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Discovery and validation of novel blood biomarkers of breast and prostate cancer

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ABSTRACT

Identification of novel biomarkers of breast and prostate cancers to supplement current diagnostic, prognostic and predictive tools is an important clinical need. Interaction between the host's immune system and tumours is reflected in detectable changes in profiles of proteins in blood cells and therefore these cells can be a source for cancer biomarkers. In a previous investigation conducted in our laboratory, a panel of proteins differentially expressed in white blood cells (WBCs) of patients with primary and advanced breast cancer (BRCA and ABC cohorts, respectively) was identified using integrated approaches. The aim of the present study was to assess the biomarker potential of five proteins: Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5LOX, using Western blot, immunofluorescence, flow cytometry and ELISA techniques. Serpin B1, Lipocalin 2, 5LOX and Copine 3 were present in neutrophils, whereas Integrin α4 was detected in T-lymphocytes. Significantly higher levels of Serpin B1 and Lipocalin 2, and lower levels of Integrin α4 were observed in WBCs in the BRCA cohort compared to healthy individuals, suggesting their utility as early breast cancer biomarkers. Levels of Serpin B1 and Lipocalin 2 increased and Integrin α4 decreased as the disease advanced, indicating their utility as biomarkers of poor prognosis. Lower basal levels of Serpin B1 in patients undergoing chemotherapy and endocrine treatment correlated with complete response to treatment. Additionally, the utility of BORIS as a diagnostic marker was investigated in plasma of breast cancer patients and urinary sediments and exosomes of prostate cancer patients. The presence of BORIS autoantibodies was demonstrated in plasma of breast cancer patients. The highest levels of *BORIS* mRNA were detected in urine of prostate cancer patient with the advanced disease. This indicates potential of the BORIS for breast cancer diagnosis and its utility for the prediction of the aggressiveness in prostate cancer.

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Chapter 1: Introduction

1.1 Cancer

Breast and prostate cancer are the two most common types of cancer in women and men, respectively. Typically, both types of cancer are sex hormone-dependent and have underlying biological similarities. The sex hormones oestrogen and androgen are key drivers of both prostate and breast cancer. Therefore, the inhibition of oestrogen and androgen receptors, as well as key enzymes involved in steroid metabolism, are some of the therapeutic strategies that can be used to combat the disease (Risbridger et al. 2010).

Cancer is a disease characterised by uncontrolled cell proliferation caused by the accumulation of multiple abnormalities that affect cell regulatory mechanisms. Mutations accumulate during the life cycle of a cell. Under normal physiological conditions, the cell recognises these mutations and undergoes programmed cell death (apoptosis) by a number of pathways (intrinsic, extrinsic and cytotoxic T-lymphocyte). However, during oncogenesis, a cell acquires a series of mutations known as “driver mutations”, which confer a selective growth advantage. To date, 140 genes that can promote driver mutations have been identified (Vogelstein et al. 2013). Following these driver mutations, the cell transitions from normal physiological, to precancerous and finally a cancerous cell. Post oncogenesis, the cell will acquire numerous other mutations, known as “passenger mutations”, irrelevant to tumour progression .

During cancer progression and therapy, tumour cells adapt to their microenvironment in the same manner in which species evolve, resulting in heterogeneity between cells of the same tumour type and subtypes. This process impacts patient prognosis, treatment response and can explain the failure of many prognostic and predictive biomarkers (Gerlinger et al. 2014).

1.1.1 Breast cancer

1.1.2 Mammary glands under normal physiological conditions

The mammary glands are the secretory organs unique to the Mammalia class. Unlike most organs of the body that fully develop during embryogenesis, breast development continues through puberty reaching maturity by the end of pregnancy and the lactation phase. The breast is a modified apocrine gland located in the chest wall, composed of glandular (secretory) and adipose (fatty) tissue supported by a fibrous connective tissue framework (Figure 1.1) The functional unit of the breast is the terminal duct lobular unit (TDLU). The mammary epithelium is composed of basal and luminal cells. The basal epithelium consists of contractile myoepithelial cells. These cells form the outer layer of the organ and function to secrete milk from the lactating gland. The luminal epithelium generates the inner layer of the breast, whilst also forming ducts and secretory alveoli (Prater et al. 2014).

There are three distinct stages of breast development– embryonic, pubertal and reproductive. The mammary gland begins to form at 4 weeks of gestation when ectoderm proliferates to form the mammary or milk line. Which is further categorised into individual placodes following the formation of mammary buds, culminating in the formation of the primary ductal structure and terminal end buds (TEB) (Hennighausen & Robinson 2005). This process is coordinated under the same signals that regulate teeth, sweat glands and hair follicle formation (Robinson 2012).

Following birth, mammary development is suspended. At puberty, under the control of oestrogen and progesterone, with each menstrual cycle the breast expands considerably, involving growth and branching of the secondary and tertiary ductal structures.

The major part of breast development takes place during pregnancy and lactation. During this stage, under the regulation of prolactin and progesterone, ducts continue proliferation forming milk secreting structures known as alveoli. The alveoli join up to form lobules. At the end of lactation, the expanded epithelium undergoes apoptosis and the mammary glands return to their pre-pregnant state known as involution.

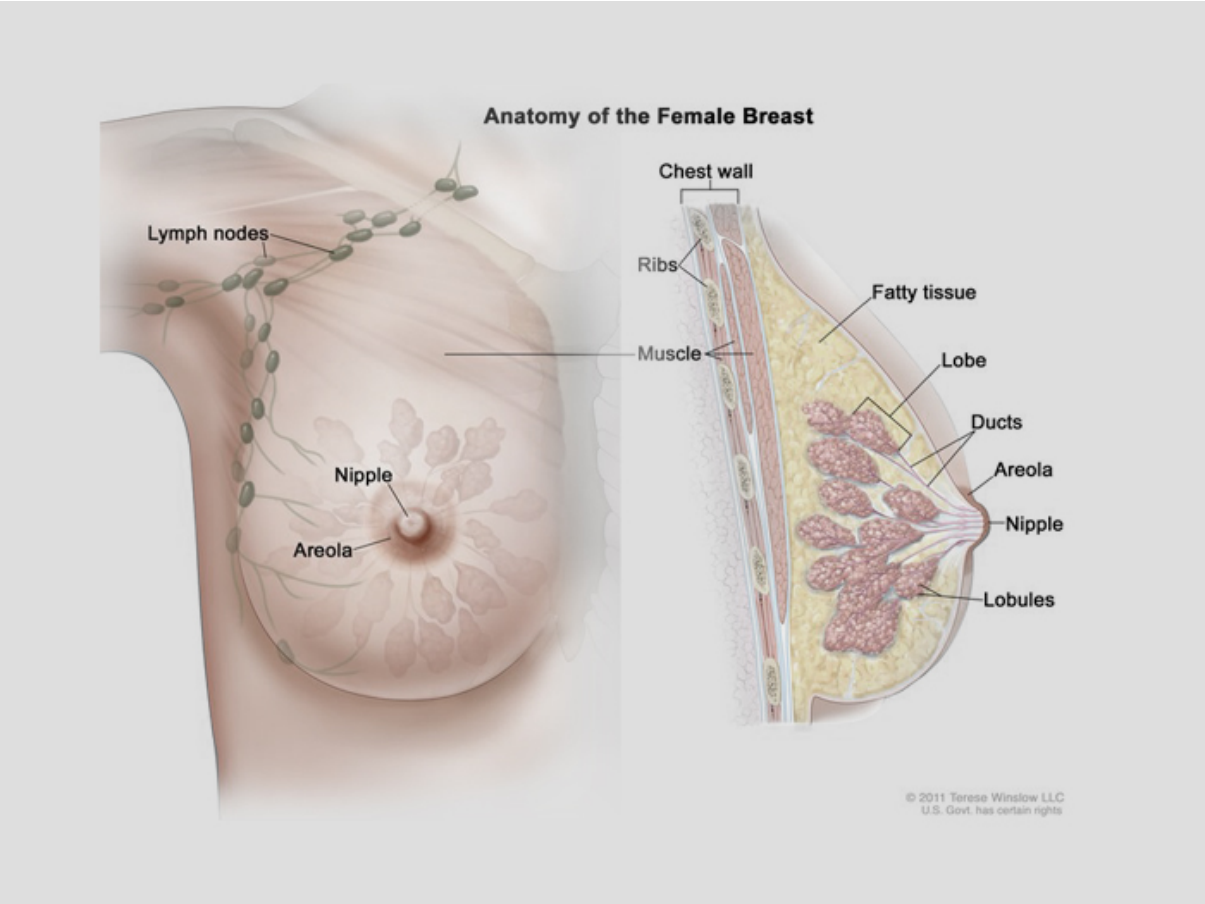


Figure 1.1 Anatomy of Breast. Front view and side view of normal breast tissue showing ducts, lobules, fatty tissue, muscle and lymph nodes. (Source https://cancer.osu.edu Last accessed 9/02/2018).

1.1.3 Breast cancer epidemiology

Breast cancer is the second most common type of cancer worldwide in women after lung cancer. In the UK, approximately one in eight women are diagnosed with breast cancer and, although the advances in diagnostics and treatment are reflected in the increasing survival after diagnosis, 17,000 women still die from breast cancer every year. The incidence of breast cancer increases with age and generally reaches a peak in the 50-60 age group. According to current national statistics, the five years survival rate for stage 1 breast cancer is 100% and 22% for stage 4 metastatic breast cancer (Cancer Research UK, 2012).

1.1.4 Risk factors

The origin of breast cancer is not known. However, many risk factors have been identified. Less than 10% of breast cancers can be attributed to an inherited genetic mutation. Examples include the *BRCA1* and *BRCA2* genes involved in DNA repair. Individuals who inherit mutant alleles of *BRCA1* and *BRCA2* possess a life-time risk of developing breast and to a lesser extent ovarian cancer (Weinberg 2014). However, the mutations in these genes only account for 40% of hereditary breast cancers. Since the discovery of *BRCA1* and BRCA2 more than 15 years ago, understanding in how cells repair DNA damage has helped to identify other genes involved in the repair network. Those that have been found associated with increased cancer risk are *ATM, CHEK2, PALB2* and *BRIP1. ATM* is the serine/threonine kinase involved in DNA repair and cell-cycle check point control (Renwick et al. 2006). *CHEK2* is a serine/threonine kinase that activates downstream of ATM and mediates cell cycle arrest following DNA damage and/or phosphorylation of BRCA1/p53 (Nevanlinna & Bartek 2006). *PALB2* modulates the recruitment of

BRCA1/BRCA2 to the site of DNA damage (Nepomuceno et al. 2017). *BRIP1* has a role in BRCA1 dependent DNA repair (Seal et al. 2006).

It is known that breast cancer is sex hormone dependent. In postmenopausal women, the positive correlation between circulating oestrogen levels and the risk of breast cancer is well established. The available data for premenopausal women shows a similar pattern, in which the breast cancer risk increases with increasing sex hormone concentration (Key 2013).

An epidemiological study published in 1969 reported a higher incidence of breast cancer among nuns (Fraumeni et al. 1969). Since then, many studies have demonstrated that early birth and increased numbers of full-term pregnancies significantly decrease a woman's risk of developing breast cancer (Clavel-Chapelon & Gerber 2002; Dall et al. 2017).

Older studies have reported an early menarche and late menopause as an important risk factor for breast cancer (Apter & Vihko 1983). However, more recent findings have not shown a significant correlation (Opdahl et al. 2011).

An increase in BMI has been reported to be positively associated with post-menopausal breast cancer (Renehan et al. 2008; Magnusson et al. 1998; Huang et al. 1997). Few mechanisms have been suggested of how obesity affects mammary tumorigenesis. One of them is that increased levels of adipose tissue serve as an additional source of oestrogen through increased activity of the enzyme aromatase.

An alternative explanation suggests that hyperinsulinemia, often associated with obesity, can stimulate the mammary epithelium via insulin like growth factor (IGF-1). IGF-1 is a known mitogen for a wide variety of cancer cell lines (Yaginuma et al. 1997; Oku et al.

1991; Macaulay 1992) and has been targeted by monoclonal antibodies (mAb) over the past decade.

Studies have shown that women who exercise 3 times a week have 30-40% reduced risk of developing breast cancer (Brown et al. 2012). Furthermore, regular physical activity can improve disease prognosis and lower disease reoccurrence in cancer patients (Lahart et al. 2015). The positive effects of exercise are linked to reduced body fat, lower insulin levels and sex hormone production. In addition, physical activity reduces cancer risk by improving immune system function (Dieli-Conwright et al. 2016).

1.1.5 Types of Breast cancer

Breast cancer originates from the transformation of breast epithelial cells, lining milk ducts and lobules of the breast. While still restricted to the to the duct or lobule, breast tumours classified as ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS is an early stage breast cancer that can progress to invasive ductal carcinoma (IDC) in which tumour cells migrate to the other parts of the breast. It is estimated that 40% of DCIS will progress into IDS (Cowell et al. 2013). However, currently it is not possible to predict which patient is at risk of developing IDC. In a recent study, by analysing individual breast cancer cells from tissue specimens obtained from patients, researches attempted to track the progression of DCIS to IDS. Following from their study, researchers came up with a model, in which clones arise from a single cell in the milk duct and escape from it to establish IDC in different regions of the breast (Figure 1.2) (Casasent et al. 2018).

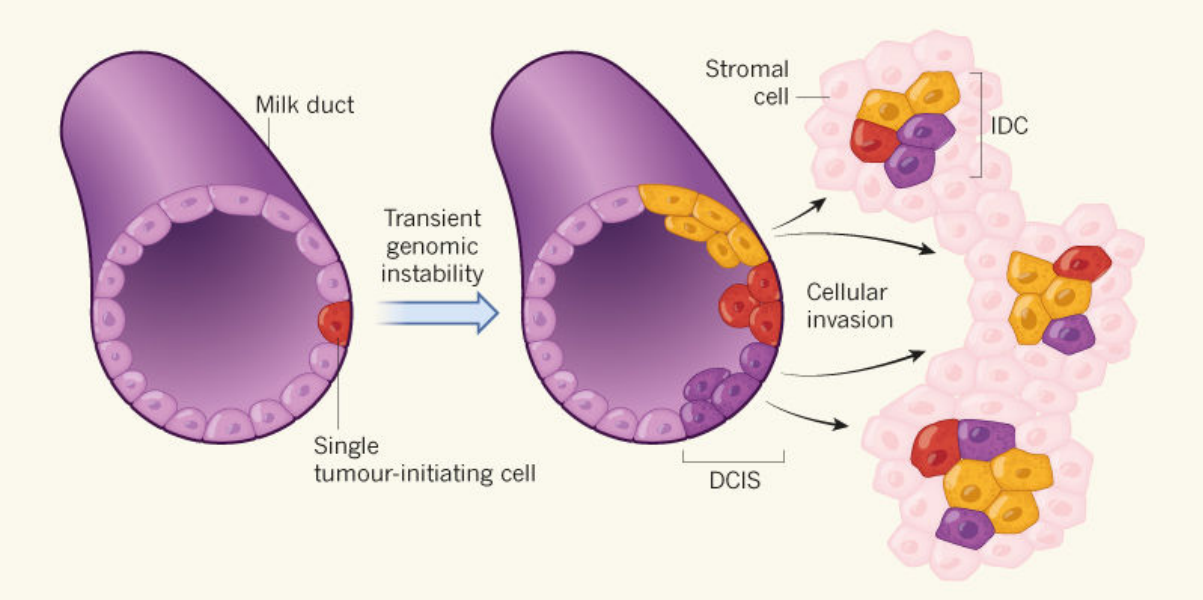


Figure 1.2 Schematic model of the transition from DCIS to invasive stage IDC. DCIS arises from a single tumour cell, in which following mutation forms different clones (purple and yellow). DCIS sometime progress to IDC and all the tumour clones present in DCIS also present on IDC. Adapted from (Casasent et al. 2018).

The traditional breast cancer classification system is based on histopathological assessment (type and grade of tumour), expression of oestrogen receptor (ER), progesterone receptor (PR) and over-expression of human epidermal growth factor receptor 2 (HER2). However, histologically identical tumours may still show different clinical behaviour due to molecular heterogeneity. Therefore, molecular classification based on gene expression profile and proteomic studies have been introduced. According to the genetic profiling, breast cancer is classified into the Luminal A, Luminal B, Basal-like, Her2 positive and Normal-like (Perou et al. 2000). However, more subtypes have now been identified based on the molecular analysis of 2,000 breast cancers (Curtis et al. 2012).

Breast cancers with positive hormonal status (oestrogen or progesterone) constitute up to 65-75 % of all breast cancers. Those that express oestrogen or progesterone, but not the HER2 belong to the Luminal A group. Marker of proliferation Ki67 is a differentiator between Luminal subtype A and B (Miller et al. 2014).

Clinically breast cancer is classified into primary breast cancer characterised by local occurrence and advanced breast cancer spread to distant organs. It is known that 30% of patients diagnosed with primary breast cancer develop metastatic disease, which is the main reason for patient mortality. The metastatic spread of breast cancer cells is achieved by two main routes; vascular and lymphatic. Tumour cells acquire changes according to their metastatic capacity in the early stages of tumorigenesis. Regional lymph nodes are often the first sites to develop metastases. Bone is the most common site of metastasis and almost 80% of patients with advances breast cancer develop these lesions (Siclari et al, 2006).

1.1.6 Metastatic breast cancer

Metastatic invasion accounts for 90 % of cancer-associated mortality. Furthermore, the survival rate for metastatic disease has not improved in the past 30 years (Tevaarwerk et al. 2013). Breast cancer can relapse in two forms; distant metastasis (bone, brain, liver, lung and distant lymph node), which happens in 30 - 60 % of cases and locoregional disease (breast, chest wall and regional lymph nodes) diagnosed in 10 % of patients (Yates et al. 2017). The tumour drained lymph nodes are the first sites of metastatic dissemination in breast cancer. About 30 - 60 % of breast cancer patients with lymph node metastasis eventually develop distant spread (Redig & Mcallister 2013).

Genome profiles of metastatic lesions resemble those of the primary tumour with some acquired genomic changes that are not present in primary lesions (Brastianos et al. 2015; De Mattos-Arruda et al. 2014).

The process of metastases known as metastatic cascade involves a sequence of steps; local invasion, intravasation, transport, extravasation, formation of micrometastases and colonization (Weinberg 2014). The first step of local invasion is associated with invasion of the tissue surrounding the primary tumour. The tumour cells then enter the circulation and disseminate via the blood stream or lymphatic system to distant organs where they undergo cell cycle arrest and adhere to the capillary bed to consequently extravasate the bloodstream, enter the hostile tissue and colonise it (Figure 1.3). Importantly, in order to survive during all those steps, malignant cells have to escape apoptotic signals and the host immune response (Scully et al. 2012).

The “seed and soil” theory postulates that all types of tumour spread in a certain organotrophic manner (Paget 1889). PR- and ER-positive tumour subtypes are more likely to develop secondary lesions in bones, whereas HER2-positive have a tendency to

spread to the liver and lung (Smid et al. 2008). For triple negative breast cancer, the most common sites for metastasis are the brain and lungs and it is less likely to spread to bone tissue (Sihto et al. 2011).

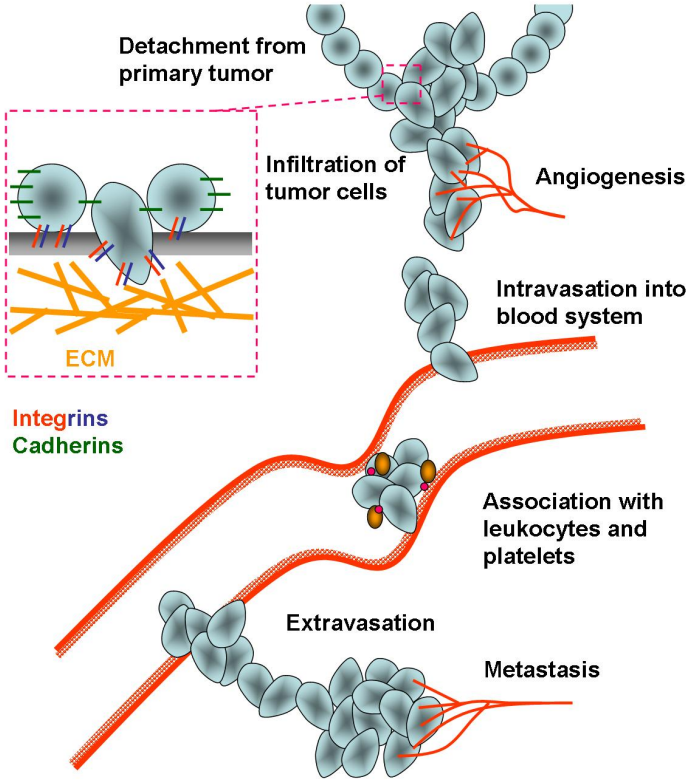


Figure 1.3 Major steps in metastatic dissemination of the tumour. The first step is detachment of the tumour cells from the primary site. The tumour cells then enter the circulation and disseminate via the blood stream or lymphatic system to distant organs where they undergo cell cycle arrest and adhere to the capillary bed to consequently extravasate the bloodstream, enter the hostile tissue and form secondary lesions. Adapted from (Guo & Giancotti 2004).

1.1.7 Breast cancer diagnosis

The important requirement for the successful breast cancer treatment lies is in its earliest possible detection. Diagnosis of breast cancer involves “triple assessment” and includes physical examination, radiological investigation (mammography or ultrasonography) and pathological investigation. Mammography is the main tool in the diagnosis of breast cancer, however, as with most medical procedures, it has limitations and as a result about 20 % of breast cancers are not detected (National Cancer Institute, 2012). On other hand, a recent study on breast cancer screening reported that women are more likely to have breast cancer that is overdiagnosed than have earlier detection of a tumour that is destined to become large. The same study reported that the reduction in breast cancer mortality was the result of improved systemic treatment and not due to the screening programme (Welch et al. 2016).

Another study suggests that the increased detection of small tumours (< 2 cm) is of somewhat limited utility, as the progression from small to large tumour (> 2 cm) would not necessarily occur during the lifetime of the patient. Furthermore, small tumours that are not detected by screening would become more readily detectible within 19 years. In contrast, tumours with a less favourable prognosis are rarely diagnosed early as they have such short lead times (Lannin & Wang 2017). Currently, there is no tool to distinguish primary tumours that are likely to become metastatic from those that will not likely to spread during the lifetime of the patient (Weinberg 2014).

1.1.8 Treatment

In the developed world, the majority of breast malignancies are diagnosed during the early stages by mammogram. Surgical removal of early stage tumour(s) followed by

chemo- or radiotherapy has been the gold standard for breast cancer treatment. Two main treatment options for breast cancer are chemotherapy and endocrine therapy. Cytotoxic chemotherapy after surgery is recommended for patients with the following parameters ER-; PR- and HER2-negative; HER2 positive, larger tumour size and lymph node positive (McDonald et al. 2016).

Cytotoxic agents used in standard chemotherapy include anthracyclines, taxanes and 5-fluorourocyl. Anthracyclines, originally derived from bacterium *Streptomyces peucetius varcaesius* have been a part of chemotherapy since their discovery in 1960. These antitumour antibiotics, being DNA intercalators, interfere with DNA transcription by binding to the unwound DNA strand and preventing cell replication. In addition, they produce free radicals to damage tumour cells. One of the major side effect of anthracyclines is their cardiotoxic effect caused by the peroxidation of the cardiac sarcoplasmic reticulum by free radicals. This in turn lead to cardiac necrosis, as a result of cardiac tissue lacking catalase, the enzyme required to neutralise free radicals (Moudgil & Yeh 2016).

Taxanes are naturally occurring compounds, isolated from the Pacific yew tree. The anti-tumour effect of taxanes occurs due to their ability to arrest the cell cycle during metaphase by the activation of mitotic checkpoints. In addition to anti-cancer treatment, taxanes are widely used in cell biology to induce cell death (Weaver 2014).

5 fluoroucyl is a part of chemotherapy agent known as anti-metabolite. It inhibits the thymidylate synthetase to create shortage of thymine required for DNA replication (Longley et al. 2003).

Patients with PR- or ER-positive breast cancer receive endocrine treatment agents such as oestrogen receptor modulators and aromatase inhibitors. Oestrogen receptor

modulators work by binding to the oestrogen receptor to interfere with DNA synthesis. Among them is Tamoxifen prescribed for pre-menopausal women. Aromatase inhibitor drugs inhibit the enzyme “aromatase” – a main source of oestrogen in post-menopausal women (Miller et al. 2014). Endocrine treatment is prescribed for at least five years. However, targeted endocrine therapy for ER-positive breast cancer is not effective in all cases. Many patients initially benefit from it, but with time develop drug resistance (Selli et al. 2016).

About 25 % of breast cancers are characterised by the overexpression of HER2 (ErbB1) protein. HER2 is the transmembrane glycoprotein that has both intracellular receptor tyrosine kinase (TK) domain and an extracellular ligand binding domain. HER2 receptors are activated via homodimerisation or heterodimerisation with its family members HER1 or HER3. Treatment of breast cancers overexpressing HER2 involves the monoclonal antibody Herceptin, also called trastuzumab in combination with chemotherapy (Weinberg 2014).

Triple negative breast cancer (TNBC) are tumours characterised by the absence of ER, PR and HER2. These tumours are biologically aggressive and do not benefit from hormonal based treatment. Chemotherapy following surgery remains the major therapeutic option for the treatment of TNBC (Wahba & El-Hadaad 2015).

The main aim of treatment for patients with metastatic disease is to prolong the survival and minimise the side effects. Treatment options for metastatic cancer patients depends on the site of the metastatic lesion and the molecular subtype of the tumour, as it has been shown to be associated with a distinct response to treatment (Rouzier et al. 2005). Treatment options are the same as those for primary tumours and include chemotherapy, hormone therapy or monoclonal antibody approaches for treatment of HER2-positive

tumours (Lambert et al. 2017). Androgens, oestrogen and progesterone are often used for the third or fourth line of treatment of metastatic disease. Medroxyprogesterone, a synthetic progesterone is administered to control the growth of breast cancer cells by inhibiting oestrogen receptor levels. It is often prescribed to patients that no longer respond to tamoxifen. High doses of artificial oestrogen, diethylstilbestrol, is used in patients that have been treated with antioestrogens. As the growth of breast cancer cells, prolonged period of time deprived from oestrogen shown to be inhibited by the high dose of oestrogen (Osipo et al. 2003). Despite androgens historically being used as treatment agents for metastatic breast cancer, due to their utility in suppressing tumour growth, they have since been rendered obsolete.

Surgery is the primary choice of treatment for locally recurred breast cancer. Reginal recurrence after mastectomy is treated with surgical excision of regional lymph nodes followed by radiotherapy. Patients with advanced disease at their first presentation with breast cancer, benefit from the surgical removal of the primary lesion. Metastatectomy can be performed for the pulmonary and liver metastases, however, it poses great risk for the patient.

Radiation therapy (RT) for the treatment of advanced breast cancer has been used in clinical practice since 1949. It is based on the use of high energy rays to kill malignant cells at various metastatic locations. This type of cancer treatment reduces local relapse of the disease by 70%. Over the years RT has been improved to reduce normal cell toxicity and overall treatment time. Local RT to the metastatic lesion is the treatment of choice for locally recurred breast cancer. A limited number of bone metastases are treated with local RT, while multiple lesions are treated with systemic RT. In the case when resection of liver metastasis is not possible, RT is applied. For patients with brain metastasis, surgery is an option if the disease is symptomatic, as it can eliminate

symptoms of intracranial hypertension, focal neurological deficit and improves overall survival. As in the case of pulmonary metastasis, inaccessible metastatic brain lesions can be treated with RT.

Successful treatment of patients with advanced breast cancer heavily depends on accurate staging of the cancer. In order to stage breast cancer, various investigative techniques are used with the focus on the presence of metastatic dissemination. During the course of treatment, the radiological investigation is used to evaluate the progression of the disease and monitor the treatment response. Radiological investigation includes X-ray, CT scan, Ultrasound scan, MRI scan and PET-CT scan. These investigations are coupled with histopathological examination of the metastatic specimen to improve the accuracy of the disease staging.

The evolutionary nature of cancer makes it very difficult to treat. However, this problem is not only attributed to cancer treatment, but also resistance to antibiotics and some viral infections such as human immunodeficiency virus (HIV). In the context of HIV treatment, this issue is successfully addressed through a combination of drugs, in conjunction with anti-HIV vaccines, are now used to combat HIV (Hammer et al. 1997; Gulick et al. 1997; Ferguson et al. 2013).

1.1.9 Vaccines

The immune response against cancer, particularly breast cancer, plays an important role in the survival of diagnosed patients. Early animal studies showed that the immune system recognises tumours and elicits an immune response against them (Foley 1953). A typical tumour contains 30 to 70 mutations, these mutations are foreign to the host's

immune system and could therefore be targeted by immunotherapy (Vogelstein et al. 2013).

Selecting a target for vaccine development is crucial, however, unlike in the case of cervical cancer, where viral aetiology is established, there is no single cause for breast cancer. There are different subsets of breast cancer such as Luminal A, Luminal B, HER2 and basal like cancers, which makes it challenging to identify a common vaccine target.

Currently several types of anti-tumour vaccines are being tested in breast cancer patients (Parmiani et al. 2014). Peptide-based vaccines work by generating a cytotoxic T-lymphocyte response against the tumour cells. One such example is the vaccine against Folate receptor (FR alpha) that is expressed in 80% of TNBCs and negatively correlated with patient survival (Cheung et al. 2016). HER2-derived peptide vaccine is another example of the therapeutic strategy against HER2 positive breast cancer being tested showing improved patient survival (Clifton et al. 2016).

Another therapeutic approach is the use of DNA-based vaccines to combat cancer. For instance, DNA vaccine INO-1400 that contains a plasmid encoding tumour associated antigen, human telomerase reverse transcriptase (hTERT), expressed in 85% of tumour cells. The result of INO-1400 vaccination is the expression of hTERT, which in turn evokes a cytotoxic T-lymphocyte immunological response against malignant cells expressing hTERT.

DNA based vaccines are also used to develop tailored treatment for TNBC patients. In this approach, the patient's own cancer cells are used to generate plasmid DNA in order to boost the anti-tumour immune response by propagating isolated T-cells. This approach is known as Adaptive T cell transfer therapy (ATC) and is currently being tested (Moreno Ayala et al. 2017).

Emerging data suggest the potential of the use of dendritic cell-based vaccines for cancer treatment. Dendritic cells (DCs) are a population of leukocytes that play a role in the initiation of the innate immune response. Once DCs recognise the antigen, they migrate to the lymphoid organs where they interact with CD4+ and CD8+ T lymphocytes resulting in activation of T cells. Activated T cells become cytotoxic T cells and generate an antigen specific immune response (Gelao et al. 2014). DCs are present in the infiltrate of breast tumours and have been found to provide a memory response to tumour antigens (Lissoni et al. 1999). The effectiveness of DCs anticancer vaccines have been demonstrated by animal studies. Immunisation of mice with tumour antigen loaded DCs have been reported to provoke a cytotoxic T lymphocyte immune response against tumour cells (Zheng et al. 2013).

The Boris antigen is a new protein that is now being targeted for developing a vaccine against breast cancer. The application of DCs based vaccines against Boris is currently under investigation (Mkrtichyan et al. 2011).

A major limitation of immunotherapy is tumour-associated self-tolerance and mutation (Cheng et al. 2004). These issues are being addressed by the use of multiple immunogenic epitopes to reduce tumour immune escape variants (Buonaguro et al. 2011).

1.1.10 Biomarkers of Breast Cancer

Biomarkers, also known as molecular markers, are defined by the National Cancer Institute as a substance found in tissue, blood or other body fluids that are a sign of cancer or certain noncancerous conditions. The properties of a good cancer biomarker include high presence in cancer patients and absence or low presence in healthy individuals and

easy quantification of biological samples (Carlomagno et al. 2017). Depending on the source of biomarkers they are classified as humoral or cellular. Currently, biomarkers play a crucial role in the management of breast cancer. Depending on their properties biomarkers can be divided into three groups; diagnostic, prognostic and predictive.

A diagnostic biomarker allows for the early detection of cancer in a noninvasive way. By the time of diagnosis breast cancer grows to its critical mass which makes it difficult to treat. Currently, there are no approved biomarkers to be used for early detection of breast cancer. Therefore, there is the need to identify cancer markers to assist with the diagnosis of disease onset.

The purpose of prognostic biomarkers is to inform about disease outcome, such as recurrence, progression or death, independent of the treatment(s) received. For instance, patients with “triple-negative” breast cancer have a significantly lower survival rate than other breast cancers. As the main cause of mortality of breast cancer is metastatic dissemination, new markers are required to identify the likelihood of metastasis. (Ballman 2015). However, current prognostic tests include tumour sampling which are obtained through biopsy, yet in order to minimise the invasive procedure circulating biomarkers are urgently needed.

Predictive biomarkers provide information regarding treatment response and help to identify patients that are likely to benefit from a specific treatment. One such example of an important predictive biomarker is HER2, the overexpression of which allows clinicians to determine which patients would benefit from monoclonal antibody therapies (Duffy et al. 2017).

1.1.10.1 Humoral biomarkers

The first cancer marker was discovered in 1864 by Bense Jones, who identified the presence of a specific immunoglobulin in the urine of myeloma patients. Since then, many circulating tumour markers have been identified and characterised.

Presently there are no sensitive and specific minimally invasive biomarkers for the detection of early stage breast cancer. Currently available serum biomarkers for breast cancer include carcinoma antigen CA 15-3 and carcinoembryonic antigen (CEA) which are mainly used as predictive and prognostic biomarkers in patients with metastatic breast cancer. Unfortunately, the specificity of the aforementioned markers is low as elevated levels of both proteins have also been found in patients with inflammatory and autoimmune diseases as well as in smokers and the elderly (Lumachi & Basso 2004). In a recent study the combined use of CA 15-3 with two novel tumour associated autoantibodies was investigated in order to increase sensitivity for early breast cancer detection. Predictive accuracy was demonstrated to be considerably higher; however, most of the serum used in the study was from patients with advanced disease (Dong et al. 2013).

Epithelial cells are protected by a mucous barrier comprised of secreted and transmembrane mucins. MUC1 is a transmembrane protein that has been shown to be overexpressed in epithelial cancer cells. Overexpression can lead to the loss of epithelial polarity and activation of tyrosine kinases causing downstream signalling and cancer cell survival (Graham et al. 2014). In order to detect the MUC1 protein, several antibodies are used, such as CA15-3 and CA27.29. Determining the concertation of CA15-3 in blood is used for screening, not only for breast cancer, but for pancreatic, lung, ovarian, colon and liver cancers. In breast cancer, CA15-3 is utilised to predict the relapse of disease (Lee et al. 2013). A recent study reported that serum levels of CA15-3 can be used as an independent prognostic factor in patients with advanced breast cancer (Darlix et al. 2016).

In another study on the correlation of the molecular subtype of tumour and CA15-3 levels, researchers found that the high levels of the marker was strongly associated with ER negative status (Shao et al. 2015). According to the current guidance of the American Society of Clinical Oncologists, the use of CA15-3 is not recommended for screening, diagnosis or staging of breast cancer.

CA27-29 is another MUC1 marker, also known as breast carcinoma-associated antigen (Rack et al. 2010). Although, elevated levels of CA27-29 are present in serum of 80 % of breast cancer patients, it is also found in patients with benign breast conditions as well as in other malignancies (Beveridge 1999). This marker has similar utility to CA15-3.

Another widely used serum marker in breast cancer is the oncofoetal glycoprotein CEA, the first discovered in human colon carcinoma in 1965. Various studies confirmed that high serum concentration of CEA in breast cancer patients correlates with metastasis and poor disease outcome. In primary breast cancer, high pre-treatment levels of CEA were found to be useful criteria to identify node negative breast cancer patients with high risk of disease recurrence (Molina et al. 2010). In a recent study, high levels of CEA in preoperative primary breast cancer was detected in patients with HER2 positive tumours (Shao et al. 2015). Currently CEA is recommended to be used in clinical settings in combination with digital imaging to assess treatment efficacy (Harris et al. 2007). However, it is not specific for breast cancer as elevation of CEA has also been detected in other malignancies such as colon, lung, thyroid, pancreas, liver, stomach, ovary, and bladder (Thompson et al. 1991). Further work, although challenging, is necessary to improve the limitations for early breast cancer diagnosis.

Currently few promising circulating biomarkers of breast cancer are in the development pipeline. These include mammaglobin and MicroRNAs (miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-182) (George & Mittal 2010; Mar-Aguilar et al. 2013).

Mammaglobin is a 14-22 kDa protein found in the plasma of over 70 % of breast cancer patients and not detected in patients with other types of cancer. To investigate the levels of this protein, an ELISA technique was applied and was able to differentiate between breast cancer patients and healthy volunteers. Furthermore, it showed high sensitivity and specificity of the potential biomarker (Bernstein et al. 2005).

miRNAs are a type of short interfering RNAs that function in post-transcriptional regulation of gene expression. The changes in miRNA are reported to be strongly associated with cancer development and progression (Vasudevan et al. 2007; Alajez et al. 2011). Therefore, the potential use of miRNA as circulating cancer biomarkers is currently a popular area for clinical investigation. For instance, increased levels of miRNA-10b and miRNA-373 is found in the plasma of lymph node positive breast cancer patients (W. Chen et al. 2013).

1.1.10.2 Cellular biomarkers

The cellular markers routinely used in clinical practice are oestrogen receptor (ER), progesterone receptor (PR) and HER2. These are important markers of treatment response in breast cancer.

Oestrogen receptor (ER) is a member of the nuclear steroid family of receptors and is controlled by the female sex hormone oestrogen. ER measurement in breast cancer tumours is used to identify patients that benefit from endocrine treatments such as

tamoxifen in both local and metastatic diseases. Clinically, ER-positive breast tumours indicate a more favourable prognosis for patients (Lumachi et al. 2013).

Progesterone receptor (PR) is nuclear hormone receptor that binds to progesterone. PR status is analysed in local and metastasised breast cancer lesion to identify patients that would benefit from hormonal treatment and in determining a treatment plan.

HER2 is a protein encoded by the *erbB* oncogene and is a truncated version of the epidermal growth factor receptor (EGF). Healthy cells require growth factors in order to grow, whereas cancerous cells are less dependent on growth factor stimulation. The oncogene *erbB* lacks the N-terminal and this mutation causes abhorrent growth signalling independent of ligand binding (Weinberg 2014). The presence of HER2 in breast cancers are associated with poor prognosis. Overexpression of HER2 is detected in 15-30 % of invasive breast cancers (Rimawi et al. 2015). Detection of HER2 in clinical settings is determined by IHC and fluorescence *in situ* hybridisation (FISH) techniques (Petroni et al. 2016). HER2 expression patterns are evaluated in both primary and metastatic breast cancer patients. Determining HER2 status is important to determine patients that would benefit from anti-HER2 monoclonal antibody therapy. However, anti-HER2 treatment is not exclusive, patients with HER2 positive disease also benefit from anthracycline based therapy.

The proliferation marker Ki67 is another prognostic parameter that influence treatment regimes in the management of breast cancer. Ki67 is the nuclear protein that has a role in cell proliferation. Its status in tumour mass is evaluated using immunohistochemistry. Positive staining in early stage breast cancer correlates with high risk of breast cancer recurrence and poor survival rates in both node negative and node positive patients (de Azambuja et al. 2007). Another study reported that Ki67 positive cancer significantly

correlates with shorter overall patient survival (Stuart-Harris et al. 2008). The predictive value of Ki67 in response to treatment was evaluated in several recent studies. In one of them, high Ki67 was indicative of improved relapse-free survival in an ER positive cohort treated with both chemotherapy and endocrine treatment (Niikura et al. 2012). For the treatment of locally advanced breast cancer, chemotherapy is often administered before surgery. In this cohort of patients, high pre-treatment Ki67 was shown to be predictive of complete response to chemotherapy (Brown et al. 2014).

Urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PaI-1) are a class of plasminogen activating proteins. High levels of uPa and PaI-1 in tumour tissues such as breast, prostate, endometrial, colon, lung and stomach are associated with poor disease prognosis (Stillfried et al. 2007). UPa is protease that converts the plasminogen into plasmin and PaI-1 is an inhibitor of the activity of uPa. Both uPa and PaI-1 are directly involved in metastasis and therefore regarded as a prognostic marker of cancer. Their elevated levels in breast cancer tissue is associated with poor prognosis for patients with node negative disease (Lampelj et al. 2015).

Tumour suppressor protein P53 is a nuclear protein involved in the regulation of the cell cycle. Mutation of p53 is frequently observed in a wide variety of human cancers. In breast cancer, mutation of p53 is a strong indicator of the aggressiveness of the tumour and is associated with poor survival probability (LI 2008). A recent study shows that p53 status may be used as a predictive factor for the response to chemotherapy, as it has been shown that cancer cells treated with chemotherapy agents undergo p53 mediated apoptosis. Reduced levels of p53 could be a marker of resistance to chemotherapy (Parrales & Iwakuma 2015).

Cyclin E, a cell cycle regulator, is expressed in late phases. Deregulation of cell cycle proteins is a frequent event in tumorigenesis. Overexpression of Cyclin E is associated with poor prognosis and disease recurrence (Bi et al. 2015).

1.1.10.3 Monitoring treatment response

Monitoring treatment response is crucial in the management of metastatic breast cancer. It helps clinicians to identify patients that benefit from the treatment received and those that require a change of treatment. Traditionally, measurement of the levels of CA15-3 and CA 27.29 are used to monitor the response to treatment in patients with advanced disease. Elevation in the levels of CA15-3 and CA 27.29 indicate treatment failure. However, this method is not recommended to be used alone, but in combination with diagnostic imaging as levels of CA15-3 are shown to rise spuriously in the first 4-6 weeks of treatment (Graham et al. 2014).

1.1.10.4 Multigene prognostic assays

1.1.10.4.1 Oncotype DX

Oncotype DX is a 21-gene assay that is used to evaluate breast cancer recurrence. The test includes 16 cancer related genes and 5 reference genes from primary breast cancer tissue. The result of the test is a score from 0-100 reflecting the likelihood of disease recurrence within 10 years (McVeigh et al. 2014). This assay has prognostic and predictive value for ER positive breast cancer. More specifically, the test provides information whether patient will benefit from chemotherapy.

1.1.10.4.2 MammaPrint assay

The MammaPrint assay is a prognostic microarray-based multigene assay for patients younger than 61 years of age with stage I-II node negative or I-III node positive breast cancer (Mook et al. 2009). The assay measures the mRNA expression of 70 genes involved in the regulation of cell proliferation in order to identify patients with a very good or very poor prognosis. Originally the test required freshly frozen tissue (within an hour from the surgery), however, recently the assay was improved to allow the use of formalin fixed paraffin embedded tissue (Mittempergher et al. 2011).

1.1.10.4.3 Genomic grade index

The genomic grade index is a microarray test that measures the expression of 97 genes to establish the molecular grade of the tumour. The assay is based on the comparison of gene expression profiles of grade I and grade III tumours (Gyorffy et al. 2015).

The breast cancer index is an RT-PCR based test that is designed to predict early and distant disease recurrence in ER positive node negative breast cancer. In addition, it helps identify patients who would benefit from extended endocrine therapy (Sgroi et al. 2013).

1.1.10.4.4 EndoPredict assay

The EndoPredict assay is an RT-PCR assay based on measurement of the expression of eight cancer and three control genes. The test is used to predict disease recurrence in ER positive breast cancer patients undergoing endocrine treatment (Filipits et al. 2011).

1.1.10.5 Liquid biopsy

Liquid biopsy is an approach, alternative to tissue biopsy, that involves analyses of tumour tissue material found in blood and other body fluids. The test is based on the knowledge that during tumorigenesis tumour components are shed into the circulation. The notion of liquid biopsy is not new, it was first established to detect foetal DNA in maternal blood (Lo et al. 1997). Recently though many techniques have been developed to study circulating tumour cells, cancer stem cells and circulating tumour DNA. Cancer is caused by a series of mutations in oncogenes that affect the function of certain pathways potentially taking several decades to develop. Therefore, the majority of cancers are not detected in the first 90% of the "cancer lifetime" (Vogelstein et al. 2013). As most of the cancer driver genes and pathways affected by them are well studied, liquid biopsy may serve as a tool for early diagnosis (Heitzer et al. 2017).

1.1.10.5.1 Circulating tumour cells

Circulating tumour cells (CTC) are released by tumours into the circulation. The presence of the CTC in the blood of cancer patients was first reported in 1869 by Thomas Ashforth (Ferreira et al. 2016). Advanced tumours release millions of cells into the circulation each day, but these cells have short half-lives (Vogelstein et al. 2013). Therefore, the main challenge in the field of CTC research lies in the low number of CTC found in the blood of cancer patients (one CTC is detected among millions of healthy blood cells) (Nelson 2010). CTCs have distinct physiological characteristics that help to distinguish them from normal blood cells. Like most epithelial cells they are of large size, different density, charge and express epithelial cell surface markers not present on leukocytes (Yu et al. 2011). Based on these properties, various strategies have been developed to capture CTCs (Andreopoulou et al. 2012; Campton et al. 2015; Sollier et al. 2014). The prognostic value of CTCs in the circulation of breast cancer patients has been studied extensively. Numerous studies have reported the correlation between a high CTC count and poor

prognosis for breast cancer patients (Botteri et al. 2010; Zhao et al. 2011; Munzone et al. 2012). In a recent study on how ER positive cancer cells acquire resistance to endocrine treatment, a population of ER negative CTCs were reported in ER positive tumours, which might be able to escape hormonal treatment (Babayan et al. 2013). Furthermore, a special test based on the detection of the panel of CTC was developed to identify patients with advanced disease that resist endocrine treatment (Paoletti et al. 2015)

1.1.10.5.2 Cancer stem cells

Cancer stem cells were first identified in human breast cancers in 2003 (Al-Hajj et al. 2003). The discovery of cancer stem cells (CSC) changed the way researchers look at the mechanism of tumorigenesis. According to the CSC hypothesis, cancers originate from progenitor cells, or CSC. Organ specific stem cells are characterised by their ability to self-renew and differentiate into cell types that form the organ. The ability of female breast tissue to expand during puberty and pregnancy suggests the presence of cells with proliferative properties that are typically attributed to stem cells. It is believed that CSC are shed by the primary tumour and metastatic lesion into the circulation and therefore, considered as important prognostic biomarkers to study disease progression. For example, in metastatic breast cancer the baseline CSC count of >5 was identified as a risk factor for disease progression as well as an indicator of poor survival (Lv et al. 2016). Similarly, in patients with primary breast cancer, the detection of CSC before and after chemotherapy indicated an increased risk of disease recurrence and worse patients survival (Rack et al. 2014). CSCs are detected through the use of epithelial markers such as cytokeratins that are not expressed in blood cells (Alix-Panabieres & Pantel 2014).

1.1.10.5.3 Circulating tumour DNA

Circulating tumour DNA (ctDNA) are derived from the dying tumour cells that release their DNA fragments into the blood of cancer patients. Despite their short half-lives they are more abundant in blood than CTCs (Cai et al. 2015). The detection of ctDNA was reported in 75 % of metastatic breast cancer patients and 50 % of patients with a primary tumour (Bettegowda et al. 2014). The increased level of ctDNA in patients with metastatic disease was found to be associated with progressiveness of the disease. Furthermore, the changes in ctDNA levels in the response to treatment were found to be indicative of the changes in tumour mass (Dawson et al. 2013). In addition, sequencing of ctDNA reflects the specific mutation acquired by the tumour in response to treatment (Murtaza et al. 2013). A recent study reported that ctDNA is detectable in 50 % of patients with early stage breast adenocarcinoma (Bettegowda et al. 2014).

1.1.10.6 Proteomics in biomarker discovery

Proteomic approaches are regarded as a powerful and popular tool to study tumorigenesis. Although using genomic approaches have resulted in the discovery of several cancer related genes, in order to learn about their function proteomics is required. Furthermore, mRNA based studies whilst being informative do not always correlate with the corresponding protein concentration (Schwanhüusser et al. 2011). Proteins are highly dynamic molecules that are regulated by various processes such as proteolytic degradation, post-translational modification and composition of complex structures. Therefore, studying proteomic profiles of different specimens from cancer patients is a frequently used tool to identify cancer biomarkers for disease progression and treatment monitoring. Moreover, proteomics is a more favourable technique then genomics as it can

be applied to diagnostic and prognostic purposes within clinical settings (Sallam 2015). Proteins can be detected by various assays such as western blot and ELISA.

The ultimate goal in biomarker discovery is the development of blood-based assays, as blood reflects information regarding all physiological and biological processes in the body.

Since the invention of mass spectrometry (MS) more than 15 years ago, many potential candidate cancer biomarkers have been identified. However, to date only 15 plasma/serum biomarkers have been approved by the United States FDA (Table 1.1) Although the discovery of novel biomarkers is not problematic, it is the validation process where obstacles occur (Makawita & Diamandis 2010). The most commonly used proteomic techniques are tissue protein microarray, 2D-gel electrophoresis and mass spectrometry.

1.1.10.7 Mass spectrometry overview

Mass spectrometry is a technique used to detect, identify and quantify molecules based on their mass-to-charge ratio. The technique was originally developed almost 100 years ago to measure masses of charged atoms and separate elemental isotopes, which contributed to the development of the nuclear bomb during WWII (Griffiths 2008). Later MS was applied to study protein structures.

MS has become a method of choice for cancer biomarker discovery, as it is used to identify unknown compounds within complex samples. The first step of MS analysis is the generation of ions from the studied substance by electron ionisation. These ions are then fragmented and separated according to their mass/charge ratio. The data is displayed in the form of a plot of ion abundance versus mass/charge ratio. The identified mass is then

compared with a known substance using a database thus allowing for protein identification.

To ionise proteins different ion sources are used. MALDI (Matrix Assisted Laser Desorption/Ionisation) is the ionisation technique used to analyse DNA, proteins and sugars, where the sample is mixed with a laser absorbing matrix to create ions. SELDI (surface enhanced laser desorption ionisation) is a variant of MALDI that relies on the use of a ProteinChip coated with specific matrices (hydrophobic, cationic or anionic) that can trap proteins selectively. To avoid unspecific binding, the chip is washed after (Vertes 2008). LS-MS/MS method is the combination of mass spectrometry and liquid chromatography. This technique offers higher accuracy in analysis of larger biological molecules.

1.1.10.8 2D-polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE is a key technique in comparative proteomic research, the use of which allows thousands of proteins to be separated in a single gel resulting in the generation of a protein profile. This technique is used to find differentially expressed protein in samples healthy versus diseased individuals. The technique involves two steps to separate cell lysate according to two protein properties; molecular mass and charge. In the first dimension, proteins are solubilised in urea and are separated on the basis of their isoelectric point focusing on a pH gradient. Proteins migrate until they have no net charge (Saraswathy et al. 2011). In the second dimension, proteins are loaded onto a polyacrylamide gel containing sodium dodecyl sulphate (SDS) under an electric field. The proteins are denatured and due to the presence of SDS become negatively charged allowing them to migrate under the electric field according to the molecular weight

(O'Farrell 1975). The result is proteins are separated in distinct regions on the acrylamide gel, which is then stained. Following staining, proteins can be excised from the gel, digested with proteases and identified using mass spectrometry.

Table 1.1 list of United States Food and Drug Administration approved plasma/serum biomarkers (Chambers et al. 2014).

Biomarker	**Cancer**	**Sample**	**Year**
Pro2PSA	Prostate	Serum	2012
ROMA(HE4+CA-125)	Ovarian	Serum	2011
OVA1 (multiple proteins)	Ovarian	Serum	2009
Fibrin degradation product	Colorectal	Serum	2005
AFP-L3%	Hepatocellular	Serum	2005
CA19-9	Pancreatic	Serum/Plasma	2002
CA-125	Ovarian	Serum/Plasma	1997
CA15-3	Breast	Serum/Plasma	1997
CA27.29	Breast	Serum	1997
Free PSA	Prostate	Serum	1997
Thyroglobulin	Thyroid	Serum/Plasma	1997
α-fetoprotein	Testicular	Serum/Plasma	1992
Total PSA	Prostate	Serum	1986
Carcinoembryonic antigen	Not specified	Serum/Plasma	1985

1.1.10.9 Biomarker development pipeline

The biomarker development pipeline represents a sequence of phases that include biomarker discovery, verification and validation before approval for use in clinical settings (Figure 1.4). In the discovery phase, a list of potential protein biomarkers is generated using mass-spectrometry based experiments performing sample fractionation (gel electrophoresis, iso-electric focusing and liquid chromatography). The result is tens to hundreds of differentially expressed proteins that are discovered. In a review focused on existing biomarker discoveries, a list of 1261 putative proteins cancer markers was generated, showing that there is no shortage of proteins in the discovery phase (Polanski & Anderson 2007). However, there is discrepancy between biomarker discovery and clinical validation.

In the qualification step, given the high number of discovered candidate biomarkers, these have to be filtered to select for the most promising targets. In the verification and validation phase the selected biomarkers undergo quantification using various techniques such as RT-QPCR and protein assays to exclude false-positive candidates. As a result, the number of target proteins is dramatically decreased. The pre-clinical steps involve expression and purification of protein biomarkers to develop antibodies against them. Finally, in the clinical stage also known as clinical trials, candidate biomarkers undergo validation on a large number of clinical samples. In the outcome at this stage surrogate and clinical endpoint are evaluated. The clinical endpoint reflects on how patients feel, function or how long a patient survives, whilst the surrogate endpoint is a biomarker substitute for a clinical endpoint (Atashpaz-Gargari et al. 2014).

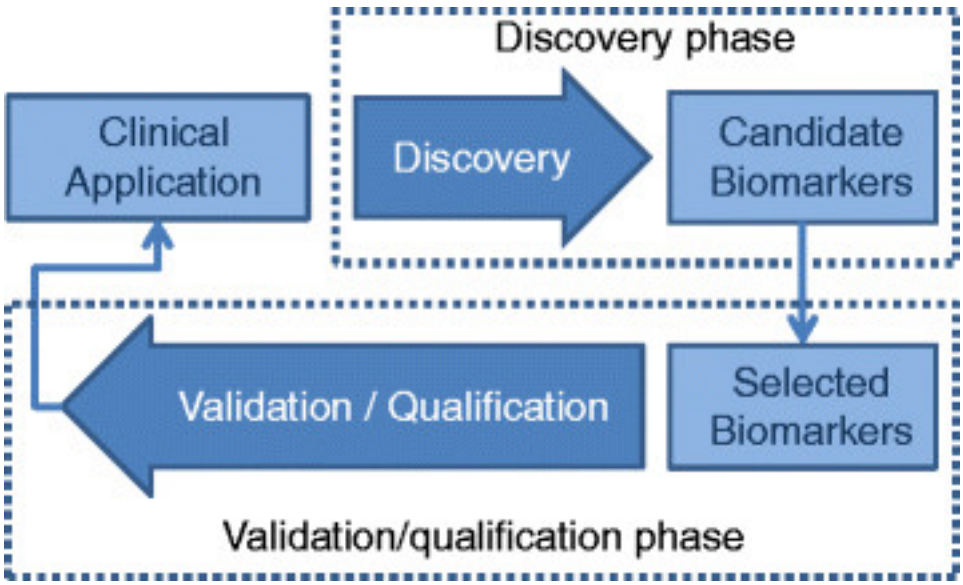


Figure 1.4 Main stages of the biomarker development pipeline. The discovery phase involves MS experiment to generate a list of potential biomarkers which are then selected to be validated and used for clinical applications (Atashpaz-Gargari et al. 2014).

1.2 Cancer and immunity

1.2.1 Relationship between cancer and inflammation.

The relationship between the development of cancer and inflammation was first suggested by Dr Rudolph Virchow in 1863. He identified leukocytes in cancerous tissues and discovered the link between inflammation and cancer. In another publication in 1986, Harold Dvorak described tumours as wounds that do not heal (Flier et al. 1986). The researcher found that the same processes are required for wound-healing and cancer progression. Cancerous cells release growth factors and cytokines in the same way as wounded cells do to recruit leukocytes and initiate an inflammatory response that includes proliferation of epithelial cells and formation of blood vessels near the wound (Weinberg 2014). A recent study by Feng and co-workers looking into parallels between tumorigenesis and wound inflammation using live imaging techniques also revealed the same pattern of leukocyte recruitment in both processes (Feng et al. 2010).

1.2.2 Neutrophils

Neutrophils, also referred to as polymorphonuclear leukocytes are the most common type of white blood cells, comprising 50-70 % of all leukocytes. They represent the first line of immune defence against invading pathogens and along with other leucocytes they are directed to repair sites of damaged tissues. Neutrophils functions by releasing enzymes, anti-bacterial agents and cytokines. In the blood of healthy individual these haematopoietic cells exist in a non-active state, ensuring that their intracellular content is not released to damage host tissue (Wright et al. 2010).

Neutrophils develop in the bone marrow. During infection, resting neutrophils become activated (a process referred to as priming) and recruited to the site of infection by

selectin- mediated rolling, integrin- mediated adhesion and transmigration through the vessel wall. At the site of inflammation neutrophils release cytokines and chemokines to recruit other immune cells, engulf pathogens via phagocytosis and form neutrophil extracellular traps (NETs) in order to prevent bacterial spread. In the absence of inflammation, neutrophils die apoptosis (Figure 1.5) (Geering & Simon 2011; Amulic et al. 2012; Mócsai 2013).

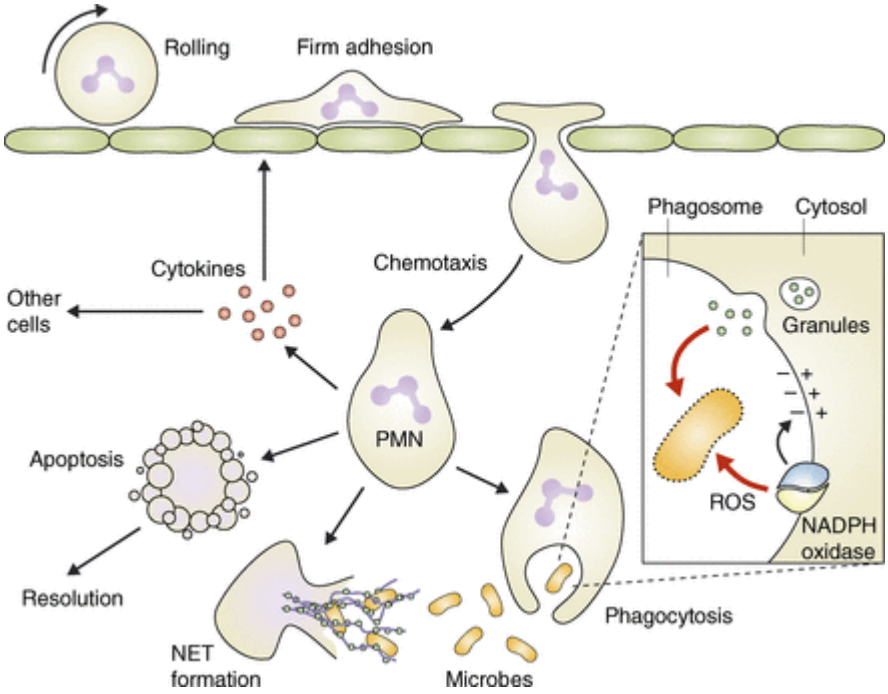


Figure 1.5 Neutrophil functions. After migrating to the site of inflammation, neutrophils phagocytose and digest the invading microbes; release NETs, which trap bacteria; and produce cytokines, which contribute to the inflammatory reaction. Once infection is cleared, neutrophils die by apoptosis and promote a program to resolve inflammation. Inset, the destruction of the pathogens inside the phagosome occurs by ROS generated by the NADPH oxidase, as well as granule enzymes released from intracellular granules. Illustration is taken from (Mócsai 2013).

1.2.2.1 Role of neutrophils in cancer

In addition to their antimicrobial function, a new role for neutrophils as modulators of anti-tumour immunity has been proposed (Mantovani et al. 2011) It is recognised that leukocyte infiltration is one of the main characteristics of a wide variety of human tumours. Tumour associated neutrophils, referred to as TANs, are known to be present in tumour leukocyte infiltrate and it has been shown that cancerous cells themselves attract neutrophils by secreting one of the major neutrophil attractants, interleukin 8 (Figure 1.7) (Gregory & Houghton 2011). It has been established that many tumour cell lines including breast, colon, cervical, lung, brain, ovarian display elevated levels of interleukin 8 (Xie 2001).

Recent clinical studies indicate that high levels of neutrophils in tumours correlate with poor disease outcome. For example, the presences of TAN in metastatic renal cell carcinoma is associated with poor patient survival (Jensen et al. 2009). Furthermore, it has been revealed that an elevated count of neutrophils in whole blood, indicating an acute inflammation, is also reported in cancer patients and together with a reduced number of lymphocytes is associated with poor survival in patients with metastatic melanoma and metastatic renal cancer (Schmidt et al. 2005; Negrier et al. 2002). More recently, the neutrophil to lymphocyte ratio (LNR) has been introduced as a prognostic marker for colorectal cancer and non-small cell lung cancer (Tomita et al. 2011).

How neutrophils contribute to carcinogenesis has only been partially explained. ROS released by neutrophils are thought to induce DNA damage at the sites of chronic inflammation (Güngör et al. 2010). Neutrophils have four types of granules in their cytoplasm containing different proteinases that participate in normal cell proliferation. A

growing body of evidence suggests that products derived from neutrophils have roles in tumour cell proliferation, angiogenesis and metastasis (Figure 1.6) (Houghton 2010). The ability of proteinases to activate gene expression is well documented in the literature. Furthermore, tumour invasion involves proteolytic cascades and a positive correlation between the aggressiveness of tumour and the secretion of various proteases has been found. Due to their wide variety of functions, extracellular proteases and their inhibitors have great potential as clinical biomarkers for predicting outcome disease outcome (Roy & Walsh, 2014).

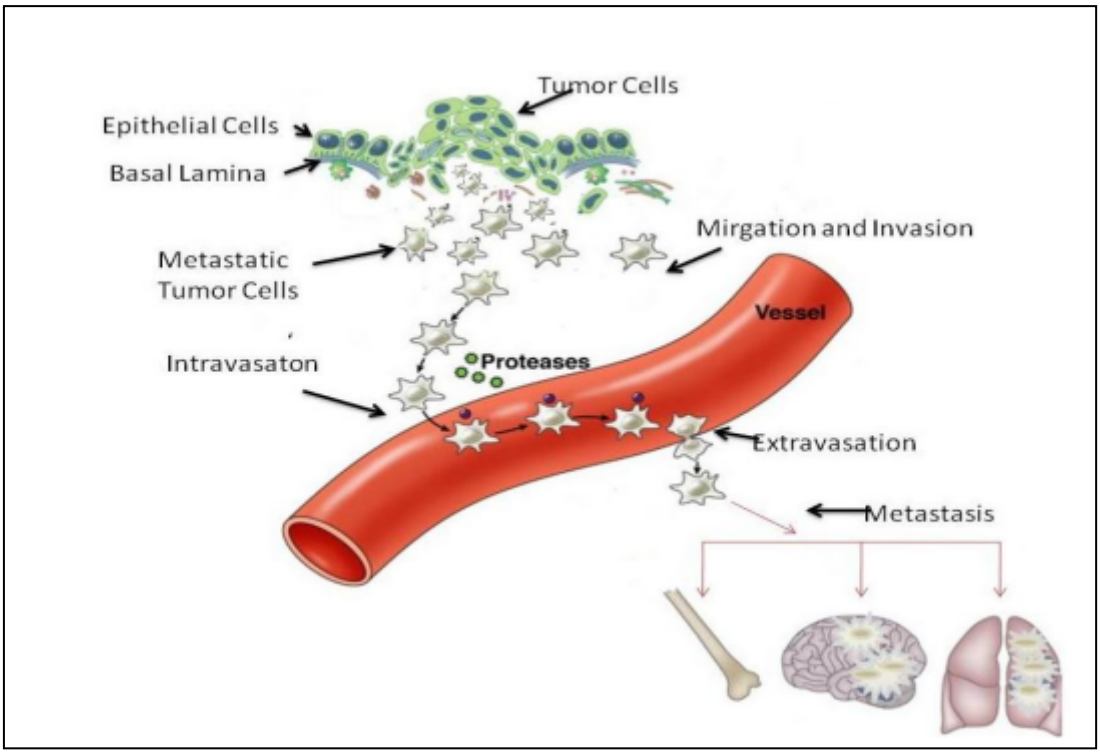


Figure 1.6 Schematic representation of tumour dissemination and the supporting role of proteases in this process. In order to spread, tumour cells must leave the actual tumour and enter the bloodstream or lymphatic system. For this purpose, they use certain enzymes (proteases), that break down the tissue surrounding the tumour (Rakash 2012).

Angiogenesis is the formation of new blood vessels from the existing vascular beds that occurs normally in the human body during embryogenesis. In adults, the process of the formation of new blood vessels is restricted to physiological situations, such as wound healing and female reproductive cycling. During the menstrual cycle, angiogenesis occurs in order to support the growth of the endothelium and neutrophils are found to be the source for pro-angiogenic vascular endothelium growth factor (VEGF) in these tissues (Mueller et al. 2000).

During tumour progression the formation of new capillaries is continued in order to supply growing tumour cells with oxygen and nutrition and the positive correlation between intensity of angiogenesis and tumour invasiveness has been reported (Folkman 2006).

Recent studies investigating the role of neutrophils in angiogenesis suggest the existence of an angiogenic subtype of neutrophils that is distinct from inflammatory neutrophils. Pro-angiogenic neutrophils are thought to be recruited to the site of malignancy via interleukin 8 (also known as CXCL) where they, together with tumour associated macrophages, release Oncostatin M and metalloproteinase 9 (MMP-9) that induce VEGF to promote the development of new capillaries (Figure 1.8) (Tazzyman et al. 2009).

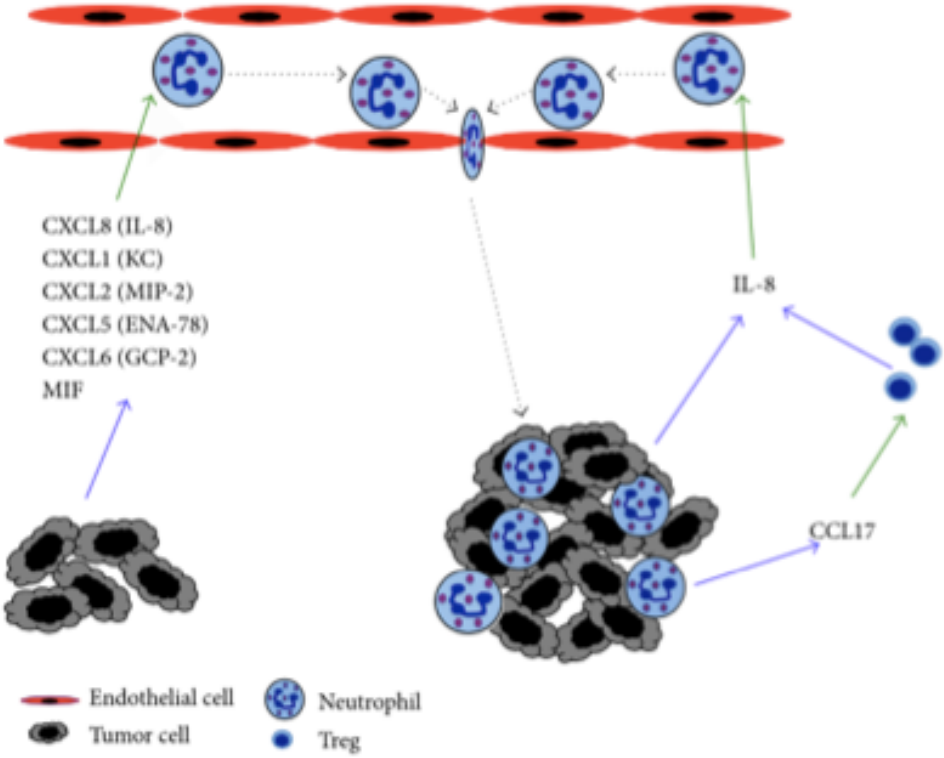


Figure 1.7 Mechanism of neutrophil recruitment to tumours. Tumour cells produce chemokines (CXCL1,CXCL2 etc.), which are chemoattractants for neutrophils . the cell then migrate out of the blood circulation into the tumour. Where they produce chemoattractant for regulatory T cells (CCL17) (Uribe-Querol & Rosales 2015).

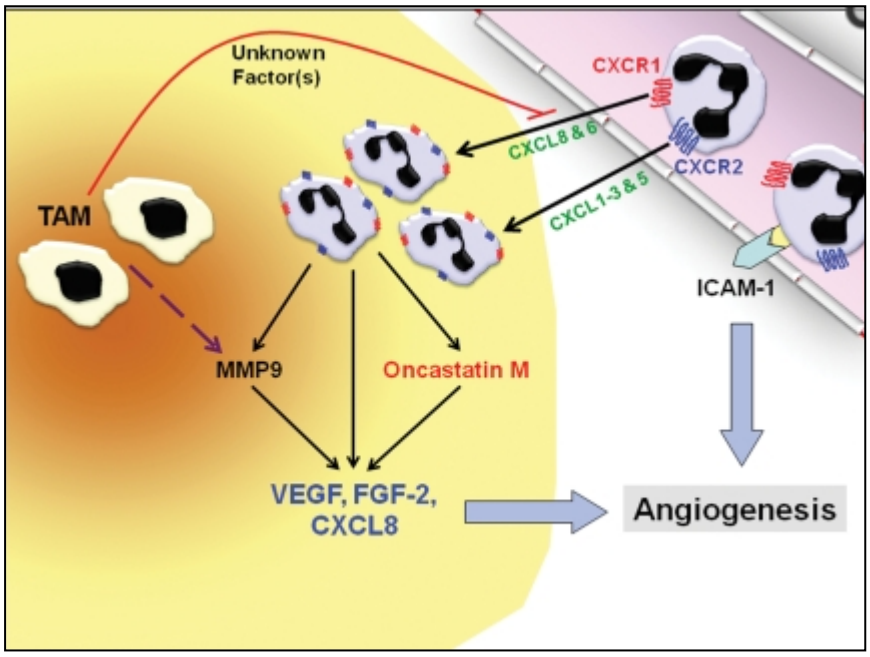


Figure 1.8 Angiogenic function of neutrophils. Neutrophils are recruited by malignant cells via the secretion of Interleukin 8 (CXCL). Once inside the tumour, they release factors such as Oncostatin M and MMP-9 to activate the production of VEGF by the tumour in order to promote angiogenesis (Tazzyman et al. 2009).

1.2.3. Discovery of novel white blood cells-based cancer biomarkers

Previous studies have found that peripheral blood cells are a valuable source for breast cancer biomarkers, as cancer provokes an immune response reflecting in detectable changes in circulating blood cells. Blood biomarkers have a high potential for early cancer diagnosis and prognosis, and could be used to aid or even replace mammography (Aarøe et al. 2010).

It is recognised that cancer cells interact with the host immune system and it is reflected in changes in gene expression profiles in peripheral blood cells. A wide range of techniques including microarrays and proteomic methods were used for biomarker discovery in the blood of cancer patients. For example, it was suggested that whole blood can be used as a diagnostic tool for detection of cancer during early stages. In these studies the cancer-related gene expression patterns in the blood of breast cancer patients were analysed and groups of genes with altered expression in cancer patients were discovered (Lönneborg et al. 2009; Aarøe et al. 2010). These genes were related to various functional processes such as defence response, translation, and metabolic processes (e.g. lipid and steroid metabolism).

In agreement with the above study, work conducted previously within the Klenova group has demonstrated that white blood cells (WBCs) are a potential source for cancer biomarkers (D'Arcy et al. 2008). A number of candidate blood-based biomarkers were identified to be used for early breast cancer diagnosis, prognosis and treatment monitoring. In these studies, proteomic approaches were applied; they included two approaches (1) 2D gel electrophoresis followed by mass spectrometry and (2) the proteome-wide profiling of membrane fractions of WBCs using the Orbitrap Velos mass spectrometer. The protein profiles of WBCs from breast cancer patients were analysed

and compared to those of healthy donors. As a result, a panel of candidate proteins were selected based on various criteria for further investigation. Among those criteria were the proteins role in the tumour immune response, increase or decrease in protein levels, presence in WBCs in healthy donors and others (Many, J., PhD thesis, 2015).

To identify the differences between different grades and stages, samples were chosen in patients with either Grade1-Stage1 tumours (favourable prognosis) or Grade3-Stage3 tumours (less favourable prognosis). We reasoned that by choosing samples with these characteristics the differences in proteome would be significant. Orbitrap LFQ mass spectrometer was used to generate proteome profiles of WBCs. Protein profiles were then compared between different patients' cohorts. The pipeline of biomarkers discovery is presented in Figure 1.9. After a rigorous process of shortlisting fifteen candidates were selected for further validation (see Table 1.2 for details).

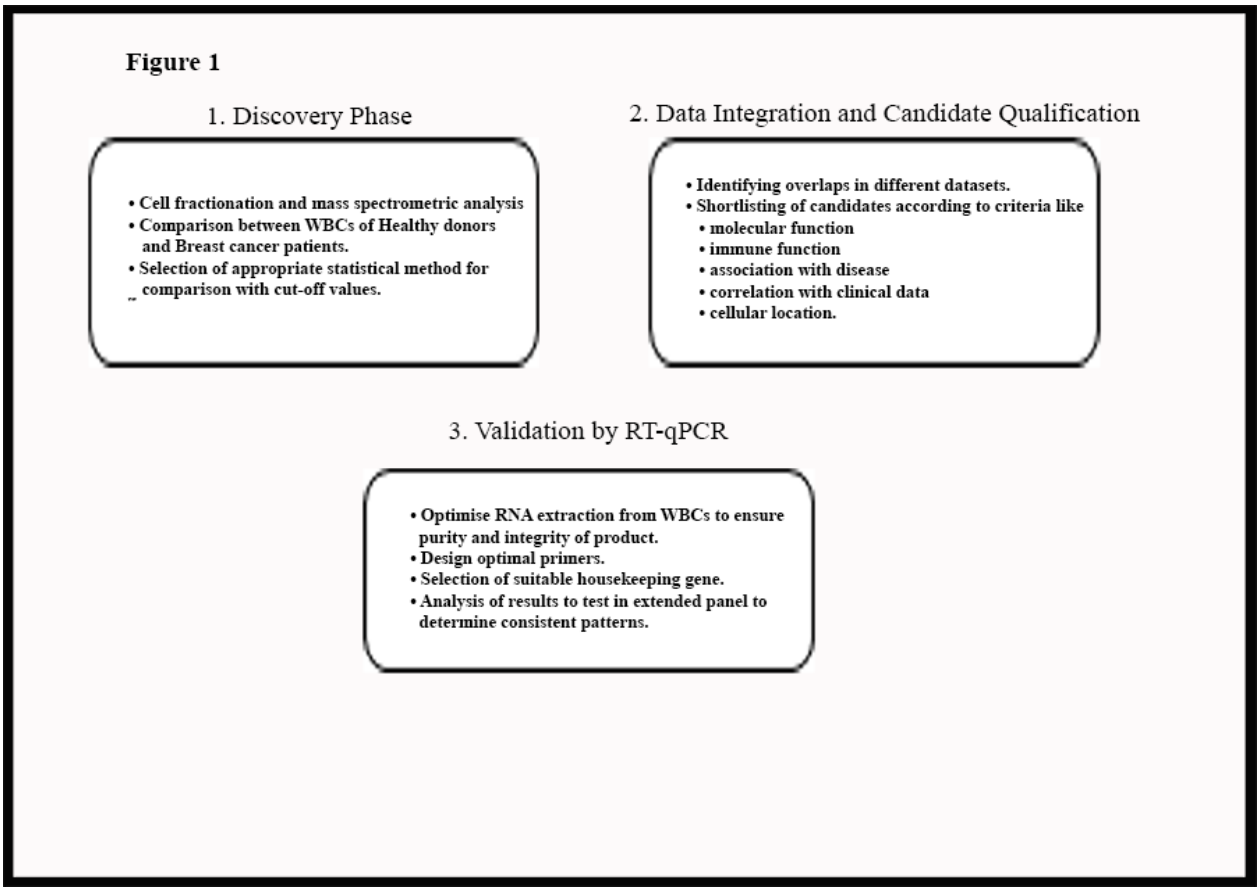


Figure 1.9 Workflow of proteomics biomarker development pipeline. To identify the differences between different grades and stages, samples were chosen in patients with either Grade1-Stage1 tumours (favourable prognosis) or Grade3-Stage3 tumours (less favourable prognosis). We reasoned that by choosing samples with these characteristics the differences in proteome would be significant. Orbitrap LTQ mass spectrometer was used to generate proteome profiles of WBCs. Protein profiles were then compared between different patients' cohorts. After a rigorous process of shortlisting fifteen candidates were selected for further validation (see Table 1.2 for details).

Table 1.2 Short listed biomarker candidates identified in the previous study for further validation (Mani, PhD thesis, 2015): Genes common from different datasets comparing healthy donors, primary breast cancer patients and metastatic breast cancer patients were obtained. The overlapping genes were further shortlisted based on several criteria; normal presence in WBC, correlation with clinical response/data, relative levels to healthy donor, function in immune response and association with cancer through literature search to give a 15 panel gene list to be further validated. Red coloured cells indicate overexpression; green indicates underexpression; pink overexpression only in low grade breast cancer.The presence in metastatic breast cancer cohort is denoted by 'X' and the fold change is not included since the initial analysis did not include comparison with healthy donors.

Candidates	Primary Breast cancer-2D-gel electrophoresis	Primary Breast cancer-High throughput proteomics	Primary Breast cancer - Microarray (GSE16443)	Metastatic Breast cancer-2D-gel electrophoresis	No of categories of the overlap
SERPINB1	+1.3	+1.46		X	3
CALR			+1.4	X	2
YWHAE		+1.46		X	2
CPNE3			+1.2	X	2
ANXA3			+1.97	X	2
ANXA1	+1.3	+1.49			2
LTF			+2.37	X	2
NONO			+1.21	X	2
ITGA4		-3.4	-1.28		2
WDR1			-1.23	X	2
RHOA		+1.4	+1.5		2
LCN2			+2.16	X	2
ALOX5		+1.6			1
FGL2		+			1
OSTF1		+1.23			1

Our current laboratory studies analysing protein profiles of WBCs from breast cancer patients show promising results for five of the identified proteins. Their use for early breast cancer diagnosis, prognosis and treatment monitoring is currently being investigated.

1.2.3.1 Biological function of Serpin B1

One of the short-listed candidate biomarker is Serpin B1, a member of serpin family of proteinase inhibitors that maintain homeostasis by neutralising overexpressed protease activity. Serpins, the largest family of protease inhibitors that include several hundred members was first described in 1980. These proteins are encoded by genes located on 10 different chromosomes and characterised by the absence of the N-terminal signal peptide required for the secretion pathway and therefore reside intracellularly. Despite their exclusively cytoplasmic distribution, extracellular localization of serpins has been reported. For instance, SerpinB5 was found in secretory vesicles at the cell surface (Pemberton et al. 1997). Although, the exact mechanism remains unknown, a study aiming to investigate the secretion of serpin by blood monocytes revealed alternative, endoplasmic reticulum ER-Golgy-independent pathway (Ritchie & Booth 1998). Serpin exist in two forms, a native metastable form that changes into a relaxed stable form after binding to the protease (Gatto et al. 2013). The interaction of serpins with proteases happens through inhibitory and substrate pathways and results in protease degradation. Proteases play roles in the regulation of a variety of biological processes including angiogenesis, apoptosis, cell migration and blood coagulation (Mangan et al. 2008). The prevention of unwanted proteolytic activity of proteases is regulated by inhibitors such as the serine protease family (Bots & Medema 2008).

The Serpin B1 protein is expressed in the cytoplasm of granulocytes, monocytes and macrophages where it inhibits several target molecules; neutrophil elastase (NE),

cathepsin G, proteinase-3 and regulates NET-osis. NE is secreted by activated neutrophils during inflammatory processes with a role in the degradation of foreign organic molecules phagocytised by neutrophils. It has been reported that NE induces lung tumour growth and proliferation (Houghton et al. 2010).

Serpin B1 has been shown to be associated with tumour progression. Increased levels of this protein are reported in 62.5 % of invasive oral squamous cell carcinomas (Tseng et al. 2009). In our study, we observed elevated levels of Serpin B1 in leukocytes of breast cancer patients. One can hypothesise that in order to initiate the metastatic process cancer cells must degrade the extracellular matrix (ECM) barrier. This is achieved by overexpression of several families of proteases including serine proteases and metalloproteinases (Roy & Walsh 2014).

1.2.3.2 Biological function of Lipocalin 2

Lipocalin 2 (LCN2), also referred to as neutrophil gelatines associated Lipocalin (NGAL) belongs to the family of small proteins characterised by their ability to bind and transport lipophilic molecules such as retinols, steroids and iron. The LCN2 gene is located at 9qN34 and contains 7 exons. Recently, several new properties attributed to Lipocalin have been reported such as prostaglandin synthesis, protease inhibition and modulation of cell growth. The expression of NGAL is induced by cytokines and was found to be upregulated under inflammatory conditions (Bauer et al. 2008).

NGAL is secreted by neutrophils in few conformations, as a monomer or as a complex with matrix-metalloproteinese-9 (MMP-9). This enzyme belongs to the family of zinc-dependent endopeptidases comprising of 23 members with different substrate specificity and tissue specific expression patterns (Roy & Walsh 2014). MMPs have various roles in the regulation of signalling pathways that control cell growth, inflammation and

angiogenesis. In cancer MMPs are thought to have multiple functions. The central roles are promotion of cancer cell invasiveness via degradation of the ECM and the support of angiogenesis via liberation of VEGF (Bergers et al. 2000). Recent studies investigating the prognostic utility of MMPs in breast cancer patients revealed that MMP-9 expression in breast tumours is highly associated with higher tumour grade and ER-negative status (McGowan & Duffy 2008).

The role of Lipocalin 2 in the regulation of the innate immune response is attributed to its iron-binding property. Iron is necessary for all living organism. During infection, cells of the innate immune response stimulate the secretion of Lipocalin 2 in order to limit the availability of this nutrient resulting in inhibition of bacterial activity (Reilly et al. 2012).

Heightened expression of Lipocalin 2 has been reported in many epithelial cancers including breast carcinomas. For example, Lipocalin 2 was found to be up-regulated in the plasma of a mouse model of breast cancer (Pitteri et al. 2008). In human studies, Bauer *et al*. examined the localization of NGAL in tumours of 208 primary breast cancer patients using an immunostaining technique and compared the results with clinical outcome. The researchers detected strong correlation between cytoplasmic location of NGAL and HER2 overexpression, lymph node metastasis and decrease disease-free survival (Bauer et al. 2008).

1.2.3.3 Biological role of Copine 3 (CPNE3)

Another protein identified by previous work within the Klenova group is CPNE3- a calcium-dependant phospholipid-binding protein that exists in the cytosol of human neutrophils. This protein belongs to a group of proteins characterised by two calcium binding C-2 domains at the N-terminus and an A-domain at the C-terminus. The A-domain

shared distant similarity with the A-domain of certain integrins which can bind other proteins in a Ca^{2+} and Mg^{2+} dependent manner which are involved in cell migration (Perestenko et al. 2010). Proteins that exhibit C2 domains that are required for calcium dependent binding to phospholipids include enzymes such protein kinases. Interestingly some studies based on phosphorylation experiments have demonstrated that CPNE3 may function as a protein kinase (Caudell et al. 2000). CPNE3 was found to be ubiquitously expressed in mammalian tissues.

In primary breast cancer, elevated levels of CPNE3 was found to correlate with levels of HER-2 and suggested that Copine-3 has a role in the regulation of HER-2 dependant tumour progression. The exact mechanism of the regulation is unknown, however, bioinformatics analysis suggested that CPNE3 does not bind HER-2 directly. Furthermore, CPNE3 expression was detected in prostate and ovarian tumours suggesting its more general role in carcinogenesis (C Heinrich et al. 2010).

Abnormally low expression of CPNE3 has been reported in the peripheral blood of patients with stable coronary artery disease and has been proposed to be used as a genetic marker on the risk of recurrence of the disease (Tan et al. 2018). For patients with acute myeloid leukaemia high levels of CPNE3 in monocytes were identified as poor a prognostic factor as it was found to correlate with shorter overall survival (Lin Fu et al. 2017).

1.2.3.4 Biological role of Integrin α4

Integrins are the family of 24 heterodimeric adhesion receptors required for the interaction of cells with the extracellular matrix (ECM), cell migration, growth and differentiation. As a result, integrins play an essential role in physiological as well as pathological events such as inflammation and tumourigenesis. Each integrin is a heterodimer composed of α

and β subunits specific for the interaction with proteins of the ECM, namely collagen, fibronectin and laminin (Hinton et al. 2008).

Integrin signalling is bidirectional allowing for a diverse cellular outcome. The inside-out signalling is directed by binding of intracellular activators such as talin and kindlins to the "tail" of the receptor, which in turn allows the cell to bind to the ECM proteins and facilitates cell adhesion and migration. The outside-in signalling enables binding of the extracellular ligands to the "head" of the receptor, allowing the transmission of the signal into the cell (Figure 1.10). The combination of both types of signalling contribute to the broad repertoire of the integrin molecules function and their ability to control the variety of processes such as cell proliferation and survival (Shattil et al. 2010). Several studies have demonstrated the crucial role of integrin signalling in each step of metastatic cascade (Akalu et al. 2005; Parise et al. 2000; Gilcrease 2007).

Integrin α4, also known as CD49d, is a cellular adhesion receptor involved in the migration of lymphocytes from blood to the sites of infected tissues. CD49d is an α chain of very-late antigen (VLA)-4 that forms a heterodimer with integrin β1 and β7 and is involved in cell adhesion to fibronectin. This receptor is primarily expressed on T- and B-lymphocytes, but not on neutrophils.

The altered expression of integrin 4 in human cancers has been associated with tumour progression (Rincon et al. 1992; Holzmann et al. 1998). For instance, low levels of integrin alpha4 have been reported in gastric carcinoma cells. In this study, following overexpression of the gene, the reduction of the invasiveness of the gastric cancer cells was observed. As the mechanism of downregulation, transcriptional silencing of *Integrin α4* by DNA methylation was identified (Park et al. 2004). Another study reported hypermethylation of the *Integrin α4* gene as a poor prognostic factor in breast cancer, as

it was shown to be associated with higher grade tumours and lymph node metastasis (Do et al. 2014).

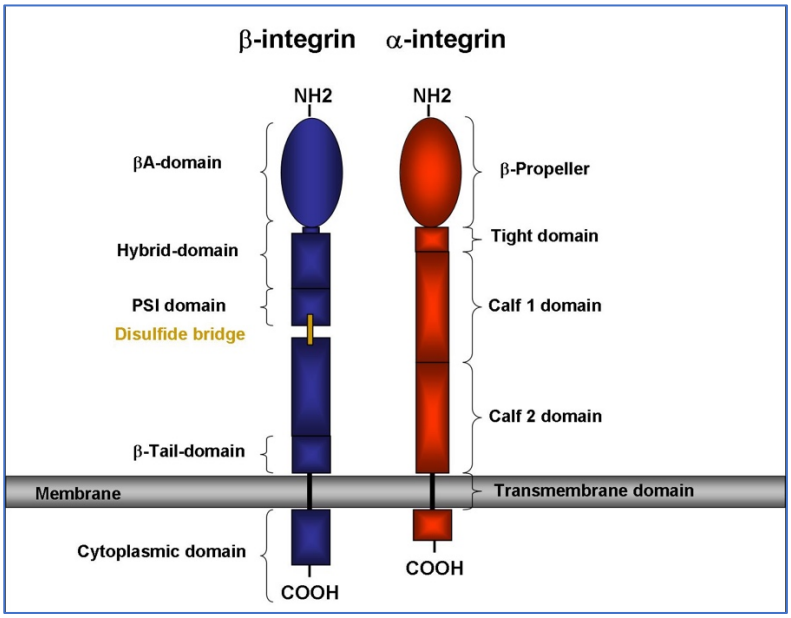


Figure 1.10 Structure of Integrin. Integrins are heterodimeric adhesive receptors designed to act as bidirectional signalling molecules. They are composed of α and β subunits. N-terminal ("head"), the ligand binding site is presented by β-propeller and βA domain. C-terminal cytoplasmic domain ("tail") (Shattil et al. 2010).

1.2.3.5 Biological role of Lipoxygenase 5 (5LOX)

Leukotrienes are pro-inflammatory regulators, their name originates from the cells which they are derived, namely leukocytes (macrophages, neutrophils and eosinophils). 5LOX is a member of a large family of enzymes required for the biosynthesis of leukotrienes. 5LOX has two structural domains, a C-terminal a-helix containing the catalytic site and an N-terminal β barrel that facilities calcium dependent activation of the enzyme (Rådmark & Samuelsson 2010). The 5LOX enzyme is usually located in the cytoplasm as soluble protein, yet in the presence of calcium it translocates to the nucleus or endoplasmic reticulum, where with the help of its activating protein FLAP it catalyses the oxygenation of arachidonic acid and therefore, synthesis of leukotrienes (Tuncer & Banerjee 2015). Furthermore, independently from calcium activation, different forms of cell stress such as oxidative stress or osmotic shock lead to the activation of 5LOX and the production of leukotrienes (Werz et al. 2002).

Leukotrienes function as anti-inflammatory molecules. It is known that chronic inflammation is a risk factor for carcinogenesis. The expression of 5LOX has been shown to increase within the tumour microenvironment (Wang & Dubois 2010). The role of 5LOX in the growth of several tumour types, including pancreatic, colorectal, prostate and breast cancer has been reported (Harris 2009). It is believed to be produced by cancerous cells as well as tumour associated macrophages (TAM) and its abhorrent expression shown to contribute to the survival and proliferation of malignant cells (Rådmark & Samuelsson 2010). Furthermore, the expression of 5LOX correlates with density of TAM in hypoxic areas of ovarian tumours (Wen et al. 2015).

Due to its heightened expression in tumours, 5LOX become an attractive therapeutic target in research. For instance, in a study on chronic myeloid leukaemia, this enzyme

was identified as a critical regulator of leukaemia stem cells (LSCs) and its inhibition was shown to cause impairment of LSCs (Chen et al. 2009). Study on the genetic deletion of 5LOX in mast cells of mice xenografted with human colon cancer showed a significant reduction of the tumour mass (Cheon et al. 2011). Whereas, in lung cancer models, mice globally deficient in 5LOX exhibited increased tumour volume and liver metastases (Poczobutt et al. 2016). Furthermore, 5LOX is proposed to be a potential serum protein marker for breast cancer, as elevated levels have been reported in the serum of breast cancer patients (Kumar et al. 2016).

1.2.4 Autoantibodies in the plasma of cancer patients

Although little is known about the origin of the immune response, it is now recognised that cancer patients produce autoantibodies to cancer proteins, particularly in breast cancer (Carney et al, 2013). Many studies have begun to emerge, showing that autoantibody production takes place many years before the diagnosis of cancer and therefore, can serve as an early marker for cancer (Frenkel et al. 1998).

For years autoantibodies (AAb) were considered to be a by-product associated with the breakdown or release of tumour proteins. Nowadays the production of AAb is believed to be triggered by the overexpression of self-antigen on the surface of malignant cells and the inflammatory reaction within tumours. Leukocyte infiltration detected in different types of tumour support the above hypothesis (Madrid & Maroun 2011).

Although autoantibodies have showed promising results as novel diagnostic biomarkers for the early detection of many cancers, the low sensitivity and specificity have limited their clinical application (Zhong et al. 2008). The presence of p53 autoantibodies has been observed in only 15% of patients with breast cancer and has been associated with other diseases as well (Lenner et al. 1999; Kulić et al. 2010).

Since a single biomarker lacks sensitivity and specificity, recent studies now concentrate on identification of a panel of autoantibodies to increase the reliability and accuracy of diagnosis (Chapman et al., 2007; Desmetz et al, 2011; Dong et al., 2013).

1.3 Prostate cancer

With more than 670,000 diagnosed cases per year prostate cancer is the second most common type of cancer in men worldwide (Van Hemelrijck et al. 2012). Early detection of the disease during stage 1 allows for almost 100% survival. However, once the cancer has metastasised outside the prostate gland, the survival rate falls significantly It is most commonly diagnosed in men aged 65 and above which account for 75% of diagnosed cases. Men under 50 years of age represent 1% of the prostate cancer diagnoses (Crawford et al. 2017).

1.3.1 The prostate gland under normal physiological conditions

The prostate gland is part of the male reproductive and urinary system (Figure 1.11). This pyramid-shaped organ is located at the base of the bladder. In younger men, the gland is approximately the size of a walnut, however, the organ enlarges with age. The main purpose of the prostate gland is the secretion of alkaline fluid that represents 20% of seminal liquid. The prostate gland is divided into a right and a left lobe composed of branching ducts surrounded by stroma made by connective tissue and muscle fibres.

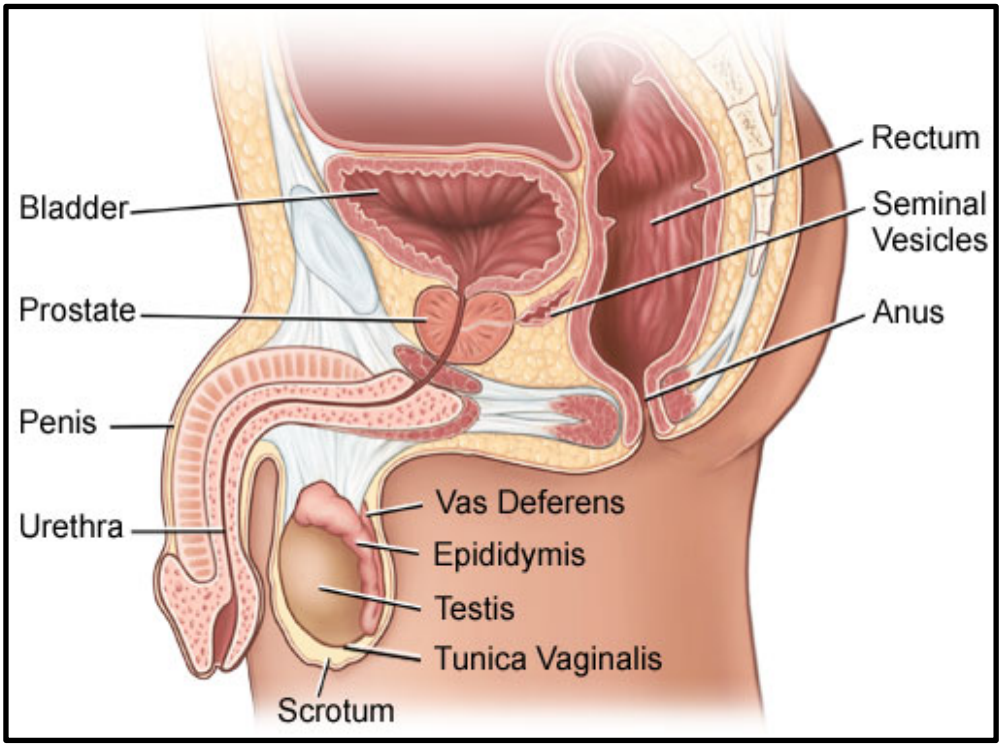


Figure 1.11 Anatomy of the male reproductive tract. Adapted from http://medicalcenter.osu.edu

13.2 Diagnosis and treatment

During early stages prostate cancer is asymptomatic, however, at the later stage painful urination, blood in urine or seminal fluid and thigh pain is reported by patients. The risk of developing prostate cancer increases with age. The incidences of prostate cancer are highest in African-American populations and lowest in Asian populations (Kinseth et al. 2014; Kimura 2012).

Diagnostic tools for prostate cancer include prostate specific antigen (PSA) test, digital rectal examination (DRE), biopsy and Magnetic Resonance Imaging (MRI) (Lavery et al. 2016). Staging and grading of the tumour are important predictors for disease outcome. Staging of the disease is evaluated by measuring tumour size/volume and malignant potential of the cancer. It involves DRE or MRI scanning and categorised into 4 stages. Grading of the prostate tumour is performed using Gleason Grading System based on the microscopic examination of the biopsy specimen. According to this method low-grade tumours are associated with small and uniformly packed glands. High-grade tumours are characterised by loose glandular architecture and more spread out cells.

Although both grading and staging of prostate cancer are very important determinants for prostate cancer management, they are associated with invasive and painful procedures and are not accurate in early detection of the disease.

Management of prostate cancer depends on the stage of the disease. For stages I-III of prostate cancer patients, treatment involves radical prostatectomy and radiotherapy. For the advanced, stage four patients endocrine therapy and chemical castration are the choice of treatment (Trewartha & Carter 2013). Endocrine therapy also called androgen deprivation therapy for the treatment of advanced prostate cancer was introduced in 1941 (Huggins & Hodges 1941). The basis of the treatment is to deprive prostate cancer cells

from androgens. Testosterone is the major androgen, in the prostate it is converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT has a higher affinity to the androgen receptor and greater stimulation of prostate tumour growth than testosterone. The 5α-reductase inhibitor is often used as a treatment option to reduce the level of circulating DHT. The vast majority of cancers respond to androgen deprivation therapy. Although, endocrine treatment improves survival, it is not curative and has an adverse effect on quality of life (Tammela 2012).

1.3.3 Established and emerging biomarkers of prostate cancer

The most commonly used serum biomarker for detection of prostate cancer is prostate specific antigen (PSA) (Gaudreau et al. 2016). The discovery of PSA in the late 1970's has revolutionised management of the disease and saved many lives (Ablin 1972). PSA is produced by prostate epithelial cells and found in semen. PSA is a 33 kDa serine protease, a member of the Kallikrein family of proteins.

At low levels, it is present in the serum of healthy men, however in the presence of prostate cancer it levels are found to be elevated. Other factors such as age, ethnicity, obesity and some medication are reported to affect levels of PSA. Those factors result in over diagnosis of the disease and lead to unnecessary biopsy investigation and over treatment causing distress among patients (Adhyam & Gupta 2012).

A major challenge in the management of prostate cancer revolves around the failure of existing diagnostic tools such as PSA to distinguish between benign hyperplasia and cancer as well as between aggressive and slow growing tumours (Romero Otero et al. 2014).

1.3.4 Urine based biomarkers of prostate cancer

Urine represents a cost-effective and non-invasive source for prostate cancer (PCa) biomarkers. Urinary cancer markers include DNA-, RNA- and protein-based biomarkers.

Epigenetic modifications, such as DNA hypermethylation occurs under the influence of stress and dietary factors and leads to increased risk of cancer formation (Estelle ret al, 2001; Jones et al, 2002). This event happens in the early stage of carcinogenesis and therefore, can serve as a biomarker for early cancer detection. Currently, the hypermethylation of over 40 genes have been investigated. One of the best studied examples is the hypermethylation of glutathione *S*-transferase P1 (*GSTP1*) which is present in 90% of the PCa and in 78% of the corresponding urine sediments (Goessl et al. 2001). Another example is the tumour suppressor gene Ras-association domain family protein isoform A (*RASSF1A)*, in which silencing is observed in 70% of prostate tumours (Kuzmin et al. 2002).

Properties of several potential protein-based biomarkers currently being investigated. Among them the serum/urine PSA ratio for early diagnosis of prostate cancer, Annexin A3 (ANXA3) calcium-binding protein production of which is decreased in malignant prostate tissue and the role of increased levels of MMP9 (Irani et al., 2005; Bolduc et al., 2007; Schostak et al., 2009; Roy et al., 2008).

PCA3 is a commercially available RNA-based prostate cancer test, discovered by comparative gene expression methodologies (Bussemakers et al. 1999).

The expression of miRNA is highly dysregulated in prostate cancer tissue, however, diagnostic properties of urinary miRNA failed to show an improvement in prostate cancer detection (Kalogirou et al., 2013; Stephan et al, 2015).

1.3.5 Exosomal biomarkers

Exosomes are the small vesicles released by various cell types into extracellular microenvironment by the endocytic recycling pathway (Figure 1.12) (Lee et al. 2012). Exosomes contain proteins, lipids, mRNA and microRNA molecules and carry tissue specific information. Exosomes function in cellular communication by merging with a recipient cell and releasing their contents resulting in altered cellular function.

Proteomic studies on exosome composition reported variation depending on the cell type origin (Mathivanan et al. 2010). The content of exosomes can reveal information about a host cell and underlying pathophysiological conditions such as cancer and inflammation. Furthermore, exosomes can be isolated from all body fluids such as blood, urine, sweat and tears. These characteristics of exosomes make them an ideal source of biomarker(s) with diagnostic and prognostic value (Rak 2013). Numerous studies analysing exosomal content revealed that exosomes derived from the fluids of cancer patients have different protein, DNA and RNA expression levels and profiles (Balaj et al. 2011; Simpson et al. 2012). To study exosomes, common markers such as CD9, CD63, Alix and TSG101 are used (Willms et al. 2016).

In the recent study, prostate cancer biomarkers, PCA3 and *TMPRSS2-ERG* have been detected in exosomes isolated from urine of prostate cancer patients (Nilsson et al. 2009). Furthermore, high levels of plasma-derived exosomal survivin have been reported in prostate cancer patients, showing potential as an early disease biomarker (Khan et al. 2012). Another study on circulating exosomal miRNAs revealed the association between miR-141 and miR-375 with metastatic prostate cancer (Bryant et al. 2012).

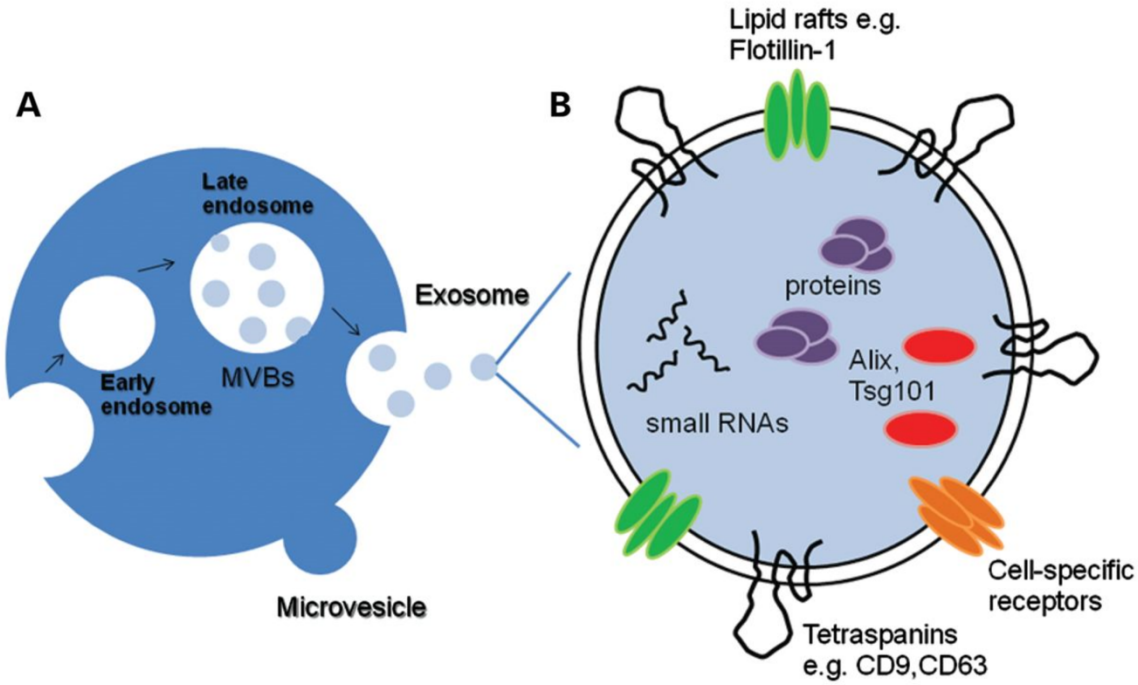


Figure 1.12 A Synthesis of exosomes. Firstly, the exosomal vesicle is formed at the plasma membrane in the form of an early endosome, which then matures into the late endosome or multivesicular bodies (MVBs). Next, MVBs merge with the plasma membrane and release exosomes into the extracellular space. **B** Structure of exosomes showing common membrane exosomal markers CD9/CD63 in addition to the intracellular markers Alix and Tsg101 (Lee et al. 2012).

1.3.6 Brother Of the Regulator of Imprinting Sites – BORIS

In a previous study, BORIS- cancer testis antigen (CTA) was detected in the leukocytes of breast cancer patients, while in normal healthy conditions it is not present in peripheral blood (D'Arcy et al. 2008; Martin-Kleiner 2012). To date BORIS is the only known paralogue of the CTCF highly conserved zinc-finger protein expressed in all cell types (Klenova, et al., 2002). For its involvement in diverse regulatory functions, including gene expression through organisation of chromatin structure and 14,000-25,000 identified potential binding sites CTCF has been named "master waiver" of the genome (Phillips & Corces 2009; Klenova et al. 2002).

Although, BORIS and CTCF have a highly conserved DNA binding domain, suggesting binding to the same sites, they are expressed in a different manner, often as antagonists resulting in opposite function. As mentioned before, CTCF's expression is ubiquitous and in cancer it appears to function as a tumour suppressor gene. BORIS on the other hand, as a member of cancer testis genes, is normally present in male germ line cells and not in other somatic cells; also it is reported to be detected in human oocytes and in embryonic stem cells (Martin-Kleiner 2012). Despite some controversy in the literature, BORIS has been shown to be abhorrently expressed in the majority of human tumours and cancer cell lines. For example, Khomanskikh and co-workers reported the presence of BORIS in melanoma (Kholmanskikh et al., 2008). In another study, expression of BORIS was detected in ovarian cancer (Woloszynska-Read et al. 2007).

Although the functional role of BORIS in cancer cell lines is not known, it has been suggested that overexpression of BORIS in tissues other than testis could indicate tumorigenesis (Klenova et al, 2002). In line with the above view, recent reports indicate that BORIS is responsible for the activation of other CTAs in tumours (Kosaka-Suzuki et

al. 2011; Kang et al. 2007). Furthermore, there is evidence that BORIS can deregulate the expression of tumour suppressor genes, such as CTCF and retinoblastoma-related protein *Rb2/130* (Fiorentino et al. 2011). Taken together, these reports reveal considerably broader roles of BORIS.

CTAs, including BORIS, for their exclusive expression in spermatocytes and frequent overexpression in tumours are considered to be an attractive target for cancer immunotherapy and BORIS antitumour vaccines based on the deletion of the ZF-region of BORIS have been tested in mice with different malignancies (Loukinov et al. 2006).

1.4 Aims of the study

The main aim of the present study is to further validate the utility of Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5LOX as biomarkers for early cancer diagnosis, prognosis and treatment monitoring. The hypothesis is that the levels of these proteins will change depending on the clinical information (Figure 1.12). To test this hypothesis the following objectives were studied:

1. Validation of novel cancer biomarkers identified from blood of breast cancer patients using RT-qPCR, Western blot analysis, Immunofluorescent staining, enzyme-linked immunosorbent assay (ELISA) and FACS techniques.
2. Correlate the finding from the above objective with clinical outcome data to identify possible diagnostic, prognostic and predictive factors.

The secondary aim of this study was to investigate the property of BORIS in urine sediment as a biomarker of prostate cancer and anti-BORIS autoantibody (AAb) in plasma as a breast cancer biomarker.

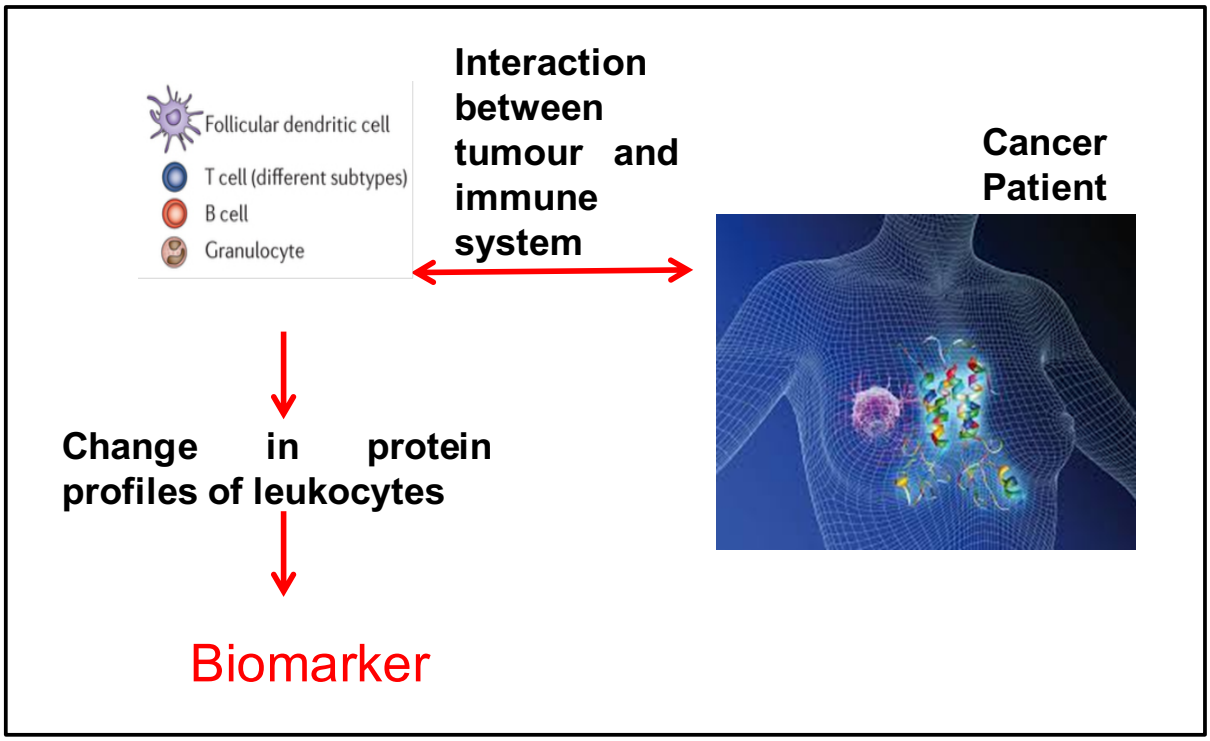


Figure 1.12 The rationale of the study. Growing tumour(s) interact with the hosts immune system, which is reflected in changes in both gene and protein expression profiles within white blood cells. These changes appear to be present before a tumour is detected and can serve as biomarkers for early cancer diagnosis.

Chapter 2 Materials and methods

2.1 Participants

2.1.1 Samples from prostate cancer patients

The total of 67 cDNA samples from urine sediment and urinary exosomes from prostate cancer patients was received from the University of East Anglia (Pellegrini et al. 2017). Patients were with different parameters of the disease**: HG-PIN**- atypia, **P**- prostatitis, **CBN- PSA** normal to age and clinical benign; **CB<1**- PSA<1 clinically benign, **Sneg-** raised PSA negative biopsy; **L**- Low risk PSA<10, **IL** – intermediate risk PSA>10; **I** Intermediate risk PSA≤20, **HL-**High risk, PSA>20;**Hh** High risk, PSA<100, **Hhh-**High risk, PSA>100.

2.1.2 Blood samples from breast cancer patients

The ethical approval for this study was obtained from NRES Essex Research Ethics Committee [MH 363 (AM03)] and the Research & Development Department of Colchester Hospitals University NHS Foundation Trust. Women diagnosed with advanced breast cancer who were about to undergo chemotherapy or endocrine treatment in Colchester General Hospital were recruited to participate in this study. Written consent was obtained from all participants. Patients commencing chemotherapy have six cycles of treatment. The blood was taken from those patients six times; at the start of the treatment (baseline) and before every subsequent cycle. Patients undergoing endocrine treatment had their blood taken three times; at the beginning of the treatment, after six weeks and after twelve weeks. Clinical response of these patients to the treatment was assessed by the clinician after each treatment.

The efficacy of the treatment is evaluated using Response Evaluation Criteria In Solid Tumour (RECIST). This procedure is based on the monitoring of the changes in tumour

size after each treatment and assessment of disease progression (Litière et al. 2017). According to RECIST, the clinical response is categorised into four groups:

- CR (complete response) - disappearance of all lesions

- PR (partial response) – reduction of the lesion diameter by 30%

- SD (stable disease) – no significant change of the size of the lesion.

- PD (progressive disease) – the appearance of new lesions and an enlargement of the existing one by at least 20%

2.2 Cell culture

2.2.1 Cell lines

Cell lines used in the present study include human embryonic kidney cell line 293T, human breast cancer cell line MCF7, human leukaemia cell line K562, human B lymphocyte cell line Raji and human T lymphocyte cell line Jurkat.

2.2.2 Media and cell growth conditions

The growth medium used for 293T cells was DMEM (Dulbecco's Modified Eagles Medium) with L-Gutamine, glucose, Pyridoxine-HCl, $NaHCO_3$ (PAA-GE Healthcare) supplemented with 10% of FBS (Biosera) and 50µg/ml gentamicin (Life Technologies-Invitrogen). All cells were maintained at 37°C and 5% CO_2.

The MCF7, K562, Raji and Jurkat T cell lines were cultured in RPMI 1640 (Roswell Park Memorial Institute medium 1640 (1X) with L-Glutamine) medium (PAA-GE Healthcare) supplemented with 10% of fetal bovine serum (FBS) (Biosera) and 50µg/ml gentamicin (Life Technologies-Invitrogen) and were maintained at 37°C in presence of 5% CO_2.

Table 2.1 Cell lines used in the present study.

Cell line	Description	Reason for choosing
293T	Human embryonic kidney cell line	Highly transfectable cells
MCF7	Human breast cancer cell line	Used for overexpression of BORIS
K562	Human leukaemia cell line	Cell line positive for CD15
Raji	Human B lymphocyte cell line	Cell line positive for CD43 and CD21
Jurkat	Human T lymphocyte cell line	Cell line negative for CD43

2.2.3 Trypsinization

All cell lines used in this study grow as an adherent monolayer and require passaging when they are approximately 70-80% confluent. To trypsinize, the spent media was removed, and cells were washed with 2ml of warm EDTA (1X). Cells were detached by adding 1ml of warm 10% trypsin (Sigma)/EDTA (PAA-GE Healthcare) to the cell monolayer and incubating the flasks in the incubator for 2-10 minutes (min). After examining the cells under the microscope, the trypsin/EDTA in the flask was diluted ten times with warm complete media. The media was carefully pipetted to collect all the cells stuck to the flask. The cell suspension was then centrifuged at 2000 rpm for 5 min and the supernatant was aspirated off. The cell pellet obtained was re-suspended in fresh complete media and one-tenth of this final cell suspension was transferred to the original flask for future culture and the rest was used for other experiments as described

in the following sections. The cells were maintained in culture for up to one month after which they were replaced with a fresh stock.

2.2.4 Counting and seeding of cells

For counting, 10µl of the final cell suspension was obtained as described in 2.1.3.1 and cell number was counted using a haemocytometer. Average of number of cells present in the four corner squares multiplied by 10^4 is equal to the concentration of cells per ml. The cells were diluted to the required concentration (normally between $1.0x10^5$-$1.5x10^5$ cells/ml) using appropriate media and were seeded in a 12- or 6-well plates or flasks as per the experimental set up.

2.2.5 Freezing and defrosting cell stocks was followed by standard procedure

Cells were trypsinized as described in Section counted and 0.8ml of cell suspension (concentration: $1x10^6$ cells/ml) were diluted with 0.8ml of freezing mix composed of 20% Dimethyl Sulfoxide (DMSO) (Sigma) and 80% FBS. The cell mix was stored at -80°C freezer in a cryotube wrapped in insulating material to prevent rapid cooling and after a week it was moved to liquid nitrogen container for longer storage.

To defrost cells, the cryogenic tubes were removed from liquid nitrogen on dry ice and rapidly defrosted under warm water. The defrosted cell suspension was added slowly into 10ml of pre-warmed growth media followed by centrifugation at 2000 rpm for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 9ml of RPMI 1640 complete medium and transferred into a small flask. The flask was maintained at 37°C in the presence of 5% CO_2 until the cells were confluent enough to passage.

2.2.6 Preparation of cell lysates for SDS page

Cells were grown in an adherent monolayer. Pre-warmed 2mL of Trypsin/EDTA was added to the cells to dislodge them and neutralised by the addition of 9mL of pre-warmed

culture medium. Cells were counted using haemocytometer, required number of cells were centrifuged at 2000rpm for 3 minutes and the obtained cell pellet was lysed immediately using SDS lysis buffer at the ratio of 1x10 of cells per 20 μL of lysis buffer (Table 2.1).

2.3 Collection of blood samples and blood procession

The blood samples were collected from two cohorts of breast cancer patients (primary and advanced) treated at Colchester General Hospital (Essex United Kingdom). As a control blood samples from healthy individuals, not diagnosed with cancer were collected. All blood samples were obtained with previous written consent.

The blood was processed by buoyancy density method (D'Arcy et al., 2006). The experiment involved separating the blood constituents namely cells (erythrocytes, leukocytes, platelets) and plasma. Erythrocytes (RBCs) were discarded, but the leukocytes (WBCs) were used for experiments and plasma was stored at -80°C. Histopaque 1119-1 (Sigma) and Red Cell Lysis Buffer (Roche) were stored at 4°C. Blood, Histopaque and Red Cell Lysis Buffer must be at room temperature prior to fractionation and HBSS should be kept on ice before use. Blood samples were stored in an Ethylenediaminetetraacetic acid (EDTA) tube. Each tube contained 5 mL of blood. Blood was mixed well before the experiment.

Histopaque1119-1(equal to the volume of blood i.e. 5 mL) was taken in a 15mL falcon tube. The blood was then carefully layered onto the Histopaque by gentle pipetting by the side of the tube taking care to avoid mixing with the Histopaque. It was centrifuged at 1900 rpm for 30 minutes at room temperature (18-22°) without using the brake of the centrifuge.

The sample got separated into varying bands based on their densities (Figure 2.1). The

top layer was the plasma fraction, which was separated and stored at -80°C after adding EDTA (2 mM) and Triton (0.5%) to it. The buffy layer containing leukocytes was transferred to a new falcon tube and the erythrocyte layer was discarded. Cold HBSS was then added to the leukocyte fraction to give a final volume of 12 mL and was centrifuged at 1400 rpm for 5 minutes at 4°C to obtain a pellet. The supernatant was removed leaving 2 mL of HBSS (to prevent disturbing the pellet) and cold HBSS was again added to get a final volume of 10 mL. It was again centrifuged at 1400 rpm for 5 minutes at 4°C. The pellet obtained may contain erythrocytes, which were lysed using 1-2 mL of red cell lysis buffer. After centrifuging at 1400 rpm for 5 minutes at 4°C and removing the supernatant, a white blood cells pellet was obtained. This was again washed with 10 mL cold HBSS and centrifuged. Leukocyte pellet thus obtained was re-suspended by adding 500µl of cold HBSS. The WBC were counted using a haemocytometer.

Cell suspension was divided into five fractions each of which was used for the following tests: 1) Cell smears ($3x10^4$cells per well of a chamber slide), 2) RNA analysis ($2x10^6$cells), 3) Western Blot analysis ($2x10^6$ cells) and 4) ELISA ($1x10^7$cells). These cell suspension fractions were centrifuged, and the pellets were obtained. Cell pellet for RNA was re-suspended in 20 µl cold HBSS and 100 µl RNA-later solution was added and stored at -80°C. Cell pellet for Western blot was lysed using 200 µl of SDS lysis buffer (0.1M Tris pH6.8, 7M urea, 10% mercaptoethanol, phenol red dye and 4% SDS) and stored at -20°C or -80°C. The remaining two cell pellets were stored at -80°C.

The slide was coated with 2 µl leukocyte suspension per well. Once the smears were dried, they were fixed with 100% methanol (pre-cooled to -20°C) for 5 minutes. Fixing the cells was essential to maintain the cellular architecture and avoid loss of cells in the subsequent washing steps during staining. Fixed smears were washed twice with cold 1

X PBS. Slides were dried, put into a cardboard folder and stored at -80°C. The smears can be stored at 4°C after adding 400 µl 1X PBS, which is supplemented with 0.01% Sodium Azide. Sodium Azide is a preservative as it has a bactericidal property.

Table 2.2 Reagents and buffers used for blood processing. Reagents used for white blood cells isolation.

Histopaque 1119-1 (Sigma)	
Triton 0.5%final	
HBSS (Hank's Balanced Salt Solution)	
EDTA 0.5M (Sigma)	
Red cell lysis buffer	
SDS lysis buffer	0.1 M Tris pH 6.8, 7M urea, 1% β-mercapthoethanol, phenol red dye, 4%SDS
RNA later (Qiagen)	
Methanol 100%	
Buffer 1 for ELISA	25mM Tris/Hepes pH 8.0, 2mM EDTA, 0.5%Tween 20, 1mM PMSF
Buffer 2 for ELISA	25mM Tris/Hepes pH 8.0, 2mM EDTA, 0.5%Tween 20, 0.5M NaCl, 1mM PMSF

2.4 Methods for protein extraction and analysis

2.4.1 Preparation of White Blood Cell Lysates and Western Blot

Total cell lysates were prepared from $2x10^6$ whole white blood cells in SDS lysis buffer. The composition of resolving (8.1%) and stacking (4%) gel is given in Table 2.2. Each gel contained one well with cell lysate from healthy donor 2 as the control and remaining wells with white blood cells lysates from breast cancer patients. White blood cell lysates were loaded onto the casted polyacrylamide gel and were resolved in the gel running buffer (see Table 2.3.1) for 2 hrs under 40mA/125V. A pre-stained standard protein marker containing proteins of known molecular weight ranging from 6kDa to 175kDa (New England Biolabs) was always run parallel to the protein samples to be analysed.

The resolved proteins from the SDS-PAGE gel were transferred onto the Polyvinylidene difluoride (PVDF) membrane (Immobilion P Millipore Inc.) using a semi-dry electro blotting apparatus (Bio Rad) in the following manner. Prior to the transfer, the gel was incubated in gel running buffer with 1% methanol for 15 min and the membrane was hydrated in absolute methanol for 10 seconds and immediately washed with RO H_2O. A stack consisting of 5 pieces of Whatman papers soaked in transfer buffer (see Table 2.5A) followed by the membrane, then the gel and finally 5 more pieces of soaked Whatman papers on the top was prepared and placed carefully on the surface of the semi-dry electro blotter. Bubbles were removed from the stack by gently rolling a glass rod over it and the apparatus was set for transfer for 2 hrs at 100mA/35V. After transfer, the membrane was washed with 20% methanol, rinsed thoroughly with RO H_2O and was incubated in blocking buffer (see Table 2.5A) for at least 40 min at room temperature (RT) or overnight at 4°C. After the blocking step, the membrane was washed once with washing buffer (see Table 2.5A) and was then incubated with the primary antibody prepared in blocking buffer (antibody against the protein of interest) for 2 hrs at RT.

Membrane was washed thrice with washing buffer (5 min each wash) before incubating with the secondary antibody (prepared in blocking buffer) for another 2 hrs at RT. In this study, all the secondary antibodies used were conjugated with horse radish peroxidise (HRP). Signal detection was performed using Luminata Forte (Millipore).

Table 2.3 Composition of buffers used for western blot analysis.

Buffer	**Composition**
Resolving buffer	2M Tris/HCl, 0.2%SDS (pH8.9)
Stacking buffer	0.1MTris/HCl, 0.1%SDS (pH6.8)
Running buffer	0.025M Tris/HCl, 0.192M Glycine, 0.1%SDS
Transfer buffer	20mM Na2PO4, 2% Methanol, 0.05%SDS
Blocking buffer	0.1% Tween, 5% dry skimmed milk powder, 1xPBS
Washing buffer	0.1% Tween, 1xPBS

2.4.2 Immunofluorescent staining (IF) of fixed White Blood Cells

Antigen retrieval was performed in a citrate buffer (10mM, 6.0pH) on full power for 1 minute to retrieve the antigens. Endogenous peroxidise activity was blocked by incubating the slides in 3% in H_2O_2 for 15 minutes. The slides were then permeabilised in PBSx1-0.26% Triton for 20 minutes at room temperature and blocked with serum from the same species as the secondary antibody in the following buffer: PBS, 0.05% Tween, 2% serum, 1%BSA for 30 minutes minimum. Serpin B1 and Lipocalin 2 expression was detected by incubating the slides with goat anti-rabbit Serpin B1 diluted in PBSx1, 0.05% Tween, 1% BSA (1:200 dilution, Abcam) for 2 hours at RT and washed with PBSx3. Slides were then incubated in the dark for 1 hour with anti-IGg-FITC conjugated secondary antibody diluted in the same buffer as primary (1:400 dilution), washed PBS 3x and mounted on the microscope slides with Fluoro-Gel mounting media containing DAPI (Interchim) to identify the nuclei. Negative controls were prepared by incubation of slides with secondary antibody only. Images were taken using Confocal Laser Scanning Microscopy (BioRad Hercules).

2.4 Antibodies used in immunological techniques and their application

Primary antibodies	**Supplier**	**Application**	**Secondary Antibodies**	**Supplier**
Rabbit anti-SerpinB1	Abcam	WB, IF, FACS	Anti-rabbit HRP	Abcam
			Goat anti-rabbit IgG FITC labelled	Abcam
Rabbit anti-Lipocalin2	Sigma	WB, IF, FACS	Anti-rabbit HRP	Abcam
			Goat anti-rabbit IgG FITC labelled	Abcam
Anti-Boris				
Rabbit anti Copine 3	Abcam	WB, IF	Anti-rabbit HRP	Abcam
			Goat anti-rabbit IgG FITC	
Mouse Anti-Actin	Abcam	WB	Anti-mouse HRP	Abcam
Rabbit anti-Integrin α4	Abcam	WB, IF, FACS	Anti-rabbit HRP	Abcam
			Goat anti-rabbit IgG FITC	
Rabbit anti-Lipoxygenase	Abcam	WB, IF, FACS	Anti-rabbit HRP	Abcam
			Goat anti-rabbit IgG FITC	

2.4.3 Protein quantification ImageJ analysis WB

The image obtained from the PVDF membrane was then analysed with ImageJ analysis software which can be downloaded freely. The software analyses the band intensity and gives values. The target protein molecule was normalised between samples using the signal obtained from β-Actin antibody probing. Protein density values from different gels were normalised based on the value obtained from a common sample used.

2.4.4 Flow cytometry

Freshly isolated WBCs were stained for 20 minutes with pre-labelled neutrophils cell surface markers (CD15/CD66b) at room temperature. Cells were washed with the staining buffer (PBS/Serum/BSA) and fixed with cold 4% formaldehyde on ice for 20 minutes. Cells were permeabilised in 0.25% tween/PBS for 20 minutes on ice, washed twice with the staining buffer and incubated with antibody against Serpin B1, Lipocalin 2, Integrin α4, Lipoxygenase (1:200) o/n at 4°C. The cells were then stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) for 45 minutes in the dark to detect protein of interest and analysed using flow cytometer (BD Accuri C6). For a negative control, cells were stained with an isotype control antibody.

Table 2.5 Antibody used for the FACS experiment.

Primary antibody	Supplier
APC conjugated anti-human CD3	BioLegend
APC mouse IgM Isotype control	BioLegend
PE anti-human CD4	BioLegend
PE mouse IgM Isotype control	BioLegend
APC anti-human CD15	BioLegend
PE anti-human CD66b	BioLegend

2.4.5 Enzyme-linked immunosorbent assay (ELISA)

Preparation of cell lysates

The human embryonic kidney cell line 293T was used for this experiment. The growth medium used for 293T cells was DMEM (Dulbecco's Modified Eagles Medium) with L-Gutamine, glucose, Pyridoxine-HCl, $NaHCO_3$ (PAA-GE Healthcare) supplemented with 10% of FBS (Biosera) and 50µg/ml gentamicin (Life Technologies-Invitrogen). All cells were maintained at 37°C and 5% CO_2.

Transfection protocol was followed as per the instruction manual. $1.2x10^5$ cells were seeded in a 12-well plate approximately 20 hours (hrs) before transfection. The cells should be about 60-80% confluent at the time of transfection. The old medium was aspirated from the wells and fresh growth medium containing serum and antibiotics was added just before transfection. Cells were transfected with 1µg of pCMV6 BORIS and pCI-CTCF plasmid constructs using 4µl of jetPRIME transfection reagent and were incubated at standard growth conditions (37°C, 5% CO_2). Fresh growth medium was added to the wells after 6 hrs of transfection. The cells were collected after 48 hrs of

transfection and lysed with equal volumes of Buffer1 and Buffer2 (Table 2.3.1). Halt protease inhibitor (2%) from Thermo Scientific was added, cells were incubated on ice for 20 minutes and centrifuged at 13000rpm at 4°C for 15 minutes. The resulted supernatant was used for the ELISA experiment.

Table 2.6 Plasmids used in DNA transfection experiments.

Plasmid	Description	Source
pCMV6-BORIS	pCMV6-XL4 vector cloned with *BORIS* cDNA	Loukinov *et al.*, 2002)
pCI-CTCF	pCI vector cloned with His-tagged *CTCF* cDNA	(Farrar *et al.*, 2010)

2.4.5.1 In -house ELISA

ELISA was performed using QED's GEM®ELISA Kit. Antigen (lysate from transfected 293T cells) was bound to the wells of ELISA plates in 50 µL/well of coating buffer. Antigen coated plates were sealed with plastic wrap and incubated overnight at room temperature. The next day, the coating buffer was removed by inverting the plates and the wells were blocked for 30 minutes with 200 µL/well of 1X blocking solution, prepared by diluting 10X blocking solution in distilled water. The blocking solution was removed by inverting the plates. Pure plasma samples were added and incubated for 1 hour with gentle agitation. Plates were washed 3 times with 1X washing buffer (prepared by diluting 10X wash buffer in distilled water) by filling all wells and then inverting the plates. Secondary antibody, was diluted an antibody diluent (1:15000 mouse monoclonal secondary antibody to human IgG HRP ab99765). Diluted secondary antibody was added to each well for 30 minutes at room temperature with gentle agitation. Plates were washed 3 times with 1X wash

buffer. 100 µL/well of substrate solution were added and plates were incubated for 30 minutes at room temperature. Optical density readings were taken at single wavelength of 405 nm.

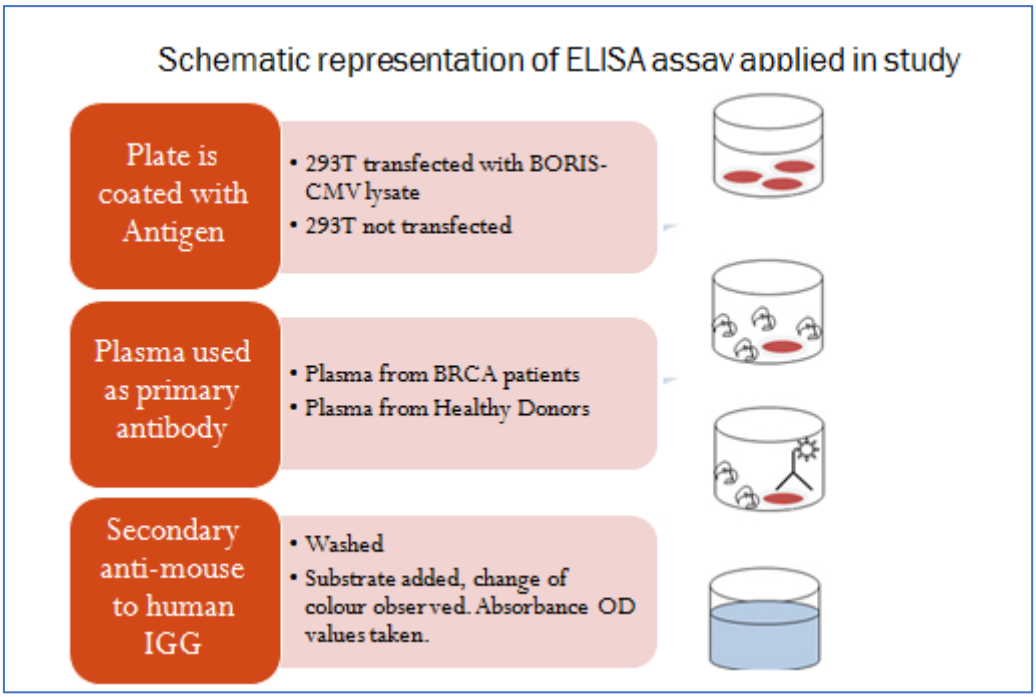


Figure 2.1 Schematic representation of the ELISA technique used to measure the levels of BORIS in plasma of breast cancer patients. The main steps of the protocol.

2.4.5.2 ELISA commercial kits

Cell lysates were prepared from $10x10^6$ white blood cells in equal volumes of Buffer 1 and Buffer 2 (Table 2.3.1). Halt protease inhibitor (2%) from Thermo Scientific was added, cells were incubated on ice for 20 minutes and centrifuged at 13000rpm at 4°C for 15 minutes. The resulted supernatant was used for the ELISA experiment.
ELISA was performed with Lipocalin2/NGAL Human ELISA kit (Abcam, Cambridge, UK), Human SERPINB1 ELISA kit and Human INTEGRIN α4 ELISA kit (Neo-Biotech according to the manufacturer's recommendation.

The assays for Serpin B1 and Integrin α4 employ the quantitative sandwich enzyme immunoassay technique. Antibody specific for Integrin α4 and Serpin B1 have been pre-coated onto a microplate. In the first step of the experiment, 100μL of standards and samples were pipetted into the wells and incubated for 2 hours at 37°C. After the plate was inverted to remove any unbound substances and 100 μl of biotin-conjugated antibody specific to the protein of interest were added to the wells following 1-hour incubation at 37°C. in the next step of the experiment, antibodies were aspirated, and wells were washed 3x with wash buffer provided in the kit. After washing, 100 μL of avidin conjugated Horseradish Peroxidase (HRP) was added to the wells followed by 1-hour incubation at 37°C. following 3x wash with wash buffer, a 90μL of TMB substrate solution was added to each well and incubated for 30 minutes at 37°C. The colour developed was in proportion to the amount of protein of interest present in the sample. In the final step, the colour development was stopped by addition of 50μL of Stop Solution and optical density was determined using microplate reader set to 450 nm.

Human Lipocalin-2 ELISA Kit (Abcam) employs sandwich (quantitative) assay. To perform the assay, 50μL of samples and standards were added to the pre-coated wells, followed by the addition of 50μL of antibody mix (capture antibody and detector antibody). After 1-hour incubation at room temperature on a shaker, the wells were washed with 3x350 μL 1x wash buffer to remove unbound material. After, 100μL of TBM Substrate was added to each well and incubated for 10 minutes in the dark on a shaker. To stop the reaction, 100μL of Stop Solution was added, and the optical density was measured at a single wavelength of 450nm.

2.5 RNA extraction and quality control

2.5.1 RNA extraction

In the presented study, total RNA was extracted from blood of healthy donors and MCF7 cell line transfected with pCMV6 BORIS plasmid to serve as negative and positive control for RT-qPCR reaction respectively. RNA was extracted using Trisure (Bioline). All purified RNA samples were subjected to treatment with DNase to remove contaminating DNA. For this purpose, TURBO DNA-*free*™ Kit (Ambion) was used and the enzyme reaction was prepared according to manufacturer's instructions. For up to 10μg of RNA, 0.1 volume of 10x TURBO DNase buffer and 1μl of TURBO DNase were added in a 50μl reaction. The reaction mix was incubated at 37°C for 30 min. Enzyme was then inactivated by incubating the reaction with 0.1 volume of DNase inactivation reagent for 5min at RT and finally the contents of the tube were centrifuged at 10300 rpm for 2 min. DNA-free RNA was transferred to a fresh tube and stored at -80°C.

2.5.2 RNA quality control

After extraction, the concentration of total RNAs was determined using NanoDrop® ND-1000 UV/VIS Spectrophotometer. Although, the procedure for quantification of RNA using Nanodrop gives some idea about the contaminants present in the RNA, it is not a reliable method to assess the integrity of RNA and to identify any genomic DNA contamination present in the RNA sample. To assess the integrity of total RNA isolated from white blood cells and MCF7 breast cancer cell line Agilent Bioanalyser 2100 was used.

Quantification of RNA using Nanodrop® ND-1000 UV/VIS Spectrophotometer

Purified RNA samples were quantified using NanoDrop® ND-1000 UV/VIS Spectrophotometer (LabTech International Ltd, UK) following the manufacturer's guidelines. The nucleic acid concentration was determined by Beer's law and the absorbance was measured at 260, 280 and 230nm. The ratios of 260nm/280nm and 260nm/230nm were obtained to assess the purity of RNA. Both ratios had to be above 1.8 to be considered pure. Although Nanodrop measurements indicate the purity of RNA it does not indicate the integrity of RNA or indicate presence of DNA in the RNA sample.

Analysis of RNA quality using Agilent 2100 Bioanalyzer

Quality of all RNA samples was assessed using Agilent 2100 Bioanalyzer instrument according to the manufacturer's instruction. The Agilent Bioanalyzer performs capillary electrophoresis to analyse RNA, DNA and proteins. It is a very efficient method used to perform absolute RNA quality control before downstream applications such as gene expression analysis or microarrays. All the reagents of Agilent RNA 6000 Nano kit were incubated at RT for 30 minutes before the start of the procedure. The Gel matrix was filtered through the column provided in the kit. The gel-dye mix was prepared by mixing 1µl of the RNA 6000 Nano Dye with 65µl of the Agilent RNA 6000 Nano Gel matrix according to the manufacturer 's instructions. The 6000 NanoChip was primed by placing

a 9µl of this mixture into the specified well on the NanoChip. The gel was dispersed across the chip with a help of a plunger. The RNA samples were denatured for 2minutes at +70°C and loaded into the NanoChip alongside 5µl of the RNA Nano Marker. The NanoChip was then vortexed for 60 seconds at 2499xg on a vortex mixer and applied for analysis by the Bioanalyser. To start the experiment, Eukaryote Total RNA assay was selected on the program and run. After the run was complete the data was saved as a pdf file and the RNA quality was assessed from the electropherograms obtained. Only RNA of acceptable quality was used in downstream applications.

2.5.3 Synthesis of cDNA and RT-qPCR

RNA prepared and analysed as described above was reverse-transcribed to cDNA using VERSO cDNA Synthesis kit (Thermo Fisher) according to the manufacturer's instructions. Real time qPCR was performed in LightCycler®96 Roche using the SensiMixPlus SYBR kit (Quantance). To analyse the results, comparative C_T method was used. First the levels of BORIS mRNA in samples from urinary sediment were analysed using SYBR qPCR. Next, the levels of BORIS mRNA were evaluated in total urinary exosomal mRNA. To detect all the 23 known mRNA variants of *BORIS* its expression was analysed by quantitative RT-PCR with two sets of primers encompassing 1–2 and 3–4 exon boundaries, respectively. For this experiment TaqMan gene expression assays (Life Technologies, Applied Biosystems, Carlsbad, CA) was used. The amplification reaction was performed using the TaqMan Universal PCR master Mix (Life Technologies, Invitrogen, Carlsbad, CA). All samples were run in duplicate in 96-well plates on the LightCycler detection system (Roche). Normalization was carried out using GapDH as internal control gene. TaqMan gene expression assay IDs for each set of primers and probe were: Hs00966555_g1 (*BORIS* E1–E2); Hs00966548_g1 (*BORIS* E3–E4)

(Zampieri et al. 2014). The qPCR conditions used were as follows: 95.0°C for 3 min followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, 72°C for 20 sec.

2.6 Statistical tests

Data was plotted using the GraphPadPrism (version 4) software (San Diego, CA). Since the data obtained in the present study is not normally distributed statistical difference between groups was analysed by non-parametric Kruskal-Wallis analysis for multiple groups.

The Kaplan-Meier method was used for survival analysis (p value for log-rank test).

Chapter 3

3. 1 Validation results for Serpin B1

One of the short-listed candidate biomarkers selected for validation in this study is Serpin B1 - a member of the serpin family of proteinase inhibitors that maintain homeostasis by neutralising proteinase activity. Serpins are the largest family of proteinase inhibitors that include more than 1000 members, with roles in the regulation of a variety of biological processes including angiogenesis, apoptosis, cell migration and blood coagulation (Mangan et al. 2008). It is highly conserved in all vertebrates (Kaiserman & Bird 2005).

Serpin B1 inhibits three serine proteases: neutrophil elastase (NE), proteinase 3(PR3) and cathepsin G (CG)- major components of neutrophil azurophilic granules secreted by activated neutrophils during the inflammatory processes (Korkmaz et al. 2010). The main role of these proteases is degradation of foreign organic molecules phagocytosed by neutrophils. However, their abhorrent activity has been associated with cancer progression. One of the proposed mechanism of the action is that the increased NE activity induces the expression of matrix metalloprotease-2 (MMP)-important component for the degradation of the extracellular matrix barrier by the cancer cells and the initiation of metastatic event (Roy & Walsh 2014). The study investigating the role of Serpin B1 in the regulation of this event in hepatocellular sarcoma (HCC) reported that knockdown of *Serpin B1* gene resulted in the increase in the levels of active MMP2 and promotion of invasiveness of HCC cells (Cui et al. 2014).

Existing reports suggest the crucial role of Serpin B1 for the sustaining of the sufficient neutrophil reserve in bone marrow. It is demonstrated by the findings that Serpin B1 deficient mice are shown to have a considerable reduction in the neutrophils. The

availability of the bone marrow reserve of mature neutrophils is essential for host defense against infection (C. Benarafa et al. 2011).

The altered expression of Serpin B1 has been reported in variety of tumour tissues and shown to be associated with tumour progression. For instance, elevated levels of this protein were found in 62.5% of invasive oral squamous cell carcinomas (Tseng et al. 2009). In breast cancer tissue, upregulation of Serpin B1 was found to be associated with prolonged relapse-free survival of breast cancer patients (Sheng et al. 2015).

In our previous proteomic study Serpin B1 was found to be overexpressed in WBCs of breast cancer patients by both highthroughput proteomics (fold change +1.46) and 2D gel electrophoresis (fold change +1.46).

Given the evident important role of Serpin B1 for the neutrophil function and innate immune response and the fact that in the previous proteomic studies in the laboratory it was identified as best candidate biomarker, in the present study the utility of this protein as novel WBCs-based breast cancer biomarker was being investigated.

Objectives of the present study:

Validation of novel cancer biomarkers identified from breast and prostate tissue specimens is one of the overarching aims of this study. In this Chapter we will focus on validation of Serpin B1. The rationale behind this investigation is illustrated in Figure 3.1. Two Objectives will be pursued in this Chapter, which are as follows:

1. Assess the expression patterns of Serpin B1 the white blood cells (WBC) from large cohorts of breast cancer patients and healthy donors using a range of different techniques. They will include Western blot analysis, enzyme-linked

immunosorbent assay (ELISA), immunofluorescence and fluorescence-activated cell sorting (FACS) analyses.

2. Correlate findings from Objective 1 with clinical outcome data to identify possible diagnostic, prognostic and predictive biomarker properties of Serpin B1.

3.2 Results

3.2.1 Serpin B1 expression in the WBCs of obtained from blood of healthy donors.

Based on previous findings, showing that Serpin B1 has potential as breast cancer biomarker, here its utility was evaluated using Western blot. Levels of Serpin B1 were investigated in white blood cells (WBC) purified from blood samples obtained from 11 healthy donors. The results showed that in most of the samples, levels of Serpin B1 in healthy donors are lower in comparison with breast cancer patients (Figure 3.2, top panel). However, in some cases we observed elevated levels of the protein of interest. For example, in the sample corresponding to healthy donor 45, levels of the protein of interest were considerably higher than in other healthy donor samples. Interestingly, this individual has a family history of both, breast and prostate cancer (Table 3.1). In sample 11, levels of Serpin B1 also appeared to be higher than in the rest of the samples and correlates with a familial history of breast cancer. Thus, we can propose that high levels of Serpin B1 in healthy donors could possibly correlate with higher risk of disease development.

Table 3.1 Healthy donors Information

HD1	No history of cancer
HD2	No history of cancer
HD5	Yes, prostate
HD11	Yes, breast
HD45	Yes, breast and prostate cancer
HD46	Yes, uterine and skin cancer
HD47	Yes, colon
HD48	No history of cancer
HD49	Yes, gastric
HD50	No history of cancer
HD51	Yes, breast and lung cancer

3.2.2 Western blot analysis of Serpin B1 expression in the WBCs from breast cancer patients with different stages of breast cancer

Following from the analysis of Serpin B1 in healthy donors, the levels of Serpin B1 were evaluated in four different categories of breast cancer patients and compared with healthy donors. This was achieved by evaluating the levels of this protein in WBCs obtained from blood samples from 39 participants comprising the primary breast cancer cohort further stratified into two groups with different stages of lymph node metastasis, node negative and node positive; and 32 of advanced breast cancer patients (a cohort of breast cancer patients treated for secondary metastases).

Our results revealed that overall levels of Serpin B1 are higher in WBCs of patients with breast cancer in comparison with healthy individuals and the highest in the WBC from participants with advanced breast cancer (representative examples are shown in Figure 3.2, middle and bottom panels). Following the analysis of the images of the bands, densitometry was performed on the blots using Image J software and the results were plotted on a graph using GraphPad Prism 6 software. As shown in Figure 3.3, levels of Serpin B1 are overall higher in the combined primary breast cancer cohorts, although lymph node positive samples had more Serpin B1 than node negative samples. The highest levels of Serpin B1 was detected in the advanced breast cancer cohorts (the obtained values are given in the table 3.1). Statistical analysis of the above data revealed significant difference in the levels of Serpin B1 between all breast cancer cohorts and healthy donors.

The analysis of Serpin B1 levels between two breast cancer cohorts revealed that it is higher in advanced cohort than in primary one. The Kruskal-Wallis test of the data showed that the difference is statistically significant. In primary breast cancer cohort Serpin B1

levels were higher in node positive group than in node negative, however, these results were not statistically significant. Comparison of the node positive group from primary breast cancer cohort and advanced breast cancer cohort showed that Serpin B1 levels are significantly higher in advanced cohort. The difference in Serpin B1 levels between node negative group of primary cohort and advanced cohort also shown to be statistically significant.

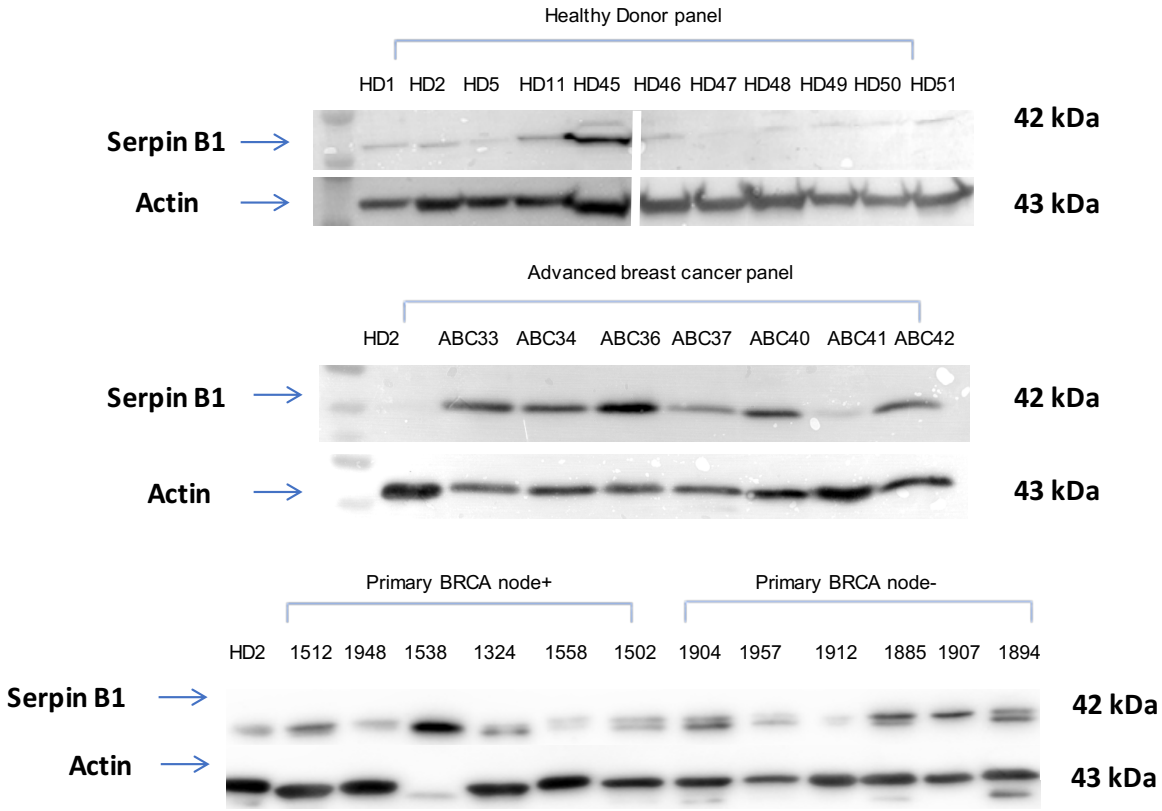


Figure 3.2 Representative western blot assays demonstrating Serpin B1 levels in WBCs from healthy donors and breast cancer patients with different disease conditions. WBCs were isolated from blood of cancer patients. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control).

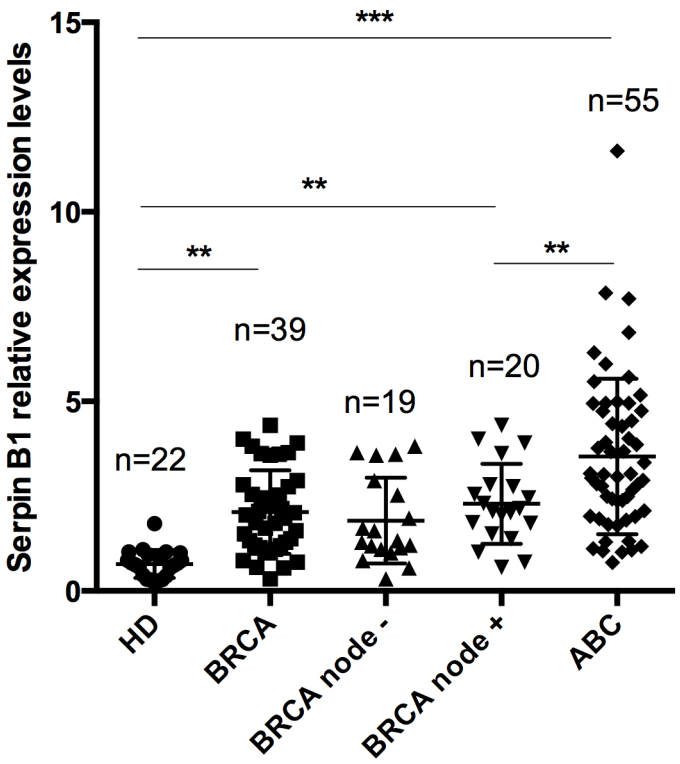


Figure 3.3 Serpin B1 levels in WBCs of breast cancer patients with different disease states. The WBCs samples were prepared and analyzed by Western blot assay as described in the text and Figure 3.2. Band corresponding to the proteins of interest were visualized using Image Analyzer. Densitometry was performed on the blots using Image J software. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-actin bands were determined. The sample from Healthy Donor 2 (HD2) was used in all Western assays to cross compare data from different gels. The results plotted on a graph using GraphPad Prism 6 software. Statistical difference between groups was determined by non-parametric Kruskal-Wallis test. $** \ P \leq 0.01$; $*** \ P \leq 0.001$

Keys describing the participants' cohorts:

HD: healthy donors;

BRCA: all participants from the primary breast cancer cohort;

BRCA node -: primary breast cancer cohort, lymph node metastasis negative;

BRCA +: primary breast cancer cohort, lymph node positive;

ABC - advanced breast cancer cohort (a cohort of breast cancer patients treated for secondary metastases).

3.2.3 Western blot analysis of Serpin B1 expression in WBCs of patients with advanced disease.

To assist the assessment of the breast cancer treatment efficacy, several approved tumour markers such as CA15-3 and CEA are used. However, the serum levels of above markers are not always elevated in patients with advanced disease (Brooks 2009). Therefore, there is a need for more reliable non-invasive biomarkers for treatment monitoring and disease progression that can assist or replace the radiological tests.

This part of the study was designed to evaluate the potential of Serpin B1 as a novel white blood cell-based biomarker for treatment monitoring in patients with metastatic (advanced) breast cancer. The aim of this part of the study was to assess the changes in the levels of Serpin B1 in blood of breast cancer patients with metastatic disease and correlate them with clinical data provided by the hospital in order to determine the clinical value of Serpin B1 as a novel white blood cells-based biomarker for treatment monitoring. According to RECIST, the clinical response is categorised into four groups:

- CR (complete response) - disappearance of all lesions

- PR (partial response) – reduction of the lesion diameter by 30%

- SD (stable disease) – no significant change of the size of the lesion.

- PD (progressive disease) – the appearance of new lesions and an enlargement of the existing one by at least 20%

Information regarding participants taken part in this part of the study is given in “Materials and Methods” section 2.1.2. Fractionation of blood and separation of white blood cells was performed as described in “Materials and Methods” section 2.5. Western blot

analysis was performed to analyse changes in Serpin B1 expression during the treatment as described in “Materials and Methods” section 2.6.

3.2.3.1 Western blot analysis of Serpin B1 expression in WBCs from breast cancer patients with metastatic disease during chemotherapy treatment in correlation with survival.

Next, we analysed whether Serpin B1 can serve as a marker for the response to the treatment. In order to achieve this, expression of Serpin B1 was assessed in WBCs of breast cancer patients with metastatic disease during chemotherapy treatment. Patients with invasive breast cancer had undergone six rounds of chemotherapy treatment. The level of Serpin B1 in WBCs was evaluated in WBCs obtained from 30 breast cancer patients prior the first (baseline), third (middle) and sixth (final) treatments. Upon evaluation, four different patterns of Serpin B1 levels in the response to chemotherapy treatment were observed and results were divided into four groups of response (Figure 3.4).

In the first group, we observed a gradual decrease in the level of Serpin B1 in response to treatment. This group comprised of 8 patients with the majority of the patients. One patients only had 1 month survival and one 48 months. The rest of the patients form this group had survival time between 12 and 23 months. In the second group, we observed increase of Serpin B1 levels in the middle of the treatment and decrease in the end. The observed pattern correlates with survival time between 9 and 48 months. Decline in the level of Serpin B1 expression in the middle of the treatment and slight rise at the end, was detected in the third group comprised of 13 patients. This pattern in changes in protein expression associates with variable survival time from 3 to 26 months and one alive patient. In the final fourth group, we observed gradual rise of the levels of Serpin B1

expression during the course of treatment. This group consist of two patients with 7 and 12 months survival time.

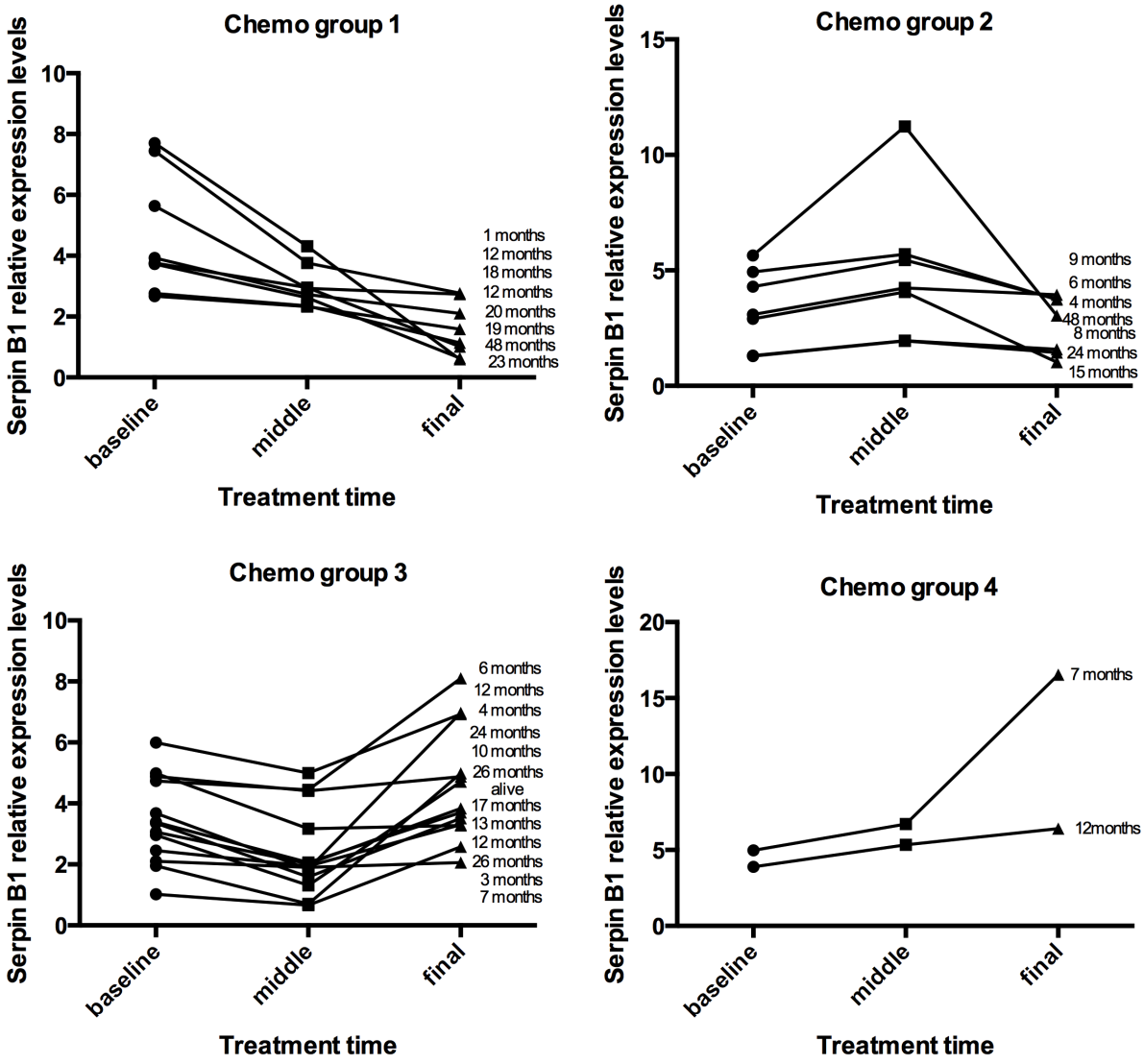


Figure 3.4 Western blot analysis of the correlation between changes in the levels of Serpin B1 during the chemotherapy treatment and survival of the patients. WBCs were extracted from blood of breast cancer patients prior the treatment-baseline, in the middle and at the final stage; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

3.2.3.2 Western blot analysis of Serpin B1 expression in WBCs from breast cancer patients with metastatic (advanced) disease during endocrine treatment in correlation with survival.

Ten patients with advanced estrogen receptor (ER) and progesterone receptor (PR) positive breast cancer had undergone three round of endocrine treatment involving different types of drugs. After each step of treatment, the level of Serpin B1 was measured and three different expression patterns were observed in the response to treatment (Figure 3.5).

In the first group, we observed a gradual decrease of the level of Serpin B1 in the response to treatment. The detected pattern of Serpin B1 expression correlates with more favourable prognosis, longer survival of the patients (24 months and one patient alive).

In the second group, we observed increase of Serpin B1 levels in the middle of the treatment and decrease in the end. This group comprised of 3 patients, one with 24 months overall survival and for two of the patients the clinical information is unknown.

Decline of the level of Serpin B1 in the middle of the treatment and rise at the end, was detected in the third group of patients. This pattern in changes in protein expression is associated with survival time from 4 to 48 months.

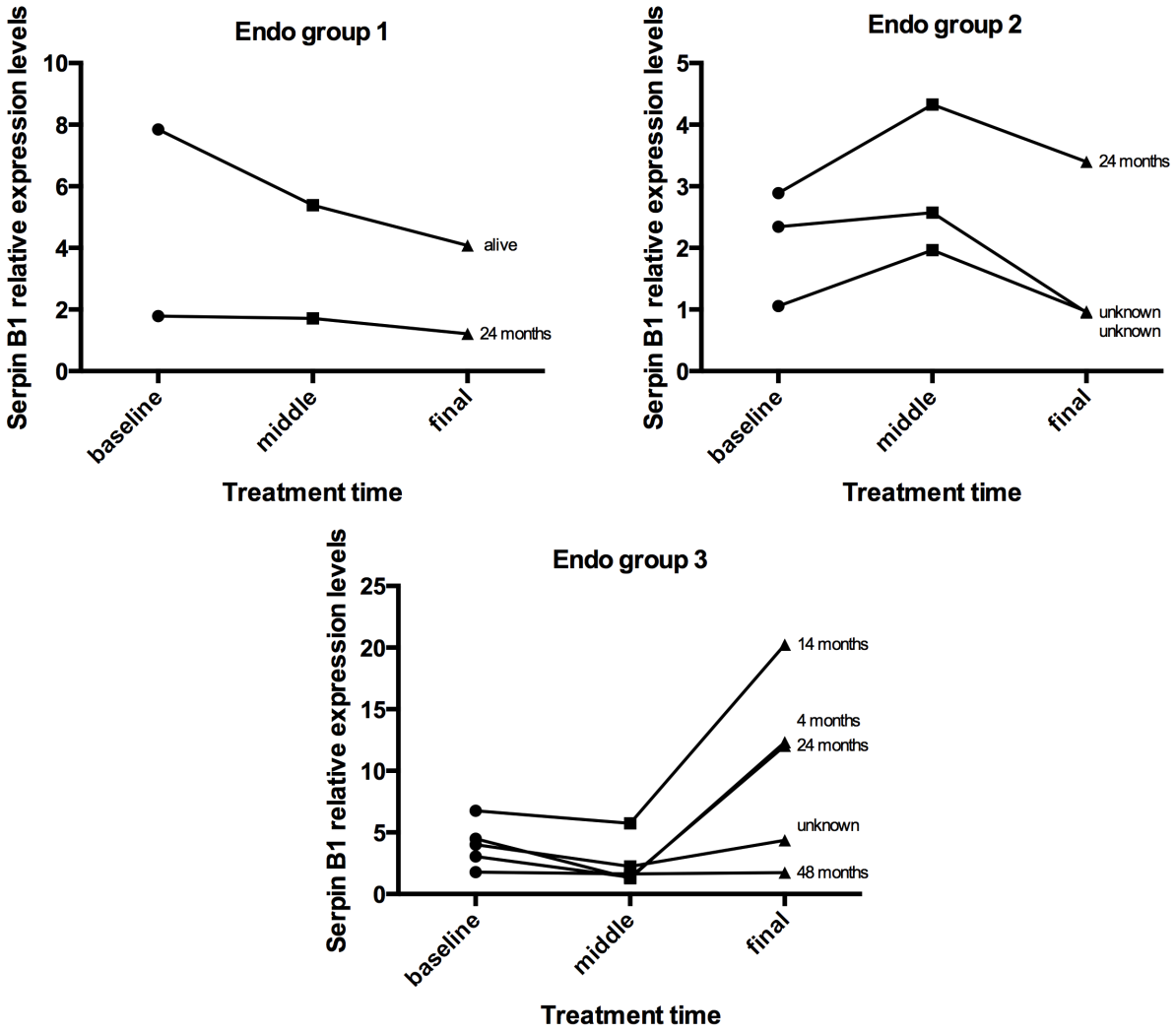


Figure 3.5 Western blot analysis of the correlation between changes in the levels of Serpin B1 during the course of endocrine treatment and survival of the patients. WBCs were extracted from blood of breast cancer patients prior the treatment-baseline, in the middle and at the final stage; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

3.2.3.3 Western blot analysis of Serpin B1 expression in WBCs from breast cancer patients with advanced disease during chemotherapy treatment in correlation with clinical response.

Next, we analysed whether Serpin B1 can serve as a marker for the response to the chemotherapy treatment. In order to achieve this, the level of Serpin B1 was evaluated in WBCs obtained from 30 breast cancer patients prior the first (baseline), third (middle) and sixth (final) treatments and grouped according to the clinical response to the treatment - CR (complete response) - PR (partial response) - PD (progressive disease) - SD (stable disease). The generated data showed that the levels of Serpin B1 during the chemotherapy are not indicative of the treatment response (Figure 3.6).

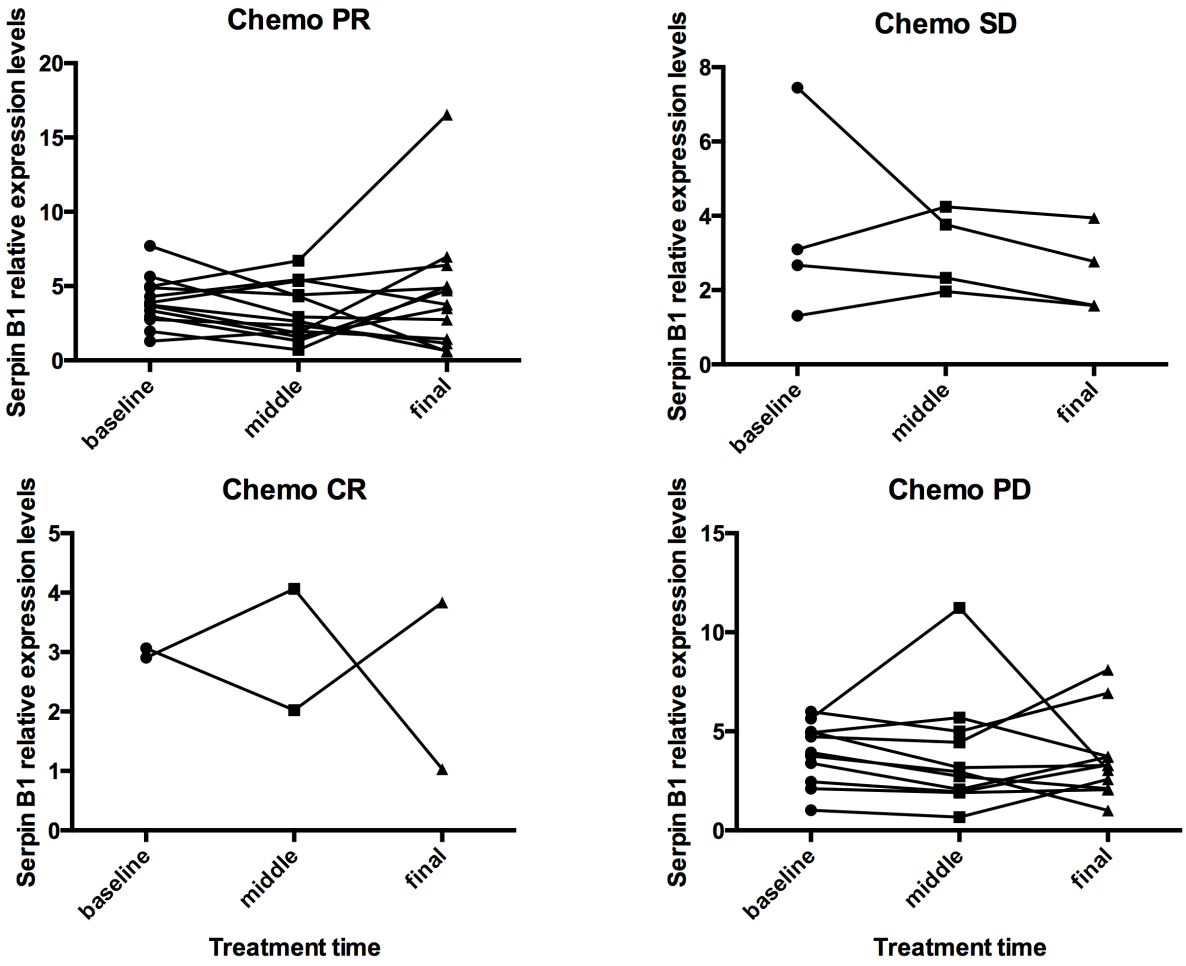


Figure 3.6 Western blot analysis of the correlation between changes in the levels of Serpin B1 during the course of chemotherapy treatment and clinical response. Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

3.2.3.4 Western blot analysis of Serpin B1 expression in WBCs from breast cancer patients with advanced disease during the course of endocrine treatment in correlation with clinical response.

Next, we analysed whether Serpin B1 can serve as a marker for the response to the endocrine treatment. In order to achieve this, the level of Serpin B1 was measured in WBCs obtained from 10 breast cancer patients prior the first (baseline), second (middle) and final third stage of the treatments and grouped according to the clinical response to the treatment - CR (complete response) - PR (partial response) - PD (progressive disease) - SD (stable disease). The generated data showed that the levels of Serpin B1 are not indicative of endocrine treatment response (Figure 3.7).

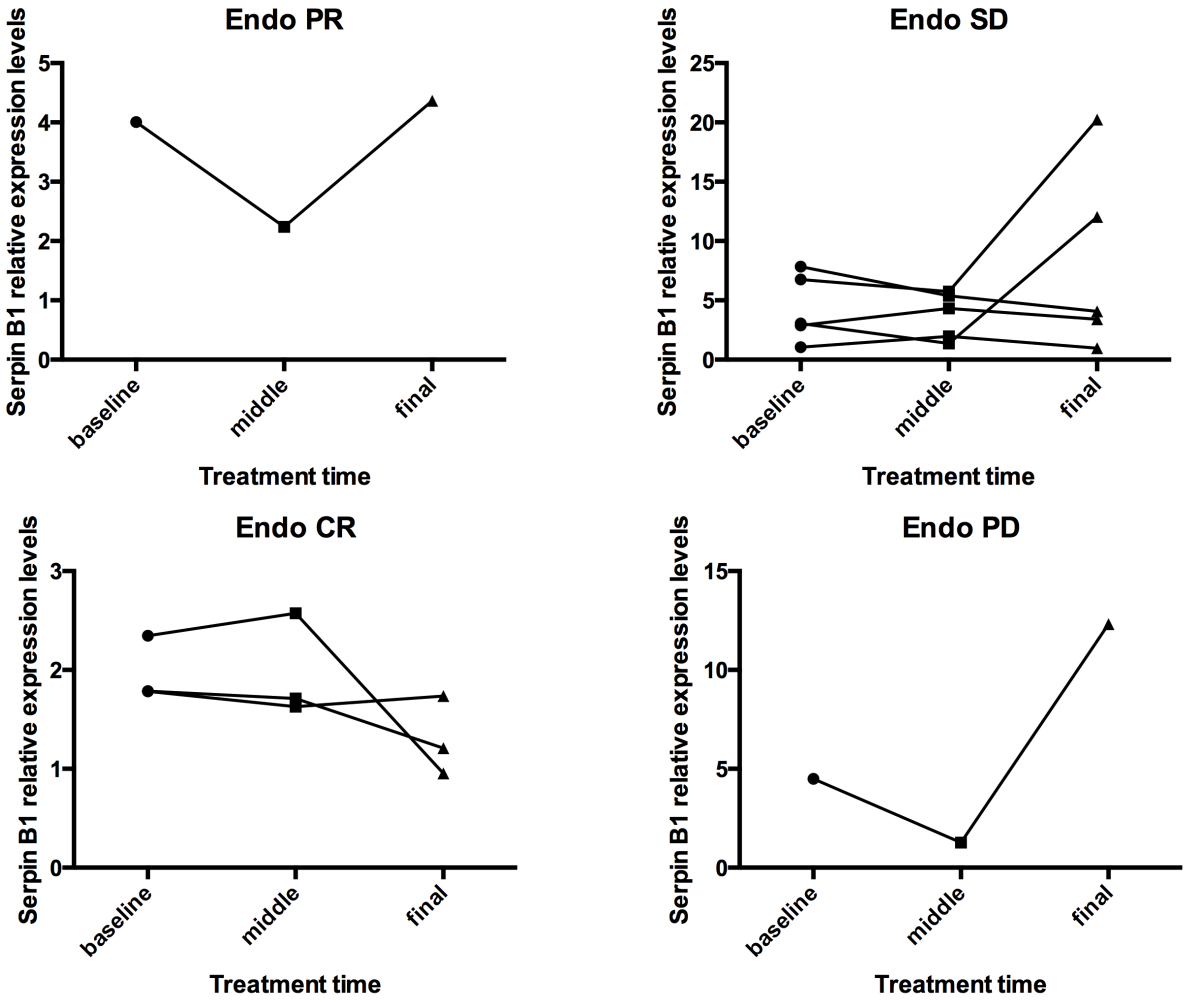


Figure 3.7 Western blot analysis of the correlation between changes in the levels of Serpin B1 during the course of endocrine treatment and clinical response. Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined, as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

Table 3.2 Initial levels of Serpin B1 and Lipocalin 2 in WBCs of advanced(metastatic) breast cancer patients prior the treatment received.

Sample ID	Initial Serpin B1 levels	Initial Lipocalin2 Levels	Treatment type	Prognosis	Survival in months
ABS2	1.746		Endocrine	PR	48 months
ABS3	2.11	1.72	Chemotherapy	CR	7 months
ABS4	3.009	1.42	Chemotherapy	PD	3 months
ABS5	1.174		Endocrine	SD	Alive
ABS6	5.98	0.96	Chemotherapy	PD	48 months
ABS8	2.98		Endocrine		4 months
ABS9	2.48		Endocrine	PD	
ABS10	2.42	1.49	Chemotherapy	PD	12 months
ABS11	2.38	1.64	Endo/Chemo	PR	13 months
ABS12	2.92	4.28	Chemotherapy	PR	Alive
ABS13			Chemotherapy		1 month
ABS14	4.02	6.83	Endocrine	PR	
ABS15			Chemotherapy	NE	1 month
ABS16	4.98		Chemotherapy	PR	10 months
ABS17	4.34	1.05	Chemotherapy	PR	12 months
ABS18	5.52		Chemotherapy	PR	12 months
ABS19			Endocrine	PR	24 months
ABS20	3.9		Chemotherapy	PD	18 months
ABS21			Endocrine	PD	23 months
ABS22	2.8	1.11	Chemotherapy	SD	24 months

ABS23	4.4	1.05	Chemotherapy	PR	6 months
ABS24		1.39	Chemotherapy	PD	4 months
ABS25	3.85	1.2	Chemotherapy	PR	12 months
ABS26	3.07	1.19	Chemotherapy	SD	4 months
ABS27			Endocrine	PD	22 months
ABS28	2.76	1.28	Chemotherapy	RIP	2 months
ABS29	4.9	1.7	Chemotherapy	PR	7 months
ABS30	3.68	1.36	Endo/Chemo	SD	23 months
ABS31	2.49	1.68	Chemotherapy	PD	19 months
ABS32	2.82	1.59	Chemotherapy		15 months
ABS33	7.7	4.38	Chemotherapy	PD	48 months
ABS34	6.8	2.14	Endocrine	SD	14 months
ABS35	1.11		Chemotherapy	PD	1 month
ABS36	11.60	3.008	Chemotherapy		3 months
ABS37	4.74	6.18	Chemotherapy	PD	6 months
ABS38			Chemotherapy	PD	4 months
ABS39			Chemotherapy	RIP	1 months
ABS40	4.95	2.7	Chemotherapy	PD	9months
ABS41	1.9	5.78	Chemotherapy	PR	24 months
ABS42	4.98	4.52	Chemotherapy	PD	3 months
ABS43	5.64	2.77	Chemotherapy	PD	7 months
ABS44	3.09	2.81	Chemotherapy	CR	17 months
ABS45	3.67	2.98	Chemotherapy	PR	
ABS46	3.088	2.63	Endocrine	SD	12 months
ABS47	1.72	1.5	Endocrine	CR	24 months
ABS48	2.38	2.3	Endocrine	CR	

ABS49			Endocrine	PR	10 months
ABS50	1.31	0.83q	Chemotherapy	SD	8 months
ABS51	1.28	0.79	Chemotherapy	PR	24 months
ABS52	1.048		Endocrine	PD	3 months
ABS53	4.488	2.15	Endocrine	PD	4 months
ABS54	2.67	1.29	Chemotherapy	SD	
ABS55	7.86	0.92	Endocrine	SD	Alive
ABS56	1.97	2.11	Chemotherapy	SD	1 months
ABS57	3.76	1.49	Chemotherapy	PD	20 months
ABS58	3.89	1.54	Chemotherapy	PD	26 months
ABS59	1.05	1.53	Endocrine	SD	
ABS60		1.68	Chemotherapy	PD	
ABS61	1.02	1.86	Chemotherapy	PD	26 months
ABS62	4.73	1.45	Endocrine	PR	12 months
ABS63	6.28	1.55	Endocrine	PR	12 months
ABS64	1.96	0.87	Chemotherapy	PR	3 months
ABS65	0.75		Chemotherapy	PR	3 months
ABS66	1.86	0.88	Chemotherapy	PD	
ABS67	5.16	0.93	Endocrine	SD	

3.2.3.5 Western Blot analysis of the basal levels of Serpin B1 in WBCs of patients with advanced breast cancer in correlation with survival.

The aim of the next experiment was to analyse whether the basal levels of Serpin B1 in WBCs of the advanced breast cancer patients before treatment have any correlation with overall survival. For this purpose, the levels of Serpin B1 were assessed by western blot analysis using hand cast gels. The obtained results showed that low levels of the protein in the beginning of the treatment correlate with longer survival of the patients with chemotherapy treatment (Figure 3.8). These results suggest that Serpin B1 has a potential to be used as a marker of survival for the patients commencing chemotherapy. The results for patients with endocrine treatment showed no correlation between basal levels of Serpin B1 and overall survival (Figure 3.9). Therefore, it can be concluded that Serpin B1 is not marker for survival in the group pf patients commencing endocrine therapy.

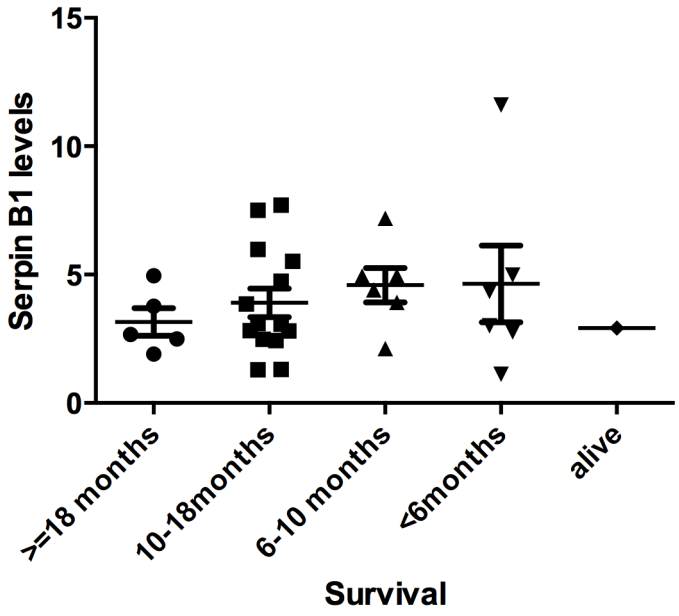


Figure 3.8 Analysis of the correlation between initial levels of Serpin B1 before treatment and response to chemotherapy treatment in advanced breast cancer patients by western blot. WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed on the blots using Image J Software. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

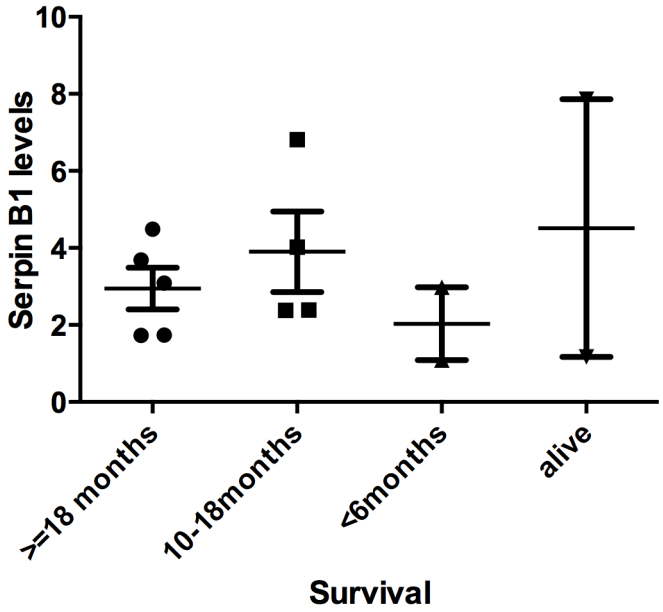


Figure 3.9 Analysis of the correlation between initial levels of Serpin B1 before treatment and response to endocrine treatment in advanced breast cancer patients by western blot. PD progressive disease; PR partial response; SD stable disease; CR complete response. WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed on the blots using Image J software. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

3.2.3.6 Western Blot analysis of the basal levels of Serpin B1 in WBCs of advanced breast cancer patients in correlation with response to treatment.

To assess whether Serpin B1 can serve as a marker of treatment response, the basal levels of the protein of interest were compared with corresponding clinical data. According to the obtained results the lower levels of Serpin B1 in the beginning of the both, chemotherapy and endocrine therapy, correlate with complete response to the treatment received (Figures 3.10, 3.11).

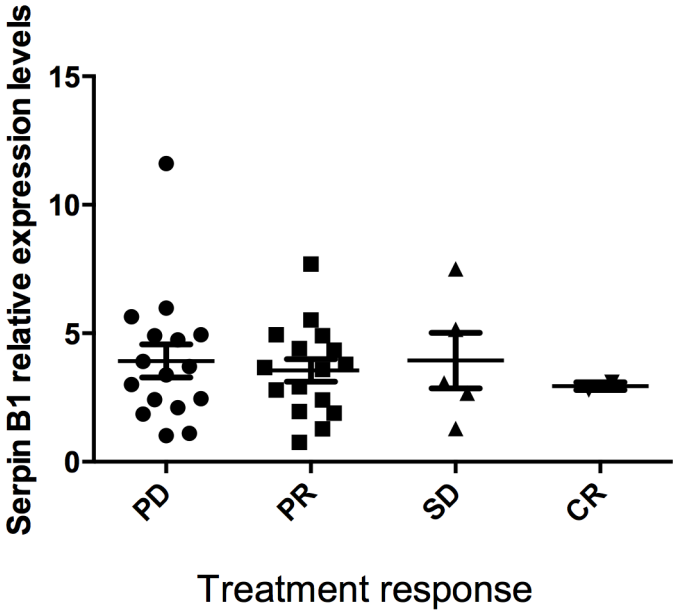


Figure 3.10 Analysis of the correlation between initial levels of Serpin B1 before treatment and response to chemotherapy in advanced breast cancer patients by western blot. PD progressive disease; PR partial response; SD stable disease; CR complete response. WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed on the blots using Image J Software.The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

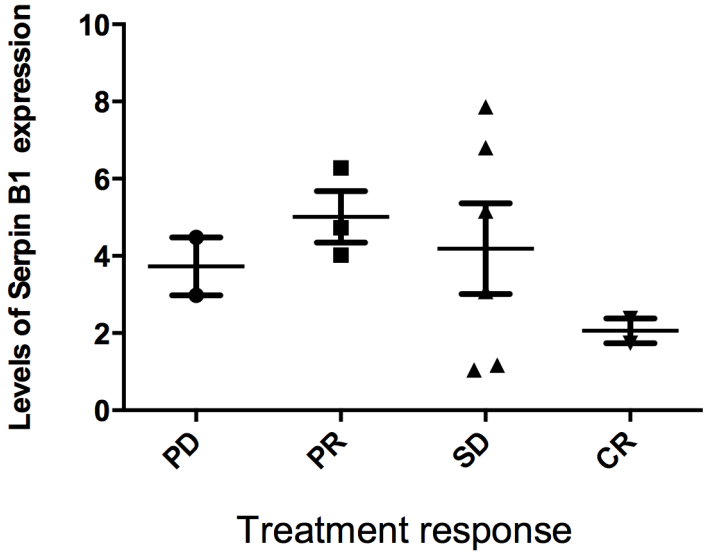


Figure 3.11 Analysis of the correlation between initial levels of Serpin B1 before treatment in correlation with response to endocrine treatment in advanced breast cancer patients by western blot. PD progressive disease; PR partial response; SD stable disease; CR complete response. WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed on the blots using Image J software. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

3.2.3.7 Kaplan-Meier survival analysis

Next, we carried out Kaplan-Meier analysis to study the correlation between high and low levels of Serpin B1 expression and patient's overall survival (Figure 3.12). The baseline protein levels of Serpin B1 were measured by Western Blot in 55 advanced breast cancer patients. The Serpin B1 signal was normalised to the β Actin signal using Image J software. Expression values between the gels were compared by normalising to the expression value of the common healthy donor 2 sample used in all experiments. The cut-off point was determined by average of all the values, as 2. The generated data shows no significant difference between these parameters (p=0.0568).

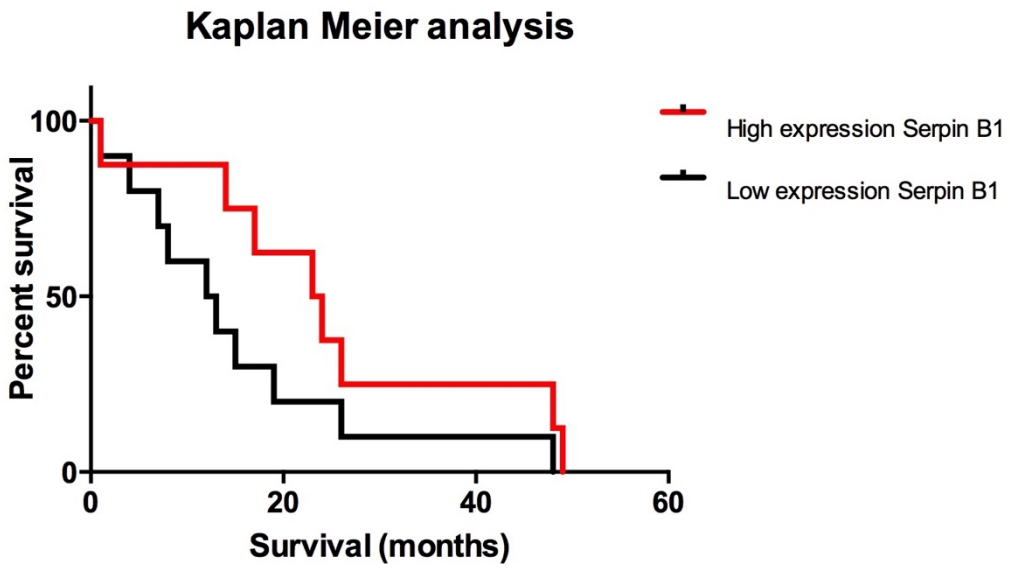


Figure 3.11 Survival curves according to Serpin B1 expression. Kaplan-Meier survival curve for high Serpin B1 expression versus low Serpin B1 expression did not show significant difference. WBCs were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Serpin B1 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Serpin B1 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

3.2.4 Immunofluorescent analysis

3.2.4.1 Distribution of Serpin B1 in WBCs of breast cancer patients

Next, the distribution of Serpin B1 protein in WBCs of breast cancer patients and healthy donors was examined by Immunofluorescent staining technique using primary anti-Serpin B1 antibody (Figure 3.13). Examination of slides revealed different distribution of Serpin B1. In leukocytes from the node negative primary breast cancer patients Serpin B1 appeared to be present in the cytoplasm and nuclei of the cells. Leukocytes from node positive breast cancer patients were characterised by higher intensity of the staining and, in addition to nuclei and cytoplasm, the extracellular localisation of Serpin B1 was detected. In leukocytes of patients with advanced breast cancer Serpin B1 protein was detected in the cytoplasm of the cells with high intensity. In the samples obtained from healthy individuals the intensity of the staining was weak. These results support the data obtained from western blot analysis.

In order to statistically compare the staining intensity between the samples ImageJ was used. Densitometric analysis of the Serpin B1 expression, showed statistically significant ($p<0.05$) increase in the Serpin B1 levels in white blood cells of breast cancer patients compared to those of healthy donors.

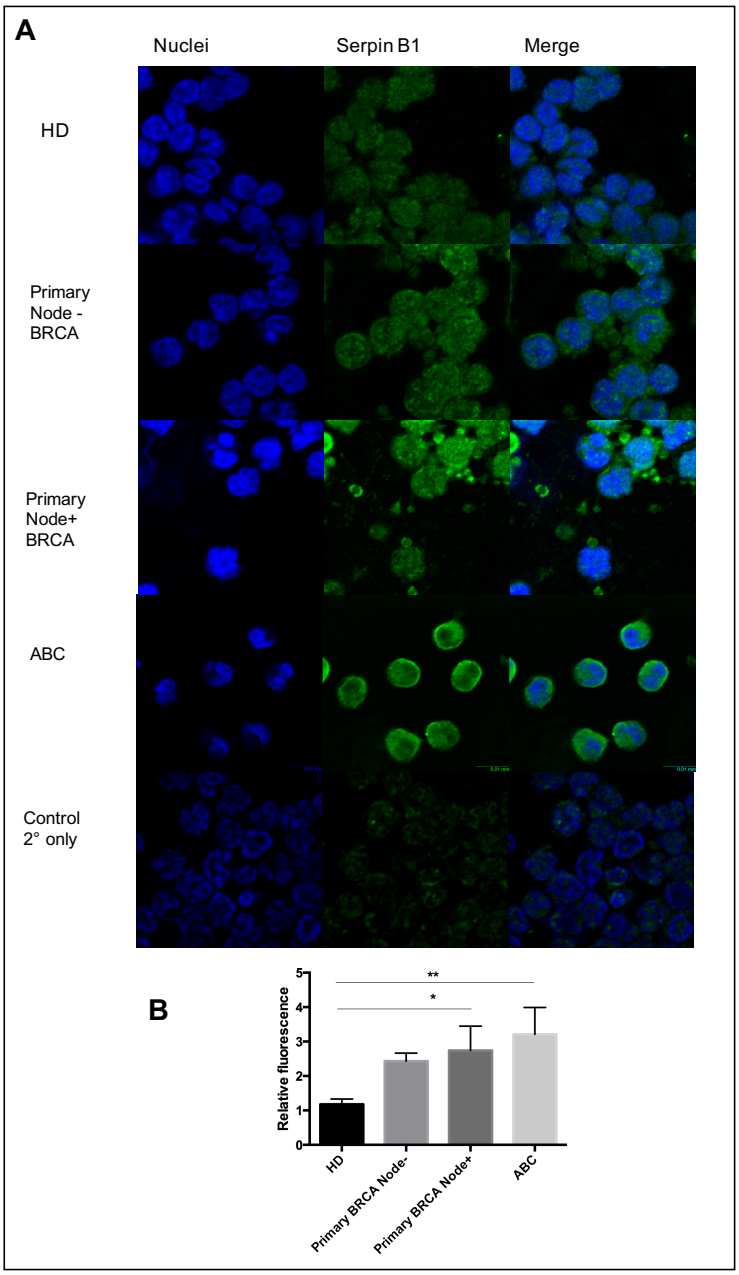


Figure 3.13 Serpin B1 protein distribution in WBCs of BRCA node negative, node positive, advanced cancer patients and healthy donors. A WBCs from breast cancer patients and healthy donors were stained with FITC-labelled anti-rabbit secondary IgG antibody (dilution 1:400) against Serpin B1 primary antibody (dilution 1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining in the cytoplasm and the green dots in the nucleus corresponds to Serpin B1 protein. All images were taken by Confocal Scanning Microscope (BioRad Hercules) at 60X magnification. **B** Bar graph represents the proportion of signal distribution among samples measured using ImageJ software.

3.2.4.2 Identification of Serpin B1 expressing cells

In order to further understand exactly which immune cells overexpress Serpin B1 double immunofluorescence procedure was carried out and analysed by laser-scanning confocal microscopy. For this purpose, white blood cells from breast cancer patients were double stained against the cluster of differentiation (CD) cell surface markers to distinguish between subpopulation of white blood cells and the protein of interest.

First leukocyte fractions were stained with CD15 antibody which recognises carbohydrate epitope expressed in mature neutrophils and eosinophils. The obtained double immunofluorescence analysis revealed that the majority of Serpin B1 positive cells were neutrophils (Figure 3.14). Staining with only the secondary antibody did not show any nonspecific background.

Next in order to determine whether populations of lymphocytes express Serpin B1, white blood cells were stained with B cells related antigen CD21 and T cells related antigen CD43. However, the staining for lymphocytes showed negative results (Figure 3.15). In order to validate antibody used in the present study western blot and immunofluorescence analysis were performed on CD15 positive K562 cell line, CD21 and CD43 positive Raji cell line. As the result, positive staining was observed in all analysed cell lines (Figures 3.16, 3.17). These results suggest that there were no population of lymphocytes on the analysed leukocyte smears.

The described findings may be associated with the fact that neutrophils are the most abundant circulating blood leukocytes that comprise up to 80% of all white blood cells (Borregaard 2010). Lymphocytes are immune cells responsible for cellular and humoral immunity. In the blood of human adults they make up 20% of the total number of white blood cells (Klaus 2001).

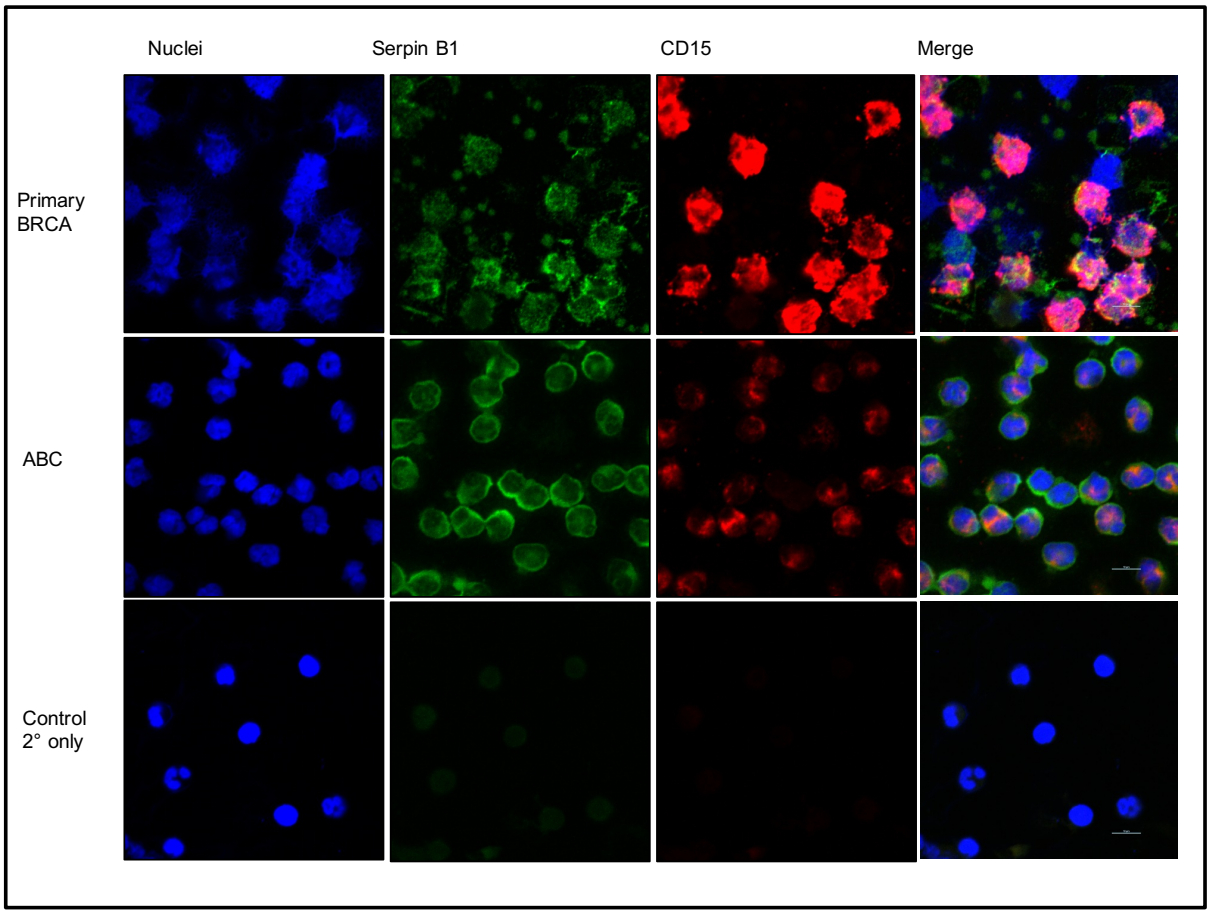


Figure 3.14 Immunofluorescence analysis of Serpin B1 protein distribution in leukocytes of primary and advanced breast cancer patients. WBCs extracted from blood of primary and advanced breast cancer patients were double stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) against Serpin B1 primary antibody (dilution 1:200) and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD15 neutrophil primary antibody (1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining corresponds to Serpin B1 protein and red staining represents Neutrophils. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

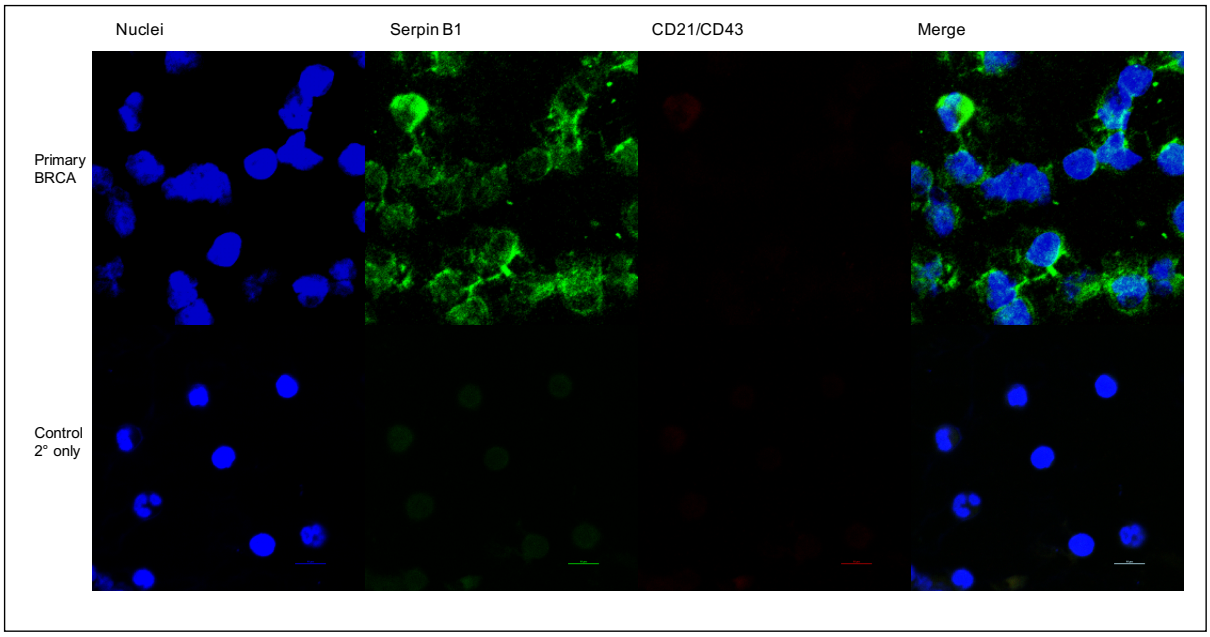


Figure 3.15 Immunofluorescence analysis of Serpin B1 protein distribution in lymphocytes of primary and metastatic breast cancer patients. WBCs extracted from blood of primary breast cancer patients were double stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) against Serpin B1 primary antibody (dilution 1:200) and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD21 positive B cells and CD43 positive T-cells primary antibody (1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining corresponds to SerpinB1. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

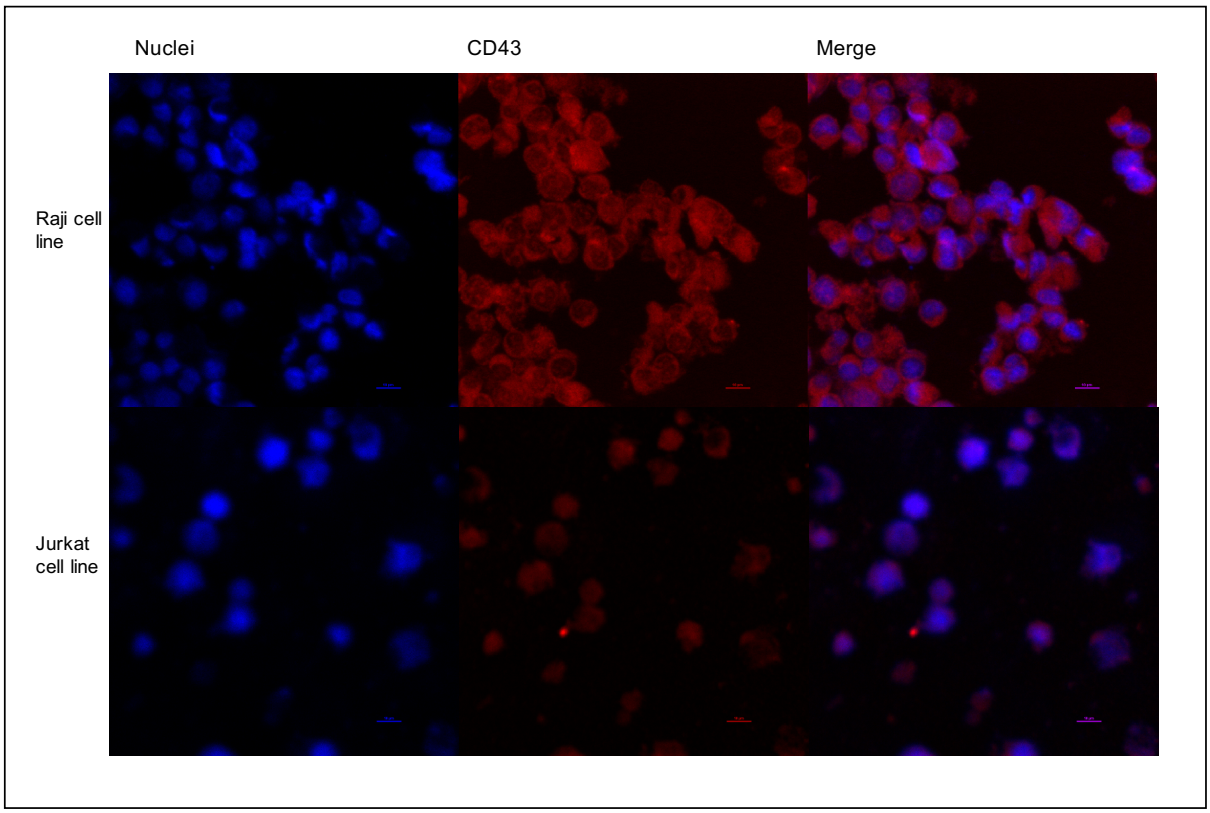


Figure 3.16 Representative immunofluorescence staining pattern of anti-CD43 antibody-positive Raji cell line and anti-CD43 antibody-negative control Jurkat cell line. Cell were fixed with cold methanol and stained with Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD43 primary antibody (1:200). The blue staining shows the position of the nucleus. The red staining corresponds to membrane cell surface marker CD43. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

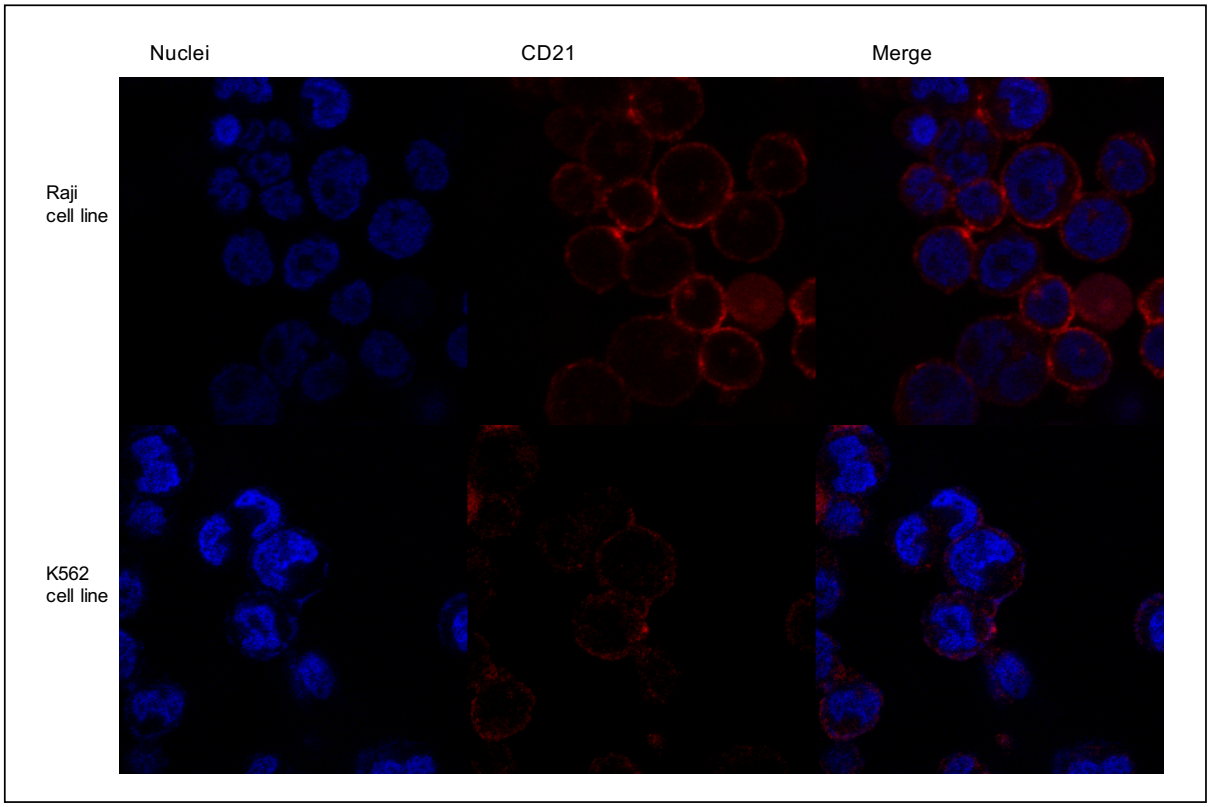


Figure 3.17 Representative immunofluorescence staining pattern of anti-CD21 antibody-positive Raji cell line and anti-CD21 antibody-negative control K562 cell line. Cell were fixed with methanol and stained with Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD21 primary antibody (1:200). The blue staining shows the position of the nucleus. The red staining corresponds to membrane cell surface marker CD21. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification

3.2.5 Evaluation of the levels of Serpin B1 in neutrophils of primary breast cancer patients using flow cytometric analysis.

Here we aimed to further validate the increased expression of the Serpin B1 using flow cytometry (FACS). Flow cytometry is the method that allows for rapid quantitative assessment of proteins within the specific cell type via cell surface marker phenotyping.

For this experiment, freshly isolated WBCs (within 6 hours from blood draining) from randomly selected primary breast cancer patient and healthy donor were stained with two pre-labelled markers expressed by mature neutrophils: CD15, CD66b (BioLegend). Serpin B1 is intracellular protein therefore pre-stained cells were fixed, permeabilised and stained with unlabeled polyclonal rabbit anti-Serpin B1 antibody followed by the incubation with FITC-labelled anti-rabbit secondary antibody.

For a negative control, cells were stained with an isotype control antibody. Isotype controls are antibodies of the same isotype as the target primary antibody. They are of unknown specificity or are raised against antigens known to be absent in target cells. Isotype controls are used to estimate non-specific staining of primary antibodies.

The results obtained for Mean fluorescent intensity (MFI) show that the levels of Serpin B1 are almost two-fold higher in the neutrophils from a primary breast cancer patient than from a healthy volunteer (Figure 3.18).

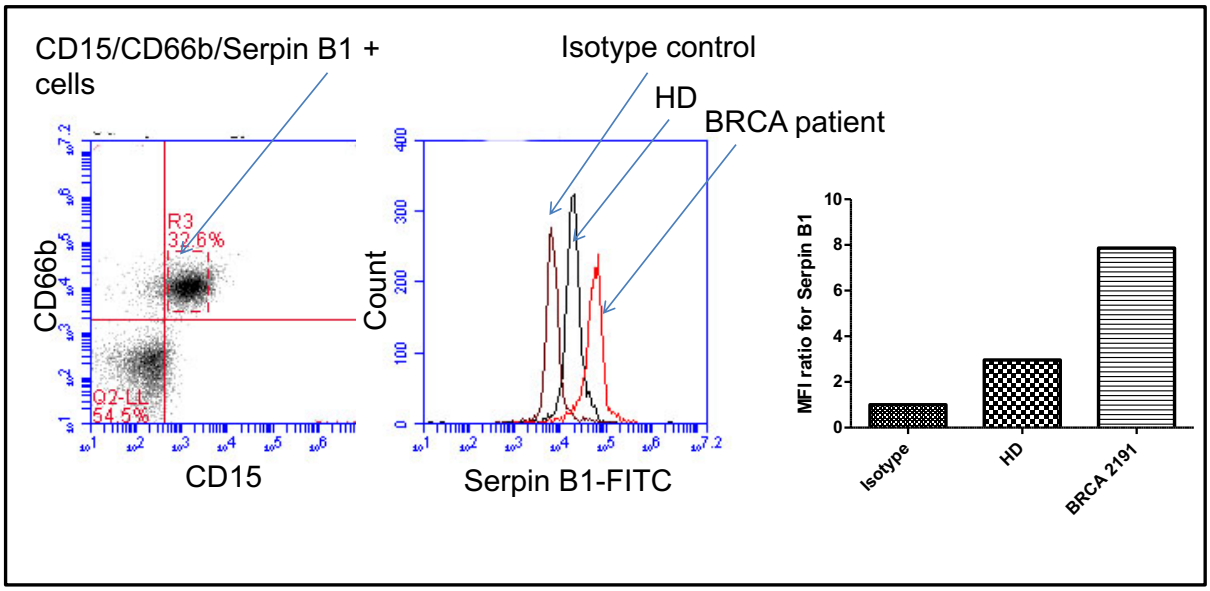


Figure 3.18 Flow cytometric analysis of the Serpin B1 levels in neutrophils of breast cancer patient and a healthy donor (randomly selected). Freshly isolated WBCs were stained for 20 minutes with pre-labelled neutrophils cell surface markers (CD15/CD66b) at room temperature. Cells were washed with the staining buffer (PBS/Serum/BSA) and fixed with cold 4% formaldehyde on ice for 20 minutes. Cells were permeabilised in 0.25% tween/PBS for 20 minutes on ice, washed twice with the staining buffer and incubated with antibody against Serpin B1(1:200) o/n. The cells were then stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) for 45 minutes in the dark to detect Serpin B1 and analysed using flow cytometer (BD Accuri C6). For a negative control, cells were stained with an isotype control antibody. **A** Gating of the CD15+CD66b+SerpinB1+ neutrophils. **B** Peaks representing fluorescent intensity of the Serpin B1 expression in BRCA, HD and background isotype control **C** Quantification of the levels of Serpin B1 expression in the neutrophils of HD and BRCA according to the Mean fluorescent intensity (MFI) of the samples and control.

3.2.6 Analysis of the expression of the Serpin B1 using ELISA

3.2.6.1 Analysis of the expression of the Serpin B1 in healthy donors and cancer patients using ELISA

In the final step of the validation experiment, the levels of Serpin B1 were analysed in the white blood cells and plasma of two cohorts of breast cancer patients (primary and advanced). This was achieved using a quantitative sandwich enzyme immunoassay technique (ELISA) commercial kit. The sandwich ELISA is the immunological assay, designed to detect and quantify the presence of the specific antigen or antibody in the given sample. The technique represents the basic tool for the laboratory diagnosis in hospitals. Given that Serpin B1 is a promising marker of breast cancer prognosis, the use of the ELISA kit in medical practice to quantitatively evaluate Serpin B1 levels in the blood of cancer patients would be of particular interest.

White blood cell pellets ($10x10^6$) obtained from the blood of breast cancer patients and healthy donors using a fractionation method as described in section 2.1. The resulting cell pellet was lysed by the addition of equal volume of Buffer 1 and Buffer 2 (Table 2.1) with the volume ratio of $1mL/4x10^6$ and incubated on ice for 20 minutes. The cell lysate was centrifuged at 13000 rpm and at 4°C for 15 minutes. The pellet was discarded and the supernatant (cell lysate) was used for the experiment.

The aim was to analyse the expression pattern and concentration of Serpin B1 in plasma and WBC of healthy donors and two cohorts of breast cancer patients. To calculate the concentration of Serpin B1 in plasma and white blood cells of breast cancer patients (primary and advanced) a standard curve method was used. The results demonstrate a significant increase of the Serpin B1 levels in white blood cells from primary and advanced breast cancer patients in comparison with healthy individuals (Figure 3.19). Furthermore,

the white blood cell levels of the analysed protein correlate with their respective serum levels (Figure 3.20). Upon measurement of Serpin B1 levels, it was observed that its concentration in WBCs of healthy individuals ranges between 1.12ng/mL and 10ng/mL. In primary breast cancer patients, it was detected to be greater, between 3.44ng/mL and 20ng/mL, and in advanced cancer patients even greater, between 5ng/mL and 27.24ng/mL.

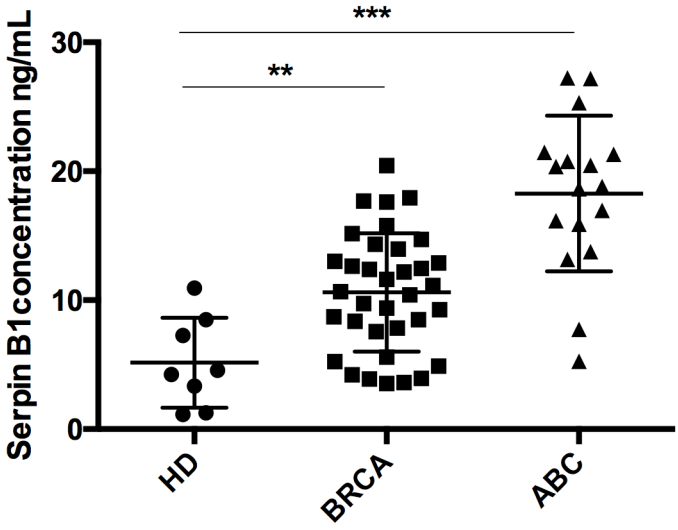


Figure 3.19 Analysis of the concentration of Serpin B1 levels in WBCs of breast cancer patients using ELISA. WBCs and plasma were isolated from blood of cancer patients and healthy donors. Cell lysates were prepared from $1x10^7$ cells. To calculate the concentration of Serpin B1 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients, ABC- advanced breast cancer patients. The concentration of Serpin B1 in WBC of breast cancer patients differ significantly from that of healthy donors. Data analysed by Kruskal-Wallis test. *P<0.05; **P<0.01; ***P<0.001.

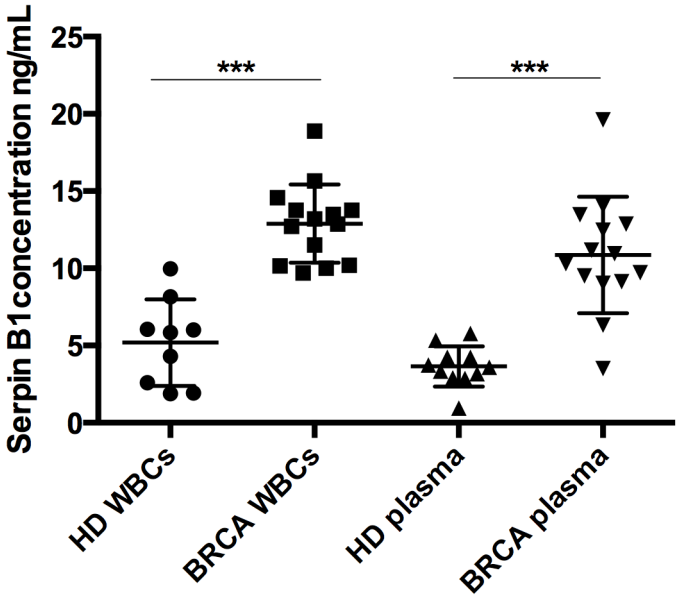


Figure 3.20 Concentration of serum and WBCs levels of Serpin B1 using quantitative sandwich ELISA commercial kit. WBCs and plasma were isolated from blood of cancer patients and healthy individuals. Each dot represents an individual patient**.** Cell lysates were prepared from 1x10^7 cells. To calculate the concentration of Serpin B1 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients, Concentration of Serpin B1 in WBCs correlates with its corresponding serum concentration. *P<0.05; **P<0.01; ***P<0.001

3.2.6.2 Analysis of the Serpin B1 levels in a cohort of patients with advanced breast cancer in response to treatment using ELISA.

To analyse whether the changes in circulating Serpin B1 concentration could be indicative of the treatment response in advanced breast cancer patients undergoing chemotherapy and endocrine treatment, the levels of the analyte were measured from the serum of 10 patients (6 chemotherapy, 4 endocrine) at three treatment time points; beginning, middle and final stage. The data revealed different patterns and were grouped according to the clinical information (Figure 3.21). The data indicates that changes in circulating levels of Serpin B1 do not correlate with treatment response.

To see if trends in Serpin B1 concentration correlate with patient survival from advanced cancer, the results were grouped according to the length of survival (Figure 3.22. The data showed no correlation.

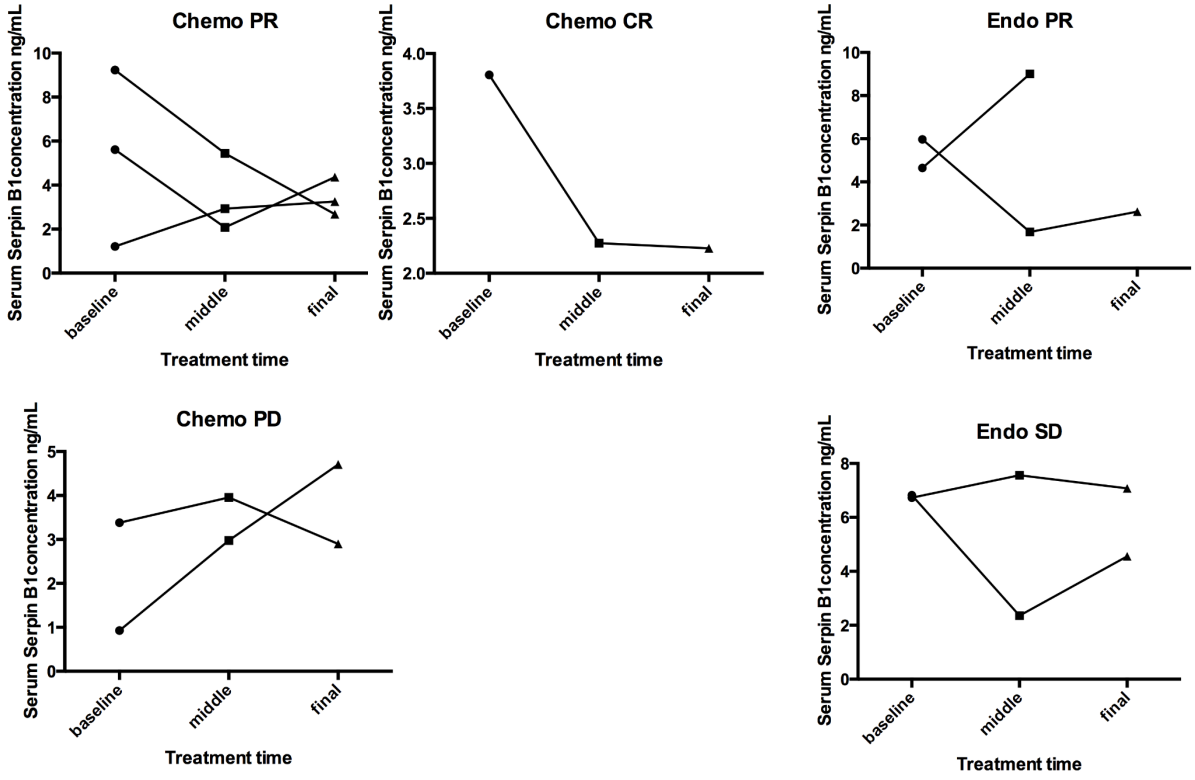


Figure 3.21 Analysis of the correlation between circulating levels of Serpin B1 in serum of advanced breast cancer patients and treatment response using sandwich ELISA. Serum was separated from blood of advanced breast cancer patients prior the first (baseline), second (middle) and final third stage of the treatments. To calculate the concentration of Serpin B1 in plasma a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. **PD** progressive disease; **PR** partial response; **SD** stable disease; **CR** complete response. **Chemo** chemotherapy treatment. **Endo** endocrine treatment.

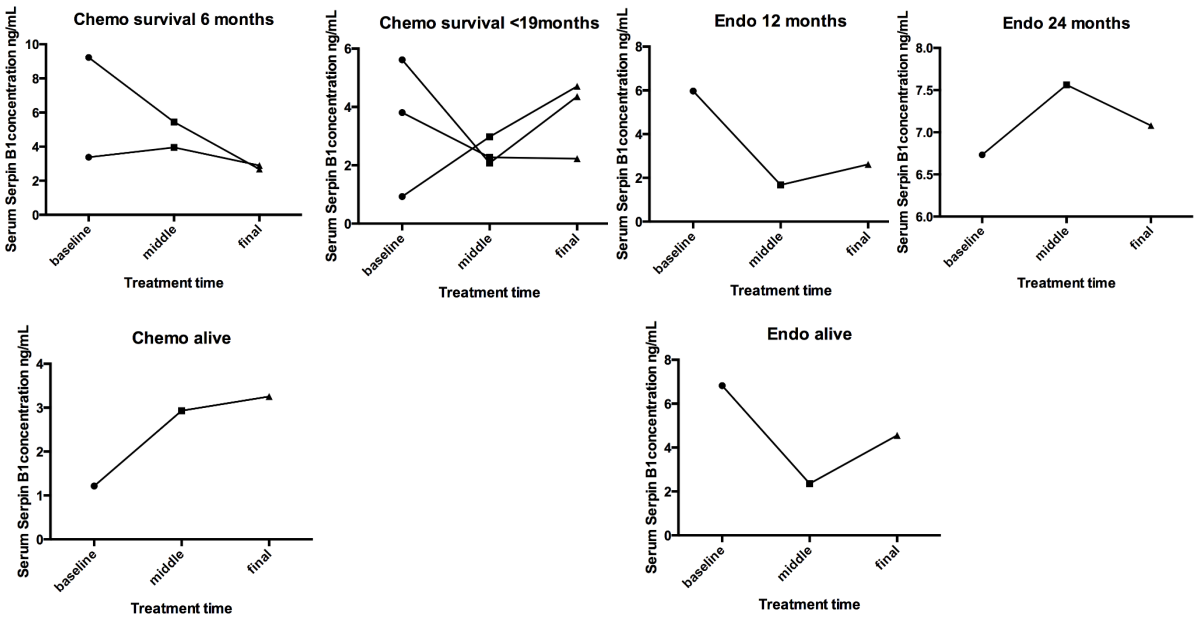


Figure 3.22 Analysis of the correlation between circulating levels of Serpin B1 in serum of advanced breast cancer patients and survival time using sandwich ELISA. Serum was separated from blood of advanced breast cancer patients prior the first (baseline), second (middle) and final third stage of the treatments. To calculate the concentration of Serpin B1 in plasma a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. **Chemo** chemotherapy. **Endo** endocrine therapy.

3.3 Discussion

Previous studies have demonstrated that the protein expression profile of WBCs from cancer patients differ from those in healthy donors (Aarøe et al. 2010; Sotiriou & Pusztai 2009). In our laboratory, several differentially expressed proteins were identified in WBCs of cancer patients. In the presented study, we aimed to analyse the expression pattern of five of the identified targets. The aim of this part of the study was to validate heightened expression of *Serpin B1* on protein level.

The levels of expression of previously identified protein Serpin B1 were analysed in WBCs of different groups of breast cancer patients: primary breast cancer patients and patients with advanced (metastatic) breast cancer; WBCs from healthy donors were used as control. We observed that the levels of Serpin B1 increases as the disease advances. The obtained data revealed that Serpin B1 levels differ significantly between two cohorts of breast cancer (primary and advanced) and healthy donor group. Furthermore, the higher levels of Serpin B1 observed in some of healthy donors correlated with familial history of breast and prostate cancer. This data suggests that Serpin B1 has features of prognostic marker for disease progression. Measurement of its levels in WBCs of primary breast cancer may be useful to distinguish between patients with node negative and node positive disease. Furthermore, as Serpin B1 levels differ significantly between primary and advanced cohorts, the high levels of the protein in samples from primary cohort may help to identify patients that have risk of development of the invasive disease.

Next, we examined whether Serpin B1 can serve as a predictive marker for the treatment response. In order to achieve this, the expression levels of the target protein were measured by western blot in the group of patients with advanced (metastatic) disease.

The obtained data revealed the most favourable pattern in protein expression in correlation with survival.

The results from Western blot were supported by immunofluorescent staining, where higher degree of staining was observed in white blood cells of advanced and node positive primary breast cancer patients in comparison with node negative primary breast cancer and healthy donor's samples. Moreover, distinct distribution of Serpin B1 protein in WBCs of patients in association with different stages of the disease was detected. In leukocytes of node negative and node positive breast cancer patients Serpin B1 was appeared to be present in vesicles at the cell surface. This observation was the interesting one as it was previously reported that Serpin B1 is cytoplasmic proteins characterised by the absence of N-terminal signal peptide that is required for the secretion pathway and therefore reside intracellularly (Pemberton et al. 1997). Since the main known role for Serpin B1 appears to protect granulocytes by the inhibition of their own granule proteases a possible explanation of the overexpression of Serpin B1 in leukocytes of breast cancer patients would be the protection of these cells (Zhao et al. 2014 & Benarafa et al. 2011).

The nuclei localisation of SerpinB1 observed in the present study is also reported in other studies. For example, Farley and co-workers showed that in resting neutrophils Serpin B1 localises in the cytoplasm and translocates into the nucleous during NETosis (neutrophil extracellular trap generation). These structures are sought to be released by dying neutrophils in order to trap and kill pathogens and reported be negatively regulated by Serpin B1 (Farley et al. 2012; Remijsen et al. 2011).

Measurement of the levels of Serpin B1 in WBCs of the patients with advanced disease undergoing chemotherapy and endocrine treatment revealed that lower levels of the protein in the beginning of the both treatments correlate with complete response to the

treatment. Furthermore, lower initial levels of the protein were shown to associate with longer survival of the group of patients received chemotherapy treatment. This trend was not observed for the patients received endocrine treatment. Kaplan-Meier test for combined chemotherapy and endocrine patients did not show the significant difference between lower/higher levels of Serpin B1 and overall survival of the patients. Interestingly, the lower initial Serpin B1 levels showed association with complete response to treatment in both chemotherapy and endocrine treatment groups of patients.

Consistent with the above results, the flow cytometric analysis of the Serpin B1 expression in randomly selected sample from primary breast cancer patient shows elevated levels of the protein and supports the overall validation data generated in this study. However, these results are based on one sample analysed, therefore the panel needs to be expanded.

Finally, analysis of the circulating levels of Serpin B1 expression using ELISA revealed heightened patterns of the protein expression in WBCs of both (primary and advanced) cohorts of breast cancer patients. Furthermore, the serum Serpin B1 levels showed the same trend as previously observed WBCs one. In serum of primary breast cancer patients significantly higher concentration of Serpin B1 was detected.

Serpin B1 primarily acts as intracellular protein as it lacks the hydrophobic signal peptide, the typical feature of the secretory proteins (Pemberton et al. 1997). Here consistent with the IF results its extracellular localisation was detected using ELISA. The high levels of Serpin B1were also reported in serum of the patients with type 2 diabetes (Takebayashi et al. 2016).

Taken together these results demonstrate the potential of Serpin B1 as a biomarker for breast cancer diagnosis and prognosis.

Chapter 4

4.1 Lipocalin 2

Lipocalin 2, also referred to as neutrophil gelatines associated lipocalin (NGAL) belongs to the family of small proteins characterised by their ability to bind and transport lipophilic molecules such as retinols, steroid and iron (Xiao et al. 2017). Recently, several new properties attributed to lipocalins have been reported, such as prostaglandin synthesis, protease inhibition and modulation of cell growth. The expression of NGAL is induced by cytokines and found to be upregulated under inflammatory conditions (Bauer et al. 2008).

Heightened expression of Lipocalin 2 has been reported in many epithelial cancers including breast carcinomas. For example, Lipocalin 2 was found to be up-regulated in plasma in a mouse model of breast cancer (Pitteri et al. 2008). In human studies, Bauer et al (2008) examined localization of NGAL in tumours of 208 primary breast cancer patients using immunostaining and correlated the results with clinical outcome. The researchers detected a strong correlation between cytoplasmic localisation of NGAL and HER2 overexpression, lymph node metastasis and decreased disease-free survival (Bauer et al. 2008). More information about Lipocalin 2 is given in the section 1.2.4.2.

Objectives of the present chapter:

Our previous proteomic studies reported heightened expression of Lipocalin 2 in WBCs of breast cancer patients. We hypothesised that Lipocalin 2 expression in WBCs will correlate with clinical outcomes (e.g. survival and therapy response). This chapter comprises of two major aims. The first one is to validate the heightened expression pattern of Lipocalin 2 in WBCs from breast cancer patients in comparison with healthy individuals. The second aim is to study the changes in Lipocalin 2 levels in a cohort of advanced breast cancer patients undergoing treatment in order to evaluate the prognostic

and predictive biomarker value of Lipocalin 2. To achieve this, target protein levels were assessed in plasma and WBCs of breast cancer patients using western blot, Immunofluorescence, flow cytometry and ELISA techniques.

4.2 Results

4.2.1 Western blot analysis of Lipocalin 2 expression in WBCs obtained from blood of healthy donors and breast cancer patients

Proteomic analysis revealed elevated levels of Lipocalin 2 in WBCs of individuals diagnosed with breast cancer (Many, J., PhD thesis, 2015). In order to further verify this data, protein levels of Lipocalin 2 were measured by western blot in 13 healthy donors, 11 primary and 47 patients with advanced breast cancer. In agreement with the expected size, a band of 25 kDa was observed (Figure 4.1) Densitometry analysis was then performed using the Image J software and the Lipocalin 2 signal was normalised to β Actin. Expression levels between the gels was normalised to the expression value of the common healthy donor 2 (HD2) sample used in all experiments (Figure 4.2). The results revealed significant overexpression of Lipocalin 2 protein levels in WBCs of both primary and advanced breast cancer patients in comparison with healthy donors.

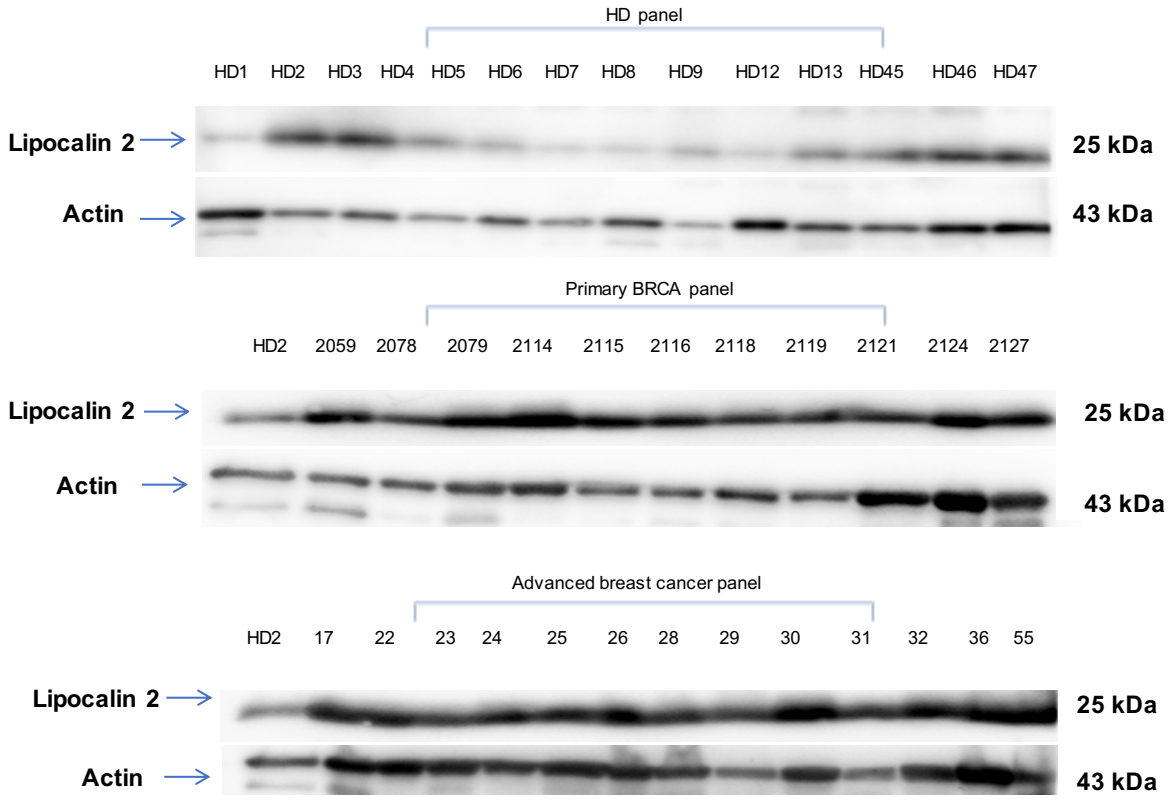


Figure 4.1 Representative western blots of Lipocalin 2 levels in breast cancer patients with different disease conditions. WBCs were isolated from blood of cancer patients. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin 2 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin 2 bands over the intensity of the corresponding β-actin bands were determined as a control healthy donor 2 was used in all the western blots and the results plotted on a graph using GraphPad Prism 6 software.

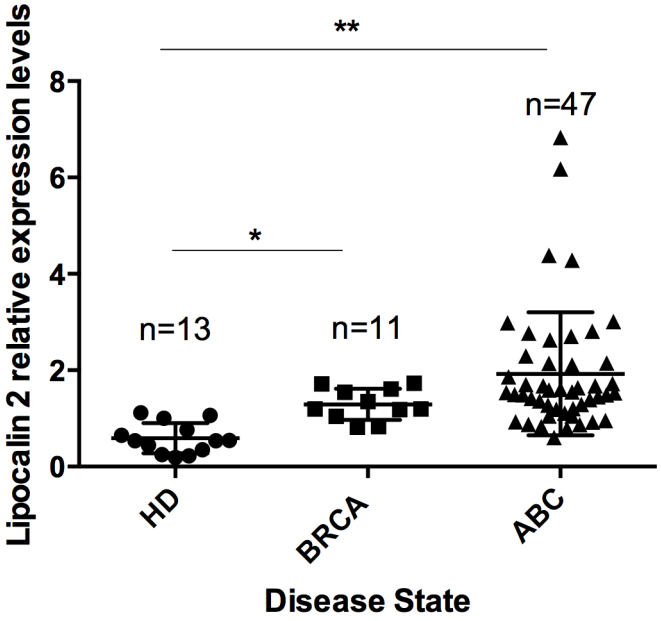


Figure 4.2 Lipocalin 2 protein levels in breast cancer patients with different disease states. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with the anti-Lipocalin 2 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Band corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed (Image J) and the ratios of the intensity of the Lipocalin 2 bands over the intensity of the corresponding β-actin bands were determined as a control healthy donor 2 was used in all the western blots HD- healthy donors, BrCa- primary breast cancer, ABC- breast cancer patients with advanced. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test. *P<0.05, ** P<0.01. The difference between primary and advanced cohorts are not significant

4.2.2 Western blot analysis of Lipocalin 2 expression in WBCs of patients with advanced breast cancer.

4.2.2.1 Western blot analysis of Lipocalin 2 expression in WBC from breast cancer patients with advanced disease during treatment in correlation with clinical response.

Advanced/metastatic breast cancer is when the disease has spread beyond the breast to other organs in the body. Biomarkers that predict response to therapy may eliminate unnecessary treatment or could be used to guide therapeutic strategies. Currently the assessment of response to therapy is based upon both subjective and objective measures. Objective assessment is evaluated on the basis of measurement of the tumour size before and after treatment. However, the response monitoring is recommended after 2-3 cycles of chemotherapy or several months of endocrine therapy. As the aim of the treatment for advanced breast cancer patients is to provide prolongation of good quality of life. The subjective response to treatment is based on estimation of the balance of benefit and side-effects of the therapy given. The currently available serum markers CA15-3 and CEA have their limitations. For example, these biomarkers do not reliably show an early response to treatment (Kabel 2017).

In this part of the project the level of Lipocalin 2 was assessed in a cohort of patients with advanced breast cancer undergoing chemotherapy and endocrine treatment. These analyses are important to determine whether Lipocalin 2 could be a potential prognostic biomarker to predict treatment response. In order to achieve this, western blotting was performed to evaluate the expression levels of Lipocalin 2 were measured in WBCs taken from patients at the beginning, middle and end of treatment. The ratios of the intensity of

the Lipocalin 2 bands over the intensity of the corresponding β-Actin bands were determined using densitometry, as a control healthy donor 2 was used in all the westerns. These trends were then correlated with data for the patients' treatment response, which was divided into four categories: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD).

Lipocalin 2 levels, in response to chemotherapy, declined in stable disease, although these results are not reliable as only one sample was analysed within this category (Figure 4.3). The rest of the response categories showed mixed trends of the changes in protein expression. The results obtained for patients with endocrine treatment indicate that the levels of Lipocalin 2 decline in the response to treatment in all four categories of the patient, with the exception for one patient with stable disease which shows a slight increase in Lipocalin 2 levels in the middle of the treatment (Figure 4.4).

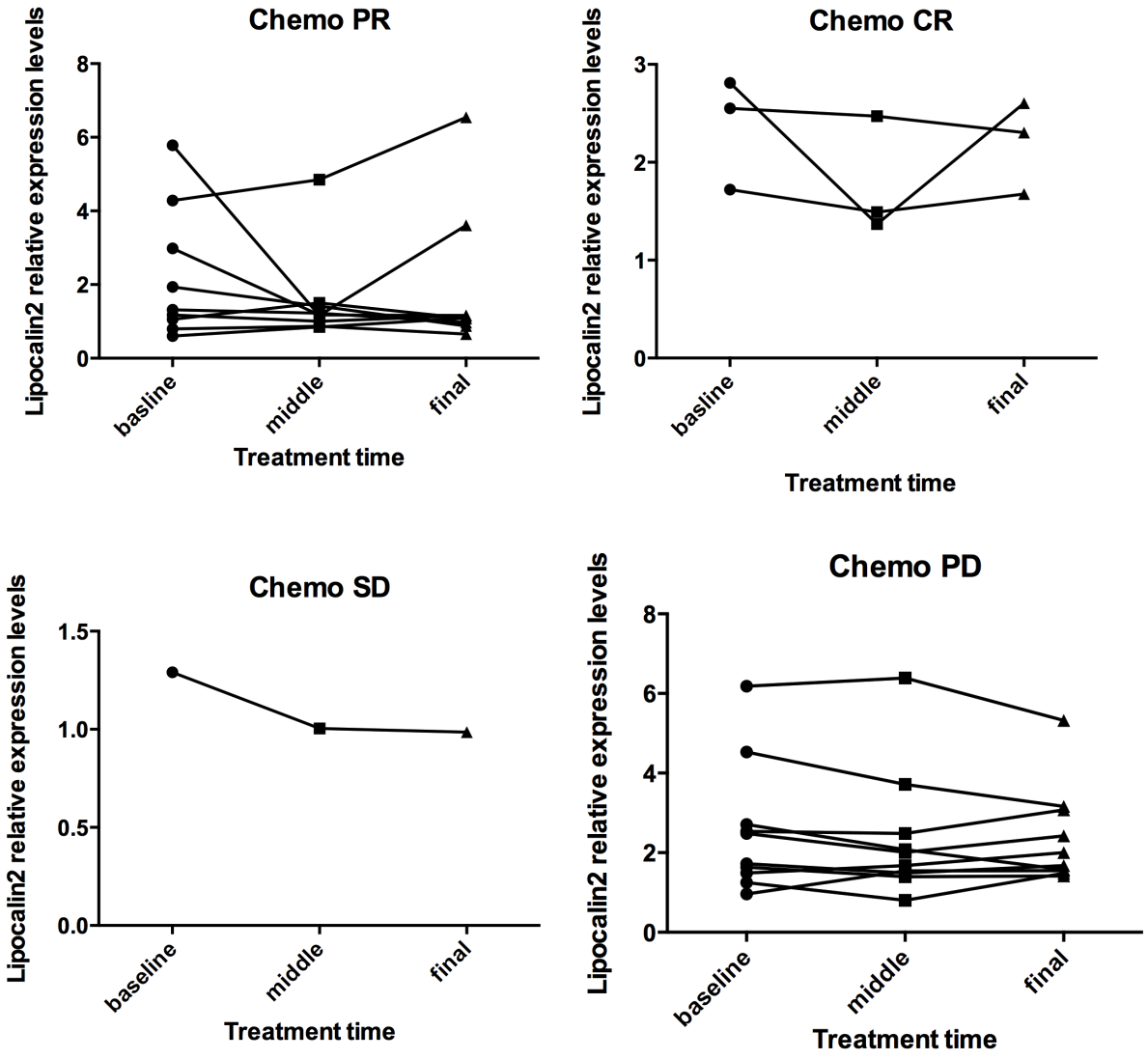


Figure 4.3 Western blot analysis of the correlation between changes in the levels of Lipocalin2 during the course of chemotherapy treatment and clinical response. Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). WBC were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

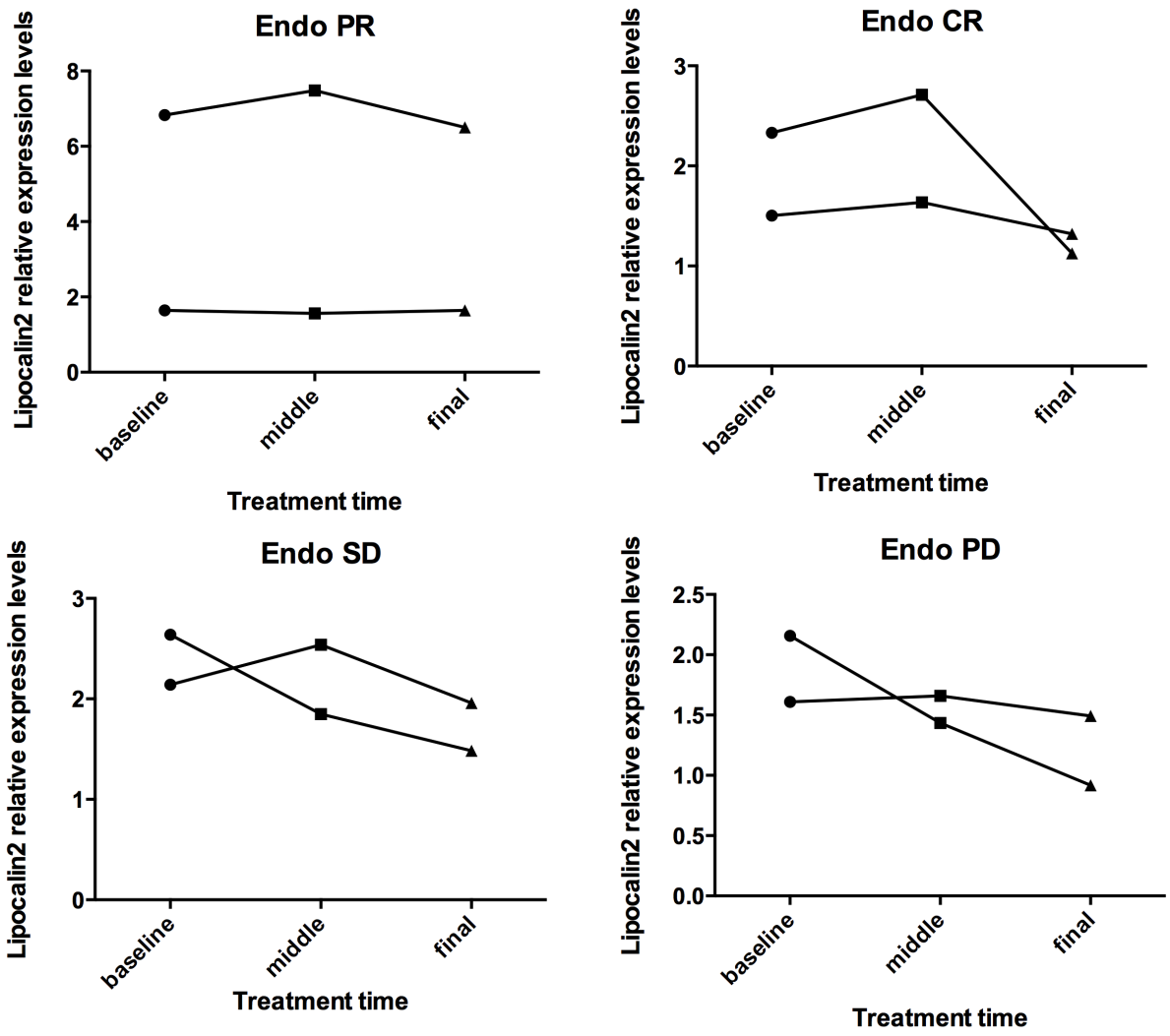


Figure 4.4 Western blot analysis of the correlation between changes in the levels of Lipocalin 2 during the course of endocrine treatment and clinical response. Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). WBC were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined using densitometry, as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

4.2.2.2 Western Blot analysis of the basal levels of Lipocalin 2 in WBCs of advanced breast cancer patients in correlation with response to treatment.

Next, assessed the basal levels of the protein of interest was assessed at the beginning of treatment in correlation with corresponding clinical data for the treatment response. The aim of this experiment was to assess if Lipocalin 2 could be used as a biomarker to predict response to treatment. According to the obtained results lower initial levels of Lipocalin 2 associate with stable disease in the cohort of chemotherapy patients (Figure 4.5). Although, Kruskal-Wallis test of the data showed that the p value is not significant, the median of the patients with stable disease was lower than of the patients with partial response, complete response and progressive disease.

The results for patients with endocrine treatment showed a similar trend, with lower initial levels of Lipocalin 2 correlating with stable disease (Figure 4.6). Statistical test showed that the p value was not significant, but the median for stable disease cohort was lower than for partial response, progressive disease and complete response.

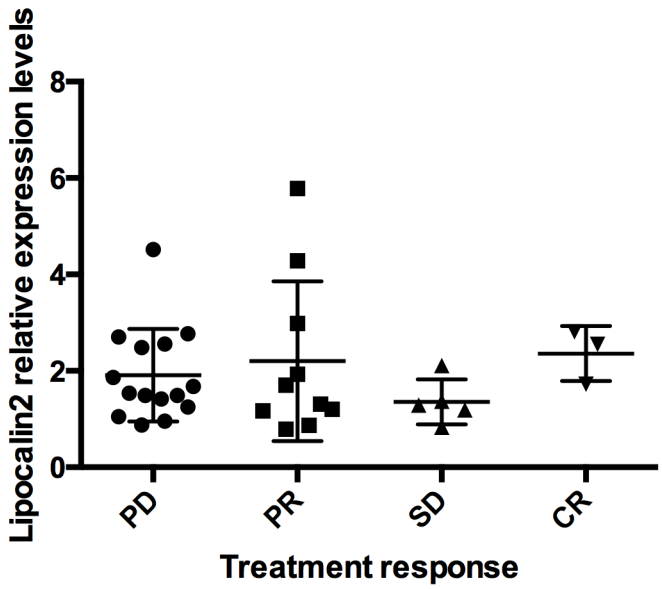


Figure 4.5 Analysis of the correlation between initial levels of Lipocalin 2 before treatment and response to chemotherapy in advanced breast cancer patients by western blot. PD progressive disease; PR partial response; SD stable disease; CR complete response. Leukocytes were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

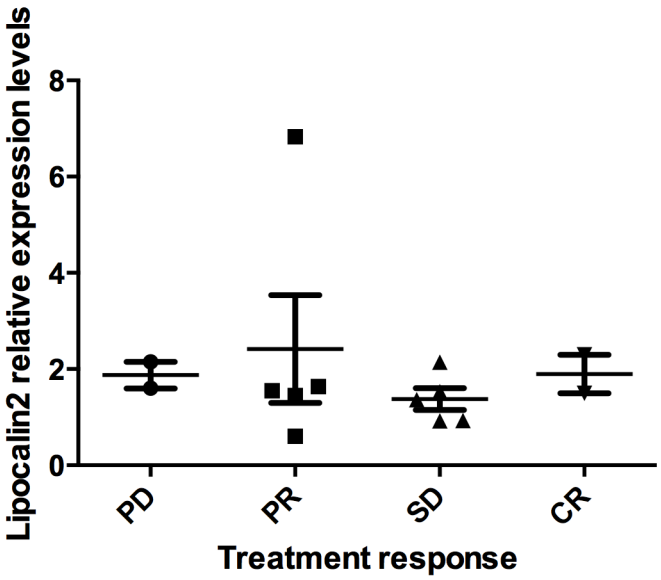


Figure 4.6 Analysis of the correlation between initial levels of Lipocalin 2 before treatment in correlation with response to endocrine treatment in advanced breast cancer patients by western blot. Leukocytes were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

4.2.2.3 Western blot analysis of the changes in the Lipocalin 2 expression levels in WBCs from breast cancer patients with metastatic disease during chemotherapy treatment in correlation with survival.

Next, we analysed whether Lipocalin 2 can serve as a marker for the response to treatment in patients with advanced disease. In order to achieve this, Lipocalin 2 expression was assessed in WBC of breast cancer patients with metastatic disease during chemotherapy treatment. Patients with invasive breast cancer had undergone six rounds of chemotherapy treatment. The level of Lipocalin 2 in WBC was evaluated in WBC obtained from 18 breast cancer patients prior the first (baseline), third (middle) and sixth (final) rounds of treatment using western blot technique. Upon evaluation, four different patterns of Lipocalin 2 levels in the response to chemotherapy treatment were observed and results were divided into four groups of response (Figure 4.7).

In patient group 1, Lipocalin 2 levels gradually decreased and survival times ranged from 3 to 15 months. In group 2 Lipocalin 2 levels increased in the middle of the treatment and declined at the end; survival times ranged from 4 to 24 months. Group 3 represents the majority of the patients treated with chemotherapy. In this group, Lipocalin2 levels declined in the middle of the treatment and increased in the end; survival time ranged from 4 to 48 months. In group 4 Lipocalin 2 levels increased during the course of treatment. The group comprised of 2 patients and correlates with more favourable prognosis for the patients: one with 48 months survival time and alive patient

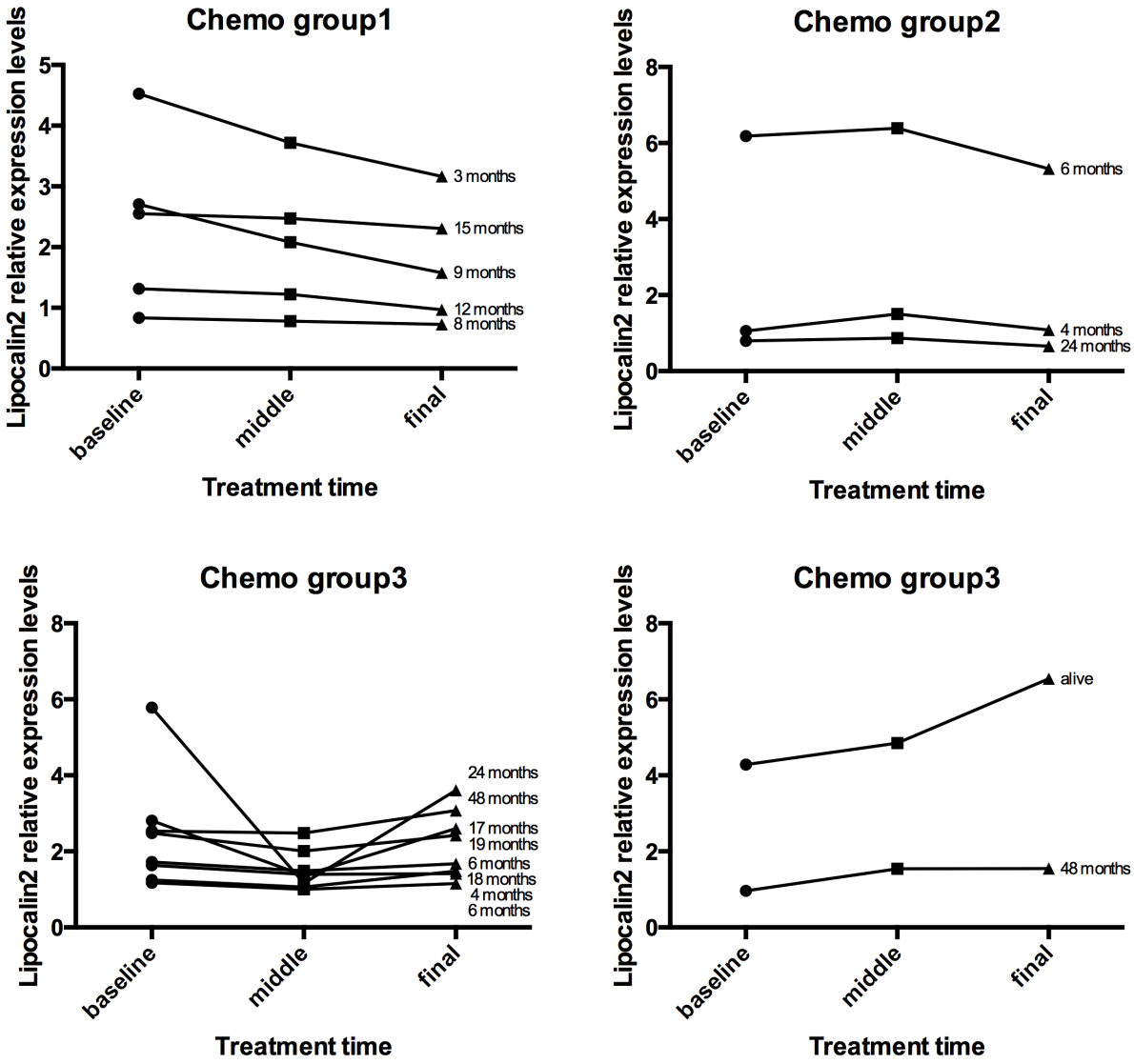


Figure 4.7 Western blot analysis of the correlation between changes in the levels of Lipocalin2 during the chemotherapy treatment and survival of the patients. WBC were extracted from blood of breast cancer patients prior the treatment-baseline, in the middle and at the final stage; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software.

4.2.2.4 Western Blot analysis of the basal levels of Lipocalin 2 in WBCs of advanced breast cancer patients in correlation with survival.

The aim of the next experiment was to analyse whether the initial levels of Lipocalin 2 in WBCs of the advanced breast cancer patients before chemotherapy and endocrine treatment have any correlation with overall survival. For this purpose, the levels of Lipocalin 2 were assessed by Western Blot analysis using hand cast gels. The obtained data showed that the levels of protein in the beginning of the treatment do not correlate with the survival of the patients and therefore Lipocalin2 cannot be used as a marker of survival (Figure 4.8, 4.9).

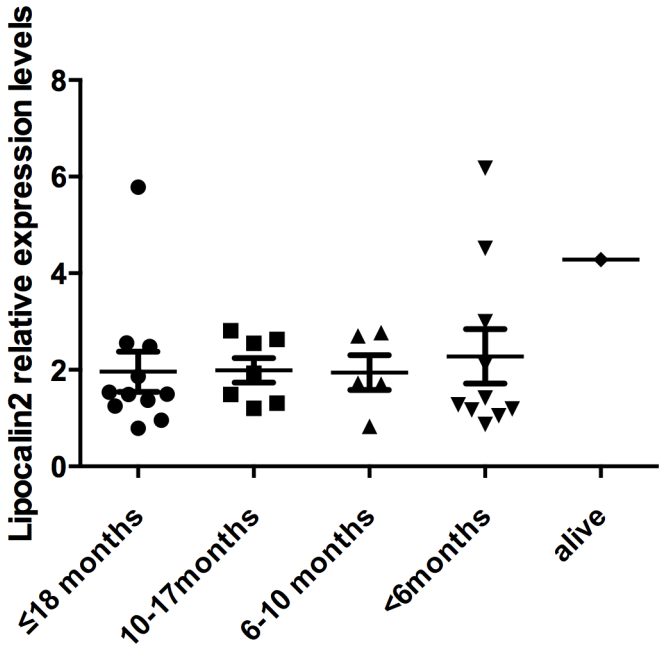


Figure 4.8 Analysis of the correlation between initial levels of Lipocalin2 before treatment and response to chemotherapy in advanced breast cancer patients by western blot. Leukocytes were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

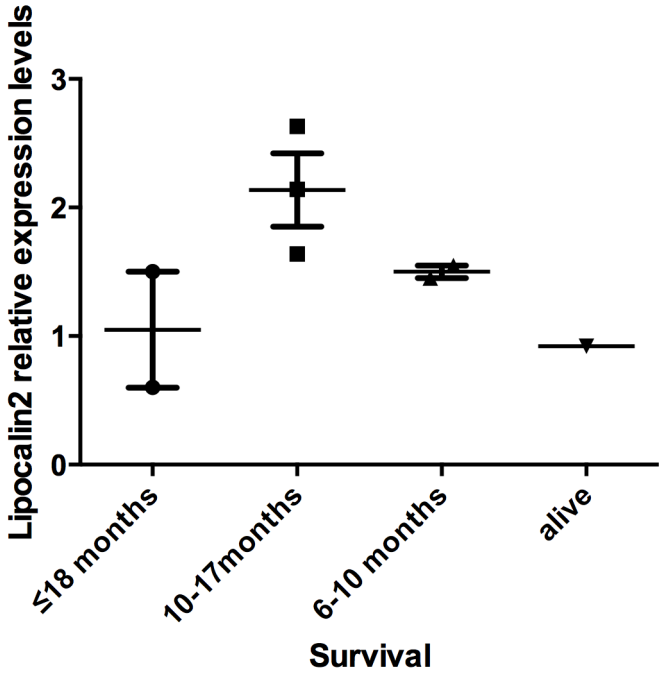


Figure 4.9 Analysis of the correlation between initial levels of Lipocalin2 before treatment and response to endocrine treatment in advanced breast cancer patients by western blot. Leukocytes were extracted from blood of breast cancer patients; cell lysates were prepared from 2x10^6 cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test.

4.2.2.5 Kaplan-Meier survival analysis

Next, we carried out Kaplan-Meier analysis to study the correlation between high and low levels of Lipocalin 2 expression and patient's overall survival (Figure 4.10). The baseline protein levels of Lipocalin 2 were measured by Western Blot in 47 advanced breast cancer patients. The Lipocalin 2 signal was normalised to the β Actin signal using Image J software. Expression values between the gels were compared by normalising to the expression value of the common healthy donor 2 sample used in all experiments. The cut-off point was determined by average of all the values, as 2. The generated data shows no significant difference between these parameters ($p=0.7$).

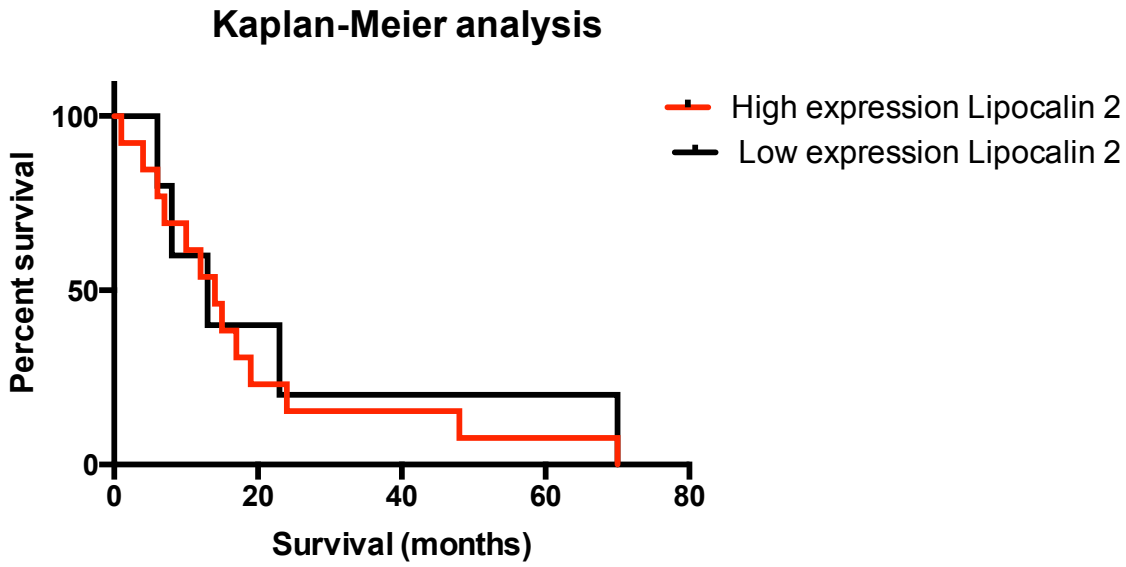


Figure 4.10 Survival curves according to Lipocalin 2 expression. Kaplan-Meier survival curve for high Lipocalin 2 expression versus low expression did not show significant difference. The cut-off point was determined by average of all the values, as 2 WBCs were extracted from blood of advanced breast cancer patients; cell lysates were prepared from $2x10^6$ cells. Samples were separated using SDS-PAGE, blotted and probed with anti-Lipocalin 2 antibody. The same membrane was then re-probed with anti-β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyser. The ratios of the intensity of the Lipocalin 2 bands over the intensity of the corresponding β-Actin bands were determined as a control healthy donor 2 was used in all the westerns and the results plotted on a graph using GraphPad Prism 5 software

4.2.3 Immunofluorescent analysis

4.2.3.1 Distribution of Lipocalin 2 in WBCs of breast cancer patients

Next, the distribution of Lipocalin2 protein in WBC of breast cancer patients and healthy donors was examined by immunofluorescent staining using primary anti-Lipocalin 2 antibody (Figure 4.10 A). Examination of slides revealed different distribution of Lipocalin 2. In the healthy donor samples Serpin B1 appeared to be present in the cytoplasm of the cells. WBCs samples from primary breast cancer patients were characterised by higher intensity of the nuclei and cytoplasmic staining. In leukocytes of patients with advanced breast cancer Lipocalin 2 protein was detected in the cytoplasm of the cells with high intensity. In the samples obtained from healthy individuals the intensity of the staining appears to be lower. These results support the data obtained from Western Blot analysis. The proportion of the distribution of the Lipocalin 2 signal among the samples was measured using Image J software. The results show, that that the distribution of the signal corresponding to the Lipocalin 2 protein is significantly higher in the WBCs samples from primary and advanced breast cancer patients in comparison to healthy donors.

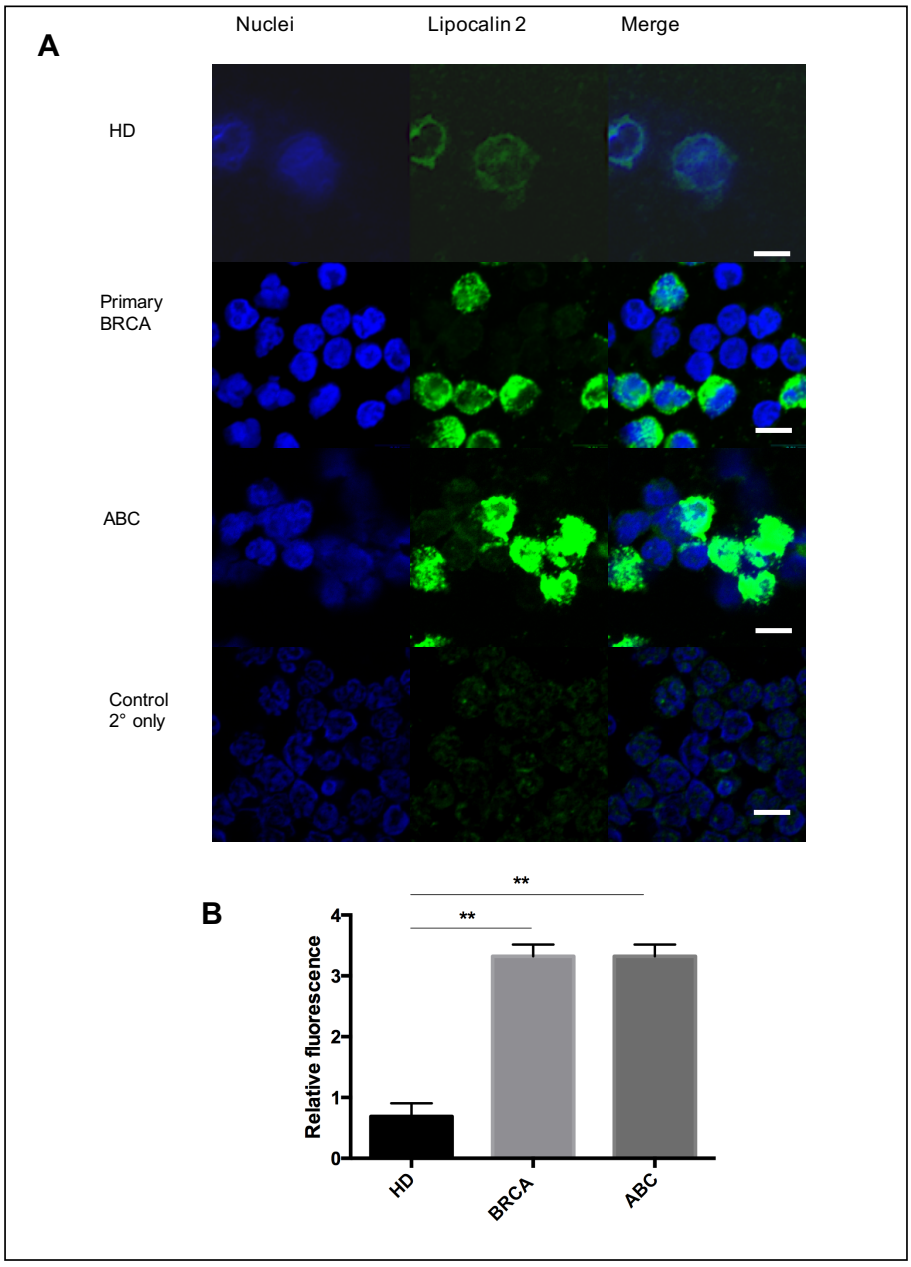


Figure 4.10 Immunofluorescent analysis of Lipocalin 2 protein distribution in WBC of BRCA node negative, node positive, advanced cancer patients and healthy donors. A WBC from breast cancer patients and healthy donors were stained with FITC-labelled anti-rabbit secondary IgG antibody (dilution 1:400) against Lipocalin2 primary antibody (dilution 1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining in the cytoplasm corresponds to Lipocalin2 protein. Scale bar 0.1mm. All images were taken by Confocal Scanning Microscope (BioRad Hercules) at 60X magnification. **B** Bar graph represents the proportion of signal distribution among samples measured using ImageJ software. ** P<0.01

4.2.3.2 Lipocalin 2 expression in immune cells

In order to further understand exactly which immune cells overexpress Lipocalin 2, cells were co-stained with antibodies for Lipocalin 2 and immune cell specific markers. For this purpose, WBCs from breast cancer patients were double stained against the cluster of differentiation (CD) cell surface markers to distinguish between subpopulation of white blood cells and the protein of interest.

First leukocyte fractions were stained with CD15 antibody, which recognises a carbohydrate epitope expressed in mature neutrophils and eosinophils. The obtained double immunofluorescence analysis revealed that the majority of Lipocalin 2 positive cells were neutrophils (Figure 4.11). The results show, that the cells negative for CD15 were also negative for Lipocalin 2. The secondary antibody control did not show any nonspecific background staining.

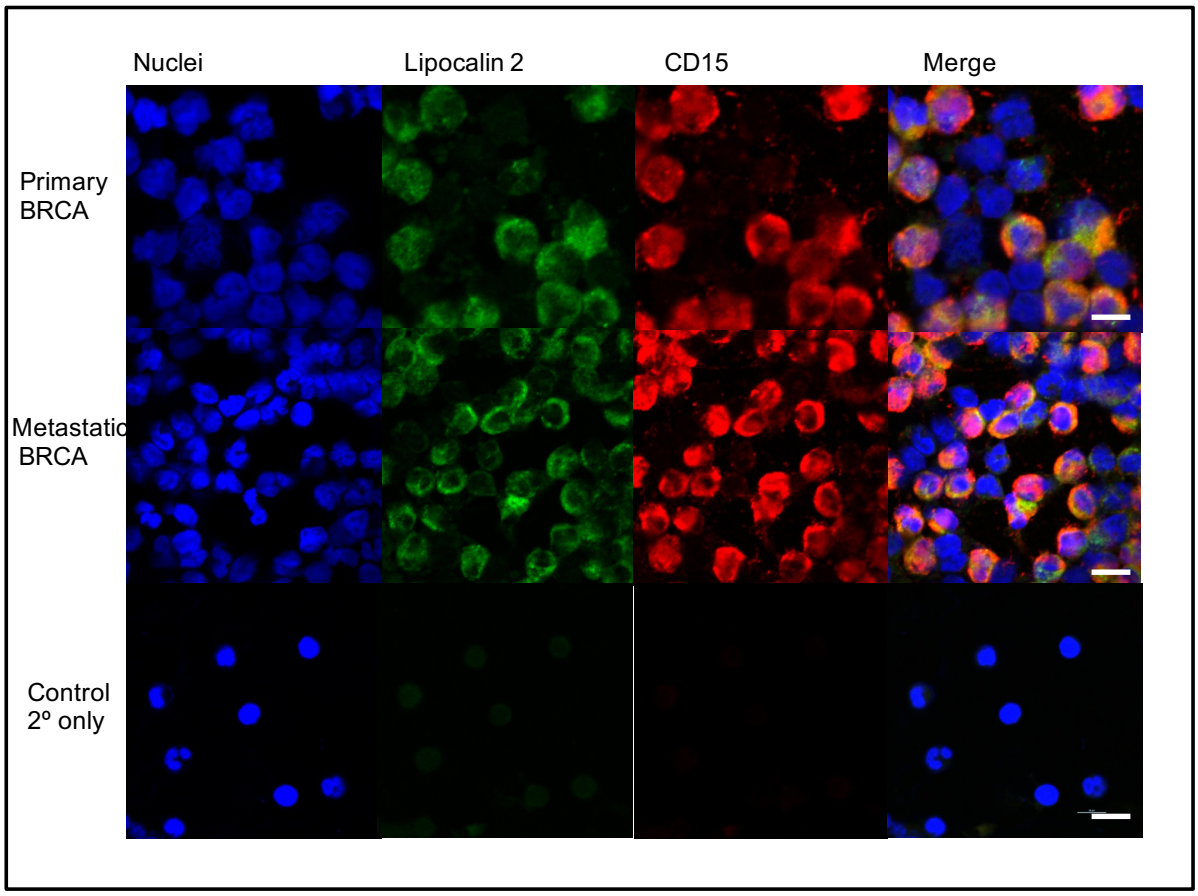


Figure 4.11 Immunofluorescence analysis of Lipocalin2 protein distribution in leukocytes of primary and advanced breast cancer patients. WBC extracted from blood of primary and advanced breast cancer patients were double stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) against Lipocalin2 primary antibody (dilution 1:200) and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD15 neutrophil primary antibody (1:200), plus secondary only as the control. The blue staining (DAPI) shows the position of the nucleus. The green staining corresponds to Lipocalin2 protein and red staining represents Neutrophils. Scale bar 0.01mm. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

4.2.4 Evaluation of the levels of Lipocalin 2 in neutrophils of primary breast cancer patients using flow cytometric analysis.

Previously we reported elevated levels of Lipocalin 2 in neutrophils of breast cancer patients in comparison with healthy individuals. Here we aimed to further validate the increased expression of the above protein using flow cytometry (FACS). Flow cytometry is the method that allows for rapid quantitative assessment of proteins within the specific cell type via cell surface marker phenotyping.

Because granulocytes rapidly degenerate soon after collection, WBCs were isolated from the blood sample within 6 hours from the blood draining and immediately used for the experiment (Chue & Neagu 2014). For this experiment, freshly isolated WBCs randomly selected from a primary breast cancer patient and a healthy donor were stained with two pre-labelled markers expressed by mature neutrophils: CD15, CD66b. The combination of these two markers is commonly used to identify the mature neutrophils. Lipocalin 2 is intracellular protein therefore pre-stained cells were fixed, permeabilised and stained with unlabelled polyclonal rabbit anti-Lipocalin 2 antibody followed by the incubation with FITC-labelled anti-rabbit secondary antibody to detect Lipocalin 2.

For a negative control, cells were stained with an isotype control antibody. Isotype controls are antibodies of the same isotype as the target primary antibody. They are of unknown specificity or are raised against antigens known to be absent in target cells. Isotype controls are used to estimate non-specific staining of primary antibodies.

The results obtained for Mean Fluorescent Intensity (MFI) show that the levels of Lipocalin 2 in WBCs of randomly selected primary breast cancer patient is two-fold higher than in healthy donor (Figure 4.12).

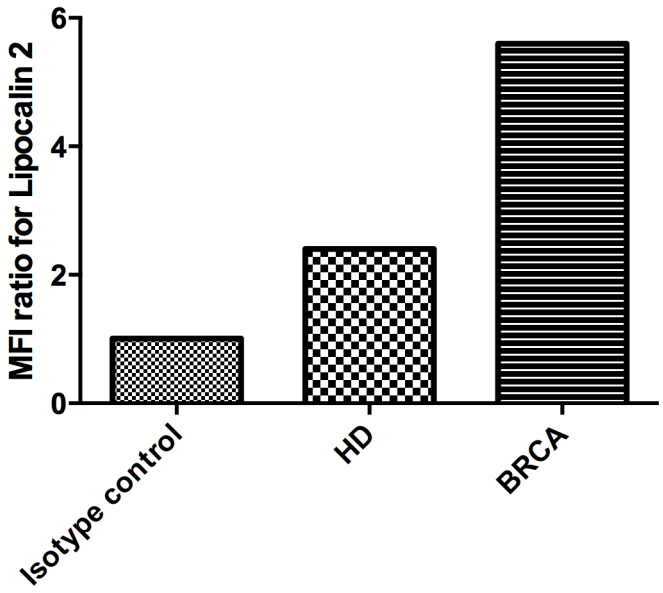


Figure 4.12: Flow cytometric analysis of the Lipocalin 2 levels in neutrophils of breast cancer patient and a healthy donor (randomly selected). Quantification of the levels of Lipocalin 2 expression in the neutrophils of HD and BRCA according to the Mean Fluorescent Intensity (MFI) of the samples and control. Freshly isolated WBCs were stained for 20 minutes with pre-labelled neutrophils cell surface markers (CD15/CD66b) at room temperature. Cells were washed with the staining buffer (PBS/Serum/BSA) and fixed with cold 4% formaldehyde on ice for 20 minutes. Cells were permeabilised in 0.25% tween/PBS for 20 minutes on ice, washed twice with the staining buffer and incubated with antibody against Lipocalin 2 (1:200) o/n. The cells were then stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) for 45 minutes in the dark to detect Lipocalin 2 and analysed using flow cytometer (BD Accuri C6). For a negative control, cells were stained with an isotype control antibody.

4.2.5 Analysis of the expression of the Lipocalin 2 using ELISA

In the final step of the validation experiment, the levels of Lipocalin 2 were analysed in the WBCs and plasma of two cohorts of breast cancer patients (primary and advanced). This was achieved using a quantitative sandwich enzyme immunoassay technique (ELISA) commercial kit. The technique represents the basic tool for the laboratory diagnosis in hospitals. Given that Lipocalin 2 is a promising marker of breast cancer prognosis, the use of the ELISA kit in medical practice to quantitatively evaluate Lipocalin 2 levels in the blood of cancer patients would be of particular interest.

WBC pellets ($1x10^7$) were obtained from the blood of breast cancer patients and healthy donors using a fractionation method as described in section 2.3. The resulting cell pellet was lysed by the addition of equal volume of Buffer 1 and Buffer 2 (Table 2.3.) with the volume ratio of $1mL/4x10^6$ and incubated on ice for 20 minutes. The cell lysate was centrifuged at 13000 rpm and at 4°C for 15 minutes. The pellet was discarded and the supernatant (cell lysate) was used for the experiment.

The aim was to analyse the expression pattern and concentration of Lipocalin 2 in plasma and WBC of healthy donors and two cohorts of breast cancer patients. To calculate the concentration of Lipocalin 2 in plasma and white blood cells of breast cancer patients (primary and advanced) a standard curve method was used. The obtained results demonstrate that the concentration of Lipocalin 2 in WBCs of primary breast cancer patients is significantly higher than in those of healthy donors ($p<0.05$) (Figure 4.19). In WBCs of advanced breast cancer patients, the concentration of Lipocalin 2 was also shown to be higher, but these results were not statistically significant.

The measurement of the levels of Lipocalin 2 concentration in WBCs and plasma of cancer patients revealed that while its levels correlate in samples from primary breast

cancer patients, in samples from healthy donors, the WBCs concentration of Lipocalin 2 is lower than in the corresponding serum samples (Figure 4.20).

To assess the changes in the serum concentration of Lipocalin 2 in advanced breast cancer patients in the response to treatment, its levels were measured in samples obtained in the beginning, middle and the final stage of the treatment. In the results, no significant change in the serum concentration of Lipocalin 2 in response to treatment was detected (Figure 4.21).

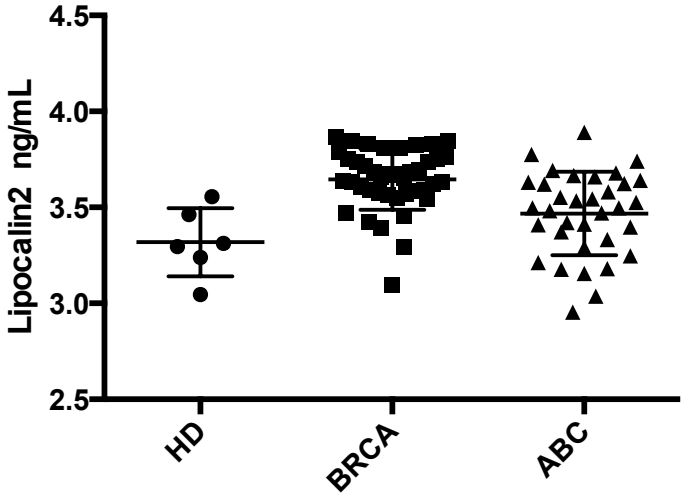


Figure 4.19 Analysis of the concentration of Lipocalin 2 levels in WBCs of breast cancer patients using sandwich ELISA. WBCs were isolated from blood of cancer patients and healthy individuals (Each dot represents an individual patient). Cell lysates were prepared from 1x10^7 cells. To calculate the concentration of Lipocalin 2 in white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients, ABC- advanced breast cancer patients. The concentration of Lipocalin 2 in WBCs of primary breast cancer patients differ significantly from that of healthy donors ($p<0.05$). Data analysed by Kruskal-Wallis test.

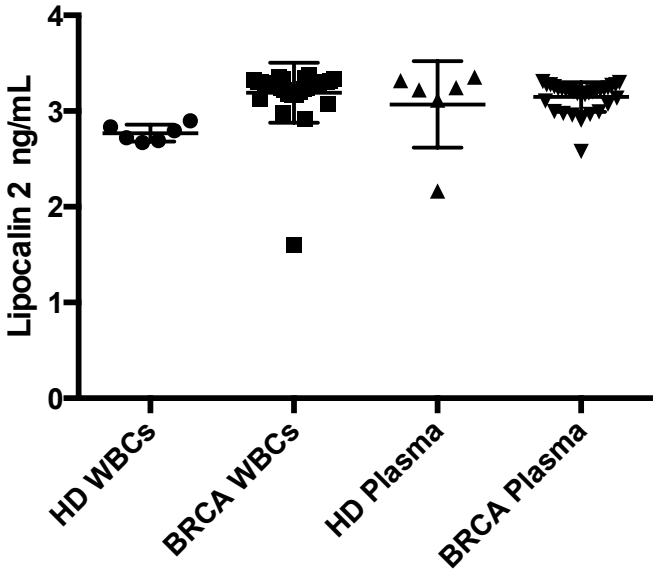


Figure 4.20 Concentration of Lipocalin 2 in WBCs and plasma of healthy donors and primary breast cancer patients using quantitative sandwich ELISA commercial kit. WBCs and plasma were isolated from blood of cancer patients and healthy individuals (Each dot represents an individual patient). Cell lysates were prepared from 1x10^7 cells. To calculate the concentration of Lipocalin 2 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients. The concentration of Lipocalin 2 in WBCs of breast cancer patients differ significantly from that of healthy donors ($p<0.05$). Data analysed by Kruskal-Wallis test. Concentration of Lipocalin 2 in WBCs correlates with its corresponding serum concentration.

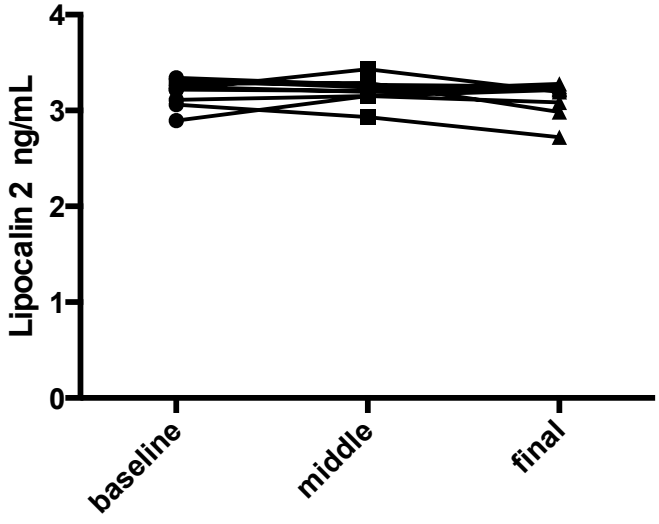


Figure 4.21 Analysis of the correlation between circulating levels of Lipocalin 2 in serum of advanced breast cancer patients and treatment response using ELISA. quantitative sandwich ELISA commercial kit. Serum was separated from blood of advanced breast cancer patients prior the first (baseline), second (middle) and final third stage of the treatments. To calculate the concentration of Lipocalin 2 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm.

4.3 Conclusion

Proteomic analysis revealed that Lipocalin 2 (NGAL) is up-regulated in leukocytes of individuals diagnosed with breast cancer. Analysis of corresponding mRNA expression demonstrated stable coordinate changes with protein levels (Mani, J PhD thesis. 2015). In order to further verify the reported findings, the levels of Lipocalin 2 protein expression in leukocytes were measured by Western blot and the distribution of the protein within the cells was analysed by Immunofluorescent staining (IF). The data obtained from Western blot analysis confirms the upregulation of Lipocalin 2 protein levels in WBCs of breast cancer patients in comparison with healthy donors' data. Furthermore, advanced breast cancer WBCs samples have more Lipocalin 2 than primary breast cancer samples. These findings correlate with studies on cell lines where highly metastatic cell lines are shown to express higher amount of the protein than benign (Stoesz et al. 1998). Suggesting that Lipocalin 2 could provide benefit for cancer cell growth (Shi et al. 2008). The IF staining of WBCs using anti-Lipocalin 2 antibodies revealed strong elevated cytoplasmic presence of NGAL in the inspected WBCs from breast cancer patients and this data is consistent with report obtained from the Western blot analysis. Lipocalin 2 was first discovered as a component of neutrophil granules (Le Cabec et al. 1997).

The data from flow cytometry experiment showed that the levels of the analysed protein is increased in WBCs of randomly selected primary breast cancer patient, when compared with healthy volunteer.

Furthermore, analysis of the data obtained by ELISA, revealed that concentration of Lipocalin 2 in WBCs of breast cancer patients is higher than in those of healthy donors, supporting the prognostic value of the candidate biomarker. The serum concentration of the above protein showed no changes in the response to the treatment in the analysed

samples from advanced breast cancer cohort. Therefore, the use of Lipocalin 2 as the predictive marker of treatment response is not possible.

Here, the elevated levels of Lipocalin 2 in WBCs of breast cancer patients were confirmed by three independent techniques. These findings are in line with the existing literature reports indicating that the expression of Lipocalin 2 is significantly increased in several solid tumours including breast and endometrium (Bauer et al. 2008). Whether immune cells have the same trend is unknown. Lipocalin 2 is a secreted protein (Zhang et al. 2014). A recent study indicate that Lipocalin 2 could be secreted by hematopoietic cells into tumour microenvironment to promote breast tumour growth (Ören et al. 2016).

The overexpression of NGAL in leukocytes of breast cancer patients could be explained by its' well documented ability to bind and transport iron to the cytoplasm which results in the increased intracellular iron concentration (Flo et al. 2004). The beneficial role of iron uptake for the cancer cells is widely recognised. Cancer cells require more iron than normal cells to sustain their rapid growth. For instance, in breast cancer cells increased concentration of Lipocalin 2 in a complex with iron was found to serve as a source of iron (Torti & Torti 2013).

Furthermore, most commonly used biomarkers in cancer diagnosis are glycoproteins such as CAE and CA125. Lipocalin 2 being a small glycoprotein has drawn much attention during the recent years as potential biomarker due to its differential expression in several epithelial malignancies. For instance, in gastric and ovarian cancers, serum levels of Lipocalin 2 could distinguish between cancer patients and healthy individuals with high accuracy (Argani et al. 2001; Wang et al. 2010).

Chapter 5 Validation results for Integrin α4

5.1 Integrin α4

Integrin α4 is the cell adhesion receptor required for the migration of immune cells. It belongs to a large family of heterodimer molecules composed of 24 members (Johnson et al. 2009). More information about Integrin α4 is given in the section 1.2.3.3.

Objectives of the present chapter:

Our previous studies of proteome profiles demonstrated that Integrin α4 protein levels are decreased in WBCs of breast cancer patients in comparison with healthy donors (Many, J., PhD thesis, 2015). We hypothesised that Integrin α4 expression in WBCs will correlate with clinical outcomes (e.g. survival and therapy response). This chapter comprises of two major aims. The first one is to validate the heightened expression pattern of Integrin α4 in WBCs from breast cancer patients in comparison with healthy individuals. The second aim is to study the changes in Integrin α4 levels in a cohort of advanced breast cancer patients undergoing treatment in order to evaluate the prognostic and predictive biomarker value of Integrin α4 . To achieve this, target protein levels were assessed in plasma and WBCs of breast cancer patients using western blot, Immunofluorescence, flow cytometry and ELISA techniques.

5.2 Results

5.2.1 Western blot analysis of Integrin α4 expression in the WBCs from breast cancer patients with different stages of breast cancer

Integrin α4 levels were compared in two cohorts of breast cancer patients' samples (primary and advanced) and healthy donors using Western blot analysis. The membranes were probed with the anti-Integrin α4 antibody and anti-β Actin antibody used as a loading control. The results are presented in Figure 5.1. Densitometry was performed on the blots using Image J software. The values for integrin α4 were normalised to β Actin and the values between different gel images were normalised using the expression values of the common sample (Healthy Donor 4, HD4) run on all gels. The results are presented in Figure 5.2. For the statistical analysis Kruskal-Wallis test was performed using GraphPad Prism software. The obtained results showed significantly ($p<0.05$) lower levels of Integrin α4 expression in WBCs of primary and advanced breast cancer cohorts in comparison with healthy donors.

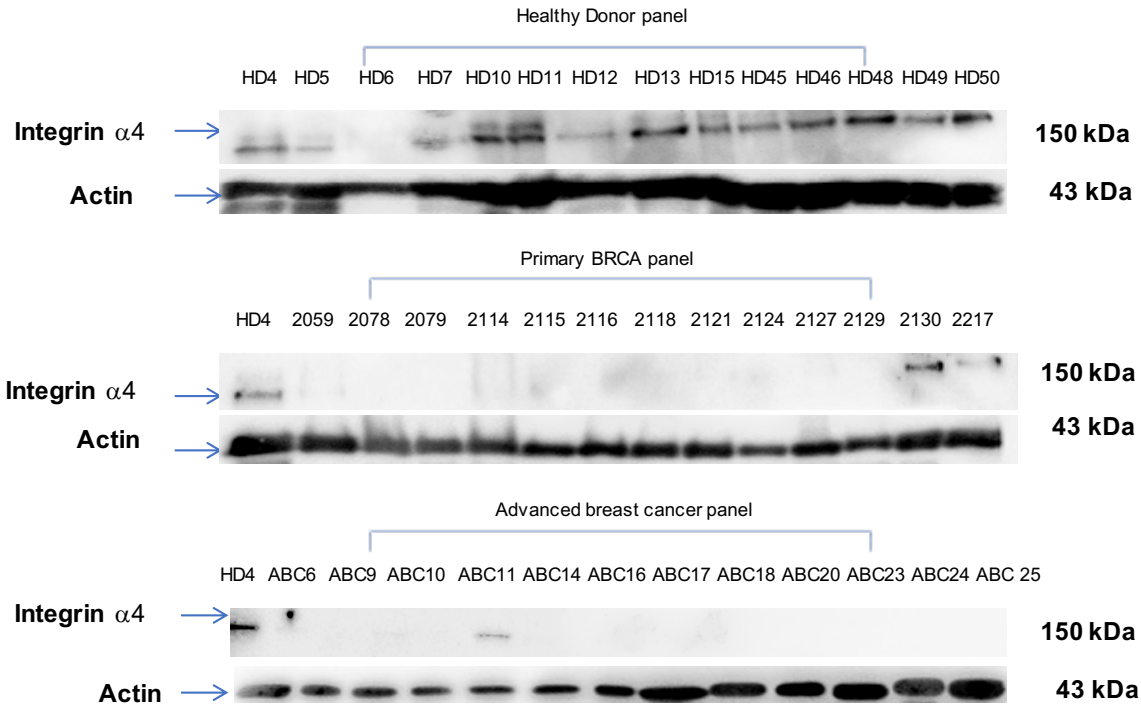


Figure 5.1 Representative western blot assays demonstrating Integrin α4 levels in WBCs from healthy donors and breast cancer patients with different disease conditions. WBCs were isolated from blood of cancer patients or healthy donors. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Integrin α4 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyzer. The results of the quantitative analysis are presented in the next Figure.

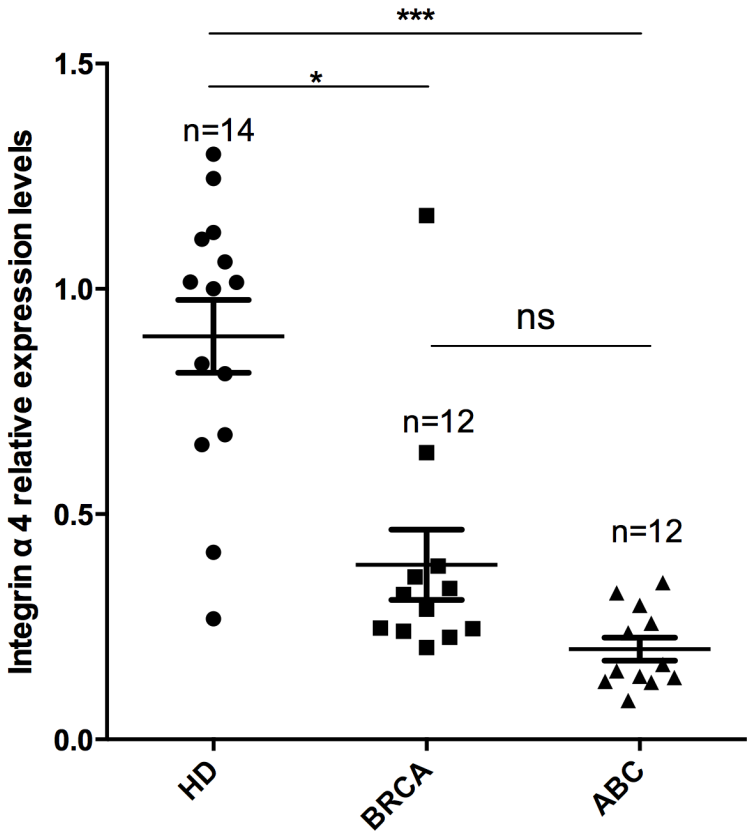


Figure 5.2 Quantitative analysis of the Western blot data for Integrin α4 levels in healthy donors and breast cancer patients with different states of the disease. The ratios of the intensity of the Integrin α4 bands over the intensity of the corresponding β-actin bands were determined; as a control healthy donor 4 was used in all the westerns and the results plotted on a graph using GraphPad Prism 6 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test. HD-healthy donors, BRCA- primary breast cancer patients, ABC-advanced breast cancer patients. ns not significant, P>0.05; * P<0.05; *** P<0.0001

5.2.2 Western blot analysis of Integrin α4 expression in WBCs of advanced breast cancer patients during treatment

To further investigate the apparent absence of Integrin α4 signal in WBCs samples from advanced breast cancer patients, it was decided to run samples taken during the treatment. The samples from four patients were resolved on the gel (ID numbers 3, 16, 18 and 20) and the results are shown in Figure 5.3. The cell lysates from the WBCs from the ABC cohort were prepared from the blood samples taken at the beginning (1), middle (3) and final (6) stage of the chemotherapy treatment. Interestingly, Integrin α4 expression was more highly detected toward the end of the treatment.

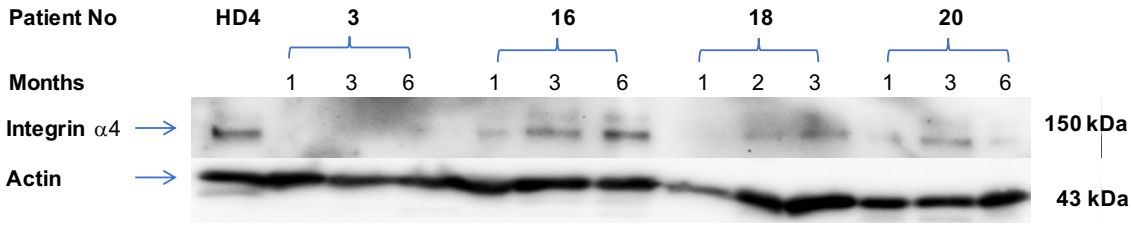


Figure 5.3 Western blots analysis of Integrin α4 levels in WBCs of advanced breast cancer patients during the chemotherapy treatment. WBCs were isolated from blood of advanced breast cancer patients during the treatment. 3, 16, 18, 20 represent patients' number. Numbers in the brackets represent treatment number of the treatment cycle. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Integrin α4 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Band corresponding to the proteins of interest were visualised using Image Analyzer.

5.2.3 Immunofluorescence analysis

Immunofluorescence analysis of Integrin α4 in WBCs of breast cancer patients and HD using Confocal Scanning Microscope (Nikon) at 60X magnification. Integrin α4 is reported to be expressed in lymphocytes and involved in lymphocyte migration and T cell activation. Therefore, in this experiment CD3 and CD4 markers of T cells differentiation were used to study the distribution of Integrin α4 in WBCs. According to the obtained results, the expression of Integrin α4 is not exclusive to lymphocytes. The positive staining for the protein of interest was detected in all WBCs of breast cancer patients and healthy donors. However, in WBCs of healthy donors, the staining appears to be stronger than in breast cancer patients, supporting the findings of the western blot analysis (Figure 5.4).

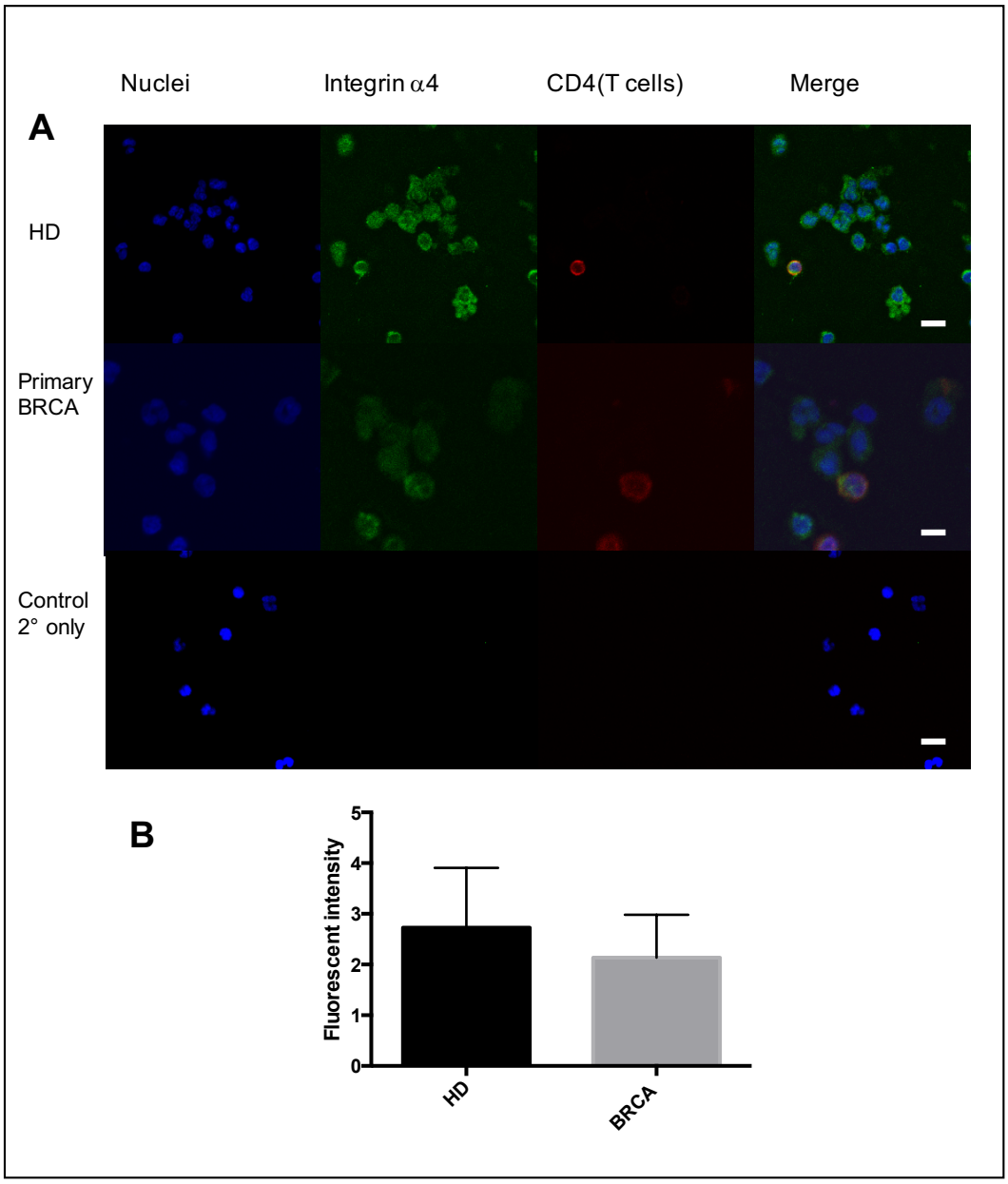


Figure 5.4 Immunofluorescence analysis of Integrin α4 in WBCs of primary breast cancer patients (BRCA) and healthy donors (HD) using Confocal Scanning Microscope (Nikon) at 60X magnification. A WBC from breast cancer patients and healthy donors were stained with FITC-labelled anti-rabbit secondary IgG antibody (dilution 1:400) against Integrin α4 primary antibody (dilution 1:200), plus secondary only as the control and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD4 T-cell primary antibody (1:200), plus secondary only as the control. The blue staining (DAPI) shows the position of the nucleus. The green staining corresponds to Integrin α4 protein and the red to T-cells. All images were taken by Confocal Scanning Microscope (BioRad Hercules) at 60X magnification. Scale bar 10 μm. **B** Bar graph represents the Integrin α4 expression (fluorescent intensity) in the immunofluorescent images, as measured using ImageJ software.

5.2.4 Evaluation of the levels of Integrin α4 in T cells of primary breast cancer patients using flow cytometric analysis

Here we aimed to further validate the decreased expression of the Integrin α4 using flow cytometry. For this experiment, freshly isolated WBCs (within 6 hours from venipuncture) from a randomly selected primary breast cancer patient and healthy donor were stained with two pre-labelled markers expressed by T cells CD3/CD4. Integrin α4 is an intracellular protein and therefore pre-stained cells were fixed, permeabilised and stained with unlabeled polyclonal rabbit anti-Integrin α4 antibody followed by incubation with FITC-labelled anti-rabbit secondary antibody. For a negative control, cells were stained with an isotype control antibody.

The results obtained for Mean Fluorescent Intensity (MFI) show that the levels of Integrin α4 is lower in randomly selected WBCs sample obtained from primary BRCA in comparison with healthy donor (Figure 5.5). However, these results are based on the analysis of the one primary breast cancer sample. Therefore, the panel should be expanded.

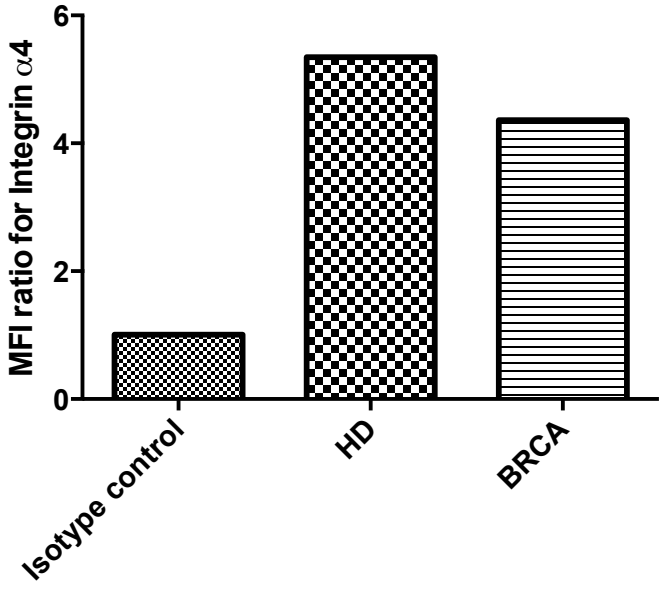


Figure 5.5 Flow cytometric analysis of the Integrin α4 levels in neutrophils of breast cancer patient and a healthy donor (randomly selected). Freshly isolated WBCs were stained for 20 minutes with pre-labelled T cell surface markers (CD3/CD4) at room temperature. Cells were washed with the staining buffer (PBS/Serum/BSA) and fixed with cold 4% formaldehyde on ice for 20 minutes. Cells were permeabilised in 0.25% tween/PBS for 20 minutes on ice, washed twice with the staining buffer and incubated with antibody against Integrin α4 (1:200) o/n. The cells were then stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) for 45 minutes in the dark to detect Integrin α4 and analysed using flow cytometer (BD Accuri C6). For a negative control, cells were stained with an isotype control antibody. Quantification of the levels of Integrin α4 expression in the neutrophils of HD and primary BRCA according to the Mean Fluorescent Intensity (MFI) of the samples and control.

5.2.5 Analysis of the expression of the Integrin α4 using ELISA

5.2.5.1 Analysis of the expression of the Integrin α4 in healthy donors and breast cancer patients using ELISA

In the final step of the validation experiment, the levels of Integrin α4 were analysed in the white blood cells and plasma of two cohorts of breast cancer patients (primary and advanced) using an alternative method, namely, the quantitative sandwich enzyme immunoassay technique (ELISA). Given that Integrin α4 is a promising marker of breast cancer prognosis, the use of the ELISA kit in medical practice to quantitatively evaluate Integrin α4 levels in the blood of cancer patients would be of particular interest.

White blood cell pellets ($1x10^7$) were obtained from the blood of breast cancer patients and healthy donors. The resulting cell pellet was lysed by the addition of equal volume of Buffer 1 and Buffer 2 (Table 2.3.1) with the volume ratio of $1mL/4x10^6$ and incubated on ice for 20 minutes. The cell lysate was centrifuged at 13000 rpm and at 4°C for 15 minutes. The pellet was discarded and the supernatant (cell lysate) was used for the experiment.

The aim was to analyse the expression pattern and concentration of Integrin α4 in plasma and WBC of healthy donors and two cohorts of breast cancer patients. To calculate the concentration of Integrin α4 in plasma and white blood cells of breast cancer patients (primary and advanced) a standard curve method was used. The obtained results revealed that high variation of the protein concentration in the plasma samples (Figure 5.6). Although the concentration of Integrin α4 was found to be higher in WBCs samples from primary and advanced breast cancer patient in comparison with healthy donors, many samples were found to be negative for Integrin α4. Further, Integrin α4 levels in

WBCs of healthy donor samples varied from 0 to 2.1ng/mL, in primary breast samples from 0 to 9 ng/mL and in advanced breast cancer samples from 0 to 7ng/mL. Overall, these results are not statistically significant ($p>0.05$).

Next, the measurement of the Integrin $\alpha 4$ concentration was performed in WBCs of cancer patients and the corresponding plasma samples. The results revealed that the Integrin $\alpha 4$ in WBCs correlates with plasma concentration. In the case of primary breast cancer samples, results showed that Integrin $\alpha 4$ levels are higher in WBCs than in plasma (Figure 5.7). The inconsistency with Western blots and ELISA results observed in the presented study can be explained by the use of different antibodies in these assays. Unfortunately, we have little control over this factor

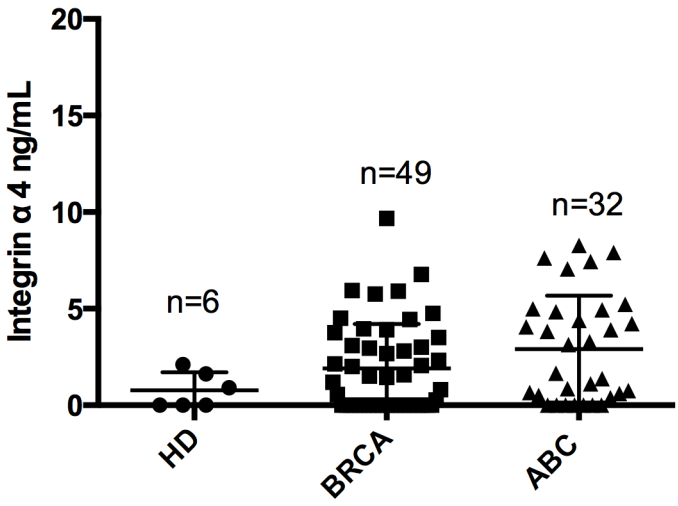


Figure 5.6 Concentration of Integrin α4 in WBCs in different participants' cohorts using quantitative sandwich ELISA commercial kit. WBCs and plasma were isolated from blood of cancer patients and healthy individuals (Each dot represents an individual patient). Cell lysates were prepared from 1x10^7 cells. To calculate the concentration of Integrin α4 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients, ABC- advanced breast cancer patients. Results are not statisticaly significant.

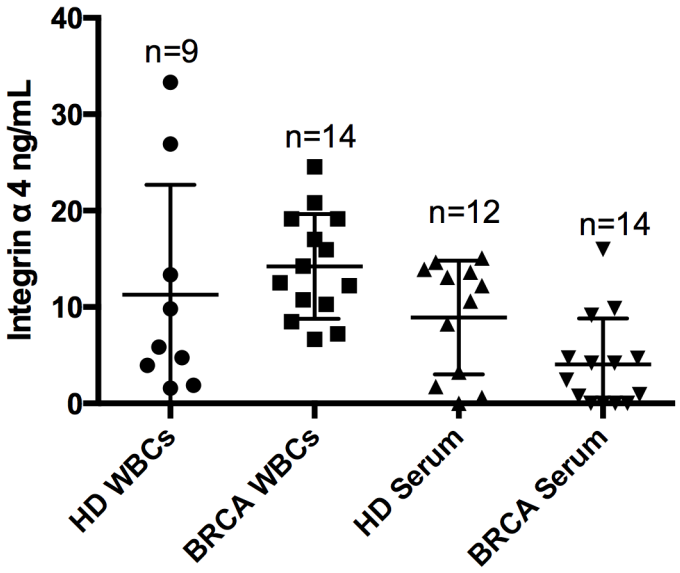


Figure 5.7 Concentration of Integrin α4 in WBCs and plasma in different participants' cohorts using quantitative sandwich ELISA commercial kit. Each dot represents an individual patient. WBCs and plasma were isolated from blood of cancer patients and healthy individuals. To calculate the concentration of Integrin α4 in plasma and white blood cells a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. HD- healthy donors, BRCA- primary breast cancer patients, results are not statisticaly significant.

5.2.5.2 Analysis of the Integrin α4 levels in cohort of advanced breast cancer patients in response to treatment using ELISA.

To analyse whether the changes in circulating Integrin α4 concentration could be indicative of the treatment response in advanced breast cancer patients undergoing chemotherapy and endocrine treatment, the levels of the analyte were measured in serum of 10 patients (6 chemotherapy, 4 endocrine) at three treatment time points; beginning, middle and final stage of treatment.

The data revealed different patterns and the results were grouped according to the clinical information. According to the obtained data, the levels of Integrin α4 decrease in the middle of the chemotherapy treatment and increase in the end (Figure 5.8). The data for the patients with endocrine treatment showed different trends. For the patients with stable disease, plasma levels of Integrin α4 increased in the middle of the treatment and then declined at the end for one patient, and gradually declined during the treatment for another.

To see if trends in Integrin α4 concentration correlate with advanced cancer patient survival, the results were grouped according to the length of survival (Figure 5.9). The data showed that in the category of patients with chemotherapy, the levels of Integrin α 4 declines in the middle of the treatment and rises in the end in all analysed samples, the patient with 6 months survival time showed the same trend as the alive patient. The results for the patients with endocrine treatment indicate that in patients that are still alive, that the levels of the protein of interest increased in the middle and decreased at the end of the treatment. In the 2 patients with 12 months survival, the levels of analyte declined during the treatment

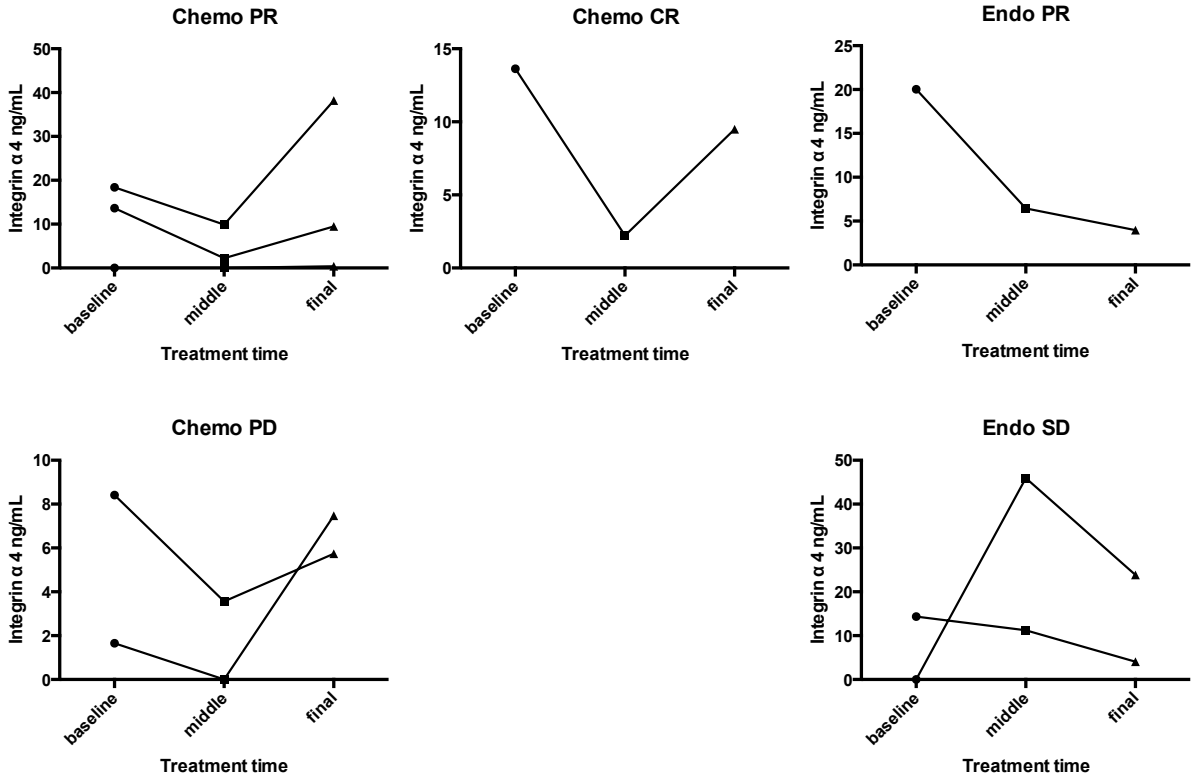


Figure 5.8 Analysis of the correlation between plasma levels of Integrin α4 in serum of advanced breast cancer patients and treatment response using sandwich ELISA. To calculate the concentration of Integrin α4 in plasma a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. **PD** progressive disease; **PR** partial response; **SD** stable disease; **CR** complete response. **Chemo** chemotherapy treatment. **Endo** endocrine treatment. Serum was separated from blood of advanced breast cancer patients prior the first (baseline), second (middle) and final third stage of the treatments.

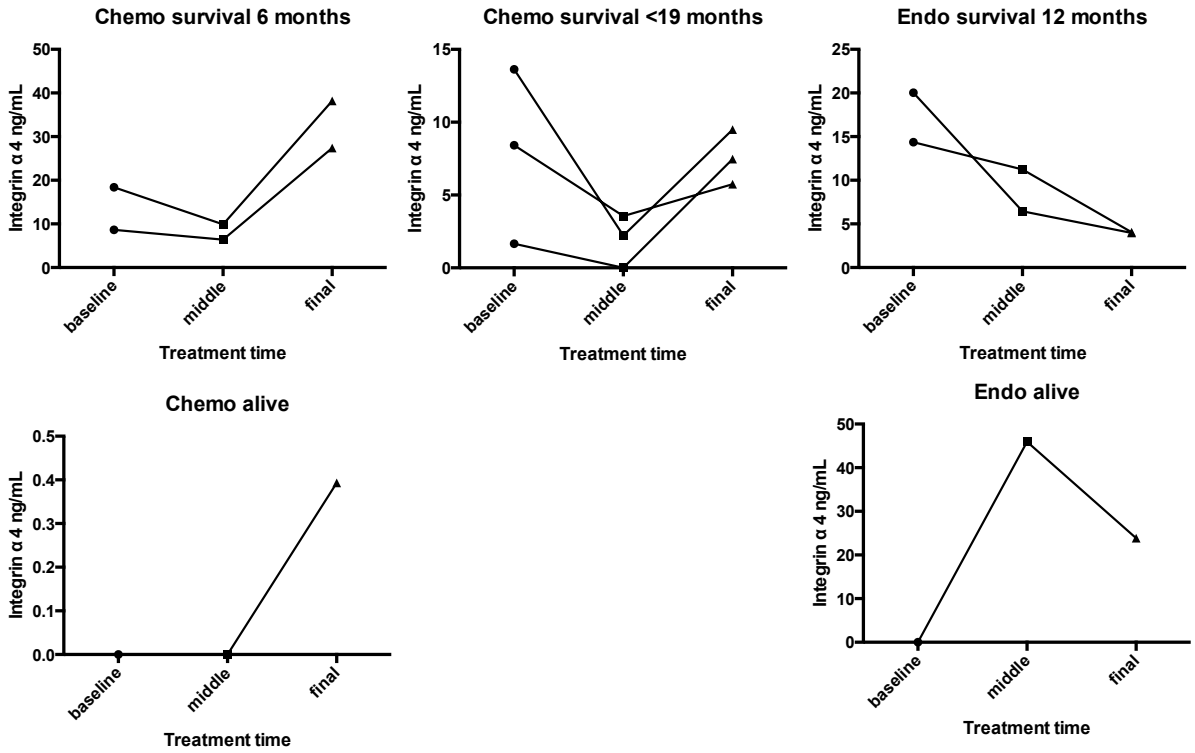


Figure 5.9 Analysis of the correlation between circulating levels of Integrin α4 in serum of advanced breast cancer patients and survival time using sandwich ELISA. To calculate the concentration of Integrin α4 in plasma a standard curve method was used. Optical density readings were taken at single wavelength of 450 nm. **Chemo** chemotherapy. **Endo** endocrine therapy.

5.3 Discussion

In the experiments conducted in this chapter the early biomarker, prognostic and predictive values of previously identified candidate breast cancer biomarker Integrin $\alpha 4$ was evaluated. A value of this protein as possible early biomarker of breast cancer was confirmed by a Western blot assay, whereby the decreased levels Integrin $\alpha 4$ were detected in WBCs of two cohorts of breast cancer patients, primary and advanced in comparison with healthy donors. The results of this experiment were significant ($p < 0.0107$ for the primary breast cancer cohort). The low expression of the Integrin $\alpha 4$ was confirmed by IF study and FACS analysis using WBCs from a randomly selected participant with primary breast cancer.

In the WBCs samples of the advanced breast cancer cohort, the signal was very difficult to distinguish from the background. We reasoned that if treatment is successful, Integrin $\alpha 4$ could re-appear in the WBCs. To test this possibility, the WBCs isolated from blood taken in the beginning, middle and end of the treatment were tested. In the results, the signal for the protein of interest was detected in the late stage of the treatment in some of the patients. These findings however disagree with the observations that chemotherapy has direct effect on immune cells, resulting in depletion of lymphocytes population decreased expression levels of Integrin $\alpha 4$ (Bracci et al. 2014). These results need to be explored further.

The data obtained from ELISA experiment showed different results. The Integrin $\alpha 4$ protein concentration was found to be higher in breast cancer samples in comparison with healthy donors. However, it should be noted that in many analysed samples the concentration was not measurable.

Interestingly, the analysis of the protein expression in plasma obtained from patients with advanced breast cancer undergoing chemotherapy and endocrine treatment showed higher concentration of Integrin α4. Thus, in response to chemotherapy treatment the levels of Integrin α4 in plasma raised and in response to endocrine treatment declined.

The results of ELISA might have been compromised by the presence of additional forms of Integrin α4, cross-reactivity with other proteins or some technical problems with the kit or protocol. The ELISA experiments will need to be repeated in the future and a different kit for Integrin α4 measurements should be used.

Due to such conflicting results, it is difficult to conclude whether Integrin α4 has a potential a value as a biomarker of successful treatment and cancer progression.

Integrin α4 mediates lymphocyte migration to the sites of infection (Wang et al. 2009).

The decreased expression of Integrin α4 revealed by the present study correlates with the findings that during HIV infection detectable loss of circulating CD4+T cells expressing Integrin α4 is observed. Furthermore, throughout the infection, these cells are reported to be eventually eliminated. However, in animal controlling the infection the levels of CD+4 T cells positive for Integrin α4 were eventually restored (Wang et al. 2009). The decrease of this particular subset of T cells has been suggested to have a role in the development of immunosuppression, as the recruitment of immune cells to the site of inflammation and potentially cancer is limited (Wang et al. 2009)

In the present study, the decreased Integrin α4 levels were observed in WBCs of breast cancer patients using western blot, flow cytometry and IF. Accordingly, as integrin are required for the adhesion of immune cell to the endothelium and migration for the subsequent elimination of pathogens or malignant cells, the decreased levels of this

protein could be one of the strategy of cancer cells to escape the immune response. Escape from immune control is recognised as a one of the “Hallmarks of Cancer” (Hanahan & Weinberg 2011).

Another explanation of the observed decreased levels of Integrin α4 protein in WBCs of breast cancer patients is that chemotherapy has direct effect on immune cells, resulting in depletion of lymphocytes population (Bracci et al. 2014)Therefore, resulting in the decreased expression levels of Integrin α4.

The question whether the immune system has positive, negative or none effect on tumour development is still open (Mittal et al. 2014) The findings presented in this chapter may help understand the extent of the immune’s system interaction with cancer.

Chapter 6 Validation results for Lipoxygenase 5 (LOX5)

6.1 Lipoxygenase 5

Lipoxygenase 5, or 5LOX, is an inflammatory response protein related to leukotriene synthesis. This enzyme catalyses first two steps in leukotriene formation from arachidonic acid (Steinhilber et al. 2010). Overexpression of lipoxygenases and their products has been implicated in acute and chronic inflammatory diseases and in cancer as these two processes are closely linked. Various types of malignant cells are reported to have elevated levels of 5LOX (Erler et al. 2009; Le et al. 2009; Sundaram & Ghosh 2006). Furthermore, the 5LOX gene-silencing is shown to reduce viability of cancer cells, suggesting that arachidonic acid metabolites play important role in cancer progression (Sundaram & Ghosh 2006). It has been linked to the promoting prostate cancer and breast cancer cells survival (Sarveswaran et al. 2011; Borin et al. 2017). The overexpression of 5LOX protein has been previously reported in the WBCs of breast cancer patients, especially in high grade category. More information about 5LOX is given in section 1.2.3.5.

Objectives of the present chapter:

Our previous proteomic studies reported heightened expression of 5LOX in WBCs of breast cancer patients. We hypothesised that 5LOX expression in WBCs will correlate with clinical outcomes (e.g. survival and therapy response). This chapter comprises of two major aims. The first one is to validate the heightened expression pattern of 5LOX in WBCs from breast cancer patients in comparison with healthy individuals. The second aim is to study the changes in 5LOX levels in a cohort of advanced breast cancer patients undergoing treatment in order to evaluate the prognostic and predictive biomarker value

of 5LOX. To achieve this, target protein levels were assessed in plasma and WBCs of breast cancer patients using western blot, Immunofluorescence and flow cytometry.

6.2 Results

6.2.1 Western blot analysis of 5LOX expression in WBCs of breast cancer patients and healthy donors

The levels of 5LOX were measured by Western blot assay in WBCs from 13 healthy donors, and 20 and 13 patients with primary and advanced breast cancer, respectively. A representative Western blot image is shown in Figure 6.1. For quantitative analysis of these data, the values obtained from the bands of 78 kDa corresponding to 5LOX were normalised to the values from β-Actin signals using Image J software. The values between the gels were compared by normalising to the expression value of the common healthy donor 4 (HD4) sample used in all experiments. The results revealed overexpression of 5LOX protein levels in WBCs of primary breast cancer cohort in comparison with healthy donors. In the advanced breast cancer cohort, 5LOX levels was shown to be lower than in healthy donors. However, the Kruskal-Wallis test showed that these results are not statistically significant ($p>0.05$) (Figure 6.2).

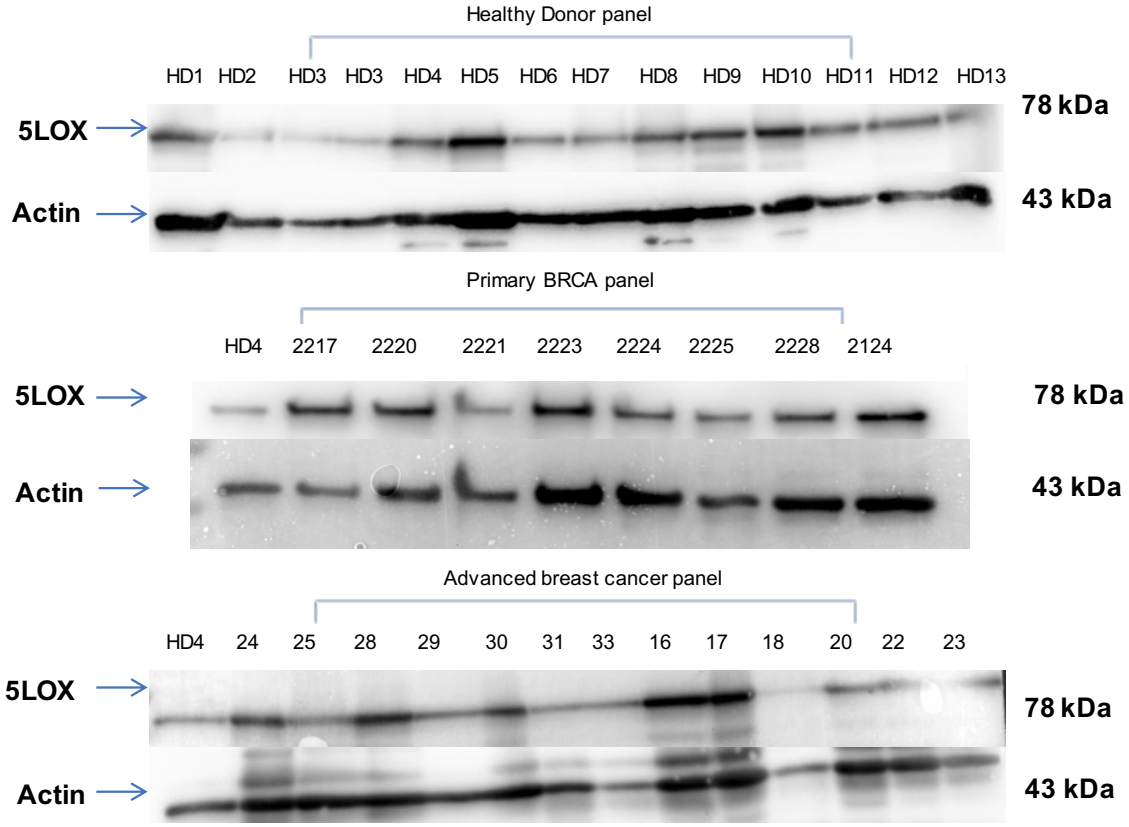


Figure 6.1 Representative western blots of 5LOX levels in breast cancer patients with different disease conditions. WBC were isolated from blood of cancer patients. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-5LOX antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Band corresponding to the proteins of interest were visualised using Image Analyzer. The ratios of the intensity of the 5LOX bands over the intensity of the corresponding β-actin bands were determined as a control healthy donor 4 was used in all the westerns and the results plotted on a graph using GraphPad Prism 6 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test (see next Figure).

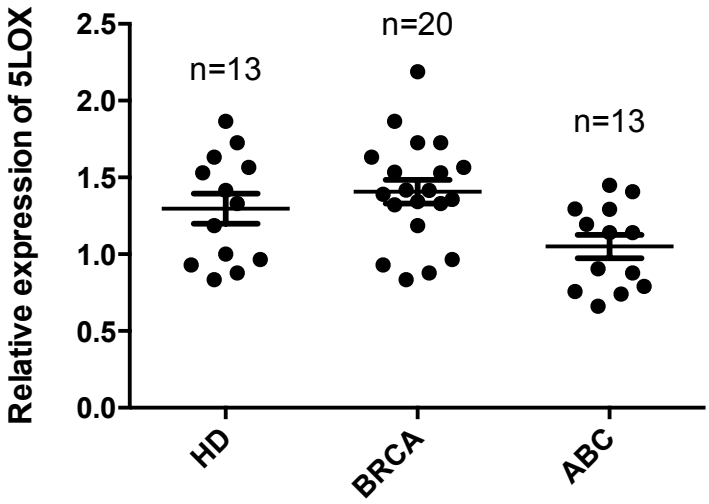
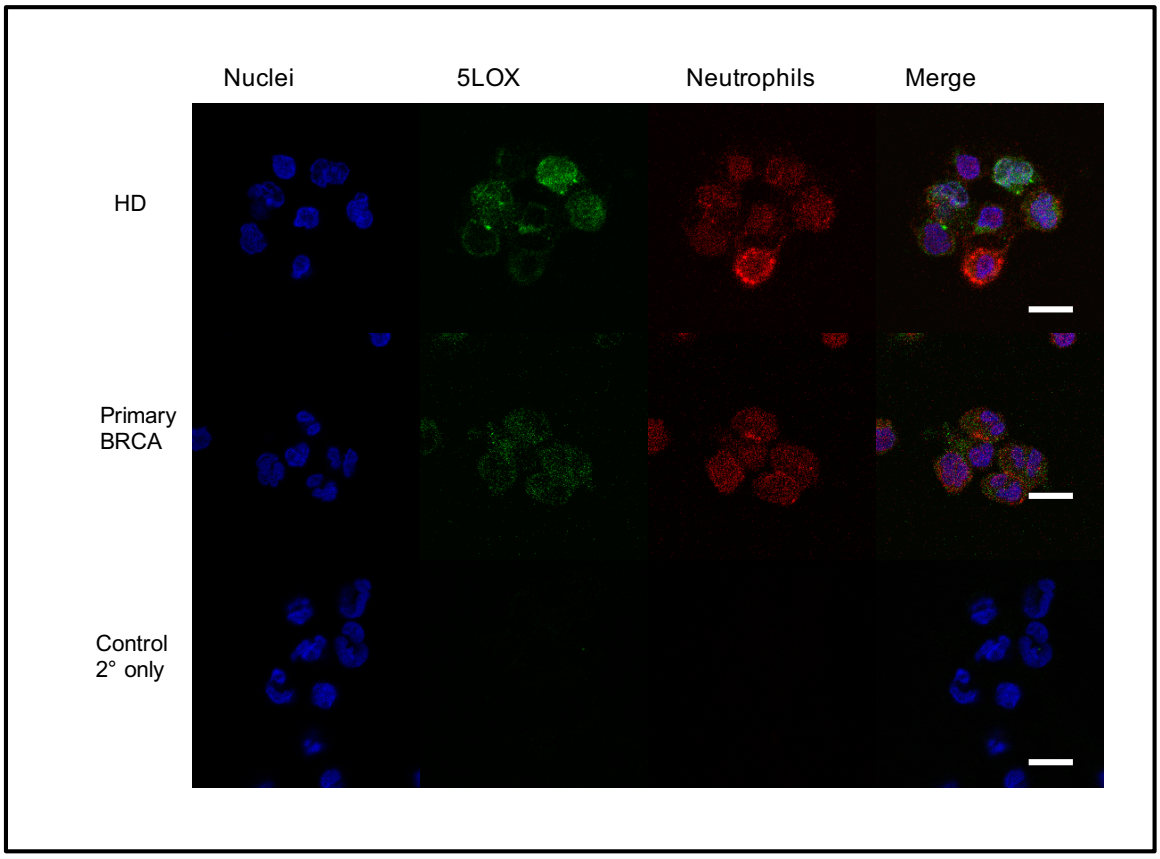


Figure 6. 2: Western blot analysis of 5LOX levels in breast cancer patients with different disease conditions. WBC were isolated from blood of cancer patients. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-5LOX antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Band corresponding to the proteins of interest were visualised using Image Analyzer. The ratios of the intensity of the 5LOX bands over the intensity of the corresponding β-actin bands were determined as a control healthy donor 4 was used in all the westerns and the results plotted on a graph using GraphPad Prism 6 software. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test. Results are not statistically significant. HD- healthy donors, BRCA- primary breast cancer patients, ABC- advanced breast cancer patients.

6.2.2 Immunofluorescent analysis of 5LOX distribution in immune cells

Next, the distribution of 5LOX protein in WBCs of breast cancer patients and healthy donors was examined by Immunofluorescent staining technique using primary anti-5LOX antibody (Figure 6.3). This analysis revealed higher intensity of the 5LOX in the WBCs of healthy donor, than in breast cancer patient. It should be noted that WBCs from HD5 that was used in the present experiment also showed stronger signal in Western blot analysis. The WBCs from the breast cancer patient 2228, on the other hand, showed lower signal. The pattern of protein expression correlated with previously observed Western blot results. Later on, the healthy donor 5 was diagnosed with cancer, which may explain the elevation of LOX5.

In order to determine which immune cells overexpress LOX5 the WBCs were also stained with the CD15 antibody which recognises carbohydrate epitope expressed in mature neutrophils and eosinophils. The obtained double immunofluorescence analysis revealed that the majority of 5LOX positive cells were neutrophils (Figure 6.3). Staining with only the secondary antibody did not show any nonspecific background.



6.3 Immunofluorescence analysis of 5LOX protein distribution in leukocytes of primary and advanced breast cancer patients. WBC extracted from blood of healthy donor 5 and primary cancer patient 2228 were double stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) against 5LOX primary antibody (dilution 1:200) and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD15 neutrophil primary antibody (1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining corresponds to 5LOX protein and red staining represents Neutrophils. Scale bar 0.01mm. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

6.2.3 Evaluation of the levels of 5 LOX in neutrophils of primary breast cancer patients using flow cytometric analysis.

Data presented in this Chapter suggested that levels of 5LOX are elevated in WBCs of breast cancer patients in comparison to healthy donors (Figures 6.1 and 6.2), and it is expressed in neutrophils (Figure 6.3). In our next experiments we aimed to confirm the expression pattern of 5LOX using flow cytometry (FACS). Flow cytometry is the method that allows for rapid quantitative assessment of proteins within the specific cell type via cell surface marker phenotyping.

Because granulocytes rapidly degenerate soon after collection, WBCs were isolated from the blood sample within 6 hours from blood collection and immediately used for the experiment. Furthermore, due to their large size, these cells are the most unstable type of WBCs, therefore accurate pipetting required in order to avoid cell burst.

For this experiment, freshly isolated WBCs from a randomly selected primary breast cancer patient (2225) and healthy donor 5 were stained with two pre-labelled markers expressed by mature neutrophils: CD15, CD66b. 5LOX is an intracellular protein therefore pre-stained cells were fixed, permeabilised and stained with unlabeled polyclonal rabbit anti-5LOX antibody followed by the incubation with FITC-labelled anti-rabbit secondary antibody.

For a negative control, cells were stained with an isotype control antibody, which are of the same isotype as the target primary antibody. The Isotype controls are used to estimate non-specific staining of primary antibodies; they are of unknown specificity or are raised against antigens known to be absent in target cells.

Surprisingly, the levels of 5LOX in a neutrophils from a breast cancer patient were lower than in a healthy donor (Figure 6.4). For this experiment, the WBC sample from the same

healthy donor 5 (HD5) was used. The higher 5LOX levels observed in this sample in comparison with BRCA sample can be explained by the fact, that this donor was diagnosed with cancer later on.

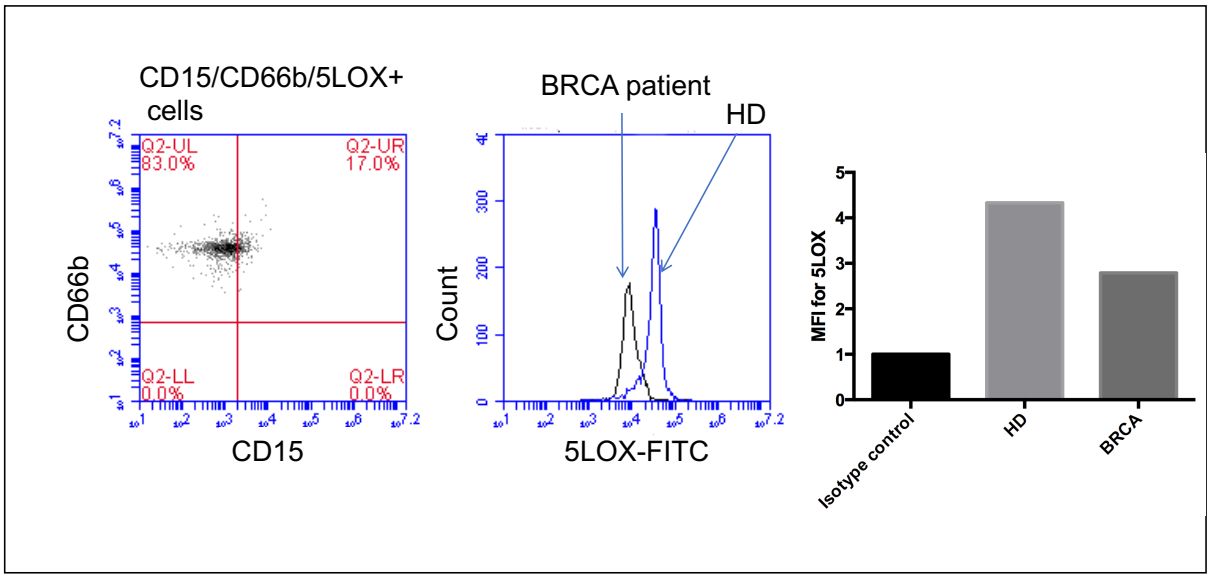


Figure 6.4: Flow cytometric analysis of the 5LOX levels in neutrophils of breast cancer patient and a healthy donor (randomly selected). Freshly isolated WBCs were stained for 20 minutes with pre-labelled neutrophils cell surface markers (CD15/CD66b) at room temperature. Cells were washed with the staining buffer (PBS/Serum/BSA) and fixed with cold 4% formaldehyde on ice for 20 minutes. Cells were permeabilised in 0.25% tween/PBS for 20 minutes on ice, washed twice with the staining buffer and incubated with antibody against 5LOX (1:200) o/n. The cells were then stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) for 45 minutes in the dark to detect 5LOX and analysed using flow cytometer (BD Accuri C6). For a negative control, cells were stained with an isotype control antibody. **A** Gating of the CD15+CD66b+5LOX. **B** Peaks representing fluorescent intensity of the 5LOX expression in BRCA, HD and background isotype control **C** Quantification of the levels of 5LOX expression in the neutrophils of HD and BRCA according to the Mean fluorescent intensity (MFI) of the samples and control.

6.3 Discussion

The aim of the present study was to validate the expression patterns of 5LOX using Western blot assay, IF and FACS techniques.

According to the data obtained by Western blot analysis the levels of 5LOX protein are elevated in the WBCs of primary and decreased in advanced breast cancer patients in comparison with healthy donors, supporting previously reported proteomic findings. FACS and IF analysis however, showed that it's levels are higher in healthy donor than in primary breast cancer patient. It is important to mention, that for FACS and IF only one, randomly selected sample from the cancer patients was used. Healthy donor 5 sample that was used in these experiments showed the highest signal on Western blot. This volunteer was later diagnosed with lung cancer, confirming that heightened expression of 5LOX is specific for cancer related changes in protein expression.

Collectively, the data generated in the present study is in agreement with findings that elevated levels of 5LOX may be a biomarker of various cancers in particular prostate cancer (Sarveswaran et al. 2011).

Malignant cells grow more rapidly than normal cells and require more nutrients and oxygen. A connection between fat and various types of cancer is recognised, as fatty acids produce energy and metabolites important for cell growth and proliferation Arachidonic acid is metabolised via 5LOX to generate series of eicosanoids, which are essential for cancer cells to survive (Sarveswaran et al. 2010; Sarveswaran et al. 2011). Due to the low number of samples analysed, the correlation of 5LOX expression with clinical data for advanced breast cancer patients was not performed.

Chapter 7 Validation results for Copine 3

7.1 Copine 3

Copine 3 is calcium-dependent membrane binding protein. The function of Copine protein family have been shown to be related to cell growth and cancer development (Yang et al. 2006; Ramsey et al. 2008). The exact mechanism is unknown, but existing reports show that Copine 3 expression correlates with ErbB2 amplification in breast cancer (C Heinrich et al. 2010). Due to its previously reported elevated expression in WBCs of breast cancer patients and the role in breast cancer progression, it was chosen for further validation. More information about Copine 3 is provided in section 1.2.4.4.

Objectives of the present chapter:

Proteomic studies revealed that the protein levels of Copine 3 is upregulated in WBCs of breast cancer patients (Many, J., PhD thesis, 2015). This chapter comprises of two major aims. The first is to validate the heightened expression pattern of Copine 3 in WBCs from breast cancer patients in comparison with healthy individuals. The second aim is to study the changes in Copine 3 levels in a cohort of advanced breast cancer patients undergoing treatment in order to evaluate the prognostic and predictive biomarker value of Copine 3. To achieve this, Copine 3 levels were assessed in WBCs of breast cancer patients using western blot and Immunofluorescence techniques.

7.2 Results

7.2.1 Western blot analysis of Copine 3 expression in the WBCs from breast cancer patients with different stages of breast cancer

In previous study microarray data and 2D gel electrophoresis showed +1.2-fold change in the expression of Copine 3 in WBCs of primary and advanced breast cancer samples in comparison to healthy control group (Many, J., PhD thesis, 2015). Based on this finding, in the present study the biomarker property of Copine 3 was initially assessed using a western blot technique. In these experiments, the levels of Copine 3 were evaluated in different categories of breast cancer patients (primary and advanced) in comparison with healthy donors.

The membranes with different samples were probed with anti-Copine 3 antibody, and then with the anti- β-Actin antibody to detect β-Actin served as a loading control. The gels were digitalised and the intensity values were obtained using Image J software. The values for Copine 3 were normalised to the values for β Actin for each sample. The values between different gel images were normalised with each other using the expression values of common sample (HD2) run on all gels. These results are presented in Figure 7.1. For statistical analysis Kruskal-Wallis test was performed using GraphPad Prism software (data are shown in Figure 7.2). The observed data revealed conflicting and inconsistent results for the protein expression in different participants' groups. In particular, levels of Copine 3 differ within the same cohorts of breast cancer patients and healthy donors. Levels of Copine 3 expression in samples from patients with advanced breast cancer also varied.

According to the literature, the Copine 3 expression in breast cancer tissue correlates with HER2 expression (C. Heinrich et al. 2010) We asked whether a similar trend can be

observed in WBCs of breast cancer patients and levels of Copine 3 were linked to the tumour HER2 status. However, no correlation was observed following this analysis (data is not presented).The expression pattern did not show correlation with the patients' clinical data provided by the hospital. Therefore, it can be concluded that Copine 3 cannot be used as a biomarker for diagnostic, prognostic or predictive purpose.

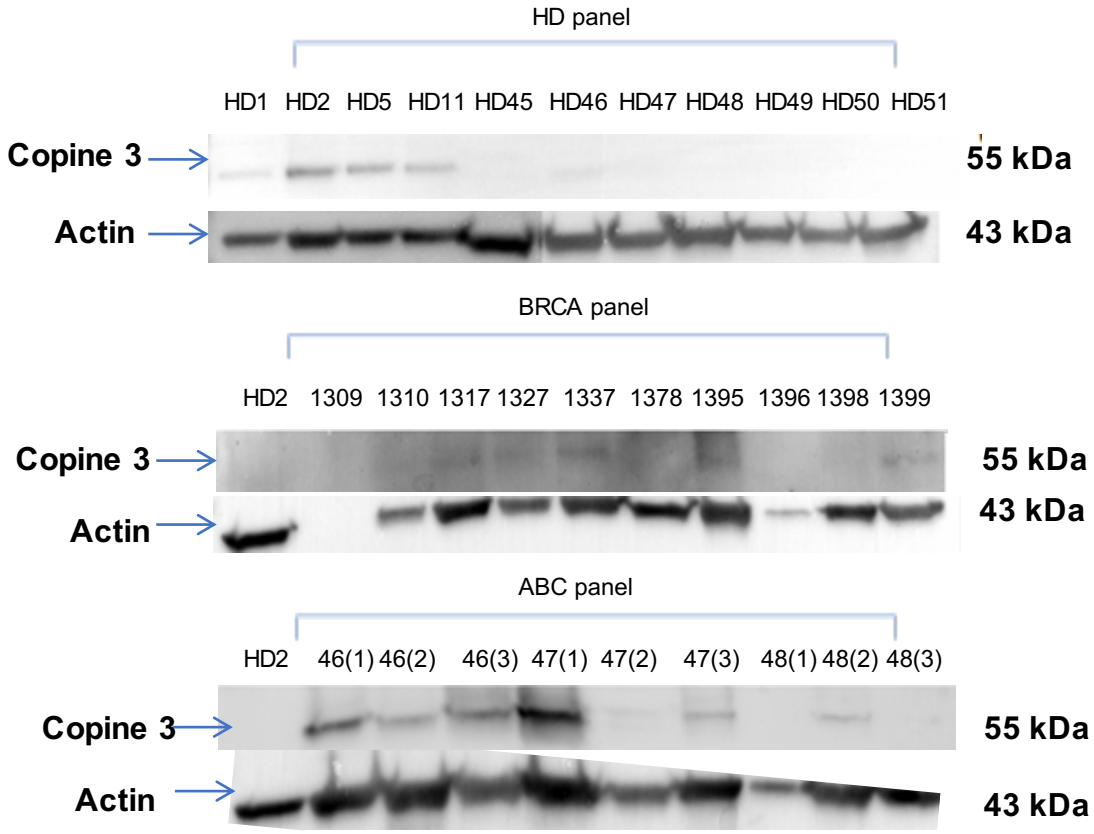


Figure 7.1 Representative western blots of Copine 3 expression in WBCs of breast cancer patients with different disease conditions and healthy donors. WBCs were isolated from blood of cancer patients or healthy donors. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Copine 3 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Bands corresponding to the proteins of interest were visualised using Image Analyzer.

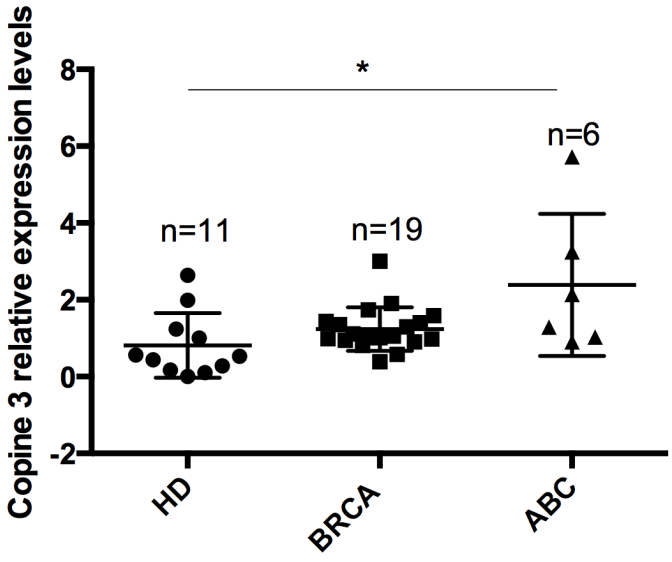


Figure 7.2 Copine 3 protein levels in breast cancer patients with different disease conditions. Cell lysates were prepared from 2x10^6 cells. The samples were separated using SDS-PAGE, blotted and probed with anti-Copine 3 antibody. The same membrane was then re-probed with anti- β-Actin antibody (loading control). Band corresponding to the proteins of interest were visualised using Image Analyser. Densitometry was performed (Image J) and the ratios of the intensity of the Copine 3 bands over the intensity of the corresponding β-actin bands were determined as a control healthy donor 2 was used in all the western blots.

HD- healthy donors, BRCA- primary breast cancer, ABC- breast cancer patients with advanced. Statistical difference between groups was analysed by non-parametric Kruskal-Wallis test. *P<0.05,

7.2.2 Immunofluorescence analysis of Copine 3 expression in the WBCs of healthy donors, primary and advanced breast cancer patients

In order to visualise the distribution of Copine 3 in the WBCs of healthy donors and primary and advanced breast cancer patients the immunofluorescent staining was performed. As shown in Figure 7.3, Copine 3 is present in the WBCs of healthy donor at relatively low levels compared to the primary BRCA sample and it is restricted to the nucleus. In the primary BRCA sample Copine 3 is localized both in the cytoplasm and nucleus. In the WBCs sample from a patient with advanced breast cancer Copine 3 levels are higher than in the healthy donor and a patient with primary BRCA. The protein can be detected in the cytoplasm and in the nucleus. No signal was detected in the control experiments when cells were incubated with the secondary antibody only.

Therefore, based on the obtained images, it may be concluded that the localisation and level of expression of Copine 3 is different in the WBCs of breast cancer patients compared to the WBCs of healthy individuals. It should however be noted that these experiments have been limited to single WBCs samples and need to involve specimens from more participants.

In order to determine which type of blood cells express Copine 3, WBCs were co-stained with the anti- Copine 3 antibody and the antibodies against the immune-cell specific markers, i.e. cluster of differentiation (CD) cell surface antigens to distinguish between subpopulation of WBCs.

When WBCs were stained with CD15 antibody, which recognises a carbohydrate epitope expressed in mature neutrophils and eosinophils, the double immunofluorescence analysis revealed that the majority of Copine 3 positive cells were neutrophils (Figure 7.4). These results also demonstrated that cells negative for CD15 were also negative for

Copine 3. The secondary antibody control did not show any nonspecific background staining.

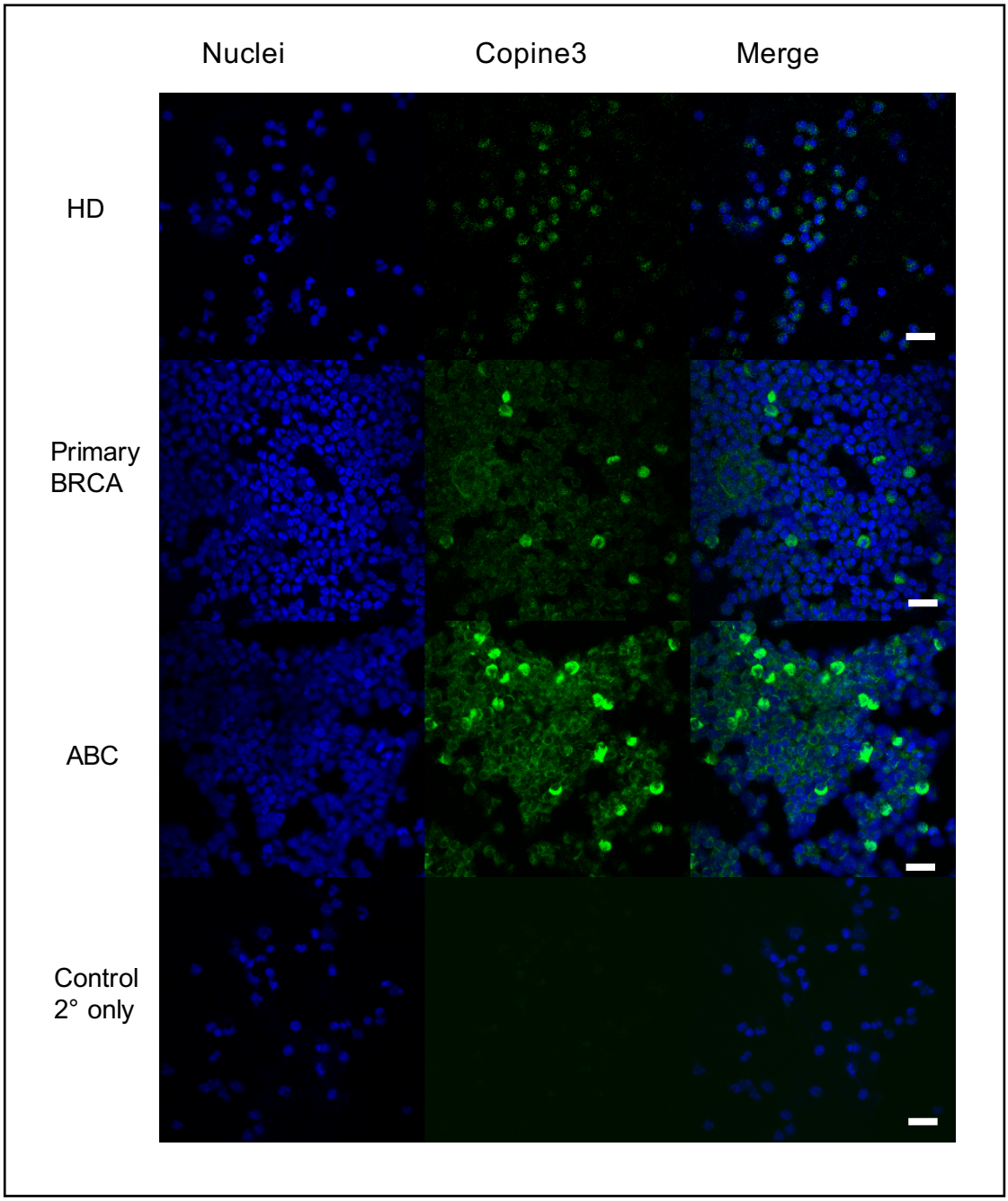


Figure 7.3 Representative Immunofluorescent analysis of Copine 3 protein distribution in WBC of BRCA node negative, node positive, advanced cancer patients and healthy donors. WBC from breast cancer patients and healthy donors were stained with FITC-labelled anti-rabbit secondary IgG antibody (dilution 1:400) against Copine 3 primary antibody (dilution 1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining in the cytoplasm corresponds to Copine 3 protein. Scale bar 0.01mm. All images were taken by Confocal Scanning Microscope (BioRad Hercules) at 60X magnification.

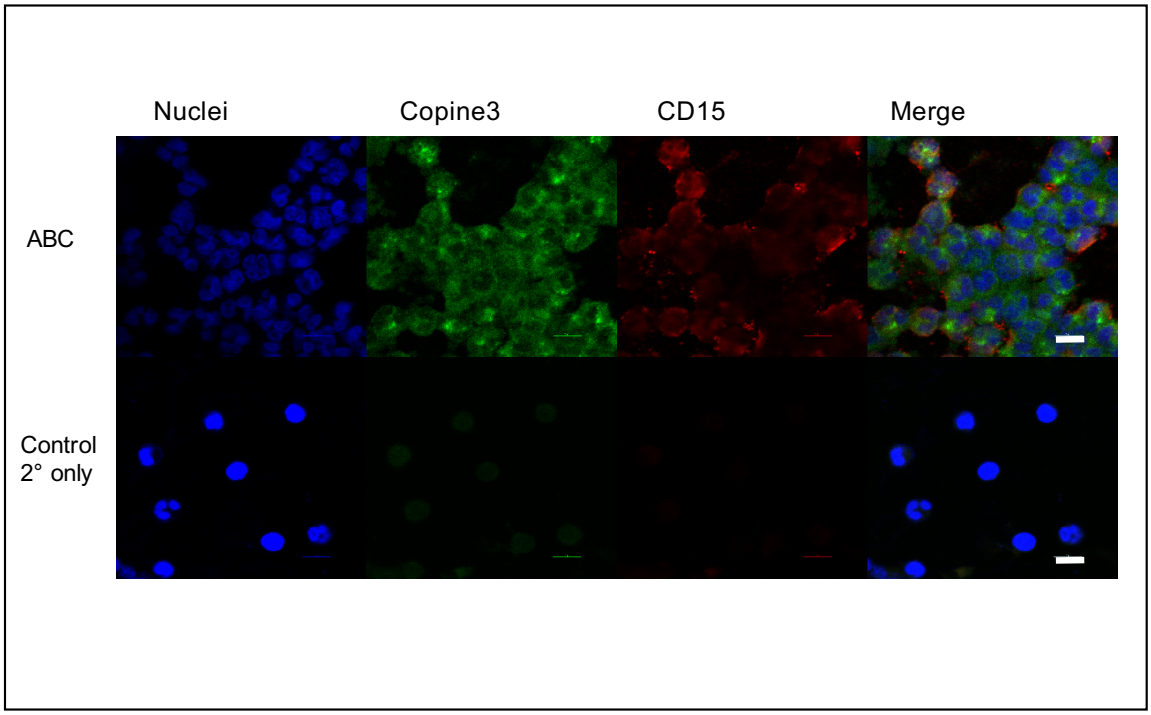


Figure 7.4 Immunofluorescence analysis of Copine 3 protein distribution in WBCs advanced breast cancer patients. WBC extracted from blood of advanced breast cancer patients were double stained with FITC-labelled anti-rabbit secondary IgG antibody (1:400) against Copine 3 primary antibody (dilution 1:200) and Tritc-labelled anti-mouse secondary IgG antibody (1:400) against CD15 neutrophil primary antibody (1:200), plus secondary only as the control. The blue staining shows the position of the nucleus. The green staining corresponds to Copine 3 protein and red staining represents Neutrophils. Scale bar 10μm. All images were taken by Confocal Scanning Microscope (Nikon) at 60X magnification.

7.3 Discussion

The precise role of the Copine family in the cells is currently unclear, although some studies reported that it influences the activities of membrane proteins and lipids (Perestenko et al. 2010). The existing reports indicate that Copine 3 interacts with ErbB2 to promote tumour metastasis and it also shown to be upregulated in breast and prostate tumours (C Heinrich et al. 2010). Recent studies demonstrate that Copine 3 is able to bind several nuclear proteins including Interleukin enhancer binding protein 2 and DNA topoisomerase 1 (Perestenko et al. 2010). As reported in the literature, normally copines show nuclear localisation, however in response to specific stimuli these proteins can re-localise to the plasma membrane (Perestenko et al. 2010) This is consistent with the IF results observed in the present study, where in WBCs of healthy donor the location of the Copine 3 appeared to be restricted to the cell nucleus, whereas in WBCs of primary and advanced breast cancer patients it showed cytoplasmic location as well as nuclear.

According to the IF results generated in the present study the majority of cells expressing Copine are neutrophils. Neutrophils contain toxic proteins that are securely stored in three granule types (azurophilic, specific and gelatinase granules) and secretory vesicles. During phagocytosis, the function of these organelles is highly dependent on the ions of calcium (Nunes & Demaurex 2010). The one of the proposed role for copines is providing calcium regulation of intracellular signaling pathways (Creutz & Edwardson 2009). Copine 3 is reported to translocate to the cell membrane in the response to the increase in intracellular calcium, where it interacts with various binding partners including ErbB2 (C. Heinrich et al. 2010).

The presence of Copine 3 in neutrophils was previously demonstrated. this study isolated Copine 3 from the cytosol of human neutrophils, where it exists as monomer and undergo conformation changes upon calcium binding (Cowland et al. 2003).

The results obtained in the present investigation show higher levels of Copine 3 in WBCs samples from advanced breast cancer patients, but not consistently. In the majority of the samples from cohorts of breast cancer patients as well as healthy donors it was not detected at all. The high variation in the levels of protein in different participant within the same group could be associated with difference in age, gender or ethnicity. Furthermore, lifestyle factors (e.g. diet, smoking, alcohol intake, obesity), underlying pathological conditions or certain medicine can have an effect on protein concentration (Rifai et al. 2006). Due to the inconsistency of the generated data for this target, its application as biomarker for breast cancer diagnosis and prognosis is questionable.

Differential expression of Copine 3 in peripheral blood was reported previously. In these studies, low expression of Copine 3 in peripheral blood was suggested to be an independent risk factor of occurrence of acute myocardial infarction among patients with stable coronary artery disease (Tan et al. 2018).

Copines are evolutionary conserved proteins present in protozoa, nematodes, plants and Mammalia (Creutz et al. 1998). Their ubiquitous expression in different organisms and high sequence conservation suggest the important role of these proteins in cellular processes (Li et al. 2010). The nuclear localisation of the majority of the copines points out their role in nuclear processes (Perestenko et al. 2010). The importance of studying this family of proteins is also supported by the recent report demonstrating interaction of Copine 3 with ErbB2 to promote breast tumour migration (C Heinrich et al. 2010). Furthermore, general role of Copine 3 in carcinogenesis is proposed as differential

expression of the protein is reported in normal tissue against breast, prostate and ovarian cancer (C Heinrich et al. 2010).

Chapter 8 Assessment of the presence of anti-BORIS autoantibody in the plasma of breast cancer patients and its potential as an early cancer biomarker

8.1 Background

The quest for novel cancer biomarkers is a continuing process, which involves huge efforts at different stages of the pipeline (discovery, validation and implementation). In addition, new molecular components and sources are considered in the search for biomarkers. The final Chapters 8 and 9 of this study represent pilot experiments to explore potential new biomarkers in plasma and urine samples from breast and prostate cancer patients, respectively.

BORIS, a paralogue of the transcription factor CTCF, is a member of the cancer-testis antigen (CTA) family (D'Arcy et al. 2008) CTCF is 11-ZF DNA binding protein that has been named "master waiver of the genome" due to its diverse role in the regulation of the chromatin structure (Kim et al. 2015). CTCF has been considered as tumour suppressor whereas BORIS demonstrates features of an oncogene (Marshall et al. 2014). BORIS is normally present at high levels in the testis; however, it is aberrantly expressed in various tumors and cancer cell lines. Example includes breast, prostate, ovarian, endometrial and other cancers (Kholmanskikh et al. 2008; Cuffel et al. 2011; Makovski et al. 2012). The presence of BORIS in tumours suggested that it can be potential biomarker of cancerous condition, which has been which has been supported by experimental findings (Cheema et al. 2014; K. Chen et al. 2013). However, there has been some controversy in the literature regarding BORIS function and expression in breast cancer and normal tissue (Tiffen et al. 2013; Rosa-Garrido et al. 2012; Hines et al. 2010). Interestingly, the circulating cell-free BORIS mRNA was recently reported in the blood of patients with breast cancer (Joosse et al. 2014).

In a previous study conducted in our laboratory, BORIS was detected in leukocytes of breast cancer patients, but it was absent in healthy donors (D'Arcy et al. 2008; Martin-Kleiner 2012).

Objectives of the present chapter:

The observation that BORIS is aberrantly expressed in various tumours but not in normal tissues led to a proposition that the appearance of BORIS may lead to generation of anti-BORIS autoantibodies which can be detected in blood plasma. The phenomenon of the autoantibody generation has been described previously for a number of proteins such as p53, HER2 and epithelial mucin 1 (MUC1) (Goodell et al. 2006; Disis et al. 1997; von Mensdorff-Pouilly et al. 1996). Furthermore, since their appearance is associated with the development of cancer they may be considered as potential cancer biomarkers (Zaenker et al. 2016). In this study, the presence of anti-BORIS autoantibody in plasma of breast cancer patients was evaluated with the aim to investigate whether anti- BORIS autoantibodies can serve as an early marker of breast cancer.

8.2 Results

Generation of specific autoantibodies in response of the appearance of tumour-associated antigens is one of the mechanisms of anti-tumour immune response, although its molecular basis is not well understood (Zaenker et al. 2016). We reasoned that anti-BORIS autoantibodies may be generated as part of such response mechanism at the early stages of cancer development and hence may be potential biomarkers for cancer detection. In order to explore this, we applied direct enzyme-linked immunosorbent assay (ELISA) with several optimization steps applied to measure the levels of anti-BORIS antibodies in plasma of breast cancer patients and healthy donors. The layout of ELISA assay is presented in Figure 8.1. It relies on BORIS protein expressed in cultured cells transfected with the BORIS-expressing plasmid (the results of transfection of 293T cell line with pCMV6 BORIS plasmid are presented in Figure 8.2). Anti-BORIS autoantibodies (AAb) were detected in all analysed plasma samples from breast cancer patients and healthy donors. The highest level of anti-BORIS AAb was detected in plasma from primary breast cancer cohort. Between healthy donors' samples and samples from the patients' cohort with metastatic (advanced) breast cancer no significant differences were observed (Figure 8.3).

Next, the presence of anti-CTCF autoantibodies was assessed in the same plasma samples as BORIS and CTCF share a similar zinc finger domain and therefore can potentially cross-react. First, the 293T cells were transfected with CTCF expressing vector, pCI-CTCF vector using JetPRIME transfection reagent; the results of the transfection are presented in Figure 8.4. The ELISA analysis of cell lysates with and without exogenous CTCF revealed the presence of the anti-CTCF antibodies at high levels in plasma from primary breast cancer cohort, but not in healthy donors and patients with metastatic (advanced) breast cancer (Figure 8.5).

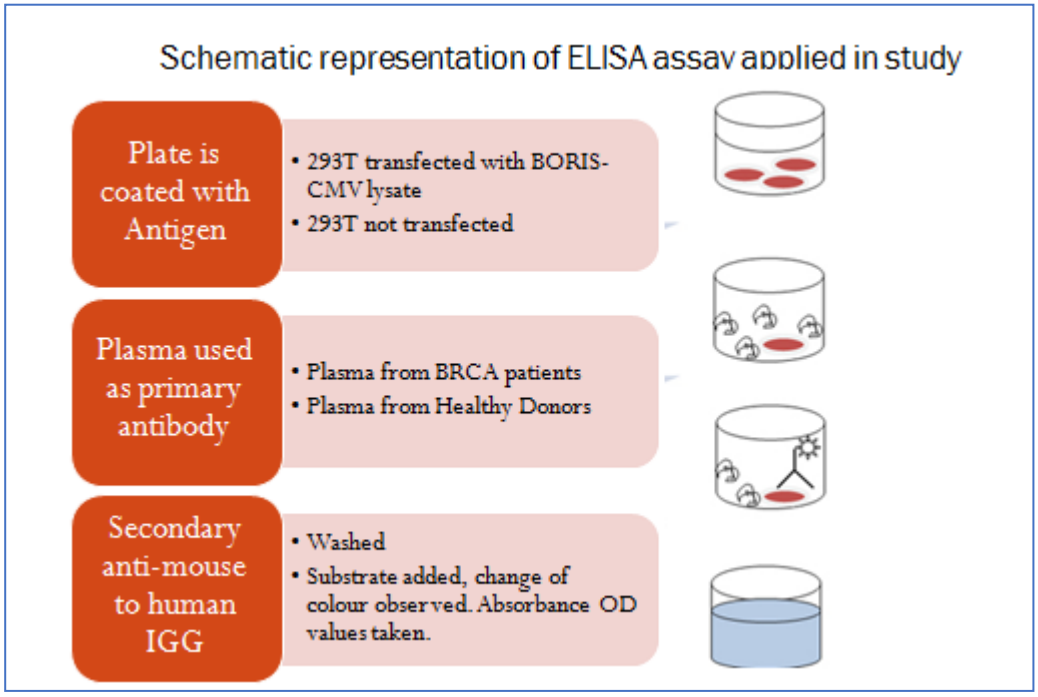


Figure 8.1 Represents ELISA assay employed in the preset study. Antigen was bound to the wells of ELISA plates in 50 µl/well of coating buffer. Antigen coated plates were sealed with plastic wrap and incubated overnight at room temperature. The next day, the coating buffer was removed by inverting the plates and the wells were blocked for 30 minutes with 200 µl/well of 1X blocking solution, prepared by diluting 10X blocking solution in distilled water. The blocking solution was removed by inverting the plates. Pure plasma samples were added and incubated for 1 hour with gentle agitation. Plates were washed 3 times with 1X washing buffer (prepared by diluting 10X wash buffer in distilled water) by filling all wells and then inverting the plates. Secondary antibody, was diluted an antibody diluent (1:15000 mouse monoclonal secondary antibody to human IgG HRP ab99765). Diluted secondary antibody was added to each well for 30 minutes at room temperature with gentle agitation. Plates were washed 3 times with 1X wash buffer. 100 µl/well of substrate solution were added and plates were incubated for 30 minutes at room temperature. Optical density readings were taken at single wavelength of 405 nm.

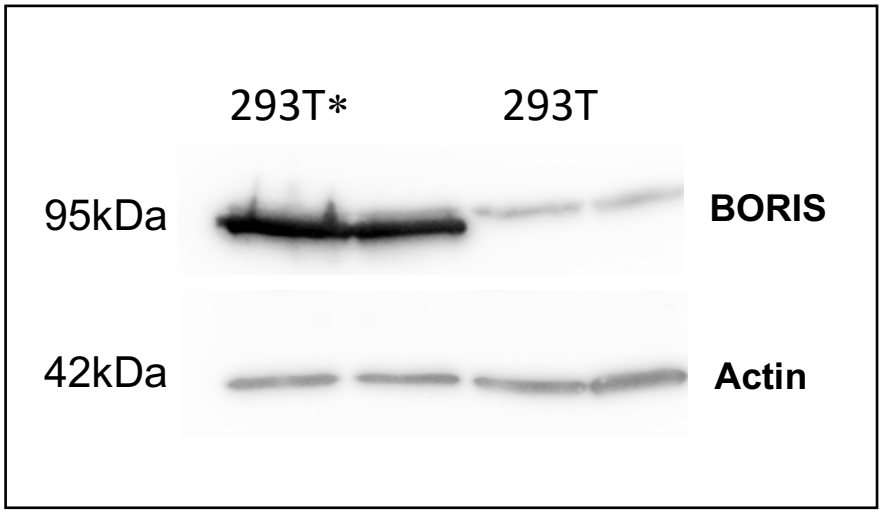


Figure 8.2 Western blot analysis of the transfection of 293T cells with pCMV6 BORIS plasmid. 293T∗ transfected with pCMV6 BORIS, 293T cells lysate non-transfected. For transfection jetPRIME transfection reagent from PolyPlus transfection was used. For the western blot hand-cast 8% polyacrimydil gel from BioRad was used. Total cell lysates were prepared and resolved in SDS-PAGE. Samples were heated prior the loading to a 90°C for 5 min, loaded in a volume of 20 µL per sample and separated by electrophoresis. In the next step, proteins were transferred from the gel to Immobilon-P nitrocellulose membrane (Millipore) with transfer buffer by semi-transfer method (10V, 200mA, 30 min) and membrane was incubated overnight in blocking solution (5% Tween, 50mM Tris Base). Next, membrane was probed with 1:1000 monoclonal anti- BORIS and 1:5000 anti-actin primary antibodies for two hours. Signal detection was performed using Luminata Forte (Millipore)

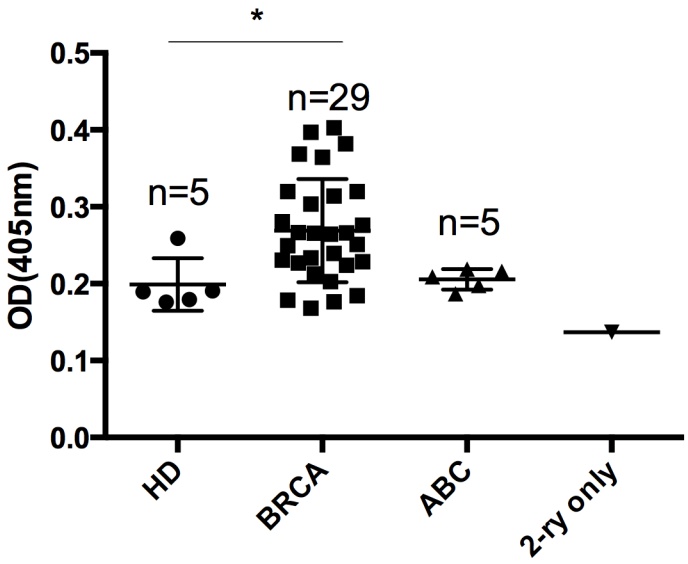


Figure 8.3 Presence of anti-BORIS autoantibody in plasma of breast cancer patients.

Represents ELISA results and shows the presence of anti-BORIS autoantibody in plasma of breast cancer patients and healthy donors. As control, secondary antibody only and lysate from transfected 293T cells were used. Absorbance was taken at 405 nm (measured as Optical Density, or OD).*P<0.05, HD – healthy donor, BRCA- primary breast cancer patients, ABC- advanced breast cancer cohort (a cohort of breast cancer patients treated for secondary metastases), 2-ry only -control, secondary antibody only

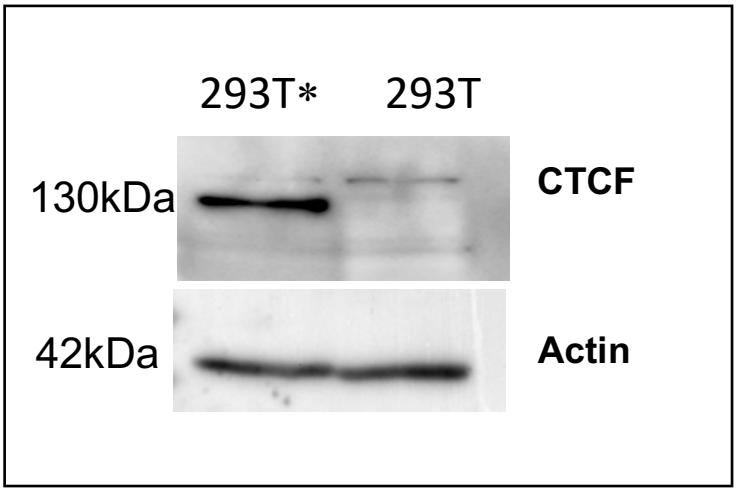


Figure 8.4 Western blot analysis of 293T cells transfected with the CTCF – expressing plasmid, pCI-CTCF. For transfection jetPRIME transfection reagent from PolyPlus transfection was used. For the Western blot hand-cast 8% polyacrimydil gel from BioRad was used. Total cell lysates were prepared and resolved in SDS-PAGE. Samples were heated prior the loading to a 90°C for 5 min, loaded in a volume of 20 µL per sample and separated by electrophoresis. In the next step, proteins were transferred from the gel to Immobilon-P nitrocellulose membrane (Millipore) with transfer buffer by semi-transfer method (10V, 200mA, 30 min) and membrane was incubated overnight in blocking solution (5% Tween, 50mM Tris Base). Next, membrane was probed with 1:1000 monoclonal anti-His Tag and 1:5000 anti-actin primary antibodies for two hours. Signal detection was performed using Luminata Forte (Millipore). **293T*** cell lysate from cells transfected with pCI-CTCF plasmid, **293T** cells lysate non-transfected.

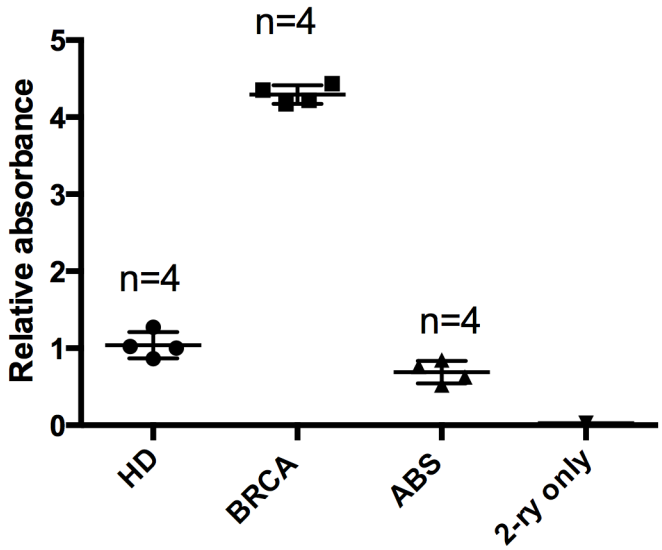


Figure 8.5 Presence of anti-CTCF autoantibody in plasma of breast cancer patients.

Represents ELISA results and shows the presence of anti-CTCF autoantibody in plasma from breast cancer patients and healthy donors. As control, secondary antibody only and lysate from non-transfected 293T cells were used.HD – healthy donor, BRCA- primary breast cancer patients, ABC- advanced breast cancer cohort (a cohort of breast cancer patients treated for secondary metastases).2-ry only -control, secondary antibody only

8.4 Discussion

It is recognised that autoantibodies (AAb) to tumour antigens are found in plasma of breast cancer-bearing patients (Tabernero et al. 2009; Anderson et al. 2010; Zhong et al. 2008; Wang et al. 2015). Although the process of their formation is not understood it is believed to be a part of natural immune response of the immune system to the appearance of the new antigen or antigen mutation, overexpression or degradation related to cancer. It is of particular of interest in breast cancer, as females are known to generate greater immune response than males.

BORIS has a potential for being biomarker for early diagnosis of breast cancer and since its appearance is linked to cancer it could induce an immune response leading to production of the anti-BORIS autoantibodies. The quantitative technique such as ELISA could be useful in medical practice for detection of such antibodies in plasma samples.

In the present experiment, the sandwich ELISA assay has been employed to assess the presence of anti-BORIS autoantibody in plasma samples from two cohorts of breast cancer patients (primary and advanced) and healthy donors. It was observed that the levels of autoantibody are higher in samples obtained from primary breast cancer patients in comparison with healthy donors'. Interestingly, level of anti-BORIS autoantibody in plasma samples from patients with advanced cancer appeared to be lower than in samples from primary breast patients.

These results correlate with data from similar studies. For example, in lung cancer the levels of AAb against Annexin1 in samples collected 1 year before the diagnosis were considerably higher than in already diagnosed individuals. Moreover their levels reported to increase with proximity to disease diagnosis (Brichory et al. 2001).

To explain the low levels of AAb in samples from patients with advanced stages of breast cancer, we hypothesize that anti-BORIS antibodies are generated only as an initial bodily response to cancer and its levels decrease with tumour progression. These results support previous findings conducted in our laboratory, measuring the expression of BORIS in leukocytes of cancer patients using western blotting technique (D'Arcy et al. 2008).

Similar results reported in reports studied antitumor immune responses against glioma by measuring serum anti-FLNC and demonstrating the decrease of the above AAb in high-grade gliomas or in a late stage of tumour progression. To explain these results, researches also measured numbers of the peripheral blood lymphocyte in both advanced and early stages of cancer and detected overall decrease in lymphocyte count in patients with higher grade of glioma. They proposed that lymphocytopenia may contribute to decrease in AAb formation (Adachi-Hayama et al. 2014).

ELISA technique relies on specific interaction between epitope specific sequence of amino acids found on antigen and corresponding to the antibody binding site. As BORIS and CTCF share a similar zinc finger domain and therefore can potentially cross-react, we analysed the presence of anti-CTCF autoantibodies in the same plasma samples. Data generated in our experiment indicate cross reactivity thus in a future to improve experiment reliability generation of mutant BORIS construct lacking ZF domain may be needed.

Chapter 9 Analysis of BORIS as urine based prostate cancer biomarker

9.1 BORIS (Brother Of the Regulator of imprinted Sites)-a potential cancer biomarker

BORIS, a paralogue of the transcription factor CTCF, is a cancer-testis antigen. Under normal condition is expressed during embryonic male germ development and in testis. *BORIS* transcript has been shown be aberrantly expressed in the majority of human tumours including prostate cancer (Kholmanskikh et al. 2008; Cuffel et al. 2011; Makovski et al. 2012; Cheema et al. 2014). Adding to the complexity of this gene, recent study identified 23 mRNA splice variants potentially encoding 17 variants of the BORIS isoform proteins (Pugacheva et al. 2010). In the study, conducted in our laboratory the BORIS expression at the protein and mRNA levels was reported in prostate tumours (Cheema et al. 2014). Urine is considered as a viable source of prostate-derived RNAs for biomarker discovery since it may contain materials (cells or exosomes) shed from tumours. As a part of the study conducted at the University of East Anglia (Pellegrini et al. 2017), the urine samples from prostate cancer patients were provided and tested in our laboratory to measure the levels of *BORIS* mRNA in these specimens.

9.2 Experimental Aim

This Chapter represents pilot experiments to explore the potential of *BORIS* mRNA as a urine biomarker for early diagnosis and prognosis of prostate cancer. In these experiments, the levels of *BORIS* mRNA were evaluated using RT-qPCR in total mRNA obtained from urine sediments and urinary exosomes of prostate cancer patients. The results were then correlated with the clinical information.

9.3 Results

9.3.1 Characterisation of RNA samples

In the present study, total RNA was isolated from white blood cells of the healthy donors and MCF7 breast cancer cell line to be used as negative and positive controls, respectively. Extraction of RNA from blood can be very challenging due to several factors. First, in order to preserve RNA from degradation, the stabilising reagent RNAlater was added to the sample prior the freezing. However, we found that RNAlater interfered with the RNA precipitation step, due to its high density. To address this issue, pure ice-cold water was added to the samples to reduce their density. The second reason is the low concentration of extracted RNA (10 million cells were used to obtain only around ~100ng of RNA). To solve this, the conventional phenol-chloroform RNA extraction protocol was modified with the addition of the extra chloroform steps and ethanol washing step.

Total RNAs isolated in the presented study were then subjected to the quality control using Agilent 2100 Bioanalyser. Prior to the assessment, all the samples were heated to 75°C for 2min in order to remove secondary structure. Data obtained using the Bioanalyser show the examples of good and low quality RNAs are presented in Figure 9.1. Clear 18S and 28S peaks with low noise in between and the absence of DNA contamination can be observed indicating high quality RNA in most samples (A). However, in some of the samples the various degree of degradation can be observed (B).

The concentration of extracted RNA was determined using Nanodrop ND 1000 Spectrophotometer. RNA has the light absorbance at 260nm and the ratio of the absorbance at 260nm and 280nm is used to assess its purity. The results indicate that the RNA isolated and used for the present study is pure.

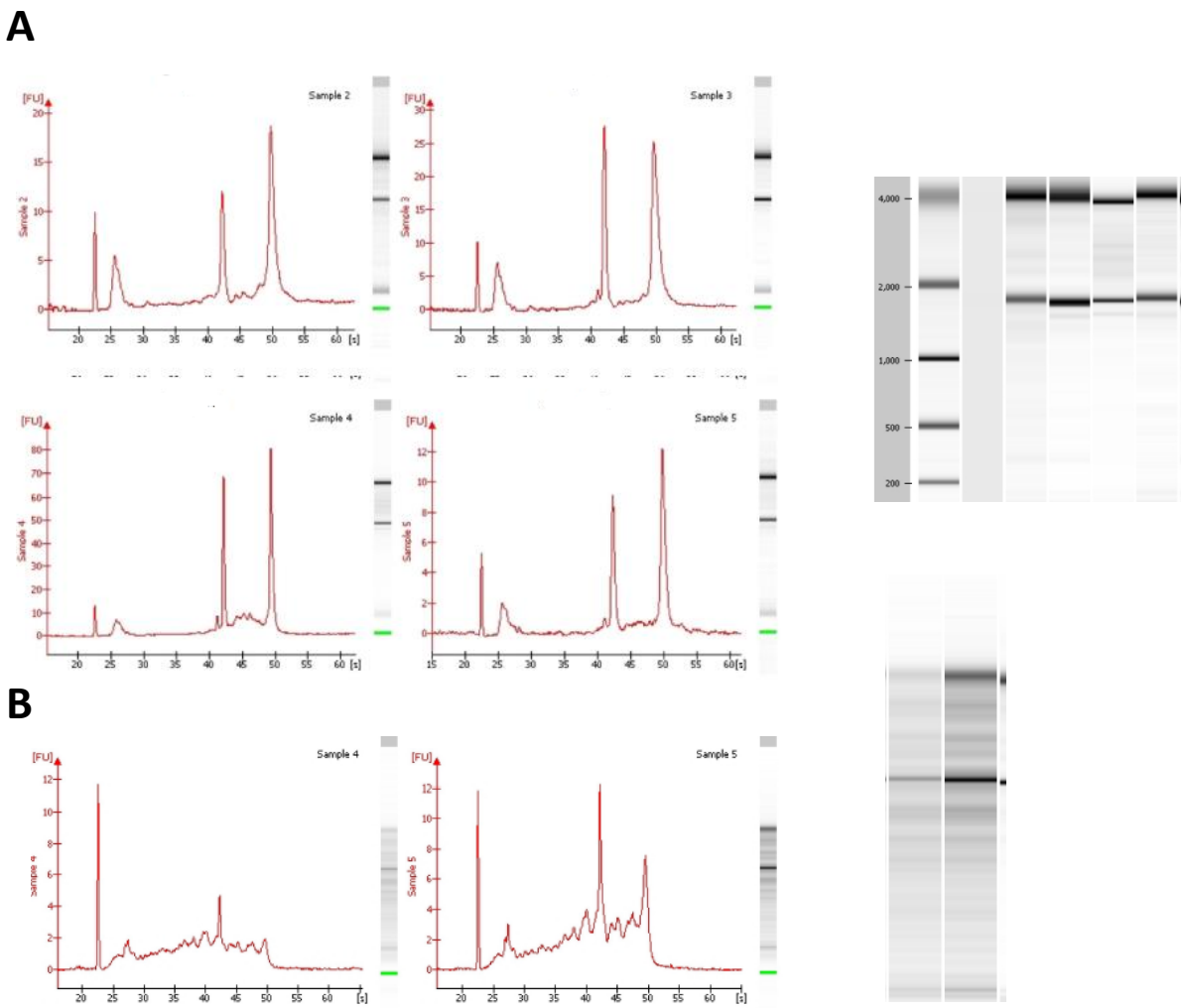


Figure 9.1 Assessment of total RNA integrity by Agilent 2100 Bioanalyser. Total RNAs were extracted from leukocytes of healthy donors and MCF7 breast cancer cell line as described in Material and Methods and analysed using Agilent 2100 Bioanalyser. **A,** electropherograms represent good quality RNA used in the present study and characterised by clear 18S and 28S peaks and two well separated bands. **B,** demonstrates results of degraded RNA characterised by the absence of clear peaks and smear like image on the gel.

9.3.2 Quantitative real time PCR analysis of BORIS mRNA expression levels in urine sediment and urinary exosomes obtained from prostate cancer patients with different disease stages.

It has been previously reported that BORIS-cancer testis antigen (CTA) is present in prostate tumours (Cheema et al. 2014). To further validate the potential practical applications of BORIS as a biomarker for early diagnosis and prognosis of prostate cancer here we employed RT-qPCR. In these experiments, the levels of BORIS mRNA were evaluated in the total mRNA obtained from urine sediment and urinary exosomes of prostate cancer patients.

The presence of BORIS was assessed in cDNA samples from urine sediment of individuals diagnosed with prostate cancer. The total of 67 samples was received from the University of East Anglia. As positive control the total RNA extracted from MCF7 cell line, MCF7 transfected with BORIS-CMV plasmid was used. It was not possible to obtain urine sediment samples from healthy donors in these experiments. Therefore, as negative control RNA extracted from the leukocytes of healthy donors, previously reported to be BORIS-negative was used. The obtained RT-qPCR results showed

First the levels of *BORIS mRNA* in samples from urinary sediment were analysed using SYBR qPCR (Figure 9.2). Next, the levels of *BORIS* mRNA were evaluated in total urinary exosomal mRNA. To detect all the 23 known mRNA variants of *BORIS* its expression was analyzed by quantitative RT-PCR with two sets of primers encompassing 1–2 and 3–4 exon boundaries, respectively (Zampieri et al. 2014).

The obtained results were grouped according to the disease stage and the levels of PSA and plotted using Prism GraphPad 5 software (Figures 9.3.and 9.4.). Consistent with the results obtained from RT-qPCR using SYBR the highest levels of BORIS mRNA were

detected in sample from patient with advanced prostate cancer. However, the rest of the data shows no correlation between the disease stage and the levels of BORIS mRNA.

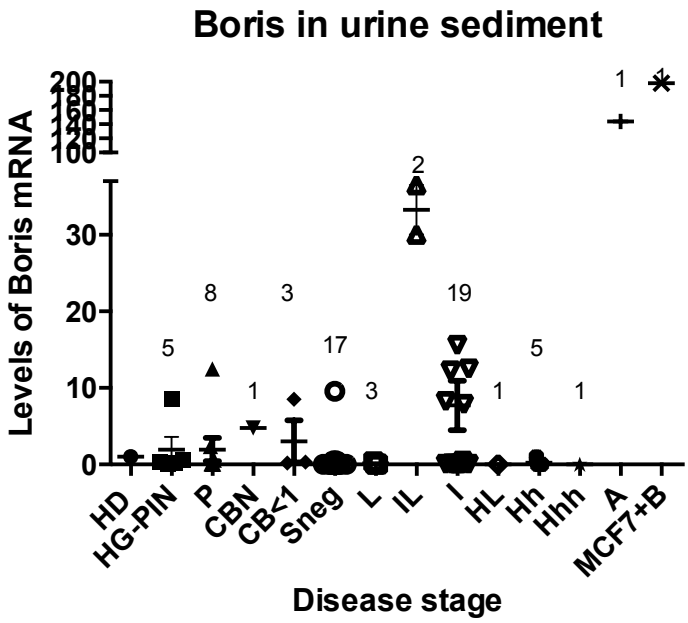


Figure 9.2 Quantitative real time PCR analysis of BORIS mRNA expression levels in urine sediment from prostate cancer patients in different disease stages. Real-time qPCR expression levels of BORIS mRNA were calculated using the comparative C_T method and normalised to GapDH expression. **HG-PIN**- atypia, **P**- prostatitis, **CBN-PSA** normal to age and clinical benign; **CB<1**- PSA<1 clinically benign, **Sneg-** raised PSA negative biopsy; **L**- Low risk PSA<10, **IL** – intermediate risk PSA>10; **I** Intermediate risk PSA≤20, **HL-**High risk, PSA>20;**Hh** High risk, PSA<100, **Hhh-**High risk, PSA>100, **A**-advanced disease. **MCF+B**-MCF7 transfected with BORIS-CMV plasmid, used as a positive control, **HD**-RNA from blood of healthy donor, used as a negative control

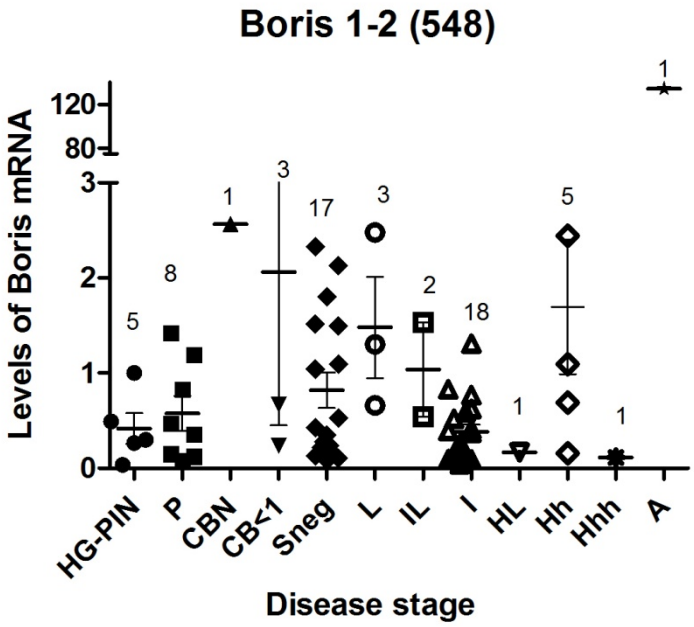


Figure 9.3 Quantitative real time PCR analysis of BORIS mRNA expression levels in urinary exosomes of prostate cancer patients. *BORIS* expression was analyzed by quantitative RT-PCR with set of primers encompassing 1–2 exon. Gap-DH was used as the reference gene in all the experiments. **HG-PIN**- atypia, **P**- prostatitis, **CBN-** PSA normal to age and clinical benign; **CB<1**- PSA<1 clinically benign, **Sneg-** raised PSA negative biopsy; **L**- Low risk PSA<10, **IL** – intermediate risk PSA>10; **I** Intermediate risk PSA≤20, **HL-**High risk, PSA>20; **Hh** High risk, PSA<100, **Hhh-**High risk, PSA>100, **A**-advanced disease

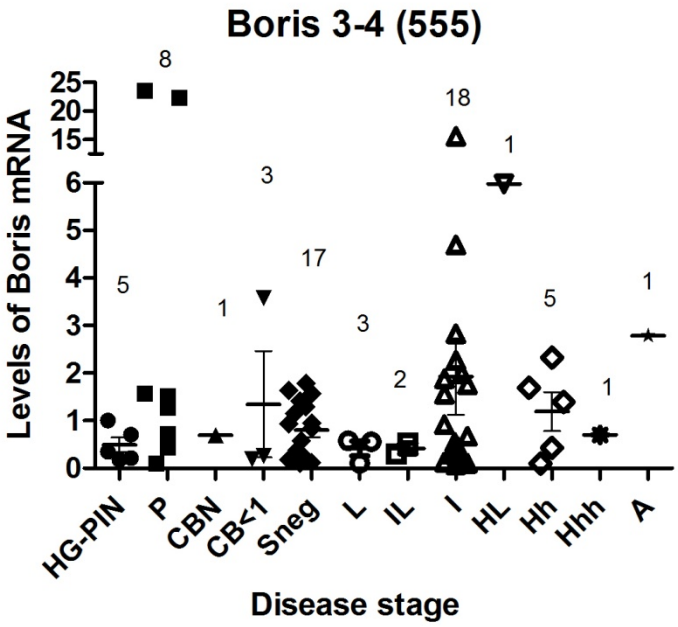


Figure 9.4 Figure: Quantitative real time PCR analysis of BORIS mRNA expression levels in urinary exosomes of prostate cancer patients. *BORIS* expression was analyzed by quantitative RT-PCR with set of primers encompassing 3-4 exon. Gap-DH was used as the reference gene in all the experiments. **HG-PIN**- atypia**; P**- prostatitis; **CBN-** PSA normal to age and clinical benign; **CB<1**- PSA<1 clinically benign, **Sneg-** raised PSA negative biopsy; **L**- Low risk PSA<10, **IL** – intermediate risk PSA>10; **I** Intermediate risk PSA≤20; **HL-**High risk, PSA>20; **Hh** High risk, PSA<100; **Hhh-**High risk, PSA>100, **A**-advanced disease.

9.4 Discussion

The aim of this chapter was to evaluate levels of *BORIS mRNA* in urine sediments and urinary exosomes of prostate cancer patients and investigate its possible utility as a prostate cancer biomarker. The qPCR data demonstrates that *BORIS* mRNA was detected in all analysed samples at different levels. The *BORIS mRNA* was present at the highest level in a sample with the advanced disease. Interestingly, both urinary exosomes and urine sediment from patient with advanced cancer show elevated levels of *BORIS mRNA*. Although these results were based on only one sample from a patient with advanced prostate cancer, they are in agreement with previous observations (Cheema et al. 2014) showing positive correlation between BORIS protein expression in prostate tumours and clinical data (tumour stage and Gleason score) and suggest potential application of BORIS as a prognostic marker of the aggressive prostate cancer. Given that there are 23 known mRNA variants of *BORIS* its expression was analyzed with two sets of primers encompassing 1–2 and 3–4 exon boundaries. The rest of the data shows no correlation between clinical information and BORIS levels.

Chapter 10: General Discussion

Breast cancer is a complex and heterogeneous disease, which makes the diagnosis and management of this condition a challenging task. Currently, mammography remains the main diagnostic tools for screening and detection of breast cancer. However, mammography has false negative rates of approximately 15 % which puts patients' lives in danger (Chan et al. 2015). False positive results are also common, causing over-diagnosis and unnecessary biopsy. In addition, mammography does not provide information regarding the potential malignancy of the tumour. Therefore, there is a need for biomarkers for screening, diagnosis and prognosis of breast cancer. Biomarkers currently used in clinical practice include cellular markers (HER2, ER and PR) that help to select for the patients who will benefit from the endocrine treatment and to predict the response to the HER2 targeting therapy. The humoral biomarkers (circulating tumour cells, CA 15-3 and CA 27-29) are used to monitor breast cancer progression and treatment efficacy. However, these biomarkers are not accurate in prediction of treatment response and are only recommended to be used in combination with cellular markers. Furthermore, they have not yet been approved for diagnostic and screening purposes. Hence, there is a continuous quest to identify novel accurate non-invasive molecular biomarkers for detection, prognosis and treatment monitoring of breast cancer.

The interaction between the tumour and the host's immune system is complex. This complex interaction has been studied extensively, largely focusing on the tumour microenvironment, which is characterised by abundant infiltration of immune cells. This study focuses on the changes in peripheral blood in the response to cancer. In particular the changes in protein profile of immune cells, which could be utilised as the source for novel breast cancer biomarkers.

The proteomics approach employed as one of the methods for identification of biomarkers for this study is very popular in finding novel cancer biomarkers and have advantage over gene expression studies as protein biomarkers are more preferable in clinical settings. The availability of well-developed antibody assays allows for their easier detection.

In the previous study conducted in Professor Klenova's laboratory, proteomic based strategies, namely 2D gel electrophoresis and the Orbitrap mass spectrometry, were applied to identify the differences in protein profiles of WBCs of breast cancer patients and healthy donors. These two techniques were used independently to identify overlapping proteins and 136 statistically significant differentially expressed proteins were discovered. Subsequent gene anthology analysis showed that 34 of those were involved in the immune response processes. Furthermore, it revealed that inflammation response was generally greater in WBCs of breast cancer patients, supporting the theory that inflammation favours tumour progression (Grivennikov et al. 2010; Elinav et al. 2013). The generated data was compared with publically available gene expression data from Gene Expression Omnibus (GEO) to identify a list of potential biomarkers. Fifteen candidate biomarkers were shortlisted involving criteria such as: normal presence in WBCs, correlation with clinical data, expression in healthy donors, association with cancer and function in immune response. To validate the biomarkers, RT-qPCR was used and the list was further reduced to five potential protein biomarkers which were subsequently chosen for extended validation. The following biomarkers were selected: Serpin B1, Lipocalin 2, Integrin α4, Copine 3 and 5LOX. It should be noted that the greatest obstacle in the validation of biomarkers is the lack of a standard protocol, while for drug discovery the validation protocols are well established. In this study, to validate the potential protein biomarkers a variety of the protein assays were employed such as Western blot, IF, FACS and ELISA. The ELISA based assay was used to measure the

expression of the above proteins in WBCs as well as in plasma obtained from the participants' blood. These techniques proved to be effective for the given task, as they successfully supported previously reported differential expression patterns of Serpin B1 and Lipocalin 2. For Copine 3, Integrin α4 and 5LOX, however, the data proved to be less consistent.

Of note, the assessment of mRNA levels of the candidate proteins is also often conducted and such experiments were performed in the previous study (Mani, PhD thesis, 2015). The summary of these analyses presented in Table 10.1 shows that mRNA levels do not correlate with protein levels in all cases. However, absence of correlation between levels of proteins and corresponding mRNAs is not surprising and has been reported in the literature (Tian et al. 2004; Vogel et al. 2010; Lundberg et al. 2010). In this study, the functional significance of the candidates and the initial analysis at protein levels, but not RT-qPCR data, were important for the selection of the candidates for further validation

Table 10.1 The summary of RT-qPCR results for five WBC biomarkers (Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5-Lipoxygenase). Genes common from different datasets comparing healthy donors, primary breast cancer patients and metastatic breast cancer patients were obtained. Fold change refers to fold change in primary breast cancer cohort. Red coloured cells indicate overexpression; green indicates under-expression. *T-test p value<0.05. The presence in metastatic breast cancer cohort is denoted by 'X' and the fold change is not included since the initial analysis did not include comparison with healthy donors. Adapted from J. Mani (J.Mani, PhD thesis, University of Essex, 2015) and Table 1.2 (this thesis).

Candidates	Primary Breast cancer-2D-gel electrophoresis	Primary Breast cancer-High throughput proteomics	Primary Breast cancer -Microarray (GSE16443)	Metastatic Breast cancer-2D-gel electrophoresis	Fold Change in RT-qPCR
Serpin B1	+1.3	+1.46		X	-1.16
Copine 3 (CPNE3)			+1.2	X	-1.47*
Integrin α4 (ITGA4)		-3.4	-1.28		-1.4*
Lipocalin 2 (LCN2)			+2.16	X	-1.35*
5-Lipoxygenase (5LOX)		+1.6			+1.28

Serpin B1 as a prospective biomarker

Using proteomic and genomic approaches Serpin B1 was identified as potential biomarker for breast cancer. Comparison of the expression profile of the WBCs of the healthy donors and breast cancer patients using 2D-PAGE revealed +1.3 and +1.46-fold change in the expression of the Serpin B1 protein in breast cancer patients respectively. Validation of these results using RT-qPCR showed downregulation of Serpin B1 on mRNA levels (Table 10.1) (Mani, PhD thesis, University of Essex, 2015). Data generated in the present study suggests that Serpin B1 has features of diagnostic and prognostic biomarker. Quantification of the above protein by the Western blot showed its elevation in WBCs of breast cancer patients. Serpin B1 levels were shown to be higher in patients with advanced disease. Furthermore, its expression levels were higher in patients with node positive status than in node negative participants. These results were statistically significant ($p<0.05$). Higher levels of Serpin B1 in primary breast cancer patients than in healthy donors indicate that this feature of Serpin B1 is associated with the presence of breast cancer and hence it can serve as an early biomarker of cancer. It will be important to test this property of Serpin B1 in the cohort of donors which include the pre-diagnostic samples; this will allow an unbiased validation approach.

Assessment of the predictive value of Serpin B1 in WBCs from patients with advanced breast cancer showed that low levels of the protein in the beginning of the treatment correlate with longer survival of the patients with chemotherapy treatment. These results suggest that Serpin B1 has a potential to be used as a marker of survival. However, Kaplan-Meier analysis of the combined data for the patients received chemotherapy and endocrine data did not show significant difference between lower or higher levels of the protein and the survival of the patients.

Analysis of the changes in the levels of Serpin B1 during the treatment did not show correlation with clinical response. Therefore, it can be concluded that Serpin B1 cannot be used as a predictive marker for the treatment efficacy.

The results from western blot analysis were confirmed by IF, were higher staining intensity was detected in WBCs of node positive primary breast cancer and advanced breast cancer cohorts. Interestingly, different distribution of the Serpin B1 was detected in WBCs of patients with different stages of the disease. In leukocytes of node negative and node positive breast cancer patients Serpin B1 was appeared to be present in vesicles at the cell surface. In samples from patients with advanced disease strong cytoplasmic localisation of the protein was observed.

The data from FACS analysis, measuring the expression levels of Serpin B1 in randomly selected primary breast cancer sample show almost twofold higher expression in comparison with a healthy donor. Furthermore, as determined by ELISA, Serpin B1 was elevated in plasma and WBCs from patients with both, primary and advanced breast cancer, in comparison with healthy donors.

The existing literature reports may help to explain the heightened expression of Serpin B1 in response to cancer. Serpin B1 belongs to the class of serine protease inhibitors. Proteases in healthy cells are important in carrying out biological processes. Their proteolytic activity is carefully regulated and balanced by the work of protease inhibitors, which if disturbed often leads to disease like cancer (Rakash 2012). The abhorrent activity of serine proteases contributes to pathology of immune diseases and cancer metastasis (Grassi et al. 2009; Heutinck et al. 2010; Blanco et al. 2005). Therefore, serine proteases are attractive targets in the field of biomarker research. One of the successful example of

the existing cancer biomarker that belong to the subgroup of serine proteases is PSA, used for the screening for prostate cancer.

Serine proteases that are expressed in endosomal vesicle of granulocytes are involved in different immune processes, such as inflammation, pathogen clearance and apoptosis. The release of neutrophil proteases during infection causes damage to the inflamed tissue. Given that proteases required for cell death, the role of serpins is to protect cells by inhibiting serine proteases. In particular, neutrophils express Serpin B1 that protect leukocytes from self-induced damage (Ashton-Rickardt 2010). Therefore, the data from the presented research, showing overexpression of Serpin B1 in WBCs of breast cancer patients, suggest that upregulation of this protease inhibitor may be a strategy to protect neutrophils from self-inflicting damage. These results are supported by studies on Serpin B1-deficient mice, which exhibit decreased viability of neutrophils (Charaf Benarafa et al. 2011; Burgener et al. 2016).

Collectively the data generated in the present study show that levels of Serpin B1 rises as disease progress, suggesting that it could be a marker of lymph node metastasis for primary breast cancer and a general advanced breast cancer marker of disease prognosis. The results confirm that Serpin B1 could be taken to the clinical validation phase of biomarker development pipeline. To determine the specificity of the Serpin B1 its expression has to be evaluated in other types of cancer. Functional studies may help to explain the molecular basis of the generated data.

Lipocalin 2 as prospective biomarker

Using proteomic and genomic approaches Lipocalin 2 was identified as potential biomarker for breast cancer. Comparison of the expression profile of the WBCs of the healthy donors and breast cancer patients using 2D-PAGE revealed +2.16-fold change

in the expression of the Lipocalin 2 protein in breast cancer patients. Validation of these results using RT-qPCR showed downregulation of Lipocalin 2 on mRNA levels (Table 10.1) (Mani, PhD thesis, University of Essex, 2015). The aim of the present study was to validate the previously reported findings. Prognostic value of Lipocalin 2 was evaluated using Western blot technique. The generated data showed significant overexpression of the target protein in both cohorts of breast cancer patients (primary and advanced) in comparison with healthy donors suggesting that Lipocalin 2 has properties of a diagnostic and prognostic breast cancer marker.

To assess the value of the Lipocalin 2 as the predictive biomarker of treatment efficacy in the cohort of advanced breast cancer patients, the initial levels of Lipocalin 2 was correlated with clinical response to treatment. The obtained results indicate that lower initial levels of Lipocalin 2 associate with better prognosis for the patients, as it correlated with the stable disease. Although the p value was not significant, the median of the category of patients with stable disease was lower when compared with less favourable clinical response categories. The evaluation of the changes in the Lipocalin 2 expression during the treatment indicate that the levels of protein decrease in response to endocrine treatment.

The comparison between initial levels of Lipocalin 2 and overall survival of the patients showed no correlation for both, chemotherapy and endocrine treatment.

The results from IF experiment, confirmed the data from Western blot analysis. The higher degree of staining for Lipocalin 2 was observed in patients with primary and advanced breast cancer in comparison with healthy donors. The quantification of the relative intensity revealed that these results are significant for both cohorts ($p<0.05$).

FACS analysis of the expression of Lipocalin 2 in neutrophils showed +2-fold higher expression ratio in randomly selected primary breast cancer sample when compared with healthy donor.

The data generated using ELISA technique supports the diagnostic and prognostic value of Lipocalin 2 protein. It showed significantly elevated levels of the protein of interest in primary breast cancer samples. Although, its levels in advanced breast cancer samples was shown to be elevated, but not significantly.

The reports available in the literature suggest that Lipocalin 2 has an oncogenic role. However, this role is contrasting and location dependant. This innate immune protein found to be upregulated in various inflammatory disorders. As a component of neutrophil granules, its required for the neutrophil function and plays essential role in innate immune response against bacterial infection (Liu et al. 2013; Toyonaga et al. 2016). Indeed, Lipocalin 2 deficient mice are shown to exhibit higher susceptibility to pathogens and higher mortality rate than the wild type (Flo et al. 2004). Furthermore, in the state of colonic inflammation, Lipocalin 2 deficiency contributes to intestinal carcinogenesis. This may be explained that in the absence of Lipocalin 2, the iron availability is increased facilitating the growth advantage for the certain pathogenic bacteria that promote inflammation and tumorigenesis (Moschen et al. 2016).

In colorectal cancer, the loss of Lipocalin 2 associates with increased tumour multiplicity, suggesting that it has a tumour suppressive role (Reilly et al. 2012). In contrast, in mice model of mammary tumorigenesis, inhibition of Lipocalin 2 supresses tumour formation and metastasis (Berger et al. 2010; Leng et al. 2011).

Integrin α4 as prospective biomarker

In the proteomic study previously conducted in our laboratory, Integrin α4 expression was shown to be decreased in WBCs of breast cancer patients. Validation of these results using RT-qPCR confirmed the downregulation of Integrin α4 on mRNA levels (Table 10.1) (Mani, PhD thesis, University of Essex, 2015).

The aim of the present study was to validate previous findings using various protein assays. The data generated using Western blot technique confirmed previously reported changes. The protein levels of Integrin α4 were found to be significantly lower in WBCs of primary and advanced breast cancer cohorts in comparison with healthy donors. Furthermore, in WBCs samples from advanced breast cancer the signal for Integrin α4 was not detected at all. It might be due to the very low levels of the above protein present in WBCs at the advanced stage of the disease. To further investigate these finding, the samples taken at different treatment time points were run on a gel to see whether signal will appear after the treatment received. As the result, the signal was detected at the later stages of the treatment. Integrin α4 is expressed in T- lymphocytes playing the key role in the immune response against tumour. The increased levels of T cells in breast cancer tissue is considered as a prognostic factor for disease outcome and it is positively associated with overall patient survival (Bates et al. 2006; Mahmoud et al. 2011). It is known that during the DNA- damaging chemotherapy the absolute number of lymphocytes is decreased (Onyema et al. 2015). Therefore, from the biological sense, the not detectable signal of Integrin α4 in western blot of advanced breast cancer patients could be explained by the low number of T cells in the WBCs sample.

The results from IF experiment indicate lower fluorescent intensity for the Integrin α4 in the samples from primary breast cancer patients, supporting the finding from Western

blot analysis. Similar trend was observed using FACS analysis. The expression of Integrin $\alpha 4$ in T cells of cancer patient was shown to be 1.22-fold change lower when compared to healthy donor.

Although the data generated so far showed that the protein levels of Integrin $\alpha 4$ is decreased in WBCs of breast cancer patients, the data obtained by ELISA experiment contradict the above results indicating the increased levels of Integrin $\alpha 4$ in the samples from breast cancer patients. The observed discrepancy in the results could be explained by the different antibody used in the commercial assays and, naturally, this aspect is beyond our control. Furthermore, the results of ELISA could be affected by the technical problem with the kit or protocol, or both. In the future, these experiments should be repeated with the use of another kit.

The inconsistency between the Western blot and ELISA data is not a new challenge. The combined usage of these two techniques provides more confidence in the positive results than either of them independently, especially if different antibody are used for each assays. The advantage of Western blot is that it less likely to give false positive results, in contrast, false-positive results are often observed in ELISA due to the cross reactivity or high background.

In summary the validation results for Integrin $\alpha 4$ showed conflicting results therefore it is difficult to evaluate biomarker property of this protein. It still remains an interesting candidate biomarker as its levels were shown to be decreased in the WBCs of cancer patients. The majority of the investigated biomarkers show elevated levels which makes it easier to investigate their levels in biological specimens. Specifically, decreased levels of Integrin $\alpha 4$ in primary breast cancer patients than in healthy donors indicate that this feature of Integrin $\alpha 4$ may be associated with the presence of breast cancer and hence

it can serve as an early biomarker of cancer. It will be important to test this property of Integrin $\alpha 4$ in the cohort of donors which include the pre-diagnostic samples; this will allow an unbiased validation approach.

5LOX as prospective biomarker

Using proteomic and genomic approaches 5LOX was previously identified as potential biomarker for breast cancer. Validation of these results using RT-qPCR confirmed the upregulation of 5LOX on mRNA levels (Table 10.1) (Mani, PhD thesis, University of Essex, 2015). In this study the heightened levels 5LOX in WBCs from breast cancer patients were confirmed by the Western blot analysis. However, the FACS and IF analyses showed the opposite results. The observed discrepancy in the data could be explained by the fact, that for FACS and IF experiments, the WBCs from a healthy donor with the highest 5LOX expression was used. It should be noted that later this person was later diagnosed with cancer. The use of the samples from other healthy participants in further experiments would be preferable for the validation of 5LOX as biomarker of breast cancer.

The importance of 5LOX in tumorigenesis has been well documented. For example, heightened expression of 5LOX by malignant cells has been reported to promote cell survival and proliferation and elevated levels of 5LOX have been shown in human cancer cell lines (colorectal, pancreatic, breast and prostate) (Gupta et al. 2001; Matsuyama et al. 2007; Wasilewicz et al. 2010; Jiang et al. 2006; Hennig et al. 2005) The knockdown of 5LOX gene induce cell death and 5LOX inhibitors have been demonstrated to induce apoptotic death in various cancer cells (Avis et al. 2001; Sarveswaran et al. 2011; G.-X. et al. 2015). Elevated levels of 5LOX in the plasma of patients at the late stages of breast cancer have been recently reported (Kumar et al. 2016). These observations and the

findings of the present study suggest the properties of 5LOX as a potential prognostic biomarker for breast cancer.

Copine 3 as prospective biomarker

Using proteomic and genomic approaches Copine 3 was previously identified as potential biomarker for breast cancer. The levels of this protein was found to be up-regulated in WBCs of breast cancer patients. Validation of these results using RT-qPCR showed downregulation of Copine 3 on mRNA levels (Table 10.1) (Mani, PhD thesis, University of Essex, 2015). To further verify previously reported findings, in this study Western blot and IF techniques were employed to study differential expression of Copine 3 in two breast cancer cohorts (primary and advanced) and healthy donors. The Western blot results revealed overexpression of Copine 3 in the advanced cohort. However, in almost 50% of the samples Copine 3 was not detected. In the primary breast cancer cohort, Copine 3 levels did not change when compared to healthy donors. Furthermore, the levels of this protein differ within the same cohorts. Due to this fact, the levels of Copine 3 was not measured in correlation with clinical outcome (e.g. survival and therapy response).

The data from IF, shows different localisation of Copine 3 in WBCs sample of both breast cancer cohorts (primary and advanced) and control, healthy donor sample. In WBCs sample of healthy donor the protein appeared to be present mainly in nucleus of the cell. Whereas in the primary and advanced WBCs breast cancer samples its cytoplasmic localisation can be observed. In the sample from the advance cancer patient, the intensity of the staining for Copine 3 seems to be higher than in healthy donor and primary patient. Based on these observations we conclude that due to the lack of consistency in the data obtained for Copine 3 levels in WBCs, this protein is unlikely to be a suitable biomarker

for breast cancer. However, Copine 3 still remains an interesting molecule to study in relation to mechanisms of breast tumorigenesis.

Copine 3 is a member of Ca^{++} dependent lipid-binding protein family, which in response to increase in intracellular Ca^{++} translocate to plasma membrane where they interact with the range of cell signalling proteins (Perestenko et al. 2010). One of the interacting partner of Copine 3 in breast cancer tumour is ErbB2 protein. It was suggested that Copine 3 is involved in ErbB2- dependent cancer cell motility because breast cancer cells decrease in ErbB2 dependent wound healing following Copine 3 knock down (C. Heinrich et al. 2010).

Cancer cells proliferation and apoptosis depend of intracellular Ca^{++} concentration. Furthermore, an elevation of intracellular Ca^{++} concentration is also an important requirement for the function of immune cells for the elimination of their target pathogens and cancer cells (Schwarz et al. 2013). These observation and the finding from the present study suggest that increased WBCs levels of Copine 3 in immune cells of cancer patients might be attributed to the accumulation of Ca^{++} in these cells with the aim to eliminate cancer cells.

Comparison of the biomarker properties of Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5-Lipoxygenase: Conclusions

The aim of this investigation was to identify, validate and assess the biomarker potential of five proteins differentially expressed in WBCs of patients with primary and advanced breast cancer. The results of the assessment of these proteins are summarised in Table 10.2. We conclude that despite the promise these proteins initially showed, not all of them passed more rigorous validation tests for different clinical utilities in breast cancer. Due to time constraints it was also not possible to characterise all five proteins to the same

extent. Since levels of Serpin B1, Lipocalin 2 and Integrin α4 change in the BRCA cohort in comparison with HD, they may serve as potential biomarkers for early diagnosis of breast cancer (individually or in a panel). The increase in the levels of Serpin B1 and Lipocalin 2 in the ABC cohort in comparison with the BRCA cohort suggests that these proteins can be used as biomarkers of disease progression. Furthermore, lower levels of Serpin B1 in the beginning of both chemotherapy and endocrine treatment correlate with complete response to treatment received. The lower initial levels of Lipocalin 2 can predict favourable response to chemotherapy and endocrine treatment received. These results suggest their utility as predictive biomarkers.

Table 10.2 The summary of the characteristics of five WBC biomarkers (Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5-Lipoxygenase). Levels of the proteins in BRCA and ABC cohorts are presented in comparison with the HD cohort. Red coloured cells indicate over-expression; green indicates under-expression. Increased intensity of the colours reflects the increased levels of the protein.
Adapted from J. Mani (Mani, PhD thesis, University of Essex, 2015).
Keys: BRCA – patients with primary breast cancer; ABC – patients with advanced breast cancer; HD – healthy donors; N.D. – not determined

Candidates	Cells positive for the biomarker	Levels in the BRCA cohort/diagnostic biomarker	Levels in the ABC cohort/biomarker of disease progression	Predictive biomarker	Biomarker of more favourable prognosis (survival)
Serpin B1	**Neutrophils**	**Yes**	**Yes**	**Yes**	**No**
Copine 3 (CPNE3)	**N.D**	**No**	**Yes**	**N.D**	**N.D**
Integrin α4 (ITGA4)	**T-cells**	**Yes**	**Yes**	**No**	**N.D**
Lipocalin 2 (LCN2)	**Neutrophils**	**Yes**	**Yes**	**Yes**	**No**
5-Lipoxygenase (LOX5)	**N.D.**	**No**	**No**	**N.D**	**N.D**

Utility of anti-BORIS autoantibody in the plasma of breast cancer patients

Early detection of breast cancer offers the patients greater chance of cure. In the present study, the presence of anti-BORIS autoantibody was assessed in plasma of primary breast cancer cohort to assess whether it can be used clinically as diagnostic marker of the disease. In this experiment, the quantitative analysis of BORIS expression using ELISA was applied. As the antigen, the lysate from 293T cells transfected with pCMV6 BORIS and overexpressing BORIS protein was used. The reason to use overexpressed protein instead of the recombinant was that it creates a model to study the antibody-antigen interaction in more biologically natural way. The plasma samples from primary breast cancer patients showed higher absorbance when compared to healthy donors, which was similar to the control (secondary antibody only). The obtained results demonstrate that using ELISA assay the anti-BORIS autoantibody can be detected in plasma of primary breast cancer patients and not in healthy donors. Knowing, that BORIS and CTCF share a similar zinc finger domain and therefore can potentially cross-react, to prove that the detected signal is specific to BORIS we analysed the presence of anti-CTCF autoantibodies in the same plasma samples. Data generated in our experiment indicate cross-reactivity therefore in the future the generation of mutant BORIS construct lacking ZF domain will be required to improve reliability of these experiments.

Potential application of *BORIS* as a biomarker of prostate cancer in urinary sediments and exosomes.

BORIS is a member of the cancer-testis antigen (CTA) family that is encoded by genes that are normally expressed only in germ line, but are also expressed in various tumours. CTA represent similarity between the processes of spermatogenesis and tumorigenesis (Wang et al. 2016). Aberrant *BORIS* expression is reported in several cancers and is

required for the proliferation of the malignant cells (Dougherty et al. 2008; Mkrtichyan et al. 2011; Gaykalova et al. 2012; Tiffen et al. 2013). Furthermore, *BORIS* expression is detected in the leukocytes of the breast cancer patients, suggesting a potential clinical significance of *BORIS* as a marker of tumorigenesis (D'Arcy et al. 2006).

According to reported findings, *BORIS* has 23 mRNA isoforms variants classified by 6 subfamilies and encoding 17 different peptides (Pugacheva et al. 2010). The expression of the isoform variants of *BORIS* appears to differ between the cancers (Asano et al. 2016; Yoon et al. 2016).

Expression of *BORIS* has also been reported in prostate tumours indicating potential diagnostic application of the protein as a biomarker of prostate cancer. Furthermore, the ability of *BORIS* to activate the AR gene is described as the mechanism for the growth and development of prostate tumours (Cheema et al. 2014).

Based on potential clinical significance of *BORIS* in prostate tumour tissue, in the present study the presence of BORIS mRNA in urinary sediment and exosomes of 67 prostate cancer patients was evaluated. The obtained data indicate high *BORIS* expression levels in the urine sediment from a patients with advanced prostate cancer. These results are consistent with the previously reported findings demonstrating positive correlation between *BORIS* protein expression in prostate tumour and the aggressiveness of the disease (Cheema et al. 2014). Elevated *BORIS* mRNA levels, which were 30 folds higher than in the negative control, were also detected in the group of patients with intermediate risk (PSA>10).

The qPCR data for the exosomal *BORIS* mRNA using set of primers encompassing 1–2 exon boundaries revealed that *BORIS* present at its highest in the sample from a patient

with the advanced prostate cancer. Two other groups that display high *BORIS* levels were intermediate (PSA>10) and high risk (PSA<100) groups.

The results from qPCR analysing *BORIS* mRNA in urinary exosomes using set of primers encompassing 3–4 exon boundaries showed high levels of *BORIS* in the groups of patients with intermediate (PSA<20), high (PSA>20) and advanced disease.

In conclusion the data presented here indicate that *BORIS* present at its highest in urine sediment and urinary exosomes of patients with advanced PCa and therefore has a potential as a PCa urine biomarker of the aggressiveness of the disease.

Several promising RNA-based urine PCa biomarkers are described in the literature. However only PCA3 is FDA approved and commercially available diagnostic test to be used in clinical settings. However, it is not routinely used in the hospitals as the accuracy of the test is not significant enough to warrant a biopsy (Mengual et al. 2016).

To improve the diagnostic accuracy of the disease, many studies now focus panel of biomarkers and prove that combined multiple markers outperform the use of the single marker (Laxman et al. 2008; Mengual et al. 2014; Rigau et al. 2010). Given the heterogeneity of PCa, the use of the multiple markers is a reasonable solution to improve current diagnostic limitations of the disease.

Conclusion for all aims

Aim 1

The main aim of the presented study was to assess the biomarker properties of five proteins: Serpin B1, Lipocalin 2, Copine 3, Integrin α4 and 5LOX using Western blot, IF, flow cytometry and ELISA techniques. In the results, significantly higher levels of Serpin B1 and Lipocalin 2, and lower levels of Integrin α4 were observed in WBCs of the BRCA

cohort compared to healthy donors suggesting their utility as early breast cancer biomarkers. Furthermore, levels of Serpin B1 and Lipocalin 2 increase and Integrin α4 decreased as the disease advanced, indicating their utility as biomarkers of poor prognosis.

Aim 2

In the secondary aim of the study, the utility of BORIS as diagnostic marker was investigated in plasma of breast cancer patients and urinary sediment and exosomes of prostate cancer patients. In the result, the presence of BORIS was demonstrated in plasma of breast cancer patients. Furthermore, the highest levels of BORIS mRNA were detected in urine of prostate cancer patient with the advanced disease. Suggesting, the potential of the BORIS for breast cancer diagnosis and its utility for prediction of the aggressiveness in prostate cancer.

Future work

Given that currently there are no ideal biomarkers available for diagnosis, prognosis and treatment monitoring of breast cancer, the quest to identify biomarkers for the above purposes continues. In the complex disease such as cancer a single biomarker only represents a part of the disease pathogenesis. Therefore, the use of a panel of biomarkers increase sensitivity and specificity of the test. In the present study, the panel of differentially expressed WBCs based biomarkers was validated.

In order for the most promising biomarkers to be applied in the clinical setting a series of human studies must be conducted (phase 1-4 trials). During phase 1 (exploratory phase) of clinical trials the diagnostic accuracy, sensitivity and specificity of a biomarker is determined from the receiver operating characteristic (ROC) curve. ROC is used to determine appropriate cut-off points. During phase 2 (challenge phase) the same group

of patients are traditionally used as in phase 1, but the given biomarker is assessed using the cut-off points determined from the phase 1 trials. The third phase aims to assess the performance of the biomarker in the target population across different geographical regions. At this stage the diagnostic value of the biomarker has to be evaluated by several independent laboratories. Phase 4 (outcome phase) assesses the utility of the biomarker with regard to the long-term health outcome of patients following clinical acceptance and/or commercial availability (Rifai et al. 2006).

In addition, clinical assessment of the protein assay will need to be applied to determine whether lifestyle factors (e.g. diet, smoking, obesity, alcohol intake, exercise) or the use of certain medication influence the change in biomarker concentration (Rifai et al. 2006).

Since levels of Serpin B1, Lipocalin 2 and Integrin α4 were significantly altered in WBCs of the BRCA cohort compared to healthy individuals, their utility as early breast cancer biomarkers should be tested in the cohort of donors which include the pre-diagnostic samples to allow an unbiased validation approach. Usually diagnostic biomarker studies utilise samples collected after diagnosis, hence the results could be affected by late-stage response to cancer. One of the examples of the current study using this approach is the assessment of plasma CA 125 ability to detect ovarian cancer (Menon et al. 2014).

Selected assays for optimisation of WBCs detection from whole blood

Immunohistological method. Whole blood smears from breast cancer patients and control group will be immunostained using the target biomarker-specufic monoclonal antibodies. This assay provides information about cell-sub-populations and phenotype of biomarker-positive cells. The disadvantage of this method is that it may not be easily adapted for clinical practice due to it slow throughput. Nevertheless such cell-based techniques are preferred by some diagnostic companies (e.g. DakoCytomation).

Immunofluorometric method. A flow cytometry approach can be applied to detect biomarkers in whole blood. The assay has an advantage of being high throughput and sensitive, however, this assay system would require rapid processing of fresh samples and would not be applicable to frozen samples. The protocol for such procedures are widely available and their potential utility has been demonstrated in the current investigation http://www.chemicon.com/resource/ANT101/a2C.asp

ELISA Detection of biomarker in whole blood using an ELISA format has a clinical advantage for high throughput test. Sandwich ELISA is a preferred option in clinical immunology and commercial kits are widely available (JOHNSTONE, A. THORPE 1987; Kemeny & Challacombe 1988). They were used in this investigation with varied results. Since this method detects solubilised antigens from purified WBCs, it will be important to further optimise the protocols for extraction of the antigen from WBCs. The alternative method is to solubilise the whole blood; in this case frozen samples can be used. If commercial kits are not available, the ELISA kit will need to be developed in house or in collaboration with commercial companies

In addition, in the future it would be interesting to investigate the changes in the functions of WBCs. This could be done by utilising primary breast cancer tissue and WBCs samples. The extract from tumour tissue could be applied to WBCs to induce changes related to cancer. To study these changes migration, apoptosis, survival assays could be used. Furthermore, the protein profile of the components of the tissue supernatant could be determined to identify molecules responsible for the induction of WBCs.

In addition, functional studies on the deletion of identified WBC-based protein candidate biomarkers maybe useful to explain molecular basis by which differential expression of identified proteins contribute to breast cancer tumorigenesis.

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