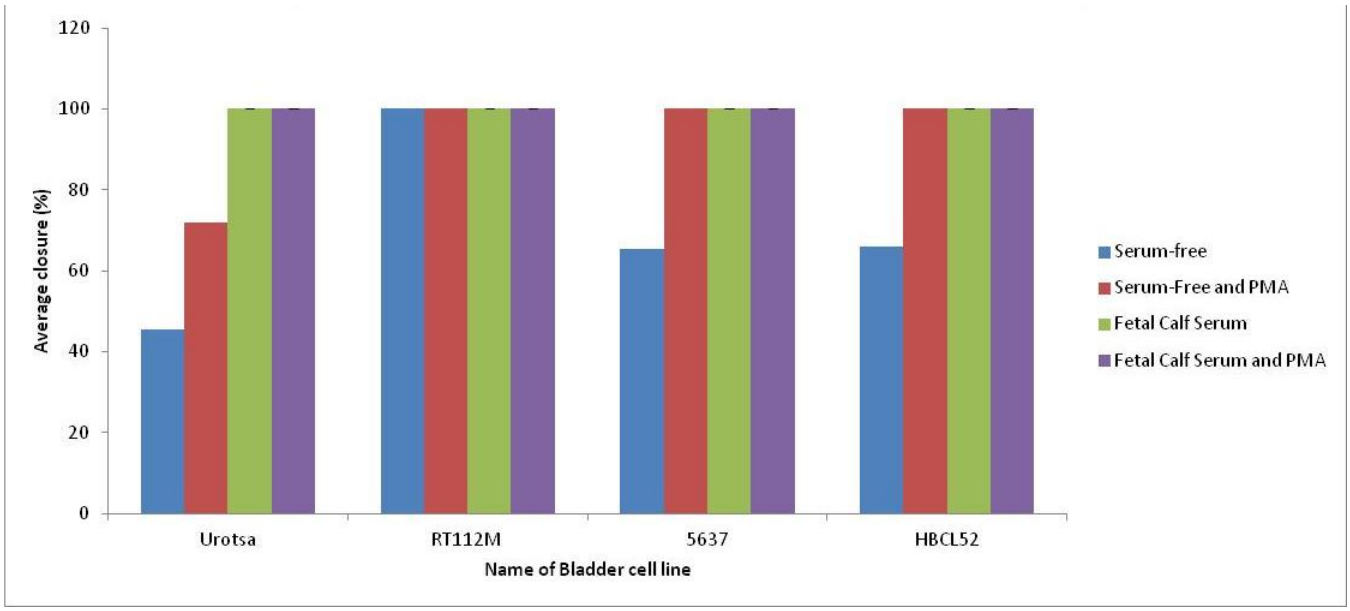
It is apparent that in presence of PMA in both SF and FCS, it increased cell migration than that of controls. This is consistent throughout normal and different grade of UBC cell lines at 5 and 20 hours. However, at 5 hours, there is more closure in RT112M (Grade II) in FCS than that with and without PMA than that of HB-CLS-2 (Grade III) and UROTsa (normal) and 5637 (Grade II). At 24 hours, for SF, the normal urothelial cell in presence of PMA in SF media did not have 100% wound closure whereas of RTT12M (Grade II), 5637(Grade II), HB-CLS-2 (Grade III); there was 100% confluency. This emphasises the variability in how PMA influences cell migration as for a normal bladder cell line there was still a wound present unlike UBC cell lines.

Figure 17: The effect of PMA on cell migration of bladder cell lines. (A) 5 hours, (B) 20 hours. Error bars were plotted based upon standard deviation of the data. It is apparent that PMA induces cell migration in serum-free and fetal calf serum (more in fetal calf serum) and the controls in serum-free and fetal calf serum. This pattern occurs across the normal urothelium and the different grades of bladder cancer cell lines.

(A)



(B)



4.5.2 *MTT Assay*

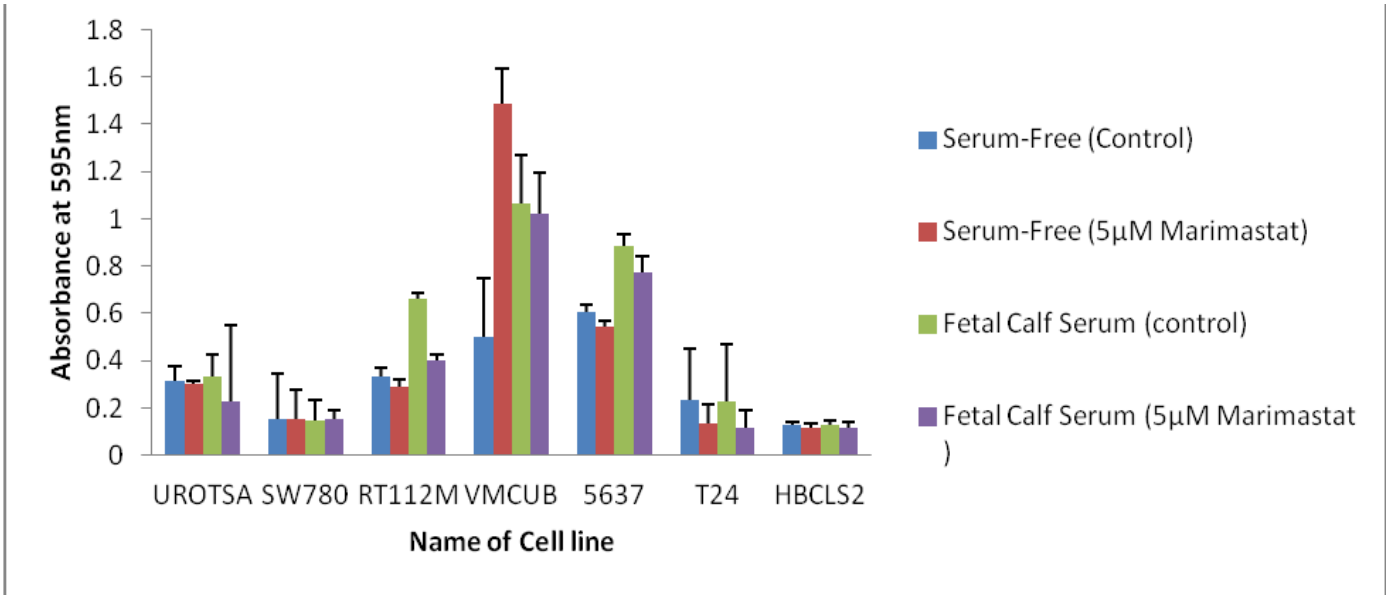
This was performed to see whether Marimastat and PMA effects cell viability. The MTT assay measured the in vitro cytotoxic effects of Marimastat and PMA on bladder cell lines.

4.5.2.1 There is limited inhibitory effect of Marimastat on cell viability.

5μM of Marimastat was used to see its affect on normal urothelial and different grades of UBC lines. This is because a preliminary experiment revealed there seems to be no difference doubling the concentration to 10μM [data not shown]. It is apparent there is a slight lower viability in presence of 5μM Marimastat than that of control whether Serum-free or Fetal Calf Serum across all bladder cell lines shown in Figure 19. T-test values had all more than 0.05; except for 5637 (Fetal Calf Serum and Serum-Free with and without Marimastat), RT112M (Serum-free with and without Marimastat) and VMCUB (serum-free with and without Marimastat) as shown in Appendix D.

Figure 19 The effect of Marimastat on cell viability

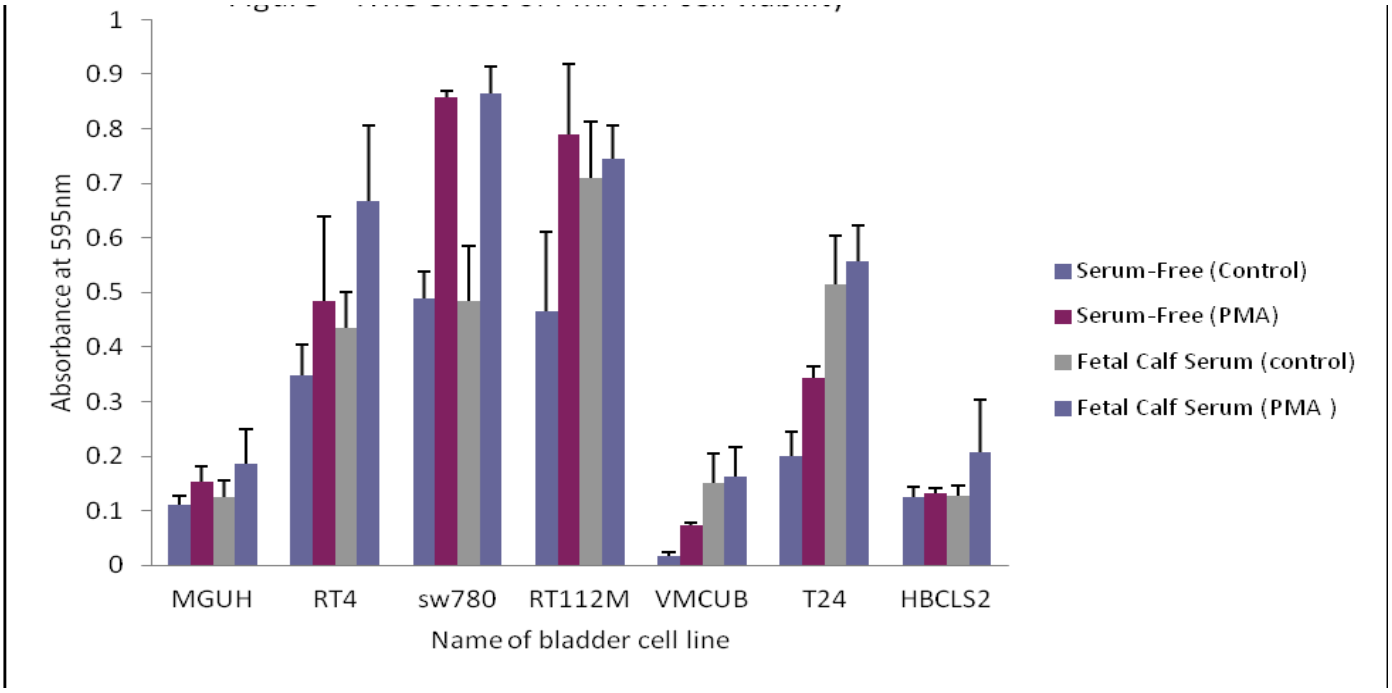
All cell lines in serum-free and fetal calf serum were treated with and without 5μM of Marimastat and were incubated for 24 hours before doing the MTT assay for cell viability. The bar graph shows the absorbance measured at 595nm on a ELISA plate reader. This experiment had three trials for each concentration. The averages were plotted, whereas the error bars were plotted based upon standard deviation. The bars represent the number of viable cells.



4.5.2.2 PMA stimulates cell viability.

PMA stimulates cell viability and this is seen across all bladder cell lines as shown in Figure 20. T-test values had all more than 0.05; except for RT4 (Fetal Calf Serum with and without PMA), MGUH (Serum-Free with and without PMA) and RT112M (Serum-Free with and without PMA) as shown in Appendix E.

Figure 20: The effect of PMA on cell viability. All cell lines in serum-free and fetal calf serum were treated with and without PMA and were incubated for 24 hours before doing the MTT assay for cell viability. The bar graph shows the absorbance measured at 595nm on an ELISA plate reader. This experiment had three trials for each concentration. The averages were plotted, whereas the error bars were plotted based upon standard deviation. The bars represent the number of viable cells.



4.6 Selecting biomarkers

Two methods were utilized to selective candidate biomarkers: HPA (www.proteinatlas.org) and IPA (www.ingenuity.com).

4.6.1 Candidate Biomarkers based on HPA analysis

HPA project was established in 2003 and is a publically available source that contains 11,200 proteins that complement more than 50% of genes (Asplund et al. 2011). The HPA immunohistochemistry candidate biomarkers were used to compare with the proteomic data.

4.6.1.1. PMA –HPA comparison

A Venn diagram was created to compare the HPA immunohistochemistry candidate biomarkers with the proteins that were present in 2 or more cell lines of PMA experiments. This was performed using the programme Venny (bioinfogp.cnb.csic.es/tools/venny/). 83 potential candidates have been revealed to be present in both the PMA proteomic data and HPA as shown in Figure 21. Appendix F shows a summary of the proteins including the function and their location using the gene ontology tool Panther database (www.pantherdb.org/).

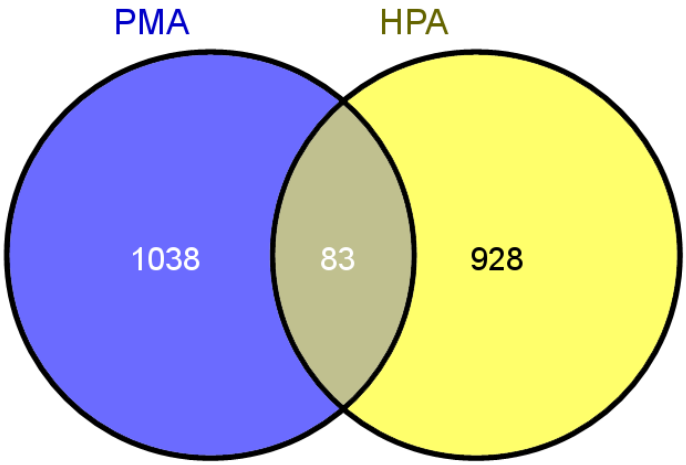


Figure 21: A Venn diagram comparing the PMA proteomic data with the HPA immunohistochemistry candidate biomarkers.

83 proteins were present in both PMA and HPA data. 1038 proteins were present only in the proteomic data and not in HPA data. 928 proteins were presentonly in the HPA data and not the proteomic data.

The PMA proteomic data consists of secreted and shedded proteins as PMA was added to whole secretomes. This was further investigated in relation to presence in high grade (T24, HB-CLS-2, SW780, RT112M and 5637) and not low-grade (RT4, MGH-U3 and VMCUB), up-regulation by PMA by two-fold and down-regulation by Marimastat by half- fold [data not shown]. Results revealed that all 83 proteins were upregulated by PMA. Ephrin-B1 and Fibroblast growth factor 19 were the only two proteins down-regulated by Marimastat. 17 proteins were higher in low grade UBC than high grade cancers (such as CD9, EXT1, GSTP1), 12 proteins were equivalent in both high grade (such as IL6, GSTM1, G6PD) and low grade and the remainder were elevated in high grade cancers than low grade cancers (such as ANXA, APOE, CALR, CD, CSF, CUL2, EGFR, EPCAM, EPHA HSPB1, FAS). This summarised in Appendix G.

4.6.1.1. PMA –Marimastat comparison

A Venn diagram was created to compare the HPA immunohistochemistry candidate biomarkers with the proteins that were present in 2 or more cell lines of Marimastat experiments. This was performed using the programme Venny (bioinfogp.cnb.csic.es/tools/venny/). 60 potential candidates have been revealed to be present in both the Marimastat proteomic data and HPA as shown in Figure 22. Using Panther database (www.pantherdb.org/), the locations and roles of these proteins have been summarised in Appendix H.

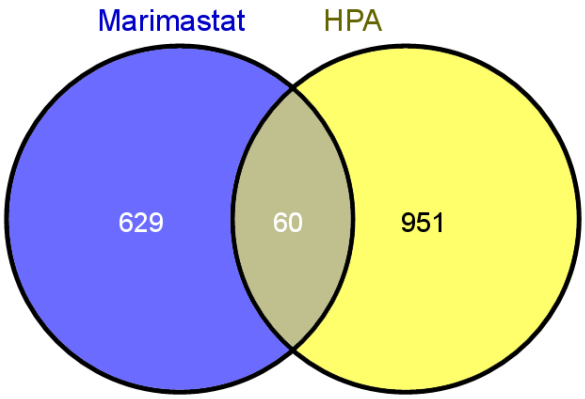


Figure 22: A Venn diagram comparing the Marimastat proteomic data with the HPA immunohistochemistry candidate biomarkers.

60 proteins were present in both PMA and HPA data. 629 proteins were present only in the proteomic data and not in HPA data. 951 proteins were presentonly in the HPA data and not the proteomic data.

4.6.2 Candidate Biomarkers based on IPA analysis

IPA-Biomarkers Filter was used to provide potential and relevant biomarker candidates from the experimental data based upon biological characteristics, disease and mechanism (Ingenuity n.d.). The following options were selected [Species – Human], [other cell lines], [not detected in biofluids] and [diagnostic biomarkers].

4.6.2.1 PMA-IPA analysis

Proteins that were present in two or more cell lines from the PMA proteomic data were subjected to analysis using IPA. Four potential candidate biomarkers were revealed: Glutathione S-transferase Mu 1 (GSTM1), Laminins (LAMC2), alpha-methylacyl-CoA racemase (AMARC) and APEG growth factor. GSTM1 is an enzyme located in cytoplasm. LAMC2 is a family consisting of extracellular matrix glycoproteins and are an important non-collagenous component of the basement membranes (National Center for Biotechnology Information 2014). They are located in the extracellular space. The core analysis revealed that the proteins predicted to be related to cancer are involved in a number of carcinogenic events such as metastasis, progression, invasion and proliferation.

4.6.2.2 Marimastat-IPA analysis

Proteins that were present in two or more cell lines from the Marimastat proteomic data were subjected to analysis using IPA. Two potential candidates were revealed: GSTM1 and LAMC. These proteins were also present in the PMA-IPA analysis. When Marimastat-based proteins were present in two or more cell lines was subjected to analysis using IPA. In relation to the core analysis, lots of proteins were predicted to be related to carcinogenic events such as metastasis, progression, invasion and proliferation.

4.6.3 Validation of Biomarkers

MMP-1 an interstitial collagenase was measured in urine to investigate whether it could be a potential diagnostic biomarker. The urine samples were randomly selected from the BCPP study and approximate to the population presenting with primary UCB and are summarised in Table 4 showing the number of patients in each group and number of males, females and each grade of UCB. All other data are presented as medians (lower quartile-upper quartile).

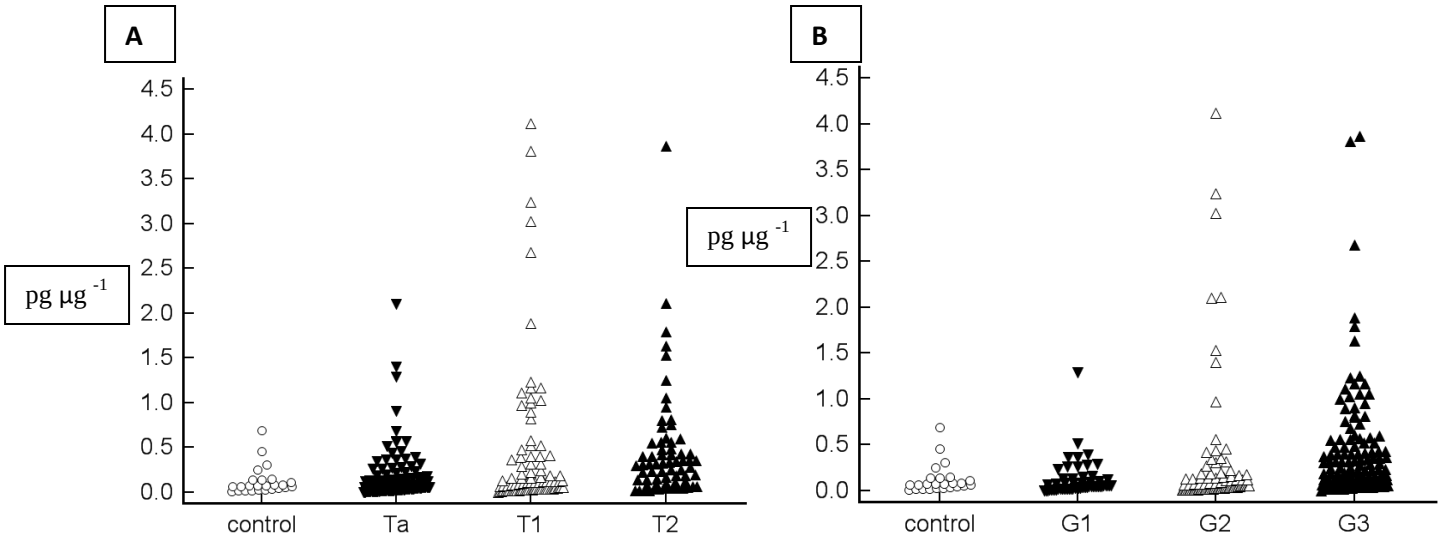
Table 4. The table presents the number of patients in each group based on the stage of their cancer. The gender and grade of UCB are also presented. The Creatinine (μg ml^{-1}), MMP1 (pg/ml) and MMP1 C were presented as medians (lower quartile-upper quartile)

Stage	n	Gender Male/female	Grade 1/2/3/4	Age (years)	Creatinine (μg ml-1)	MMP1 pg/ml	MMP1 C
Control	25	N/A	N/A	N/A	N/A	N/A	0.077146 (0.038167 – 0.137921)
pTis	4	4/0	0/0/4	66.28 (60.49418 - 74.68036)	2771.08 (2497.696 – 2965.455)	160.67 (127.1412 – 200.8163)	0.05 (0.044197 – 0.075344)
pTa	135	108/27	57/63/14	70.38 (61.73511 – 77.46749)	1718.59 (1033.993 - 2315.38)	132.751 (43.49681 - 364.6141	0.074698 (0.035811 - 0.232378)
T1	31	26/5	0/10/21	11.315 (67.04-78.355)			
T2+	67	51/16	0/4/62/1	75.10 (67.06502-80.83504)	364.22 (119.0459 - 913.254	1451.98 (995.6129 - 2117.455)	0.31 (0.105568-0.518854)

4.6.3.1 The relationship between Urinary MMP1 levels with tumour stage and grade.

To determine and analyse whether the levels of MMP-1 in patients (UBC and non-cancer controls) has a possible correlation with grade and tumour stage. The distribution of urinary MMP-1 levels in the control subjects and UBC patients with tumour stage (Ta, T1, T2) is shown in Figure 23a. Mann-Whitney test showed there was no significant difference found in Ta and T1. However, there was a significant difference found in T2 where the p-value was 0.0001. The relationship between the urinary MMP-1 levels of patients with tumour grade is shown in Figure 23b. Mann-Whitney test showed there was no significant difference for G1 and G2, however there was a significant difference for G3.

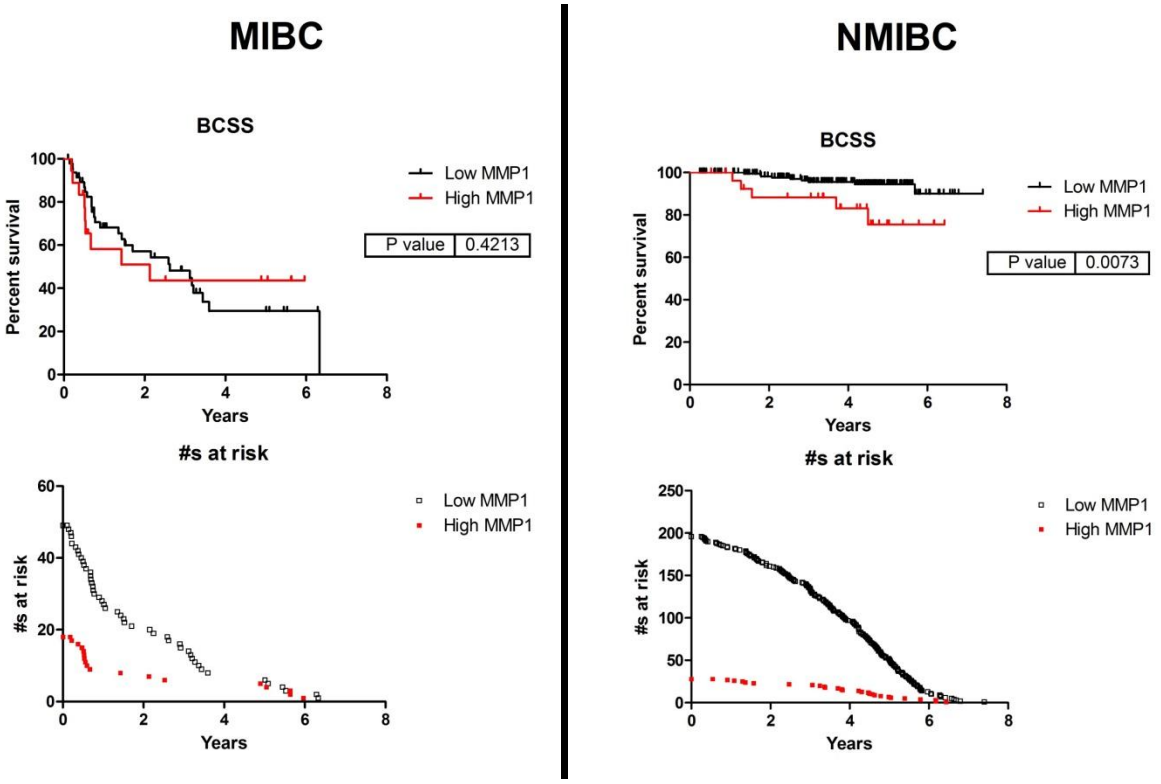
Figure 23: Urinary levels of MMP-1 in patients (UBC and non-cancer controls) correlation with stage and grade. A) stage B) grade. The data are normalised to urinary creatinine (pg µg $^{-1}$).



4.6.3.2 MMP-1 as a prognostic biomarker

A Kaplan-Meier curve was plotted to determine the variation in prognosis. A comparison was conducted between high and low urinary MMP-1 levels using a threshold determined as mean +2SD in the controls. There is no significant difference for MIBC but there is a significant difference for NMIBC as presented in Figure 24. The results emphasises it is not a good prognostic biomarker.

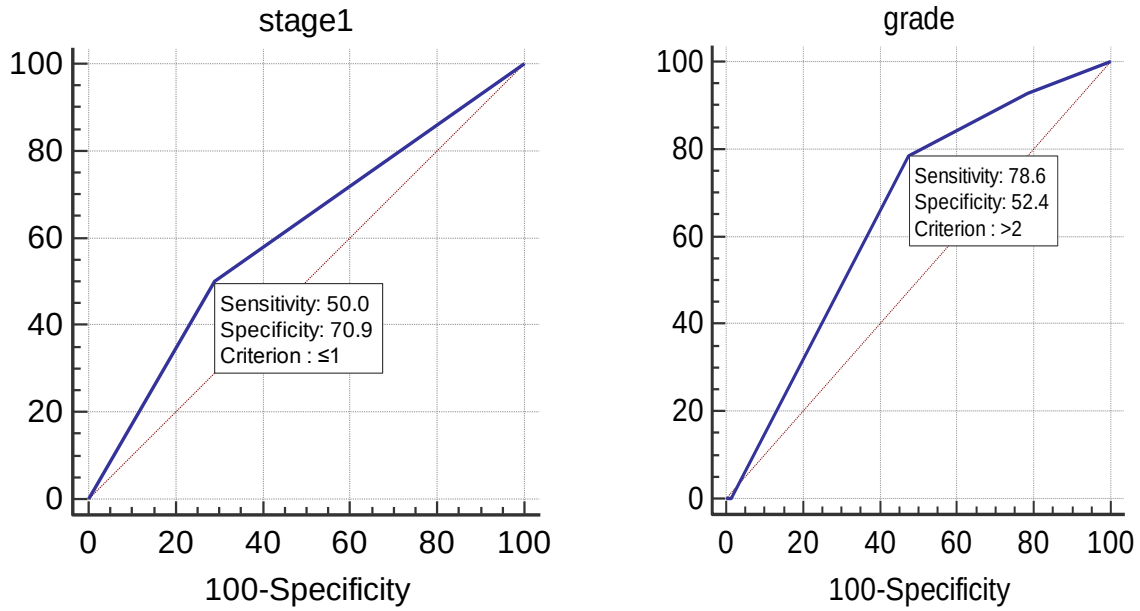
Figure 24: Kaplan-Meier curves comparing high versus low Urinary MMP-1 levels of UBC subjects A) MIBC p value >0.05 no significant difference B) NMIBC. P value <0.05 significant difference



4.6.3.3 MMP-1 as a diagnostic biomarker

To determine and evaluate whether MMP-1 is a suitable diagnostic biomarker for UBC, a Reciever Operating Characteristic (ROC) curve and a sensitivity/specificity report was created using the MedCalc software. The ROC curves for both tumour grade (low grade vs high grade) and tumour stage (low stage vs high stage) are shown in Figure 25 and were plotted based upon Clarke-Pearson et al. The ROC curve consists of sensitivity (true positive rate) plotted with 100-specificity (false positive rate) for varied cut-off points (MedCalc n.d.). Each point corresponds to sensitivity/specificity pair of a specific decision threshold (Med Calc n.d.).

Figure 25: Reciever Operating Characteristic analysis of MMP-1. (A) Tumour stage (low stage vs high stage) (B) Tumour grade (low grade vs high grade). For (A) the sensitivity is 50.0 whereas specificity is 70.9. For (B) the sensitivity is 78.6 whereas specificity was 52.4. It is a poor diagnostic biomarker.



The area under the ROC curve (AUC) for each ROC curve was calculated to determine how well MMP-1 can differentiate between the two diagnostic groups for grade and stage. Campbell revealed that if the test went across the chart (upper left corner) with 100% in both sensitivity and specificity,

this emphasises that it is a reliable and accurate diagnostic test and the AUC will be 1. Thus, the closer the ROC curve to that direction, the more accurate the test is. However, if the variable cannot distinguish between the groups being investigated, the AUC score will be 0.5. The results for this study show that when comparing low grade and high grade groups (Figure 25a), the AUC score was 0.653 with 95% confidence interval whereas when comparing low stage and high stage groups (Figure 25b), the AUC score was 0.605 with 95% confidence interval. The p-value was <0.05 and this emphasises that the AUC is not significantly different from 0.5. Therefore, this is evidence that it is a poor diagnostic marker that is unable to distinguish between the two groups for grade and stage and in turn does not have potential to detect UBC.

The Youden index was also calculated. Youden, defined it as:

$$J = \max \{\text{sensitivity}c + \text{specificity}c - 1 \}$$

This is where *c* consists of potential criterion values. *J* is the Youden index and is maximum vertical distance between the ROC curve and the diagonal line. For the ROC curve that corresponds to low grade vs high grade, the Youden index is 0.3094. For the ROC curve that corresponds to low grade vs high grade, the Youden index is 0.3094.

5.0 Discussion

This project utilized a shotgun proteomic approach to identify potential candidate biomarkers from secretomes of a panel of cell lines for urinary biomarker discovery. We have successfully identified over 2,242 proteins in the PMA-conditioned media of all 9 cell lines combined whereas in Marimastat-based experiments 1879 proteins have been identified. Proteins are identified by Demethyl labelling. PMA was added to whole secretomes to observe all proteins (secreted and shedded) released by cells whereas Marimastat, a broad-spectrum MMP inhibitor, was added to ultracentrifuged secretomes to focus on shedded proteins. Proteomic analysis on membrane proteins post-ultracentrifugation also took place as they have the potential to be biomarkers. Omics technology creates an extensive list of candidate markers and many techniques to select potential ones have been developed (Chen et al. 2013).

5.1 Proteomic analysis of UBC whole PMA-treated secretomes

As shown in Figure 4 and 5, whole secretomes were enriched with extracellular proteins and the predominant biological function is metabolism. This reflects the functional role of the bladder and emphasises that secretomes contain cytoplasms. The extracellular matrix (ECM) is secreted by cells and provides structural and functional support (Blonder et al. 2009). Another feature of the extracellular space is vesicles and there are two types intracellular that facilitate in many cellular processes such as protein-trafficking in the cell, whereas extracellular vesicles exert their roles outside of the cells (Blonder et al. 2009). The ECM is synthesised by various cells in the tumour environment. In addition, tumour vesicles are linked to secretion of growth factors in the tumour environment such as TGFβ cytokine that increase tumour progression. Vesicles are also linked to inducing invasion and ECM degradation (Blonder et al. 2009). ECM degradation facilitates

metastasis. Studies have revealed that metalloproteinases such as MMP-9 and MMP-2 are connected with vesicles and play a role in ECM degradation (Blonder et al. 2009). Shedding of vesicles has been discovered in many cancers that can allow tumours to evade host immune response (Blonder et al. 2009). This may explain as to why MMPs have been upregulated by PMA that stimulates shedding (Chen et al. 2010, Blonder et al. 2009).

5.2 Upregulated proteins by PMA

Upregulated proteins emphasises regulated release by PMA. After ultracentrifugation, there were still plasma-membrane bound proteins present as shown in Figure 6. Besides proteolytic enzymes such as MMPs that have been upregulated by PMA, other proteases were also upregulated such as Complement protease (C1r), Tissue plasminogen activator (TPA) and ADAM9. In addition, other proteins that are involved in proteasome activator complex and detoxification enzymes are also up-regulated.

5.2.1 *C1R*

The C1r is part of the C1 complex (C1q, C1r and C1s subunits) whose role is to commence the downstream classic complement cascade (Dobo et al. 2009, Kane et al. 2010). There is evidence that the complement increases tumour growth by aiding in cell proliferation and regeneration (Kane et al. 2010). Other way of how complement proteins encourages carcinogenesis is by migration, angiogenesis, invasion, and evading immunosurveillance (Kane et al. 2010).

5.2.2 *TPA*

Tissue polypeptide antigen (TPA) is one of the constituents of the cytoskeleton of non-squamous epithelia. It is found in the cytokeratin's proteolytic fragments (8, 19, and 8). Ecke et al. revealed

that its levels are linked with stages and grades of UBC and can be used to distinguish between control and UBC patients. However, it cannot be used to distinguish between MIBC and NMIBC.

5.2.3 *ADAM-9*

ADAM-9 is a member of the ADAM family that all are vital for altering cellular responses (Cho et al. 2005). Previous studies have shown that ADAM-9 promotes tumour cell invasion (Cho et al. 2005). Invasion is regulated by matrix-degrading enzymes, contact with stromal cells and integrins (Cho et al. 2005). Cho et al. revealed that in order to promote invasion, ADAM-9 specifically associates with $\alpha_6\beta_4$ and $\alpha_2\beta_1$ on cell surface and can cleave laminin.

5.2.4 *Detoxification enzymes*

Detoxification enzymes such as Glutathione S-transferase P (GSTP1), Lactoylglutathione lyase (LGUL), Peroxiredoxin-4 (PRDX4) and Superoxide dismutase (SODC)) have also been enhanced by PMA. Normally, these detoxification enzymes shield cells against oxidative stress and for them to be released from UBC secretomes implies that they protect tumour cells which are frequently under oxidative stress. Another enzyme is Triosephosphate isomerase (TPIS) that have been linked with metastasis (Karsani et al. 2014).

It is important to note that the proportion of intracellular proteins does not solely indicate that it is due fetal bovine serum (FBS) or apoptosis. Another explanation for their presence is due to a recent finding where intracellular proteins have other functions in the extracellular space (Villanueva et al. 2013). Further evidence from non-classical secretory pathways and analysis of exosomes from cancer cells have revealed their presence (Mendez et al. 2013). Thus, ECM is a useful source to discover biomarkers for early UBC diagnosis.

5.2.5 Other upregulated proteins

CUL2 was also present which correlates with previous studies as Fernandez-Gomez et al. discovered CUL3 that also shows potential for aggressiveness in bladder cancer.

Dahse et al. revealed that when a biomarker panel that consisted of DSG-2 with 5 other genes (BRCA2, ICAM-1, KRT-18, TNFRSF6 and LIG-3) it significantly identified tumour progression in NMIBC patients.

5.3 Ectodomain shedding

Ectodomain shedding is the proteolytic cleavage of the extracellular domain of membrane proteins. Proteases that induce this mechanism are known as 'sheddases' and are of two types: a distegrin and metalloproteinase (ADAMs) and metalloproteases (MMPs) (Chow 2007). There was no presence of ADAMs and MMPs (except for MMP-10 that was down-regulated). This is because Marimastat inhibits these sheddases and is a broad spectrum MMP inhibitor that has potency against majority of MMPs (Rasmussen 1999). It covalently associated with the zinc atom at the active site of MMPs (Eckhardt 2001). In addition there were a low number of proteins identified and the data was more variable. Possible reasons for this include spanning of some proteins down that is partly due to Marimastat and partly due to lack of PMA, and also ultracentrifugation. Other reasons include experiments (digestions, mass spectrometry) that did not seem to be working as well later. Therefore, if there is less data and it is more variable then differences between the cell lines may not be detected.

5.4 Selecting potential biomarkers

5.4.1 *Potential biomarkers based upon HPA comparison with proteomic data*

For the HPA-PMA analysis, 83 proteins were present in both whereas for the HPA-Marimastat analysis, 60 proteins were present as shown in Figure 21 and 22 respectively. The 83 proteins from the HPA-PMA analysis underwent further investigation as the PMA was added to whole secretomes to reveal both the secreted and shedded proteins. This was done in relation to whether they are present in high grade (T24, HB-CLS-2, SW780, RT112M and 5637) and not low-grade (RT4, MGH-U3 and VMCUB), up-regulation by PMA by two-fold and down-regulation by Marimastat by half- fold.

Previous studies have been conducted with some of these proteins in relation to cancer. Amongst these are EphrinB. This was upregulated by PMA and one of the proteins that were down-regulated by Marimastat. It was found in high grade cancers but not low grade cancers. It is a membrane bound protein ligand that in combination with the Eph family of receptor tyrosine kinases plays a pivotal role in carcinogenesis (Lu et al. 2013). A recent study has shown it to be associated with metastasis and its increased expression signifies poor prognosis in breast cancer.

Fas was one of the proteins up-regulated by PMA but was not down-regulated by Marimastat. It was found in high grade cancers but not low grade cancers. Kamp et al. investigated whether Fas and Fas ligand expression and synthesis on UBC cell lines of different grades is how they secret. They utilized RT112 (Grade 1), RT4 (Grade 1), SUP (Grade 4) and T24 (Grade 3). RT4 (Grade I) and T24 (Grade III) was also used in this present study. Results found that Fas was expressed in all UBC cell lines however its ligand was expressed in SUP and T24 but not in RT112 and RT4. However,

there was no correlation between Fas and its ligand and concluded that they have little clinical revelence.

In addition, GSTM1 is found in both HPA-PMA and HPA-Marimastat data. It was upregulated by PMA but not down-regulated by Marimastat. Safarinejad et al. revealed that glutathione-S-transferases (GSTs) such as GSTM1 and GSTP1, detoxifying enzymes that bind to glutathione to aid in removing carcinogenic substances may be linked with bladder cancer susceptibility.

Furthermore, Busch et al. revealed that APOE a bladder cancer-linked urinary protein correlates with increased tumour stage and can be a potential diagnostic biomarker.

5.4.2 *Potential biomarkers based upon IPA comparison with proteomic data*

Using the Biomarker Filter tab on IPA software, four proteins were elucidated: GSTM1 and AMARC, cytsolic enzymes, and LAMC2 and APEG growth factor in extracellular space were also revealed. In IPA core analysis, many proteins were predicted to be related to carcinogenic events such as invasion, proliferation, progression, metastasis and hyperplasia.

5.4.2.1 GSTM1

This was also revealed in HPA analysis. GSTM1 is one of GST's dimeric enzymes that reside in the cytosol (Bandyopadhyay et al. 2013). Carrato et al. revealed that GSTM1 null genotype enhances the risk of bladder cancer.

5.4.2.2 AMACR

AMACR enzyme is a biomarker found to be overexpressed in colorectal, renal and prostate cancer (Dietel et al. 2008). In relation to bladder cancer, Dietel et al. revealed it is expressed in high grade UBC and may have a role in tumour cell metabolism.

5.4.2.3 LAMC2

El-Rifai et al. revealed that LAMC is a potential biomarker of bladder cancer and is linked with tumour grade and metastasis.

5.4.3 Potential candidate biomarkers

The potential biomarkers from selective methods: EGFR, EpCAM, CUL-3, GSTM1 and PTX3 appear to be promising. This complements some of the results achieved from our group previously and other studies. Bryan et al. discovered that urinary levels of EpCAM are elevated and are dependent on the grade and stage of UBC but is a poor prognostic biomarker. Recent studies have shown CUL-3 to be a significant prognostic marker (Theodorescu 2013).

EGFR is a 170 kDa transmembrane glycoprotein and a member of tyrosine kinase receptors that allows ligands to bind to the extracellular domain (Agarawal et al. 2013). It is overexpressed and is a good predictor of poor survival and cancer progression especially in high grade tumours (Agarawal et al. 2013).

Furthermore, GSTM1 is a potential candidate as it was amongst those present in both selection methods: HPA and IPA. When further analysis took place with the HPA-PMA data, it was found to be present in both high and low grade cancers. It was also up-regulated by PMA, however it was not down-regulated by Marimastat.

5.5 An introduction to EpCAM biology

EpCAM is a potential biomarker based on our group's previous findings relating to EpCAM ectodomain shedding that is currently on-going investigations. We decided to investigate this process in more detail. PMA and Marimastat and a combination of both were utilized to see their effect on EpCAM release from bladder cell secretomes and how tumor necrosis factor α-converting enzyme (TACE or ADAM17) a reported protease can specifically inhibit EpCAM shedding. Results reveal important aspects of EpCAM biology and also provide interesting insights into the mechanisms of morphogenesis.

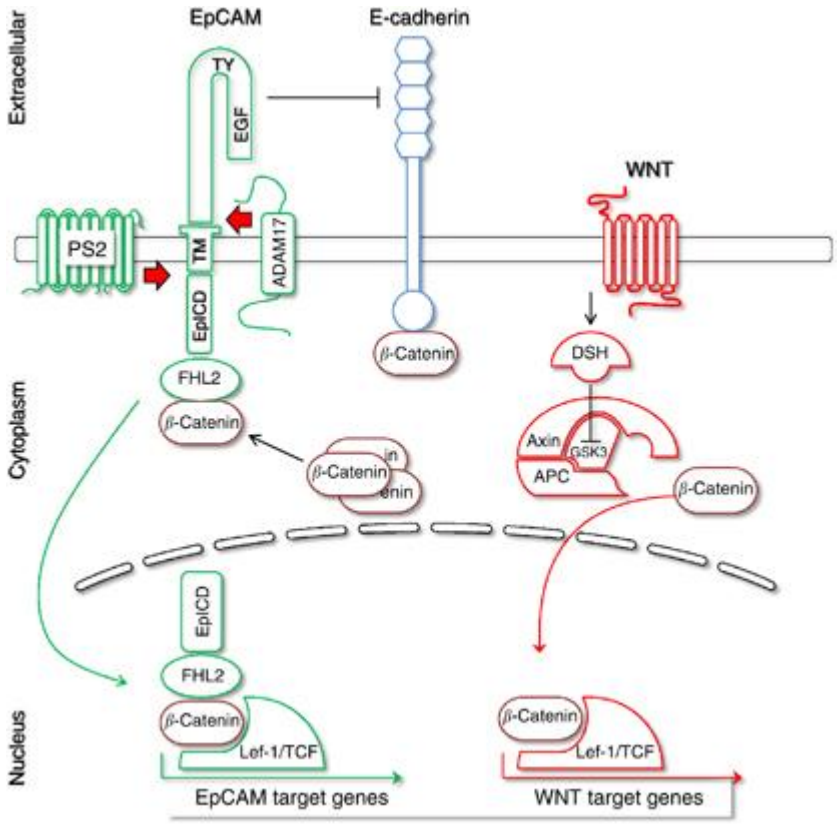
5.5.1 *PMA stimulates shedding of EpCAM via PKC activation*

PMA stimulates an increased release of ectodomain shedding EpCAM from bladder cancer cell secretomes as well as reducing proteolytic processing (Hahn et al. 2003). This stimulatory effect is inhibited by peptide hydroxamate MMP1 inhibtors such as Marimastat that inhibit PKC suggesting that activation of Protein kinase C (PKC) mediates PMA-induced EpCAM shedding (Parker et al 2010). PKC consists of a family of serine/threonine kinases (Toker 1998). ADAM 17 mediates the PMA-induced release.

5.5.2 *The effect of ADAM-17 on EpCAM release*

Evidence suggests that ADAM17 induces proteolytic cleavage of EpCAM via its extracellular site. This increases shedding of EpEX ectodomain. This is subsequently followed by cleavage of the transmembrane domain by γ-secretase. This causes EpICD (soluble cytoplasmic peptide) release (Giepmans 2013, Gires et al. 2009). EpICD then creates a complex with β-catenin, Lef-1 and FHL2 to initiate gene transcription as shown in Figure 22 (Gires et al. 2009)

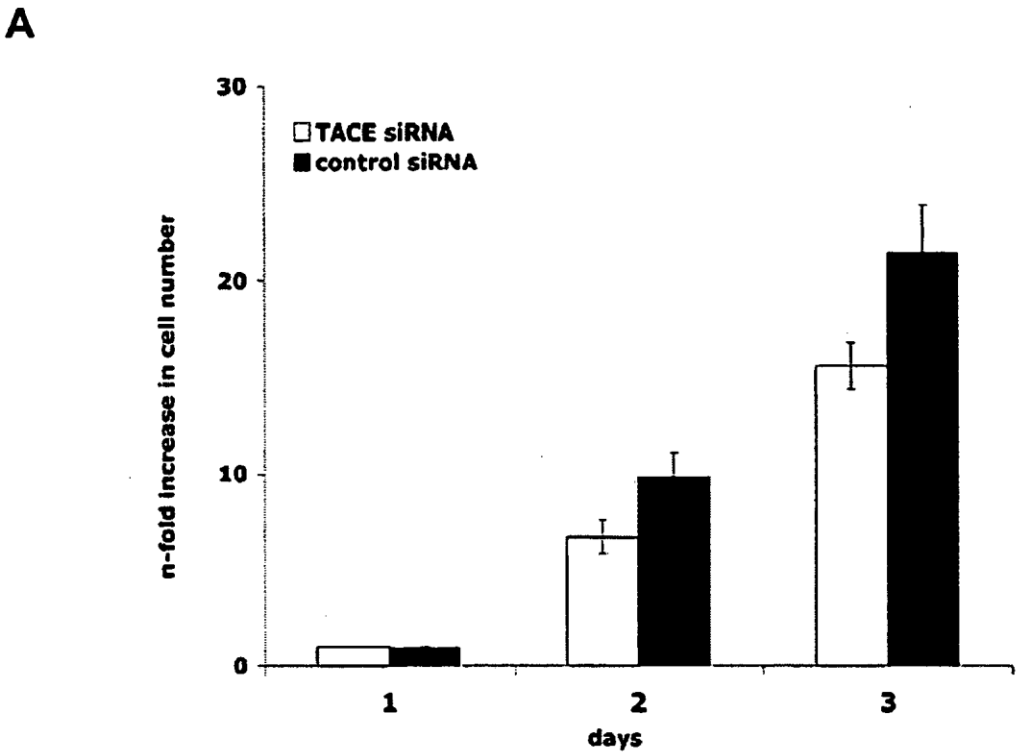
Figure 22: The cross talk between EpCAM and WNT signalling pathways (Gires et al. 2009)



However, according to this present study, the ADAM 17 siRNA did not work despite previous studies have confirmed it can knock-down EpCAM as shown in Figure 23 (Gires 2011). This emphasises that siRNA did not knock down ADAM17.

Figure 23 ADAM-17 siRNA knockdown using FaDu carcinoma cells.

An ELISA experiment took place and revealed how the cells that were transfected with ADAM17 siRNA reduces the number of cells over a period of 3 days than the control siRNA. The mean and standard deviations are shown for two independent experiments (Gires 2011)



5.5.3 *The role of ADAM-17 in cancer*

ADAM-17 is the strongest evidence of the role of ADAMs in cancer. Other ADAMs involved are ADAM-15, ADAM-12, ADAM-10 and ADAM-9. Down-regulation or knocking down ADAM-17 expression reduces growth of cancer cells and has been reported by previous studies. Baldys portrayed that knockdown of ADAM17 using siRNA decreased endothelial cell proliferation and invasion *in vitro*. Blobel et al. revealed that it reduced tumour growth and pathological neovascularisation. There are other ADAM proteases that are linked with pathological neovascularisation such as ADAM9 and ADAM-15 (Crown et al. 2011).

Increased ADAM expression promotes tumour formation, invasion and proliferation (Crown et al. 2011). One of the predominant mechanisms in increasing invasion is by activating growth factors such as EGFR/HER membrane-bound ligands into their active state by shedding their ectodomains. The ligands such as epiregulin, TGF-alpha are released and freely associate with EGFR/HER receptors. After receptor dimerisation, down-stream signalling pathways such as janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, phosphatidylinositol 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAPK) pathway are then activated. This can lead to many hallmarks of cancer such as enhanced cell survival, motility and proliferation (Crown et al. 2011). In particularly, ADAM-17 via the PI3K pathway was reported to increase cell proliferation (Chopp et al. 2009).

Another study revealed that ADAM-17 increases migration via the platelet derived growth factor receptor beta (PDGFRβ) that stimulates ADAM-17 that in turn releases EGFR ligands and the EGFR/ERK signalling pathway (Blobel et al. 2010). This led to ADAM-17 activation, release of EGFR ligands and EGFR/ERK signalling.

Furthermore, stimulating positive growth factors are not the only growth factors that lead to such carcinogenic events. Inhibitory growth factors such as TGFβ inhibit cell proliferation in normal and benign cells. However, as the cancer progresses, it increases proliferation (Ikushima 2010).

Thus, findings suggested that PMA stimulates EpCAM release whereas Marimastat has an inhibitory effect on EpCAM release. This led to investigations on their effect on cell phenotype where Marimastat did not have an inhibitory effect on cell migration but to a limited extent on cell viability.

5.6 PMA stimulates cell migration and viability.

PKC is first upregulated but subsequently downregulated which may explain the underlying reasons for the results obtained. Charvart et al. states that cell migration is an essential process in physiological and pathological conditions such as wound healing and tumour invasion. PKC regulates cell migration by stimulating signal transduction that regulate actin cytoskeleton (Parkos 2013).

Fagotto et al. revealed that high levels of EpCAM are linked to increased proliferation in normal or tumour tissues. EpCAM can act as a cell-cell adhesion molecule and bind to the actin cytoskeleton via α-actinin to regulate cell movement. Other possible reasons due to high proliferation rates are due to separating α-catenin from E-cadherin (Fagotto et al. 2010). Another reason for the increasing effects of EpCAM on proliferation is due to signalling activity of its intracellular domain that can be cleaved and can subsequently associate with β-catenin, Lef-1 to stimulate gene transcription of oncogenes such as C-myc and cyclins A/E (Fagotto et al. 2010).

5.7 Marimastat has limited effect on cell viability and migration

Marimastat did not have much inhibitory effect on cell viability and migration. Previous studies have shown that Marimastat does inhibit proliferation and migration using human keratinocyte line, HaCaT; demonstrating the role of MMPs in cell migration (Auffinger et al. 2013, Charvart et al. 1999). Gao et al revealed that integrin-linked kinase (ILK) a serine-threonine protein kinase that has a major role in many cellular mechanisms increases cell migration via nuclear factor-κB (NF-κB) upregulation of MMP-9. Reasons why Marimastat has worked in Charvart et al. study and not this present study leads to further investigation where higher concentrations more than 5μM of Marimastat will be used.

5.8.MMP1 as a prognostic and diagnostic biomarker

MMP-1 is interstitial collagenases and the structure of its catalytic domain is similar to other MMPs (Acharya et al 2006, Chaplain 2013). Urine analysis of UBC patients revealed that it is a poor diagnostic and prognostic marker. It was elevated in a proportion of patients. Mellon et al. discovered the role of MMP-1 in UBC and revealed similar results to this study where urinary MMP-1 was detectable more at higher grade tumours. This opposes Durkan et al. study who discovered that MMP1 is a useful prognostic indicator for bladder tumour progression.

MMP3 may be the recommended urinary metalloproteinases as early diagnostic biomarker in the early stages of both types of bladder cancer although both MMP9 and MMP3 can be used in the diagnosis of advanced stages. Further studies are required on large number of urine samples to confirm these results (El-Sharkawi et al. 2014)

5.9 Further experiments

If time was extended, I would like to work on siRNA and get results on how ADAM siRNA effects EpCAM release via ELISA and Western blotting. I would also like to measure the following potential candidates further: EGFR, PTX3, GSTM1 and CUL-3. I would also like to investigate cell surface proteomics where cells are treated with a membrane-impermeant biotinylating reagent to label surface-exposed proteins. The cell surface proteome will notify what sheddases are present and may identify cancer-specific proteins useful as biomarkers or drug targets.

6.0 Conclusion

Ultimately, many findings can be extracted from this study; firstly, PMA stimulates shedding of EpCAM ectodomain whereas Marimastat decreases EpCAM release. In relation to investigations conducted on the cell phenotype, Marimastat had minimal inhibitory effect on on cell viability and migration. On the other hand, PMA induced cell migration and viability. Many potential biomarkers for UBC have been revealed via the two selections: IPA and HPA analysis. From these, potential candidates that would be taken further are where EpCAM, EGFR, PTX3, GSTM1 and CUL-3 are potential candidates. MMP-1 was detected in urine from UBC patients, with a significant increase in concentration with increased stage and grade of tumour. However it is a poor diagnostic and prognostic biomarker. We have comprehensively utilized UBC secretomes and further bioinformatic analyses e.g. comparison with gene expression data which will facilitate in urinary biomarker discovery.

7.0 References

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8.0 Appendices

Appendix A

137 proteins found in all 9 secretomes from the PMA proteomic data. The names of the proteins from the accession names were revealed using Panther database (http://www.pantherdb.org/)

Accession	Name of Protein
K2C1	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
INHBA	Inhibin beta A chain OS=Homo sapiens GN=INHBA PE=1 SV=2
AGRIN	Agrin OS=Homo sapiens GN=AGRN PE=1 SV=4
A4	Amyloid beta A4 protein OS=Homo sapiens GN=APP PE=1 SV=3
TENA	
LAMB3	
HS90B	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4
HSP7C	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1
HS90A	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5
JAG1	Protein jagged-1 OS=Homo sapiens GN=JAG1 PE=1 SV=3
ACTN4	Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2
ACTB	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1
APLP2	
K1C10	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
K22E	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
K1C9	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
TIMP1	
K2C6A	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3
ACTN1	Alpha-actinin-1 OS=Homo sapiens GN=ACTN1 PE=1 SV=2
A1AT	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3
GRP78	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2
HSP71	Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A PE=1 SV=5

K1C16	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4
MMP13	
PTK7	
TPA	
PTX3	
ACLY	ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=1 SV=3
TR11B	
AHNK	Neuroblast differentiation-associated protein AHNAK OS=Homo sapiens GN=AHNAK PE=1 SV=2
1433E	14-3-3 protein epsilon OS=Homo sapiens GN=YWHAE PE=1 SV=1
HSP13	Heat shock 70 kDa protein 13 OS=Homo sapiens GN=HSPA13 PE=1 SV=1
IL6	Interleukin-6 OS=Homo sapiens GN=IL6 PE=1 SV=1
K1H1	Keratin, type I cuticular Ha1 OS=Homo sapiens GN=KRT31 PE=1 SV=3
SPIT1	
A2MG	Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=2
IF4A1	Eukaryotic initiation factor 4A-I OS=Homo sapiens GN=EIF4A1 PE=1 SV=1
1433Z	14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1
ACTC	Actin, alpha cardiac muscle 1 OS=Homo sapiens GN=ACTC1 PE=1 SV=1
CTGF	
ICAM1	Intercellular adhesion molecule 1 OS=Homo sapiens GN=ICAM1 PE=1 SV=2
1433T	14-3-3 protein theta OS=Homo sapiens GN=YWHAQ PE=1 SV=1
EPHA2	
IBP7	Insulin-like growth factor-binding protein 7 OS=Homo sapiens GN=IGFBP7 PE=1 SV=1
LSR	
ALDOA	Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2
FST	
HSP74	Heat shock 70 kDa protein 4 OS=Homo sapiens GN=HSPA4 PE=1 SV=4
K2C6C	
PLOD2	
CO6A2	
GANAB	Neutral alpha-glucosidase AB OS=Homo sapiens GN=GANAB PE=1 SV=3
HSP72	Heat shock-related 70 kDa protein 2 OS=Homo sapiens GN=HSPA2 PE=1 SV=1
ALBU	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
CO4A2	
CTNA1	
ITB4	
IQGA1	Ras GTPase-activating-like protein IQGAP1 OS=Homo sapiens GN=IQGAP1 PE=1

	SV=1
ITB1	Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2
LIF	
TACD2	
ACTBL	Beta-actin-like protein 2 OS=Homo sapiens GN=ACTBL2 PE=1 SV=2
GELS	Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1
GBLP	Guanine nucleotide-binding protein subunit beta-2-like 1 OS=Homo sapiens GN=GNB2L1 PE=1 SV=3
GSLG1	Golgi apparatus protein 1 OS=Homo sapiens GN=GLG1 PE=1 SV=2
IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Homo sapiens GN=IDH1 PE=1 SV=2
PTPRF	
4F2	4F2 cell-surface antigen heavy chain OS=Homo sapiens GN=SLC3A2 PE=1 SV=3
AMD	Peptidyl-glycine alpha-amidating monooxygenase OS=Homo sapiens GN=PAM PE=1 SV=2
H4	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2
ITA6	Integrin alpha-6 OS=Homo sapiens GN=ITGA6 PE=1 SV=4
PAI1	
ECM1	
ITA2	Integrin alpha-2 OS=Homo sapiens GN=ITGA2 PE=1 SV=1
K2C5	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3
MMP3	
PCDGK	
UPAR	
CATD	
HEXB	Beta-hexosaminidase subunit beta OS=Homo sapiens GN=HEXB PE=1 SV=3
HNRPK	Heterogeneous nuclear ribonucleoprotein K OS=Homo sapiens GN=HNRNPK PE=1 SV=1
IMB1	Importin subunit beta-1 OS=Homo sapiens GN=KPNB1 PE=1 SV=2
1433S	14-3-3 protein sigma OS=Homo sapiens GN=SFN PE=1 SV=1
CDCP1	
CYR61	
GNAI3	Guanine nucleotide-binding protein G(k) subunit alpha OS=Homo sapiens GN=GNAI3 PE=1 SV=3
CAB45	
GDIB	Rab GDP dissociation inhibitor beta OS=Homo sapiens GN=GDI2 PE=1 SV=2
GOLM1	Golgi membrane protein 1 OS=Homo sapiens GN=GOLM1 PE=1 SV=1
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens GN=ITIH2 PE=1

	SV=2
CADH1	
GALT7	N-acetylgalactosaminyltransferase 7 OS=Homo sapiens GN=GALNT7 PE=1 SV=1
GDF15	Growth/differentiation factor 15 OS=Homo sapiens GN=GDF15 PE=1 SV=2
ILF2	Interleukin enhancer-binding factor 2 OS=Homo sapiens GN=ILF2 PE=1 SV=2
PVRL1	
STC1	
TNF6B	
5NTD	5'-nucleotidase OS=Homo sapiens GN=NT5E PE=1 SV=1
DJB11	
GRP75	Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9 PE=1 SV=2
GSTO1	Glutathione S-transferase omega-1 OS=Homo sapiens GN=GSTO1 PE=1 SV=2
GSTP1	Glutathione S-transferase P OS=Homo sapiens GN=GSTP1 PE=1 SV=2
HS105	Heat shock protein 105 kDa OS=Homo sapiens GN=HSPH1 PE=1 SV=1
IGSF8	Immunoglobulin superfamily member 8 OS=Homo sapiens GN=IGSF8 PE=1 SV=1
IPO5	Importin-5 OS=Homo sapiens GN=IPO5 PE=1 SV=4
IPO7	Importin-7 OS=Homo sapiens GN=IPO7 PE=1 SV=1
MET	
RHOA	
1A01	HLA class I histocompatibility antigen, A-1 alpha chain OS=Homo sapiens GN=HLA-A PE=1 SV=1
1C12	HLA class I histocompatibility antigen, Cw-12 alpha chain OS=Homo sapiens GN=HLA-C PE=1 SV=2
AATC	Aspartate aminotransferase, cytoplasmic OS=Homo sapiens GN=GOT1 PE=1 SV=3
ARPC2	Actin-related protein 2/3 complex subunit 2 OS=Homo sapiens GN=ARPC2 PE=1 SV=1
CATB	
ESM1	
HNRPQ	Heterogeneous nuclear ribonucleoprotein Q OS=Homo sapiens GN=SYNCRIP PE=1 SV=2
HS74L	Heat shock 70 kDa protein 4L OS=Homo sapiens GN=HSPA4L PE=1 SV=2
N2DL2	
2AAA	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform OS=Homo sapiens GN=PPP2R1A PE=1 SV=4
6PGD	6-phosphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE=1 SV=3
AKA12	A-kinase anchor protein 12 OS=Homo sapiens GN=AKAP12 PE=1 SV=3

ARP3	Actin-related protein 3 OS=Homo sapiens GN=ACTR3 PE=1 SV=3
GBB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Homo sapiens GN=GNB1 PE=1 SV=3
GDIA	Rab GDP dissociation inhibitor alpha OS=Homo sapiens GN=GDI1 PE=1 SV=2
GDIR1	Rho GDP-dissociation inhibitor 1 OS=Homo sapiens GN=ARHGDIA PE=1 SV=3
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2 OS=Homo sapiens GN=GNAI2 PE=1 SV=3
GNAS2	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short OS=Homo sapiens GN=GNAS PE=1 SV=1
H2AY	Core histone macro-H2A.1 OS=Homo sapiens GN=H2AFY PE=1 SV=4
H2B1J	Histone H2B type 1-J OS=Homo sapiens GN=HIST1H2BJ PE=1 SV=3
HEXA	Beta-hexosaminidase subunit alpha OS=Homo sapiens GN=HEXA PE=1 SV=1
K1C13	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4
K1C14	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4
ACTBM	Beta-actin-like protein 3 OS=Homo sapiens GN=ACTBL3 PE=1 SV=1
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9 OS=Homo sapiens GN=ADAM9 PE=1 SV=1
GGH	Gamma-glutamyl hydrolase OS=Homo sapiens GN=GGH PE=1 SV=2
GLU2B	Glucosidase 2 subunit beta OS=Homo sapiens GN=PRKCSH PE=1 SV=2
HNRPR	Heterogeneous nuclear ribonucleoprotein R OS=Homo sapiens GN=HNRNPR PE=1 SV=1
ITAV	Integrin alpha-V OS=Homo sapiens GN=ITGAV PE=1 SV=2

Appendix B Proteins upregulated by PMA

The name of the proteins upregulated by PMA with their cellular locations, molecular functions and biological functions have been determined using the Panther database (http://www.pantherdb.org/)

Accession Name	Name	Molecular function	Biological function	Cellular component
1433S	14-3-3 protein sigma;SFN;ortholog		cell cycle;cell communication	
2A5G	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit gamma isoform;PPP2R5C;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	protein phosphorylation;cell communication	
2AAA	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform;PPP2R1A;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	metabolic process	
ABCF1	ATP-binding cassette sub-family F member 1;ABCF1;ortholog			
ACLY	ATP-citrate synthase;ACLY;ortholog			
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9;ADAM9;ortholog		fertilization;apoptotic process;neurological system process;mesoderm development;apoptotic process;heart development	
AGRIN	Agrin;AGRN;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development	extracellular region;extracellular matrix
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1;AHSA1;ortholog	catalytic activity;protein binding;enzyme activator activity	immune system process;protein folding;response to stress;regulation of catalytic activity	
AIP	AH receptor-interacting		visual perception;sensory perception	

	protein;AIP;ortholog			
ANXA1	Annexin A1;ANXA1;ortholog		fatty acid metabolic process;cell communication	
ARPC4	Actin-related protein 2/3 complex subunit 4;ARPC4;ortholog	structural constituent of cytoskeleton	cellular component movement;cellular component organization	actin cytoskeleton;intracellular
BLVRB	Flavin reductase (NADPH);BLVRB;ortholog	oxidoreductase activity	metabolic process	
BROX	BRO1 domain-containing protein BROX;BROX;ortholog			
C1R	Complement C1r subcomponent;C1R;ortholog	serine-type peptidase activity;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	gamete generation;complement activation;proteolysis;cellular process;blood coagulation	
CADH2	Cadherin-2;CDH2;ortholog	binding	cellular process;visual perception;sensory perception of sound;mesoderm development;nervous system development;heart development;muscle organ development	plasma membrane;cell part
CADH3	Cadherin-3;CDH3;ortholog	binding	cellular process;visual perception;sensory perception of sound;mesoderm development;nervous system development;heart development;muscle organ development	plasma membrane;cell part
CALX	Calnexin;CANX;ortholog	calcium ion binding	protein folding;intracellular protein transport;exocytosis	
CAPG	Macrophage-capping protein;CAPG;ortholog	structural constituent of cytoskeleton;calcium ion binding;actin binding	cellular process;cellular component morphogenesis;cellular component organization	actin cytoskeleton;intracellular
CATL2	Cathepsin L2;CTSL2;ortholog	cysteine-type peptidase activity	proteolysis;cell adhesion	
CAYP1	Calcyphosin;CAPS;ortholog	catalytic activity;calcium ion binding;receptor binding;calmodulin	metabolic process;cell cycle;cell communication;cation transport;regulation of	

		binding;enzyme regulator activity	catalytic activity	
CEL	Bile salt-activated lipase;CEL;ortholog	lipase activity	metabolic process	extracellular region;cytoplasm
CFAB	Complement factor B;CFB;ortholog			
CG050	Uncharacterized protein C7orf50;C7orf50;ortholog			
CHSTB	Carbohydrate sulfotransferase 11;CHST11;ortholog	transferase activity	sulfur compound metabolic process	
CLIC1	Chloride intracellular channel protein 1;CLIC1;ortholog			
CLIC1	Chloride intracellular channel protein 1;CLIC1;ortholog			
CN37	2',3'-cyclic-nucleotide 3'-phosphodiesterase;CNP;ortholog	phosphoric diester hydrolase activity	cyclic nucleotide metabolic process	
CNDP2	Cytosolic non-specific dipeptidase;CNDP2;ortholog	metallopeptidase activity;deacetylase activity	cellular amino acid biosynthetic process;protein phosphorylation;proteolysis	
CNOT3	CCR4-NOT transcription complex subunit 3;CNOT3;ortholog			
CO5A2	Collagen alpha-2(V) chain;COL5A2;ortholog	receptor activity;extracellular matrix structural constituent;transmembrane transporter activity	macrophage activation;cell communication;cell-cell adhesion;blood circulation;ectoderm development;mesoderm development;cellular component morphogenesis;response to stimulus;intracellular protein transport;receptor-mediated endocytosis;regulation of liquid surface tension;cellular component organization	extracellular region;extracellular matrix
COR1C	Coronin-1C;CORO1C;ortholog	actin binding	cellular process;cytoskeleton organization	cytoskeleton;intracellular

CPNE 3	Copine-3;CPNE3;ortholog		intracellular protein transport	
CTNA 1	Catenin alpha-1;CTNNA1;ortholog	structural constituent of cytoskeleton;actin binding	cell communication;cell adhesion;cellular component morphogenesis;cellular component organization	intracellular
CUL2	Cullin-2;CUL2;ortholog	ubiquitin-protein ligase activity	proteolysis;cell cycle	
CYTM	Cystatin-M;CST6;ortholog	cysteine-type peptidase activity;protein binding;cysteine-type endopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
DDR1	Epithelial discoidin domain-containing receptor 1;DDR1;ortholog			
DDX5	Probable ATP-dependent RNA helicase DDX5;DDX5;ortholog	RNA helicase activity;translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	nucleobase-containing compound metabolic process;translation;regulation of translation	
DEK	Protein DEK;DEK;ortholog			
DESP	Desmoplakin;DSP;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement;cellular component morphogenesis;cellular component organization	actin cytoskeleton;intracellular
DLDH	Dihydrolipoyl dehydrogenase, mitochondrial;DLD;ortholog	oxidoreductase activity	respiratory electron transport chain;nitrogen compound metabolic process;ferredoxin metabolic process	
E41L1	Band 4.1-like protein 1;EPB41L1;ortholog			
EPCR	Endothelial protein C receptor;PROCR;ortholog	receptor activity;protein binding	immune system process;cell communication;blood coagulation	
ERAP 2	Endoplasmic reticulum aminopeptidase 2;ERAP2;ortholog	metallopeptidase activity	proteolysis	
F10A	Hsc70-interacting		protein folding;response to	

1	protein;ST13;ortholog		stress	
FHL2	Four and a half LIM domains protein 2;FHL2;ortholog	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity	mesoderm development;muscle organ development	
FRPD1	FERM and PDZ domain-containing protein 1;FRMPD1;ortholog			
FST	Follistatin;FST;ortholog	protein binding		
FUBP1	Far upstream element-binding protein 1;FUBP1;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell communication;neurological system process;induction of apoptosis;intracellular protein transport;nuclear transport	ribonucleoprotein complex
FUBP2	Far upstream element-binding protein 2;KHSRP;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell communication;neurological system process;induction of apoptosis;intracellular protein transport;nuclear transport	ribonucleoprotein complex
FZD6	Frizzled-6;FZD6;ortholog	guanylate cyclase activity;receptor activity;protein binding	reproduction;phosphate-containing compound metabolic process;nitrogen compound metabolic process;biosynthetic process;cyclic nucleotide	plasma membrane;intracellular;neuron projection

			metabolic process;cell communication;cell proliferation;single-multicellular organism process;cellular component morphogenesis;embryo development;cell differentiation;nervous system development;response to stimulus;regulation of nucleobase-containing compound metabolic process;regulation of phosphate metabolic process;regulation of catalytic activity;cellular component organization	
G3P	Glyceraldehyde-3-phosphate dehydrogenase;GAPDH;ortholog	oxidoreductase activity	glycolysis;glycolysis	
GLYG	Glycogenin-1;GYG1;ortholog	transferase activity, transferring glycosyl groups	glycogen metabolic process	
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2;GNAI2;ortholog			
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2;GNAI2;ortholog	pyrophosphatase activity;adenylate cyclase activity;receptor binding	phosphate-containing compound metabolic process;nitrogen compound metabolic process;catabolic process;biosynthetic process;cyclic nucleotide metabolic process;cell communication;response to stimulus;regulation of nucleobase-containing compound metabolic process;regulation of phosphate metabolic process;regulation of catalytic activity	plasma membrane;protein complex;intracellular
GRN	Granulins;GRN;ortholog			
H4	Histone H4;HIST1H4A;ortholog			

H4	Histone H4;HIST1H4A;ortholog			
HCFC1	Host cell factor 1;HCFC1;ortholog	nucleic acid binding;chromatin binding	spermatogenesis	
HEXB	Beta-hexosaminidase subunit beta;HEXB;ortholog	hydrolase activity, hydrolyzing N-glycosyl compounds	polysaccharide metabolic process;lipid metabolic process	
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;ortholog			
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;ortholog			
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;ortholog			
HSP74	Heat shock 70 kDa protein 4;HSPA4;ortholog		immune system process;protein folding;protein complex assembly;response to stress;protein complex biogenesis	
HSP7C	Heat shock cognate 71 kDa protein;HSPA8;ortholog		immune system process;protein folding;protein complex assembly;response to stress;protein complex biogenesis	
HSPB1	Heat shock protein beta-1;HSPB1;ortholog	structural molecule activity	immune system process;protein folding;muscle contraction;visual perception;sensory perception;response to stress	
HTSF1	HIV Tat-specific factor 1;HTATSF1;ortholog	catalytic activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions	
HXK2	Hexokinase-2;HK2;ortholog	carbohydrate kinase activity	generation of precursor metabolites and energy;phosphate-containing compound metabolic process;catabolic process;monosaccharide metabolic process;cellular	cytoplasm

			process;homeostatic process	
HYOU1	Hypoxia up-regulated protein 1;HYOU1;ortholog			
ICOSL	ICOS ligand;ICOSLG;ortholog		system development;cellular defense response	
IF2A	Eukaryotic translation initiation factor 2 subunit 1;EIF2S1;ortholog	translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	translation;regulation of translation	
IF4A2	Eukaryotic initiation factor 4A-II;EIF4A2;ortholog	RNA helicase activity;translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	nucleobase-containing compound metabolic process;translation;regulation of translation	
IMPA1	Inositol monophosphatase 1;IMPA1;ortholog	binding	sulfur compound metabolic process;phospholipid metabolic process;nucleobase-containing compound metabolic process;phospholipid metabolic process;cell communication	
INAR2	Interferon alpha/beta receptor 2;IFNAR2;ortholog	receptor activity	natural killer cell activation;macrophage activation;apoptotic process;cell communication;apoptotic process;cellular defense response;blood coagulation;negative regulation of apoptotic process	
INHBA	Inhibin beta A chain;INHBA;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
IPO7	Importin-7;IPO7;ortholog	GTPase activity;transmembrane transporter	metabolic process;intracellular protein transport;nuclear transport	

		activity;protein binding		
ITA2	Integrin alpha-2;ITGA2;ortholog		cellular process;cell adhesion	
K22E	Keratin, type II cytoskeletal 2 epidermal;KRT2;ortholog	structural constituent of cytoskeleton	cellular process;cellular component morphogenesis;cellular component organization	intermediate filament cytoskeleton;intracellular
KPYM	Pyruvate kinase isozymes M1/M2;PKM;ortholog			
LTBP4	Latent-transforming growth factor beta-binding protein 4;LTBP4;ortholog	extracellular matrix structural constituent;calcium ion binding;receptor binding;calmodulin binding;calcium-dependent phospholipid binding	cell communication;skeletal system development	extracellular region;extracellular matrix
LYPA2	Acyl-protein thioesterase 2;LYPLA2;ortholog	phospholipase activity	lipid metabolic process;protein lipidation;cell communication	
LYPD3	Ly6/PLAUR domain-containing protein 3;LYPD3;ortholog			
MCM2	DNA replication licensing factor MCM2;MCM2;ortholog	DNA helicase activity;hydrolase activity;nucleic acid binding	DNA replication;cell cycle	
MCM4	DNA replication licensing factor MCM4;MCM4;ortholog	nucleic acid binding	DNA replication;cell cycle	
MCTS1	Malignant T-cell-amplified sequence 1;MCTS1;ortholog	receptor activity	cell cycle	
METK2	S-adenosylmethionine synthase isoform type-2;MAT2A;ortholog	nucleotidyltransferase activity	cellular amino acid metabolic process	
MICB	MHC class I polypeptide-related sequence B;MICB;ortholog			
MMP	Stromelysin-	metallopeptidase	proteolysis	extracellular

10	2;MMP10;ortholog	activity		region;extracellular matrix
MMP 9	Matrix metalloproteinase-9;MMP9;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
MTN A	Methylthioribose-1-phosphate isomerase;MRI1;ortholog	isomerase activity	sulfur compound metabolic process;nitrogen compound metabolic process;biosynthetic process;cellular amino acid biosynthetic process;cellular process	intracellular
MVP	Major vault protein;MVP;ortholog	RNA binding		ribonucleoprotein complex
NEUR 1	Sialidase-1;NEU1;ortholog			
NMT1	Glycylpeptide N-tetradecanoyltransferase 1;NMT1;ortholog	transferase activity	protein lipidation	
NP1L 4	Nucleosome assembly protein 1-like 4;NAP1L4;ortholog	phosphatase activity;phosphatase activity;protein binding;phosphatase inhibitor activity;phosphatase regulator activity	apoptotic process;DNA replication;cell cycle;apoptotic process;regulation of catalytic activity;chromatin organization	
NRG1	Pro-neuregulin-1, membrane-bound isoform;NRG1;ortholog	growth factor activity	cell-cell signaling;ectoderm development;nervous system development	
OAF	Out at first protein homolog;OAF;ortholog			
OFUT 1	GDP-fucose protein O-fucosyltransferase 1;POFUT1;ortholog			
OSTP	Osteopontin;SPP1;ortholog	cytokine activity	immune system process;cellular process;cell adhesion;cellular component morphogenesis;cellular component organization	extracellular region;extracellular matrix
OXSR 1	Serine/threonine-protein kinase OSR1;OXSR1;ortholog			
PAI1	Plasminogen activator inhibitor	serine-type peptidase	proteolysis;regulation of biological process;regulation of	

	1;SERPINE1;ortholog	activity;peptidase inhibitor activity	catalytic activity	
PARK 7	Protein DJ-1;PARK7;ortholog	sequence-specific DNA binding transcription factor activity;cysteine-type peptidase activity;sequence-specific DNA binding transcription factor activity;RNA binding	transcription from RNA polymerase II promoter;proteolysis;response to stress;regulation of transcription from RNA polymerase II promoter	
PCBP 3	Poly(rC)-binding protein 3;PCBP3;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell cycle;cell communication;neurological system process;induction of apoptosis;intracellular protein transport;nuclear transport	ribonucleoprotein complex
PDC6I	Programmed cell death 6-interacting protein;PDCD6IP;ortholog		induction of apoptosis;induction of apoptosis	
PDIA3	Protein disulfide-isomerase A3;PDIA3;ortholog	protein disulfide isomerase activity	protein folding;cellular protein modification process	
PFD4	Prefoldin subunit 4;PFDN4;ortholog			
PFD5	Prefoldin subunit 5;PFDN5;ortholog			
PKN2	Serine/threonine-protein kinase N2;PKN2;ortholog	protein kinase activity;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	protein phosphorylation;cell communication	
PLD3	Phospholipase D3;PLD3;ortholog	phospholipase activity	lipid metabolic process	
PODX L	Podocalyxin;PODXL;ortholog			

PRIO	Major prion protein;PRNP;ortholog			
PRS4	26S protease regulatory subunit 4;PSMC1;ortholog	hydrolase activity	proteolysis	
PSA1	Proteasome subunit alpha type-1;PSMA1;ortholog	peptidase activity	proteolysis	
PSA4	Proteasome subunit alpha type-4;PSMA4;ortholog	peptidase activity	proteolysis	
PSB1	Proteasome subunit beta type-1;PSMB1;ortholog	peptidase activity	proteolysis	
PTN11	Tyrosine-protein phosphatase non-receptor type 11;PTPN11;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	cellular protein modification process	cytoplasm
PUR8	Adenylosuccinate lyase;ADSL;ortholog	lyase activity	purine nucleobase metabolic process;cellular amino acid metabolic process	
PVRL1	Poliovirus receptor-related protein 1;PVRL1;ortholog	receptor activity	cell communication;cell-cell adhesion	
PVRL2	Poliovirus receptor-related protein 2;PVRL2;ortholog	receptor activity	cell communication;cell-cell adhesion	
PVRL4	Poliovirus receptor-related protein 4;PVRL4;ortholog	receptor activity	cell communication;cell-cell adhesion	
PYR1	CAD protein;CAD;ortholog	transferase activity;ligase activity	nitrogen compound metabolic process;pyrimidine nucleobase metabolic process;cellular amino acid biosynthetic process	
PZP	Pregnancy zone protein;PZP;ortholog	peptidase activity;cytokine activity;serine-type endopeptidase inhibitor activity	complement activation;proteolysis;cellular process;response to stimulus;regulation of catalytic activity	
RAB13	Ras-related protein Rab-13;RAB13;ortholog			
RHOA	Transforming protein RhoA;RHOA;ortholog	GTPase activity;protein binding	metabolic process;cell communication;intracellular protein transport;receptor-mediated endocytosis	

RIC8A	Synembryn-A;RIC8A;ortholog			
RL19	60S ribosomal protein L19;RPL19;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RL23A	60S ribosomal protein L23a;RPL23A;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RLA2	60S acidic ribosomal protein P2;RPLP2;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RNT2	Ribonuclease T2;RNASET2;ortholog	endoribonuclease activity;nucleic acid binding	RNA catabolic process	
ROAA	Heterogeneous nuclear ribonucleoprotein A/B;HNRNPAB;ortholog	catalytic activity;structural constituent of ribosome;poly(A) RNA binding	DNA replication;RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;mRNA polyadenylation;RNA splicing, via transesterification reactions;rRNA metabolic process;protein metabolic process;cell cycle;neurological system process;ectoderm development;nervous system development	ribonucleoprotein complex
RPN1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1;RPN1;ortholog	transferase activity, transferring glycosyl groups	translation;protein glycosylation	
RS27A	Ubiquitin-40S ribosomal protein S27a;RPS27A;ortholog	structural constituent of ribosome;nucleic acid binding	proteolysis	
RS9	40S ribosomal protein S9;RPS9;ortholog			
RSMB	Small nuclear ribonucleoprotein-associated proteins B and B';SNRPB;ortholog	catalytic activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions	
S10A6	Protein S100-A6;S100A6;ortholog	calcium ion binding;growth	macrophage activation;DNA replication;cell cycle;cell	

		factor activity;calmodulin binding	communication	
S30BP	SAP30-binding protein;SAP30BP;ortholog	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity	transcription from RNA polymerase II promoter	
SAFB1	Scaffold attachment factor B1;SAFB;ortholog			
SC23A	Protein transport protein Sec23A;SEC23A;ortholog	catalytic activity;protein binding;small GTPase regulator activity	metabolic process;cell communication;intracellular protein transport;exocytosis;regulation of catalytic activity	
SDC1	Syndecan-1;SDC1;ortholog	structural constituent of cytoskeleton;receptor binding	macrophage activation;cell communication;cell adhesion;skeletal system development	extracellular region;cytoskeleton;intracellular
SEM4C	Semaphorin-4C;SEMA4C;ortholog	receptor binding	immune system process;cell communication;neurological system process;ectoderm development;mesoderm development;angiogenesis;nervous system development;heart development	
SEM5A	Semaphorin-5A;SEMA5A;ortholog	receptor binding	immune system process;cell communication;neurological system process;ectoderm development;mesoderm development;angiogenesis;nervous system development;heart development	
SK2L2	Superkiller viralicidic activity 2-like 2;SKIV2L2;ortholog	DNA helicase activity;RNA helicase activity;hydrolase activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;cell cycle	
SLIT1	Slit homolog 1 protein;SLIT1;ortholog	receptor activity	immune system process;transcription from RNA polymerase II	extracellular region;extracellular matrix

			promoter;protein phosphorylation;cellular component movement;cell-cell signaling;cell-cell adhesion;sensory perception;ectoderm development;nervous system development;regulation of transcription from RNA polymerase II promoter	
SMD2	Small nuclear ribonucleoprotein Sm D2;SNRPD2;ortholog	mRNA binding	mRNA processing	
SMRC2	SWI/SNF complex subunit SMARCC2;SMARCC2;ortholog	transcription cofactor activity;sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity;chromatin binding;protein binding	transcription from RNA polymerase II promoter;regulation of transcription from RNA polymerase II promoter	
SNAA	Alpha-soluble NSF attachment protein;NAPA;ortholog		intracellular protein transport	
SPIT1	Kunitz-type protease inhibitor 1;SPINT1;ortholog	peptidase activity;protein binding;serine-type endopeptidase inhibitor activity	immune system process;proteolysis;blood coagulation;regulation of catalytic activity	
SPSY	Spermine synthase;SMS;ortholog	transferase activity	metabolic process	
SRRM2	Serine/arginine repetitive matrix protein 2;SRRM2;ortholog			
STK24	Serine/threonine-protein kinase 24;STK24;ortholog			

SYK	Lysine--tRNA ligase;KARS;ortholog	aminoacyl-tRNA ligase activity;RNA binding	translation	
TB182	182 kDa tankyrase-1-binding protein;TNKS1BP1;ortholog		mitosis;chromosome segregation	
TBB5	Tubulin beta chain;TUBB;ortholog			
TBB5	Tubulin beta chain;TUBB;ortholog			
TBB5	Tubulin beta chain;TUBB;ortholog			
TBL1R	F-box-like/WD repeat-containing protein TBL1XR1;TBL1XR1;ortholog			
TCP4	Activated RNA polymerase II transcriptional coactivator p15;SUB1;ortholog			
TCPE	T-complex protein 1 subunit epsilon;CCT5;ortholog		protein folding	
TGFB1	Transforming growth factor beta-1;TGFB1;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
TIMP1	Metalloproteinase inhibitor 1;TIMP1;ortholog	peptidase activity;protein binding;metalloendopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
TPA	Tissue-type plasminogen activator;PLAT;ortholog	serine-type peptidase activity	proteolysis;blood coagulation	extracellular region
TPD52	Tumor protein D52;TPD52;ortholog			
TSN1	Tetraspanin-1;TSPAN1;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system	

			process;blood coagulation	
TTL12	Tubulin--tyrosine ligase-like protein 12;TTLL12;ortholog	ligase activity;structural constituent of cytoskeleton	protein metabolic process	microtubule;cytoskeleton;intracellular
TXD17	Thioredoxin domain-containing protein 17;TXNDC17;ortholog	transmembrane transporter activity	respiratory electron transport chain;transport	
U520	U5 small nuclear ribonucleoprotein 200 kDa helicase;SNRNP200;ortholog	DNA helicase activity;RNA helicase activity;hydrolase activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;meiosis	
UBQL1	Ubiquilin-1;UBQLN1;ortholog		proteolysis	
UN45A	Protein unc-45 homolog A;UNC45A;ortholog		immune system process;protein folding;response to stress	
VAS1	V-type proton ATPase subunit S1;ATP6AP1;ortholog	hydrolase activity;cation transmembrane transporter activity;proton-transporting ATP synthase activity, rotational mechanism	nucleobase-containing compound metabolic process;cation transport	proton-transporting ATP synthase complex;intracellular
XPO1	Exportin-1;XPO1;ortholog	receptor activity	transcription from RNA polymerase II promoter;mRNA transcription;mRNA transcription;protein metabolic process;intracellular protein transport;nucleobase-containing compound transport	
XPOT	Exportin-T;XPOT;ortholog	RNA binding	tRNA metabolic process	
ZN207	Zinc finger protein 207;ZNF207;ortholog	DNA binding	nucleobase-containing compound metabolic process	

Appendix C

Proteins down-regulated by Marimastat half-fold. Their cellular location, biological function and molecular function have been determined using Panther database (http://www.pantherdb.org/)

Accession Name	Name	Molecular function	Biological function	Cellular component
1433S	14-3-3 protein sigma;SFN;ortholog		cell cycle;cell communication	
2A5G	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit gamma isoform;PPP2R5C;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	protein phosphorylation;cell communication	
2AAA	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform;PPP2R1A;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	metabolic process	
ABCF1	ATP-binding cassette sub-family F member 1;ABCF1;ortholog			
ACLY	ATP-citrate synthase;ACLY;ortholog			
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9;ADAM9;ortholog		fertilization;apoptotic process;neurological system process;mesoderm development;apoptotic process;heart development	
AGRIN	Agrin;AGRN;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development	extracellular region;extracellular matrix
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog	catalytic activity;protein binding;enzyme	immune system process;protein folding;response to	

	1;AHSA1;ortholog	activator activity	stress;regulation of catalytic activity	
AIP	AH receptor-interacting protein;AIP;ortholog		visual perception;sensory perception	
ANXA1	Annexin A1;ANXA1;ortholog		fatty acid metabolic process;cell communication	
ARPC4	Actin-related protein 2/3 complex subunit 4;ARPC4;ortholog	structural constituent of cytoskeleton	cellular component movement;cellular component organization	actin cytoskeleton;intracellular
BLVRB	Flavin reductase (NADPH);BLVRB;ortholog	oxidoreductase activity	metabolic process	
BROX	BRO1 domain-containing protein BROX;BROX;ortholog			
C1R	Complement C1r subcomponent;C1R;ortholog	serine-type peptidase activity;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	gamete generation;complement activation;proteolysis;cellular process;blood coagulation	
CADH2	Cadherin-2;CDH2;ortholog	binding	cellular process;visual perception;sensory perception of sound;mesoderm development;nervous system development;heart development;muscle organ development	plasma membrane;cell part
CADH3	Cadherin-3;CDH3;ortholog	binding	cellular process;visual perception;sensory perception of sound;mesoderm development;nervous system development;heart development;muscle organ development	plasma membrane;cell part
CALX	Calnexin;CANX;ortholog	calcium ion binding	protein folding;intracellular protein transport;exocytosis	
CAPG	Macrophage-capping	structural	cellular process;cellular	actin

	protein;CAPG;ortholog	constituent of cytoskeleton;calcium ion binding;actin binding	component morphogenesis;cellular component organization	cytoskeleton;intracellular
CATL2	Cathepsin L2;CTSL2;ortholog	cysteine-type peptidase activity	proteolysis;cell adhesion	
CAYP1	Calcyphosin;CAPS;ortholog	catalytic activity;calcium ion binding;receptor binding;calmodulin binding;enzyme regulator activity	metabolic process;cell cycle;cell communication;cation transport;regulation of catalytic activity	
CEL	Bile salt-activated lipase;CEL;ortholog	lipase activity	metabolic process	extracellular region;cytoplasm
CFAB	Complement factor B;CFB;ortholog			
CG050	Uncharacterized protein C7orf50;C7orf50;ortholog			
CHSTB	Carbohydrate sulfotransferase 11;CHST11;ortholog	transferase activity	sulfur compound metabolic process	
CLIC1	Chloride intracellular channel protein 1;CLIC1;ortholog			
CLIC1	Chloride intracellular channel protein 1;CLIC1;ortholog			
CN37	2',3'-cyclic-nucleotide 3'-phosphodiesterase;CNP;ortholog	phosphoric diester hydrolase activity	cyclic nucleotide metabolic process	
CNDP2	Cytosolic non-specific dipeptidase;CNDP2;ortholog	metallopeptidase activity;deacetylase activity	cellular amino acid biosynthetic process;protein phosphorylation;proteolysis	
CNOT3	CCR4-NOT transcription complex subunit 3;CNOT3;ortholog			
CO5A2	Collagen alpha-2(V) chain;COL5A2;ortholog	receptor activity;extracellular matrix structural constituent;transmembrane transporter activity	macrophage activation;cell communication;cell-cell adhesion;blood circulation;ectoderm development;mesoderm development;cellular	extracellular region;extracellular matrix

			component morphogenesis;response to stimulus;intracellular protein transport;receptor-mediated endocytosis;regulation of liquid surface tension;cellular component organization	
COR1C	Coronin-1C;CORO1C;ortholog	actin binding	cellular process;cytoskeleton organization	cytoskeleton;intracellular
CPNE3	Copine-3;CPNE3;ortholog		intracellular protein transport	
CTNA1	Catenin alpha-1;CTNNA1;ortholog	structural constituent of cytoskeleton;actin binding	cell communication;cell adhesion;cellular component morphogenesis;cellular component organization	intracellular
CUL2	Cullin-2;CUL2;ortholog	ubiquitin-protein ligase activity	proteolysis;cell cycle	
CYTM	Cystatin-M;CST6;ortholog	cysteine-type peptidase activity;protein binding;cysteine-type endopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
DDR1	Epithelial discoidin domain-containing receptor 1;DDR1;ortholog			
DDX5	Probable ATP-dependent RNA helicase DDX5;DDX5;ortholog	RNA helicase activity;translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	nucleobase-containing compound metabolic process;translation;regulation of translation	
DEK	Protein DEK;DEK;ortholog			
DESP	Desmoplakin;DSP;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement;cellular component morphogenesis;cellular	actin cytoskeleton;intracellular

			component organization	
DLDH	Dihydrolipoyl dehydrogenase, mitochondrial;DLD;ortholog	oxidoreductase activity	respiratory electron transport chain;nitrogen compound metabolic process;ferredoxin metabolic process	
E41L1	Band 4.1-like protein 1;EPB41L1;ortholog			
EPCR	Endothelial protein C receptor;PROCR;ortholog	receptor activity;protein binding	immune system process;cell communication;blood coagulation	
ERAP2	Endoplasmic reticulum aminopeptidase 2;ERAP2;ortholog	metallopeptidase activity	proteolysis	
F10A1	Hsc70-interacting protein;ST13;ortholog		protein folding;response to stress	
FHL2	Four and a half LIM domains protein 2;FHL2;ortholog	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity	mesoderm development;muscle organ development	
FRPD1	FERM and PDZ domain-containing protein 1;FRMPD1;ortholog			
FST	Follistatin;FST;ortholog	protein binding		
FUBP1	Far upstream element-binding protein 1;FUBP1;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell communication;neurological system process;induction of	ribonucleoprotein complex

			apoptosis;intracellular protein transport;nuclear transport	
FUBP2	Far upstream element-binding protein 2;KHSRP;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell communication;neurological system process;induction of apoptosis;intracellular protein transport;nuclear transport	ribonucleoprotein complex
FZD6	Frizzled-6;FZD6;ortholog	guanylate cyclase activity;receptor activity;protein binding	reproduction;phosphate-containing compound metabolic process;nitrogen compound metabolic process;biosynthetic process;cyclic nucleotide metabolic process;cell communication;cell proliferation;single-multicellular organism process;cellular component morphogenesis;embryo development;cell differentiation;nervous system development;response to stimulus;regulation of nucleobase-containing compound metabolic process;regulation of phosphate metabolic process;regulation of	plasma membrane;intracellular;neuron projection

			catalytic activity;cellular component organization	
G3P	Glyceraldehyde-3-phosphate dehydrogenase;GAPDH;ortholog	oxidoreductase activity	glycolysis;glycolysis	
GLYG	Glycogenin-1;GYG1;ortholog	transferase activity, transferring glycosyl groups	glycogen metabolic process	
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2;GNAI2;ortholog			
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2;GNAI2;ortholog	pyrophosphatase activity;adenylate cyclase activity;receptor binding	phosphate-containing compound metabolic process;nitrogen compound metabolic process;catabolic process;biosynthetic process;cyclic nucleotide metabolic process;cell communication;response to stimulus;regulation of nucleobase-containing compound metabolic process;regulation of phosphate metabolic process;regulation of catalytic activity	plasma membrane;protein complex;intracellular
GRN	Granulins;GRN;ortholog			
H4	Histone H4;HIST1H4A;ortholog			
H4	Histone H4;HIST1H4A;ortholog			
HCFC1	Host cell factor 1;HCFC1;ortholog	nucleic acid binding;chromatin binding	spermatogenesis	
HEXB	Beta-hexosaminidase subunit beta;HEXB;ortholog	hydrolase activity, hydrolyzing N-glycosyl compounds	polysaccharide metabolic process;lipid metabolic process	
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;orthol			

	og			
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;ortholog			
HSP71	Heat shock 70 kDa protein 1A/1B;HSPA1A;ortholog			
HSP74	Heat shock 70 kDa protein 4;HSPA4;ortholog		immune system process;protein folding;protein complex assembly;response to stress;protein complex biogenesis	
HSP7C	Heat shock cognate 71 kDa protein;HSPA8;ortholog		immune system process;protein folding;protein complex assembly;response to stress;protein complex biogenesis	
HSPB1	Heat shock protein beta-1;HSPB1;ortholog	structural molecule activity	immune system process;protein folding;muscle contraction;visual perception;sensory perception;response to stress	
HTSF1	HIV Tat-specific factor 1;HTATSF1;ortholog	catalytic activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions	
HXK2	Hexokinase-2;HK2;ortholog	carbohydrate kinase activity	generation of precursor metabolites and energy;phosphate-containing compound metabolic process;catabolic process;monosaccharide metabolic process;cellular process;homeostatic process	cytoplasm
HYOU1	Hypoxia up-regulated protein			

	1;HYOU1;ortholog			
ICOSL	ICOS ligand;ICOSLG;ortholog		system development;cellular defense response	
IF2A	Eukaryotic translation initiation factor 2 subunit 1;EIF2S1;ortholog	translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	translation;regulation of translation	
IF4A2	Eukaryotic initiation factor 4A-II;EIF4A2;ortholog	RNA helicase activity;translation initiation factor activity;translation initiation factor activity;translation initiation factor activity	nucleobase-containing compound metabolic process;translation;regulation of translation	
IMPA1	Inositol monophosphatase 1;IMPA1;ortholog	binding	sulfur compound metabolic process;phospholipid metabolic process;nucleobase-containing compound metabolic process;phospholipid metabolic process;cell communication	
INAR2	Interferon alpha/beta receptor 2;IFNAR2;ortholog	receptor activity	natural killer cell activation;macrophage activation;apoptotic process;cell communication;apoptotic process;cellular defense response;blood coagulation;negative regulation of apoptotic process	
INHBA	Inhibin beta A chain;INHBA;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	

IPO7	Importin-7;IPO7;ortholog	GTPase activity;transmembrane transporter activity;protein binding	metabolic process;intracellular protein transport;nuclear transport	
ITA2	Integrin alpha-2;ITGA2;ortholog		cellular process;cell adhesion	
K22E	Keratin, type II cytoskeletal 2 epidermal;KRT2;ortholog	structural constituent of cytoskeleton	cellular process;cellular component morphogenesis;cellular component organization	intermediate filament cytoskeleton;intracellular
KPYM	Pyruvate kinase isozymes M1/M2;PKM;ortholog			
LTBP4	Latent-transforming growth factor beta-binding protein 4;LTBP4;ortholog	extracellular matrix structural constituent;calcium ion binding;receptor binding;calmodulin binding;calcium-dependent phospholipid binding	cell communication;skeletal system development	extracellular region;extracellular matrix
LYPA2	Acyl-protein thioesterase 2;LYPLA2;ortholog	phospholipase activity	lipid metabolic process;protein lipidation;cell communication	
LYPD3	Ly6/PLAUR domain-containing protein 3;LYPD3;ortholog			
MCM2	DNA replication licensing factor MCM2;MCM2;ortholog	DNA helicase activity;hydrolase activity;nucleic acid binding	DNA replication;cell cycle	
MCM4	DNA replication licensing factor MCM4;MCM4;ortholog	nucleic acid binding	DNA replication;cell cycle	
MCTS1	Malignant T-cell-amplified sequence 1;MCTS1;ortholog	receptor activity	cell cycle	
METK2	S-adenosylmethionine synthase isoform type-2;MAT2A;ortholog	nucleotidyltransferase activity	cellular amino acid metabolic process	
MICB	MHC class I polypeptide-related sequence			

	B;MICB;ortholog			
MMP10	Stromelysin-2;MMP10;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
MMP9	Matrix metalloproteinase-9;MMP9;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
MTNA	Methylthioribose-1-phosphate isomerase;MRI1;ortholog	isomerase activity	sulfur compound metabolic process;nitrogen compound metabolic process;biosynthetic process;cellular amino acid biosynthetic process;cellular process	intracellular
MVP	Major vault protein;MVP;ortholog	RNA binding		ribonucleoprotein complex
NEUR1	Sialidase-1;NEU1;ortholog			
NMT1	Glycylpeptide N-tetradecanoyltransferase 1;NMT1;ortholog	transferase activity	protein lipidation	
NP1L4	Nucleosome assembly protein 1-like 4;NAP1L4;ortholog	phosphatase activity;phosphatase activity;protein binding;phosphatase inhibitor activity;phosphatase regulator activity	apoptotic process;DNA replication;cell cycle;apoptotic process;regulation of catalytic activity;chromatin organization	
NRG1	Pro-neuregulin-1, membrane-bound isoform;NRG1;ortholog	growth factor activity	cell-cell signaling;ectoderm development;nervous system development	
OAF	Out at first protein homolog;OAF;ortholog			
OFUT1	GDP-fucose protein O-fucosyltransferase 1;POFUT1;ortholog			
OSTP	Osteopontin;SPP1;ortholog	cytokine activity	immune system process;cellular process;cell adhesion;cellular component morphogenesis;cellular component organization	extracellular region;extracellular matrix
OXSR1	Serine/threonine-protein kinase			

	OSR1;OXSR1;ortholog			
PAI1	Plasminogen activator inhibitor 1;SERPINE1;ortholog	serine-type peptidase activity;peptidase inhibitor activity	proteolysis;regulation of biological process;regulation of catalytic activity	
PARK7	Protein DJ-1;PARK7;ortholog	sequence-specific DNA binding transcription factor activity;cysteine-type peptidase activity;sequence-specific DNA binding transcription factor activity;RNA binding	transcription from RNA polymerase II promoter;proteolysis;response to stress;regulation of transcription from RNA polymerase II promoter	
PCBP3	Poly(rC)-binding protein 3;PCBP3;ortholog	catalytic activity;mRNA binding;protein binding	induction of apoptosis;RNA splicing, via transesterification reactions;transcription from RNA polymerase II promoter;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;protein metabolic process;cell cycle;cell communication;neurological system process;induction of apoptosis;intracellular protein transport;nuclear transport	ribonucleoprotein complex
PDC6I	Programmed cell death 6-interacting protein;PDCD6IP;ortholog		induction of apoptosis;induction of apoptosis	
PDIA3	Protein disulfide-isomerase A3;PDIA3;ortholog	protein disulfide isomerase activity	protein folding;cellular protein modification process	
PFD4	Prefoldin subunit 4;PFDN4;ortholog			
PFD5	Prefoldin subunit 5;PFDN5;ortholog			
PKN2	Serine/threonine-protein kinase N2;PKN2;ortholog	protein kinase activity;calcium ion binding;calmodulin binding;calcium-	protein phosphorylation;cell communication	

		dependent phospholipid binding		
PLD3	Phospholipase D3;PLD3;ortholog	phospholipase activity	lipid metabolic process	
PODXL	Podocalyxin;PODXL;ortholog			
PRIO	Major prion protein;PRNP;ortholog			
PRS4	26S protease regulatory subunit 4;PSMC1;ortholog	hydrolase activity	proteolysis	
PSA1	Proteasome subunit alpha type-1;PSMA1;ortholog	peptidase activity	proteolysis	
PSA4	Proteasome subunit alpha type-4;PSMA4;ortholog	peptidase activity	proteolysis	
PSB1	Proteasome subunit beta type-1;PSMB1;ortholog	peptidase activity	proteolysis	
PTN11	Tyrosine-protein phosphatase non-receptor type 11;PTPN11;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity	cellular protein modification process	cytoplasm
PUR8	Adenylosuccinate lyase;ADSL;ortholog	lyase activity	purine nucleobase metabolic process;cellular amino acid metabolic process	
PVRL1	Poliovirus receptor-related protein 1;PVRL1;ortholog	receptor activity	cell communication;cell-cell adhesion	
PVRL2	Poliovirus receptor-related protein 2;PVRL2;ortholog	receptor activity	cell communication;cell-cell adhesion	
PVRL4	Poliovirus receptor-related protein 4;PVRL4;ortholog	receptor activity	cell communication;cell-cell adhesion	
PYR1	CAD protein;CAD;ortholog	transferase activity;ligase activity	nitrogen compound metabolic process;pyrimidine nucleobase metabolic process;cellular amino acid biosynthetic process	
PZP	Pregnancy zone protein;PZP;ortholog	peptidase activity;cytokine	complement activation;proteolysis;c	

		activity;serine-type endopeptidase inhibitor activity	ellular process;response to stimulus;regulation of catalytic activity	
RAB13	Ras-related protein Rab-13;RAB13;ortholog			
RHOA	Transforming protein RhoA;RHOA;ortholog	GTPase activity;protein binding	metabolic process;cell communication;intracellular protein transport;receptor-mediated endocytosis	
RIC8A	Synembryn-A;RIC8A;ortholog			
RL19	60S ribosomal protein L19;RPL19;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RL23A	60S ribosomal protein L23a;RPL23A;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RLA2	60S acidic ribosomal protein P2;RPLP2;ortholog	structural constituent of ribosome;nucleic acid binding	translation	
RNT2	Ribonuclease T2;RNASET2;ortholog	endoribonuclease activity;nucleic acid binding	RNA catabolic process	
ROAA	Heterogeneous nuclear ribonucleoprotein A/B;HNRNPAB;ortholog	catalytic activity;structural constituent of ribosome;poly(A) RNA binding	DNA replication;RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;mRNA polyadenylation;RNA splicing, via transesterification reactions;rRNA metabolic process;protein metabolic process;cell cycle;neurological system process;ectoderm development;nervous system development	ribonucleoprotein complex
RPN1	Dolichyl-diphosphooligosaccha	transferase activity, transferring glycosyl	translation;protein glycosylation	

	ride--protein glycosyltransferase subunit 1;RPN1;ortholog	groups		
RS27A	Ubiquitin-40S ribosomal protein S27a;RPS27A;ortholog	structural constituent of ribosome;nucleic acid binding	proteolysis	
RS9	40S ribosomal protein S9;RPS9;ortholog			
RSMB	Small nuclear ribonucleoprotein-associated proteins B and B';SNRPB;ortholog	catalytic activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions	
S10A6	Protein S100-A6;S100A6;ortholog	calcium ion binding;growth factor activity;calmodulin binding	macrophage activation;DNA replication;cell cycle;cell communication	
S30BP	SAP30-binding protein;SAP30BP;ortholog	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity	transcription from RNA polymerase II promoter	
SAFB1	Scaffold attachment factor B1;SAFB;ortholog			
SC23A	Protein transport protein Sec23A;SEC23A;ortholog	catalytic activity;protein binding;small GTPase regulator activity	metabolic process;cell communication;intracellular protein transport;exocytosis;regulation of catalytic activity	
SDC1	Syndecan-1;SDC1;ortholog	structural constituent of cytoskeleton;receptor binding	macrophage activation;cell communication;cell adhesion;skeletal system development	extracellular region;cytoskeleton;intracellular
SEM4C	Semaphorin-4C;SEMA4C;ortholog	receptor binding	immune system process;cell communication;neurological system process;ectoderm	

			development;mesoderm development;angiogenesis;nervous system development;heart development	
SEM5A	Semaphorin-5A;SEMA5A;ortholog	receptor binding	immune system process;cell communication;neurological system process;ectoderm development;mesoderm development;angiogenesis;nervous system development;heart development	
SK2L2	Superkiller viralicidic activity 2-like 2;SKIV2L2;ortholog	DNA helicase activity;RNA helicase activity;hydrolase activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;cell cycle	
SLIT1	Slit homolog 1 protein;SLIT1;ortholog	receptor activity	immune system process;transcription from RNA polymerase II promoter;protein phosphorylation;cellular component movement;cell-cell signaling;cell-cell adhesion;sensory perception;ectoderm development;nervous system development;regulation of transcription from RNA polymerase II promoter	extracellular region;extracellular matrix
SMD2	Small nuclear ribonucleoprotein Sm D2;SNRPD2;ortholog	mRNA binding	mRNA processing	
SMRC2	SWI/SNF complex subunit SMARCC2;SMARCC2;ortholog	transcription cofactor activity;sequence-specific DNA binding transcription factor	transcription from RNA polymerase II promoter;regulation of transcription from RNA polymerase II promoter	

		activity;sequence-specific DNA binding transcription factor activity;chromatin binding;protein binding		
SNAA	Alpha-soluble NSF attachment protein;NAPA;ortholog		intracellular protein transport	
SPIT1	Kunitz-type protease inhibitor 1;SPINT1;ortholog	peptidase activity;protein binding;serine-type endopeptidase inhibitor activity	immune system process;proteolysis;blood coagulation;regulation of catalytic activity	
SPSY	Spermine synthase;SMS;ortholog	transferase activity	metabolic process	
SRRM2	Serine/arginine repetitive matrix protein 2;SRRM2;ortholog			
STK24	Serine/threonine-protein kinase 24;STK24;ortholog			
SYK	Lysine--tRNA ligase;KARS;ortholog	aminoacyl-tRNA ligase activity;RNA binding	translation	
TB182	182 kDa tankyrase-1-binding protein;TNKS1BP1;ortholog		mitosis;chromosome segregation	
TBB5	Tubulin beta chain;TUBB;ortholog			
TBB5	Tubulin beta chain;TUBB;ortholog			
TBB5	Tubulin beta chain;TUBB;ortholog			
TBL1R	F-box-like/WD repeat-containing protein TBL1XR1;TBL1XR1;ortholog			
TCP4	Activated RNA polymerase II transcriptional coactivator p15;SUB1;ortholog			
TCPE	T-complex protein 1 subunit		protein folding	

	epsilon;CCT5;ortholog			
TGFB1	Transforming growth factor beta-1;TGFB1;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
TIMP1	Metalloproteinase inhibitor 1;TIMP1;ortholog	peptidase activity;protein binding;metalloendopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
TPA	Tissue-type plasminogen activator;PLAT;ortholog	serine-type peptidase activity	proteolysis;blood coagulation	extracellular region
TPD52	Tumor protein D52;TPD52;ortholog			
TSN1	Tetraspanin-1;TSPAN1;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system process;blood coagulation	
TTL12	Tubulin--tyrosine ligase-like protein 12;TTLL12;ortholog	ligase activity;structural constituent of cytoskeleton	protein metabolic process	microtubule;cytoskeleton;intracellular
TXD17	Thioredoxin domain-containing protein 17;TXNDC17;ortholog	transmembrane transporter activity	respiratory electron transport chain;transport	
U520	U5 small nuclear ribonucleoprotein 200 kDa helicase;SNRNP200;ortholog	DNA helicase activity;RNA helicase activity;hydrolase activity;mRNA binding	RNA splicing, via transesterification reactions;mRNA splicing, via spliceosome;RNA splicing, via transesterification reactions;meiosis	
UBQL1	Ubiquilin-1;UBQLN1;ortholog		proteolysis	
UN45A	Protein unc-45 homolog A;UNC45A;ortholog		immune system process;protein folding;response to	

			stress	
VAS1	V-type proton ATPase subunit S1;ATP6AP1;ortholog	hydrolase activity;cation transmembrane transporter activity;proton-transporting ATP synthase activity, rotational mechanism	nucleobase-containing compound metabolic process;cation transport	proton-transporting ATP synthase complex;intracellular
XPO1	Exportin-1;XPO1;ortholog	receptor activity	transcription from RNA polymerase II promoter;mRNA transcription;mRNA transcription;protein metabolic process;intracellular protein transport;nucleobase-containing compound transport	
XPOT	Exportin-T;XPOT;ortholog	RNA binding	tRNA metabolic process	
ZN207	Zinc finger protein 207;ZNF207;ortholog	DNA binding	nucleobase-containing compound metabolic process	
1433F	14-3-3 protein eta;YWHAH;ortholog		cell cycle;cell communication	

Appendix D

The mean, standard deviation and T-test value of the Marimastat MTT data.

Conditions	Name of Cell	Mean	SD	t test
Serum-Free (Control)	SW780	0.1535	0.193	
Serum-Free (5µM M)		0.1548	0.1216	0.991
Fetal Calf Serum (control)		0.1493	0.0858	
Fetal Calf Serum (5µM M)		0.1553	0.0377	0.9193
Serum-Free (Control)	VMCUB	0.501	0.2499	
Serum-Free (5µM M)		1.4876	0.1484	0.0215
Fetal Calf Serum (control)		1.0644	0.2031	
Fetal Calf Serum (5µM M)		1.0243	0.1679	0.8616
Serum-Free (Control)	UROTsa	0.314	0.0628	
Serum-Free (5µM M)		0.302	0.012	0.8282
Fetal Calf Serum (control)		0.334	0.0913	
Fetal Calf Serum (5µM M)		0.228	0.3202	0.7221
Serum-Free (Control)	T 24	0.2333	0.2203	
Serum-Free (5µM M)		0.1338	0.0797	0.6664
Fetal Calf Serum (control)		0.2282	0.2414	
Fetal Calf Serum (5µM M)		0.1142	0.0777	0.5058
Serum-Free (Control)	5637	0.606	0.0326	
Serum-Free (5µM M)		0.545	0.0242	0.0009
Fetal Calf Serum (control)		0.883	0.0496	
Fetal Calf Serum (5µM M)		0.771	0.0726	0.0033
Serum-Free (Control)	rt112m	0.331	0.0401	
Serum-Free (5µM M)		0.291	0.0305	0.0417
Fetal Calf Serum (control)		0.662	0.0273	
Fetal Calf Serum (5µM M)		0.404	0.0234	######
Serum-Free (Control)	HBCLS2	0.126	0.0167	0.3442
Serum-Free (5µM M)		0.1148	0.022	
Fetal Calf Serum (control)		0.1275	0.0181	0.8861
Fetal Calf Serum (5µM M)		0.1145	0.0275	

Appendix E

The mean, standard deviation and T-test value of the PMA-MTT data.

Name of cell line	Condition	Mean	SD	t test
MGUH	Serum free	0.1105	0.01587	0.00531
	Serum free and PMA	0.15359	0.02755	
	Fetal calf serum	0.12549	0.03105	0.05452
	Fetal calf serum and PMA	0.18542	0.06425	
RT4	Serum free	0.3482	0.05571	0.06568
	Serum free and PMA	0.48316	0.15617	
	Fetal calf serum	0.4343	0.06697	0.00352
	Fetal calf serum and PMA	0.66687	0.13959	
SW780	Serum free	0.4893	0.05043	2.39E-06
	Serum free and PMA	0.85758	0.09996	
	Fetal calf serum	0.48466	0.01284	3.33E-08
	Fetal calf serum and PMA	0.86443	0.04992	
RT112M	Serum free	0.46599	0.14541	0.00089
	Serum free and PMA	0.78975	0.12919	
	Fetal calf serum	0.71077	0.1012	0.45959
	Fetal calf serum and PMA	0.74518	0.06129	
VMCUB	Serum free	0.01625	0.00279	1.45E-08
	Serum free and PMA	0.07446	0.00784	
	Fetal calf serum	0.15141	0.05432	0.6627
	Fetal calf serum and PMA	0.16341	0.05339	
T24	Serum free	0.19921	0.19921	2.39E-06
	Serum free and PMA	0.34308	0.34308	
	Fetal calf serum	0.51463	0.06394	0.74488
	Fetal calf serum and PMA	0.55825	0.08953	
HB-CLS-2	Serum free	0.126	0.01669	0.39734
HB-CLS-2	Serum free and PMA	0.133	0.00772	
	Fetal calf serum	0.127	0.01814	0.09751
	Fetal calf serum and PMA	0.20753	0.096121754	

Appendix F

The 83 proteins that were present in both HPA and PMA proteomic data. The Panther database (http://www.pantherdb.org/) was used to determine their molecular function, biological function and cellular component.

Name of protein	Molecular function	Biological function	Cellular component
ATP-citrate synthase;ACLY;ortholog			
Disintegrin and metalloproteinase domain-containing protein 9;ADAM9;ortholog		fertilization;apoptotic process;neurological system process;mesoderm development;apoptotic process;heart development	
Annexin A1;ANXA1;ortholog		fatty acid metabolic process;cell communication	
Caveolin-1;CAV1;ortholog	catalytic activity;structural molecule activity;protein binding;small GTPase regulator activity	lipid metabolic process;cellular process;extracellular transport;lipid transport;intracellular protein transport;endocytosis;regulation of catalytic activity	
Cullin-2;CUL2;ortholog	ubiquitin-protein ligase activity	proteolysis;cell cycle	
Protein DEK;DEK;ortholog			
Fibroblast growth factor 19;FGF19;ortholog	growth factor activity	cell cycle;cell-cell signaling;mesoderm development;angiogenesis;nervous system development	
Follistatin;FST;ortholog	protein binding		
Growth factor receptor-bound protein 2;GRB2;ortholog	receptor binding	immune system process;cellular component movement;cell communication;ectoderm development;mesoderm development;nervous system development;cellular defense response	
Heme oxygenase 1;HMOX1;ortholog	oxidoreductase activity	porphyrin-containing compound metabolic process	
Heat shock protein beta-1;HSPB1;ortholog	structural molecule activity	immune system process;protein folding;muscle contraction;visual	

		perception;sensory perception;response to stress	
Inhibin beta A chain;INHBA;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
Fructose-bisphosphate aldolase A;ALDOA;ortholog			
Apolipoprotein E;APOE;ortholog			
Calreticulin;CALR;ortholog	calcium ion binding	protein folding	
Macrophage colony-stimulating factor 1;CSF1;ortholog	cytokine activity	immune response;macrophage activation;cell communication;mesoderm development;hemopoiesis;response to stimulus	
Ephrin type-A receptor 2;EPHA2;ortholog		nervous system development	
Fascin;FSCN1;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement	actin cytoskeleton;intracellular
Glucose-6-phosphate 1-dehydrogenase;G6PD;ortholog	oxidoreductase activity	monosaccharide metabolic process	
Interleukin-11;IL11;ortholog			
Interleukin enhancer-binding factor 3;ILF3;ortholog	kinase activity;hydrolase activity;deaminase activity;DNA binding;RNA binding;protein binding;kinase activator activity;kinase regulator activity	spermatogenesis;response to interferon-gamma;apoptotic process;purine nucleobase metabolic process;protein metabolic process;cell cycle;neurological system process;anterior/posterior axis specification;anterior/posterior axis specification;apoptotic process;response to stimulus;RNA localization;regulation of	

		catalytic activity	
L-lactate dehydrogenase A chain;LDHA;ortholog	oxidoreductase activity	glycolysis;glycolysis;tricarboxylic acid cycle	
CD9 antigen;CD9;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system process;blood coagulation	
Ephrin-A5;EFNA5;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	
Receptor tyrosine-protein kinase erbB-2;ERBB2;ortholog		apoptotic process;cell proliferation;cell-cell adhesion;apoptotic process;nervous system development	
Laminin subunit beta-1;LAMB1;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development;nervous system development	extracellular region;extracellular matrix
Leukemia inhibitory factor;LIF;ortholog	cytokine activity	immune system process;induction of apoptosis;cell-cell signaling;neurological system process;induction of apoptosis;response to stress;negative regulation of apoptotic process	
Bleomycin hydrolase;BLMH;ortholog			
Eukaryotic translation initiation factor 3 subunit H;EIF3H;ortholog	sequence-specific DNA binding transcription factor activity;sequence-specific DNA binding transcription factor activity	primary metabolic process	
Ephrin type-A receptor 7;EPHA7;ortholog		nervous system development	
Protein ETHE1, mitochondrial;ETHE1;ortholog	hydrolase activity	metabolic process;response to toxic substance	
Fatty acid-binding	lipid binding	lipid metabolic process;cell	

protein, adipocyte;FABP4;ortholog		communication;ectoderm development;lipid transport;vitamin transport	
Growth/differentiation factor 15;GDF15;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
Glutathione S-transferase Mu 3;GSTM3;ortholog			
Kallikrein-6;KLK6;ortholog	serine-type peptidase activity	proteolysis;response to stimulus	
ATP-citrate synthase;ACLY;ortholog	transferase activity;lyase activity;ligase activity	generation of precursor metabolites and energy;coenzyme metabolic process;carbohydrate metabolic process;tricarboxylic acid cycle;lipid metabolic process	
Annexin A4;ANXA4;ortholog		fatty acid metabolic process	
Apolipoprotein D;APOD;ortholog			
Breast cancer type 2 susceptibility protein;BRCA2;ortholog	DNA binding		
Monocyte differentiation antigen CD14;CD14;ortholog			
Epidermal growth factor receptor;EGFR;ortholog		apoptotic process;cell proliferation;cell-cell adhesion;apoptotic process;nervous system development	
Ephrin type-A receptor 4;EPHA4;ortholog		nervous system development	
Ephrin type-B receptor 2;EPHB2;ortholog		nervous system development	
Ephrin type-B receptor 6;EPHB6;ortholog		nervous system development	
Fatty acid synthase;FASN;ortholog	oxidoreductase activity;methyltransferase activity;transferase activity, transferring	cellular amino acid metabolic process;fatty acid biosynthetic process	

	acyl groups;hydrolase activity, acting on ester bonds;ligase activity		
Fructose-bisphosphate aldolase C;ALDOC;ortholog			
Annexin A2;ANXA2;ortholog		fatty acid metabolic process;mesoderm development	
AP-2 complex subunit beta;AP2B1;ortholog		intracellular protein transport;vesicle-mediated transport	
DNA-(apurinic or apyrimidinic site) lyase;APEX1;ortholog			
Brain-specific angiogenesis inhibitor 1;BAI1;ortholog	G-protein coupled receptor activity	spermatogenesis;immune response;synaptic transmission;neurotransmitter secretion;mesoderm development;heart development;response to stress;intracellular protein transport;synaptic vesicle exocytosis	
Caspase-3;CASP3;ortholog	cysteine-type peptidase activity;protein binding;peptidase inhibitor activity	apoptotic process;proteolysis;apoptotic process;regulation of catalytic activity	
CD44 antigen;CD44;ortholog			
CD59 glycoprotein;CD59;ortholog	receptor binding	immune system process	
CD70 antigen;CD70;ortholog			
CD82 antigen;CD82;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system process;blood coagulation	
CAP-Gly domain-containing linker protein 1;CLIP1;ortholog	structural constituent of cytoskeleton;microtubule binding	protein folding;cellular process;cellular component morphogenesis;intracellular protein transport;vesicle-mediated transport;cellular	microtubule;cytoskeleton;intracellular

		component organization	
Connective tissue growth factor;CTGF;ortholog	growth factor activity	cellular component movement;cell cycle;cell-cell signaling;cell-matrix adhesion;mesoderm development;angiogenesis	
C-X-C motif chemokine 5;CXCL5;ortholog	chemokine activity	immune response;macrophage activation;cell communication;angiogenesis;cellular defense response;response to external stimulus	
DNA damage-binding protein 2;DDB2;ortholog	damaged DNA binding	DNA repair	
Ephrin type-B receptor 4;EPHB4;ortholog		nervous system development	
Flap endonuclease 1;FEN1;ortholog			
Insulin-like growth factor II;IGF2;ortholog	hormone activity;growth factor activity	carbohydrate metabolic process;cell cycle;carbohydrate transport	
Annexin A7;ANXA7;ortholog		fatty acid metabolic process	
Ephrin-A1;EFNA1;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	
Ephrin-B1;EFNB1;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	
Exostosin-1;EXT1;ortholog	transferase activity, transferring glycosyl groups	polysaccharide metabolic process;protein glycosylation	
Glutathione S-transferase P;GSTP1;ortholog			
Interleukin-1 receptor type 2;IL1R2;ortholog	receptor activity	cellular process	
Laminin subunit gamma-1;LAMC1;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development;nervous system development	extracellular region;extracellular matrix
Legumain;LGMN;ortholog	cysteine-type peptidase activity	proteolysis	
Apolipoprotein A-I;APOA1;ortholog		blood circulation	

Aspartyl/asparaginyl beta-hydroxylase;ASPH;ortholog	oxidoreductase activity	cellular protein modification process;cell communication	
Complement component 1 Q subcomponent-binding protein, mitochondrial;C1QBP;ortholog			
Hsp90 co-chaperone Cdc37;CDC37;ortholog	kinase activity;protein binding;kinase activator activity;kinase regulator activity	protein folding;cytokinesis;mitosis;cell communication;cellular component morphogenesis;regulation of catalytic activity;cellular component organization	
Granulocyte colony-stimulating factor;CSF3;ortholog	cytokine activity	immune response;cell-cell signaling;response to stimulus	
Epithelial cell adhesion molecule;EPCAM;ortholog	receptor activity	cell communication	
Ephrin type-A receptor 1;EPHA1;ortholog		nervous system development	
Fatty acid-binding protein, epidermal;FABP5;ortholog	lipid binding	lipid metabolic process;cell communication;ectoderm development;lipid transport;vitamin transport	
Tumor necrosis factor receptor superfamily member 6;FAS;ortholog	cytokine receptor activity;tumor necrosis factor-activated receptor activity	B cell mediated immunity;macrophage activation;induction of apoptosis;cell-cell signaling;ectoderm development;induction of apoptosis;nervous system development;cellular defense response;negative regulation of apoptotic process	
Filamin-B;FLNB;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement;cellular component morphogenesis;cellular component organization	actin cytoskeleton;intracellular
Glutathione S-transferase Mu 1;GSTM1;ortholog			
Intercellular adhesion	receptor binding	immune system	

molecule 1;ICAM1;ortholog		process;cellular process;cell adhesion	
Interleukin-6;IL6;ortholog	cytokine receptor binding;cytokine receptor binding	immune system process;apoptotic process;cell-cell signaling;apoptotic process;negative regulation of apoptotic process	
Kallikrein-10;KLK10;ortholog	serine-type peptidase activity;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	gamete generation;immune system process;proteolysis;cellular process;blood coagulation	
Lipocalin-1;LCN1;ortholog	isomerase activity;binding	fatty acid biosynthetic process;cell communication;response to pheromone;transport	

Appendix H

The 83 proteins that were present in both HPA and PMA proteomic data underwent further investigation. Here,the Panther database (http://www.pantherdb.org/) was used to determine their molecular function, biological function and cellular component and whether they were present in higher grade but not lower grade or both. In addition, whether they have been down-regulated by Marimastat is also included. EFNB1 and FGF19 were the only two proteins down-regulated by Marimastat

Accession name	Name	Molecular function	Biological function	HIGH grade but not low grade
ACLY	ATP-citrate synthase;ACLY;ortholog	transferase activity;lyase activity;ligase activity	generation of precursor metabolites and energy;coenzyme metabolic process;carbohydrate metabolic process;tricarboxylic acid cycle;lipid metabolic process	HIGH
ANXA1	Annexin A1;ANXA1;ortholog		fatty acid metabolic process;cell communication	HIGH
ANXA2	Annexin A2;ANXA2;ortholog		fatty acid metabolic process;mesoderm development	HIGH
AP2B1	AP-2 complex subunit beta;AP2B1;ortholog		intracellular protein transport;vesicle-mediated transport	HIGH
APEX1	DNA-(apurinic or apyrimidinic site) lyase;APEX1;ortholog			HIGH
APOA1	Apolipoprotein A-I;APOA1;ortholog		blood circulation	HIGH
APOE	Apolipoprotein E;APOE;ortholog			HIGH
ASPH	Aspartyl/asparaginyl beta-hydroxylase;ASPH;ortholog	oxidoreductase activity	cellular protein modification process;cell communication	HIGH
BAI1	Brain-specific angiogenesis inhibitor 1;BAI1;ortholog	G-protein coupled receptor activity	spermatogenesis;immune response;synaptic transmission;neurotransmitter secretion;mesoderm development;heart development;response to stress;intracellular protein transport;synaptic vesicle exocytosis	HIGH
BRCA2	Breast cancer type 2	DNA binding		HIGH

	susceptibility protein;BRCA2;ortholog			
C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial;C1QBP;ortholog			HIGH
CALR	Calreticulin;CALR;ortholog	calcium ion binding	protein folding	HIGH
CASP3	Caspase-3;CASP3;ortholog	cysteine-type peptidase activity;protein binding;peptidase inhibitor activity	apoptotic process;proteolysis;apoptotic process;regulation of catalytic activity	HIGH
CAV1	Caveolin-1;CAV1;ortholog	catalytic activity;structural molecule activity;protein binding;small GTPase regulator activity	lipid metabolic process;cellular process;extracellular transport;lipid transport;intracellular protein transport;endocytosis;regulation of catalytic activity	HIGH
CD14	Monocyte differentiation antigen CD14;CD14;ortholog			HIGH
CD44	CD44 antigen;CD44;ortholog			HIGH
CD59	CD59 glycoprotein;CD59;ortholog	receptor binding	immune system process	HIGH
CD70	CD70 antigen;CD70;ortholog			HIGH
CD82	CD82 antigen;CD82;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system process;blood coagulation	HIGH
CD9	CD9 antigen;CD9;ortholog	receptor activity;receptor binding	gamete generation;immune system process;cell communication;cell-cell adhesion;neurological system process;blood coagulation	LOW GRADE THAN HIGH GRADE
CDC37	Hsp90 co-chaperone Cdc37;CDC37;ortholog	kinase activity;protein binding;kinase activator activity;kinase regulator activity	protein folding;cytokinesis;mitosis;cell communication;cellular component morphogenesis;regulation of catalytic activity;cellular component organization	HIGH
CLIP1	CAP-Gly domain-containing linker protein	structural constituent of	protein folding;cellular process;cellular component	slight high

	1;CLIP1;ortholog	cytoskeleton;microtubule binding	morphogenesis;intracellular protein transport;vesicle-mediated transport;cellular component organization	
CSF1	Macrophage colony-stimulating factor 1;CSF1;ortholog	cytokine activity	immune response;macrophage activation;cell communication;mesoderm development;hemopoiesis;response to stimulus	HIGH
CSF3	Granulocyte colony-stimulating factor;CSF3;ortholog	cytokine activity	immune response;cell-cell signaling;response to stimulus	HIGH
CTGF	Connective tissue growth factor;CTGF;ortholog	growth factor activity	cellular component movement;cell cycle;cell-cell signaling;cell-matrix adhesion;mesoderm development;angiogenesis	HIGH
CUL2	Cullin-2;CUL2;ortholog	ubiquitin-protein ligase activity	proteolysis;cell cycle	HIGH
CUL4A	Cullin-4A;CUL4A;ortholog	ubiquitin-protein ligase activity	induction of apoptosis;proteolysis;cell cycle;induction of apoptosis	HIGH
CXCL5	C-X-C motif chemokine 5;CXCL5;ortholog	chemokine activity	immune response;macrophage activation;cell communication;angiogenesis;cellular defense response;response to external stimulus	HIGH
DDB2	DNA damage-binding protein 2;DDB2;ortholog	damaged DNA binding	DNA repair	Low grade than high grade
DEK	Protein DEK;DEK;ortholog			high
EFNA1	Ephrin-A1;EFNA1;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	high
EFNA5	Ephrin-A5;EFNA5;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	high
EFNB1	Ephrin-B1;EFNB1;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	high
EGFR	Epidermal growth factor receptor;EGFR;ortholog		apoptotic process;cell proliferation;cell-cell adhesion;apoptotic process;nervous system development	high
EIF3H	Eukaryotic translation initiation factor 3 subunit	sequence-specific DNA binding	primary metabolic process	Low grade than high

	H;EIF3H;ortholog	transcription factor activity;sequence-specific DNA binding transcription factor activity		grade
EPCAM	Epithelial cell adhesion molecule;EPCAM;ortholog	receptor activity	cell communication	high
EPHA1	Ephrin type-A receptor 1;EPHA1;ortholog		nervous system development	high
EPHA2	Ephrin type-A receptor 2;EPHA2;ortholog		nervous system development	high
EPHA4	Ephrin type-A receptor 4;EPHA4;ortholog		nervous system development	high
EPHA7	Ephrin type-A receptor 7;EPHA7;ortholog		nervous system development	high
EPHB2	Ephrin type-B receptor 2;EPHB2;ortholog		nervous system development	high
EPHB4	Ephrin type-B receptor 4;EPHB4;ortholog		nervous system development	high
ERBB2	Receptor tyrosine-protein kinase erbB-2;ERBB2;ortholog		apoptotic process;cell proliferation;cell-cell adhesion;apoptotic process;nervous system development	high
ETHE1	Protein ETHE1, mitochondrial;ETHE1;ortholog	hydrolase activity	metabolic process;response to toxic substance	high
EXT1	Exostosin-1;EXT1;ortholog	transferase activity, transferring glycosyl groups	polysaccharide metabolic process;protein glycosylation	Low grade than high grade
FABP4	Fatty acid-binding protein, adipocyte;FABP4;ortholog	lipid binding	lipid metabolic process;cell communication;ectoderm development;lipid transport;vitamin transport	high
FAS	Fatty acid synthase;FASN;ortholog	oxidoreductase activity;methyltransferase activity;transferase activity, transferring acyl groups;hydrolase activity, acting on ester bonds;ligase activity	cellular amino acid metabolic process;fatty acid biosynthetic process	high
FAS	Tumor necrosis factor receptor superfamily member 6;FAS;ortholog	cytokine receptor activity;tumor necrosis factor-activated receptor activity	B cell mediated immunity;macrophage activation;induction of apoptosis;cell-cell signaling;ectoderm	high

			development;induction of apoptosis;nervous system development;cellular defense response;negative regulation of apoptotic process	
FEN1	Flap endonuclease 1;FEN1;ortholog			high
FGF19	Fibroblast growth factor 19;FGF19;ortholog	growth factor activity	cell cycle;cell-cell signaling;mesoderm development;angiogenesis;nervous system development	high
FSCN1	Fascin;FSCN1;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement	high
FST	Follistatin;FST;ortholog	protein binding		high
G6PD	Glucose-6-phosphate 1-dehydrogenase;G6PD;ortholog	oxidoreductase activity	monosaccharide metabolic process	equal
GDF15	Growth/differentiation factor 15;GDF15;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	high
GRB2	Growth factor receptor-bound protein 2;GRB2;ortholog	receptor binding	immune system process;cellular component movement;cell communication;ectoderm development;mesoderm development;nervous system development;cellular defense response	high
GSTM1	Glutathione S-transferase Mu 1;GSTM1;ortholog			equal
GSTM3	Glutathione S-transferase Mu 3;GSTM3;ortholog			high
GSTP1	Glutathione S-transferase P;GSTP1;ortholog			equal
HSPB1	Heat shock protein beta-1;HSPB1;ortholog	structural molecule activity	immune system process;protein folding;muscle contraction;visual perception;sensory perception;response to stress	high
IGF2	Insulin-like growth factor II;IGF2;ortholog	hormone activity;growth factor activity	carbohydrate metabolic process;cell cycle;carbohydrate transport	high
IL11	Interleukin-			high

	11;IL11;ortholog			
IL1R2	Interleukin-1 receptor type 2;IL1R2;ortholog	receptor activity	cellular process	high
IL6	Interleukin-6;IL6;ortholog	cytokine receptor binding;cytokine receptor binding	immune system process;apoptotic process;cell-cell signaling;apoptotic process;negative regulation of apoptotic process	equal
ILF3	Interleukin enhancer-binding factor 3;ILF3;ortholog	kinase activity;hydrolase activity;deaminase activity;DNA binding;RNA binding;protein binding;kinase activator activity;kinase regulator activity	spermatogenesis;response to interferon-gamma;apoptotic process;purine nucleobase metabolic process;protein metabolic process;cell cycle;neurological system process;anterior/posterior axis specification;anterior/posterior axis specification;apoptotic process;response to stimulus;RNA localization;regulation of catalytic activity	equal
INHBA	Inhibin beta A chain;INHBA;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	high
KLK10	Kallikrein-10;KLK10;ortholog	serine-type peptidase activity;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	gamete generation;immune system process;proteolysis;cellular process;blood coagulation	slight high
KLK6	Kallikrein-6;KLK6;ortholog	serine-type peptidase activity	proteolysis;response to stimulus	high
LAMB1	Laminin subunit beta-1;LAMB1;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development;nervous system development	high
LCN1	Lipocalin-1;LCN1;ortholog	isomerase activity;binding	fatty acid biosynthetic process;cell communication;response to pheromone;transport	high
LDHA	L-lactate dehydrogenase A chain;LDHA;ortholog	oxidoreductase activity	glycolysis;glycolysis;tricarboxylic acid cycle	low

LGMN	Legumain;LGMN;ortholog	cysteine-type peptidase activity	proteolysis	HIGH
LIF	Leukemia inhibitory factor;LIF;ortholog	cytokine activity	immune system process;induction of apoptosis;cell-cell signaling;neurological system process;induction of apoptosis;response to stress;negative regulation of apoptotic process	HIGH

Appendix H

The 60 proteins that were present in both HPA and Marimastat proteomic data underwent further investigation. Here,the Panther database (http://www.pantherdb.org/) was used to determine their molecular function, biological function and cellular component.

Name of protein	Molecular function	Biological function	Cellular component	
TIMP1	Metalloproteinase inhibitor 1;TIMP1;ortholog	peptidase activity;protein binding;metalloendopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
ANXA1	Annexin A1;ANXA1;ortholog		fatty acid metabolic process;cell communication	
ADAM9	Disintegrin and metalloproteinase domain-containing protein 9;ADAM9;ortholog		fertilization;apoptotic process;neurological system process;mesoderm development;apoptotic process;heart development	
INHBA	Inhibin beta A chain;INHBA;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
ACLY	ATP-citrate synthase;ACLY;ortholog			
MCM2	DNA replication licensing factor MCM2;MCM2;ortholog	DNA helicase activity;hydrolase activity;nucleic acid binding	DNA replication;cell cycle	
TGFB1	Transforming growth factor beta-1;TGFB1;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
FST	Follistatin;FST;ortholog	protein binding		
HSPB1	Heat shock protein beta-1;HSPB1;ortholog	structural molecule activity	immune system process;protein folding;muscle contraction;visual perception;sensory perception;response to stress	

ALDOA	Fructose-bisphosphate aldolase A;ALDOA;ortholog			
LDHA	L-lactate dehydrogenase A chain;LDHA;ortholog	oxidoreductase activity	glycolysis;glycolysis;tricarboxylic acid cycle	
FSCN1	Fascin;FSCN1;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement	actin cytoskeleton;intracellular
G6PD	Glucose-6-phosphate 1-dehydrogenase;G6PD;ortholog	oxidoreductase activity	monosaccharide metabolic process	
UBA1	Ubiquitin-like modifier-activating enzyme 1;UBA1;ortholog	ligase activity	coenzyme metabolic process;cellular protein modification process;proteolysis;cell communication;intracellular protein transport;nuclear transport	
CALR	Calreticulin;CALR;ortholog	calcium ion binding	protein folding	
ILF3	Interleukin enhancer-binding factor 3;ILF3;ortholog	kinase activity;hydrolase activity;deaminase activity;DNA binding;RNA binding;protein binding;kinase activator activity;kinase regulator activity	spermatogenesis;response to interferon-gamma;apoptotic process;purine nucleobase metabolic process;protein metabolic process;cell cycle;neurological system process;anterior/posterior axis specification;anterior/posterior axis specification;apoptotic process;response to stimulus;RNA localization;regulation of catalytic activity	
NUCB2	Nucleobindin-2;NUCB2;ortholog	nucleic acid binding;calcium ion binding;calmodulin binding;calcium-dependent phospholipid binding	cellular calcium ion homeostasis	
LAMB1	Laminin subunit beta-1;LAMB1;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development;nervous system development	extracellular region;extracellular matrix
NRP1	Neuropilin-1;NRP1;ortholog	oxidoreductase activity;serine-type peptidase activity;metallopep	immune system process;proteolysis;synaptic transmission;cell-cell adhesion;visual	extracellular region;extracellular matrix

		tidase activity;receptor activity;lipid transporter activity;transmembrane transporter activity;receptor binding;enzyme regulator activity	perception;sensory perception;ectoderm development;mesoderm development;response to external stimulus;lipid transport;intracellular protein transport;endocytosis;vitamin transport;regulation of catalytic activity	
TIMP2	Metalloproteinase inhibitor 2;TIMP2;ortholog	peptidase activity;protein binding;metalloendopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
LIF	Leukemia inhibitory factor;LIF;ortholog	cytokine activity	immune system process;induction of apoptosis;cell-cell signaling;neurological system process;induction of apoptosis;response to stress;negative regulation of apoptotic process	
MMP1	Interstitial collagenase;MMP1;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
MMP13	Collagenase 3;MMP13;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
GDF15	Growth/differentiation factor 15;GDF15;ortholog	growth factor activity	female gamete generation;cell communication;ectoderm development;mesoderm development;skeletal system development;heart development;muscle organ development	
NEO1	Neogenin;NEO1;ortholog	phosphoprotein phosphatase activity;phosphoprotein phosphatase activity;receptor activity	immune system process;induction of apoptosis;cellular protein modification process;cell cycle;cell-cell signaling;cell-cell adhesion;muscle contraction;neurological system process;ectoderm development;mesoderm development;induction of apoptosis;angiogenesis;nervous system development;muscle organ development	
GST	Glutathione S-transferase			

M3	Mu 3;GSTM3;ortholog			
KLK6	Kallikrein-6;KLK6;ortholog	serine-type peptidase activity	proteolysis;response to stimulus	
LMNA	Prelamin-A/C;LMNA;ortholog	structural constituent of cytoskeleton	cellular process;cellular component morphogenesis;cellular component organization	intermediate filament cytoskeleton;intracellular
FAS	Fatty acid synthase;FASN;ortholog	oxidoreductase activity;methyltransferase activity;transferase activity, transferring acyl groups;hydrolase activity, acting on ester bonds;ligase activity	cellular amino acid metabolic process;fatty acid biosynthetic process	
NAMPT	Nicotinamide phosphoribosyltransferase; NAMPT;ortholog	cytokine activity	cellular process	
RBBP4	Histone-binding protein RBBP4;RBBP4;ortholog	receptor activity	DNA repair;mRNA splicing, via spliceosome;cell communication;intracellular protein transport;peroxisomal transport;chromatin organization	
EGFR	Epidermal growth factor receptor;EGFR;ortholog		apoptotic process;cell proliferation;cell-cell adhesion;apoptotic process;nervous system development	
MMP13	Collagenase 3;MMP13;ortholog			
ACLY	ATP-citrate synthase;ACLY;ortholog	transferase activity;lyase activity;ligase activity	generation of precursor metabolites and energy;coenzyme metabolic process;carbohydrate metabolic process;tricarboxylic acid cycle;lipid metabolic process	
APOD	Apolipoprotein D;APOD;ortholog			
SLPI	Antileukoproteinase;SLPI;ortholog	peptidase activity;protein binding;serine-type endopeptidase inhibitor activity	proteolysis;regulation of catalytic activity	
ST14	Suppressor of tumorigenicity 14	peptidase activity;receptor	fertilization;immune system process;apoptotic process;lipid	

	protein;ST14;ortholog	activity;calcium ion binding;hormone activity;calmodulin binding;calcium-dependent phospholipid binding;peptidase inhibitor activity	metabolic process;blood circulation;mesoderm development;apoptotic process;angiogenesis;blood coagulation;lipid transport;regulation of catalytic activity	
APEX1	DNA-(apurinic or apyrimidinic site) lyase;APEX1;ortholog			
CD44	CD44 antigen;CD44;ortholog			
ANXA2	Annexin A2;ANXA2;ortholog		fatty acid metabolic process;mesoderm development	
ALDOC	Fructose-bisphosphate aldolase C;ALDOC;ortholog			
NQO1	NAD(P)H dehydrogenase [quinone] 1;NQO1;ortholog			
STRAP	Serine-threonine kinase receptor-associated protein;STRAP;ortholog		cellular process	
FEN1	Flap endonuclease 1;FEN1;ortholog			
PRDX2	Peroxiredoxin-2;PRDX2;ortholog	oxidoreductase activity;peroxidase activity	immune system process;metabolic process	
GSTP1	Glutathione S-transferase P;GSTP1;ortholog			
LAMC1	Laminin subunit gamma-1;LAMC1;ortholog	receptor activity	cell communication;cell-matrix adhesion;cell-cell adhesion;neurological system process;ectoderm development;nervous system development	extracellular region;extracellular matrix
LGMN	Legumain;LGMN;ortholog	cysteine-type peptidase activity	proteolysis	
MET	Hepatocyte growth factor receptor;MET;ortholog			
EFNA1	Ephrin-A1;EFNA1;ortholog	receptor binding	cell-cell signaling;ectoderm development;nervous system development	
EXT1	Exostosin-1;EXT1;ortholog	transferase activity, transferring glycosyl groups	polysaccharide metabolic process;protein glycosylation	
PIGR	Polymeric immunoglobulin receptor;PIGR;ortholog	receptor activity	immune system process;response to stimulus;intracellular protein	

			transport;receptor-mediated endocytosis	
RAC 1	Ras-related C3 botulinum toxin substrate 1;RAC1;ortholog	GTPase activity;protein binding	metabolic process;cell communication;intracellular protein transport;receptor-mediated endocytosis	
FLNB	Filamin-B;FLNB;ortholog	structural constituent of cytoskeleton;actin binding	cellular component movement;cellular component morphogenesis;cellular component organization	actin cytoskeleton;intracellular
FAS	Tumor necrosis factor receptor superfamily member 6;FAS;ortholog	cytokine receptor activity;tumor necrosis factor-activated receptor activity	B cell mediated immunity;macrophage activation;induction of apoptosis;cell-cell signaling;ectoderm development;induction of apoptosis;nervous system development;cellular defense response;negative regulation of apoptotic process	
MMP2	72 kDa type IV collagenase;MMP2;ortholog	metallopeptidase activity	proteolysis	extracellular region;extracellular matrix
SRC	Proto-oncogene tyrosine-protein kinase Src;SRC;ortholog	non-membrane spanning protein tyrosine kinase activity	apoptotic process;protein phosphorylation;cell-cell signaling;cell proliferation;cell adhesion;apoptotic process;cell differentiation	cytoplasm
FABP5	Fatty acid-binding protein, epidermal;FABP5;ortholog	lipid binding	lipid metabolic process;cell communication;ectoderm development;lipid transport;vitamin transport	
IL6	Interleukin-6;IL6;ortholog	cytokine receptor binding;cytokine receptor binding	immune system process;apoptotic process;cell-cell signaling;apoptotic process;negative regulation of apoptotic process	
PARP1	Poly [ADP-ribose] polymerase 1;PARP1;ortholog	transferase activity, transferring glycosyl groups	DNA repair;protein ADP-ribosylation;protein ADP-ribosylation;response to stress	
CSF3	Granulocyte colony-stimulating factor;CSF3;ortholog	cytokine activity	immune response;cell-cell signaling;response to stimulus	
GSTM1	Glutathione S-transferase Mu 1;GSTM1;ortholog			
LCN1	Lipocalin-1;LCN1;ortholog	isomerase activity;binding	fatty acid biosynthetic process;cell communication;response to pheromone;transport	