Joana Flores Cordeiro

Licenciada em Bioquímica

Pinpointing new urinary biomarkers for bladder cancer detection and stage differentiation by label-free quantitative mass spectrometry

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Doutor Hugo Santos, Investigador Auxiliar, FCT-NOVA

Co-orientador: Professor José Luís Capelo-Martínez, Professor Associado, FCT-NOVA

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Vogal: Doutor Hugo Santos

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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“Grounded apples have fallen,
Unnoticed to most sights,
Yet gravity was perceived,
Through Newton’s eyes.”

Joana Flores, biochemist aspirant

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Resumo

O cancro da bexiga é o quarto mais comum em países desenvolvidos e o sétimo mais comum em todo o mundo em homens. É também uma das neoplasias mais caras considerando o custo por paciente, devido aos métodos de diagnósticos e monitorização atuais terem elevado custo-benefício. A cistoscopia é um método invasivo, com baixa sensibilidade e contribui grandemente para os custos do cancro da bexiga, uma vez que é o método de eleição para monitorizar carcinomas uroteliais recorrentes. Assim, é necessário desenvolver novos métodos de deteção, não invasivos e mais fidedignos.

O proteoma da urina pode ser um bom início para o desenvolvimento de um método de deteção para o cancro da bexiga. Mutações genéticas em células tumorais, assim como a resposta das células vizinhas e do sistema imunitário à neoplasia vão originar diferenças ao nível da expressão e ativação proteica, originando, em consequência, um proteoma diferente. Como a urina está em contacto direto com as células tumorais e as células da bexiga, infere-se que alterações na bexiga se reflitam na urina. Proteínas indicadoras de processos normais ou de processos patológicas são denominadas de biomarcadores proteicos.

Este projeto teve como principais objetivos desenvolver um painel de biomarcadores capaz de detetar a presença de cancro de bexiga e de medir o estadiamento do cancro de bexiga no proteoma da urina. O Hospital São José providenciou amostras de urina de pacientes com cancro da bexiga: estágios não invasivos Ta e T1 e estágios T2-T4 (T2+) e de outros pacientes para serem usadas como controlo. As amostras foram selecionadas e cinco grupos foram formados: cancro da bexiga-Ta; T1; T2+; voluntários sem qualquer condição urotelial e voluntários com sintomas do trato urinário inferior. As amostras de urina foram tratadas e digeridas pelo método de preparação de amostras auxiliado por filtro e analisadas por cromatografia líquida acoplada a espetrometria de massa tandem. Os péptidos digeridos foram identificados e quantificados através de quantificação sem usar marcação, e através da identificação de péptidos únicos, as proteínas foram identificadas e posteriormente quantificadas. A bioinformática foi uma ferramenta utilizada para manusear e interpretar os dados obtidos. Dois painéis de biomarcadores proteicos foram desenvolvidos: um com 35 proteínas para identificar a presença de cancro da bexiga e outro com 76 proteínas para medir o estadiamento do cancro da bexiga.

Perspetivas futuras abrangem efetuar um estudo de validação para ambos os painéis, de forma a determinar valores de especificidade e sensibilidade para cada painel e, em consequência avaliar a capacidade deste método de substituir a cistoscopia.

Palavras-chave: Biomarcador, Cancro de Bexiga, Cromatografia Líquida, Espetrometria de massa, Proteómica, Urina

Abstract

Bladder cancer is the fourth most common *neoplasia* in more developed countries and the seventh worldwide, in the male gender. On cost per patient, it is also one of the most expensive malignancies at patient level, because current diagnostics, follow-up, and treatment are not cost-effective. Cystoscopy, the regular method to diagnose bladder cancer, is invasive, causes pain and it has a low sensibility. Furthermore, it is used to monitor recurrent urothelial carcinomas, contributing significantly to rise bladder cancer costs. Therefore, new non-invasive and more reliable methods of diagnosis and prognosis are needed.

Genetic mutations in bladder tumour cells, as well as tumour response from neighbouring cells and from the immune system, implicate protein expression and activation different from healthy people, therefore originating new features in the urine proteome. As urine is in direct contact with these tumour and urothelial cells, it is expected that changes in the bladder are reflected in the urine content. Therefore, the urinary proteome is an excellent biopsy for finding protein biomarkers of diagnosis and prognosis.

This work has the primary goal of finding a urine-based panel of biomarkers of diagnosis and staging for bladder cancer. The Hospital São José provided urine samples from patients who had Bladder Cancer as follows: (i) non-muscle invasive stages Ta and T1, and (ii) muscle invasive T2-T4 (T2+) and (iii) from other patients used as controls. So far, five groups were formed as indicated next: (a) bladder cancer-Ta; (b) T1; (c) T2+ (d) volunteers with no urothelial conditions and (e) volunteers presenting lower urinary tract symptoms.

The methodology selected to find the biomarkers was free-label quantification of peptides by High-Resolution Mass Spectrometry. To this end, the urine proteome was first separated and then digested using the Filter Aided Sample Preparation -FASP- method. The pools of peptides obtained were used to identify and quantify the proteins present in the urine samples. Then, using bioinformatics, data was interpreted, and two biomarker panels were obtained. The first panel consists of 35 proteins to diagnostic bladder cancer. The second panel consists of 76 proteins to stage bladder cancer.

Keywords: Biomarker, Bladder Cancer, Liquid Chromatography-Mass Spectrometry, Proteomics, Urine

Poster Presentation from present Master Thesis

"Pinpointing new biomarkers of bladder cancer in liquid biopsies"

Joana Flores, Juliana João, Francisco Fernandes, Elisabete Oliveira, Javier Fernández-Lodeiro, Gil Falcão, Pedro Baltazar, Luís Campos Pinheiro, Fernando Calais da Silva, Peter Horvatovich, Carlos Lodeiro-Espiño, José Luís Capelo, Hugo Miguel Santos

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Table III.3: Table listing each protein identified as potential biomarkers for measuring progress, the response (upregulation, downregulation when compared to other BCa stages) and the stage identified (T1). Abbreviations: AMBP, α-1-microglobulin/bikunin precursor; HSPG2, Basement membrane-specific heparan sulfate proteoglycan core protein; Ig, Immunoglobulin; LAIR1, Leukocyte-associated immunoglobulin-like receptor 1; LYVE1, Lymphatic vessel endothelial hyaluronan receptor 1; SH3BGRL3, SH3 domain-binding glutamic acid-rich-like protein 3; TNF, Tumour Necrosis Factor. 48

Abbreviations

AmBic	Ammonium Bicarbonate
ACN	Acetonitrile
AMBP	α-1-microglobulin/bikunin precursor
BCa	Bladder Cancer
BSA	Bovine Serum Albumin
CHCA	α-cyano-4-hydroxycinnamic acid
DDA	Data Dependant Acquisition
DIA	Dara Independent Acquisition
DTT	1,4-Dithiothreitol
EAU	European Association of Urology
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1
ESI	Electrospray ionization
FASP	Filter Aided Sample Preparation
FDR	False Discovery Rate
FmA	Formic Acid
HLB	Hydrophilic-Lipophilic-Balanced
HG	High Grade
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein
IAA	Iodoacetamide
Ig	Immoglobulin
LC	Liquid Chromatography
LAIR1	Leukocyte associated immunoglobulin like receptor 1
LFQ	Label-Free Quantification
LG	Low Grade
LMWP	Low-Molecular-Weight Proteome
LUTS	Low Tract Urinary Symptoms
LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1
MALDI	Mass-assisted Laser Desorption/Ionization
MIBC	Muscle Invasive Bladder Cancer
MMP	Matrix Metalloproteinase
MS	Mass Spectrometry
NMIBC	Non-Muscle Invasive Bladder Cancer
nUC	no Urologic Conditions
PTPN1	Tyrosine-protein phosphatase non-receptor type 1
Q	Quadrupole
SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3
SPE	Solid Phase Extraction
T2+	T2, T3, T4

TFA Trifluoracetic Acid

TNF Tumour Necrosis Factor

TOF Time-of-Flight

TuRB Transurethral Resection of Bladder

Chapter I. Introduction

1. Bladder Cancer

Bladder is a muscular organ of the urinary system and has the function of collecting and storing urine. Urine is a fluid that results from the metabolism of the body and enters the bladder through the ureters and exits through the urethra, as shown in **Figure I.1**. The wall of the bladder consists of the urothelium and the lamina propria, followed by a muscular layer. The outer wall is composed by peritoneum on the upper surface of the bladder and by connective tissue on the under surface and is surrounded by perivesical tissue[1]. Urothelium is the tissue that covers the bladder inner wall and is composed of multiple layers of epithelial cells, which allow the bladder to have high elasticity[2].

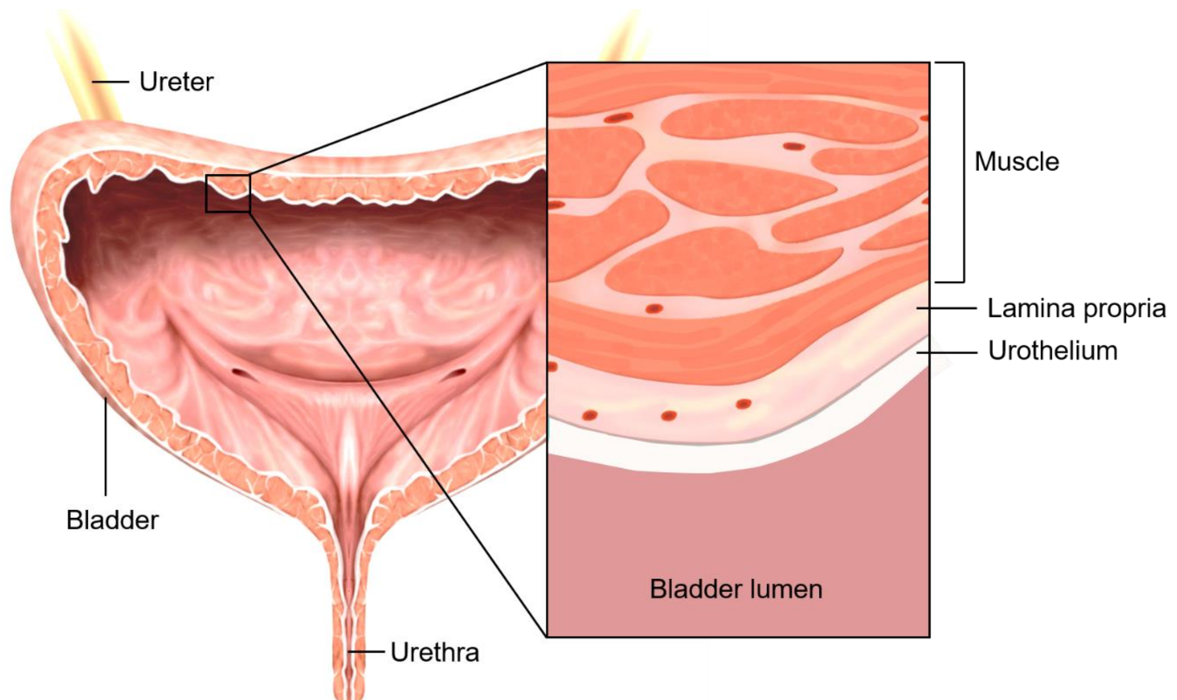


Figure I.1: Summary representation of the anatomy and histology of a female urinary bladder (Adapted from Winslow Scientific Illustrations[3]).

It is in the urothelium that bladder cancer develops. Bladder cancer (BCa) comprises any abnormal cell growth developing in the tissues of the urinary bladder, with the potential of spreading to nearby tissues or other parts of the body. It is the most common cancer of the urinary tract, and it ranks fourth among cancers in more developed countries and seventh worldwide, in the male gender. Men are more likely to get BCa than women, on a ratio near 4 to 1[4]. The average age of patients when bladder cancer is detected is approximately 70 years, with rare occurrences in individuals younger than 40 years[5].

Tobacco smoking is the most well-established risk factor for bladder cancer, causing approximately 50%–65% of male cases[6]. The present relation between occupational exposure to aromatic amines and polycyclic hydrocarbons and bladder cancer[7] was first described by Doctor Ludwig Rehn in 1895 when working on the prevalence of bladder cancer amongst workers of a fuchsine producing factory[8]. Consumption of arsenic-contaminated water[9] and therapeutic abuse of phenacetin-containing

analgesics[10] are others risk factors. Chronic infection with *Schistosoma haematobium* is a risk factor related to squamous cell bladder cancer development[11], a histological type of bladder cancer.

Genetic predisposition plays a small role in bladder cancer incidence, as urothelial carcinoma is not considered a familial disease[12]. Nevertheless, epidemiological studies showed that first-degree relative of patients has an almost 2-fold increased risk to develop bladder cancer[13]. Furthermore, the genetic mutations in *HRAS*, *Rb1*, *PTEN/MMAC1* increase the risk to develop bladder cancer[14–16] as well as two genes involved in carcinogen metabolism: *GSTM1* null phenotype and the *NAT2* slow acetylator phenotype[17]. Glutathione S-transferase is encoded by GSTM1 and detoxifies carcinogenic polycyclic aromatic hydrocarbons[18] and N-acetyltransferase 2 is encoded by NAT2 and detoxifies arylamine and hydrazine carcinogens[19].

Urothelial carcinoma accounts for 90-95% of bladder cancers, and it will be the histological type studied in this work. Other histological types are squamous cell bladder cancer and adenocarcinoma[20].

1.1 Staging and Characterising the Pathology

Grading and staging the tumour are mandatory to diagnose and select the ideal treatment for a patient affected by urothelial carcinoma. The grade of the tumour is based on the degree of nuclear anaplasia and some architectural abnormalities. According to the 2004/2016 WHO Grading System, papillary lesions can be defined as Low-Grade (LG), when cells are well differentiated and High-Grade (HG), when cells are poorly differentiated. The 1973 WHO grading system considers three grades: Grade 1 (G1) corresponds to LG, Grade 3 (G3) to HG, and Grade 2 (G2) is an intermediate grade[21].

The T stage of the bladder tumour is attributed depending on the spreading of the tumour through the bladder tissue layers, N describes the absence or presence and extent of regional lymph node metastasis and M the absence or presence of distant metastasis. **Figure I.2** schematizes bladder cancer stages defined by the International Union Against Cancer, not including T0, attributed when there is no evidence of a primary tumour, and Tx, that is attributed to primary tumours that cannot be accessed[22].

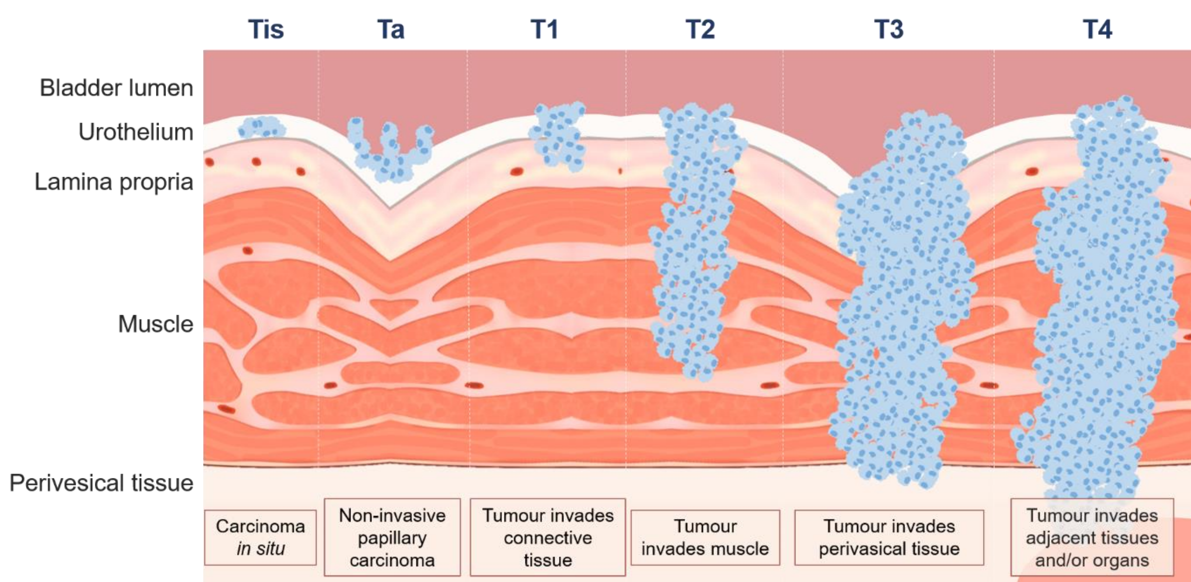


Figure I.2: Representation of the different stages of tumour invasion throughout bladder tissue (Adapted from Knowles et al.[23])

Carcinomas *in situ* (CIS) are staged as Tis and consist on flat tumours confined to the urothelium and constituted by poorly differentiated cancer cells. Non-invasive papillary carcinomas are papillary lesions, developing into the bladder wall in the shape of the papilla, that show variations in nuclear polarity, size, shape and chromatin pattern and are staged as Ta. Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP) is another type of papillary lesion, that exceeds the thickness of normal urothelium and presents increased cellular proliferation but no cellular atypia[24]. It is not considered a tumour and is considered to have a negligible risk for progression, yet it still tends to recur[25]. Muscle Invasive Bladder Cancer (MIBC) comprehends the stages T2, T3 and T4, most being nonpapillary and high-grade[26].

Low-grade disease, such as Low-Grade Ta and PUNLMP, demonstrates a high rate of recurrence but a low risk of progression to MIBC[27]. CIS, TaHG and T1HG have a high rate of recurrence and a high risk of progression[28]. Muscle invasive bladder cancer has the worst outcome for patients, as near a half dies from cancer in five years[29].

As the stage and other features of a tumour have a significant influence on the prognosis, it is important to find which patients have a worse forecast and therefore must be more frequently monitored. To define the prognosis, European Organisation for Research and Treatment of Cancer has defined six risk factors that predict the tumour recurrence and progression to muscle-invasive disease in the following five years: number of tumours, tumour size, prior recurrence rate, stage, grade and if it is carcinoma *in situ*[30]. European Association of Urology (EAU) has written guidelines on Non–Muscle Invasive Urothelial Carcinoma of the Bladder[25] and in 2011, made an update that categorizes patients into three risk groups. These groups divide the different bladder tumours from a low risk of progression to muscle-invasive to a high risk one, therefore simplifying the treatment selection[31]. In the last update, EAU has redefined the groups stratification[21], which is presented in **Table I.1**.

Table I.1: Risk groups stratification, defined by European Association of Urology in 2016

Risk group	Characteristics
Low Risk	Primary, solitary, Ta, LG/G1, <3 cm
Intermediate Risk	All tumours not defined in the other two categories
High Risk	T1 tumour
	CIS
	HG/G3 tumour
	Ta G1G2 tumours that are multiple, recurrent and >3 cm

1.2 Diagnosis and Surveillance

Haematuria, that consists of the presence of blood in urine, is the most common symptom of patients with BCa. Irritative bladder symptoms, such as pain during urination or frequent urination, may be present, particularly in the presence of CIS[21]. Nevertheless, BCa might not cause symptoms right away, delaying its detection.

The performance of a clinical test is defined by two statistical measures: sensitivity and specificity. The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with the disease, meaning that a high sensitivity has a low number of false negatives: individuals with the disease that were identified as not having the disease. The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease, meaning that a high specificity has a low number of false positives: individuals without the disease identified as having the disease[32].

Cystoscopy is the recommended technique to evaluate patients with symptoms of BCa[21]. A cystoscope is an instrument that is inserted into the urethra to allow the observation of the urothelium, through a camera. Flat lesions, like CIS, are difficult to detect[33] and papillary lesions (Ta and T1) are often missed[34]. Voided urine cytology is advocated as a complimentary exam to cystoscopy to detect high-grade tumours[21]. A cytology test consists of analysing a urine specimen under the microscope to look for cancer cells. Bladder tumours are in direct contact with urine and tumours usually have less cell-cell interaction, which causes tumour cells to leak into the urine[35]. Cytology is a highly specific test, especially for HG urothelial carcinoma. However it has a low sensitivity to low-grade cancer[36], and inflammatory conditions can misperceive its results[37]. A transurethral resection of bladder (TuRB) is recommended for patients whose cystoscopy and cytology results suggest BCa, being the only technique that allows tumour full characterisation. TuRB has the following goals: removal of all visible tumour, assessment of size, location, aspect, and multiplicity of the tumour, and obtainment of adequate tissue for pathological assessment to establish tumour type, stage, and grade[38].

As a result of the high risk of recurrence and progression of bladder cancer, patients with NMIBC need to be followed up, varying the frequency and duration of cystoscopy with the patient's degree of risk[21]. BCa is one of the most expensive malignancies at patient level, because current diagnostics, follow-up and treatment are not cost-effective[39], being cystoscopy a monitorisation technique that contributes considerably to bladder cancer expenses[40]. Although bladder cancer presents significant financial and social, governmental authorities in USA and European Union have provided research funding uneven to the burden of the disease[41,42]. Early detection of bladder cancer would reduce cancer-related mortality based on the five-year survival rates of superficial versus muscle-invasive disease. Non-invasive tools that accurately detect BCa or which can distinguish the stages Ta and T1 from stage T2 would have a profound effect on quality of life and healthcare costs[43].

2. Proteomics

Proteins are the functional molecules of the cell, being responsible for almost all the biochemical activity. Protein properties, namely concentration levels, protein synthesis and degradation rates, protein-protein and protein-substrate interaction, subcellular localisation, are hugely dynamic and can change quickly. As proteins are functionally the most relevant components of biological systems and their properties change as a response to a certain stimulus[44], a direct study of proteins can disclose a true understanding of the biologic system in study as well as to give information on biologic processes occurring in it, as embodied in **Figure I.3**.

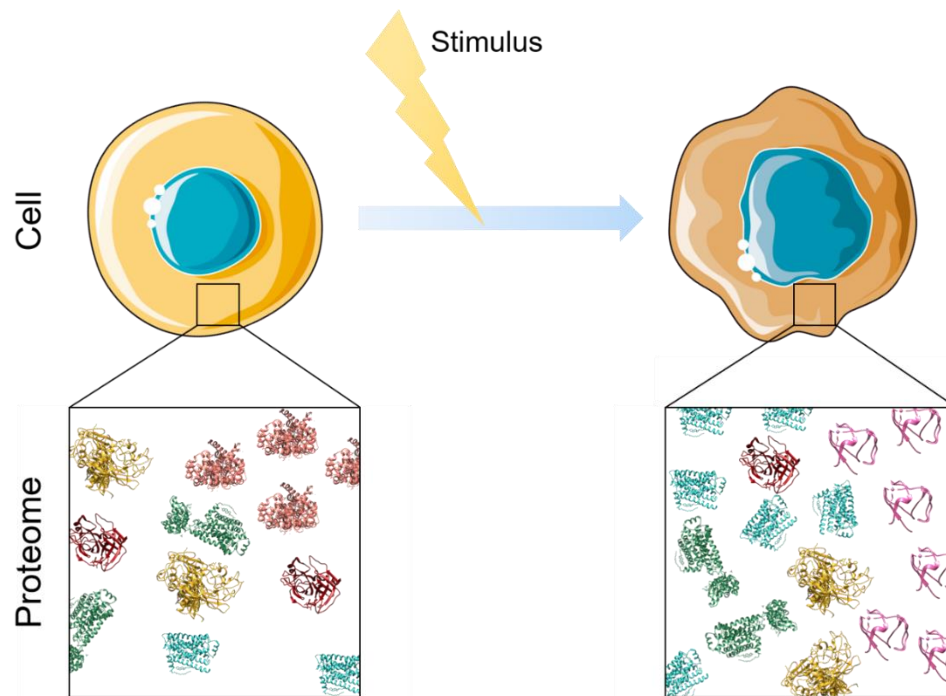


Figure I.3: Representation of the cell proteome changes as response to a certain stimulus.

In the last decades, novel approaches have emerged to allow the qualitative and quantitative measurements of complex interactions in biological systems, referred as “omics” technologies, and proteomics is one of the most developed ones. Proteomics is defined as the study of the total protein content of a cell or an organism, encoded by the genome[45], and is an approach capable of elucidating on protein-protein interactions and protein networks, protein expression, biomarkers research, proteogenomics and structural proteomics[44]. Proteomics can be divided in two approaches: top-down proteomics, which consists on separating some proteins and analysing each one in separate and bottom-up, which consists on digesting a mixture of proteins, typically with trypsin, and analysing the resulting peptides. While top-down is extremely useful for analysing single proteins or simple mixtures, it lags behind bottom-up in terms of proteome coverage, sensitivity and throughput when analysing a whole proteome[46]. As, in this work, an entire proteome will be analysed, the best method is bottom-up proteomics.

Within clinical applications, proteomics has been explored mostly as a tool for biomarker research, as they are the most objective, quantifiable medical signs modern science allows measuring. A biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”, definition created by the National Institutes of Health Biomarkers Definitions Working Group, in 1998[42]. The ideal biomarker to detect a given pathology should be measurable via a simple, reliable, and affordable method and a high diagnostic sensitivity and specificity[43]. Biomarkers typically differentiate an affected patient from a person without the disease.

The alterations can result from diverse factors, such as germline or somatic mutations, transcriptional changes, and post-translational modifications[47]. Biomarkers can be used for diagnosis, monitoring a certain disease progression, predicting recurrence and assessing the efficacy of a treatment[48]. Cancer biomarkers have been heavily searched, motivated by the widespread use of prostate-specific antigen in prostate cancer screening. At the present moment, there is a need for cancer biomarkers with more accurate diagnostic capability, particularly for early-stage cancer[49], as detecting cancer in early stages improves treatment success. To search for new biomarkers, a biomarker study must be developed and some guidelines should be pursued[50].

2.1 Designing a biomarker study

A biomarker study should have samples from the disease in study and controls. Controls should not be exclusively healthy subjects, but patients with related or similar diseases, to avoid low specificity when doing blind tests. In this work, volunteers with Low Urinary Tract Symptoms (LUTS) and with no urothelial conditions (nUC) will be the controls. LUTS includes storage and voiding symptoms and postmicturition, such as urgency, frequency, poor and intermittent stream[51]. These symptoms can be similar to bladder cancer ones[21], therefore using this condition as a control may contribute to find a BCa specific biomarker.

Samples from individuals with unclear or mixed diagnoses may be omitted, and clinical information on volunteers should be known, namely age, gender, ethnic background, and detailed status of the disease or condition under investigation, as well as relevant physiological parameters, comorbidities, and current medications or treatment[52].

Choosing the biologic sample to be analysed is also an important step. The biologic sample used to detect possible biomarkers should be stable, easy to collect and comfortable for the patients. Serum, plasma and urine are some of the used biological samples, being urine the most used for biomarker research on bladder cancer, as it can be seen in **Figure I.4**. Search parameters and exclusion criteria are presented on **Supplementary Table VI.1**, **Supplementary Table VI.2** and **Supplementary Table VI.3.**

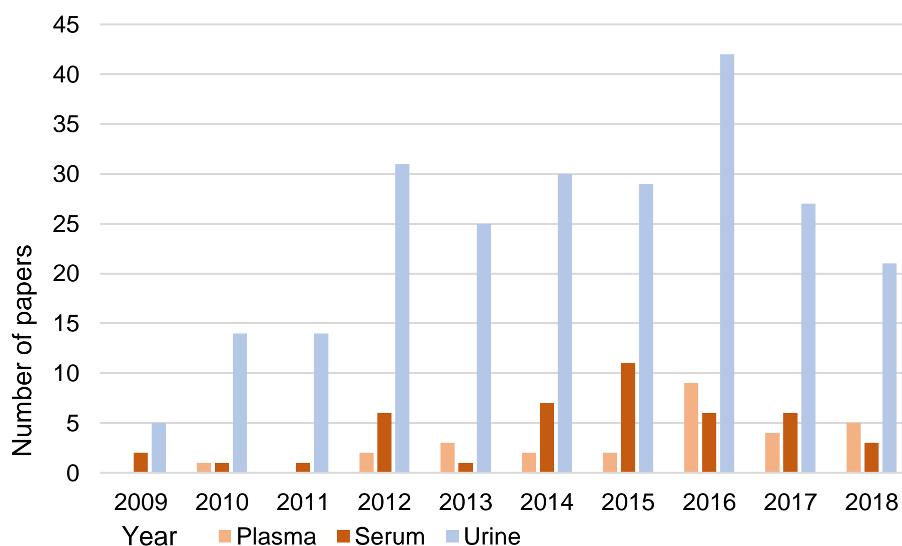


Figure I.4: Column graph showing number of studies on biomarkers for bladder cancer detection or recurrence using plasma, serum or urine, per year in the last ten years. This was the result of a bibliographic search on Scopus.

Urine is a non-invasive sample, stable, as samples can be stored for several years at -20°C[53], available in high quantity, and it involves simple sample preparation procedures[54]. In the search for BCa biomarkers, urine could be a good biological sample, as is in direct contact with tumour cells. Furthermore, as 70% of proteins in urine are derived from the urogenital tract[55], is to be expected that, if some changes occur in the bladder cells, those changes will reflect in urine.

2.2 Mass Spectrometry as a tool for proteomics studies

Mass spectrometry has been widely used to analyse biological samples and has evolved into an indispensable tool for proteomics research[56]. Currently, mass spectrometry is the main technique being applied to proteomic studies and it will also be used in this work. The elementary principle of mass spectrometry (MS) is to generate ions from a sample and sort these ions by their mass-to-charge ratio (m/z). A mass spectrometer consists of a sample introduction system, an ion source, a mass analyser and a detector, and the results are displayed as spectra of the relative abundance of detected ions as a function of m/z, under high vacuum conditions.

The functions of sample introduction systems are to produce ions in gas phase from a solid or liquid sample and to introduce enough quantity of the sample into the ion source in such a way that its composition represents that of the original sample. Recent techniques have the sample introduction and ionization process occurring simultaneously. Some sample introduction methods are gas chromatography, liquid chromatography (LC), capillary electrophoresis and having the sample in a solid probe, ionizing the sample is the next step. As proteins and peptides are easily degraded during ionization, only soft ionization methods as Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI) can be used. Illustrated in **Figure I.5 A**, ESI ionizes the analytes out of a liquid solution and is therefore readily coupled to liquid-based separation tools, such as liquid chromatography.

*Figure I.5: Depiction of two of the most common sources of ionization in mass spectrometry: **A**. Electrospray ionization (ESI) and **B**. Matrix-assisted laser desorption/ionization (MALDI). (Adapted from Aebersold et al.[57])*

MALDI is extremely sensitive, easy applicated, relatively tolerant to contamination and allows the acquisition of huge data in a short time, being represented in **Figure I.5 B**. This technique desorpts and ionizes the samples out of a dry and crystalline matrix, and is usually used to analyse relatively simple peptide mixtures. The matrix absorbs laser energy and transfers it to the sample. In this technique is essential to co-crystallize the sample within the lattice of matrix crystals, thus protecting the samples from degradation. The most commonly used matrices for MALDI of proteins, whose structures are in **Figure I.6**, are 3,5-dimethoxy-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. For peptides up to 6kDa, α-cyano-4-hydroxycinnamic acid (CHCA) is an advantageous choice[58].

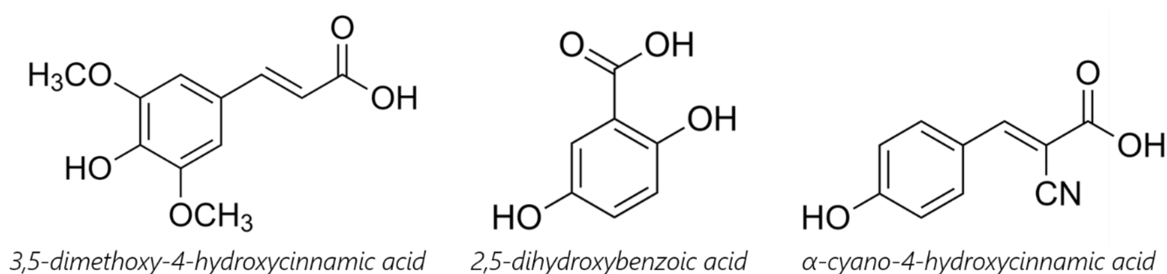


Figure I.6: Molecular structure of the most used matrixes for protein and peptide analysis by MALDI.

Current mass analysers include quadrupole (Q), quadrupole ion trap, Fourier transformed based and time-of-flight (TOF). It is now common to combine two or more analysers within a single instrument (MS/MS) to improve and extend analytical capabilities, such as peptide quantification. The combinations may involve similar analysers, as in a TOF/TOF system, or be of mixed types as in a QTOF instrument.

The time-of-flight instrument is based on the principle that ions of different m/z are dispersed in time, during their flight along a field-free drift path of known length. During a journey of the same length and starting at the same time, lighter ions will arrive earlier at the detector and by measuring the time taken, it is possible to determine the m/z ratio. Ion mirrors, named reflectrons, are applied in TOF instruments, to increase their resolution, and are presented in **Figure I.7 A.**

A linear quadrupole mass analyser consists of four-rod electrodes extending in the z-direction and mounted in a square configuration, as presented in **Figure I.7 B**. As an ion enters the quadrupole assembly in the z-direction, an attractive force is exerted on it by one of the rods with a opposite charge to the ionic one. Only ions of a certain mass-to-charge ratio will reach the detector for a given ratio of voltages: other ions have unstable trajectories and will collide with the rods. This allows the selection of

an ion with a particular *m/z* or the scanning of a range of *m/z*-values by continuously varying the applied voltage. A quadrupole time-of-flight instrument, presented in **Figure I.7 C**, comprehends a quadrupole, operated in either a wide or narrow band pass mode, that determines which ions are passed into a collision cell, being the ions introduced into a time-of-flight analyser through a pusher.

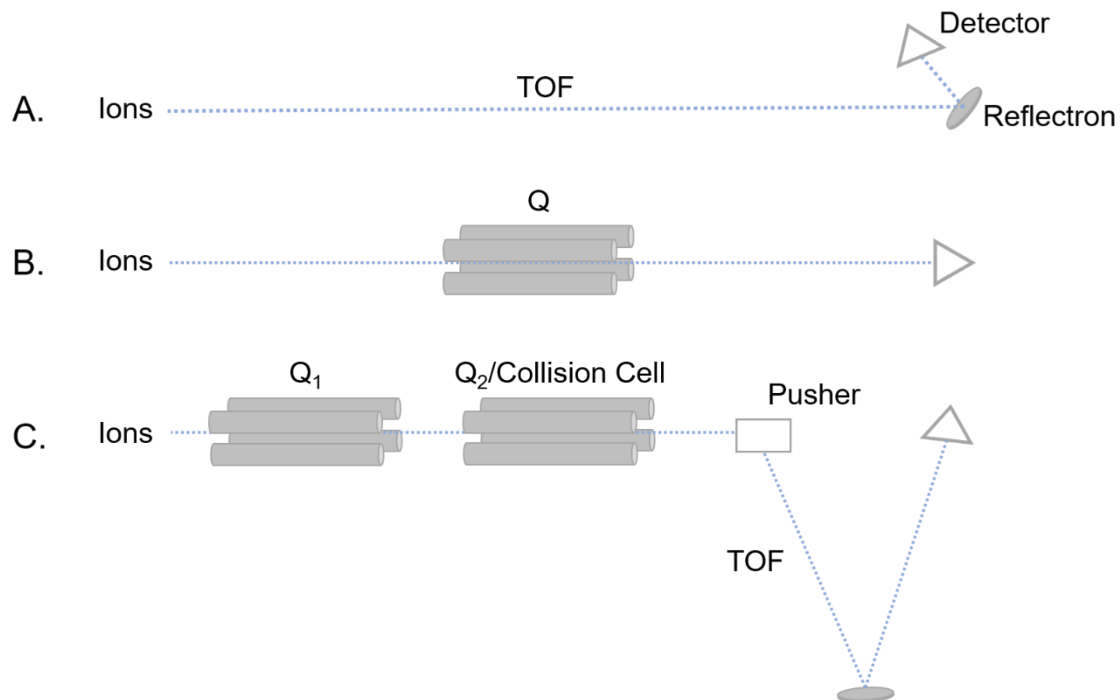


*Figure I.7: Common mass analysers used in proteome research: **A**. Time-of-flight instrument; **B**. Quadrupole analyser and **C**. Quadrupole time-of-flight instrument. (Adapted from Aebersold et al.[57])*

A detector is the component of a mass spectrometer that determines the abundance of the ions of different m/z after they have been sorted by the mass analyser. Electron multipliers are the detectors more common and their basic principle is to convert the ion current into an electric one and to amplify the electric current in order to be measured[59].

2.2.1 Quantitative proteomics

Mass spectrometry is not inherently quantitative, since proteolytic peptides show considerable variability in physiochemical properties, that in turn result in variability in mass spectrometric response between runs. Also, only a small percentage of the total digested peptides in a sample is analysed[60]. Thus, various approaches have been developed to perform relative and absolute proteomic quantitation, namely metabolic labelling, isotopic and isobaric tags, selective reaction monitoring and label-free quantification (LFQ).

Label-free quantitation is an ideal strategy for large-sample analyses in clinical screening or biomarker discovery experiments, because of the ease of experimental setup, relatively low cost and it allows to identify a larger number of proteins with a wider dynamic range of detection in comparison to

labelling methods[61]. The strategy consists of analysing samples sequentially and discretely, relying the relative quantitation on ion-counting or intensity measurements by the MS.

There are two main approaches for label-free quantification: (i) ion counting-based LFQ and (ii) intensity-based LFQ.

(i) In ion counting LFQ or spectral counting method, protein quantification is determined by counting the number of MS/MS spectra identified for a particular protein. The rationale behind ion-counting LFQ is that more abundant proteins will produce a larger number of peptides, which are more likely to be sampled resulting in a higher number of spectra[62]. Despite the strong correlation between protein abundance and spectral count, the use of dynamic exclusion to increase the chance of detecting low-abundance peptides has a deleterious effect on protein quantification by spectral counting[61].

(ii) In intensity-based LFQ quantification is performed on the precursor signal intensity from the extracted ion chromatogram (XIC). Therefore, robust and reproducible chromatographic separation is required for more accurate peptide assignment and correct quantification. For this reason, retention time alignment is a critical step in intensity-based LFQ especially when a large number of samples are analysed[62]. Intensity normalization based on total ion count is another required step to minimize bias in signal intensity. A critical issue with intensity-based LFQ is that peak identification; noise reduction, retention time alignment, peak intensity calculations, and normalization require significant computational power.

There are increasing literature comparing ion counting-based and intensity-based LFQ showing that intensity-based LFQ methods are consistently more sensitive and more accurate than ion counting LFQ [60]. For this reason, in this work label-free quantification will be carried out by measuring the signal intensities of the precursor ions.

Another important issue that affects the performance of LFQ is the method used for data acquisition. Nowadays there are two methods for LC-MS/MS proteomics analysis: Data Independent Acquisition (DIA) and Data Dependent Analysis (DDA). In DIA, the full m/z range is divided in smaller 25 m/z windows, and all the ions within the selected m/z range are fragmented and analysed in the second stage of tandem mass spectrometry. DIA comprehensively and repeatedly samples every peptide in a protein digest, producing a complex set of mixed fragmentation patterns, where a single spectrum contains fragments from multiple peptides. This type of spectra cannot be interrogated by search engines such as MASCOT or Andromeda. For this case, spectral libraries are required to retrieve identification. These spectra libraries are constituted by sets of annotated and refined peptide-MS/MS spectrum matches from DDA experiments. There are human spectral libraries available: the 2010 Human Plasma PeptideAtlas is a comprehensive collection of high-confidence peptide and protein identifications and more biofluids' libraries are being developed, namely urine[63,64].

In DDA, Peptide signals that rise above a determined threshold are selected in the first mass analyser, fragmented in the collision cell, and their MS/MS data is obtained in a second mass analyser. In DDA, each MS/MS spectra only contain fragments of a single peptide, facilitating identification through search engines and *in silico* databases. Relative quantification is achieved either by ion counting-based or intensity-based LFQ. Based on the observed linear correlation between the peak area of measured

peptides and their abundances, peptides can be quantified through the signal intensity ratio of their corresponding counterparts compared among MS runs[65].

In DDA, MS and MS2 spectra are obtained in the same run, with MS2 acquisition being dependent on MS results. In a typical DDA, it is often observed several molecular features that cannot be identified, just because they were not selected for fragmentation. On the other hand, because precursor selection obeys to a predetermined intensity threshold, it is common to observe missing values for some proteins. These issues are less observed in DIA. However, as DIA depends on pre-existing spectra libraries, it is limited to detect peptides that were previously detected by DDA, therefore decreasing its application for biomarker discovery in biological samples that are not human serum. As this work will be focused on biomarker discovery in urine, LC-MS/MS label-free quantification will be applied using DDA.

3. Application of proteomic approaches to detect bladder cancer

Proteomic approaches are promising techniques for research on biomarker candidates, due to high sensitivity, outstanding analytical performance and the ability to generate large datasets through the identification of high number of proteins. During the past years, searching for novel candidates for early detection, monitoring, and prognosis of bladder cancer has received a rapidly growing interest, as proteomic patterns in body fluids, including in urine, present new opportunities for identification of novel, highly sensitive specific markers for early detection of cancer[66].

A review of the literature was conducted using an electronic search to identify urinary proteomic biomarkers studies to detect BCa. On 19th April 2018, there were 125 records on Web of Science retrieved from the following search: "TOPIC: bladder cancer" OR "urothelial cancer" OR "urothelial carcinoma", TOPIC: urine, TOPIC: proteom*, DOCUMENT TYPE: article, published between 2008 and 2018. From that, 19 manuscripts were selected as they were studies employing peptidomic approaches to search for urinary biomarkers on bladder cancer (listed on **Supplementary Table VI.4**).

Within the selected studies, MALDI-TOF and LC-MS/MS were the used techniques and urine samples were from volunteers with BCa, healthy individuals and sometimes, volunteers with other urinary conditions. All the proteins listed as differentially expressed between BCa patients and controls were grouped, and proteins that were increased in BCa patients in two or more studies are presented in **Figure I.8**.

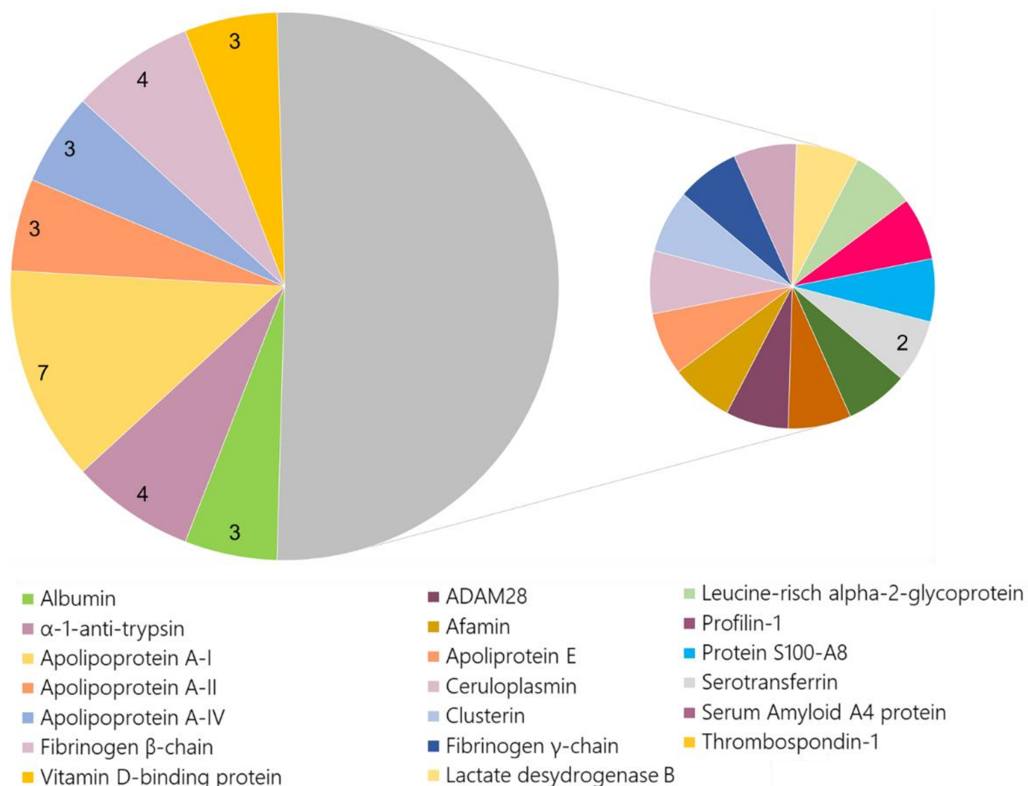


Figure I.8: Candidate Urinary Proteomic Biomarkers of Bladder Cancer; each candidate biomarker was reported at least by two urinary proteomic studies, and the charts pie number corresponds to the number of papers.

Apolipoprotein A-I, Fibrinogen β chain and α-1-antitrypsin are the most reported candidates on urinary proteomic biomarkers of bladder cancer, yet none has a straight connection with the disease molecular physiology. Lipoproteins are considered to have an indirect role in promoting tumour survival through kinase activation, and in the development of tumour angiogenesis[67,68]. However, lipoproteins were linked to hepatocellular carcinoma[69], and breast cancer[70]. Fibrinogen has been confirmed as a bladder malignancy associated protein as it is related with metastatic potential[71], though studies reported that its specificity was lower than urine cytology[72,73]. Alpha 1-antitrypsin plays a critical role in modulating immunity, inflammation, apoptosis, and possibly cellular senescence programs[74], but as lipoproteins, it has been reported as a candidate biomarker for other *neoplasias*[75,76] already published studies is that the goal is only to detect bladder cancer, while a biomarker or a biomarker-panel capable of distinguishing stages Ta, T1 and T2 would be valuable, as the treatment for these stages is different[21].

4. Aims of this Project

This work aims:

- ✓ to continue the reception of urine, collected at the Hospital São José from volunteers;
- ✓ to design a protein biomarker study to search for urothelial carcinoma biomarkers in urine;
- ✓ to treat and digest urine samples, using filter aided sample preparation;
- ✓ to analyse samples by liquid chromatography coupled to high-resolution tandem mass spectrometry;
- ✓ to determine significant changes across biological condition using MaxQuant and Perseus;
- ✓ to interrogate data with Cytoscape to disclose samples most active pathways;
- ✓ to develop a protein biomarker panel to detect bladder cancer's presence;
- ✓ to develop a protein biomarker panel that can measure bladder cancer staging.

Chapter II. Methods

1. Material

1.1 Apparatus

An analytical scale from Adam equipment (Oxford, United Kingdom), model PW124 was used to measure reagents weight. A minicentrifuge, model Spectrafuge-mini, from Labnet (Madrid, Spain), and a minicentrifuge-vortex, model Sky Line, from ELMI (Riga, Latvia) were used throughout the sample treatment. A centrifuge from MPW (Warsaw, Poland), model MPW-350 was used to centrifuge urine and a centrifuge Labnet Prism Microcentrifuge C2500-R-230V from Labnet (New Jersey, USA) was used to centrifuge urinary proteins. Centrifugal filters from Sartorius (Göttingen, Germany), model Vivaspin 15R, 10,000 MWCO Hydrosart and model Vivaspin 500, 10,000 MWCO PES were used to filter proteins and peptides higher than 10kDa from urine. A vacuum concentrator centrifuge from UniEquip (Martinsried, Germany) model UNIVAPO 150 ECH with a refrigerated aspirator vacuum pump model Unijet II was used for sample drying.

Quantification was performed using a 96-well plate (Digilab-Genomic Solutions, USA) and microplate reader ultraviolet/visible radiation spectrometer from BMG Labtech (Offenburg, Germany), model CLARIOstar. A liquid chromatograph from Thermo Fischer Scientific (Massachusetts, USA), model EASY-nLC 1200 was used to separate peptides within a sample and a mass analyser from BRUKER DALTONIK GmbH Life Sciences (Bremen, Germany), model Ultrahigh Resolution Quadrupole Time-Of-Flight (UHR-QTOF) IMPACT HD was used to analyse the peptide content of the samples.

1.2 Standards and reagents

Water was purified in Millipore's Milli-Q Synthesis system. All reagents used were LC-MS grade. Acetonitrile (ACN) from Carlo Erba Reagents (Val-de-Reuil, France), Formic Acid (FmA) from Honeywell Fluka (New Jersey, USA) and Trifluoroacetic acid (TFA) from Sigma-Aldrich (Missouri, USA) were used almost through all the experimental procedures. Ammonium Bicarbonate (AmBic), Bovine Serum Albumin (BSA) from Sigma-Aldrich (Missouri, USA) Bradford Reagent from Sigma-Aldrich (Missouri, USA), Dithiothreitol (DTT) from Nzytech (Lisbon, Portugal), Iodoacetamide (IAA) from Sigma-Aldrich (Missouri, USA) and Pierce Trypsin Protease, MS Grade from Thermo Scientific (Massachusetts, USA) were used in protein digestion steps. α-cyano-4-hydroxycinnamic acid (CHCA) and $(NH_4)H_2PO_4$ were purchased from Sigma–Aldrich (Missouri, USA), being necessary for the MALDI matrix preparation. Boric Acid was purchased from Sigma-Aldrich (Missouri, USA).

Pierce Quantitative Colorimetric Peptide Assay from Thermo Scientific (Massachusetts, USA), was purchased to quantify the total amount of peptides in each sample.

2. Patient and tumour characteristics

A total of 175 urine samples have been collected at Urology Department of Hospital São José since 2015 and continuously during this thesis, from adult volunteers with bladder cancer and volunteers with other urological conditions: LUTS and nUC, being all groups used on this work presented on **Table II.1**.

TURB was used to assess the presence of bladder cancer as well as its stage and grade. Exclusion criteria included urinary cancer history, unclear bladder cancer diagnosis or mixed bladder cancer (adenoma and papillary), HIV affected and organ transplant receivers, in recent chemo/radiotherapy (6 months) and these criteria were developed based on another biomarker studies[77–81]. Urine samples with an observable presence of blood were also excluded, to avoid a major presence of blood proteins in samples results. Only men were selected, as the urinary proteome between man and woman is different[82] and BCa is more prevalent in man[4].

Table II.1: Groups of the study

Group		Description
BCa	Ta	Only Low-Grade Tumours
	T1	Only High-Grade Tumours
	T2+	T2, T3, T4 Patients which T2 stage was assessed by TURB but it was not possible to assess if a higher stage was present
Controls	nUC	With no urinary condition
	LUTS	

After exclusion criteria, only 6 volunteers with BCa stage T1 remained, and to be easier to compare statistically, only 6 volunteers were selected *per* group and their average age is presented in **Table II.2**. More detailed information of the volunteers is present in **Supplementary Table VI.5.**

Table II.2: Patient cohort and sample sizes involved in the biomarker study

Group	Sample size n=	Medium Age (Age Interval)	
BCa:	**18**	**74 ± 2**	**(61 – 91)**
Ta	6	72 ± 10	(63 – 84)
T1	6	75 ± 6	(65 – 81)
T2+	6	76 ± 11	(61 – 91)
Controls:	**12**	**69 ± 2**	**(56 – 78)**
nCU	6	70 ± 9	(56 – 78)
LUTS	6	68 ± 6	(61 – 75)

3. Urine Sample Preparation

Urine was collected by nurse Juliana João, at São José Hospital, since 2015. Urine was usually collected between 9 AM and 2 PM, as it depended on the time of the day that the volunteers entered the hospital. The samples were collected to 50 mL tubes, where previously 38 mg of boric acid was

added, to a final concentration of 20 mM of boric acid in urine[83]. Urine samples presenting gross haematuria were excluded. Urine samples were refrigerated until they were centrifuged at 5,000 g for 20 minutes, to remove cellular components. 30 mL of supernatant was collected to Falcon tubes, 10mL per tube. The samples were kept in the freezer (-60ºC).

10 mL of miliQ water (MQ-H_2O) was added to Vivaspin tubes and centrifugation at 5000 g for 10 minutes was applied to remove traces of glycerine. After discarding the flow-through, 10 mL of urine sample was added and centrifugation at 5000 g for 20 minutes was applied. The retentate and the flow-through were stored at -80 ºC separately. The retentate, corresponding to proteins and peptides higher than 10 kDa, was quantified using the Bradford Method and BSA to make the standard curve.

4. Filter Aided Sample Preparation

To purify and digest proteins, a developed protocol of Filter Aided Sample Preparation (FASP) was applied[84–86]. Urine protein was quantified using the well-known Bradford Method, using Bovine Serum Albumin to perform the standard curve. Depending on urine's availability, between 50 and 100 µg of protein was diluted with MQ-H_2O to perform 200uL, 200 µL of Urea 2 M, Tris-HCl 75 mM, NaCl 100 mM, 0.02% SDS was added and they will be named as diluted urine. Two replicates of each urine sample were prepared. Centrifugation filters of 400 µL and 10 kDa cut-off were used to filtrate proteins from urine samples, and the method is presented in **Figure II-1**.

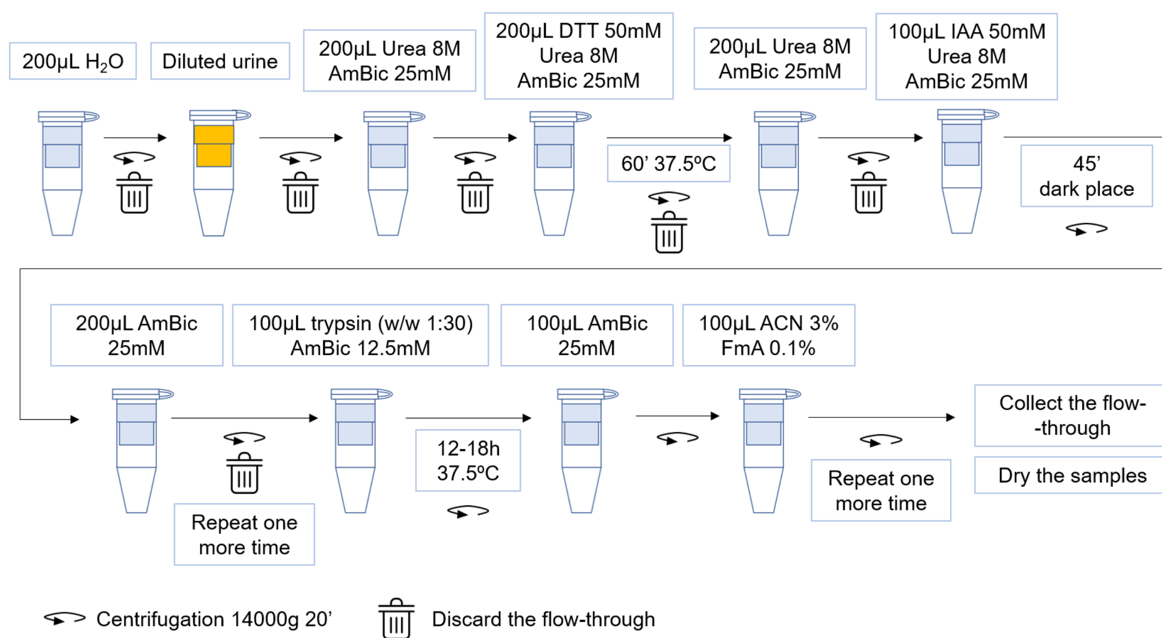


Figure II.1: Workflow of Filter Aided Sample Preparation step

1,4-Dithreithol (DTT) was used to reduce disulphide bonds and Iodoacetamide (IAA) was used to alkylate proteins thiol groups. When needed, centrifugation was applied for more than 20 minutes, to assure that all volume was drained. As in **Figure II-1**, after centrifugation, the flow-through was collected and evaporated in a vacuum centrifuge.

The peptide content of samples was identified using the Pierce Quantitative Colorimetric Peptide Assay, that bases on copper reduction by peptides and colourimetric absorption of a chelator when complexed with cuprous ions. The method was done following the assay instructions[87]. Standard solutions were prepared using the peptide digest assay standard (1000 µg/mL), according to **Table II-3**. As there was no interference with ACN and FmA, the standards were prepared in water.

Table II.3: Preparation of diluted peptide digest assay standards

Standard	Concentration (µg/µL)	Volume of Reagent (µL)	Volume of MQ-H_2O (µL)
A	Not prepared, as its concentration is far superior to digested protein content		
B	500	60	60
C	250	60 of B	60
D	125	60 of C	60
E	65	60 of D	60
F	31.3	60 of E	60
G	15.6	60 of F	60
Blank	0	0	120

The working reagent was prepared combining 50 parts of reagent A, 48 of reagent B and 2 parts of reagent C, all from the assay. 20 µL of each standard and sample were pipetted two times into the 96-well plate, followed 180 µL of working reagent. The plate was incubated at 37.5 ºC for 15 minutes and the absorbance at 480 nm was read.

Freeze-thaw cycles should be avoided and even as 4 cycles don't change urine proteome[88], freeze-thawing was avoided when possible. Urine samples were exposed to no more than three freeze-thaw cycles. After FASP, digested peptides samples were exposed from one to two freeze-thaw cycles, due to variations of drying time in the vacuum centrifuge.

5. LC-MS/MS Analysis

MS analysis was performed using UltiMate 3000 ultra-high performance liquid chromatographer, from Thermo Scientific, coupled to UltraHigh Resolution Quadrupole Time-Of-Flight (UHR-QTOF) IMPACT HD mass spectrometer, from Bruker.

3 µL of the sample with a total peptide concentration of 0.1875 µg/µL were loaded onto a Trap column Acclaim PepMap100, 5 µm, 100 Å, 300 µm i.d. × 5 mm and desalted for 5 min from 3% to 5% B (B: 90% acetonitrile 0.08% FA) at a flow rate of 15 µL min^{-1}. Then the peptides were separated using an analytical column Acclaim™ PepMap™ 100 C18, 2 µm, 0.075 mm i.d x 150 mm with a linear gradient at 300 nL min^{-1} (mobile phase A: aqueous FA 0.1% (vol/vol); mobile phase B 90% (vol/vol) acetonitrile and 0.08% (vol/vol) FA) 5-90 min from 5% to 35% of mobile phase B, 90-100 min linear gradient from 35% to 95% of mobile phase B, 100-110 95% B. Chromatographic separation was carried out at 35 ºC. MS acquisition was set to cycles of MS (2 Hz), followed by MS/MS (8–32Hz), cycle time 3.0 seconds, active exclusion, exclude after one spectrum, release after 2 min. The precursor was reconsidered if its current intensity was 3.0 higher than the previous intensity and intensity threshold for fragmentation of 2500 counts.

All spectra were acquired in the range 150–2200 m/z. LC-MS/MS data were analysed using Data Analysis 4.2 software (Bruker). Proteins were identified using Mascot (Matrix Science, UK). MS/MS spectra were searched against the Swiss-Prot database 57.15 (515,203 sequences; 181,334,896 residues), setting the taxonomy to *Human* (20,266 sequences). The following parameters were applied: precursor mass tolerance of 20 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation set as fixed modification and methionine oxidation, as variable modification. False discovery rate (FDR) was estimated by running the searches against a randomized decoy database. Quality control of the LC-MS/MS was performed every week using the HeLa cells protein digest, to assure excellent chromatographic conditions, and therefore to minimize shifts in elution time of the same peptide between analytical runs. Results of the identification step were filtered to proteins with a FDR below 0.01.

Label-free quantification was carried out using MaxQuant software V.1.6.0.16. All raw files were processed in a single run with default parameters[89]. Database searches are performed using Andromeda search engine with the UniProt-SwissProt Human database as a reference and a contaminants database of common contaminants. Data processing was performed using Perseus 1.6.2.2[90]. In brief, protein group LFQ intensities were log2-transformed to reduce the effect of outliers. To overcome the obstacle of missing LFQ values, missing values were imputed before fitting the models. Log ratios were calculated as the difference in average log2 LFQ intensity values between the two digestion methods tested (two-tailed, Student's t-test). A protein was considered statistically significant if its fold change was ≥ 1.5 and FDR ≤ 0.05[91].

6. Data analysis

Perseus 1.6.2.2 was used to transform, by Log 2, normalize Label-Free Quantification (LFQ) intensities using the Z-score and to obtain clusters, using average linkage, no constraint, preprocess with k-means and euclidean distance between column trees. Two-sample Student's T-tests, both sides, were performed to identify significant proteins that are upregulated and proteins that are downregulated between the two groups being compared. The proteins were considered significant if the False Discovery Rate (FDR) was lower to 0.05 and if S0 was lower than 1.5. Volcano plots were plotted to present significant proteins, using Student's T-test difference between two groups as x axis, and -Log Student's T-test p-value as y axis.

ClueGO v 2.5.1, a plugin of Cytoscape 3.6.1, was used to unveil the most active pathways. The following analysis parameters were defined for ClueGO: function; two sample lists were loaded, one per cluster; the organism selected was Homo sapiens and the type of ids used was Accession ID. Clusters were used in order to obtain a deep comparison between the different clusters; The Ontologies/Pathways selected were: GO_BiologicalProcess and KEGG_pathways. "All Evidence codes" box was chosen; Regarding network specificity, it was set as "detailed"; The box of "Use GO Term Fusion" and "Show only pathways with p-value ≤ 0.05" was also chosen. GO Tree Interval it was set to Min Level 3 and Max Level 8. Selection criteria for the terms that have associated genes from cluster 1 was set as min 3 genes/term and minimum 4% from all the Genes associated with the term.

The same values were chosen for the cluster, and OR was selected and “is specific” was set to 60%. The kappa score of GO Term/Pathway Network Connectivity was set to 0.4. Within the statistical options: Enrichment/Depletion (Two-sided hypergeometric test), and Bonferroni step down were selected. Within the grouping options, the “Use GO term grouping” option was selected, the group colouring chosen was fix, kappa score selected, Leading group term based on highest significance, kappa score was selected, the initial group size was set to 1, and percentages of genes and terms for group to merge were both set to 50%. Single nodes, with no connection to another node, were not considered for analysis.

To perform heat maps, normalized Z-score values of LFQ intensities of each sample were used. The normalization was applied to each row of intensities for one protein, within each comparison. Colouration was applied to be blue for the negative relative intensity (most intense blue for -2.5 or less, red for 2.5 and more, white for 0) and red for the positive relative intensity. The average value of LFQ intensity per protein was determined for each stage group, as well as the correspondent confidence interval at 95%.

Chapter III. Results and Discussion

A typical shotgun proteomics workflow involves protein extraction from tissues, cells, or body fluids, enzymatic digestion and LC-based peptide fractionation in one or multiple dimensions followed by MS-based protein identification. To extract and solubilise proteins, detergents, namely SDS, and chaotropic reagents, such as urea, are generally used. Solubilized protein mixtures are not directly applied to in-solution digestion because the presence of detergents and chaotropic reagents reduces proteolytic enzyme's activity, which, in consequence, decreases the number of available peptide analytes and significantly suppresses signal in LC-MS experiments. By using a membrane to separate the proteins contained in a liquid biopsy, the Filter Aided Sample Preparation -FASP- method facilitates removal of detergents and chaotropic reagents, as well as it allows to use the solutions for reduction, alkylation and digestion steps[86] is an easy and straightforward way as the solutions can be easily removed by centrifugation. The first step before digesting the proteome, samples were quantified via a Bradford protein assay. Quantification results can be seen in **Supplementary Table VI.6**, and the corresponding calibration curves can be seen in **Supplementary Table VI.7**. The total proteome content was determined in order to determine the appropriate amount of trypsin for efficient digestion. After protein digestion the peptide concentration in each sample was determined by Pierce Quantitative Colorimetric Peptide Assay, using a peptide calibration curves shows in **Supplementary Table VI.8**. The results of peptide quantification can be seen in **Supplementary Table VI.9**.

Figure III.1 shows the cluster of all samples used in this study. BCa stage T1 and LUTS are all grouped, BCa stage T2+ is all group except for JM-39, BCa T1 has two samples in separate, RM-55 and AR-35, both having as first neighbour a nUC sample.

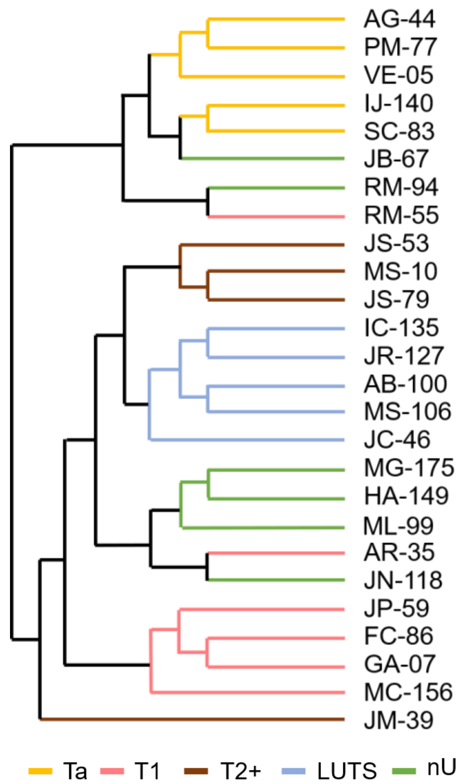


Figure III.1: Hierarchical-cluster of all samples used in this biomarker study.

After protein digestion, the resulting pools of peptides were quantified and analysed by nanoLC-MS/MS. During, LC-MS data acquisition, some samples did not originate an adequate total ion chromatogram and therefore were discarded. The samples discarded were: JS-04 replicate 1 and 2, MM-45 replicate 1 and 2, OR-169 replicate 1 and 2, AB-37 replicate 1 and PM-77 replicate 1.

1. Biomarkers detecting the presence of cancer

When developing a biomarker panel to detect BCa, the first goal is to pinpoint biomarkers capable of identifying the presence of BCa. To accomplish that, two control groups, nUC and LUTS, will be compared to BCa samples.

1.1 nUC and LUTS *vs.* Ta

LFQ data resulting from MaxQuant analysis were interrogated using Perseus software in order to determine the statistical differences between nUC *vs.* Ta and LUTS *vs.* Ta. The Student's T-Test and the –Log Student's T-test p-value were plotted to generate volcano plots.

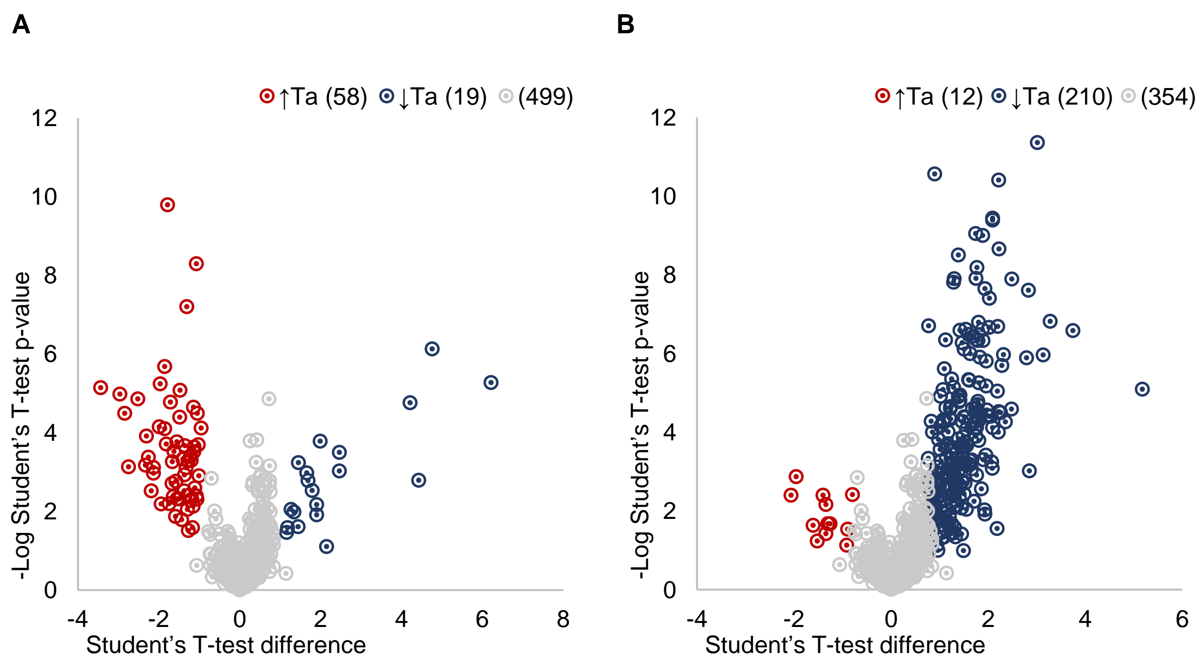


Figure III.2: Volcano plots showing the differences between proteins quantified by MaxQuant of two groups; ***A:*** *nUC and Ta;* ***B:*** *LUTS and Ta. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.*

In **Figure III.2**, it is possible to observe that the majority of protein content is not significantly different. There are several proteins whose abundance is different if BCa stage-Ta is compared to nUC and also different if BCa stage-Ta is compared to LUTS (s0 ≥ 1.5 and FDR ≤ 0.05). Those are the proteins that could be potential biomarkers. **Figure III.2, B**, shows that when cancer is compared to LUTS the number of up-regulated proteins is larger than when cancer samples are compared to healthy individuals.

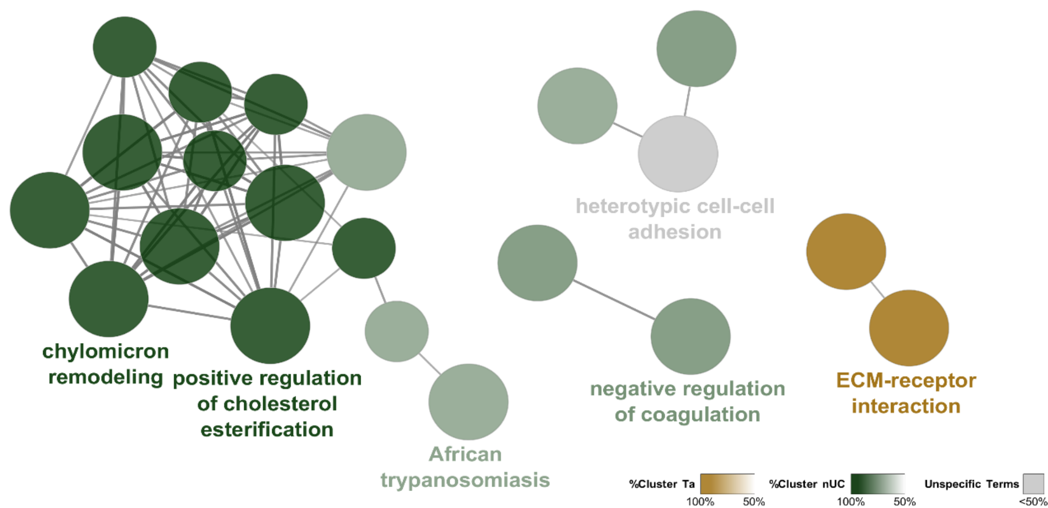


Figure III.3: Comparison of nUC versus Ta. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster Ta stage of bladder cancer are marked yellow and for cluster nUC are marked green. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison nUC vs. Ta (Figure III.2, A) were used to generate this clusters.

Observing the volcano plot in **Figure III.2**, there are 210 proteins upregulated in Ta when compared to nUC. **Figure III.3** shows more upregulated pathways in nUC when compared to Ta, that is, more downregulated proteins in Ta when compared to nUC. Several nodes that were the result of upregulated proteins in Ta were single and therefore discarded. As Ta is an initial stage of BCa, there is a smaller contribution from tumour cells to urine proteome than in later stages. Thus, proteins from single nodes could be associated with other proteins that were not identified. Extracellular Matrix (ECM) is one of the main components affected by cancer because its dysregulation allows cancer cells survival and proliferation[92]. Thus, it is not unexpected to have some changes on ECM level on Ta samples.

In nUC, there is negative regulation of coagulation and response to wounding comparing to Ta, which means that proteins related to negative regulation of coagulation are decreased in nUC when compared to Ta. This may suggest that there is an increase in coagulation activation in BCa. The pathogenesis of blood coagulation activation in cancer is complex and multifactorial. Nevertheless, a unique feature in malignancy is the role played by the expression of tumour cell-associated clot promoting properties[93].

Dysregulation of cholesterol is a common characteristic of cancer[94]; thus it is expected to see differences on cholesterol metabolism, which includes chylomicrons remodelling, as chylomicrons are constituted by triglycerides, phospholipids, cholesterol, and proteins, and they transport dietary lipids.

African trypanosomiasis is identified together with two more GO terms: hydrogen peroxide metabolic process and cellular oxidant detoxification. African trypanosomiasis is the GO term within the group with more proteins associated, but that does not mean that the individuals belonging to nUC group have this pathology. This results from ClueGO always showing the GO term with more proteins associated, within a group of GO terms. Looking at oxidants' metabolism, a GO Term linked to African trypanosomiasis,

cancer has been long linked to oxidative stress, due to an increase in DNA mutations or damage, genome instability and cell proliferation[95].

Heterotypic cell-cell adhesion is a term identified as being present in both clusters. Thus, it is not a relevant GO term to link to BCa or to look for biomarkers.

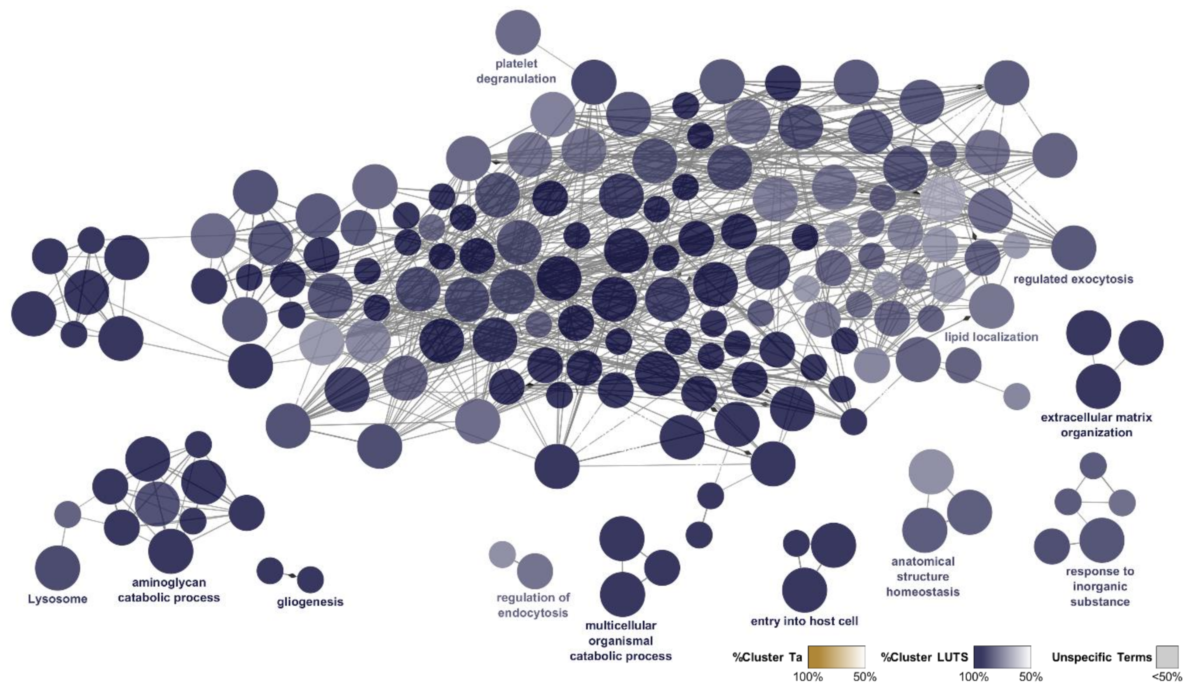


Figure III.4: Comparison of cancer LUTS versus Ta. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster Ta stage of bladder cancer are marked yellow and for cluster LUTS are marked blue. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison LUTS vs. Ta (Figure III.2, B) were used to generate this clusters.

In the integrative analysis presented in **Figure III.4**, only GO terms from upregulated proteins in LUTS, when compared to Ta, appear on ClueGO analysis. Regulation of immune response, negative regulation of leukocyte activation and cytokine production are all changes related to the immune system. As cancer is an inflammatory pathology, and uses immune system characteristics to its advantage, namely to promote tumour growth and to aid in tumour immune evasion[96]. Negative regulation of blood coagulation is downregulated in Ta when compared to LUTS as when compared to nUC. More GO terms related to coagulation, such as complement, and coagulation cascades and plasminogen activation are present. These pathways were already referred as altered in cancer cells. Zymogen activation, which consists of activating enzyme inactive precursors is one pathway altered in BCa. One example of proteins that are produced as zymogens, requiring a reaction to being active, are proteases. Several proteases' activity is severely changed in cancer cells, as a result of the progression to malignancy being frequently related with dysregulation of the normal mechanisms that regulate proteolysis[97]. As such, zymogen activation's dysregulation may reflect a proteolytical dysregulation. Lipid localisation is related to cholesterol and chylomicron remodelling, such as in the comparison nUC *vs.* Ta. Regulation of endocytosis and exocytosis is as well upregulated in LUTS. Alterations of endo/exocytic proteins have long been

associated with malignant transformation[98], which explains the difference in proteins associated with mechanisms of bulk transport's abundance in the two groups. Also, the existence of dysregulations on the extracellular matrix organisation and on the aminoglycan process will affect the cell capacity to engulf and expel molecules.

Even if the principal GO term does not seem related to the comparisons between nUC *vs.* Ta and LUTS *vs.* Ta, in the secondary GO terms, there is a lot of overlapping. Cellular oxidant detoxification, aminoglycan process, ECM-related regulation, chylomicron remodelling, and lipids regulation are some of the biologic processes in common. Proteins common to both comparisons, Ta *vs.* nUC and Ta *vs.* LUTS, with the same behaviour and present at least in one of the pathways obtained by ClueGO, were selected and heat maps were generated, being presented in **Figure III.5.**

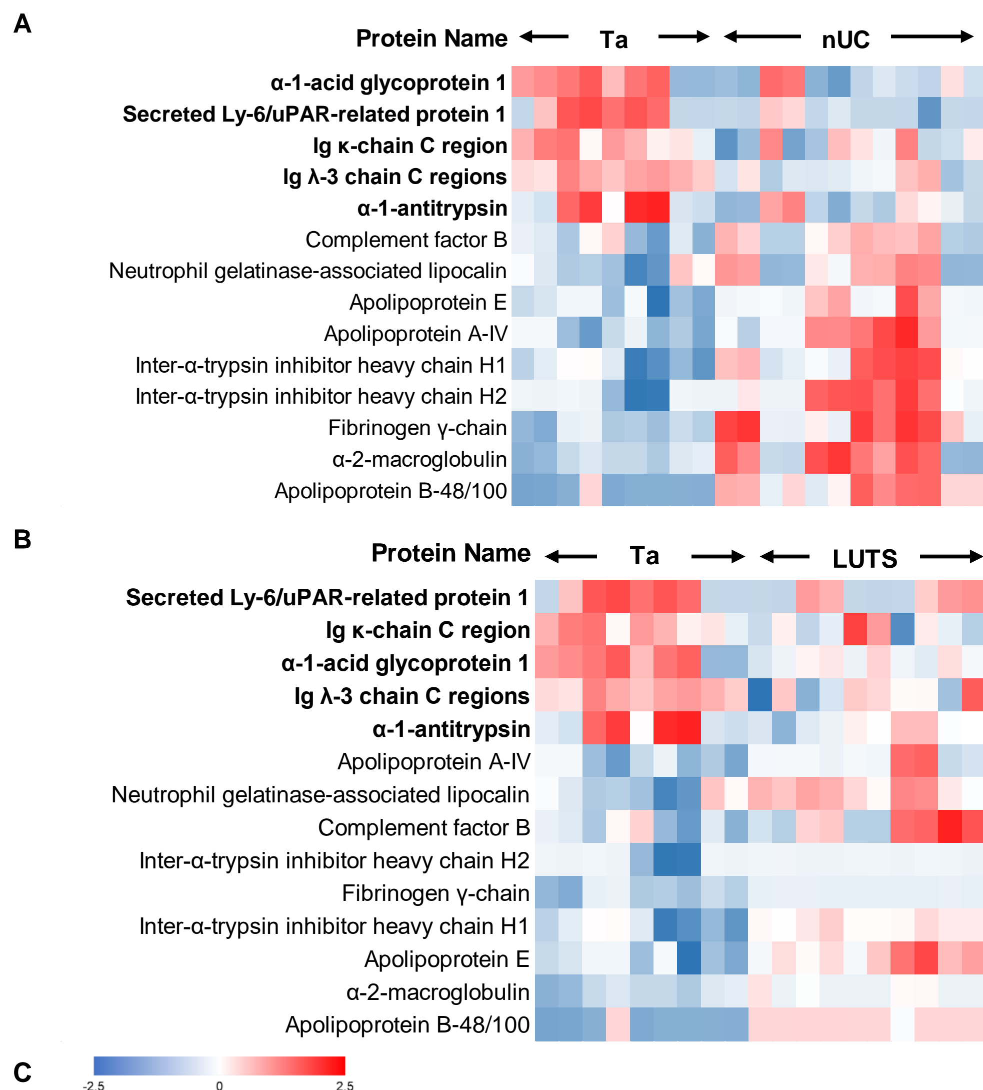


Figure III.5: Heat maps of transformed and normalized LFQ intensities (using Z-score) of significant proteins which were identified using the Student's T-test, and that were common to comparisons nUC vs. Ta and LUTS vs. Ta. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. ***A:*** *Relative intensities of proteins in nUC and Ta samples;* ***B:*** *Relative intensities of proteins in LUTS and Ta samples.* ***C:*** *Heat map scale; red marks the upregulated proteins and blue the downregulated proteins.*

Figure III.5 shows the heat maps for the proteins selected as mentioned in the previous paragraph. Such maps address that the proteins indicated behave similarly when TA cancer samples are compared with healthy or LUTS samples.

1.2 nUC and LUTS *vs.* T1

Once the Ta stage was compared with nUC and LUTS we move forward to compare T1 versus nUC and LUTS. Next, controls were compared to the intermediary stage, T1 and they are presented in **Figure III.6.**

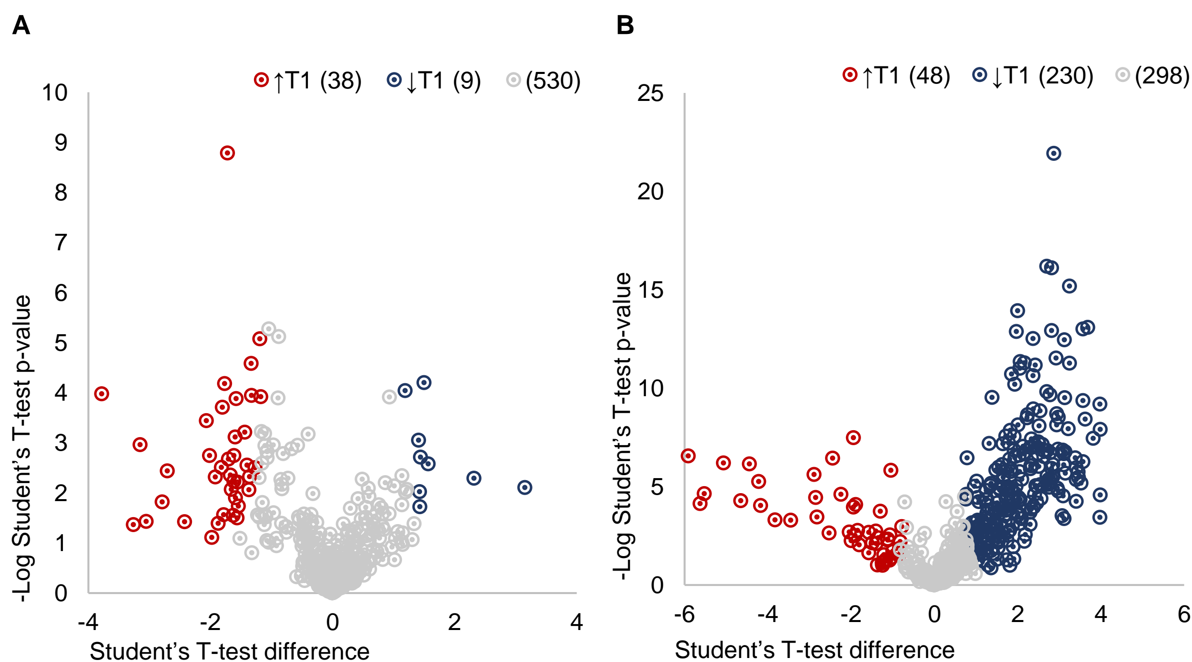


Figure III.6: Volcano plots showing the differences between proteins quantified by MaxQuant of two groups; ***A:*** *nUC and T1;* ***B:*** *LUTS and T1. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.*

Volcano plots presented in **Figure III.6** disclose significant proteins abundance in both comparisons. There are not many proteins distinguishing nUC from T1. Nevertheless, there are 47 proteins, and each one of those could be a potential biomarker. In the comparison LUTS *vs.* T1, there are more proteins whose abundance is significantly different.

Figure III.7 shows the results of the ClueGO analysis, and only pathways upregulated in T1 samples when compared to nUC ones, gathered 3 or more significant proteins.

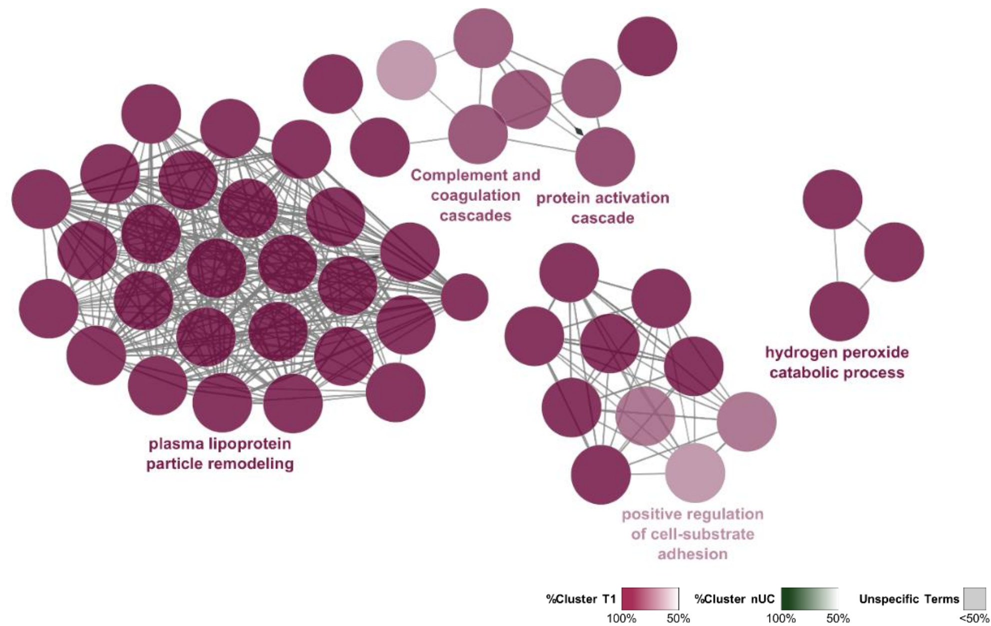


Figure III.7: Comparison of nUC versus T1. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster T1 stage of bladder cancer are marked pink and for cluster nUC are marked green. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison nUC vs. T1 (Figure III.6, A) were used to generate this clusters.

Pathway Plasma lipoprotein particle remodelling is associated with cholesterol and lipids dysregulation. Pathway Complement and coagulation cascades and protein activation cascade associated proteins are also upregulated in T1. Hydrogen peroxide catabolic process is present as well. Hydrogen peroxide is the product of reactive oxygen species dismutation[99] and its dysregulation in cancer could be related to a high concentration of reactive oxygen species that have been detected in almost all cancers[100].

The comparison of LUTS *vs.* T1 resulted in several pathways changes, as presented in **Figure III.8**.

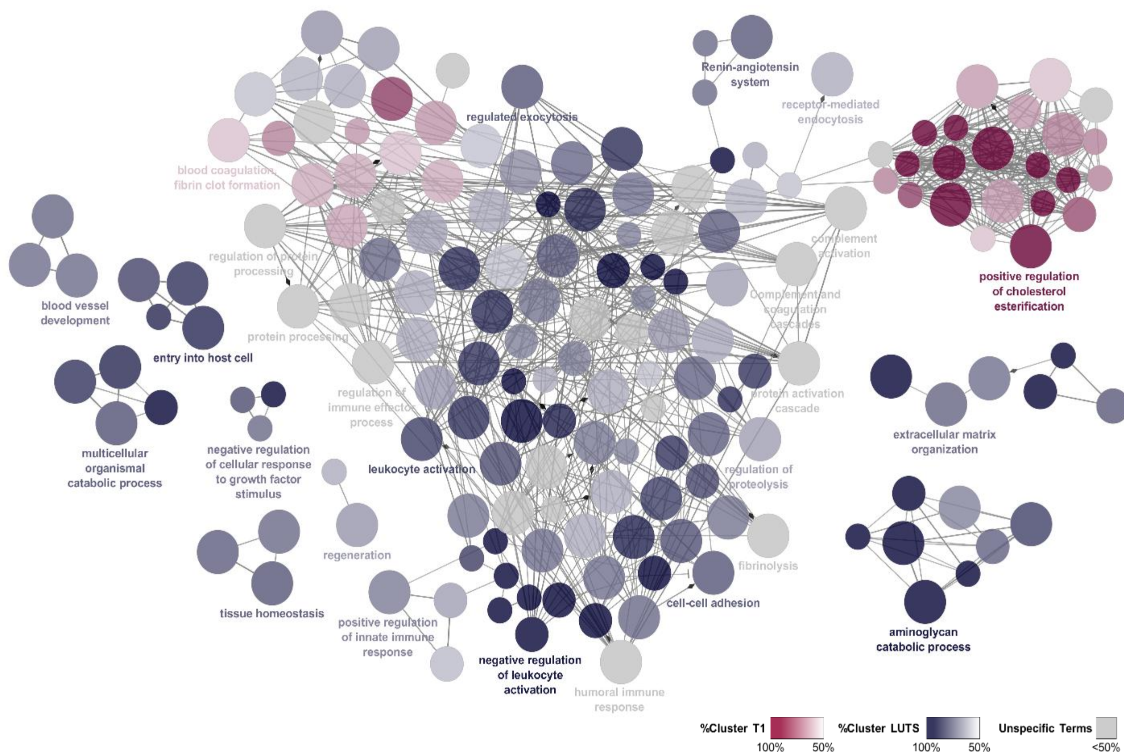


Figure III.8: Comparison of LUTS versus T1. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster T1 stage of bladder cancer are marked pink and for cluster LUTS are marked blue. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison LUTS vs. T1 (Figure III.6, B) were used to generate this clusters.

Positive regulation of cholesterol esterification is upregulated in T1, which is related to cholesterol and lipid regulation, pathway altered in cancer cells as referred before, in section **1.1 nUC and LUTS *vs.* Ta**. Blood coagulation and fibrin clot formation are also upregulated. On the T1 downregulated proteins, several are associated with immune response regulation. ECM regulation is affected as well. Cancer cells secrete growth factors allowing them to disrupt the basement membrane, penetrate into neighbouring tissues, and into the vascular system. Furthermore, cancer cells are more responsive to growth factors than normal cells[101]; thus it is possible that LUTS, in comparison to T1, presents a negative regulation of cellular response to growth factor stimulus.

There is an upregulation of similar GO terms in T1 in both comparisons, such as regulation of lipid transport, of lipoprotein lipase activity, chylomicron remodelling, positive regulation of cholesterol esterification and triglyceride metabolic process. Blood coagulation and fibrin clot formation are also upregulated in T1 comparing to nUC and LUTS. Proteins upregulated or downregulated in T1 comparing to both nUC and LUTS were selected, and they are presented in ***Figure* III.9.**

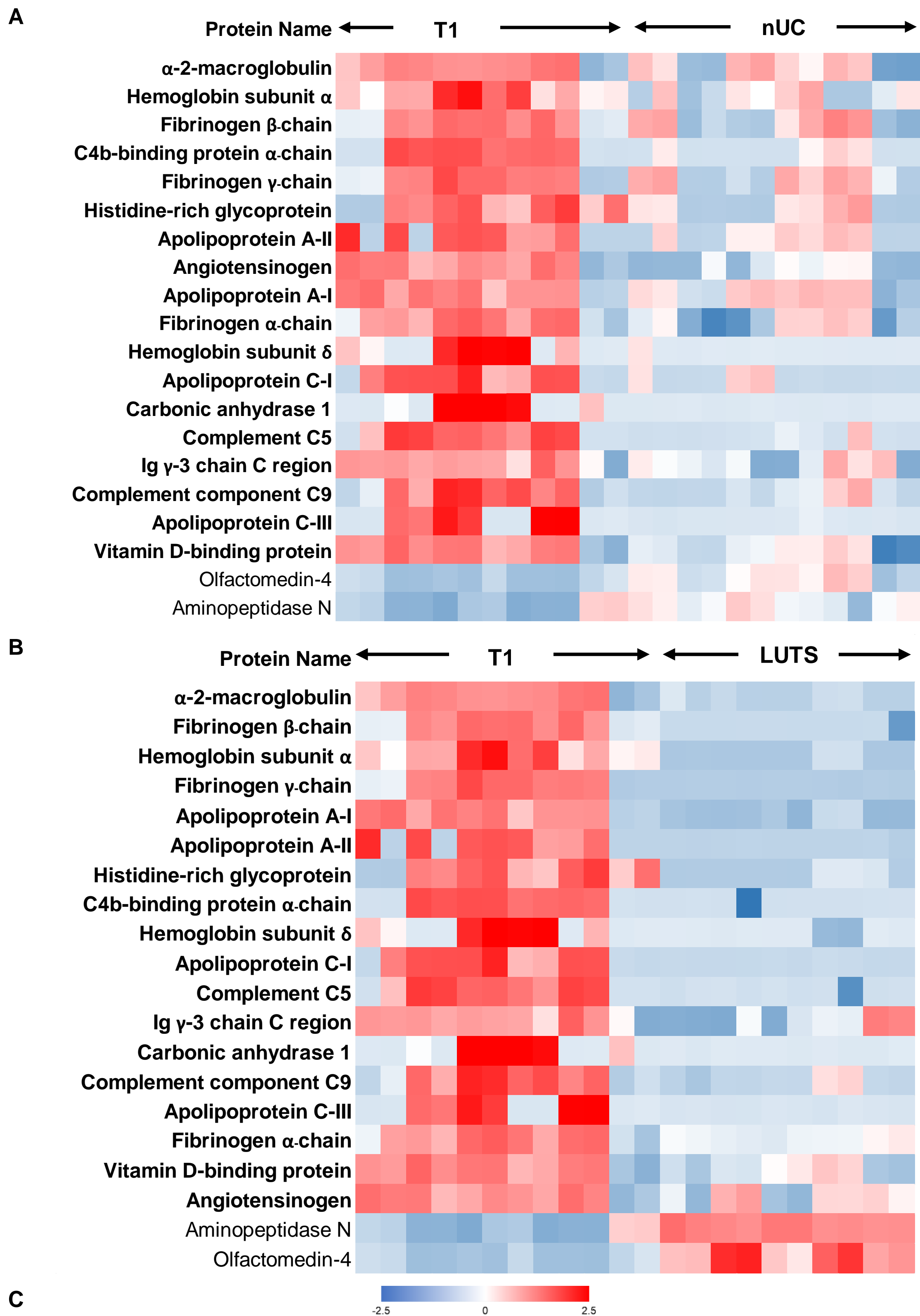


Figure III.9: Heat maps of transformed and normalized LFQ intensities (using Z-score) of significant proteins which were identified using the Student's T-test, and that were common to comparisons nUC vs. T1 and LUTS vs. T1. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. ***A:*** *Relative intensities of proteins in nUC and T1 samples;* ***B:*** *Relative intensities of proteins in LUTS and T1 samples.* ***C:*** *Heat map scale; red marks the upregulated proteins and blue the downregulated proteins.*

25 proteins have an abundance significantly different between controls and T1. The T1 sample at the right that includes two replicates, shows a colour pattern a little bit different than the rest of T1 group.

1.3 nUC and LUTS *vs.* T2+

To finish the study of biomarkers that can identify BCa presence, T2+ comparisons are presented on **Figure III.10** and forward.

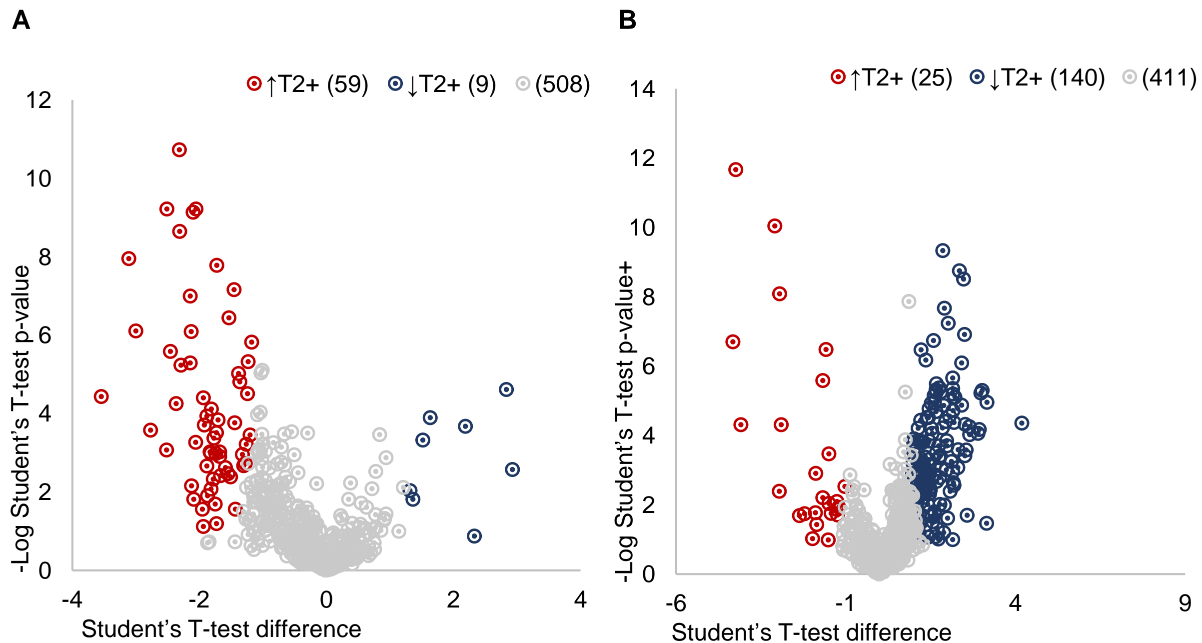


*Figure III.10: Volcano plots showing the differences between proteins quantified by MaxQuant of two groups; **A:** nUC and T2+; **B:** LUTS and T2+. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.*

T2+ has few downregulated proteins when comparing to nUC, but several upregulated, as seen in **Figure III.10**. Within the LUTS *vs.* T2+ comparison, there are a lot of proteins that can help in the biomarker panel construction. **Figure III.11** shows the comparison nUC *vs.* T2+.

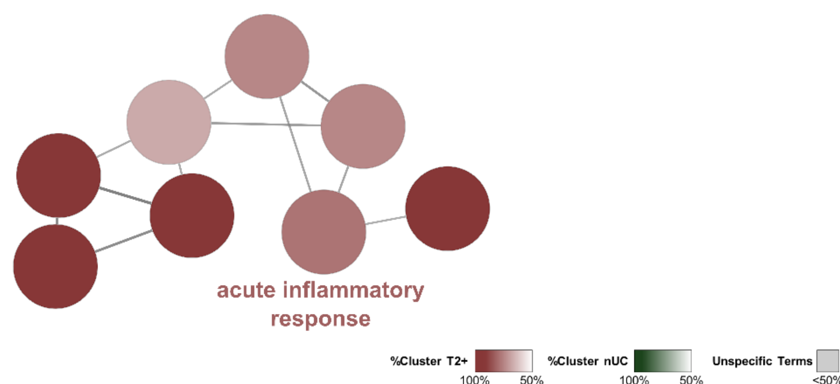


Figure III.11: Comparison of nUC versus T2+. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster T2+ stage of bladder cancer are marked brown and for cluster nUC are marked green. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison nUC vs. T2+ (Figure III.10, A) were used to generate this clusters.

Figure III.11 shows that T2+ is upregulated in acute inflammatory response, within a GO Terms group that includes complement and coagulation cascade and protein activation cascade. Once more, this is a dysregulation that can result from cancer cells' alterations in coagulation mechanisms. **Figure III.12** presents cytoscape results for the comparison LUTS vs. T2+-

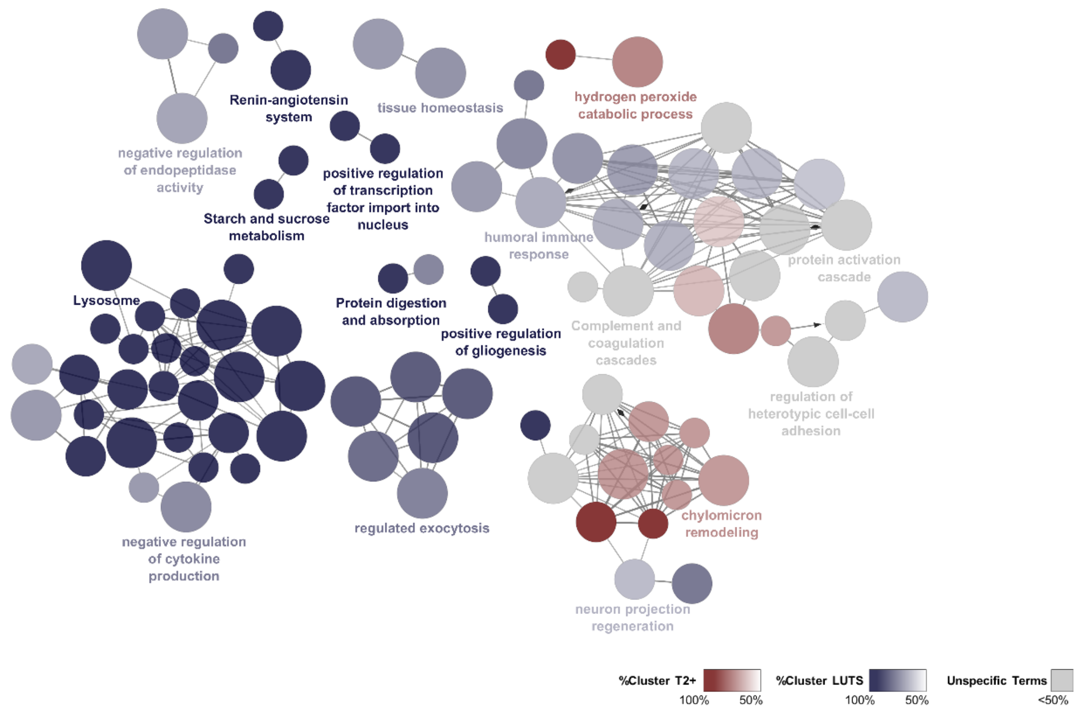


Figure III.12: Comparison of LUTS versus T2+. Representation of terms contribution from each cluster to the biological pathways. The terms for the cluster T2+ stage of bladder cancer are marked brown and for cluster nUC are marked blue. Each node represents a GO Term that gathers 3 or more proteins, all single nodes (with no connection to other node) were excluded. The protein showing significant differential in the comparison LUTS vs. T2+ (Figure III.10, B) were used to generate this clusters.

Chylomicron remodelling, cholesterol transport, and hydrogen peroxide catabolic process are upregulated in T2+, as shown in **Figure III.12**. On the downregulated pathways, regulated exocytosis, regulation of endopeptidase activity and protein digestion are some of the altered processes. Cancer cells prefer fermentation rather than respiration, and as fermentation produces less energy, cancer cells require more sugar molecules to produce the same amount of energy than normal cells, therefore having a more active glycolysis[102]. Glycolysis is a catabolism pathway of glucose and it is linked to starch catabolism and sucrose degradation, thus increased glycolysis in cancer cells may affect starch and other sugars metabolism. The heat maps of significant proteins are presented in **Figure III.13.**

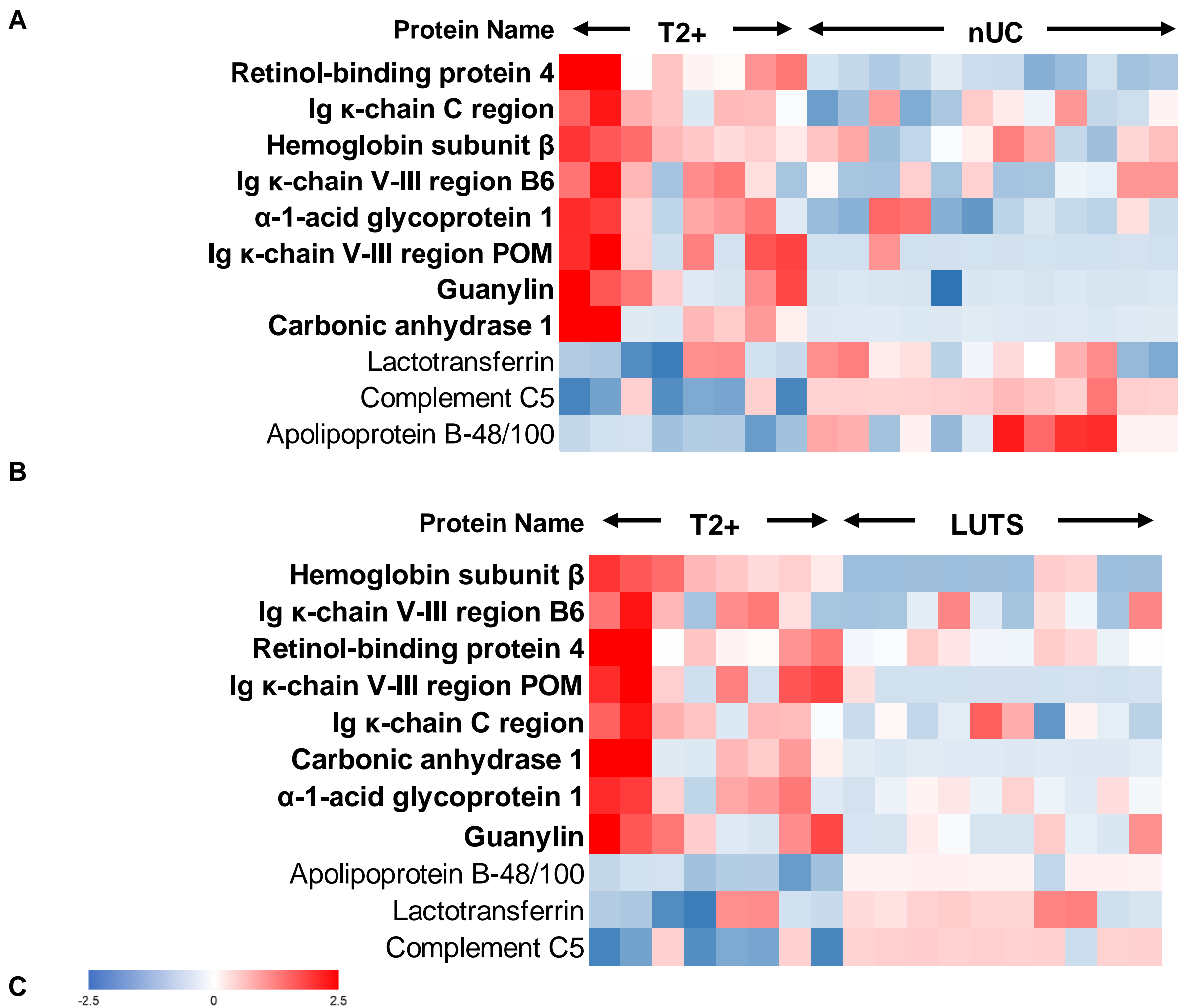


Figure III.13: Heat maps of transformed and normalized LFQ intensities (using Z-score) of significant proteins which were identified using the Student's T-test, and that were common to comparisons nUC vs. T2+ and LUTS vs. T2+. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. ***A:*** *Relative intensities of proteins in nUC and T2+ samples;* ***B:*** *Relative intensities of proteins in LUTS and T2+ samples.* ***C:*** *Heat map scale; red marks the upregulated proteins and blue the downregulated proteins.*

The small amount of potential biomarkers results from the low similarity between proteins of nUC *vs.* T2+ and LUTS *vs.* T2+; Furthermore, as T2+ presents few downregulated proteins on nUC *vs.* T2+ comparison and several upregulated proteins on the comparison LUTS *vs.* T2+, protein overlap would not be likely.

1.4 Biomarkers of Bladder Cancer Presence

All identified proteins in the comparisons: nUC and LUTS *vs.* Ta, nUC and LUTS *vs.* T1 and nUC and LUTS *vs.* T2+ were combined to generate a biomarker panel. Some proteins were identified as potential biomarkers in more than one comparison and, when aligning them, most proteins agreed on their concentration change to identify BCa. Complement C5, Fibrinogen γ-chain α-2-macroglobulin had different concentration behaviours and therefore were excluded from the panel. While BCa progresses from Ta to T1 and from T1 to T2+, there are DNA mutations that accompany the stage progress[103] and that can create proteome differences. In **Table III.1**, the panel of biomarkers we propose that can detect BCa is presented.

Table III.1: List of proteins whose abundance distinguishes BCa from nUC and LUTS.

Protein	Behaviour related to Bca presence
Angiotensinogen	↑
Apolipoprotein A-I	↑
Apolipoprotein A-II	↑
Apolipoprotein C-I	↑
Apolipoprotein C-III	↑
C4b-binding protein α-chain	↑
Carbonic anhydrase 1	↑
Complement component C9	↑
Fibrinogen α-chain	↑
Fibrinogen β-chain	↑
Guanylin	↑
Hemoglobin subunit α	↑
Hemoglobin subunit β	↑
Hemoglobin subunit δ	↑
Histidine-rich glycoprotein	↑
Ig γ-3 chain C region	↑
Ig κ-chain C region	↑
Ig κ-chain V-III region B6	↑
Ig κ-chain V-III region POM	↑
Ig λ-3 chain C regions	↑
Retinol-binding protein 4	↑
Secreted Ly-6/uPAR-related protein 1	↑
Vitamin D-binding protein	↑
α-1-acid glycoprotein 1	
α-1-antitrypsin	
Aminopeptidase N	↓
Apolipoprotein A-IV	↓
Apolipoprotein B-48/100	↓
Apolipoprotein E	↓
Complement factor B	↓
Inter-α-trypsin inhibitor heavy chain H1	↓
Inter-α-trypsin inhibitor heavy chain H2	↓
Lactotransferrin	↓
Neutrophil gelatinase-associated lipocalin	↓
Olfactomedin-4	

Some of the proteins presented have been already identified as urinary BCa biomarkers, namely afamin, apolipoproteins A-I e A-II, fibrinogen chains and Vitamin D-binding protein[104]. One way to increase the power of our biomarker panel to differentiate BCa from other non-BCa conditions would be the use of additional control groups, namely: cystitis, urinary infection and other urinary cancers, such as prostate cancer and renal carcinoma. While the idea of this work was to include some of these, the Hospital São José had some limitations on finding samples, and therefore it was not possible to add more control groups. Despite this problem, a panel of 35 proteins is a promising tool for BCa diagnosis.

2. Biomarkers addressing cancer staging

After proposing a panel of biomarkers holding the promise of diagnosis, the other main goal of this work was to identify proteins that could measure BCa progression. To accomplish this task comparisons between BCa stages were performed. The expected results would be proteins whose abundance is increased or is decreased through the three stages.

2.1 Comparison of stage Ta *vs.* stage T1

Volcano plots will be used as well to present the distribution of upregulated, downregulated and non-significant proteins between BCa stages.

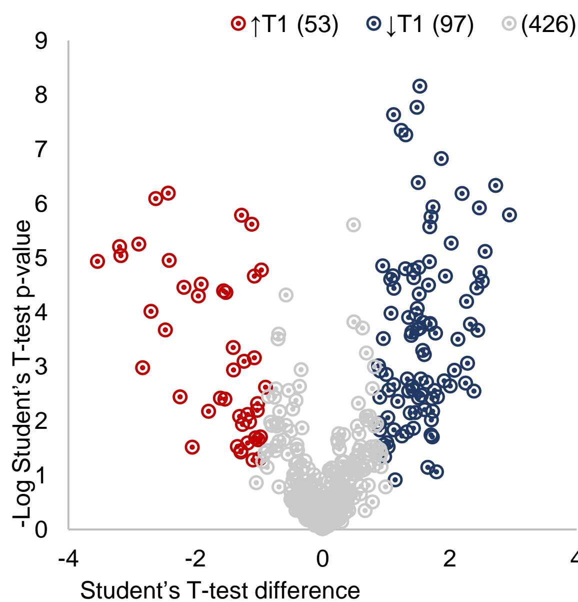
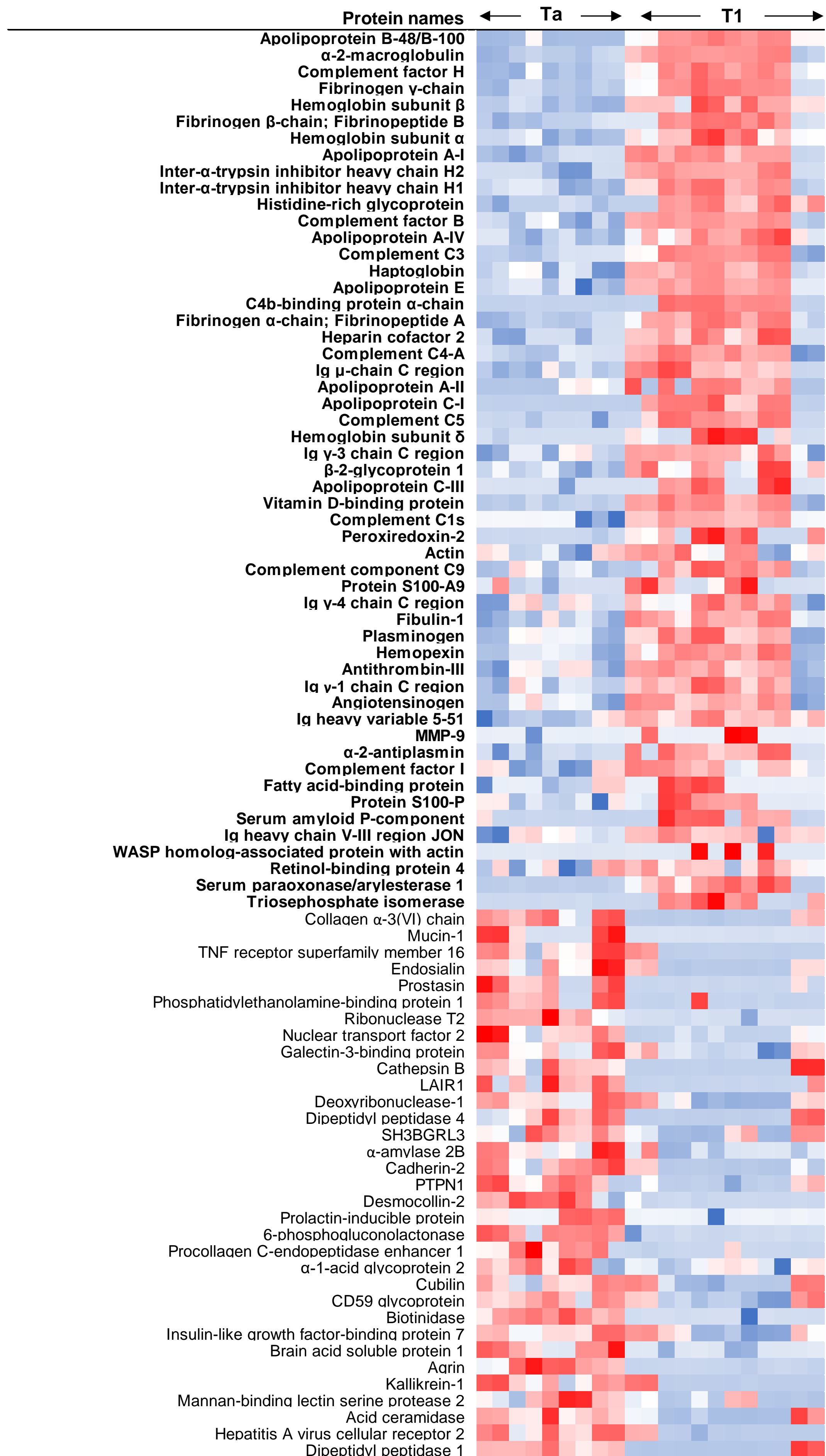


Figure III.14: Volcano plot showing the differences of proteins quantified by MaxQuant between T1 and Ta. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.

Figure III.14 shows the volcano-plot comparison between BCa stages T1 and Ta. It may be seen a total of 150 proteins listed as significantly down or over-regulated. To have a better insight into the significant proteins capability to distinguish between Ta and T1, a heatmap was performed and is presented in **Figure III.15.**


Protein names
Ta
T1
Apolipoprotein B-48/B-100
α-2-macroglobulin
Complement factor H
Fibrinogen γ-chain
Hemoglobin subunit β
Fibrinogen β-chain; Fibrinopeptide B
Hemoglobin subunit α
Apolipoprotein A-I
Inter-α-trypsin inhibitor heavy chain H2
Inter-α-trypsin inhibitor heavy chain H1
Histidine-rich glycoprotein
Complement factor B
Apolipoprotein A-IV
Complement C3
Haptoglobin
Apolipoprotein E
C4b-binding protein α-chain
Fibrinogen α-chain; Fibrinopeptide A
Heparin cofactor 2
Complement C4-A
Ig μ-chain C region
Apolipoprotein A-II
Apolipoprotein C-I
Complement C5
Hemoglobin subunit δ
Ig γ-3 chain C region
β-2-glycoprotein 1
Apolipoprotein C-III
Vitamin D-binding protein
Complement C1s
Peroxiredoxin-2
Actin
Complement component C9
Protein S100-A9
Ig γ-4 chain C region
Fibulin-1
Plasminogen
Hemopexin
Antithrombin-III
Ig γ-1 chain C region
Angiotensinogen
Ig heavy variable 5-51
MMP-9
α-2-antiplasmin
Complement factor I
Fatty acid-binding protein
Protein S100-P
Serum amyloid P-component
Ig heavy chain V-III region JON
WASP homolog-associated protein with actin
Retinol-binding protein 4
Serum paraoxonase/arylesterase 1
Triosephosphate isomerase
Collagen α-3(VI) chain
Mucin-1
TNF receptor superfamily member 16
Endosialin
Prostasin
Phosphatidylethanolamine-binding protein 1
Ribonuclease T2
Nuclear transport factor 2
Galectin-3-binding protein
Cathepsin B
LAIR1
Deoxyribonuclease-1
Dipeptidyl peptidase 4
SH3BGRL3
α-amylase 2B
Cadherin-2
PTPN1
Desmocollin-2
Prolactin-inducible protein
6-phosphogluconolactonase
Procollagen C-endopeptidase enhancer 1
α-1-acid glycoprotein 2
Cubilin
CD59 glycoprotein
Biotinidase
Insulin-like growth factor-binding protein 7
Brain acid soluble protein 1
Agrin
Kallikrein-1
Mannan-binding lectin serine protease 2
Acid ceramidase
Hepatitis A virus cellular receptor 2
Dipeptidyl peptidase 1

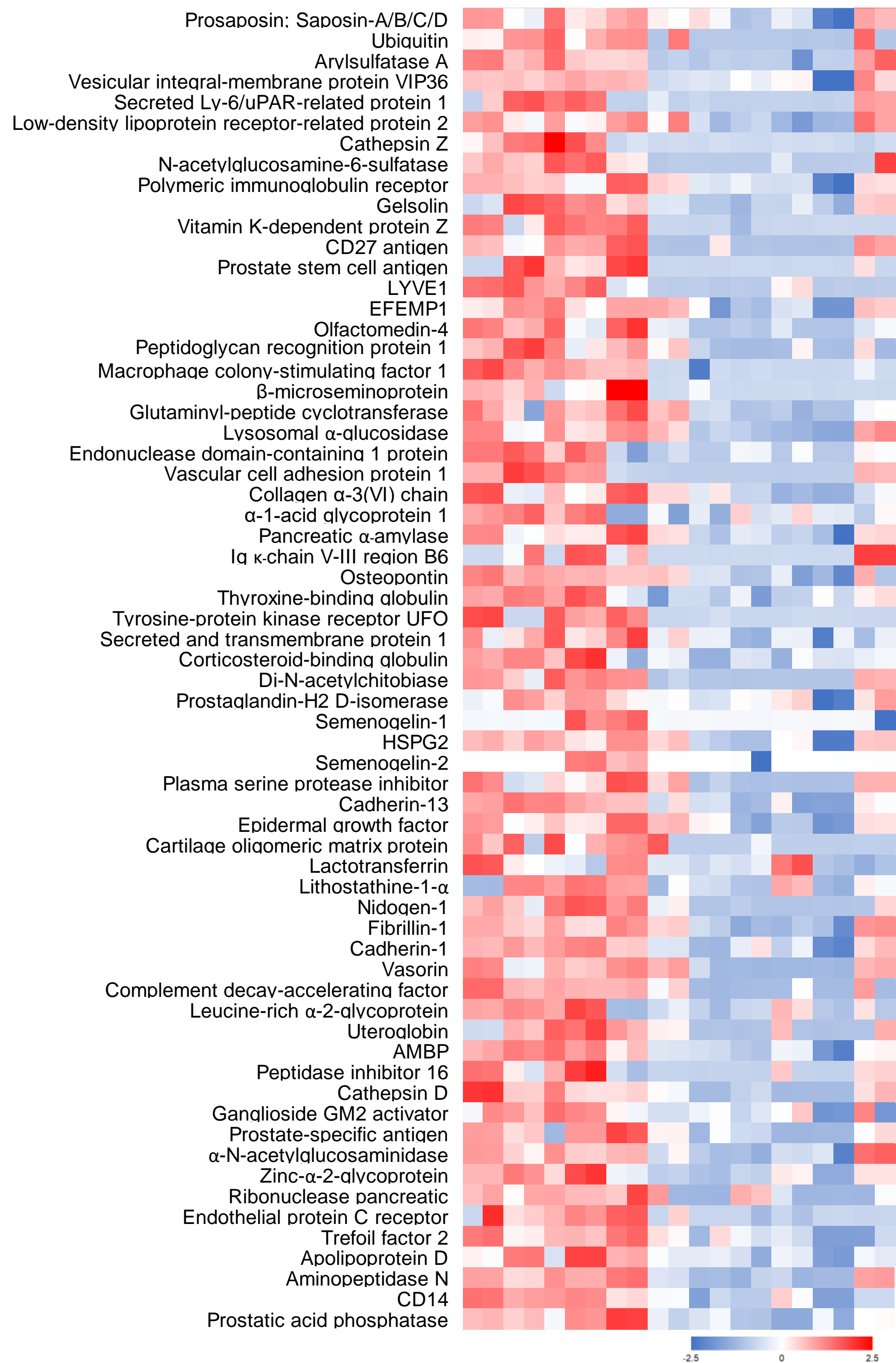


Figure III.15: Heat map of transformed and normalized LFQ intensities (using Z-score) of significant proteins between Ta and T1, which were identified using the Student's T-test. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. Abbreviations: AMBP, α-1-Microglobulin/Bikunin Precursor; EFEMP1, EGF-containing fibulin-like extracellular matrix protein 1; LYVE1, Lymphatic vessel endothelial hyaluronan receptor 1; MMP-9, Matrix metalloproteinase 9; PTPN1, Tyrosine-protein phosphatase non-receptor type 1; TNF, Tumour Necrosis Factor.

The heat map reveals that there is a good separation within the group, as it is possible to observe firstly blue on the left and red on the right, and then the inverse. The last two columns, correspondent to RM-55 replicates, do not fit as well as the other T1 samples.

2.2 Comparison of stage T1 *vs.* stage T2+

T1 and T2+ were compared, using the same method as before, being the volcano plot presented in **Figure III.16.**

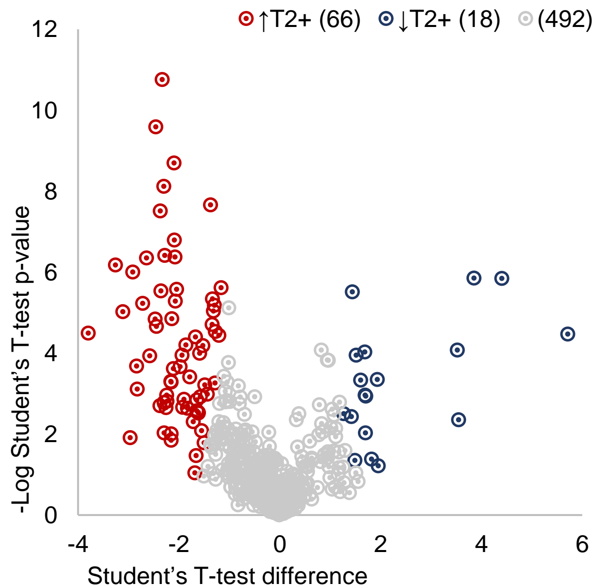
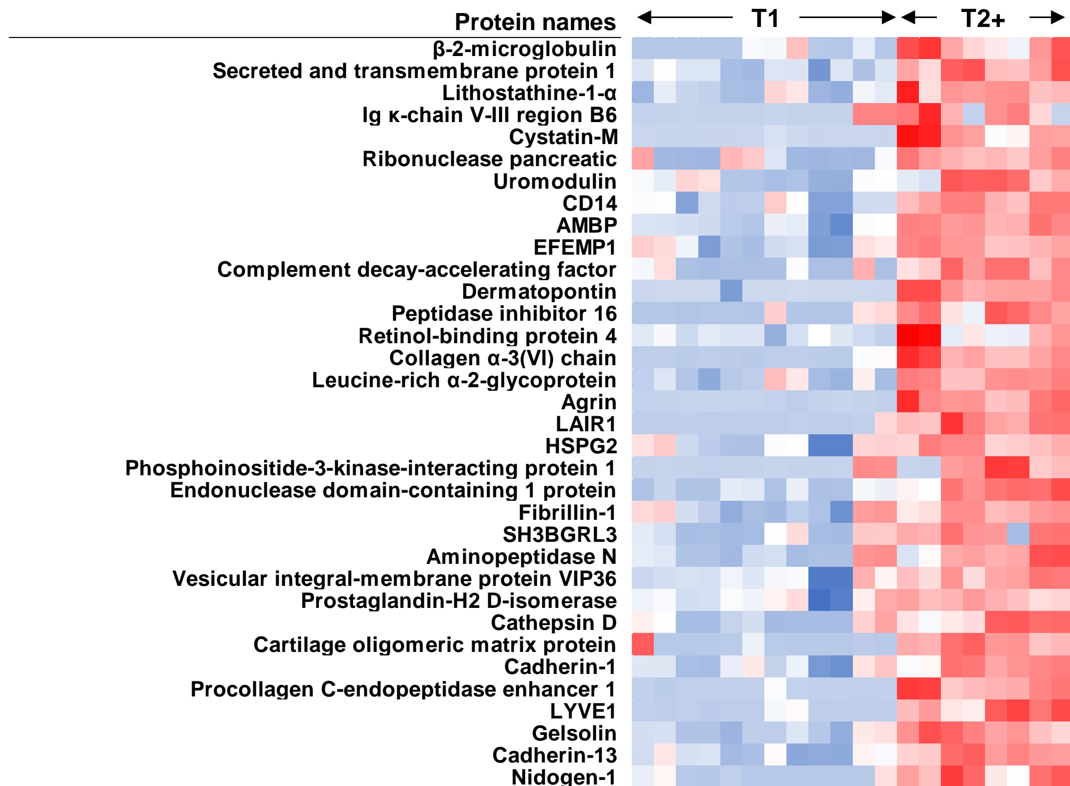


Figure III.16: Volcano plot showing the differences of proteins quantified by MaxQuant between T2+ and T1. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.

Figure III.16 presents the distribution of proteins for the comparison of T1 versus T2+. There are 84 proteins whose abundance are significantly different, and therefore could be potential biomarkers. Next, **Figure III.17** presents the heat map of significant proteins.



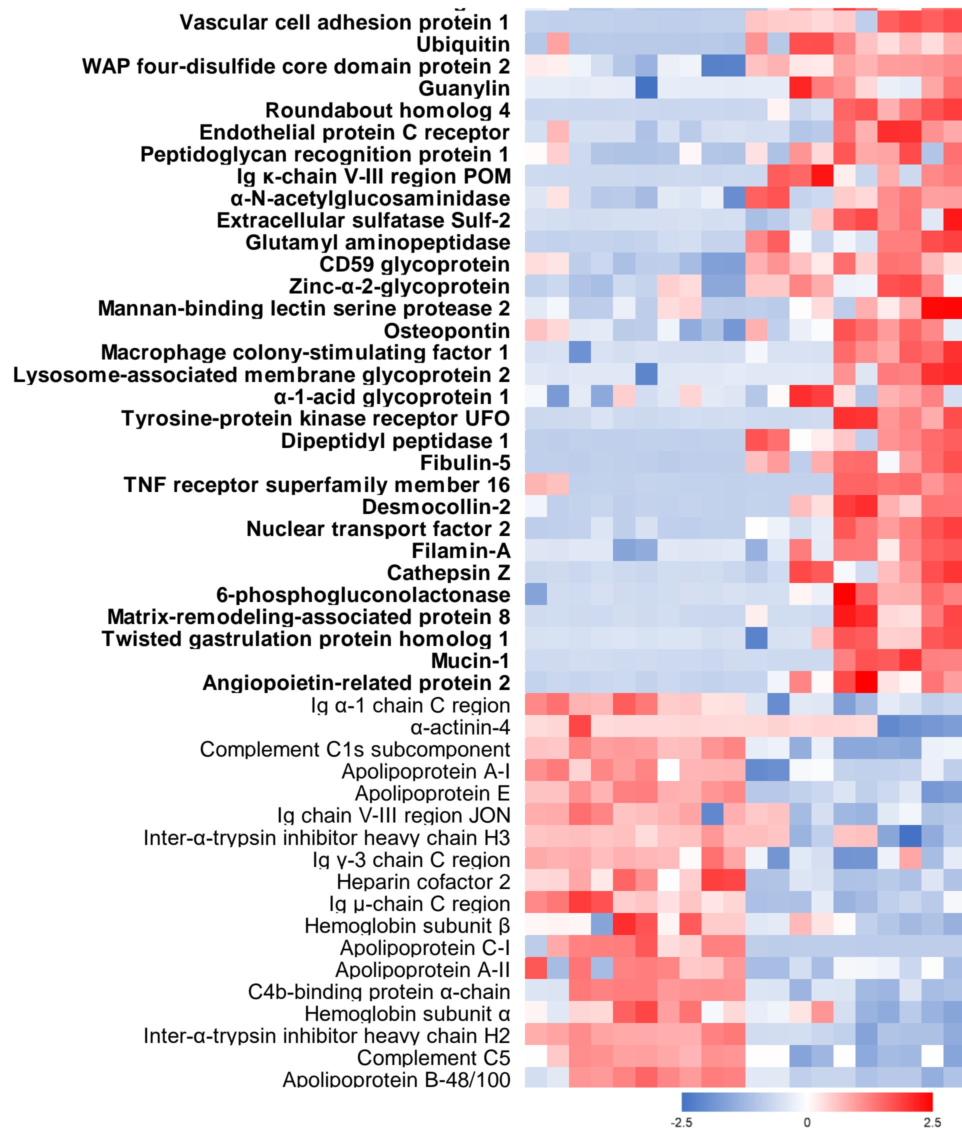


Figure III.17: Heat map of transformed and normalized LFQ intensities (using Z-score) of significant proteins between T1 and T2+, which were identified using the Student's T-test. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. Abbreviations: LYVE1, Lymphatic vessel endothelial hyaluronan receptor 1; PTPN1, Tyrosine-protein phosphatase non-receptor type 1; SH3BGRL3, SH3 Domain Binding Glutamate Rich Protein Like 3; TNF, Tumour Necrosis Factor.

The heat map presented in **Figure III.17** suggests that a good separation between BCa T1 and T2+ can be accomplished.

2.3 Comparison of stage Ta *vs.* stage T2+

At last Ta and T2+ were compared.

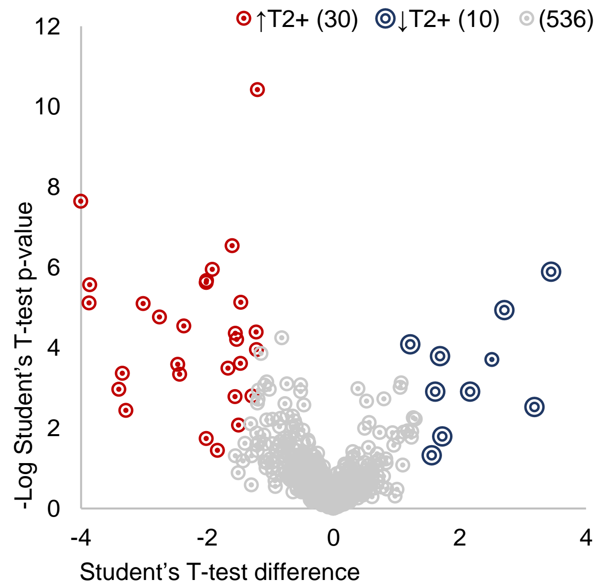


Figure III.18: Volcano plot showing the differences of proteins quantified by MaxQuant between T2+ and Ta. Significant proteins are the ones with s0 ≥ 1.5 and FDR ≤ 0.05. Red means upregulated, blue means downregulated, grey means non-significant.

Figure III.18 shows the volcano plot for the comparison of stage Ta *vs.* stage T2+. The number of significant proteins up. and down-regulated is 40. The heat map for these proteins is presented in **Figure III.19**.

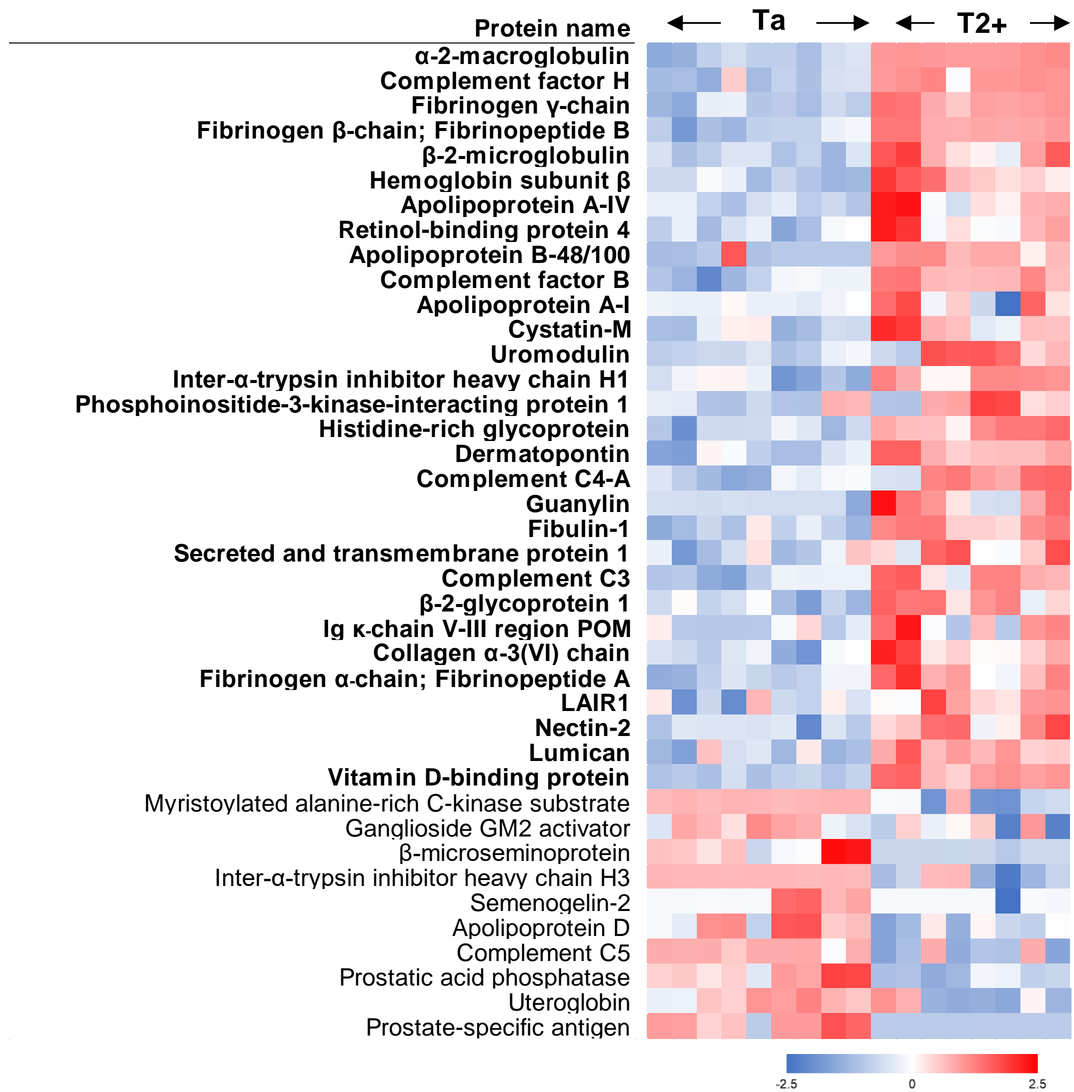


Figure III.19: Heat map of transformed and normalized LFQ intensities (using Z-score) of significant proteins between Ta and T2+, which were identified using the Student's T-test. Each column represents a replicate, and there are two replicates per sample. Proteins are ordered according to the difference between groups, from smallest to largest difference. Abbreviations: LAIR1, leukocyte associated immunoglobulin like receptor 1.

The separation between the two stages can be seen by the red and blue contrast throughout the heat map.

2.4 Biomarker of Cancer Staging

A search through heat maps revealed that Retinol-binding protein 4 abundance increases from Ta to T1 and to T2+.

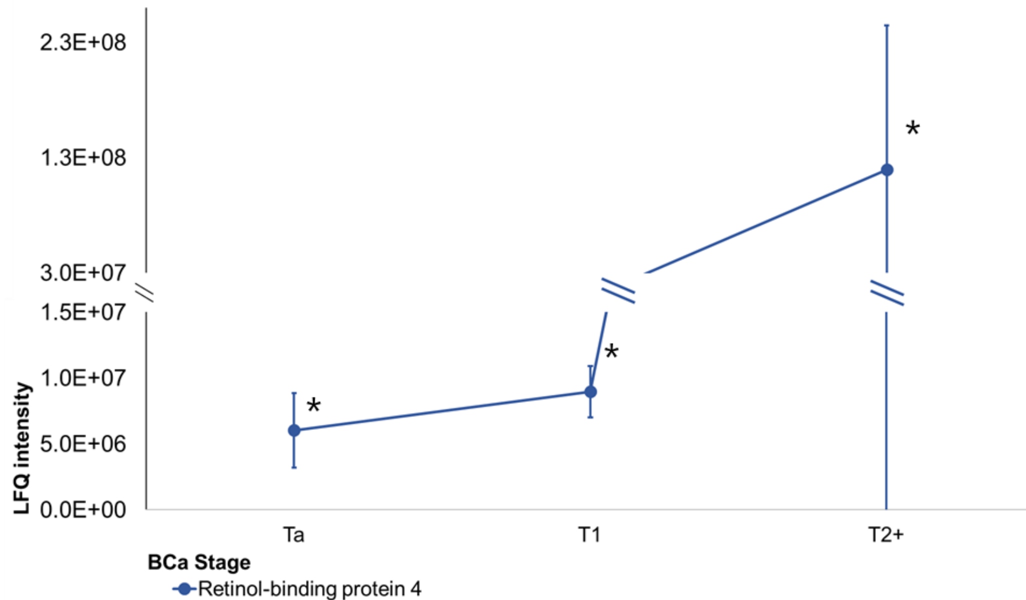


*Figure III.20: Graphic of average LFQ intensity of Retinol-binding protein 4 in each stage of BCa. Error bars correspond to Confidence Interval 95% values. * identifies proteins whose abundance in the given stage is significantly different from the other stages.*

Retinol-binding protein 4 is the only protein whose average LFQ intensity consistently increases accordingly to stage progression. As shown in **Figure III.20**, LFQ intensity average of T2+ presents a wider confidence interval, thus showing a higher biological variability consistent with individual response to cancer evolution. Retinol-binding protein 4 is involved in the transport of Vitamin A in blood. This result is in agreement with data reported in the literature, as it has been already identified as upregulated in BCa urine samples[105]. Retinol-binding protein 4 is a negative acute phase inflammatory reactant[106], and its expression is related to poor prognosis in cancer[107,108].

Despite the high number of up- and down-regulated proteins found between stages, we do not find other proteins with a constant increase or decrease over all the different stages. This fact can be explained because as the BCa is progressing, the genetic profile of the tumour also changes. Thus, we hypothesise that the number of proteins, as well as their levels change, and so finding the same proteins with a consistent increase or decrease through all the stages is difficult[23].

In an attempt to find a way to differentiate the stages we decided to select the proteins in stage Ta overexpressed when compared to stages T1 and T2+ and also to select the proteins overexpressed in T2+ when compared to Ta and T1. Such proteins are presented in Table III.2

Table III.2: Table listing each protein identified as potential biomarkers for measuring progress, the response (upregulation, downregulation when compared to other BCa stages) and the stage identified (Ta/T2+). Abbreviations: Ig, Immunoglobulin; LAIR1, Leukocyte-associated immunoglobulin-like receptor 1.

Protein	LFQ Intensity	Stage
β-2-microglobulin	↑	T2+
Collagen α-3(VI) chain	↑	T2+
Cystatin-M	↑	T2+
Dermatopontin	↑	T2+
Guanylin	↑	T2+
Ig κ-chain V-III region POM	↑	T2+
LAIR1	↑	T2+
Phosphoinositide-3-kinase-interacting protein 1	↑	T2+
Retinol-binding protein 4	↑	T2+
Secreted and transmembrane protein 1	↑	T2+
Uromodulin	↑	T2+
β-microseminoprotein	↑	Ta
Apolipoprotein D	↑	Ta
Ganglioside GM2 activator	↑	Ta
Prostate-specific antigen	↑	Ta
Prostatic acid phosphatase	↑	Ta
Semenogelin-2	↑	Ta
Uteroglobin	↑	Ta

To have a better elucidation of the capability of these proteins to identify stages Ta and T2+, and to determine if any one of those proteins increases or decreases with the disease's progression, average values of LFQ intensities of each stage group were determined and plotted.

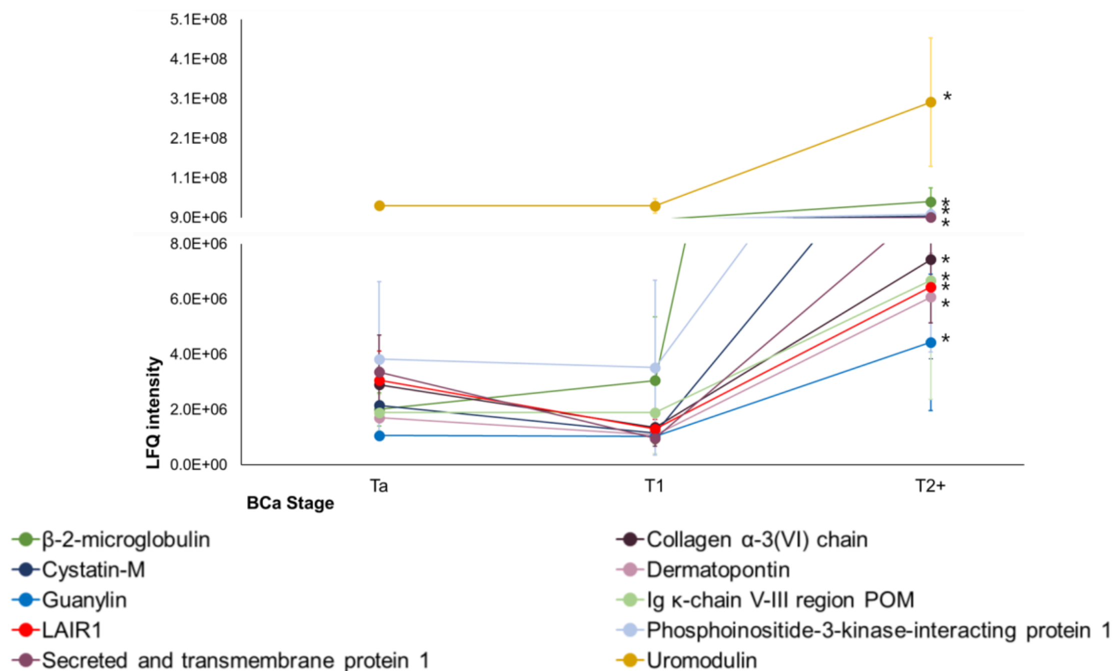


*Figure III.21: Graphic of average LFQ intensity of proteins within the same stage. Proteins presented are the ones that were significantly upregulated in T2+ in comparison to Ta and to T1. Error bars correspond to Confidence Interval 95% values. * identifies proteins whose abundance in the given stage is significantly different from the other stages. Abbreviations: LAIR1, Leukocyte-associated immunoglobulin-like receptor 1.*

Figure III.21 reveals that analysing the LFQ average intensity in each group shows some proteins whose average abundance increases or decreases along the BCa stage. Uromodulin is the protein presenting the highest LFQ average intensity on stage T2+. Uromodulin is the most abundant protein in urine, and its roles include regulation ion transport in a part of the renal tubule, immunomodulation and protection against urinary tract infections and kidney stonesand[109]. Uromodulin was found to be downregulated in cancer patients when compared to healthy volunteers[110,111], which is a result of BCa proteases hyperactivation[112]. Nevertheless, when MIBC patients were compared to NMIBC patients, uromodulin was upregulated[113], and a peptide study revealed that urine from MIBC patients has fewer uromodulin fragments than NMIBC patients[78]. While uromodulin behaviour does not keep up with the progress, Ta and T1 abundancies are similar and low compared to T2+ average abundance, so it could be a biomarker that distinguishes between NMBIC and MIBC. Phosphoinositide-3-kinase-interacting protein 1, Guanylin and Dermatopontin also have similar LFQ intensities between Ta and T1 stages, and low intensities comparing to T2+.

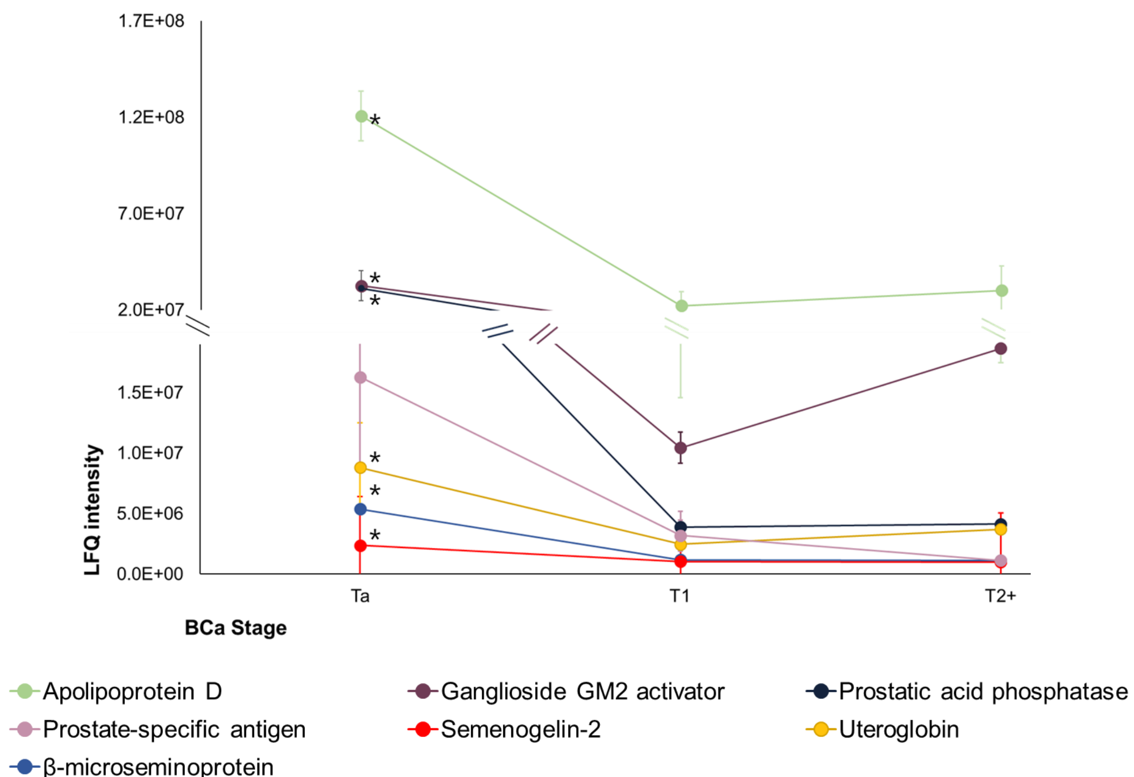


*Figure III.22: Graphic of average LFQ intensity of proteins within the same stage. Proteins presented are the ones that were significantly upregulated in Ta+ in comparison to T1 and to T2+. Error bars correspond to Confidence Interval 95% values. * identifies proteins whose abundance in the given stage is significantly different from the other stages.*

When plotting the upregulated proteins in Ta stage group, **Figure III.22** is obtained. Apolipoprotein D stood out as the one having the highest Ta LFQ intensity, and it is a secreted glycoprotein, which has many roles in cells, lipid transport being one example. Its expression correlates inversely with aggressive behaviour of several different types of malignant tumours[114,115], perhaps because Apolipoprotein D contributes to cell growth inhibition[115,116].

Prostate-specific antigen is the only protein that follows a downwards path along stage progression. This protein is expressed by cancer prostatic cancer cells, but it is as well expressed by normal cells[117]. Knowing that one of BCa hallmarks is protease hyperactivation[112], one possibility is prostate-specific antigen decrease by proteolytic degradation.

Following the search for proteins whose abundance can behave according to BCa progression, proteins that are upregulated or downregulated in T1 can also be biomarkers for stage identification. Several proteins are increased or decreased significantly in T1 compared to Ta and T2+, and they are presented in **Table III.3**.

Table III.3: Table listing each protein identified as potential biomarkers for measuring progress, the response (upregulation, downregulation when compared to other BCa stages) and the stage identified (T1). Abbreviations: AMBP, α-1-microglobulin/bikunin precursor; HSPG2, Basement membrane-specific heparan sulfate proteoglycan core protein; Ig, Immunoglobulin; LAIR1, Leukocyte-associated immunoglobulin-like receptor 1; LYVE1, Lymphatic vessel endothelial hyaluronan receptor 1; SH3BGRL3, SH3 domain-binding glutamic acid-rich-like protein 3; TNF, Tumour Necrosis Factor.

Protein	LFQ Intensity	Stage
Apolipoprotein A-I	↑	T1
Apolipoprotein A-II	↑	T1
Apolipoprotein B-48/B-100	↑	T1
Apolipoprotein C-I	↑	T1
Apolipoprotein E	↑	T1
C4b-binding protein α-chain	↑	T1
Complement C1s	↑	T1
Complement C5	↑	T1
Hemoglobin subunit α	↑	T1
Hemoglobin subunit β	↑	T1
Heparin cofactor 2	↑	T1
Ig heavy chain V-III region JON	↑	T1
Ig γ-3 chain C region	↑	T1
Ig μ-chain C region	↑	T1
6-phosphogluconolactonase	↓	T1
Agrin	↓	T1
AMBP	↓	T1
Aminopeptidase N	↓	T1
Cadherin-1	↓	T1
Cadherin-13	↓	T1
Cathepsin D	↓	T1
Cathepsin Z	↓	T1
CD14	↓	T1
CD59 glycoprotein	↓	T1
Collagen α-3(VI) chain	↓	T1
Complement decay-accelerating factor	↓	T1
Desmocollin-2	↓	T1
Dipeptidyl peptidase 1	↓	T1
Endonuclease domain-containing 1 protein	↓	T1
Endothelial protein C receptor	↓	T1

Fibrillin-1	↓	T1
HSPG2	↓	T1
Ig κ-chain V-III region B6	↓	T1
LAIR-1	↓	T1
Leucine-rich α-2-glycoprotein	↓	T1
Lithostathine-1-α	↓	T1
LYVE1	↓	T1
Macrophage colony-stimulating factor 1	↓	T1
Mannan-binding lectin serine protease 2	↓	T1
Mucin-1	↓	T1
Nidogen-1	↓	T1
Nuclear transport factor 2	↓	T1
Osteopontin	↓	T1
Peptidase inhibitor 16	↓	T1
Peptidoglycan recognition protein 1	↓	T1
Prostaglandin-H2 D-isomerase	↓	T1
Ribonuclease pancreatic	↓	T1
Secreted and transmembrane protein 1	↓	T1
SH3BGRL3	↓	T1
TNF receptor superfamily member 16	↓	T1
Tyrosine-protein kinase receptor UFO	↓	T1
Ubiquitin	↓	T1
Vascular cell adhesion protein 1	↓	T1
Vesicular integral-membrane protein VIP36	↓	T1
Zinc-α-2-glycoprotein	↓	T1
α-1-acid glycoprotein 1	↓	T1
α-N-acetylglucosaminidase	↓	T1

To have a deeper insight into the capacities of these proteins to distinguish stages, average LFQ intensities per stage were plotted.

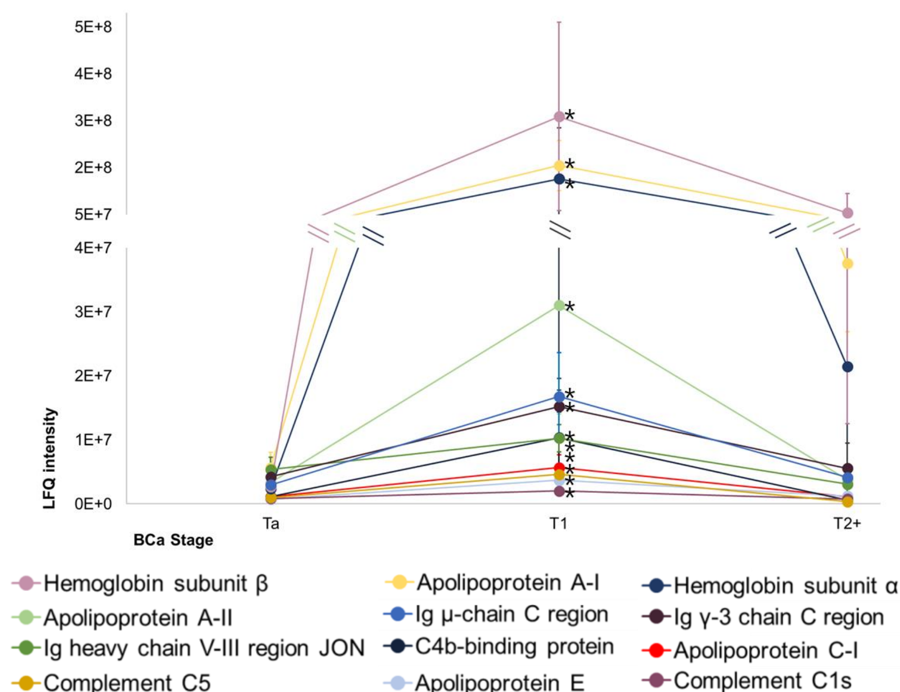


*Figure III.23: Graphic of average LFQ intensity of proteins within the same stage. Proteins presented are the ones that were significantly upregulated in T1+ in comparison to Ta and to T2+. Error bars correspond to Confidence Interval 95% values. * identifies proteins whose abundance in the given stage is significantly different from the other stages. Abbreviations: Ig, Immunoglobulin.*

In upregulated proteins in T1, presented in **Figure III.26**, several proteins stood out, such as Hemoglobin subunit β, Apolipoprotein A-I and Hemoglobin subunit α.

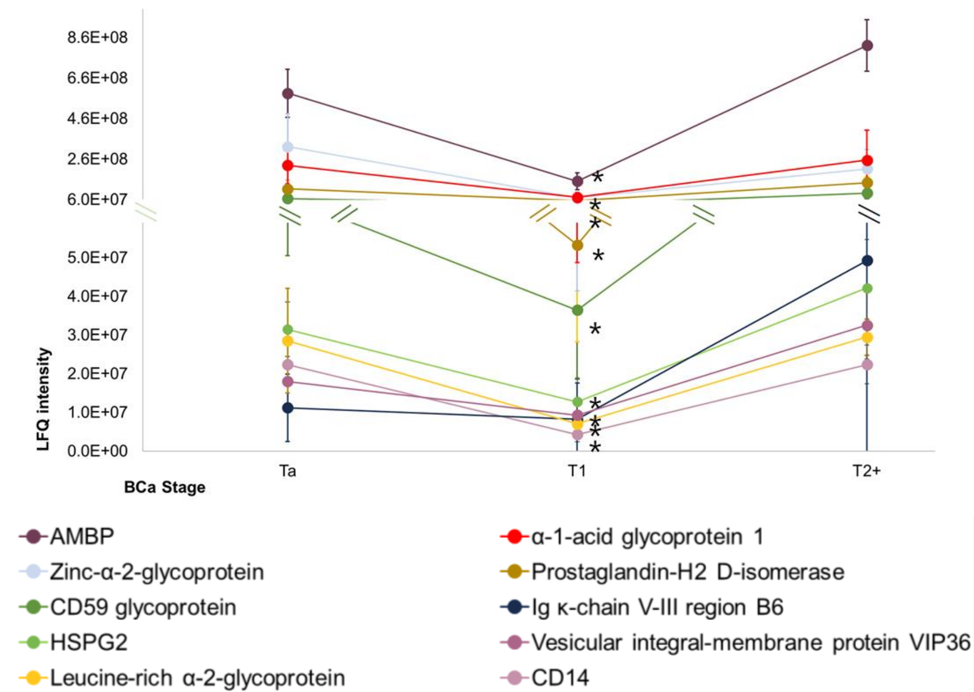


*Figure III.24: Graphic of average LFQ intensity of proteins within the same stage. Proteins presented are the ones that were significantly downregulated in T1+ in comparison to Ta and to T2+. Error bars correspond to Confidence Interval 95% values. * identifies proteins whose abundance in the given stage is significantly different from the other stages. Abbreviations: AMBP, α-1-Microglobulin/Bikunin Precursor; HSPG2, Basement membrane-specific heparan sulfate proteoglycan core protein; Ig, Immunoglobulin.*

Within the downregulated proteins in T1 stage, presented in **Figure III.27**, AMBP emerges as the protein whose abundance has the highest difference between T1 and Ta and T1 and T2+.

Chapter IV. Conclusions and Future Perspectives

This work had the primary goal of developing a biomarker panel to early diagnosis of BCa based on the urinary proteome. Also, BCa classification and tumour progression were objectives of this work.

As for the primary goal concerns, a panel of 35 proteins was found, holding the promise of an early diagnosis of BCa.

As for the classification stage concerns, a large number of proteins was identified allowing the classification of BCa stages as follows: Ta, eight proteins; T1, 41 proteins and T2+, 11 proteins.

As for the tumour progression concerns Retinol-binding protein 4 and prostatic specific antigen were the proteins found accomplishing the requisite of levels statistically different in each BCa stage.

The future perspectives of this work are performing a validation study in larger patients' cohorts, to determine the sensibility and specificity of the new panel of biomarkers using targeted LC-MS/MS and/or protein arrays. Also, the number of control diseases must be increased.

Another approach of great interest is to further investigate of the molecular mechanisms of BCa stage Ta recurrence[27], aiming to develop a method to predict recurrence, and therefore, providing sound principles to adjust therapy based on the probability of cancer recurrence. To accomplish this, urine samples from patients with Ta who had BCa previously have to be collected and analysed in a similarly fashion in order to build a panel of biomarkers for Ta recurrence.

Chapter V. References

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Chapter VI. Annexes

1. Literature Reviews

1.1 Biological Samples used to look for BCa biomarkers

Supplementary Table VI.1: Studies using plasma to search for BCa biomarkers.

Studies published since 2009 on biomarkers for bladder cancer in plasma	
Search:	TITLE-ABS-KEY("bladder cancer" AND "plasma" AND "biomarker") AND (EXCLUDE (DOCTYPE , "re ") OR EXCLUDE (DOCTYPE , "cp ") OR EXCLUDE (DOCTYPE , "le ") OR EXCLUDE (DOCTYPE , "ch "))
Year	**Study**
2018	Birkenkamp-Demtröder, K. *et al.* Monitoring Treatment Response and Metastatic Relapse in Advanced Bladder Cancer by Liquid Biopsy Analysis. *Eur. Urol.* **73,** 535–540 (2018). Araújo, J. E. *et al.* Dithiothreitol-based protein equalization technology to unravel biomarkers for bladder cancer. *Talanta* **180,** 36–46 (2018). Memon, A. A. *et al.* Soluble HER3 predicts survival in bladder cancer patients. *Oncol. Lett.* **15,** 1783–1788 (2018). Soukup, V. *et al.* Placental Growth Factor in Bladder Cancer Compared to the Diagnostic Accuracy and Prognostic Performance of Vascular Endothelial Growth Factor A. *Anticancer Res.* **38,** 239–246 (2018). Tokarzewicz, A., Guszcz, T., Onopiuk, A., Kozlowski, R. & Gorodkiewicz, E. Utility of cystatin C as a potential bladder tumour biomarker confirmed by surface plasmon resonance technique. *Indian J. Med. Res.* **147,** 46–50 (2018).
2017	Patel, K. M. *et al.* Association Of Plasma And Urinary Mutant DNA With Clinical Outcomes In Muscle Invasive Bladder Cancer. *Sci. Rep.* **7,** 5554 (2017). Christensen, E. *et al.* Liquid Biopsy Analysis of FGFR3 and PIK3CA Hotspot Mutations for Disease Surveillance in Bladder Cancer. *Eur. Urol.* **71,** 961–969 (2017). Gee, J. R. *et al.* A phase II randomized, double-blind, presurgical trial of polyphenon e in bladder cancer patients to evaluate pharmacodynamics and bladder tissue biomarkers. *Cancer Prev. Res.* **10,** 298–307 (2017). Kuppachi, S. *et al.* An Unexpected Surge in Plasma BKPyV Viral Load Heralds the Development of BKPyV-Associated Metastatic Bladder Cancer in a Lung Transplant Recipient With BKPyV Nephropathy. *Am. J. Transplant.* **17,** 813–818 (2017).
2016	Liu, J. *et al.* Elevated preoperative plasma fibrinogen level is an independent predictor of malignancy and advanced stage disease in patients with bladder urothelial tumors. *Int. J. Surg.* **36,** 249–254 (2016).

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2015	Armstrong, D. A., Green, B. B., Seigne, J. D., Schned, A. R. & Marsit, C. J. MicroRNA molecular profiling from matched tumor and bio-fluids in bladder cancer. *Mol. Cancer* **14,** 194 (2015). Du, M. *et al.* Circulating miR-497 and miR-663b in plasma are potential novel biomarkers for bladder cancer. *Sci. Rep.* **5,** 10437 (2015).
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2010	Becker, M. *et al.* Prognostic impact of plasminogen activator inhibitor type 1 expression in bladder cancer. *Cancer* **116,** 4502–4512 (2010).

Supplementary Table VI.2: Studies using serum to search for BCa biomarkers.

Studies published since 2009 on biomarkers for bladder cancer in serum	
Search:	TITLE-ABS-KEY("bladder cancer" AND "serum" AND "biomarker") AND (EXCLUDE (DOCTYPE , "re ") OR EXCLUDE (DOCTYPE , "cp ") OR EXCLUDE (DOCTYPE , "le ") OR EXCLUDE (DOCTYPE , "ch "))
Year	**Study**
2018	Tokarzewicz, A., Guszcz, T., Onopiuk, A., Kozlowski, R. & Gorodkiewicz, E. Utility of cystatin C as a potential bladder tumour biomarker confirmed by surface plasmon resonance technique. *Indian J. Med. Res.* **147,** 46–50 (2018). Qureshi, A., Fahim, A., Kazi, N., Farsi Kazi, S. A. & Nadeem, F. Expression of miR-100 as a novel ancillary non-invasive biomarker for early detection of bladder carcinoma. *J. Pak. Med. Assoc.* **68,** 759–763 (2018). Lian, J. *et al.* Serum microRNAs as predictors of risk for non-muscle invasive bladder cancer. *Oncotarget* **9,** 14895–14908 (2018).
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Supplementary Table VI.3: Studies using urine to search for BCa biomarkers.

	Studies published since 2009 on biomarkers for bladder cancer in urine
Search:	TITLE-ABS-KEY("bladder cancer" AND "urine" AND "biomarker") AND (EXCLUDE (DOCTYPE , "re ") OR EXCLUDE (DOCTYPE , "cp ") OR EXCLUDE (DOCTYPE , "le ") OR EXCLUDE (DOCTYPE , "ch "))
Year	**Study**
2018	Tokarzewicz, A., Guszcz, T., Onopiuk, A., Kozlowski, R. & Gorodkiewicz, E. Utility of cystatin C as a potential bladder tumour biomarker confirmed by surface plasmon resonance technique. *Indian J. Med. Res.* **147,** 46–50 (2018). Van Der Heijden, A. *et al.* Urine cell based DNA methylation classifier for monitoring bladder cancer. *Eur. Urol. Suppl.* **17,** e1415 (2018). Kim, W. T. *et al.* Urinary cell-free nucleic acid IQGAP3: A new non-invasive diagnostic marker for bladder cancer. *Oncotarget* **9,** 14354–14365 (2018). Oyeyemi, O. *et al.* Urinary bladder thickness, tumor antigen, and lower urinary tract symptoms in a low Schistosoma haematobium-endemic rural community of Nigeria. *Urol. Sci.* **29,** 151 (2018). Mijnes, J. *et al.* Promoter methylation of DNA damage repair (DDR) genes in human tumor entities: RBBP8/CtIP is almost exclusively methylated in bladder cancer. *Clin. Epigenetics* **10,** 15 (2018). Wu, P. *et al.* Profiling the urinary microbiota in male patients with bladder cancer in China. *Front. Cell. Infect. Microbiol.* **8,** 167 (2018). Soukup, V. *et al.* Placental Growth Factor in Bladder Cancer Compared to the Diagnostic Accuracy and Prognostic Performance of Vascular Endothelial Growth Factor A. *Anticancer Res.* **38,** 239–246 (2018). Vadasz, Z., Rubinstein, J., Bejar, J., Sheffer, H. & Halachmi, S. Overexpression of semaphorin 3A in patients with urothelial cancer. *Urol. Oncol. Semin. Orig. Investig.* **36,** 161.e1-161.e6 (2018). Birkenkamp-Demtröder, K. *et al.* Monitoring Treatment Response and Metastatic Relapse in Advanced Bladder Cancer by Liquid Biopsy Analysis. *Eur. Urol.* **73,** 535–540 (2018). Pardini, B. *et al.* microRNA profiles in urine by next-generation sequencing can stratify bladder cancer subtypes. *Oncotarget* **9,** 20658–20669 (2018).

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1.2 Urinary proteins identified as BCa biomarkers

Supplementary Table VI.4: Urinary proteins identified as biomarkers for BCa detection.

Protein	References
Albumin	Guo, J. et al. A Comprehensive Investigation toward the Indicative Proteins of Bladder Cancer in Urine: From Surveying Cell Secretomes to Verifying Urine Proteins. J. Proteome Res. 15, 2164–2177 (2016). Li, F. et al. Identification of urinary Gc-globulin as a novel biomarker for bladder cancer by two-dimensional fluorescent differential gel electrophoresis (2D-DIGE). J. Proteomics 77, 225–236 (2012). Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010).
α-1-anti-trypsin	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Lindén, M. et al. Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 12, 135–144 (2012). Chen, Y. T. et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J. Proteomics 75, 3529–3545 (2012). Yang, M. H. et al. Characterization of ADAM28 as a biomarker of bladder transitional cell carcinomas by urinary proteome analysis. Biochem. Biophys. Res. Commun. 411, 714–720 (2011).

Apolipoprotein A-I	Li, F. et al. Identification of urinary Gc-globulin as a novel biomarker for bladder cancer by two-dimensional fluorescent differential gel electrophoresis (2D-DIGE). J. Proteomics 77, 225–236 (2012). Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Lindén, M. et al. Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 12, 135–144 (2012). Chen, C. L. et al. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. J. Proteomics 85, 28–43 (2013). Lei, T. et al. Discovery of potential bladder cancer biomarkers by comparative urine proteomics and analysis. Clin. Genitourin. Cancer 11, 56–62 (2013). Li, H. et al. Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma. Proteome Sci 9, 21 (2011). Li, C. et al. Discovery of Apo-A1 as a potential bladder cancer biomarker by urine proteomics and analysis. Biochem. Biophys. Res. Commun. 446, 1047–1052 (2014).
Apolipoprotein A-II	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Chen, Y. T. et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J. Proteomics 75, 3529–3545 (2012). Chen, C. L. et al. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. J. Proteomics 85, 28–43 (2013).
Apolipoprotein A-IV	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Lindén, M. et al. Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 12, 135–144 (2012).

Fibrinogen β-chain	Li, F. et al. Identification of urinary Gc-globulin as a novel biomarker for bladder cancer by two-dimensional fluorescent differential gel electrophoresis (2D-DIGE). J. Proteomics 77, 225–236 (2012). Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Lindén, M. et al. Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 12, 135–144 (2012). Li, H. et al. Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma. Proteome Sci 9, 21 (2011). Tyan, Y.-C. et al. Urinary protein profiling by liquid chromatography/tandem mass spectrometry: ADAM28 is overexpressed in bladder transitional cell carcinoma. Rapid Commun. Mass Spectrom. 25, 2851–2862 (2011).
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Afamin	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010).

	Chen, Y. T. et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J. Proteomics 75, 3529–3545 (2012).
Apoliprotein E	Lindén, M. et al. Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer. Proteomics 12, 135–144 (2012). Chen, C. L. et al. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. J. Proteomics 85, 28–43 (2013)
Ceruloplasmin	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Zoidakis, J. et al. Profilin 1 is a Potential Biomarker for Bladder Cancer Aggressiveness. Mol. Cell. Proteomics 11, M111.009449 (2012).
Clusterin	Li, H. et al. Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma. Proteome Sci 9, 21 (2011). Zoidakis, J. et al. Profilin 1 is a Potential Biomarker for Bladder Cancer Aggressiveness. Mol. Cell. Proteomics 11, M111.009449 (2012).
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Serotransferrin	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Yang, M. H. et al. Characterization of ADAM28 as a biomarker of bladder transitional cell carcinomas by urinary proteome analysis. Biochem. Biophys. Res. Commun. 411, 714–720 (2011).
Serum amyloid A4 protein	Chen, Y. et al. Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Discovery of Novel Bladder Cancer Biomarkers by Comparative Urine Proteomics Using iTRAQ Technology Graduate Institute of Biomedical Science, Colleague of. J. Proteome Res. 9, 5803–5815 (2010). Chen, C. L. et al. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. J. Proteomics 85, 28–43 (2013)
Thrombospondin-1	Chen, Y. T. et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J. Proteomics 75, 3529–3545 (2012). Duriez, E. et al. Large-Scale SRM Screen of Urothelial Bladder Cancer Candidate Biomarkers in Urine. J. Proteome Res. 16, 1617–1631 (2017).

2. Patients supplementary information

Supplementary Table VI.5: Details of the grade and stage of BCa volunteers, as well as smoking habits and medical conditions of every volunteer whose sample was used.

Ref. Lab.	Grade/Stage	Age	Smoking Habits	Medical Conditions
VE-05	G1/Ta	63	Smoker	Peripheral venous insufficiency; Chronic bronchitis; Psoriasis
SC-83		73	Non-smoker	Hypertension; Diabetes; Recurrent pneumonia; Benign prostatic hyperplasia (BPH)
PM-77		83	Ex-smoker	Hypertension
IJ-140		84	Non-smoker	Pre-obesity; Hypertension
AG-44		66	Smoker	Cardiac Insufficiency; Pulmonary Hypertension; Renal Insufficiency; Chronic obstructive pulmonary disease (COPD)
OR-169		60	Ex-smoker	Aortic Insufficiency; Prostate Pathology; Gastritis
GA-07	G3/T1	78	Smoker	Hypertension; Renal lithiasis; Venous Insufficiency
RM-55		74	Smoker	Hypertension; Renal lithiasis; Renal Insufficiency; Prostate Pathology; Dyslipidaemia; COPD; BPH
FC-86		74	Non-smoker	Hypertension; Gastritis; BPH
JP-59		81	Smoker	Pulmonary fibrosis
MC-156		78	Non-smoker	Hypertension; Obesity; Dyslipidaemia; Diabetes
AR-35		65	Ex-smoker	Prostate Pathology; Glaucoma; COPD
AB-37	G3/T2	61	Smoker	Hypertension; Obesity; Dyslipidaemia; Diabetes; Renal Insufficiency; Active Tuberculosis
JS-04		77	Smoker	Hypertension; Obesity; Glaucoma; COPD; BPH
MS-10		91	Non-smoker	Hypertension; Dyslipidaemia; Cardiac Insuffiency; Chronic Kidney Disease
JS-79		72	Smoker	Hypertension; Obesity; Dyslipidaemia; Hyperuricemia; Roncopathy
JM-39		86	Non-smoker	Diabetes; Diabetic Retinopathy; BPH
JS-53	G3/T2-4	68	Ex-smoker	Hypertension; Dyslipidaemia; Prostatic Pathology; BPH
JC-46		56	Non-smoker	BHP

AB-100	78	Ex-smoker	Parkinson Disease; Rhinitis
MM-45	76	Non-smoker	Hypertension; Venous Insufficiency; Dyslipidaemia; Prostate Pathology; Roncopathy
MS-106	74	Non-smoker	Dyslipidaemia
JR-127	62	Smoker	Hypertension; Obesity; Dyslipidaemia; Diabetes; Gastritis; Roncopathy; COPD
IC-135	75	Non-smoker	Dyslipidaemia; Hypothyroidism; BHP
JN-118	61	Ex-smoker	Hypertension; Obesity; Dyslipidaemia; Diabetes; Kidney Stones
JB-67	67	Non-smoker	Hypertension; Dyslipidaemia; Asma
RM-94	75	Smoker	Hypertension; Dyslipidaemia; Renal Lithiasis; Prostate Pathology; COPD
ML-99	62	Non-smoker	Hypertension; Obesity; Dyslipidaemia; Renal Insufficiency; Prostatic Pathology
MG-175	75	Ex-smoker	Hypertension; Obesity; Dyslipidaemia; Prostatic Pathology; COPD
HA-149	67	Non-smoker	Hypertension; Dyslipidaemia

3. Protein and Peptide Quantifications

3.1 Protein Quantification

Supplementary Table VI.6: Protein Concentration in each urine samples, using Bradford Quantification Method.

Sample	Concentration (µg/mL)			Calibration Curve
VE-05	424.3	±	0.2	2
SC-83	8690	±	2	1
PM-77	1592	±	1	2
IJ-140	13021	±	15	3
AG-44	2133	±	1	3
OR-169	11749	±	4	2
GA-07	10907	±	3	1
RM-55	1610	±	2	4
FC-86	7642	±	5	5
JP-59	10341	±	27	6
MC-156	130559	±	16	5
AR-35	3749	±	1	4
AB-37	43748	±	10	1
JS-04	10397	±	3	2
MS-10	1165	±	17	3
JS-79	643.6	±	0.1	2
JM-39	7576	±	2	2
JS-53	417	±	0.1	3
JC-46	362.6	±	0.5	3
AB-100	330.8	±	0.1	4
MM-45	2431	±	27	5
MS-106	288.5	±	0.1	1
JR-127	719.1	±	0.1	1
IC-135	1771	±	22	5
JN-118	1265.7	±	0.3	4
JB-67	7430	±	7	5
RM-94	2194	±	9	6
ML-99	2683	±	3	4
MG-175	1861	±	7	4
HA-149	1915	±	1	6

Supplementary Table VI.7: Slope, intercept of the line and R square of the calibration curves used to quantify urinary proteins.

Calibration Curve	m	b	R^2
1	0.0250	0.0130	0.9905
2	0.0259	0.0164	0.9905
3	0.0248	-0.0211	0.993
4	0.0270	-0.0181	0.9921
5	0.0236	-0.0395	0.9874

6	0.0249	-0.0147	0.9905

3.2 Peptide Quantification

Supplementary Table VI.8: Slope, intercept of the line and R square of the calibration curves used to quantify urinary peptides.

Calibration Curve	m	b	R^2
1	0.0022	-0.0059	0.9998
2	0.0023	0.0072	0.9964
3	0.0022	-0.0016	0.9996
4	0.0023	-0.0061	0.9996
5	0.0021	-0.0003	0.9995
6	0.0022	-0.0016	0.9996

Supplementary Table VI.9: Peptide Concentration in each urine samples, using Pierce Quantitative Colorimetric Peptide Assay.

Sample	Replicate	Concentration (µg/mL)			Calibration Curve
VE-05	1	950	±	0*	2
	2	786	±	0*	2
SC-83	1	1087	±	12	1
	2	965	±	5	1
PM-77	1	1375	±	2	2
	2	1388	±	60	2
IJ-140	1	947	±	2	3
	2	854	±	6	3
AG-44	1	1106	±	19	3
	2	1131	±	29	3
OR-169	1	906	±	1	2
	2	939	±	14	2
GA-07	1	795	±	1	4
	2	606	±	4	4
RM-55	1	910	±	9	5
	2	1008	±	34	5
FC-86	1	513	±	10	5
	2	508	±	17	5
JP-59	1	987	±	18	5
	2	975	±	29	5
MC-156	1	917	±	21	6
	2	774	±	9	6
AR-35	1	880	±	8	6

	2	921	±	11	6
AB-37	1	771	±	8	1
	2	741	±	33	1
JS-04	1	928	±	1	2
	2	990	±	0	2
MS-10	1	666	±	18	3
	2	688	±	7	3
JS-79	1	1111	±	21	2
	2	950	±	14	2
JM-39	1	1339	±	18	2
	2	1178	±	21	2
JS-53	1	507	±	7	3
	2	1233	±	15	3
JC-46	1	481	±	4	6
	2	517	±	7	6
AB-100	1	680	±	4	5
	2	716	±	14	5
MM-45	1	672	±	3	4
	2	659	±	9	4
MS-106	1	505	±	7	4
	2	268	±	4	4
JR-127	1	417	±	8	4
	2	435	±	3	4
IC-135	1	691	±	10	4
	2	819	±	22	4
JN-118	1	924	±	19	5
	2	1027	±	9	5
JB-67	1	487	±	7	4
	2	569	±	7	4
RM-94	1	951	±	10	5
	2	805	±	7	5
ML-99	1	1178	±	12	6
	2	1142	±	28	6
MG-175	1	1356	±	14	6
	2	1248	±	4	6
HA-149	1	1255	±	12	6
	2	1244	±	8	6

* There was an error when adding the working reagent to one of the wells, so only replicate was used for quantification, so the standard deviation between replicates was zero.