THE HULL YORK MEDICAL SCHOOL &
THE UNIVERSITY OF HULL

The Role of the Kinin-Kallikrein System in Chronic Lymphocytic Leukaemia.

Being a thesis submitted for the degree of
Doctor of Philosophy

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Abstract

Background:

Chronic lymphocytic leukaemia (CLL) is an incurable heterogeneous disease. The identification of reliable and cost effective biomarkers is therefore imperative. A comparative proteomic approach was previously employed to study protein expression changes associated with *in vitro* BCR ligation. Kininogen, a critical protein of Kinin-Kallikrein System (KKS) was found to be upregulated ($p \geq 2$) in 3/3 "high risk" clinical samples upon BCR stimulation. Both High and Low Molecular Weight Kininogens (HMWK and LMWK, respectively) serve as a substrate from which Plasma and Tissue Kallikreins liberate Kinins, which in turn act upon B_1 and B_2 kinin receptors. This project aimed to investigate the role of the KKS in CLL and to identify novel proteins which may have clinical relevance in this disease.

Materials and Methods:

KKS was investigated using CLL clinical samples and a range of methods such as immunoblotting, reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and flow cytometry as appropriate.

Results:

The up regulation of LMWK upon *in vitro* BCR stimulation was confirmed by immunoblotting in 3/3 CLL samples previously used in a discovery phase proteomics. In a pilot series of 52 unselected CLL samples, 71% demonstrated basal LMWK expression. A total of 18 of these samples were also analysed for

LMWK transcript, which was not detected in any of these samples. The expression of Kallikrein 6 was confirmed in 25 CLL samples. The B_1 and B_2 receptors were identified in 15 and 3 CLL samples, respectively. An elevated Bradykinin level was demonstrated in 27/36 (75%) plasma samples from CLL patients and was found to be associated with untreated (p=0.039) stage A (*p=0.03*) CLL and increased Plasma Kallikrein *(p=0.001).*

Discussion:

It has been demonstrated for the first time that CLL cells express the components for KKS signalling pathway, which can be further investigated for clinical relevance.

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1. **KASHUBA, E**., BAILEY, J., ALLSUP, D. & CAWKWELL, L. 2013. The kinin-kallikrein system: physiological roles, pathophysiology and its relationship to cancer biomarkers. *Biomarkers,* 18**,** 279-96.
2. **KASHUBA, E**., EAGLE, G. L., BAILEY, J., EVANS, P., WELHAM, K. J., ALLSUP, D. & CAWKWELL, L. 2013b. Proteomic analysis of B-cell receptor signaling in chronic lymphocytic leukaemia reveals a possible role for kininogen. *Journal of proteomics,* 91**,** 478-85.
3. **KASHUBA, E**., EAGLE, G. L., BAILEY, J., ALLSUP, D. & CAWKWELL, L. Identifying novel prognostic biomarkers in Chronic Lymphocytic Leukemia using proteomics: a possible role for Kininogen. Haematologica 2012; 97 (suppl 1): 55. ***Abstract*** - Presented at the 17th Annual European Hematology Association (EHA) congress (June 2012, Amsterdam).
4. **KASHUBA, E**., EAGLE, G. L., BAILEY, J., ALLSUP, D. & CAWKWELL, L. The Kinin-Kallikrein System in Chronic Lymphocytic Leukemia - A Potential Target for Therapy?. Blood *2012; 97 (suppl 1): 55*. ***Abstract*** - Presented at the Annual American Society of Hematology (ASH) meeting (December, 2012, Atlanta, USA).
5. **KASHUBA, E**., EAGLE, G. L., BAILEY, J., ALLSUP, D. & CAWKWELL, L. A proteomics approach for prognostic biomarkers discovery in Chronic Lymphocytic Leukaemia: A possible role for Kininogen. ***Abstract*** for HYMS Research Network Conference (March, 2012 York, UK).

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Elena Kashuba

Declaration

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources. I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

List of Abbreviations

ACE	Angiotensin converting enzyme
ALC	absolute lymphocyte count
A	Alemtuzumab
BK	Bradykinin
ß2M	ß2-microglobulin
BTK	Bruton's tyrosine kinase
Bcl-2	Lymphoma/leukemia-2
Bcl-XL	B-cell lymphoma-extra large
BCR	B-cell Receptor
BLNK	B-cell linker protein
BM	Bone marrow
bp	Base pair
BSA	Bovine serum albumin
BSC	Biological Safety Cabinet
CD	Cluster determinant
CGH	Comparative genomic hybridization
CHOP	Cyclophosphamide, Doxorubicin, Vincristine and Prednisalone
Clb	Chlorambucil
CK1	Cytokeratin 1
CLL	Chronic lymphocytic leukaemia
CLLU1	Chronic lymphocytic leukemia up-regulated 1
CO_2	Carbon dioxide
CPN	Carboxypeptidase N
CPM	Carboxypeptidase M
CR	Complete response
CSR	Class Switch Recombination
CT	Computed tomography
CVP	Cyclophosphamide, Vincristine and Prednisalone
Del17	Deletion of chromosome 17p

DEPs	Differentially expressed proteins
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
ECACC	European Collection of Cell Cultures
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERK	Externally regulated kinase
ESMO	European Society for Medical Oncology
FACS	Fluorescence Activated Cell Sorter
FCR	Fludarabine, Cyclophosphamide, Rituximab
FCS	Foetal calf serum
FCRL2	Fc receptor-like 2
FDCs	Follicular dendritic cells
FGF	Fibroblast growth factor
FISH	Fluorescent in-situ hybridisation
FOXO	Forkhead Transcription Factors
FS	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Germinal center
GEP	Gene expression profiles
GPCR	G protein-coupled receptors
GSK3	Glycogen synthase kinase-3
GUSB	Glucuronidase-β
H_2O	Water
Hb	Haemoglobin
HCl	Hydrochloric acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMBS	Hydroxymethylbilane synthase
HMWK	High Molecular Weight Kininogen

HMDS	Haematological Malignancies Diagnostic Service
HPRT1	Hypoxanthine guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HYMS	Hull York Medical School
ICAM-1	Intercellular Adhesion Molecule 1
ICC	Immunocytochemical
IRF4	Interferon regulatory factor 4
ITAM	Immunoreceptor tyrosine based activation motif
IgV_H	Immunoglobulin heavy chain variable region
IWCLL	International Workshop on Chronic Lymphocytic Leukaemia
kDa	Kilodalton
KKS	Kinin-Kallikrein System
KLK	Kallikrein
KNG	Kininigen
LDT	Lymphocyte doubling time
LMWK	Low Molecular Weight Kininogen
Lys-BK	Lys-Bradykinin
mA	Milliamperes
Mac-1	Macrophage-1
MACS	Magnetic-Activated Cell Sorter
MALDI	Matrix assisted laser desorption / ionisation
MAPK	Mitogen-activated protein kinase
MBL	Monoclonal B-lymphocytosis
MCL	Mantle Cell Lymphoma
MDM2	Mouse double minute 2 homolog
mg	Milligram
MIF	Migration inhibitory factor
mL	Millilitre
mM	Millimolar
MRD	Minimal Residual Disease
MRPL19	Mitochondrial ribosomal protein L19 gene
MS	Mass spectrometry

MSC	Mesenchymal stromal cells
mTOR	Mammalian target of rapamycin
MW	Molecular weight
MZ	Marginal zone
m/z	Mass to charge ratio
NCI-WG	National Cancer Institute-Sponsored Working Group
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NEP	Neutral endopeptidase
NHS	National Health Service
NKG2D	The Natural Killer Group 2D
OD	Optical density
ORR	Overall response rate
OS	Overall survival
P	Progress
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate buffered saline
PD	Progressive disease
PE	Phycoerythrin
pERK	Phosphorylated Extracellular Regulated Kinase
PFS	Progression-free survival
PI3Kδ	Phosphoinositide 3-kinase δ
pI	Isoelectric point
PI3K	Phosphatidylinositol 3 kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
pg	Picogram
PK	Prekallikrein
PKC	Protein kinase C
PL	Prolymphocytes
PLC-γ2	Phospholipase C-γ2

PMA	Phorbol 12-myristate-13-acetate
PR	Partial response
PRCP	Prolylcarboxypeptidase
PTM	Post translational modification
PRMI	Roswell Park Memorial Institute
R	Rituximab
RIA	Radioimmunoassay
RIC allo SCT	Reduced-intensity conditioning allogeneic stem-cell
ROC	Receiver Operating Characteristic
RT-PCR	Reverse transcription polymerase chain reaction
SCT	Stem cell transplantation
SD	Stabile Disease
SDS	Sodium dodecyl sulphate ($CH_3(CH_2)_{11}SO_4^-Na^+$)
SEER	United States Surveillance, Epidemiology, and End Results
SHM	Somatic Hypermutation
sIgM	Surface IgM
SNPs	Single nucleotide polymorphisms
SS	Side scatter
Syk	Spleen Tyrosine Kinase
TBP	TATA box binding protein gene
TBS	Tris buffered saline
TLK	Toll-Like Receptors
TOF	Time of flight
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TSP-1	Thrombospondin-1
TTFT	Time to first treatment
TWEEN	Polyethylene glycol sorbitan monolaurate
uPAR	urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
UBC	Ubiquitin C
WCC	White cell count

WHO	World Health Organization
ZAP70	Zeta associated protein 70
µg	Microgram
µl	Microlitre
µM	Micromolar
2DE	Two-dimensional gel electrophoresis

CHAPTER 1

Understanding Chronic Lymphocytic Leukaemia

Chapter 1. Understanding Chronic Lymphocytic Leukaemia

1.1 General Introduction to Chronic Lymphocytic Leukaemia

Chronic Lymphocytic Leukaemia (CLL) is defined as a lymphoproliferative disorder characterised by the progressive accumulation of antigen-experienced malignant CD5+/CD23+ CLL-cells which genetically and immunophenotypically resemble memory B cells in the peripheral blood, bone marrow, lymphoid tissue and spleen (Klein *et al*., 2001, Eichhorst *et al*., 2011). Over the years CLL has been viewed as a B-cell malignancy associated with the accumulation of lymphocytes with a low proliferative index, blocked in early phases (G0/G1) of the cell cycle (Scielzo *et al*.) which were intrinsically resistant to senescence and apoptosis (Chiorazzi, 2007, Damle *et al*., 2010). However, more recent studies have challenged these postulates, describing CLL as a disease of both cell proliferation and cell accumulation (Messmer *et al*., 2005, Grabowski *et al*., 2005, Damle *et al*., 2010). Moreover heavy-water labelling experiments have shown that CLL is a dynamic rather than static disease and represents an example of a malignancy with a relatively fast cell turnover, where B-cells birth rate reaches up to 2% of the entire population per day (Messmer *et al*., 2005, Hillmen, 2012).

1.2 Epidemiology of Chronic Lymphocytic Leukaemia

CLL is the most common adult leukaemia in the USA and Western Europe, which is characterised by more frequent incidences in men rather than women;

significant ethnic variation towards Caucasians (Shvidel *et al*., 1998) and considerably lower rates in Asians in comparison to whites and blacks (Morton *et al*., 2006). CLL represents about 25%-30% of all adult Leukaemia cases worldwide (Keating, 2002, Chiorazzi *et al.,* 2005). The recent data from the United States Surveillance, Epidemiology, and End Results (SEER) Registry predicted that in 2013 about 15,680 cases in U.S. (9,720 men and 5,960 women) would be diagnosed with CLL and the number of deaths caused by this disease could reach 4,580 cases (National Cancer Institutes at the National Institutes of Health, 2013). Around 2,800 people are diagnosed with CLL in the UK each year (Cancer research UK, 2013) and this accounts for approximately 11% of all haematological neoplasm's newly diagnosed in the UK. The incidence of CLL varies greatly among gender, racial and ethnic groups (Table 1-1).

Table 1-1 The CLL Incidence Rates by Race in U.S.

This Table demonstrates the CLL incidence rates by race in U.S. between 2006-2010 (National Cancer Institutes at the National Institutes of Health, 2013). This data indicates that a higher incidence occurs in White males in comparison to Black/Asian males and that the ratio of females in each ethnic group is significantly less than the males.

Ethnic Group	Male per 100,000	Female per 100,000
White Race	6.3	3.3
Black Race	4.2	2.0
Asian/Pacific Islander Race	1.4	0.7

The CLL incidence rates per 100,000 populations estimated between 2004-2011 in the UK were: 8.9 per 100,000 males and 5.0 per 100,000 females with

Male/Female rate ratio of 1.8. In USA and UK the median age at diagnosis for CLL was reported to be 71 years of age (National Cancer Institutes at the National Institutes of Health, 2013, Haematological Malignancy Research Network, 2013) The age-adjusted incidence rates representative of UK CLL cases is demonstrated in Figure 1-1.

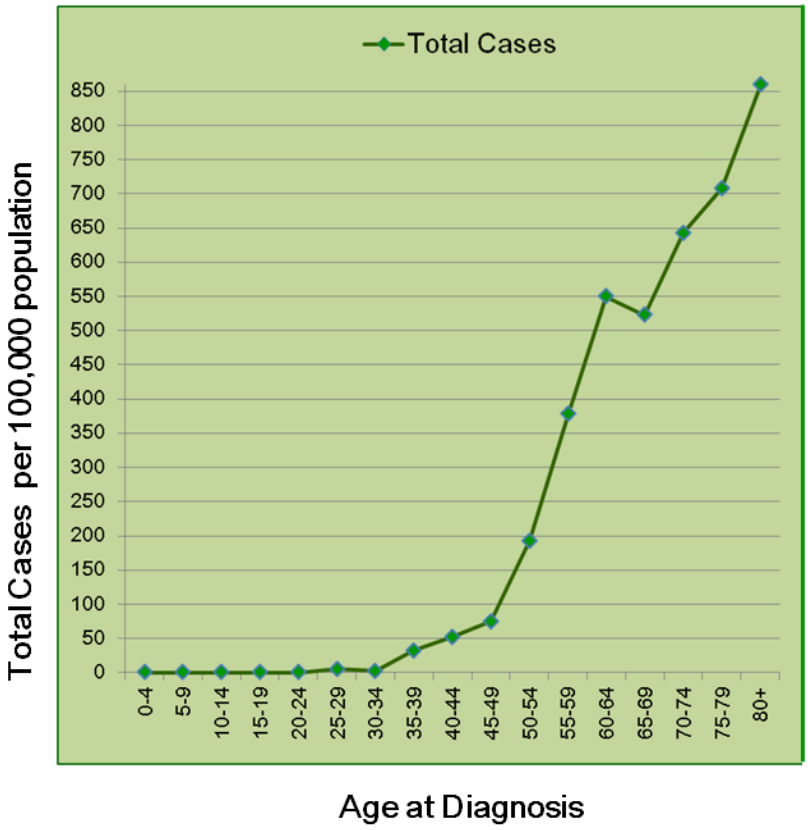


Figure 1-1 Estimated Age Specific Incidence per 100,000 UK population

The chart demonstrates the estimated age specific incidence of CLL cases in the UK, between 2004 and 2010, using data from Haematological Malignancy Research Network at www.hmrn.org [accessed 04/2013].The incidence increases dramatically over the age of 50.

Notwithstanding the therapeutic advances, early diagnosis and improved survival the disease still remains incurable and the USA data reported that the median age at death for patients with CLL is 79 years (Gribben, 2010).

1.3 An Introduction to Clinical Aspects of Chronic Lymphocytic Leukaemia

1.3.1 Clinical Presentation of Chronic Lymphocytic Leukaemia

The course of CLL resembles other chronic haematological malignancies and is characterised by a stepwise progression. It is often difficult to identify the onset of the disease. Asymptomatic disease is present in approximately 25% of CLL patients (Abbott, 2006, Inamdar and Bueso-Ramos, 2007). CLL is often diagnosed incidentally during routine medical examination or visits to physicians. The symptoms such as fever, weight loss and night sweats; mild to severe lymphadenopathy of cervical, supraclavicular and/or axillary nodes; hepatomegaly and/or splenomegaly; defective immunologic resistance are frequent complaints of CLL patients (Inamdar and Bueso-Ramos, 2007). When disease progression leads to anaemia and thrombocytopenia caused by bone marrow infiltration and replacement by tumour cells, it results in bleeding and bruising. Continuous respiratory, urinary tract bacterial and viral Infections are the leading contributing factors to death in CLL patients (Molica, 1994).

1.3.2 Criteria for diagnosis

The Guidelines for the diagnosis and treatment of CLL were originally presented by the National Cancer Institute-Sponsored Working Group (NCI-WG) in 1988. Later, when substantial progress in discovery of new prognostic tools, diagnosis and treatment was made, the Guidelines were updated in 1996 by the International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) (Hallek *et al*., 2008a). The diagnosis of CLL is based on the criteria which were framed by International Workshop on CLL guidelines and are as follows:

- The presence of ≥5x10^9/L of monoclonal B-lymphocytes in the peripheral blood sample of unexplained aetiology for at least 3 months.
- The immunophenotypic conformation of circulating B cells is required utilising flow cytometry. A CLL cell is characterised by the positive expression of CD5, CD23 antigens with low level of surface Immunoglobulin (sIg) and CD22/CD79b expressions. CLL cell is restricted to expression of either kappa (κ) or lambda (λ) Ig light chains (Table 1-2) (Hallek *et al*., 2008a).
- The malignant CLL cells found in a blood smear morphologically would be described as small, round, mature lymphocytes with compressed chromatin clumping and cyan cytoplasm or with open nuclear and narrow border of cytoplasm (Eichhorst *et al.*, 2011)

The development of flow cytometry has led to a significant lowering of the absolute lymphocyte count (ALC) required for a diagnosis of CLL (Molica *et al*., 2011a). The identification of a characteristic immunophenotype assists with

differential diagnosis between CLL and other lymphoma entities (Rawstron *et al*., 2002) including Mantle Cell Lymphoma (MCL) (Table 1-2).

Table 1-2 Immunophenotypic Characteristic of CLL B Cell and Differential Diagnosis between CLL and MCL

The data utilised in this Table was extracted from (Matutes and Polliack, 2000, Kay et al., 2002, Inamdar and Bueso-Ramos, 2007). The highlighted immunophenotypic markers are required for diagnosis of CLL. CD23 positivity can assist in making the clinically important differential diagnosis between CLL and Mantle Cell Lymphoma.

Immunophenotypic markers	CLL	MCL
Surface immunoglobulin	**Weak**	Positive
CD 5	**Positive**	Positive
CD 10	Negative	Negative
CD 19	Positive	Positive
CD 20	Positive	Positive
CD 22	**Weak or absent**	Positive
CD 23	**Positive**	Negative
CD 79b	**Weak or absent**	Positive
FMC 7	**Negative**	Positive

Immunophenotyping is an essential element in the diagnosis of CLL and other lymphoproliferative disorders. As Table 1-1 indicates CLL and MCL share the majority of phenotypic characteristics and therefore differential diagnosis can be challenging (Palumbo *et al*., 2009). Moreover in rare cases of MCL characterised by positive CD23 expression, immunohistochemical cyclin D1 staining or fluorescence in situ hybridization (FISH) are required for diagnosis (Eichhorst *et al*., 2011).

In the World Health Organization (WHO) classification, CLL and small lymphocytic lymphoma (SLL) aetiologically and pathogenetically are considered as the same underlying disease (Eichhorst *et al*., 2011), but with slightly different clinical presentation. The diagnosis of SLL is based on the presence of peripheral blood B lymphocytes, which immunophenotypically resemble CLL B-lymphocytes and do not exceed $5x10^9$/L, complemented by lymphadenopathy and / or splenomegaly (Eichhorst *et al*., 2011).

Bone marrow examination indicating the presence of more than 30% lymphocytes within all nucleated cells is no longer necessary for CLL diagnosis; but still remains as required criteria for CLL staging (Section 1.3.4) and for response assessment after therapy (Inamdar and Bueso-Ramos, 2007).

1.3.3 Morphology of CLL B-lymphocytes

CLL is characterised by the presence of mature but functionally incompetent memory B-lymphocytes (Eichhorst *et al*., 2011). The morphology of CLL cells is heterogeneous (Bennett et al., 1991) but in the vast majority of CLL cases the morphology of circulating B-cells is typical (Figure 1-2). Approximately 15% of CLL

patients express atypical morphology at the time of diagnosis or during the disease course (Matutes and Polliack, 2000). The atypical morphological repertoire is characterised by either elevated level of prolymphocytes (an immediate precursor of a lymphocyte) (CLL/PL>10%) or circulating lymphoplasmacytic (B cells developing into plasma cells) and cleaved cells (centrocytes) (>15%) (Matutes and Polliack, 2000). A number of studies suggest a negative association between atypical CLL morphology and disease outcome (Matutes and Polliack, 2000).

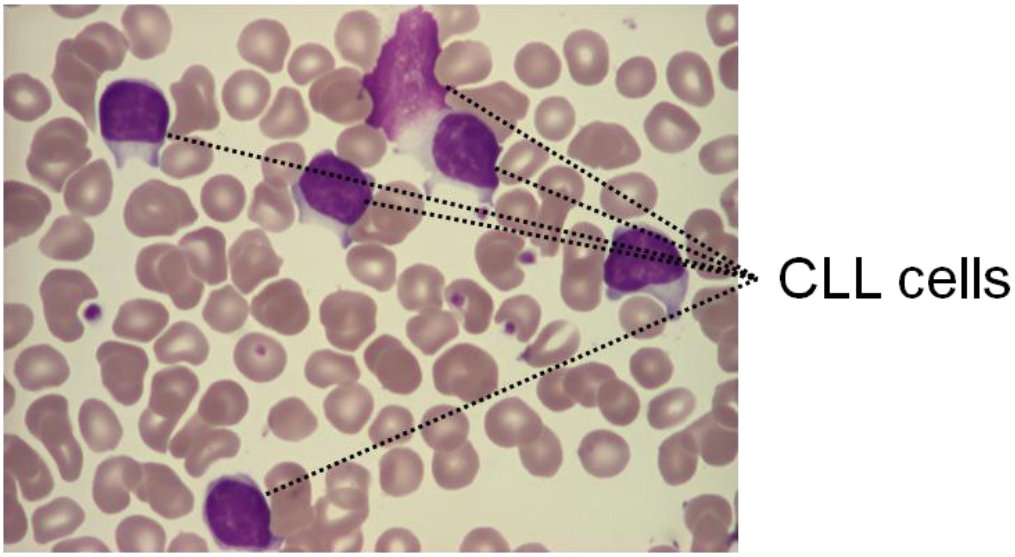


Figure 1-2 Hematoxylin & Eosin Stained Peripheral Blood Smear of CLL Patient

CLL B-cells have a large darkly stained purple nuclei designated as "soccer ball" and a little or no cytoplasm (Inamdar and Bueso-Ramos, 2007). A CLL B- lymphocyte is usually smaller and more fragile, when comparing to a normal B-lymphocyte, and when seen as a damaged cell on a blood film is described as a "smudge" cell. The smudge cells can be easily seen in the routine peripheral blood smear as their nuclei look like they are smashed against the glass slide and the percentage of identified smudge cells may have valuable correlations with CLL survival (Nowakowski et al., 2009). Images courtesy of Dr. D. Allsup (Haematology Department, Castle Hill Hospital).

1.3.4 Clinical staging in Chronic Lymphocytic Leukaemia

CLL has extremely heterogenic disease manifestation and survival times from primary diagnosis which vary between 2 to 20 years with a median survival of 8-10 years (Montserrat, 2004). Until 1975 when Dr. Rai and then later in 1981 Dr. Binet proposed clinical staging for CLL, there was not a consistent classification making it possible to prospectively separate CLL cases prognostically (Rai *et al*., 1975, Binet *et al*., 1981). Since then, two established leading staging systems of predicting the outcome in CLL have been adopted: the Binet (Table 1-3) and the Rai (Table 1-4). The Binet system is widely utilised in Europe and the UK, whereas, the Rai system is more commonly used in USA. These systems are utilised by clinicians in order to categorise patients by prognosis and assist in guidance for therapeutic strategy. Both staging systems are associated with disease outcome where survival in each disease stage may vary significantly. The Rai/Binet classification discriminates CLL patients into low-, intermediate-, and high-risk groups. In most cases, the stage is representative of the disease progressiveness (Rai et al., 1975). The Binet system groups CLL cases corresponding to the amount of lymph node areas involved and the level of anaemia or thrombocytopenia present (Binet *et al*., 1981).

Table 1-3 The Binet Clinical Staging System (1981)

This table has been modified from (Rai et al., 1975, Dighiero and Binet, 2000)

Stage	Risk	Clinical features	Survival
Stage A	Low risk	Blood and marrow lymphocytosis, <3 lymphadenopathy areas involved, no anaemia or thrombocytopenia	12+ years
Stage B	Intermediate risk	Lymphocytosis, ≤3 lymphadenopathy areas involved no anaemia or thrombocytopenia	7 years
Stage C	High risk	Lymphocytosis, ≤3 lymphadenopathy areas involved, anaemia (Hb <11g/d), Thrombocytopenia (platelets <100,000/μL	2 years

Table 1-4 The Modified Rai Clinical Staging System (1975)

This table has been modified from (Rai et al., 1975, Dighiero and Binet, 2000, Furman, 2010)

Stage	Risk	Clinical features	Survival
Stage 0	Low risk	Blood (> 5x109 Cells/L) and marrow lymphocytosis	>150 months
Stage I	Intermediate risk	Lymphocytosis (> 5x109 Cells/L) Lymphadenopathy	101 months
Stage II		Lymphocytosis (> 5x109 Cells/L) Spleno- or Hepatomegaly with/ without Lymphadenopathy	71 months
Stage III	High risk	Lymphocytosis (> 5x109 Cells/L) and Anaemia (Hb <11g/d)	19 months
Stage IV		Lymphocytosis (> 5x109 Cells/L) and Thrombocytopenia(platelets <100,000/μL With/without organomegaly and/or anaemia	19 months

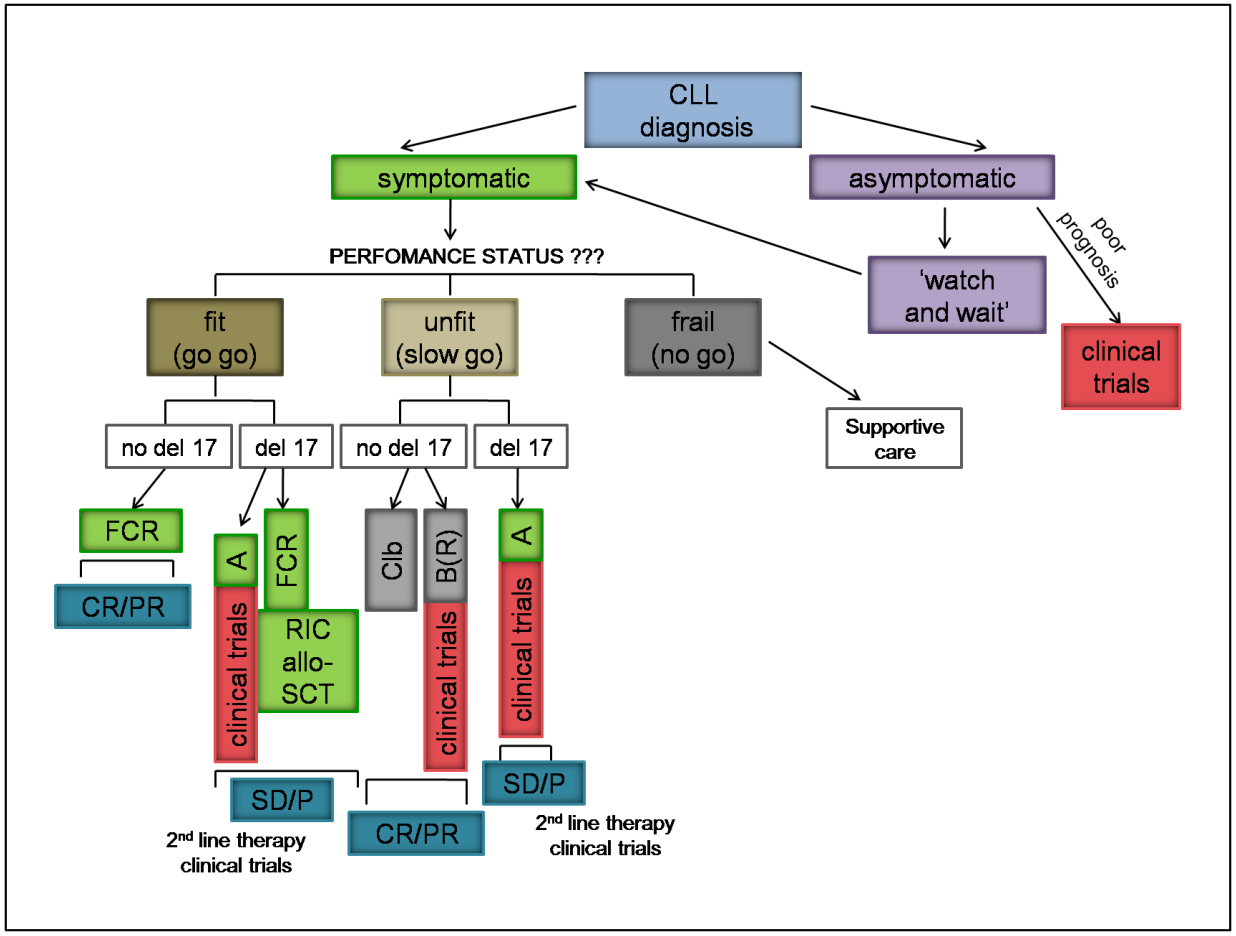
Binet stage A or Rai stage 0 indicates the absence of bone marrow failure and minimal signs of disease penetration. The patients within Binet stage B or Rai stage I/II demonstrate symptoms of nodal disease but without bone marrow failure, whereas, Binet Stage C or Rai stage III/IV indicates compromised bone marrow function.

1.3.5 Treatment of Chronic Lymphocytic Leukaemia

1.3.5.1 *Traditional Therapeutic Strategies in CLL Outlined in the 2008 IWCLL Guidelines*

Despite significant progress achieved in diagnosis, therapy and patient management in recent years the disease still remains incurable with standard therapy regimens. The treatment options for CLL patients vary greatly. The clinical course, patient performance status and disease risk generally determine the therapeutic strategy. The European and USA CLL treatment approach deviates slightly; however, the overall treatment scheme presented by Gribben and O'Brien has been integrated and shown in Figure 1-3 (Gribben and O'Brien, 2011). Upon CLL diagnosis, there are two options which can be taken, which are dependent upon either symptomatic or asymptomatic disease behaviour.

Figure 1-3 Approach to the Front Line Therapies



This is schematic presentation of the first line therapy of CLL.
A - Alemtuzumab,
RIC allo SCT - reduced-intensity conditioning allogeneic stem-cell transplantation,
B - Bendamustine,
Clb - Chlorambucil,
CR - Complete Remission,
FCR- Fludarabine, Cyclophosphamide, Rituximab;
R- Rituximab
P-Progress,
PR - Partial Remission,
SD - Stabile Disease,
del 17-Deletion of parts of chromosomes 17p
The information presented in this figure has been used from Furman, 2010, Eichhorst et al., 2011, Hillmen, 2011).

In asymptomatic cases or early stages of CLL the "watch-and-wait" strategy is applied (Figure 1-3), where patients are monitored until related symptoms manifest themselves and patients' quality of life has been affected. Typical CLL-related symptoms are listed below:

- cytopenias (excluding autoimmune phenomena)
- lymphadenopathy
- spleen and liver enlargement and associated with these symptoms
- lymphocyte doubling time (LDT) (Section 2.2.1) of < 6 months
- refractory autoimmune haemolytic anaemia and/or unresponsive to therapy with corticosteroids thrombocytopenia
- progressive symptoms of bone marrow failure such as haemoglobin level decreased below 10 g/dL or platelets below $100x10^9$/L (Molica et al., 2011b)
- severe B-symptoms such as weight loss, fatigue, fever which are of non-infectious aetiology.

However, in some cases, an asymptomatic indolent disease course, which does not require treatment intervention for several years, can be followed by a progressive and often terminal outcome within short period of time. Therefore CLL patients with high risk features may be enrolled into clinical trials (the risk factors will be discussed in greater details in Chapter 2). Up to the present time there are no convincing studies, which demonstrate survival benefit of early treatment intervention in asymptomatic CLL (Abbott, 2006). However at this present time there are several ongoing clinical trials, investigating early treatment in poor prognosis CLL patients with stage A/0. One of these trials is the German CLL

Study Group CLL7 Trial, which studies the potential benefit of combined therapies in poor risk early CLL stages (Furman, 2010).

Patients with symptomatic CLL require immediate treatment and according to ESMO Clinical Practice Guidelines for the management of CLL the following assessments are necessary prior to treatment initiation:

- detailed family and medical history
- external physical patient examination including all lymph node areas, spleen and liver
- complete blood cell and differential count
- serum chemistry tests (Eichhorst *et al*., 2011), including renal and liver function, immunoglobulins
- immunophenotypic panel analysis of 5 antibodies (CD5, CD23, FMC7, surface immunoglobulin and CD22/CD79b)
- assessment for the identification of clinical stage (Rai / Binet)
- lymphocyte doubling time test.

However, other additional investigations are suggested for the final disease diagnosis and treatment strategy identification, such as:

- a bone marrow biopsy, which can be informative and useful when initiating myelosuppressive therapies and also in CLL cases where the cause of cytopenias is unclear
- FISH analysis for the identification of cytogenetic abnormalities (Section2.1.2.4.2)
- computed tomography (CT) scans (Eichhorst *et al*., 2011).

A careful assessment of the patient fitness, presence of co-morbidities and prognostic risk are necessary for the decision making regarding the therapeutic approach (Figure 1-3).

The German CLL study group has proposed a classification of CLL patients into three groups (Goede and Hallek, 2011) (Figure 1-3). These are:

- ***Go Go***. This group usually is represented by the younger patients without significant co-morbidities or impaired performance status, which makes them suitable for intensive chemotherapy
- ***Slow Go***. CLL patients with advanced age and poor performance status/co-morbidities
- ***No Go***. Frail patients suitable for intensive care only.

Chemotherapy, immunotherapy, haematopoietic cell transplantation (HCT) and radiotherapy are the leading therapeutic approaches in CLL management. However, as Figure 1-3 demonstrates the determination of possible genetic abnormalities in CLL patients, especially chromosome 17p deletion, which is associated with *P53* gene mutation (Section 2.1.2.4.2) can play an influential role in the selection of therapeutic approach and potentially can eliminate unnecessary patient exposure to ineffective but potentially toxic chemotherapy (Furman, 2010). Until recently the primary chemotherapeutic treatment employed the alkylating agent Chlorambucil, which was successful in the induction of partial but not complete remission and is associated with a risk of the development of secondary leukaemia (Robertson *et al*., 1994). Despite this Chlorambucil still remains in significant clinical use and has a useful therapeutic role in older patients (Abbott,

2005).The combined therapeutic protocols, such as CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisalone) and CVP (Cyclophosphamide, Vincristine and Prednisalone) did not demonstrate survival benefit versus Chlorambucil in CLL treatment (Raphael *et al*., 1991). The purine nucleoside analogues Fludarabine demonstrated impressive results in treatment of indolent lymphoid neoplasm versus other agents. Noteworthy results in response rate were observed in combination regiments of Fludarabine and Cyclophosphamide (Redman et al., 1992, Abbott, 2006).

Biological therapy includes use of monoclonal antibodies such as Alemtuzumab (Campath™) (directed against CD52, a surface protein of mature lymphocytes), Rituximab (Mabthera™) (directed against CD20, an activated phosphoprotein expressed on the surface of all B-cells) and Ofatumumab (Arzerra™) (directed against CD20). Rituximab has a greater efficacy when used in combination with Fludarabine and Cyclophosphamide, producing an excellent complete remission rate in untreated (up to 85%), in previously treated (30-50%) (Pangalis *et al*., 2002) and relapsed CLL patients (Wierda *et al.*, 2005).

The use of autologous and allogeneic stem cell transplantation (SCT) has increased as treatment has the potential to induce long-term remissions with a low treatment-related mortality (5-10%) in treated CLL cases (Pangalis et al., 2002). Although the vast majority of CLL patients are of an advanced age (about >60 yrs old), there is a percentage of younger patients with poor-risk disease whose life expectancy is significantly reduced and auto and allogeneic SCT has been examined as a potentially curative option (Delgado *et al.*, 2009a).

1.3.5.2 *Novel Therapeutic Agents*

In recent years, the improved understanding of CLL on the molecular and intracellular level has driven investigation towards development of new therapeutic targets and potential therapeutic agents. In addition, a significant number of patients do not respond to conventional therapies and therefore new therapeutic direction is paramount. New therapeutic strategies have shown significant signs of promise and have the potential to revolutionise CLL treatment (Hillman, 2011). Taking into consideration the dynamics of the disease evolution, tailoring of CLL therapy towards the biology of an individual patient and individual disease behaviour is an exciting prospect. Numerous clinical trials are in progress investigating novel targeted therapeutic approaches. There are number of new directions being undertaken from inhibition of BCR signalling (Section1.5.3), apoptosis, microenvironment support, and cell cycle to development of new improved immunotherapies (Table 1-5). The inhibition of BCR signalling, which is known to be involved in the delivery of a proliferative signal, indicated the greatest potential (Hillmen, 2011). Several BCR-associated downstream molecules such as Phosphoinositide 3-kinase δ (PI3Kδ), Bruton's tyrosine kinase (BTK), Syk and Src protein-tyrosine kinases have been targeted by new therapies. Although, the most advanced BTK and PI3K inhibitors such as Ibrutinib (PCI-32765) and GS-1101 (CAL-101) are associated with an immediate resolution of the constitutional CLL symptoms such as decrease of lymph node mass (Hillmen, 2012). A trial of Ibrutinib has just been published in The New England Journal of Medicine by Byrd *et al*. and it was shown to be effective (time=26 months, the estimated progression-

free survival rate (PFS)=75% and overall survival (OS)=83%) in patients with relapsed or refractory CLL including cases with high-risk genetic mutations (Section 2.1.2.4.2) (Byrd et al., 2013). Additionally, preliminary data from PI3K inhibitor studies, which was reported in December 2013 at the ASH 55th Annual Meeting have shown a positive result with BCR-targeted therapies (Flinn, 2013). Several trials are planned to investigate the combination of these agents with conventional therapies such as Bendamustine with/without anti CD20 antibody - Rituximab (Hillmen, 2011). Dependent upon the therapeutic approach selected it should be tailored to the individual risk, patient fitness and disease biology. Although the current therapies improve CLL patient's survival, they still remain suboptimal due to associated cytopenias leading to subsequent infection, possibility of the development of refractory CLL, and inevitable relapse (Robak, 2013). This reinforces the demands for optimisation of novel therapeutic strategies, which could ultimately lead to a significantly higher rate of true complete remission.

Table 1-5 Novel Therapeutic Pathways

The information presented in this Table has been used from Furman, 2010, Eichhorst et al., 2011, Hillmen, 2011.The BCR and microenvironmental signalling play an important role in CLL cell survival (Section 1.5.4.2 and 1.5.4.1 respectively).

Target Pathway	Target Molecule	Agent	Mechanism of action
BCR	PI3Kδ BTK Syk Src	CAL-101 or GS-1101 Ibrutinib (PCI-32765) Fostamatinib, R-406 Dasatinib	Inhibition
Apoptosis	Navitoclax (ABT-263) GDC-0199 (ABT-199)	Bcl-2	Disruption of Bcl-2/Bcl-xL interactions with pro-apoptotic proteins
Microenvironment	-	Lenalidomine	Induction of tumour cell apoptosis directly and indirectly via inhibition of bone marrow stromal cell interactions
Cell cycle	CDK	Flavopiridol Dinaciclib	Inhibition of cyclin-dependent kinases, arresting cell division and causing apoptosis
Novel antibodies	CD20 antibody	Ofatumumab, GA-101	Improved antibodies

1.4 Aetiology of Chronic Lymphocytic Leukaemia

Aetiology is the study of the cause(s) or origin of a disease, disorder or pathological condition as determined by medical diagnosis. Although the aetiology of CLL is not fully understood, there is strong evidence to suggest that a combination of genetic, environmental, biochemical and psychological factors play the fundamental role in the development and evolution of CLL.

There are number of published studies strongly supporting the hypothesis of a polygenic inherited susceptibility to CLL. Two major studies by Di Bernardo *et al* and Crowther-Swanepoel *et al* have identified several single-nucleotide polymorphisms (SNPs), which are repeatedly associated with disease causation in CLL (Di Bernardo *et al*., 2008, Crowther-Swanepoel *et al.,* 2010). Table 1-6 displays several common gene loci carrying high frequency risk alleles for CLL.

The evidence for familial clustering of CLL also supports the role of genetic factoring in CLL development (Rawstron *et al.*, 2002). The age of onset in familial cases is generally reported to be earlier than in sporadic CLL cases (Goldin *et al.*, 2004). However, the mode of inheritance has not been completely established (Inamdar and Bueso-Ramos, 2007).

Previously no associations have been found between environmental factors and occupational exposures such radiation, magnetic fields, viruses, and pesticides and the risk of CLL (el-Sadek and Hassan, 1999, Goldin *et al.*, 2004).

Table 1-6 Loci Carrying High Frequency Risk Alleles for CLL

The Table shows a list of common risk loci, which were reported to influence the risk in developing CLL. The data, utilised in the Table, was extracted from Di Bernardo et al., (2008), Crowther-Swanepoel et al., (2010), who conducted genome-wide association studies in CLL and provided the primary evidence for the existence of six common, low penetrance susceptibility loci for CLL (The Gene Location for each risk locus is stated in the Table). The study of Di Bernardo et al utilised SNP data from NCB ISNP Database to analyse the observed and expected distributions of autosomal SNPs in comparison to control samples. The SNP-associated risk alleles occur near to the genes listed, and hence do not alter the protein structure. SNPs are DNA sequence variations occurring within a chromosome, in which a single nucleotide (adenine (A), cytosine (C), guanine (G) or thymine (T)) differs between different samples or paired chromosomes within one sample. Allele frequency is the proportion identified as a percentage (%) of a particular allele to all alleles considered in the study. Interestingly, pro-apoptotic BCL2L11/Bim protein expression was reported to be associated with CLL aggressiveness (Paterson et al., 2012).

Gene Location SNP	Nonrisk Allele	Risk Allele	Risk Allele Frequency	Known Name for the Nearest Gene(s)
2q13 rs17483466	A	G	0.20	*BCL2L11/Bim ACOXL*
2q37.1 rs13397985	T	G	0.19	*SP140,SP110*
1106q25.3 rs872071	A	C	0.51	*IRF4*
8q24.21 rs735665	G	A	0.21	*GRAMD1B*
15q23 rs7176508	G	A	0.37	--
19q13.32 rs11083846	G	A	0.22	*PRKD2,STRN4*

However, this understanding has been challenged by several recent investigations as there is a high degree of uncertainty around how low radiation exposures can affect the genome. The group, which has studied the incidence of

CLL among the clean-up workers exposed to the ionised radiation from the catastrophe at the nuclear power plant in Chernobyl (Ukraine) revealed that there is a significant increase in cases with CLL and that the disease has a more progressive course in comparison to normal population (Gluzman *et al.*, 2005, Hamblin, 2008). The research demonstrates that the radiation penetrates the body and damages DNA in the bone marrow and hence increases leukaemia risk. Despite this, during studies of the incidence of CLL among bomb survivors at Hiroshima and Nagasaki an increased risk of CLL had not been found (Hamblin, 2008). However, the epidemiological data demonstrates that yellow Japanese population in comparison to the white Ukrainian race is inherently less susceptible to CLL, which potentially could explain the relationship between radiation and CLL in white/Ukrainian clean up-workers.

1.5 Pathogenesis of Chronic Lymphocytic Leukaemia

1.5.1 The Origin and Development of Chronic Lymphocytic Leukaemia

The molecular pathogenesis of CLL is a complex, multistep process during which normal B-cells are transformed into the abnormal clone of B-cells via intermediate stage and which is supported by a series of cytogenetic abnormalities (Chiorazzi, 2007). However, the factors involved in the clonal expansion of malignant cells are not yet fully understood (Abrams *et al.*, 2007). Accumulating evidence indicates that CLL develops in a gradual manner in response to the clonal acquisition of a number of somatic aberrations and functional modifications, which can be inherited or acquired (Chiorazzi and Ferrarini, 2011). In most, if not

all CLL cases the normal B-cells become neoplastic via a transitional stage denoted as monoclonal B-cell lymphocytosis (MBL) (Landgren *et al.*, 2009, Stevenson *et al*., 2011, Singh and Gardinali, 2006) (Figure 1-4). MBL is characterised by the presence of monoclonal memory B-cells with a CLL phenotype ($<5 \times 10^9$/L) in peripheral blood. The pathogenesis of MBL is not fully elucidated; however, data demonstrates that MBL develops as the result of cytogenetic abnormalities, the origins of which are associated with B-cell response to antigenic stimulation. Interestingly, evidence indicates that MBL was detected in less than 1% of apparently healthy individuals with age ≥ 40 and in approximately 5% with age ≥ 60 (Marti *et al*., 2005). Furthermore, it has emerged that there is a transition from clinical MBL to fully developed CLL. The study determined that an early transformation from clinical MBL to CLL had been observed in approximately 1-2% of cases per annum (Rawstron, 2009).

The phenotype characteristics of CLL cells such as persistent use of Immunoglobulin Heavy and Light Chain Variable (IgV_H and IgV_L, respectively) *kappa/lambda* (κ/λ) genes (Chiorazzi and Efremov, 2013), differ from any normal B-cells subtype, which suggests that CLL cells are derived from rather abnormal putative progenitors (Singh and Gardinali, 2006). Multiple studies were conducted in order to identify the B-cell developmental checkpoints at which transformation of normal B-lymphocytes to CLL B-lymphocytes occurs. Notwithstanding the research results which demonstrated that oncogenic somatic mutations emerge at the late stage of B-cell development (Figure 1-5), for years it was still uncertain whether CLL originates from a single or multiple counterpart(s) (Chiorazzi and Ferrarini,

2011). Historically CLL had been defined as a single lymphoproliferative entity with variable clinical outcome. However utilising immunoglobulin (Ig) gene analysis, it has become clear that CLL is a disease of marked biological and clinical heterogeneity.

1.5.1.1 *B-Cell Receptor Complex*

The BCR is known to play a role in both normal and malignant B-cell behaviour (Young and Staudt, 2013) and in the regulation of the adaptive immune response. The BCR is a trans-membrane receptor, which consists of a ligand-binding unit, composed of a surface Immunoglobulin (Ig) and non-covalently associated signal transduction molecules designated as CD79a (Igα) and CD79b (Igβ) (Figure 1-4). Immunoglobulin is represented by the immunoglobulin isotypes IgD, IgM, IgE, IgG and composed of two identical Kappa (κ) or Lambda (λ) light (L) and two identical heavy (H) chains (Giachino et al., 1995). The C-terminus of BCR is presented by variable region (V) and N-terminus by constant (C) region. The antigen-binding (Fab) portion of Ig consists of a light chain and a fragment of a heavy chain, whereas (Fc) portion includes most of the C region of the heavy chain (C_H).

The BCR expresses a unique ability to recognise and confront the numerous diverse antigens of the different origins (either auto- or alloantigens (Catera et al., 2008)), although, the BCR specificity can differ from one B-cell to another. The evolution of such diversity is generated by the genetic mechanism, which takes place in the variable regions of the Ig light (V_L) and heavy chains (V_H) and termed VDJ rearrangements (**V**ariable, **D**iversity and **J**oining (V,D and J

respectively)). These three genes encode fundamental elements of the antigen binding region of the V_H moiety. The Human Chromosome 14 encodes multiple copies of V, D and J gene segments. During the B Cell development (Figure 1-4), one copy of each possible variant is selected, joined or recombined together to generate the required VDJ sequence utilised in the functional BCR providing the cell with a unique antigen receptor.

BCR The BCR-associated signal transduction moiety is represented by the defined amino acid sequences on the intracellular portion of CD79a that allow the association of tyrosine kinases with SH2 domains such as Lyn or Syk and is defined as intracellular Immunoreceptor tyrosine-based activation motifs (ITAMs). Both sIg and ITAM complex play a crucial role in signal transduction and BCR activation regarded as a process called phosphorylation. “Knockout“ mice with targeted deletion of either CD79a or CD79b exhibit a fundamental interruption in B-cell development in the pre-B cell stage and deficiency of functionally mature B-lymphocytes (Smith and Reth, 2004). Upon crosslinking with antigen, BCR receptor triggers the downstream activation of intracellular signalling cascades regulating the biological behaviour of B-cells in diverse ways such as growth, proliferation, differentiation (Jumaa *et al.,* 2005) and apoptosis (Eeva and Pelkonen, 2004).

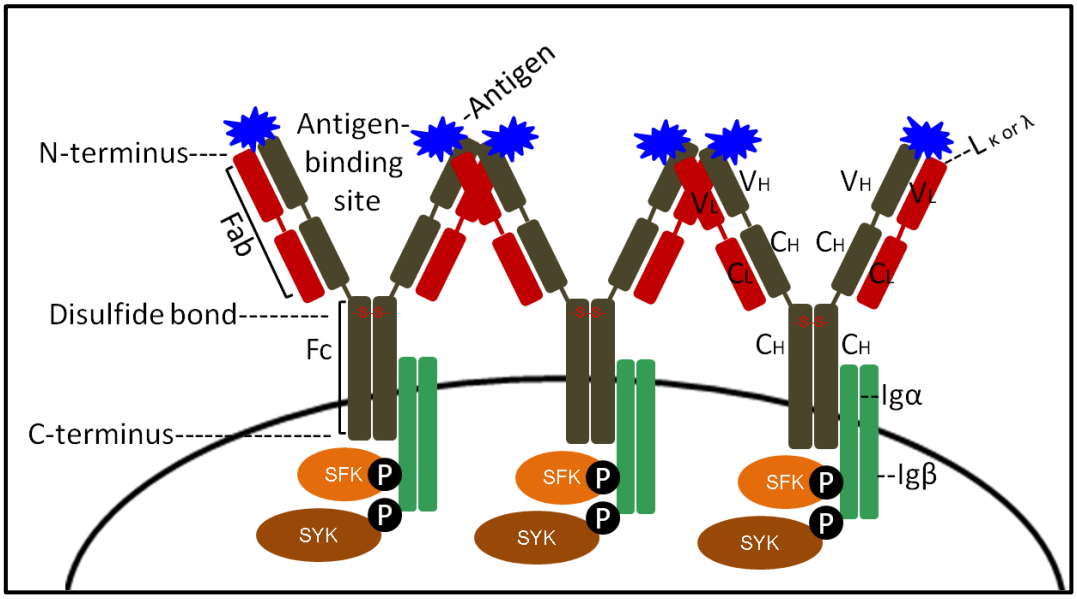


Figure 1-4 The Structure of B Cell Receptor

BCR consists of two major components, these are: antigen binding and signal transduction moieties, which are represented by the immunoglobulin associated with the transmembrane proteins CD79a and CD79b and ITAMs, respectively. Surface Ig has a Y-shape and composed of four polypeptide chains bound by disulfide bond (two heavy and two light chains). The heavy and light chains possess a variable (V) and constant (C) regions. The amino acids sequence of variable region differs between Ig isotypes and is responsible for Ig specificity. The C region possesses a relatively constant amino acid sequence. Light chain is expressed either by kappa or lambda variant.

1.5.1.2 *Somatic Hypermutation and Class Switch Recombination Processes During Normal B cell Development and Their Contribution to B-CLL pathogenesis*

1.5.1.2.1 Normal B cell Development

Normal B Lymphocyte development is a highly regulated process, which consists of a series of maturational stages initiated from a Pluripotent Hematopoietic Stem Cell, through immature Pro-and Pre-B Cells and finally

followed by subsequent commitment to a specific fate, phenotypic and functional differentiation. In a very simplified way, B Cell Development Stages are shown in Figure 1-5. During a B Cell development program, each B Cell acquires its unique receptor. In the fetal liver before birth and later in the bone marrow, Pluripotent Stem Cells react to extracellular stimuli that are delivered from stroma and are committed to differentiate, giving rise to the lymphoid progenitor. Lymphoid progenitor is a cell with no myeloid potential but able to produce B, T, and Natural Killer (NK) cells (Hirose *et al.*, 2002). The combinations of environmental signals along specific cellular pathways are known to facilitate the expression of critical transcriptional regulators that commit stem cells to a lymphoid lineage (Hardy and Hayakawa, 2001). Several molecules such as receptor tyrosine kinases (Flk2 and c-Kit), α4β1 integrin, Interleukin-7 Receptor (IL-7), chemokine receptor type 4 (CXCR4), Stromal cell-derived factor-1 (SDF-1) receptor were reported to be essential in lymphopoiesis (Burger *et al*., 2003) (Scimone *et al*., 2006). Cytokines promote terminal deoxynucleotidyl transferase (TdT) and recombinase (RAG-1 and RAG-2) production in lymphoid progenitors. The cells undergo D-J joining on the H Chain chromosome to become early Pro-B cells, which are B220 (CD45 isoform) and major histocompatibility complex (MHC) Class positive. Joining of a V segment to the $D\text{-}J_H$ concludes the late pro-B cell stage. These cells then proceed, in the bone marrow, to initiate their Ig Heavy Chain rearrangement in a process called V(D)J recombination, where V (variable), D (diversity) and J (junctional) are gene segments that together comprise the Variable domain. VDJ recombination is an intrinsic mechanism of random combination, via nucleotide deletion or insertion, of

V,D and J gene segments, which contributes to the enormous immune-receptor diversity. Pro-B cells become Pre-B cells after they express membrane μ chains with surrogate L chains in the Pre-B receptor. In the bone marrow, during early stages of B cell differentiation, after an immature B Cell encounters a multivalent self-antigen this leads to BCR/antigen interaction and subsequent cell activation. Upon such binding, in an attempt to refine and enhance cell response to the antigen, B Cells undergo rapid proliferation. During cell division, VDJ recombination may become reactivated leading to an extremely high level of somatic changes occurring in the BCR locus (Hamblin, 2007) known as "receptor editing". This self-reformation would help a cell to evade apoptosis and progress via cell development program (Figure 1-5). Utilising a mouse model, several studies demonstrated that cells, which did not undergo VDJ recombination fail to differentiate further (Hertz *et al*., 1998). However Pre-B Cells (B220 positive and TdT negative) which went through a successful VDJ rearrangement synthesise the Ig heavy chain that is retained within the endoplasmic reticulum and after follow on association with surrogate light chain, form on the cell surface pre-BCR. This receptor mediates Pre-B Cells survival and proliferation. Subsequently, light chain associates with μ chain to produce IgM, which is expressed by immature B Cells ($B220^+$ TdT^- IgM^+ IgD^-). Later, following maturation, which again requires antigen-receptor mediated signals, these cells then exit the bone marrow, enter the circulation and migrate to peripheral lymphoid organs, where they become long-lived B lymphocytes that are 220^+ TdT^- IgM^+ IgD^+ with high level of CD23 (Loffert *et al*., 1996, Hirose *et al*., 2002).

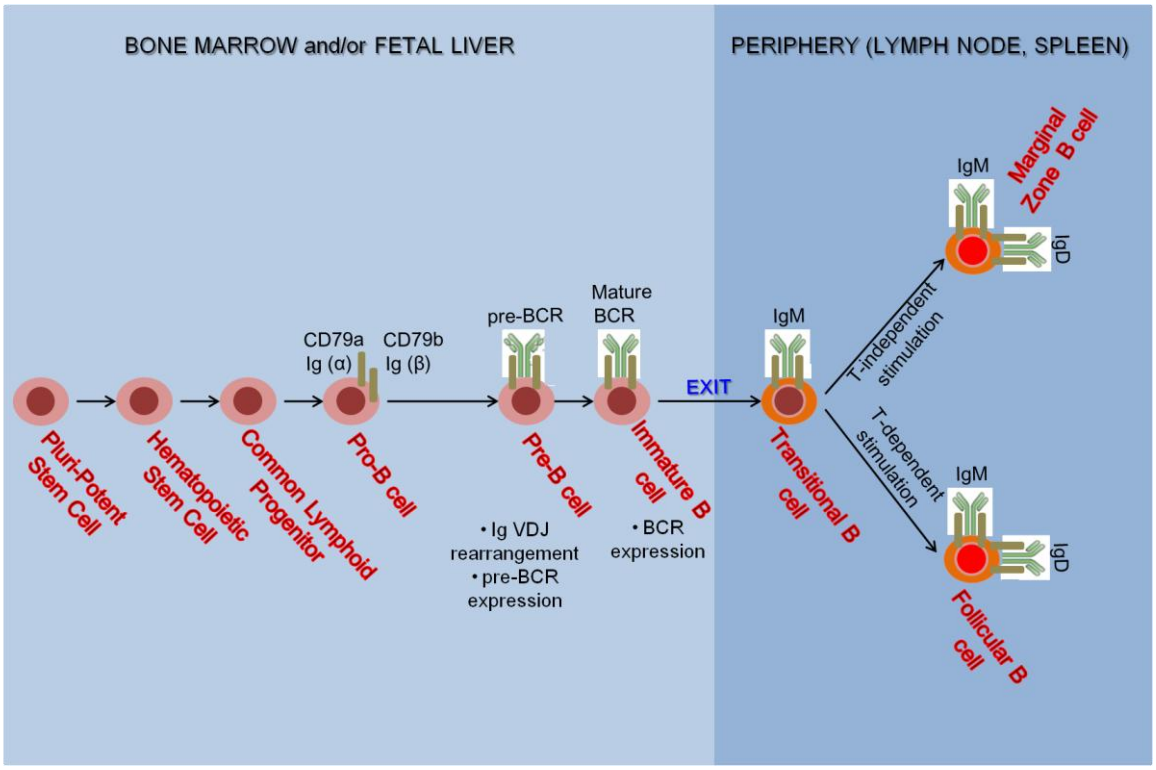


Figure 1-5 Normal B-Cell Development and Maturation

The primary part of B-cell differentiation, which originates from pro-B-cell to pre-B-cell and immature-B-cell stages occurs in bone marrow (BM), during this stage Ig undergoes VDJ recombination leading to composition of primarily pre- B Cell Receptor (BCR) and finally mature BCR structure. BCR engagement, clonal deletion and receptor editing play a critical role in positive cell selection and elimination of auto-reactive cells. The first stage of lymphoid development is in the bone marrow (primary lymphoid tissue) which is antigen independent. The cells initially express a pre-B receptor which provides antigen independent signals that promote B-cell differentiation. On exiting the bone marrow cells circulate and enter secondary lymphoid tissues (lymph node, spleen) where they encounter antigen. B-cells whose BCR interacts with antigen may receive T-cell help which then protects the B-cells from apoptosis and allows their differentiation into plasma cells or memory cells. This is T-dependent process. The cells that successfully pass this selection emigrate from the BM as transitional B cells. Subsequent differentiation categorises these cells into mature follicular B cells or marginal-zone B cells in T-cell independent and T-cell dependent manner, respectively. The post-antigen engagement regulates the B-cell population into either plasma or memory B cells. The information for the composition of this Figure was used from (Cambier et al., 2007) and (Chiorazzi, 2012).

1.5.1.2.2 Somatic Hypermutation

In the periphery, B Cells can differentiate further via different, distinct, pathways and this heterogeneity is predominantly regulated by the nature of the antigen that evokes an immune response. These pathways are known as T-dependent and T-independent. Following secondary antigenic challenge, B Cells may undergo activation (via cooperation with T Helper Cells, dendritic Cells and microphages) and subsequently migrate to the Germinal Center (GC). During an immune response to T-dependent antigens a process called Somatic Hypermutation (SHM) occurs which causes an increase in the affinity of the Ig VH region for its target antigen (Phan *et al*., 2005). SHM allows B Cells to specifically reorganise the gene encoding their V_H region to adapt their response to the encountered antigen (Di Noia and Neuberger, 2007). SHM is responsible for the production of a unique variable domain in the Ig molecule for each individual B cell, resulting in increased antibody diversity and significant affinity maturation. B Cells with an improved affinity will survive and differentiate, whereas other B Cells will undergo apoptosis within the GC (Peter J. Delves, 2011). Historically, it was believed that the process of SHM takes place exclusively within GC and was invariably a CD40, Interleukin-4 (IL-4) associated T-cell-dependent process. Nevertheless, recent work suggests that SHM may also occur in response to T Cell-independent antigen (such as the polyclonal activator, lipopolysaccharide (LPS) endotoxin), outside the GC. New evidence continues to suggest that SHM can occur in immature B cells, thus, creating a more generalised mechanism for expanding the V gene repertoire (Klein and Dalla-Favera, 2008, Mao *et al.*, 2004).

1.5.1.2.3 Class Switch Recombination

Although T-independent B Cell activation still elicits an immune reaction and antibody production, the Ig of the cognate B lymphocytes are not normally subjected to the process of isotype switching. Hence, the antibodies secreted as a result of T-independent antigen interaction, generally are of the IgM class and of relatively low affinity.

However, the production of other antibody classes requires substantial dependence on T Cell cooperation. The secondary antigenic challenge leads to antigen presentation by MHC class II molecules on specialised antigen presenting cells, which in turn activates T Cells augmented by co-stimulatory signals via CD40 and IL-4 receptors. The T-dependent activation results in the initiation of isotype switching as well as enlargement of germinal centers in secondary lymphoid organs (Pillai, 2000).

Class Switch Recombination (CSR) is a genetic cellular mechanism, which is implemented by mature B Cells in response to the immense variety of antigenic stimuli. Antibody isotype, which greatly influences the effector property and antibody stability, is determined by the C_H fragment in every individual Ig molecule. Although the signals delivered by BCR and CD40 are crucial, there is a role for cytokines expressed by T-Helper and Dendritic Cells such as IL-7 and transforming growth factor beta (TGF-β) to discern the isotype to which individual B Cells would switch. During Ig CSR, which also occurs principally within the GC, the proliferating B Cells alter the gene sequence in C_H region locus, whilst V_H region locus remains unchangeable, in order to allow switching from expressing one class of

immunoglobulin to another (for example from isotype IgM to the IgG, IgE, or IgA). The antigen-driven activation leads to the transcriptional mediation of DNA-binding proteins, those are capable of recognition of the switch sites upstream of C_H region and subsequent initiation of recombination events. This evokes the rearranged VDJ complex from its location to the site targeted for switching (Pillai, 2000). However, the nature of the antigen, which is internalised by a B Cell and the location of the antigenic provocation are of importance. These selective changes in C_H region portion initiate the antibody production.

Some activated B Cells, which have undergone CSR, differentiate into plasma cells, whilst others relocate into follicles and promote the generation of germinal centres (Pillai, 2000).

1.5.1.2.4 The Contribution to B-CLL Pathogenesis

The B Cells of >50% of CLL patients exhibit more than 2% deviation from germline in their V_H gene sequence suggesting that these B Cells have undergone SHM (Fais et al., 1998). A 2% cut-off was selected as an indicator of SHM in order to exclude the possibility of the error due to previously unrecognised polymorphisms within VH region (Matsuda and Shin, 1993). The gene expression profiles (GEP) vary between CLL cases, which suggests that CLL is not just a single disease but can be divided into two major molecular subtypes discriminated by the characteristic of expressed immunoglobulin of the BCR such as unmutated (U-CLL) (≤ 2% deviation from the germline sequence) and mutated CLL (M-CLL) (> 2% deviation from the germline sequence) (Stevenson and Caligaris-Cappio, 2004) and previous history of antigen experience (Chiorazzi and Ferrarini, 2011) (Figure

1-6). These differences in the extent of *IgV$_H$* somatic hypermutation, which are also reflected in discrimination between CLL subtypes in terms of biology and clinical behaviour (Stevenson et al., 2011), support a “multiple-cell” originating model (Singh and Gardinali, 2006). In this model some CLL cases are derived from the putative progenitor that has passed through the GC and undergone IgVH mutation (M-CLL) whilst others are derived from a cell of origin that differentiated in a T-independent manner outside of the GC microenvironment (U-CLL) (Figure 1-6).

However in challenge of the above, other GEP microarray analyses have shown that only <30 genes are differentially expressed and can distinguish M-CLL and U-CLL profiles, whereas about 12,000 genes share a common pattern of expression and differentiate between normal B-cells and CLL-cells irrespective of *IgV$_H$* mutation status (Klein et al., 2001, Rosenwald et al., 2001).

Moreover, the analyses of cellular activation and differentiation marker expressions have demonstrated that both M-CLL and U-CLL lymphocytes resemble characteristics of the activated and antigen-experienced B-cells (Damle et al., 2002), which include memory B lymphocytes and marginal zone (MZ) B lymphocytes (Chiorazzi et al., 2005). Therefore combining these findings, it is suggested that both M-CLL and U-CLL are derived from the activated and antigen-experienced B cells, where is even though U-CLL has avoided *IgV$_H$* somatic hypermutation in the GC, the B cells have still undergone activation, possibly in a GC-independent manner (Chiorazzi and Ferrarini, 2011, Singh and Gardinali, 2006).

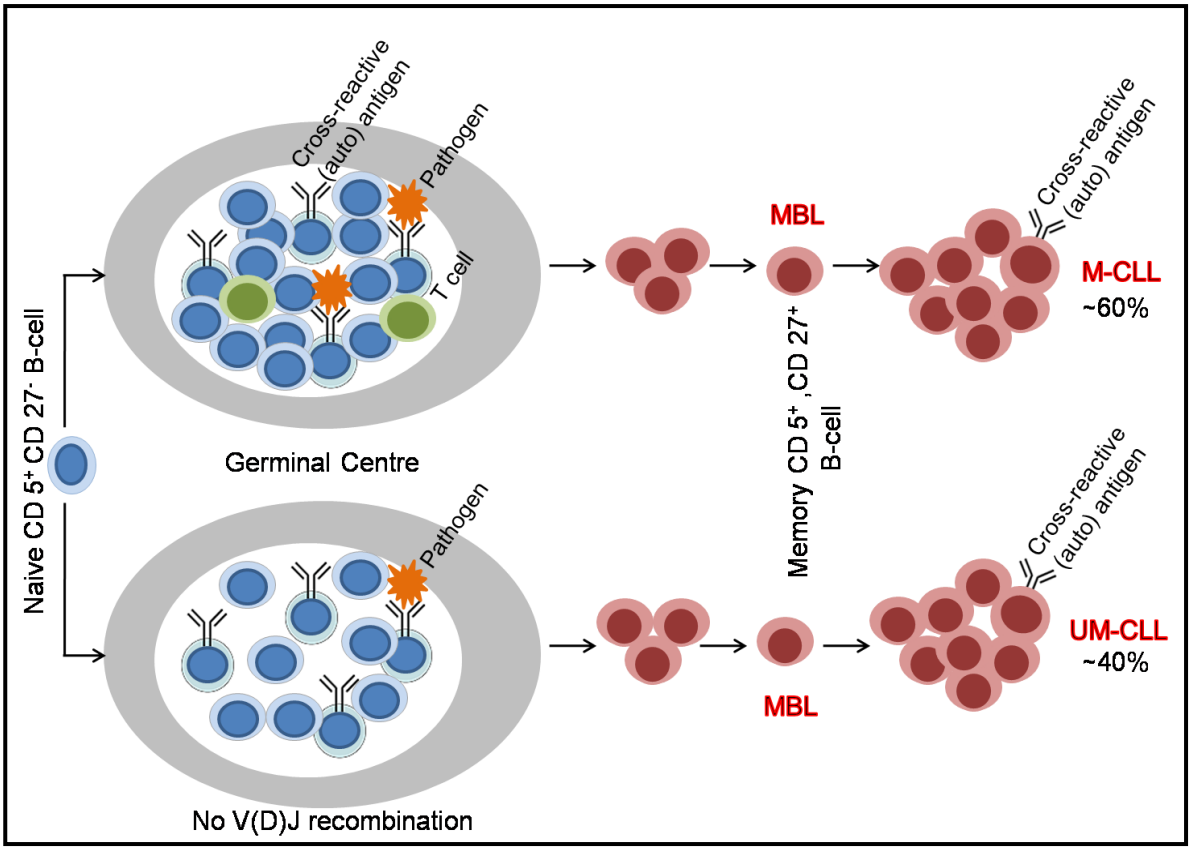


Figure 1-6 Pathogenesis of the Two Major Molecular CLL Subtypes

M-CLL and UM-CLL are two major CLL subtypes (Stevenson et al., 2011), which are expressed in 60% and 40% of all CLL cases respectively (Furman, 2010). In cases when CLL cell originates from a B cell before initiation of the rearrangement of the immunoglobulin V(D)J genes, it progresses into U-CLL. Whereas in other cases, defined as M-CLL, the transformation occurs after V(D)J recombination had taken place in the germinal center (GC) of the secondary lymphoid organs, which may altered the affinity of BCR for antigens (Stevenson et al., 2011, Singh and Gardinali, 2006). This Figure is adapted from (Singh and Gardinali, 2006), additionally information from (Forconi et al., 2010) study was used for the creation of this figure.

As previously discussed a B-CLL is a CD5+ cell, which expresses IgM/IgD; these phenotypic features resemble a normal naive B cell and therefore it is expected to express an unmutated Ig gene. However, studies have demonstrated that some CLL cells harbour somatic mutations of VH genes, associated with

specific alleles of the VH1-69 gene and the VH4-39 genes (normally used in an unmutated Ig), while CLL cells expressing the VH4-34 gene are frequently associated with somatic mutations. These investigations support the suggestion that CLL is still a single disease entity, which exhibits morphologic and phenotypic diversity (Laslo *et al*., 2006) (Singh and Gardinali, 2006).

1.5.2 BCR-Mediated Signalling in Normal B-lymphocyte

Antigen cross-linking with the V domain of Ig induces receptor clustering which promotes series of signalling events, leading to the formation of a subcellular network, termed 'signalosome' (Pierce and Liu, 2010). This signalosome complex consists of kinases and scaffold proteins including CD79a and CD79b. Phosphorylation of CD79a and CD79b by the member of Src family kinase Lyn is a central event in BCR-associated signalling (Stevenson et al., 2011). Lyn is an important signalling intermediary that transmits signals from cell surface receptors. Subsequently, phosphorylated ITAM molecules serve as the docking sites for the tandem SH2 domains of another subcellular intermediate, known as Spleen Tyrosine Kinase (Syk). Syk is a cytoplasmic tyrosine kinase that plays a critical role in the BCR-associated signal transmission. Lyn-induced phosphorylation and the subsequent auto-phosphorylation of Syk play an important role in the initiation of several interconnected pathways such as protein kinase C (PKC), mitogen-activated protein kinase / extracellular signal-related kinase (MAPK/ERK) or phospholipase Cγ2 (PLCγ2) (Stevenson et al., 2011) (Figure 1-7).

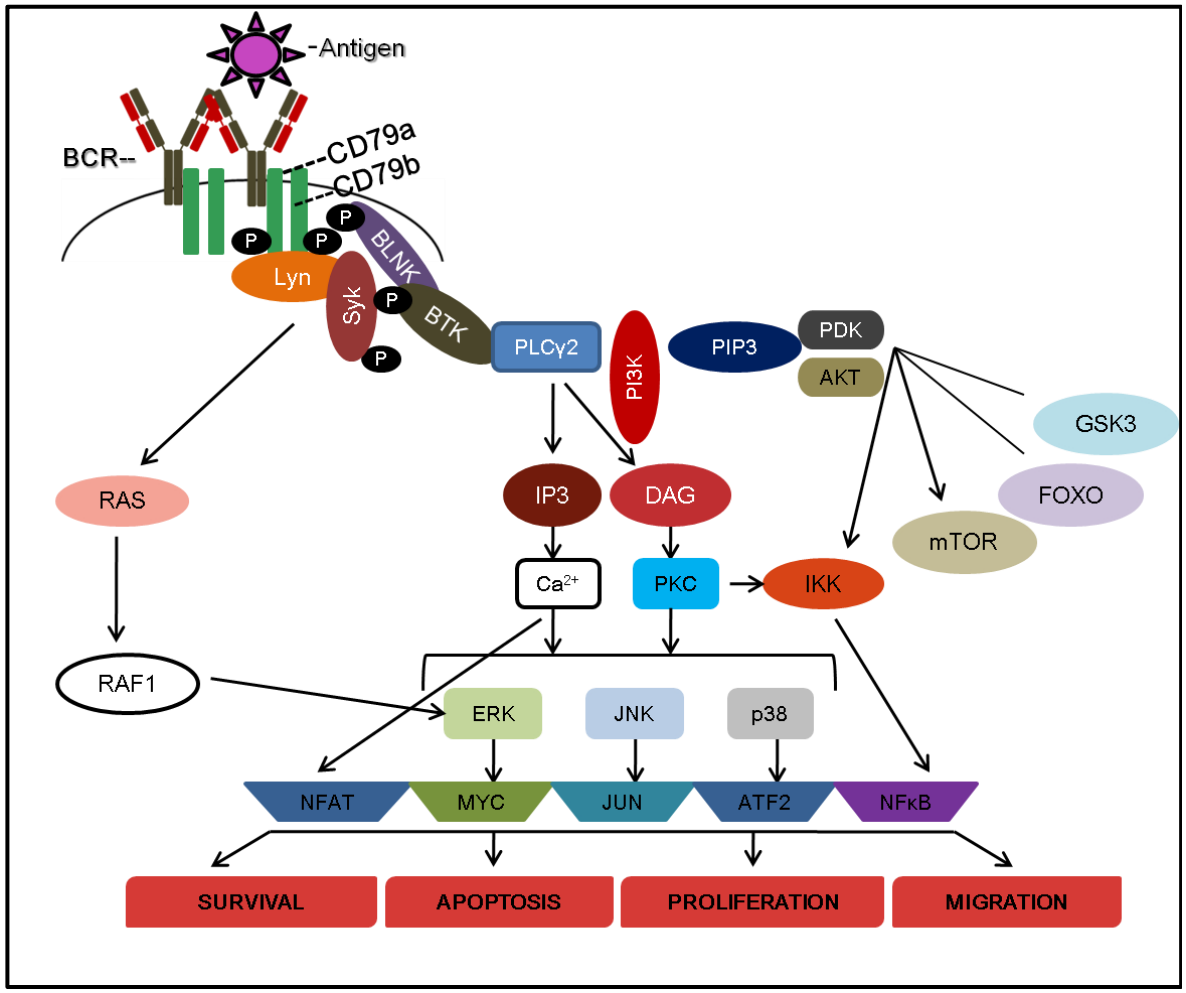


Figure 1-7 The BCR-Associated Signalling

The diagram is an oversimplified presentation of the principal BCR-mediated signalling network. Only key molecules that likely to be involved in CLL are presented. The BCR signalling involves three subsequent stages. Stage 1 is characterised by the formation of signalosome (ITAM (CD79a,b), Lyn, Syk), stage 2- by the downstream activation of BTK and BLNK resulting in triggering of PKC, PI3K, ERK, PLCγ2 interconnected signalling cascades and stage 3- by the activation of the transcription factors that are essential for the regulation of gene expression leading to the BCR-associated biologic responses such as survival, apoptosis, proliferation and migration. PIP3 recruits PDK and AKT (also known as protein kinase B), which in turn activate growth regulatory pathways (mammalian target of rapamycin (mTOR)), glycogen synthase kinase-3 (GSK3) and Forkhead Transcription Factors (FOXO). Image adapted from (International Centre for Genetic Engineering and Biotechnology (ICGEB), 2013).

The signal transmitted and amplified by the signalosome propagates downstream and enters the second stage of BCR signalling, which is characterised by the recruitment and subsequent phosphorylation of the distal signalling molecules such as Bruton Tyrosine Kinase (BTK) and B-cell Linker Protein (BLNK) (Stevenson et al., 2011). BTK possesses a Pleckstrin Homology domain which binds with high affinity to membrane-associated Phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). The interaction of BTK and PIP3 phosphorylates Phospholipase C (PLC-γ2) followed by subsequent hydrolysis of PIP2 into inositol triphosphate (IP3) and diacylglycerol (DAG) (Patterson et al., 2004) (Figure 1-7). DAG triggers PKC activation whereas IP3 interacts with the receptors on the endoplasmic reticulum leading to release of intracellular Ca^{2+} (Stevenson et al., 2011). PKC, in turn, regulates transcription factors activation, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Moreover, PLC-γ2 serves an activator for Extracellular-signal-Regulated Kinase (ERK), Jun Activation Domain Binding Protein (c-Jun) NH2-terminal and p38 kinases (Stevenson et al., 2011) (Figure 1-7). Ca^{2+}-associated signalling pathways modulate a number of downstream signalling regulators, representing the third stage of BCR-associated signalling cascade (Stevenson et al., 2011). Ultimately, this activation phosphorylates a variety of transcription factors which are involved in the regulation of gene expression (Jumaa et al., 2005, Guo et al., 2000) leading to biologic outcomes such as survival, apoptosis, proliferation and anergy (Stevenson et al., 2011).

However, the positive Lyn-associated regulation of the BCR signalling is complimented by the negative regulation via CLL surface markers such as CD22, CD5, CD72, and CD32 (or FcγRIIB) (Stevenson et al., 2011). The co-ligation of these molecules to the BCR (Sarmay et al., 1997) as well as the nature of antigen influence the duration and intensity of the BCR signalling (Stevenson and Caligaris-Cappio, 2004).

1.5.3 Molecular and Cellular Biology of CLL

Over the past years the understanding of the molecular and cellular biology of CLL has advanced significantly, indicating that tumour-microenvironment interactions and the BCR-associated signalling are the key factors promoting the proliferation, migration and survival of CLL cells.

1.5.3.1 *The Role of the Microenvironment in Pathophysiology of Chronic Lymphocytic Leukaemia*

1.5.3.1.1 Tumour-Microenvironment Proliferative Events

Recently, CLL has come to be viewed as a lymphoid malignancy with relatively high proliferation rate supported by the microenvironment (Herishanu et al., 2011, Hillmen, 2011). Data has shown that entire CLL B-population is only partially presented by the peripheral blood (PB) CLL cells as these cells migrate to the bone marrow (BM) and secondary lymphatic tissues such as lymph nodes (LN), and spleen, where they inherit a proliferative stimulus (Wiestner et al., 2005, Hillmen, 2011). Several studies utilising immunohistochemistry have distinguished the structures within LN and/or BM of CLL patients, which morphologically and

immunophenotypically resembled proliferating centers (Schmid and Isaacson, 1994, Caligaris-Cappio, 2003). Utilising proliferation markers such as Ki67, CD24 and CD43 it was demonstrated that identified proliferation centers share common features with reactive B-cell follicles (Herishanu et al., 2011, Hillmen, 2011). The findings suggested that antigen driven proliferation had originated from these proliferating centres which also were found to contain IgM and complement associated follicular dendritic cells (FDCs), enriched with T-cells and characterised by decreased expression of lymphoma/leukemia-2 (Bcl-2) protein (Schmid and Isaacson, 1994). Moreover, a recent study, which had compared the gene expression signature of CLL cells extracted from the different anatomic compartments such as PB, BM and LN and indicated that LN supports the highest CLL cells proliferation, which was also associated with clinical disease progression (Herishanu et al., 2011).

1.5.3.1.2 Microenvironmental anti-Apoptotic and pro-Survival Signalling

Over past years the role for stromal microenvironment in CLL was underestimated until recently, when studies have demonstrated that tumour cell-microenvironment interactions provide anti-apoptotic and pro-survival signals to CLL cells, which also significantly contribute to the development of drug resistance disease (Burger, 2011).

One of the characteristics of CLL cells is resistance to apoptosis, but this biologic feature is missing when CLL cells are cultured *in vitro*. Interestingly, CLL cells, in contrast to normal B cells, improve their survival when they are cultured in the presence of mesenchymal stromal cells (MSC) (Lagneaux et al., 1998). This

observation supports the concept of CLL cell-stromal interactions, indicating that CLL is a tumour which is dependent upon the host (Dal-Bo et al., 2009). The interactions of CLL cells with stromal cells such as macrophages, FDCs, T cells and nurse-like cells, collectively hereafter referred to as 'microenvironment' supports CLL cell survival via BCR and nuclear factor-B activation (Herishanu et al., 2011).

Another factor that is shown to promote CLL survival via the reciprocal interactions between CLL cells involves the activation of NOTCH pathway in BM MSCs (Nwabo Kamdje et al., 2012). This mechanism for anti-apoptotic signalling acts via the NF-κb pathway and is associated with the p53 pathway, which in turn has been linked to p53 genetic defects such as 17p deletion and *P53* mutations (Zenz et al., 2008).

CLL cells also interact with normal CD^{4+} T-helper cells which are mediated by activation of CD40 and chemokines (Hayden et al., 2012). This interaction promotes CLL-cell survival by the autocrine and paracrine production of chemokines and cytokines by CLL-cells.

Resistance to apoptosis in CLL cells has been also associated with activation of the CD74 cell surface molecule expressed on CLL cells. Macrophages express a pro-inflammatory cytokine migration inhibitory factor (MIF), which in turn serves as a ligand for CD74. Upon CD74 engagement, activation of the Syk and NFκB pathways occurs, leading to up-regulation of the *TP63* tumour promoter and subsequent CLL cell survival (Shachar and Haran, 2011).

1.5.3.1.3 Cell Trafficking

Recently, it has become more clear that tumour cell trafficking and homing, which are largely regulated by chemokines and their receptors play an important role in CLL Pathophysiology. Chemokines comprise a large family of 8-12 kDa proteins that together with their cognate receptors regulate leukocyte migration in subtype-specific fashion (Stein and Nombela-Arrieta, 2005). CLL cells were shown to liberate the inflammatory chemokines such as CCL3, CCL4, CCL22, and IL-8, which in turn attract T cells and other immune cells to the site to assist in delivering of pro-survival signalling via activation of PI3K, MAPK and Ca^{2+} mobilisation pathways (Ramsay and Rodriguez-Justo, 2013). Stromal cells in the tissue compartments release homeostatic and inflammatory CXCL12, CXCL13, CXCL 9, 10, 11, and CCL 19 and 21 chemokines, which serve as the ligands to the corresponding receptors expressed on CLL cells (Davids and Burger, 2012). These chemical stimuli enable migration of CLL cells into the tissue compartments, where via tumour cell-microenvironment interactions they will be subject to survival, apoptotic and proliferation signalling (Davids and Burger, 2012).

Multiple studies were undertaken to explore the significance of neoangiogenesis in CLL. Increased angiogenesis was observed in lymphatic tissue and the bone marrow in B-cell lymphoproliferative disorders. Angiogenesis is promoted by pro-angiogenic factors and soluble molecules such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and antiangiogenic factors such as inhibitor thrombospondin-1 (TSP-1). VEGF upon binding to its receptor effects vascular permeability and cell proliferation, migration

and survival, required for angiogenesis (Deaglio and Malavasi, 2009). Autocrine VEGF stimulation of malignant cells is recognised to be a promoting factor in tumour cell survival and disease progression (Till *et al*., 2005) (Figure 1-8).

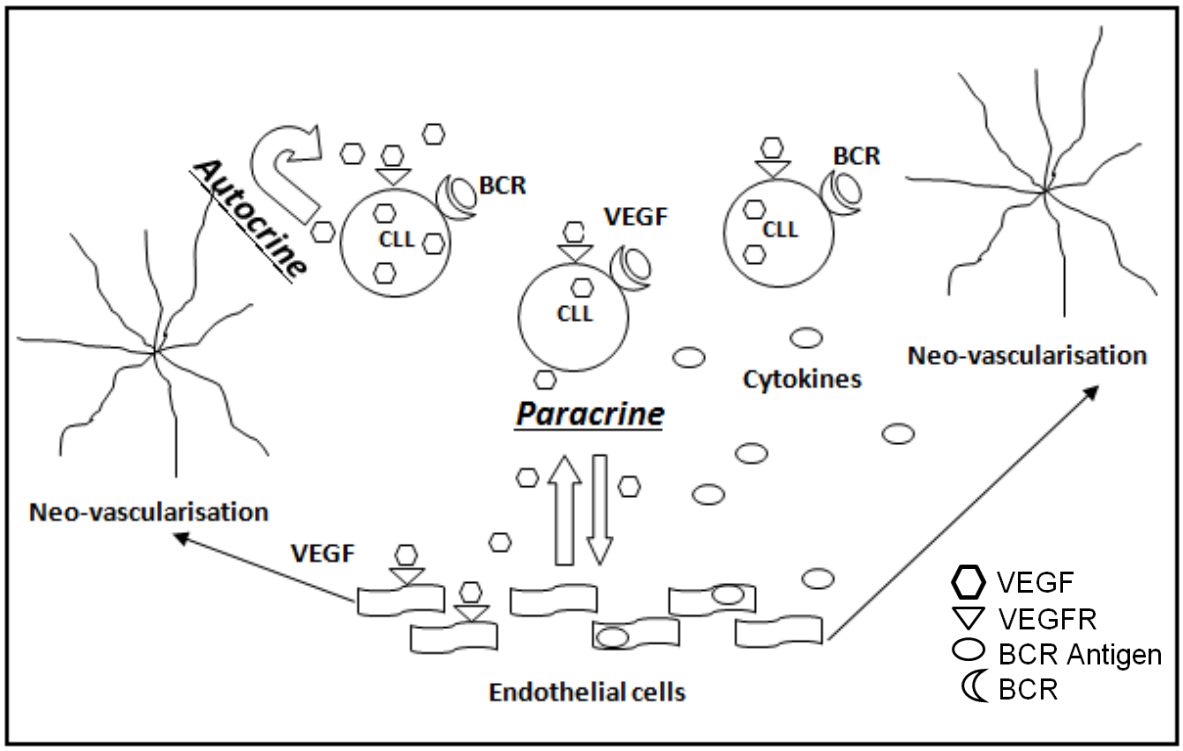


Figure 1-8 Microenvironmental Regulation of CLL Cell Survival

VEGF and other CLL cell and stromal cell-derived cytokines promote CLL cell survival. By producing VEGF, CLL cells promote proliferation of endothelial cells and induce development of neo-vascularisation in an autocrine and paracrine manner. CLL cells possess receptors for VEGF and other VEGF stimulated cytokines, thus, playing an influential part in induction of neoplastic angiogenesis in CLL. The figure is adapted from (Faguet, 2004).

The resistance of CLL cells to apoptosis and extended *in vivo* survival has consistently been linked to over expression of anti-apoptotic proteins, such as B-cell-2 (Bcl-2) protein (Packham and Stevenson, 2005). However, resistance to

apoptosis is not entirely intrinsic to CLL cells (Burger, 2007) and chronic BCR activation was found to be paramount for CLL B-cell survival and proliferation (Stevenson *et al*., 2011).

1.5.3.2 *BCR-associated signalling in Chronic Lymphocytic Leukaemia*

As it was outlined in Section 1.5.2 and 1.5.3 BCR activation and signalling upon binding to external antigen is an essential signal transduction pathway supporting normal B cell behaviour. Over the past few years multiple studies simultaneously reported that BCR signalling is principal in oncogenesis of several B-cell malignancies, although there was still an uncertainty, related to the nature of the antigen. The role for microbial or viral ligands was emphasised in the pathogenesis of some lymphomas (Hermine et al., 2002). Nevertheless both antigen-independent (tonic) and antigen-dependent BCR signalling were described in CLL (Burger and Chiorazzi, 2013). However, although CLL is considered as an antigen-driven lymphoproliferative malignancy, the ligand engagement is believed to be provided by auto-antigens (Ghia *et al*., 2008) present in the tumour microenvironment. Interestingly, recently several studies have reported that not just BCR Immunoglobulins are responsible for ligand recognition but also innate immune pattern recognition Toll-Like Receptors (TLK), which are present on the different immune cells including normal and CLL B-lymphocytes (Agathangelidis et al., 2013) (Figure 1-9).

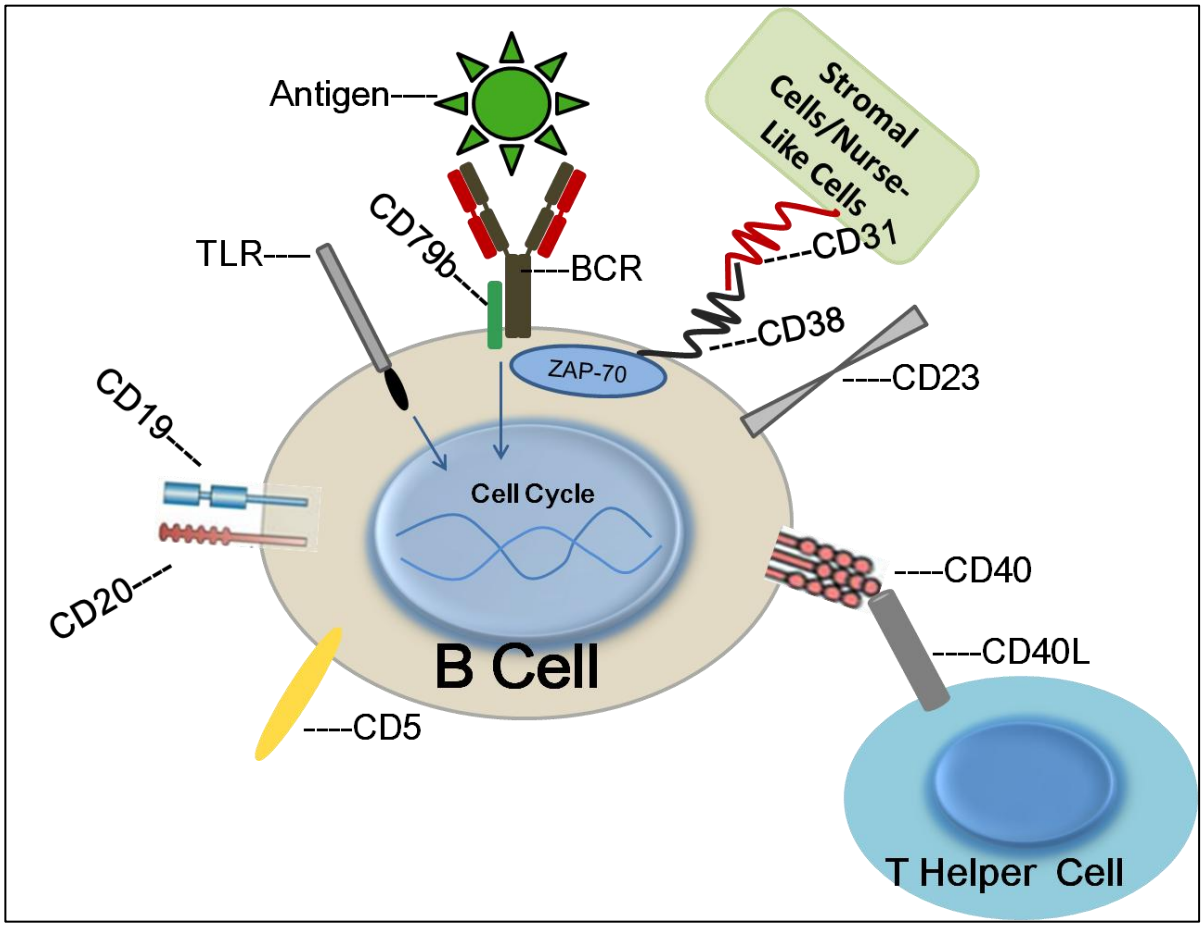


Figure 1-9 BCR Signalling and Regulation of B cell activation

BCR activation is vital for B cell survival and proliferation (Stevenson et al., 2011). Co-expressed CD5, CD19, CD20, CD23, CD38 (Section 2.1.2.6.1), CD 40, CD79 and TLR are demonstrated to play a role in B-cell development, differentiation, BCR signalling, and cell-cycle initiation events. BCR-signalling was shown to be synergetic with TLR signalling leading to initiation of class switch DNA recombination resulting in the B cell development and antibody response (Pone et al., 2012). The study demonstrated that CD40 signalling is crucial for T-cell dependent B cell activation (Foy et al., 1993). Moreover, signalling via CD40 is important in B lymphocyte activation, maturation and isotype switching (Siepmann et al., 2001). ZAP-70 integrates BCR and CD38 signalling whilst promoting ZAP-70^{+}/CD38^{+} CLL cells to home to a stromal microenvironment where they gain proliferative and survival signals. Ligation of CD38 by its ligand CD31, which is expressed on stromal and nurse-like cells, activates ZAP-70 phosphorylation (Burger 2007 Fledgling prognostic markers in CLL).

Given the findings that several BCR-associated biological variables correlate with CLL disease aggressiveness and prognosis, the role for BCR signalling in pathogenesis of CLL was considered as critical. Firstly, the use of restricted structure of IgV_H gene (Section 2.1.2.4.1) and an extreme similarity in the structure of BCR in about 20% of completely unrelated CLL patients from the different geographical areas (Oppezzo and Dighiero, 2013) are related to BCR signalling (Ramsay and Rodriguez-Justo, 2013). Additionally, the positive therapeutic effect, associated with the inhibition of signalling pathways downstream of BCR, and BCR-associated NFκB-dependant survival and proliferation of CLL B cells (Burger and Chiorazzi, 2013) ascertain the understanding about the importance of BCR signalling in CLL.

However, the cellular response to BCR ligation varies between CLL B cells. *In vitro* studies demonstrated that a portion of CLL B cells exhibit a poor responsiveness to BCR ligation (Stevenson et al., 2011), which will hereafter be referred as 'non signallers'. Non signalling CLL is characterised by a reduced signalling capacity and weak activation of the downstream signalling molecules (including MAPK pathway) upon BCR ligation. Non-signalling is a typical feature of M-CLL cells and has been associated with indolent disease behaviour (Mockridge et al., 2007). CLL signallers retain the ability to amplify and transmit the received BCR-signal, which subsequently results in the increased proliferation and survival. These features have been associated with U-CLL and progressive disease (Stevenson et al., 2011). The identification of the phosphorylation of Syk, Lyn and

ERK following in vitro ligation of the BCR have been widely utilised as the indicator for BCR responsiveness.

The biological cause of BCR-signalling heterogeneity is completely elucidated. However, the level of the surface IgM expression, which is generally weaker in CLL in comparison to normal naive B cell, was shown to be a discriminating factor for BCR responsiveness (Stevenson *et al*., 2011). Such BCR down regulation potentially could be induced by continuous antigen stimulation of the receptor in CLL microenvironment. Moreover, Syk phosphorylation (Kipps, 2007a) or mutation within the ITAM (Stevenson and Caligaris-Cappio, 2004, Allsup *et al*., 2005) as well as the mechanisms, which include Zeta-chain-associated protein kinase 70 (ZAP70) (Chen et al., 2002) expression and activity of the protein tyrosine phosphatase (PTP11B) interactions with lipid rafts (Lanham *et al*., 2003) also have been suggested as a hypothetical explanation for hypo-responsiveness of BCR.

Moreover, GEP study shown that BCR is chronically activated in CLL cells derived from BM and LN and that the level of this activation is correlated with IgV_H status and hence CLL prognosis (Herishanu et al., 2011). Similarly, proteomic studies have shown a partial association of ZAP70 and IgV_H mutations, which also related to hyperactivation of BCR (Wiestner *et al*., 2003).

CHAPTER 2

Predicting and Defining Chronic Lymphocytic Leukaemia Clinical Outcome

Chapter 2. Predicting and Defining Chronic Lymphocytic Leukaemia Clinical Outcomes

In recent years the novel understanding in the definition of molecular and cellular biology of CLL has not only begun to be translated into new therapeutic approaches but also led to a significant increase in the number of clinical and molecular factors known to be predictive of CLL outcomes (Zenz *et al.*, 2010). At this present time the only option to predict the outcome in CLL, apart from clinical staging, is to utilise biomarkers. Those novel biomarkers can be utilised to distinguish CLL patients that are more likely to develop a progressive disease and might benefit from early treatment intervention and to identify therapy targets-to stratify drug use. Additionally, using biomarkers it has become possible to predict the tendency for progression into refractory CLL and or to detect minimal residual disease after applied therapies (Hallek, 2008). Despite this, there are no established molecular biomarkers that are currently utilised in routine clinical practice under guidelines for CLL management (Hallek *et al*., 2008a). CLL patients are assessed for IgV_H mutation status (2.1.2.4.1), a presence of genetic aberrations (2.1.2.4.2) and cellular expression of Zeta (ζ)-associated protein 70 (ZAP70) (2.1.2.6.2), and CD38 (2.1.1.6.1) prior to evolving into clinical trials (Montserrat, 2012). Although the identifications of IgV_H status and genetic aberrations are shown to have the strongest prognostic value in CLL, their analyses involve the complex gene sequencing assays, which are both expensive and time consuming. In regards of ZAP-70 and CD38, these are utilised as

additional biomarkers due to the limited independent effects on prognosis; the use of conditional cut-offs and lack of markers validation in prospective studies (Malek, 2013). Therefore further research is required in order to find new prognostic markers which will be reliable, less time consuming, affordable for routine clinical testing and also will be able to predict the time to disease progression.

2.1 Prognostic and Predictive Factors in Chronic Lymphocytic Leukaemia

Prognostic markers are the tools, which whilst defining the information on the biological characteristics of CLL, also provide an estimate of the potential disease outcome in untreated patients. Whereas, predictive factors are those that predict the likely effect of given therapies on the tumour (Italiano, 2011, Montserrat, 2012). However, several factors have both prognostic and predictive relevance.

2.1.1 Traditional Clinical Prognostic Markers

At present, outside of clinical trials, clinical stage remains the first and strongest prognostic tool in CLL disease progression (Rai *et al*., 1975, Binet *et al.*, 1981, Pangalis *et al*., 2002, Montserrat, 2006) (Section 1.3.4). Despite the wide scale use by clinicians, the staging system has limitations (Montserrat, 2006) in respect of predicting therapeutic response and disease behaviour, which can be characterised as progressive (high risk) or indolent (low risk) (Furman, 2010). Moreover, when defining the patient' disease outcome, several other demographic criteria, such an advanced age and sex are important to take into account.

Mild anaemia and thrombocytopenia are common haematological features which are present in early stages in approximately 25-50% of CLL patients. As the disease progresses and the bone marrow infiltration becomes more extensive, it may result in more severe anaemia and thrombocytopenia, which consequently leads to an increased tendency for bleeding, bruising and compromised haemostasis (Inamdar and Bueso-Ramos, 2007). The pattern of bone marrow infiltration such as diffuse or non-diffuse is representative of the tumour burden, which is evidenced to be associated with disease progression (Hallek, 2008).

High Absolute lymphocyte Count (ALC) and increased LDT significantly predict CLL morbidity and mortality. LDT is identified at the time of diagnosis and is reflective of how fast the CLL cells are dividing and describes the period of time, that is required for lymphocytes to double in number (Molica and Alberti, 1987). CLL patients with a rapidly increasing lymphocyte count, which takes ≤12 months to double were found to be associated with a poorer prognosis and life expectancy; whereas patients with lymphocyte count that takes >12 months to double are correlated with a more favourable outcome (Molica and Alberti, 1987).

Atypical morphology is characterised by an increased amount of prolymphocytes (<10%) or cells with cleaved nuclei (<15%) or a combination of both and this feature correlates with rapid disease progression (Faguet, 2004).

2.1.2 New Prognostic Biomarkers

In the past two decades the direction in the field of CLL research has changed from recognition of the prognostic factors associated with clinical

characteristics to identification of the prognostic factors associated with disease biology, which are known as biomarkers (Montserrat, 2006)

Table 2-1 provides a summary of recently identified prognostic factors including traditional prognostic markers, which are separated into five different groups depending upon the origin and role in the biology of CLL.

2.1.2.1 *Response to Therapy and Duration of Response*

The response to treatment and the eradication of minimal residual disease (MRD) serve as a valuable prognostic marker and may assist in the prediction of CLL patient outcome (Burghouts *et al.*, 1980, Montserrat, 2006). The improved quality and duration of complete remission have been associated with longer survival (Montserrat, 2006). Until recently, the 1996 NCI-WG response criteria, which was designated as an overall response rate (ORR) was utilised for evaluation of the therapeutic response in CLL. According to these criteria the therapeutic response was defined as complete remission (CR), partial response (PR), stable disease (SD), and progressive disease (PD) (Cheson et al., 1996, Hallek et al., 2008b). However, recent clinical trials utilise more sensitive evaluation criteria, which depend on the quality of data analysis and include the reports on complete biological as well as clinical remission. A confirmatory computerised tomography scan, bone marrow biopsies and evaluation by a blinded independent review committee can be utilised in some clinical trials (Smith *et al.*, 1997, Kipps, 2007b, Eichhorst *et al*., 2009).

Table 2-1 Prognostic and Predictive Markers in Chronic Lymphocytic Leukaemia

The data is used from (Furman, 2010) and demonstrates only a partial list of biomarkers).

CLINICAL	CHROMOSOME GENE-ASSOCIATED	CYTOKINES AND SOLUBLE MOLECULES	CELLULAR EXPRESSION-ASSOCIATED	OTHER
Stage	IGVH status	β2-microglobulin	CD38	Bone marrow infiltration
LDT	Genetic Aberrations (FISH)	VEGF	ZAP-70	Direct anti-globulin test
ALC	Micro RNA (miRNA)	bFGF	CD49d	Re-IA
Bone Marrow infiltration	V gene usage	IL-6,IL-8	CD26	CLLU1
Age	IRF4 polymorphism	Trombospondin-1	FCRL2	Circulating endothelial cells
Gender	Deletion 6q (del 6q)	Plasma thrombopoietin	p27	
Cell morphology	MDM2 SNP	Soluble ICAM-1	HS1	
MRD status	Bcl-2 polymorphism	Soluble NKG2D ligands	P53 mutation	
Treatment response	Bcl-6 mutation	Serum thymidine kinase		
Duration of response	Telomere lengths	Soluble CD27		
Performance status	Nuclear morphology	Soluble CD23		
		Angiopoietin-2		
		Ki-67		
		Lipoprotein lipase		
		Serum-free light chain		

NKG2D- The Natural Killer Group 2D, IRF4- Interferon regulatory factor 4, MDM2- Mouse double minute 2 homolog, ICAM-1- Intercellular Adhesion Molecule 1, FCRL2- Fc receptor-like 2, Re1A- transcription factor, CLLU1- chronic lymphocytic leukemia up-regulated 1.

2.1.2.2 *Minimal Residual Disease*

MRD is the term which refers to low-level disease with no clinical symptoms or signs. MRD is represented by a number of CLL cells that failed to be eliminated and remained during treatment or during remission after treatment. MRD detection presents its potential value in the making of therapeutic decisions via discrimination between the patients who have responded well to the initial treatment and the patients who require continuation or consolidation / intensification of treatment in order to minimise the risk of disease relapse (Paietta, 2002). CLL patients, who exhibit MRD-negative status demonstrate less aggressive disease behaviour and thus have longer survival and more favourable outcome (Montserrat, 2005). There are a number of prospective trials and studies which aim to verify and establish the clinical significance of MRD quantification in CLL. The German CLL Study Group CLL8 trial demonstrated that MRD levels independently predict OS and PFS in CLL patients and thus can be utilised as a potential surrogate marker for treatment efficacy (Bottcher et al., 2012).

2.1.2.3 *Performance Status (Section 1.3.5.1)*

In order to determine the optimum treatment plan it is paramount to assess patient fitness to myelosuppressive and immunosuppressive therapies prior to the initiation of treatment. One criterion is termed performance status, a good performance status is associated with better response to treatment than those patients with poor performance status (Gribben, 2010). A number of different scoring systems are available for assessment of individual patients' fitness,

however none of the systems are CLL-specific (Oscier *et al*., 2012). One of the systems is known as the Cumulative Illness Rating Scale (CIRS). This scoring system assesses the existence of significant co-morbid conditions in different organ systems (Gribben, 2009). The German CLL Study Group recommended a scoring system, in which CLL patients are separated into three groups: “Go Go”, “Slow Go” and “No Go” as outlined in Section 1.2.5.1.

2.1.2.4 *Chromosome and Gene Associated Biomarkers*

2.1.2.4.1 Immunoglobulin Gene Mutation Status (*IgV_H* Status)

Up until 1999 several researchers simultaneously tried to determine the nucleotide structure of the immunoglobulin gene and to compare *IgV_H* DNA sequences from CLL B cells against the original germline sequence with subsequent estimation of the level of any existing deviation. In 1999 the Hamblin and Stevenson and Chiorazzi groups independently reported that *IgV_H* genes in CLL B cells can be either somatically mutated or unmutated when compared to database germline DNA sequences such as IGBLAST (IGBLAST NCBI, 2013). This is representative of mutated and unmutated status respectively (Damle *et al.,* 1999, Hamblin *et al*., 1999, Montserrat, 2006). Hamblin and colleagues studied 84 CLL patients and demonstrated that 45.2% (38 cases) expressed ≥ 98% sequence homology with germline VH gene sequence and were considered as unmutated, U-CLL. The other 54.8% (46 cases) expressed >2% of gene mutation deviation from the germline DNA sequence and were regarded as mutated, M-CLL (Hamblin *et al* 1999; DamLe *et al* 1999). Therefore, since then the cut-off value of 98 % is widely

utilised in order to discriminate between M-CLL and U-CLL, suggesting that 2% difference in the nucleotide sequence can be caused by gene polymorphism (Davis *et al*., 2003). Additionally, the correlation between CLL clinical characterisation and IgV_H mutation status has also been investigated. One interesting finding that has come out of this study is the significant correlation between IgV_H mutation status and CLL outcome (Morilla *et al.*, 2008). Unmutated IgV_H status was associated with atypical morphology, unfavourable genetic aberrations (Section 2.1.2.4.2), and disease aggressiveness and, as such, a poor prognosis (Chevallier *et al.*, 2002). Whereas CLL patients with mutated IgV_H gene have demonstrated favourable prognosis and much longer survival time of about 10-20 years (Damle *et al.*, 1999, Hamblin et al., 1999) (Figure 2-1). Subsequent research provided further evidence that unmutated IgV_H status is correlated with advanced clinical stages, clinical relapse following autologous stem cell transplantation (SCT) (Ritgen *et al*., 2003) and shorter treatment-free interval in patients with CLL (Morilla *et al*., 2008).

Following this, further study of the mutation distribution and gene usage in IgV_H, has shown that based on the normal repertoire in healthy $CD5^+$ B-cells, certain IgV_H family genes and their alleles were over or under expressed in CLL than would be normally expected (Messmer *et al.*, 2004). The data reported that *VH1-69* and *VH4-34* family genes were overrepresented whereas *VH3-30* and *VH3-09* were not expressed to the expected degree in CLL. Moreover, utilising 172 leukemic clones of B-CLL patients, this study demonstrated that, the more frequently used genes were *V1-69*, *V3-07* and *V4-34*, which were predominantly

completely unmutated (78%), predominantly mutated (93%) and usually mutated (80%) (Messmer *et al*., 2004).

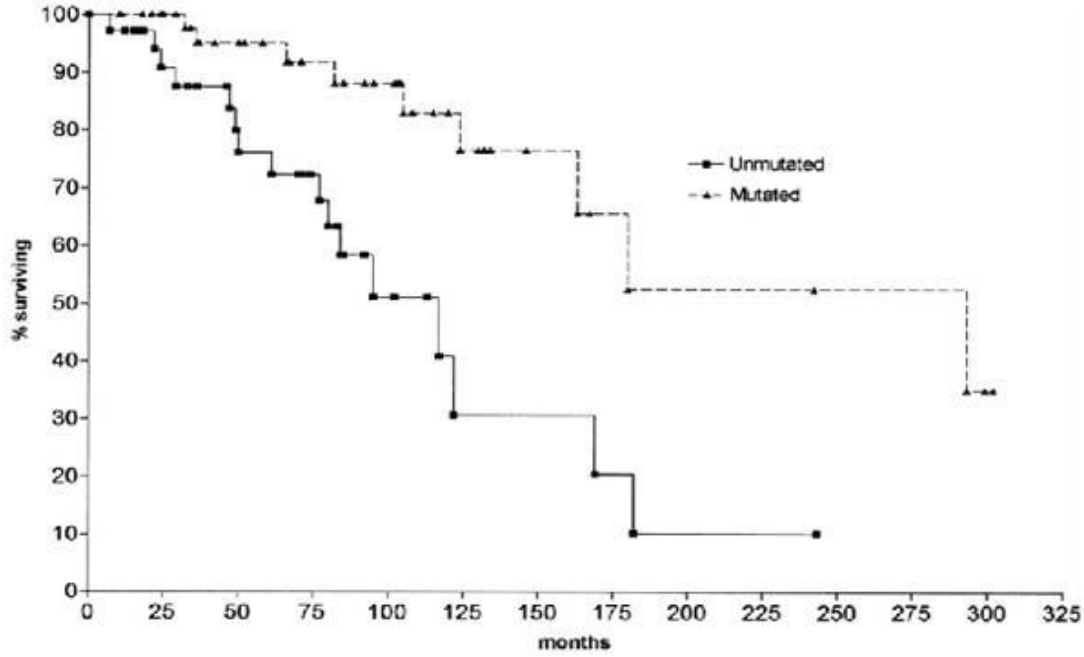


Figure 2-1 Kaplan Meier Plot Representing the Correlation between CLL Patients Survival and IGVH Mutation Status

The Kaplan Meier survival curve was adapted from (Hamblin et al., 1999). CLL patients with unmutated IGVH status (n=38) demonstrate a significantly shorter median survival (173 months) than patients with mutated IGVH status (n=46), who had a significantly longer median survival (293 months), (p=0.001). The commonly used threshold value of 98% was utilised. The difference from the germline gene of >2% was considered as "mutated".

However, it is noteworthy to mention, that the exception to these findings was originally reported by the study of Tobin and colleagues, who investigated the IgV_H mutation status and VH gene usage in 119 CLL patients and demonstrated

that a subset of mutated CLL cases which use *VH3-21* gene segment behave similarly to unmutated cases with shorter survival and unfavourable outcome, which represented the third entity in CLL (Tobin *et al*., 2002). Later, other groups had validated the data (Singh and Sahu, 2006), (Guo *et al*., 2006, Chakrabandhu and Singh, 2006).

2.1.2.4.2 Genetic Aberrations and Cytogenetic Parameters

Multiple studies have reported that the identification of genetic aberrations occurring in CLL cell genome may have diagnostic and prognostic significance in predicting survival outcomes in patient with CLL. Interphase fluorescence in situ hybridisation (FISH), a technique developed in the late 1990s, markedly enhanced chromosomal analysis of CLL cells and detection of genomic abnormalities in CLL patients (Stilgenbauer *et al.*, 2002). FISH is a very sensitive technique but has its limitations. FISH determines only those genetic aberrations that are probed for during the experiment, whereas karyotyping examines entire chromosome for any structural changes (Furman, 2010).

In a study by Dohner *et al* blood samples of 325 CLL patients were analysed for molecular cytogenetic mutations in several different regions. The data revealed that 82% (268/325) of CLL cases acquired genetic abnormalities within their malignant clone. The remaining 18% of CLL patients were referred to as cases with "normal" karyotype (Dohner *et al*., 2000b), which actually reflects the absence of the examined genetic aberrations (Furman, 2010). Moreover, the data demonstrated that 175 CLL patients acquired only one cytogenetic aberration, 67 patients exhibit two aberrations, and 26 patients were identified with more than two

aberrations (Dohner *et al.*, 2000b). The set of defined chromosomal abnormalities was correlated with CLL patients' survival and the result is outlined in Figure 2-2.

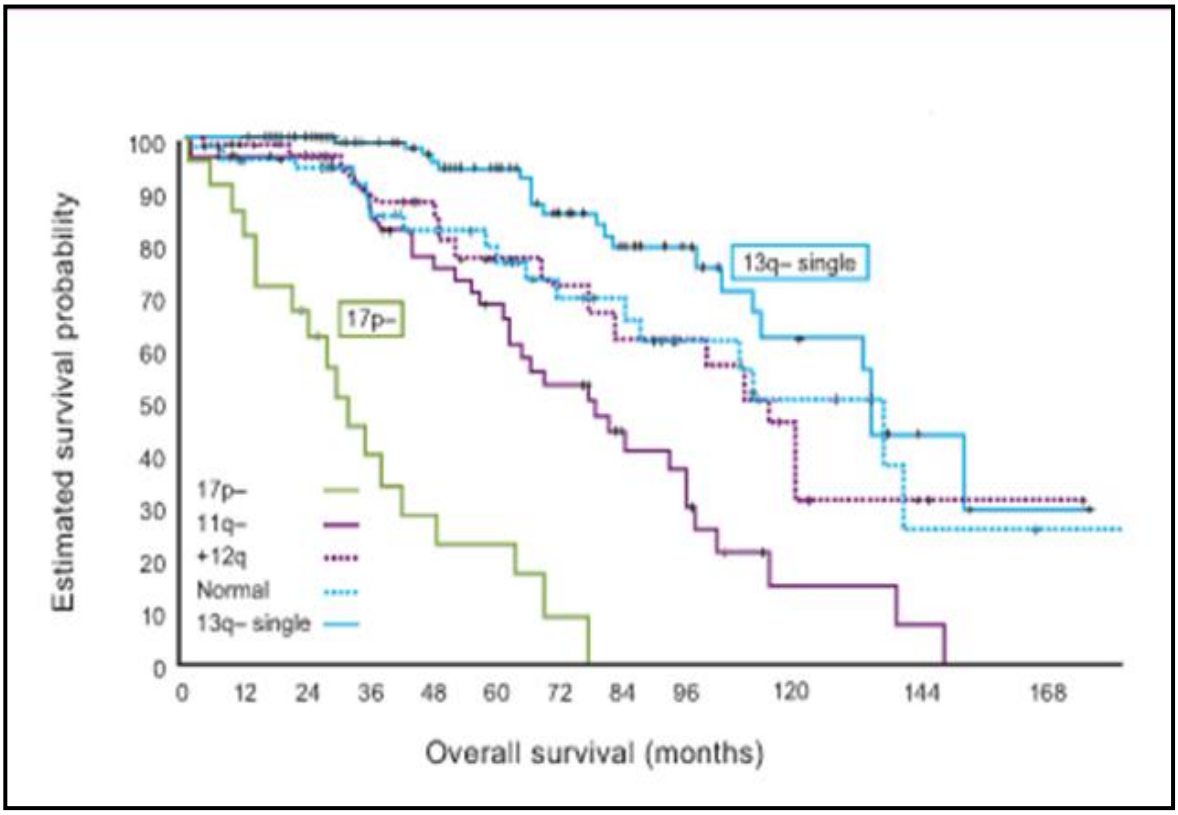


Figure 2-2 Prognostic Value of Chromosomal Abnormalities

This Kaplan Meier plot is adapted from (Dohner et al., 2000b) and demonstrates CLL survival by cytogenetic abnormalities. CLL patients examined in this study were separated into five groups and shown median survivals as follow:
del 17p (32 months)
del 11q (79 months)
trisomy 12 (114 months)
normal (111 months)
del 13q (133 months)
All groups included multiple genetic aberrations with only exception for del 13q, which was presented by a single abnormality (Dohner et al., 2000b).

Cytogenetic aberrations as determined by FISH demonstrate an independent prognostic value (Moreno and Montserrat, 2010) (Table 2-2) and may influence therapeutic decisions (Section 1.3.5). It is recommended to perform FISH prior to the treatment initiation and to subsequent, second- and third-line therapies due to the possibility of acquiring new cytogenetic defects during the course of the disease (Hallek, 2008).

Table 2-2 Genetic Aberrations and Associated Prognostic Risk

Chromosomal aberrations determined by FISH can be grouped into good, intermediate and poor prognosis. RB-1 Gene- Retinoblastoma Tumour Suppressor gene, HIP1R Gene- Huntingtin-Interacting Protein 1-Related gene, ATM Gene- Ataxia Telangiectasia Mutated gene, p53- Tumour Suppressor Gene.

Genetic Aberrations Determined by FISH	Associated Mutations	Prognosis
Deletion of 13q	*RB-1* Gene	Good
Normal karyotype	--	Good
Trisomy of 12	*HIP1R* Gene	Intermediate
Deletion of 11q	*ATM* Gene	Poor
Deletion of 17p	*p53* Gene	Poor

Although FISH is extremely useful in the prediction of the CLL clinical outcome and treatment initiation, its limitations have stimulated the development of novel techniques such as comparative genomic hybridization (CGH), a genome-wide association study of single nucleotide polymorphisms (SNPs), and whole genome sequencing or "deep sequencing" (Chiorazzi, 2012) which support the retrieval of the most comprehensive information of an individual's genetic variation.

2.1.2.5 *Cytokines and Soluble Molecules*

2.1.2.5.1 Beta2-Microglobulin (β_2M)

β_2-microglobulin is a 11.8 kDa membrane protein from the class I major histocompatibility complex and, therefore, is found on the surface of almost every nucleated cell (Delgado *et al*., 2009b). Elevated serum β_2M levels has been linked to increased proliferative activity (Simonsson et al., 1980) and subsequent high cell turnover. The up-regulation of β_2M has reproducibly been correlated with a high tumour burden and shorter progression free survival in older CLL patients (Wierda *et al.*, 2007, Tsimberidou *et al*., 2008, Parker and Strout, 2011). Moreover, decreased levels of β_2M have been associated with better remission rates and overall survival following Fludarabine-based regimens in previously untreated and treated CLL patients (Wierda *et al*., 2007). Interestingly, the discrimination between normal and elevated level of β_2M may vary between different studies and laboratories, hence the cut-off level ranges between 2 mg/L (Tsimberidou *et al*., 2008) to <4.0 mg/L (Kay *et al.*, 2002). Although, the β_2M level is clinically relevant and useful in prediction of CLL survival and can serve as a prognostic factor in CLL

(Hallek et al., 1996), when taken alone this biomarker has limited prognostic meaning. However, elevated serum β_2M level, unmutated *IgV_H*, high risk genetic aberrations such as *del 17p*, positive CD38 and ZAP 70 expression, collectively have been associated with a more aggressive form of CLL and therefore assays for β_2M level identification is utilised in prospective clinical trials (Hallek et al., 2008a).

2.1.2.6 *Cellular Expression-Associated Biomarkers*

2.1.2.6.1 CD38 as a Prognostic Marker

CD 38 is a cell surface molecule expressed by a range of haematopoietic and non-haematopoietic cells, but recent interest has been focused on the role in B lymphocytes (Hamblin, 2003). CD38 is a 45kDa transmembrane glycoprotein with receptor and enzyme properties, the expression of which is regulated by the tumour microenvironment (Patten *et al.*, 2008). CD38 expression, which can be determined utilising flow cytometry, was intensively studied in the past two decades. CD31, a platelet endothelial cell adhesion molecule, was reported to serve as an antigen for CD38 receptor (Deaglio et al., 2001). Damle and his group in 1999 reported that elevated CD38 expression correlates with unmutated *IgV_H* gene rearrangement with 92% accuracy and has a possible prognostic value in regards to disease behaviour and median survival. In this study the patients with unmutated *IgV_H* were associated with higher percentages of $CD38^+$ B-CLL cells (≥30%), poor treatment response and shorter survival (Damle *et al* 1999). Although, there is inevitable disagreement in the establishment of an absolute

value for the positive vs. negative expression with the cut-off ranging between 7% to 30%; however another study has suggested that the presence of the distinct $CD38^+$ population within CLL clone, rather than a cut-off value, is associated with poor risk CLL (Durig *et al*., 2002, Ghia *et al*., 2003). Several clinical studies observed the level of CD38 expression before and after the treatments and reported that CD38 may change its status during the disease course (Hamblin et al., 2002). Chang and Cleveland suggested that a change of CD38 expression from negative to positive phenotype is associated with disease aggressiveness and shorter median survival (Chang and Cleveland, 2002). CD38 expression was proposed as a surrogate marker for IgV_H mutation status following three models: the expressions of both markers are complementary: such as positive or negative or discordant, last was also associated with reduced OS (Damle et al., 1999, Hamblin *et al*., 2002).

2.1.2.6.2 ZAP-70 as a Prognostic Factor

ZAP70 is a CD3-associated intracellular tyrosine kinase, which is known to play an essential role in T-cell receptor complex (TCR) signalling pathway and early B-cell development (Perrot *et al*., 2011). This 70 kDa protein has been studied extensively in the last two decades due to the unusual expression in CLL cells but not in healthy mature B-lymphocytes (Hamblin 2005).

Primary data from gene expression profiling studies suggested that the ZAP70 gene was differentially expressed between M-CLL vs. U-CLL and predicted IgV_H status in 93% CLL cases (Wiestner *et al* 2003). As more research has been carried out the interest in ZAP70 expression on CLL cells continued to grow,

primarily because the expression of ZAP70 in comparison to CD38 was found to remain stable during the disease course (Crespo *et al.*, 2003, Durig *et al*., 2003) and the routine identification of ZAP70 utilising flow cytometry is less labour-intensive and more affordable than gene sequencing required for IgV_H mutation status assay (Hamblin 2007) thus can be fully standardised for clinical practice. The cut-off value for ZAP70 expression is controversial, although 20% positive CLL cells identified by FACS is commonly utilised. Using a cut-off value of 20%, ZAP70 positivity was reported to be predictive of unmutated IgV_H status (Crespo *et al.*, 2003, Orchard *et al*., 2004, Rassenti *et al.*, 2008). Del Principe *et al.* conducted the study investigating 289 CLL patients and suggested the benefit of simultaneous ZAP70 and CD38 expressions analysis and discriminated CLL patients into three groups: ZAP70 and CD38 both positive, negative or discordant, resulting in OS of 18%, 94% and 41% respectively (time=16 yrs, $p<0.001$) (Del Principe *et al*., 2006). The positive expression of CD38, ZAP70 and unmutated IgV_H as independent markers have demonstrated to be correlated with aggressive disease, advanced stages and shorter median survival, although in terms of predicting the time to first treatment (TTFT), ZAP-70 was shown to have the highest prognostic value (Rassenti *et al*., 2008).

In conclusion, ZAP70 expression certainly has valuable prognostic importance (Dighiero and Hamblin, 2008) and is used to stratify patients in the clinical trials. Multivariable combined analysis revealed the possibility of CLL patient differentiation into three prognostic subgroups according to ZAP70 and CD38 expression:

- ZAP70 and CD38 negative cases with a low risk, favourable prognosis, more indolent disease behaviour and approximately 130 months treatment-free survival,
- Discordant cases, when one of the markers is positive with intermediate risk and 43 months treatment-free survival,
- ZAP70 and CD38 positive cases with high risk, poor prognosis, progressive disease course, advanced stages and 30 months treatment-free survival, and it was reported to be irrelevant to the stage of CLL (Cramer and Hallek, 2011).

2.2 The identification of novel candidate biomarkers

Whilst the previous section highlighted the prognostic and predictive values of the various biomarkers discussed, there are however a few significant reasons that these are not widely utilised in clinical practice. Prognostic values of the majority of these biomarkers have never been validated in prospective studies. In addition the tests for identification of the prognostic and predictive biomarkers discussed previously are very costly, time-consuming, require specialised equipment, reagents and specifically skilled personnel. Moreover there are no fully optimised methodologies for robust measurement of these biomarkers which may cause discrepancies in a proportion of clinical laboratories (Binet *et al.*, 2006, Delgado *et al.*, 2009b).

As a result of the continued need for biomarkers to be identified in support of the diagnostic and management of CLL, there are numerous ongoing studies

which are investigating novel candidate biomarkers at the cellular and molecular levels in order to establish reliable clinically significant biomarkers which can be utilised and integrated into routine clinical practice.

2.2.1 Biomarker Discovery

In the past three decades biomarker discoveries have become integral to diagnostic, prognostic and treatment applications, drug discovery and development of safe drug intervention. The high-throughput search for biomarkers is spurred by the emergence of new molecular genomics-based (genomics and transcriptomics) and proteomics-based (proteomics) experimental platforms. The DNA sequencing of the human genome, which was finalised in 2001, has revealed invaluable information about complex genome architecture and mutations; however the biological and clinical significance of these structures are not fully understood. The application of new advanced molecular profiling technologies such as global mRNA-based gene expression analysis with microarrays and PCR assays and gene sequencing have led to tremendous advances in basic research and discovery and validation of novel biomarkers. Nevertheless, the study of the proteome is significantly more challenging and difficult than whole genome sequencing, however, it is important to stress that the protein biomarkers have equal if not greater potential for biomarkers identification for several important reasons:

- firstly, due to alternative splicing and heterogeneous post-translational modifications a single gene may generate several different isoforms of the corresponding protein depending on the cell or tissue type and

developmental stage (Valenzuela *et al*., 2004), which results in the expression of structurally and functionally distinct proteins;

- secondly, the human genome remains relatively static throughout the lifetime, whereas the human proteome is dynamic and varies dramatically over time depending on the cell type, physiological state and microenvironmental influences;
- thirdly, transcriptomics data does not always correlate with protein content (Rogers *et al*., 2008) and abundance. Experimental findings demonstrated that the lack of correlation may be due to not all *mRNAs* being translated into protein or translated inefficiently, resulting in a very low abundance of the corresponding protein (Mao *et al*., 2014). Moreover, some endogenous *mRNAs* may degrade rapidly and undergo such a high turnover rate that they may not express any transcript product;
- lastly, genomics and transcriptomics do not address modifications which occurred after translation. Each protein can undergo chemical modification in different ways after synthesis, which can significantly alter the structural and functional properties.

Ultimately, the protein expression profile in conjunction with gene expression and other approaches data can open a greater insight into disease aetiology, pathogenesis and development which can be used to create new diagnostic, prognostic and therapeutic tools (Boyd *et al.*, 2010).

2.2.2 The study of the proteome

The word “proteome” is originated as a term referring to ***prote***ins expressed by a gen***ome***. ***Proteins*** are macromolecules consisting of a single or multiple chains of amino residues and serve as the primary constituents of the protoplasm of all living cells. ***Genome*** is the entirety of all genetic information. The scientific study, which aim to identify, characterise and quantify the protein structure, function and interaction and subsequently translate it into an accurate and comprehensive data is known as ***comparative proteomics***.

2.2.2.1 *Proteomics Pipeline for Biomarker Discovery*

The proteomic platforms for biomarker discovery generally consist of four consequent steps leading to eventual implementation of the biomarkers in a clinical setting. These steps are graphically presented in a Figure 2-3.

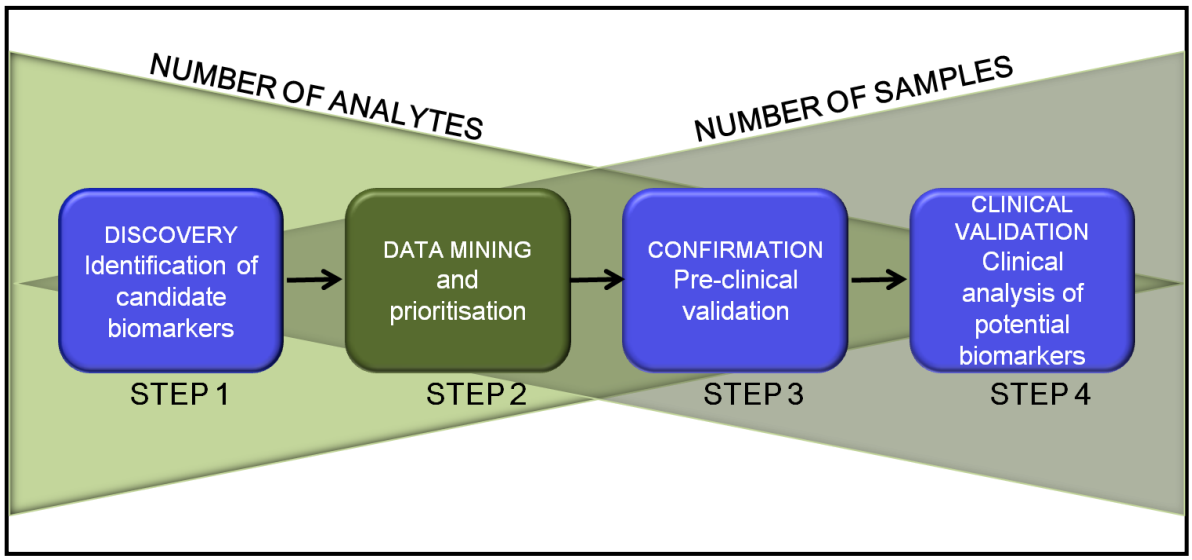


Figure 2-3 Biomarker Discovery Pipeline

The model of Biomarker Discovery Pipeline utilised in this project consists of the following steps:
Discovery step, during which the mass spectrometry (MS) or microarray-based approaches may be applied in order to generate a large list of differentially, expressed proteins (DEPs). MS is a laboratory technique, which primarily separates ions by their mass to charge ratio (m/z) and then identifies them using MS analyser, whereas the microarray-based analysis is used to interpret the data generated from protein microarrays allowing the evaluation of a large number of proteins;
Data mining step is required for analysis of a discovery-step data utilising knowledge databases and searches carried out across different data mining models for a purpose of extracting patterns, associations, relationships and for prioritisation of biomarker candidates for future research;
Confirmation step employs different protein expression assays such as western blotting or ELISA for subsequent precise measure of change in levels of proteins, confirmation of the differential expression of candidate biomarkers;
Validation is the most cost and time-consuming step requiring high sample throughput and evaluation of the putative biomarkers in the clinical context utilising immunoassay-based approaches. The number of ANALYTES generated during the discovery step anticipated to decline towards the validation step due to omission of false-positive or weaker candidate, however, the number of SAMPLES expected to increase towards clinical validation to ensure clinical or/and biological value for prime candidate proteins.

In spite of the advantages and success of the implementation of proteomics in the biomarker discovery field, it still remains a technological challenge to detect and identify the low-abundance proteins of interest, which may be often hidden by other abundant proteins (Wu *et al*., 2002b). One of those highly expressed proteins

is albumin (serum concentration is about 35–50 mg/mL) (Anderson and Anderson, 2002), which is usually a main candidate for complete selective elimination prior to performing a proteomic analysis of the circulating proteins with low level of expression in serum.

2.2.3 Applying Proteomics as a Tool to Identify Novel Prognostic Biomarkers in Chronic Lymphocytic Leukaemia

The role for BCR signalling as one of the primary regulating mechanisms in CLL pathogenesis was extensively studied utilising various experimental platforms (Perrot *et al*., 2011) including proteomics. Perrot and colleagues utilising proteomics technologies such as quantitative 2-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled to MS investigated 6 CLL samples, where 3 samples were “high risk” (UM-IgV_H and ZAP70 positive) and 3 were “low risk” (M-IgV_H and ZAP70 negative) and compared 48 proteomic expression patterns in pre-stimulated and post-anti-IgM stimulated M-CLL and UM-CLL and demonstrated that at baseline M-CLL and UM-CLL subsets express a significantly different proteomic profile and that on antigen recognition UM-CLL triggers more prominent activation of downstream signalling network when comparing to M-CLL (Perrot *et al.*, 2011).

Cochran *et al* and Scielzo *et al* employed proteomics methodologies such as two-dimensional electrophoresis coupled with mass spectrometry in order to identify the patterns of aberrant protein expression between two distinct CLL subsets, discriminated by the presence or absence of somatic hypermutation in the variable region of the immunoglobulin heavy-chain locus and other poor prognosis

factors such as CD38 expression and clinical behaviour (Cochran *et al.*, 2003, Scielzo *et al*., 2005). These studies reported that F-actin-capping protein subunit (CAPZB), conserved regulatory 14-3-3 protein, precursor of laminin receptor (LBP) (Cochran *et al.,* 2003), and hematopoietic lineage cell-specific protein-1 (HS1) (Scielzo *et al.,* 2005) were differentially expressed between two CLL subsets.

Boyd and colleagues analysed the protein content of CLL cell surface membrane fraction utilising high-throughput MALDI-TOF mass spectrometry and several novel proteins were identified including B cell-specific kinase (BLK), integral membrane proteins (MIG2B) and completely unknown B-cell novel protein #1 (BCNP1) (Boyd *et al*., 2003, Boyd *et al.*, 2010).

2.2.3.1 *A Proteomic Approach to Investigate Protein Targets Associated with B Cell Receptor Signalling in Good and Poor Prognosis Chronic Lymphocytic Leukaemia Samples*

Taking the evidence from the above studies into account and understanding the importance of a proteomics approach our group under supervision of Dr. Lynn Cawkwell *(Cancer Biology Proteomics Group, Post Graduate Medical Institute, University of Hull)* and Dr. David Allsup (*Queens Centre for Oncology and Haematology, Castle Hill Hospital, Hull*) therefore previously utilised proteomics and compared the protein expression profiles in anti-IgM stimulated *vs*. unstimulated CLL samples. The result from this work was included and published in the Journal of Proteomics (Kashuba *et al.*, 2013b). Several novel protein targets were found to be differentially expressed:

1. Kininogen-1

2. Leukotriene A4 hydrolase

3. Adenylate kinase isoenzyme 5

4. Tropomyosin 4-anaplastic lymphoma kinase fusion protein

5. Fatty acid binding protein 5

6. Aspartyl-tRNA synthetase

7. Thiopurine s-methyltransferase.

However, one particular protein of interest was found to be increased by over two-fold in 3/3 independent poor prognosis clinical samples after sustained BCR stimulation. This protein target is associated with ***Kinin-Kallikrein System*** and known as ***Kininogen.***

CHAPTER 3

The Kinin-Kallikrein system: physiological roles, pathophysiology and its relationship to cancer

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Chapter 3. The Kinin-Kallikrein System: Physiological Roles, Pathophysiology and Its Relationship to Cancer

3.1 The Kinin-Kallikrein System: The Introduction

The Kinin-Kallikrein System (KKS) is an endogenous multiprotein cascade, the activation of which leads to triggering of the intrinsic coagulation pathway and enzymatic hydrolysis of Kininogens (KNGs) with the consequent release of Bradykinin (BK) -related peptides (Kinins) (Figure 3.1). Synthesis of Kinins is known to be a predominant bio-physiological function of the KKS. Kinins are generated by proteolytic cleavage of KNGs such as High Molecular Weight Kininogen (HMWK) and Low Molecular Weight Kininogen (LMWK) by a family of serine proteases consisting of Kallikreins (KLKs) originating from plasma (pKLK) and tissue (tKLK) (Bourdet *et al.*, 2010). The liberated Kinins are the potent vasoactive peptides and display a high affinity for B_1 and B_2 receptors, which are members of the G-Protein Coupled Receptors (GPCR) family and are initiators of a complex intracellular signalling network. For the last five decades the complexity of the KKS, and the multiple interactions with other endogenous metabolic pathways such as Renin-Angiotensin system (RAS) and coagulation, has been intensively studied (Schmaier, 2003, Shariat-Madar and Schmaier, 2004). The KKS plays a crucial role in inflammation, vasodilation, smooth muscle contraction, cardioprotection, vascular permeability, blood pressure control, coagulation and pain (Colman and Schmaier, 1997, Colman, 2006, Bossi *et al.*, 2011, Marcondes and Antunes, 2005).

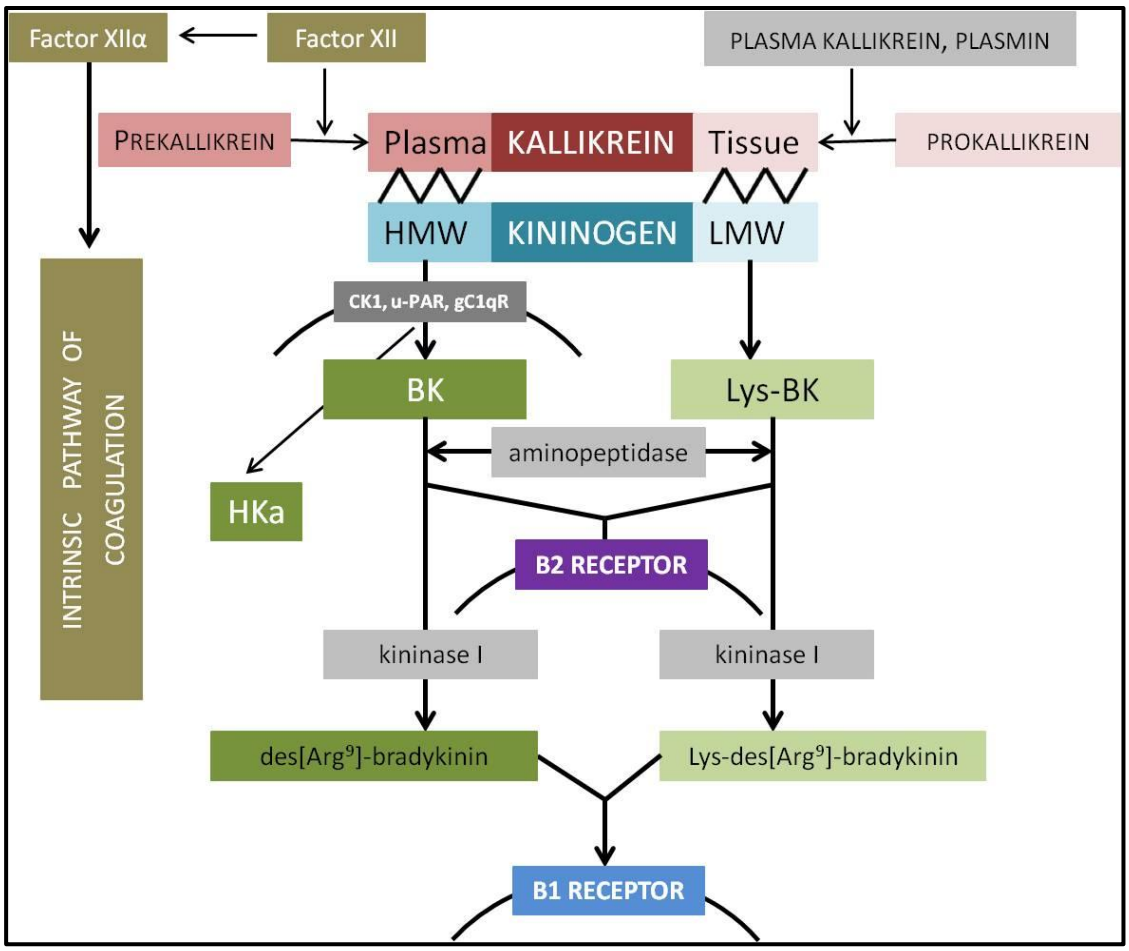


Figure 3-1 Kinin-Kallikrein System Organisation

KKS is a multiprotein metabolic pathway. The primary biologic feature of this system is hydrolysis of KNGs with subsequent liberation of vasoactive BK /Lys-BK and residual, cleaved (BK-free) KNG, which is hereafter, will be referred as HKa. Upon activation and transformation of FXII into an active FXIIa form, a series of reactions are triggered leading to activation of the coagulation pathway. BK and Lys-BK (Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9} and Lys^{1}-Arg^{2}-Pro^{3}-Pro^{4}-Gly^{5}-Phe^{6}-Ser^{7}-Pro^{8}-Phe^{9}-Arg^{10}, respectively) are the B_1 receptor ligands , whereas des-Arg-BK and Lys-des-Arg-BK (Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8} and Lys^{1}-Arg^{2}-Pro^{3}-Pro^{4}-Gly^{5}-Phe^{6}-Ser^{7}-Pro^{8}-Phe, respectively) exert their effect via the B_2 receptor.

In this chapter the physiology and pathophysiology of the KKS will be outlined and the association of this system with carcinogenesis and cancer progression will be highlighted. The information from this Chapter was published in the review paper (Kashuba *et al*., 2013a).

3.2 The Contact System and the Mechanism of Activation

KKS in multiple scientific sources is termed as the contact system. The contact system of plasma is also called the plasma kinin forming system which is initiated by intravascular activation of FXII and it has been postulated to be composed of three serine endopeptidase family proteins, namely FXII (Hageman factor), factor XI (FX1, plasma thromboplastin antecedent) and PK (Fletcher factor), with the multifunctional plasma and tissue protein KNG, which is also a kinin precursor. The interaction of all four of these proteins leads to activation of the Intrinsic Clotting Pathway, and hence to a fibrin clot organisation, and is known as “contact activation” (Kaplan *et al*., 1997). The activation of the contact system has two principal effects: mediation of the intrinsic pathway of coagulation which proceeds to fibrin formation (with associated procoagulant properties) and proteolytic cleavage of KNG leading to the release of BK–related kinins (with proinflammatory properties) (Colman and Schmaier, 1997).

3.2.1 Factor XII and Factor XI

FXII (Hageman factor) is a 596 amino acids single-chain plasma protein with a molecular weight of 80 kDa and a plasma concentration of 31 ± 8 µg/mL (0.375 µM) (Saito et al., 1976, Renné *et al.*, 2012, Marcondes and Antunes, 2005,

Colman, 2006). FXII possesses the properties of a zymogen (an inactive enzyme precursor). FXII is encoded by a 12 kb gene on chromosome 5 composed of 13 introns and 14 exons. FXII mRNA was reported to be expressed by human hepatocytes (Gordon *et al*., 1990). The gene and protein domain organisation of FXII exhibit structural homology with the serine protease family of tissue-type plasminogen activators and urokinase-type plasminogen, suggesting the allocation of FXII into the protease subfamily (McMullen and Fujikawa, 1985, Colman, 2006). FXII has a multidomain structure and possesses an Epidermal Growth Factor-like domain and a catalytic region that interacts to activate the functional site of this protein on its surface (Schmaier, 2008). The biochemistry of in vitro activation of factor XII is well studied and it was confirmed that after contact of FXII with an anionic surface (surfaces which are negatively charged due to prevalence of electrons on the surface), such as glass or silicates, dextran sulphate, heparin (Marcondes and Antunes, 2005) and kaolin, carrageenan (Moreau *et al.,* 2005), FXII undergoes conformational changes and is converted into its active form FXIIa and demonstrates procoagulant protease activity. The physiologic mechanism of FXII activation in vivo is still obscure and has continued to be a subject of speculation. Several assumptions were made in order to identify an in vivo physiologic surface for FXII activation in the intravascular department, and these included diverse cell membranes, platelets, endothelial cells and also neutrophils (Rojkjaer *et al.*, 1998). However there are studies which hypothesise that several pathogens can affect the KKS pathway via FXII activation. Morrison and Cochrane in 1974 demonstrated that injection or release of endotoxins or bacterial

lipopolysaccharide (LPS) have a direct influence on the activation of FXII (Morrison and Cochrane, 1974). In 1997, Brunnée et al demonstrated that mast cell heparin proteoglycan (MC-HepPG) isolated from a Furth mouse mastocytoma-derived cell line, which was a replica of human tissue-type mast cell HepPG, expressed the unique competence to reciprocally activate FXII and PK and could also autoactivate FXII (Brunnee *et al.*, 1997). In 2006 Frick et al described the contact system during defence against invasive bacterial infection and suggested that the contact system can be activated on the surface of bacterial pathogens, resulting in the promotion of antibacterial activity and inhibition of bacterial dissemination (Frick et al., 2006).

Once the activation of FXII and transformation into an active FXIIa form in the intravascular compartment occurs, FXIIa initiates plasma PK modification to KLK. As a result, KLK digests KNG to release BK. Although this hypothesis was universally recognised there are also substantial studies which challenge the involvement of FXII in mediation of PK to KLK conversion (Schmaier, 2002). FXII is capable of mediation of the structural transformation of PK into its active form (KLK) in the presence of KNG. However there are other studies which confirm that the conversion of PK into KLK is still possible on endothelial cell membranes cultured without serum, or in human serum lacking FXII, which suggest that FXII may not be essential for PK activation (Motta *et al*., 2001). KLK has an ability to reciprocally activate FXII, and thus supplements the KKS activation in the intravascular compartment (Rojkjaer *et al.*, 1998) and also it activates the structural transformation of a single-chained FXII into an active form called α-factor XII (XIIα)

consisting of two chains (amino terminal heavy chain connected by a disulfide bridge to a carboxy terminal light chain with molecular weight of 50 kDa and 28 kDa respectively) (McMullen and Fujikawa, 1985). Subsequently α-factor XIIα activates the transformation of FXI to Factor XIa, which in turn initiates the intrinsic pathway of coagulation (Schmaier, 2008) and the downstream activation of Factor IX leading to thrombin formation. Digestion of α-FXIIα by trypsin (McMullen and Fujikawa, 1985) or pKLK (Marcondes and Antunes, 2005) results in formation of β-factor XIIα, which is a heterodimeric molecule presented by a 28 kDa light chain connected by a SS-bond to a nonapeptide (McMullen and Fujikawa, 1985).

FXII can be autoactived upon contact with negatively charged surfaces and therefore the downstream coagulation signalling cascade in PK-deficient plasma occurs in a normal physiologic manner (Marcondes and Antunes, 2005). However, it is important to highlight that, despite the fact that multiple endogenous physiologic surfaces are available for FXII autoactivation, generally the reaction with this zymogen does not occur constitutively within normal physiological or non-pathological conditions unless it is regulated by the plasma concentration of HK and Zn2+ (Colman and Schmaier, 1997). In the intravascular compartment FXII binds to the endothelial cell receptor complex composed of uPAR, CK1 and gC1qR (Schmaier, 2008).

Activated FXII can mediate the activation of downstream FXI. However, several studies have demonstrated that FXI can be activated in a FXIIa-deficient model system, emphasising the role for thrombin in FXI activation and the subsequent coagulation cascade (Oliver *et al*., 1999). The absence of a bleeding

disorder in FXII-deficient patients lead to further investigation of the role of FXII in the process of FXI activation (Choi *et al.*, 2011). HMWK can form a complex with FXIIa or FXI, which may promote the activation of FXI and subsequent triggering of the intrinsic coagulation cascade (Thompson *et al*., 1977, Mandle et al., 1976). A new hypothesis was therefore postulated where PK and FXI become tightly bound to KNG and ligate with the multiprotein receptor complex comprising CK1, gC1qR, and uPAR on the surface of endothelial cells. This would facilitate the digestion of PK into KLK by prolylcarboxypeptidase (PRCP) (Shariat-Madar *et al.*, 2002).

3.3 The Structure and Organisation of the Kinin-Kallikrein System

In order to understand the complexity within this biologically and physiologically important system (with multiple cross-talking pathways, autocrine and paracrine activities, and multiple modes of activation and regulation), it is essential to understand the function and the properties of each constituent of the KKS and their interactions within the system. The system consists of a number of enzymes, large proteins and polypeptides which interact (intra- and inter-system) in order to activate or deactivate the bio-chemical reactions within this metabolic cascade.

3.3.1 Enzymes

3.3.1.1 *Kinin Forming Enzymes*

KLKs (also known as kinin forming enzymes) are serine proteases that liberate kinins from the KNGs. Two types of KLKs are described: pKLK and tKLK. Human plasma PK (PPK) is a zymogen precursor for pKLK. It is a single-chain polypeptide, the production of which is primarily located in hepatocytes with subsequent release into blood stream. Although the highest PPK mRNA expression levels are reported in hepatocytes, recent studies have confirmed the existence of extrahepatic PPK synthesis. PPK protein expression has been described in liver (epithelial cells), pancreas (cells of the islet of Langerhans), testicles (interstitial cells of Leydig), ovaries (follicular and granulosa cells), parotid gland, oesophagus, skin, respiratory tract, prostate and breast (Fink *et al*., 2007). PPK has been demonstrated to play a marked role in biological processes including the surface-dependent activation of blood coagulation (intrinsic pathway), the activation of FXII, kinin generation, inflammatory response, plasminogen activation, and positive regulation of fibrinolysis. PPK is secreted into plasma as an inactive molecule, either (75%) circulating as a heterodimer complex bound to HMWK or (25%) as a freely circulating protein (Moreau *et al*., 2005, Colman, 2006). PPK is a serine protease glycoprotein comprised of 619 amino acids. It exists in two molecular weight variants that circulate in plasma and vary between 85 kDa to 88 kDa (Marcondes and Antunes, 2005) and with a plasma concentration of 42 ± 3 µg/mL (0.49 µM) (Fisher *et al.*, 1982). PPK is encoded by the *KLKB1* gene (Moreau *et al*., 2005).

Upon activation of PPK, a chain of proteolytic reactions cleaves PPK within its disulphide bond to generate the double-chain variant pKLK (Yu et al., 2000). pKLK consists of a 53 kDa heavy chain (371 amino acids) and a 33-36 kDa light chain (248 amino acids) connected by a disulfide bond. The pKLK heavy chain originates from the N-terminus and is presented by 4 tandem repeats that are 90-91 amino acids long in their sequence and displays a structural resemblance to human FXI. The pKLK light chain exhibits structural homology with the trypsin family of serine proteases (Chung *et al.*, 1986). The heavy chain of pKLK interacts with the negatively charged surface and possesses a high affinity binding site for HMWK, while the light chain serves as a catalyst (Marcondes and Antunes, 2005). HMWK is a substrate for pKLK and, as a result of this interaction, the nonapeptide BK is generated. pKLK and FXII (but at a much lower rate) are both capable of proteolytic digestion of HMWK with the subsequent release of BK and residual, cleaved (BK-free) KNG (termed HKa) (Colman, 2006). The pKLK gene and protein are homologous to human FXI and both of these genes are located on chromosome 4 (Colman, 2006). The structural organisation of the pKLK gene was reported by Yu *et al* in 2000 and comprises 15 exons and 14 introns (Yu *et al*., 2000).

Human tKLK or glandular KLK is a secreted member of the S1 serine protease super family with molecular weight between 24-45 kDa. The enzyme activity of tKLK differs from pKLK and is distinguished by the catalytic mechanism and the proteins targeted for cleavage. Tissue KLK is synthesised as an inactive preproenzyme (termed proKallikrein) and is widely expressed in diverse human

tissues such as kidney, pancreas, colon, pituitary gland, erythrocytes (MacDonald *et al.*, 1988), central nervous system, spleen, adrenal and neutrophils (Marcondes and Antunes, 2005). The activation of proKallikrein *in vitro* can be achieved by trypsin-type enzymes (Frey, 1962) however, *in vivo* plasmin (Yamada and Erdos, 1982) and pKLK (Takada *et al.*, 1985) are reported to be responsible for biochemical conversion of proKallikrein into tKLK. There is a close resemblance between tKLK and trypsin, however tKLK expresses a higher specificity for interaction with cleavage sites in synthetic peptides (MacDonald *et al.,* 1988). KNGs are the principal physiological substrates for proteolytic digestion by tKLK. tKLK cleaves KNG at Met-Lys and carboxy-terminus Arg-Ser sites, resulting in the subsequent liberation of the decapeptide Lys-BK (Kallidin) (MacDonald *et al.*, 1988). Tissue KLK (as opposed to pKLK which preferentially acts upon HMWK), can generate Kallidin from either HMWK or LMWK. *In vivo*, the decapeptide Lys-BK can be easily converted into the nonapeptide BK by cleavage at the Lys-Arg bond (Erdos, 1979).

3.3.1.2 *Carboxypeptidases*

Carboxypeptidases (kininases) play the predominant role in kinin degradation. Once kinins are liberated into the circulatory system they are catalysed by kininases, which cleave and release Arg from the carboxy-terminus of BK and Lys-BK. Kininase I-type enzymes are present in two forms, namely carboxypeptidase N (CPN) in plasma (Erdos, 1979) and carboxypeptidase M (CPM) in cell membranes(Skidgel *et al*., 1984, Skidgel *et al.*, 1986, Johnson *et al.*, 1984). Although these enzymes share some properties, they are structurally and

catalytically distinct from each other and from other carboxypeptidases. Human CPM is a single-chain glycoprotein (molecular weight of 62 kDa) with maximal enzyme activity at neutral pH and is named due to its localisation on the plasma membrane. . The function of CPM is the selected cleavage of peptides containing carboxy-terminal Arg residues (whereas CPN preferentially acts on peptides containing carboxy-terminal Lys residues) (Skidgel and Erdos, 1998). CPM regulates the interaction between BK and its receptors by converting BK to des-Arg-BK and thus abrogating the unique specificity required to interact with B2 receptors, whilst producing agonise against B1 receptors (Regoli and Barabe, 1980). Zhang et al recently reported that the kinin B1 receptor possesses the ability to form a complex with CPM which potentiates B1-mediated signal transduction and that the disruption of this heterodimer can lead to inhibition of B1-dependent nitric oxide production in cytokine-treated human lung microvascular endothelial cells (Zhang et al., 2011). CPM is widely distributed in diverse human tissues.

Human CPN (plasma zinc metalloprotease) is a 280 kDa extracellular glycoprotein (Levin *et al.*, 1982) which is synthesised and secreted into the intravascular compartment by hepatocytes as a hetero-tetramer (Keil *et al.*, 2007). CPN is composed of two large domains (approximately 83 kDa each), which are responsible for protecting the protein from degradation, and two small domains (approximately 48 to 55 kDa) which exhibit enzymatic properties (Matthews and Wetsel, 2001). CPN selectively removes basic residues at the carboxy-terminus and thus regulates the activity of kinins, growth factors, cytokines (Keil *et al*., 2007) and anaphylatoxins within the blood circulation (Levin *et al.*, 1982). Several

endogenous enzymes, such as trypsin, plasmin and pKLK or urinary KLK can fragment CPN, however these newly generated active fragments are less stable at 37ºC in comparison to the original tetrameric protein (Levin *et al.*, 1982). CPN is a constitutively active enzyme and due to its ability to metabolise, and hence to protect the human body from the systemic dissemination of peptides such as vasoactive kinins, anaphylatoxins and fibrinopeptides, it is considered to be essential for sustaining life (Keil *et al.*, 2007).

3.3.1.3 *Angiotensin Converting Enzyme*

Angiotensin converting enzyme (ACE) (kininase II) is primarily synthesised as a membrane-bound protein. Due to post-translational proteolytic cleavage of the membrane anchor the ACE molecule is detached from the cell surface and liberated into the circulation as a freely-circulated enzyme (Costerousse *et al.,* 1992). There are two known isoforms of ACE, which are somatic (150 to 180 kDa) and germinal or low molecular weight (90 to100 kDa). ACE is widely distributed within diverse human tissues and found to be expressed in endothelial cells, epithelial cells, germinal cells, neurons, macrophages, T-lymphocytes (Costerousse *et al.*, 1993) and also in body fluids such as urine, cerebrospinal and amniotic fluids. The role of ACE is, firstly, to convert the inactive hormone peptide angiotensin I into the potent vasopressor angiotensin II, which is an important biologically active constituent of the RAS, which controls blood pressure and modulates fluid–electrolyte homeostasis within a human body. Secondly, ACE is involved in BK inactivation pathway (Nowak *et al.*, 2011), where ACE metabolises the vasoactive nonapeptide BK. Finally, ACE is reported to cleave Substance P,

which is a protein from the tachykinin family which expresses a broad spectrum of enzymatic properties. It is widely expressed in cells and tissues of the human peripheral and central nervous systems but is also found in extra-neuronal cells and innervated tissues.

3.3.1.4 *Neutral Endopeptidase*

Neutral endopeptidase (NEP) is a zinc-dependent metalloprotease enzyme, which is synthesised as a membrane-bound protein with a molecular weight of 89 to 96 kDa. NEP is reported to be involved in the metabolism of several biologically active secreted peptides and has a wide distribution within various human cells. Substrates for NEP activation include Angiotensin I, Angiotensin II, substance P, several vasodilator peptides (BK, Adrenomedullin) and vasoconstrictor peptides (Endothelin), the regulators of nociceptive function (enkephalins), a protein associated with Alzheimer's disease (amyloid β peptide) and chemotactic peptide (Campbell, 2003).

3.3.2 Large Proteins

3.3.2.1 *Kininogens*

KNGs are also known as the Williams-Fitzgerald-Flaujeac factor, the Fitzgerald factor and the Kallikrein factor. KNGs are large proteins and the substrate from which the serine proteases pKLK and tKLK respectively liberate the physiologically active peptides BK and Lys-BK (Sainz *et al*., 2007). Currently there are several types of human KNG which are well described: HMWK, LMWK and

HKa. T-KNG is the analogue of human KNG reported in rats (Anderson et al., 1989).

For a significant time only the released bioactive peptides BK and Lys-BK were reported to express diverse bio-physiological properties, whilst KNGs were described as precursors for proteolytic cleavage only and lacked specific activity. However, convincing studies have demonstrated that KNGs also display various biological activities. HNWK exhibits antithrombotic activity (Colman *et al*., 1999, Hassan *et al.*, 2007) and participates in cell-adhesive interactions (Motta et al., 2001). HKa is an anti-adhesive molecule which prevents cell interaction with vitronectin and also expresses antiangiogenic properties (Motta *et al.*, 2001).

KNGs are the multifunctional proteins of KKS and, up until the last two decades, it was assumed that the origin of KNGs was restricted to hepatic tissues (Okamoto *et al.*, 1996). Rat T-KNG was found to be expressed in lung, renal, neural and cardiac tissues (Mann and Lingrel, 1991). Human KNG mRNA expression was confirmed in liver, kidney (Iwai *et al*., 1988, Chao *et al.*, 1993) and endothelial cells (Schmaier *et al*., 1988). KNG synthesis was demonstrated in fibroblasts upon stimulation by mediators of inflammation such as prostaglandin-2 (PGE2), tissue necrosis factor (TNF) and Interleukin (IL)-1 and KNG expression was demonstrated in rat tarsal bones in response to in vivo mediated inflammation (Okamoto *et al.,* 1996, Takano *et al*., 1995). Taking into consideration all of the above it can be concluded that autonomous KNG synthesis is taking place in hepatic and various extrahepatic tissues and organs in humans and animals.

3.3.2.1.1 Protein Structure and Function

There are two different human protein isoforms of KNG which are recognised: HMWK (Figure 3.2) and LMWK (Figure 3.3). HK is a β-globulin with an approximate molecular weight of 120 kDa and human plasma concentration of approximately 80 mg/mL (670 nM) (Sainz *et al.*, 2007). However, there is a tendency for the increase of plasma level of KNGs with advancing age in both humans and animal models (Acuna-Castillo *et al*., 2005). LMWK is a polypeptide with a reported molecular weight of approximately 48 to 75 kDa and a human plasma concentration of approximately 60 mg/mL (Sainz *et al.,* 2007).

The sequence of HMWK and LMWK consists of 626 and 427 amino acids respectively (Figures 3.4 and 3.5). The two KNG isoforms share a common signal peptide (18 amino acids); the heavy chain (362 amino acids) and the BK sequence (9 amino acids). The unique light chain differentiates HMWK and LMWK. This consists of 255 amino acids (approximately 48 to 56 kDa) in HMWK and 38 amino acids (approximately 4 kDa) in LMWK (Colman, 1996). The shared heavy chain has an approximate molecular weight of 64 to 75 kDa (Colman, 1996).

KNGs are large multidomain molecules and each domain (D) exhibits specific and distinctive properties (Colman, 2006). There are studies which report the contrasting functions of the heavy and light chains of HMWK, represented by cysteine proteinase inhibitory activities and cofactor properties, respectively (Schmaier *et al*., 1986).

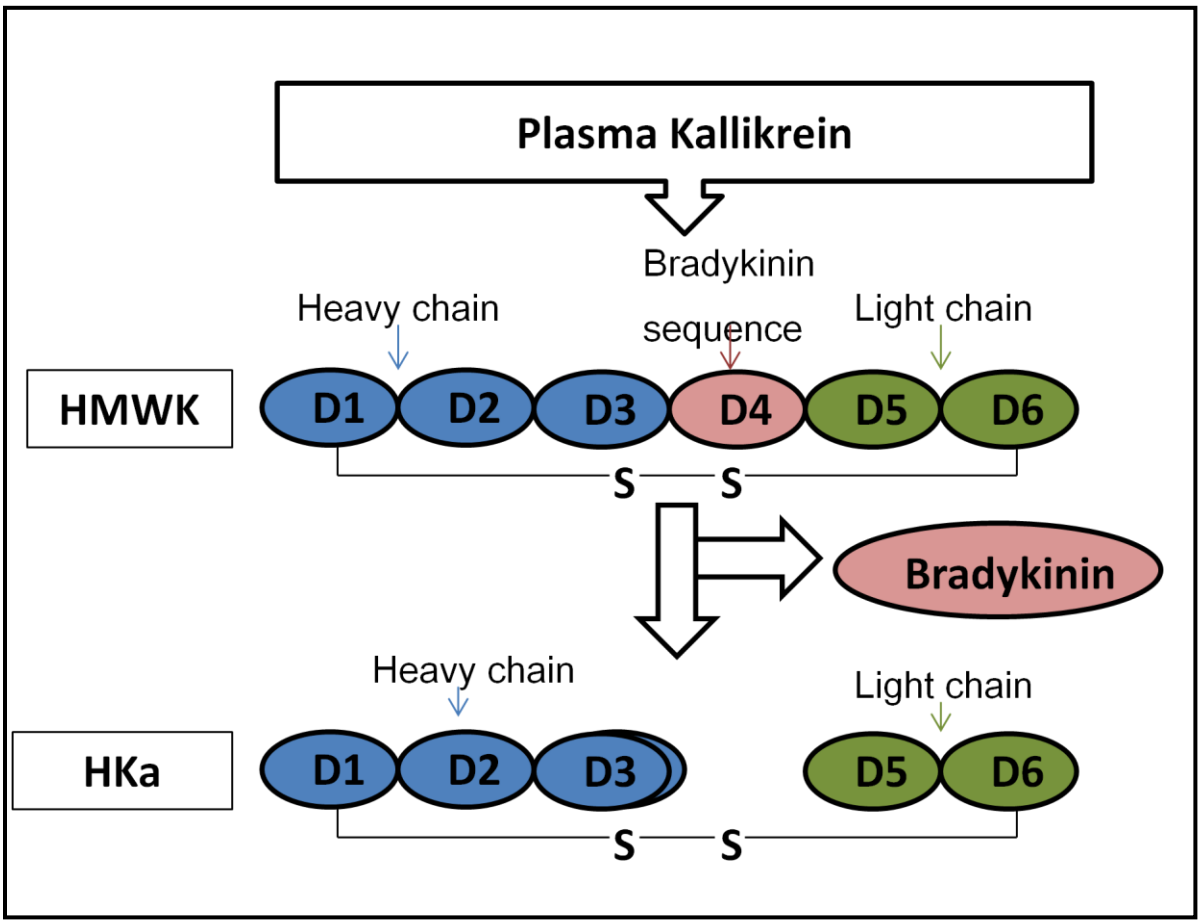


Figure 3-2 High Molecular Weight Kininogen Structure

HMWK formed by 6 single chain domains (D1-D6), represented by a heavy chain (D1-D3) and a light chain (D5h-D6).The heavy and the light chain are connected by D4, which contains the sequence of BK. After activation by KLK and the release of BK, the cleaved form HKa consists of a heavy chain and a light chain linked by a disulphide bridge.

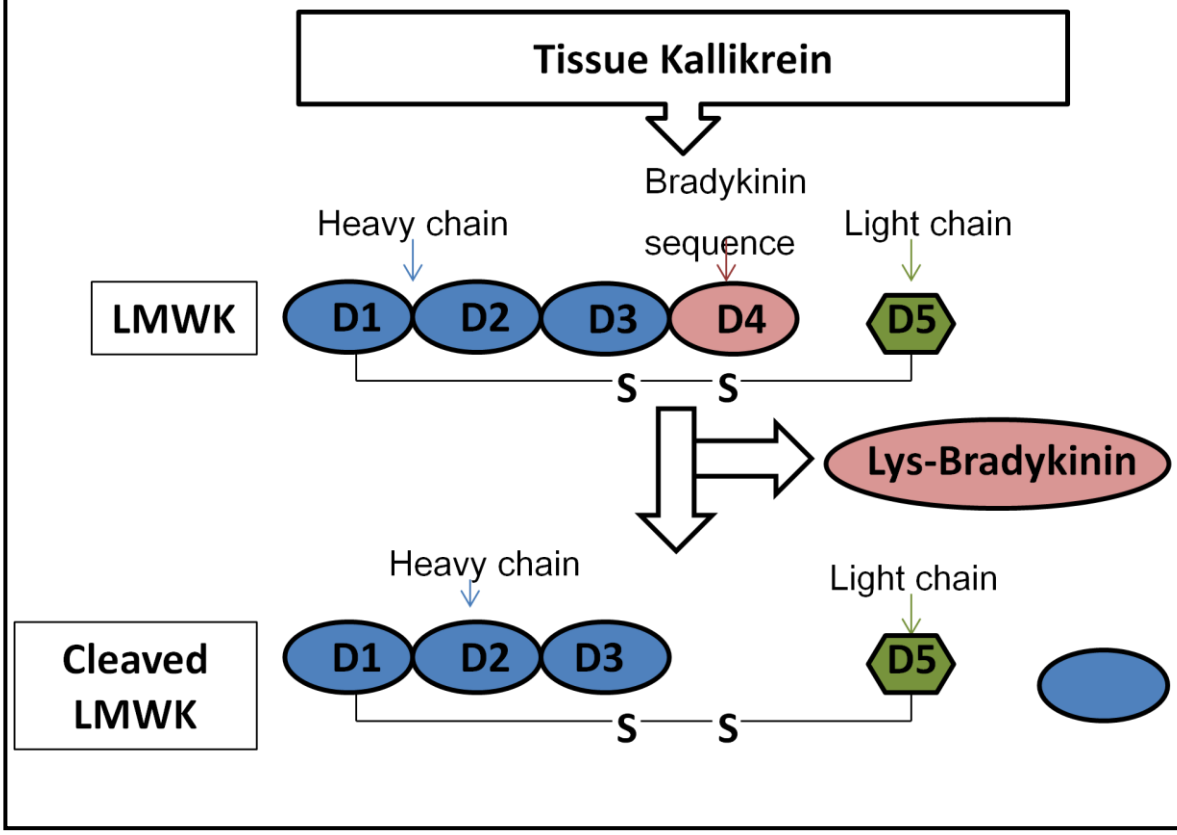


Figure 3-3 Low Molecular Weight Kininogen Structure

LMWK consists of 5 single chain domains (D1-D5), these are presented by a heavy chain (D1-D3) and a small unique light chain D5. Two chains are linked by D4, which includes the sequence of Lys-BK. After proteolytic cleavage of tissue KNG by KLK, Lys-BK is released; the cleaved form of LMWK consists of a heavy chain and a light chain linked by a S-S bond.

HMWK D6 possesses a high affinity site for binding PK and interaction of these proteins results in the formation of a HMWK/PK complex which freely circulates in plasma (Colman, 2006). D6 has the ability to down regulate urokinase-

dependent plasminogen activator and therefore inhibits HMWK and PK interaction (Colman *et al.*, 2010). Additionally, D6 is reported to express a binding site for FXII (Moreau *et al.*, 2005).

D5 of HMWK was demonstrated to be responsible for HMWK binding to hydrophilic anionic surfaces (Colman, 1996) however, the unique structure of LMWK D5 is inadequate for contact activation and lacks PK binding sites (Moreau *et al*., 2005). The function of LMWK D5 is still undefined.

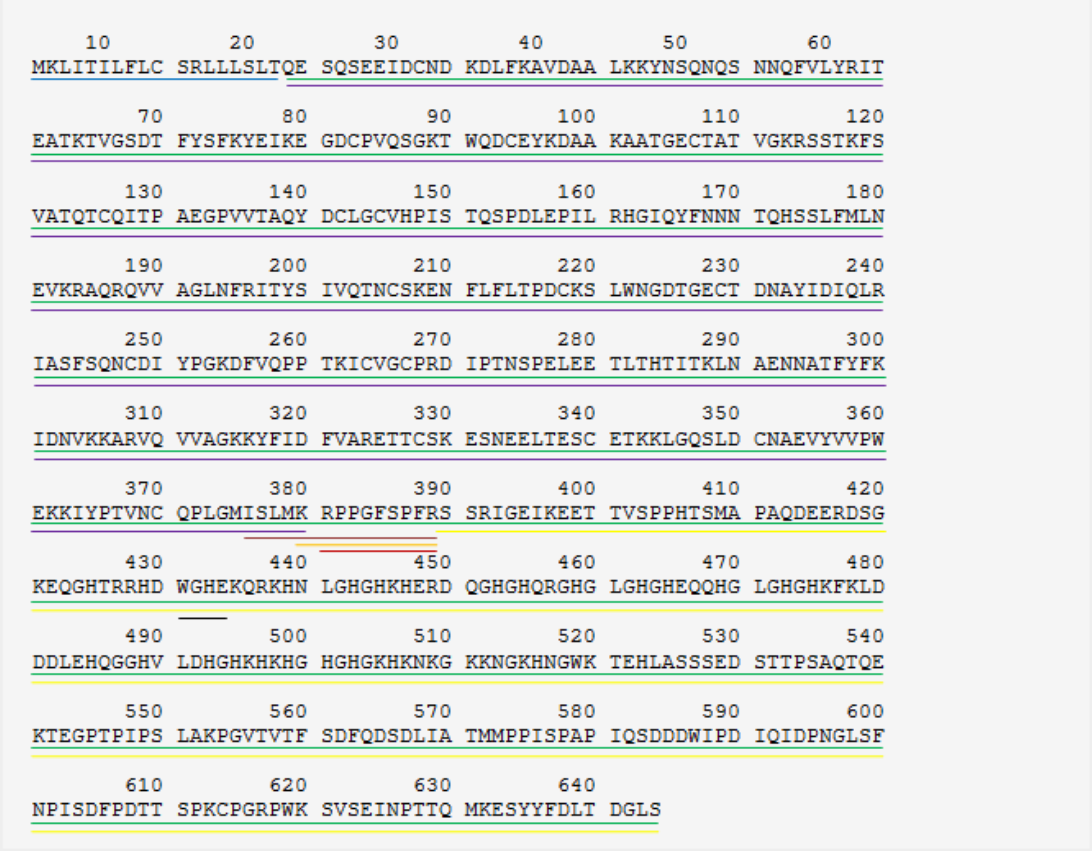
D4, which is shared by HMWK and LMWK, contains the sequence of BK and Lys-BK respectively and additionally the first 5 amino acid residues provide a low affinity binding site for platelets (Hasan *et al.*, 1996).

D3 and D2 represent the heavy chain of both HMWK and LMWK which is responsible for inhibition of cathepsin L (Colman, 2006) and platelet calpain (Schmaier et al., 1986) leading to inhibition of platelet aggregation and the suppression of platelet-thrombin binding respectively (Puri *et al.*, 1991). D3 has the ability to inhibit cathepsin L, B and H but not calpain (Colman, 2006). The specific amino acid residues Gln-Val-Val-Ala-Gly within D3 and D2 are responsible for protease inhibition activity (Salvesen *et al.*, 1986). D3 and D5 also contain neutrophil binding sites (Colman, 1996), however D3 and D5 interact with different receptors or with different sites within the same receptors (Colman, 2006). D3 binds with high affinity to monocytes, platelets and endothelial cells.

D1 is reported to exhibit an attenuated binding site for calcium (Cheung *et al*., 1993).

HMWK is characterised as a precursor for both BK and HKa. HKa is an active protein produced as a result of the proteolytic cleavage of KNG with subsequent liberation of BK. It plays a prominent role in several intracellular, molecular and signalling events. HKa suppresses angiogenesis with the anti-adhesive activity of D5 (kininostatin) leading to subsequent inhibition of endothelial cell proliferation and the facilitation of endothelial cell apoptosis (Colman *et al*., 2010). HKa promotes cell detachment by binding to the amino-terminus of vitronectin (a cell adhesion and spreading protein found in plasma and the extracellular matrix, which serves to regulate proteolysis directly mediated by plasminogen activation) and thus obstructing vitronectin interaction with uPAR, all of which takes place on the surface of endothelial cells in Zn2+ -dependant manner (Sainz *et al*., 2007). HKa freely circulates in plasma, however the persistence of HKa supplements some pathophysiological conditions such as the inflammatory response (Sainz *et al*., 2007).

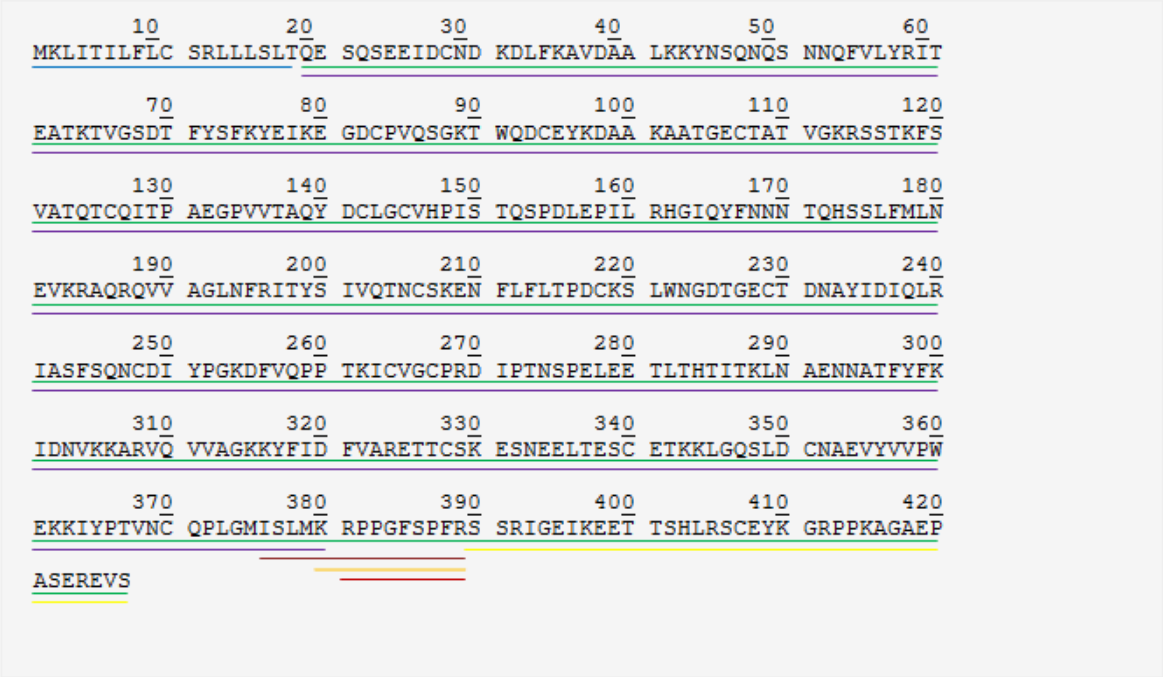
Figure 3-4 The Protein Structure of HMWK Isoform.



Subsections	Position in the chain	Length (aminoacids)	*Graphical view*
Signal peptide	*1-18*	*18*	
Peptide/Kininogen-1	*19-644*	*626*	
KNG Heavy chain	*19-380*	*362*	
Peptide/T-kinin	*376-389*	*14*	
Peptide/Lysyl-Bradykinin	*380-389*	*10*	
Peptide/Bradykinin	*381-389*	*9*	
KNG light chain	*390-644*	*255*	
Low molecular weight growth-promoting factor	*431-434*	*4*	

The information used in these tables was modified from Uniprot Database www.uniprot.org

Figure 3-5 The Protein Structure of LMWK Isoform



Subsections	Position in the chain	Length	Graphical view
Signal peptide	*1-18*	*18*	
Peptide/LMWK	*19-427*	*408*	
LMWK Heavy chain	*19-380*	*362*	
Peptide/T-kinin	*376-389*	*14*	
Peptide/Lysyl-Bradykinin	*380-389*	*10*	
Peptide/Bradykinin	*381-389*	*9*	
LMWK light chain	*390-427*	*37*	

The information used in these tables was modified from Uniprot Database www.uniprot.org

3.3.3 Polypeptides

3.3.3.1 *Kinins*

In humans, the term “kinins” refers to BK-related polypeptides such as BK and Lys-BK and their carboxy-terminal des-Arg metabolites; other kinins such as T-kinin (Ile-Ser-BK) and Met-T-kinin generally known to be expressed in rat (Moreau et al., 2005). Human Ile-Ser-BK with the amino acid sequence:

$$Ile^{1}\text{-}Ser^{2}\text{-}Arg^{3}\text{-}Pro^{4}\text{-}Pro^{5}\text{-}Gly^{6}\text{-}Phe^{7}\text{-}Ser^{8}\text{-}Pro^{9}\text{-}Phe^{10}\text{-}Arg^{11}$$

was isolated from ovarian carcinoma ascites and showed strong similarity to T-kinin, which had been previously reported in rats (Wunderer et al., 1986).

HMWK yields BK upon pKLK lysis. BK is a physiologically and pharmacologically active member of the kinin group of proteins and consists of nine amino acids with the following amino acid sequence:

$$Arg^{1}\text{-}Pro^{2}\text{-}Pro^{3}\text{-}Gly^{4}\text{-}Phe^{5}\text{-}Ser^{6}\text{-}Pro^{7}\text{-}Phe^{8}\text{-}Arg^{9}.$$

Once released locally or in plasma BK has a very short half-life and under normal physiologic conditions is rapidly degraded by plasma. There are several enzymes known to be involved in the plasma degradation of BK including CPN (kininase I), ACE (kininase II) and NEP which cleave BK at Phe8-Arg9, Phe5-Ser6 and Pro7-Phe8 positions respectively, and convert it to an inactive peptide (Kuoppala *et al*., 2000). Lys-BK (Kallidin), the decapeptide from the kinin-related group, is released from LMWK upon tKLK lysis (Sheikh and Kaplan, 1986b, Sheikh and Kaplan, 1986a). Lys-BK consists of the same amino acid sequence as BK but with an additional lysine amino acid at the N-terminus:

$$\mathbf{Lys}^{1}\text{-}Arg^{2}\text{-}Pro^{3}\text{-}Pro^{4}\text{-}Gly^{5}\text{-}Phe^{6}\text{-}Ser^{7}\text{-}Pro^{8}\text{-}Phe^{9}\text{-}Arg^{10}.$$

Kinins are implicated in many physiological and pathological events and will be discussed later in this chapter.

3.3.3.2 *Kinin Receptors*

The physiological and pathophysiological effects of BK and Lys-BK exerted via GPCR super family B_2 receptors, whereas kinin metabolites such as des[Arg9]-BK and Lys-des[Arg9]-BK through kinin B_1 receptors (Leeb-Lundberg, 2004). Last are the products of kinin degradation by kininases and are generated without the Arg residue at the carboxy-terminus of BK and Lys-BK (Drapeau *et al.*, 1991). In humans the B_1 and B_2 receptors are encoded by the BDKRB1 gene. B_2 receptors are widely distributed and ubiquitously expressed in various human tissues except liver and spleen (Kakoki *et al.*, 2007) and usually are identified under "normal" physiological conditions. B_1 receptors however are very difficult to identify under "normal" physiological conditions but are found to be significantly up-regulated by proinflammatory cytokines such as IL-1β (Marceau *et al.*, 1998) and growth factors such as epidermal growth factor (EGF) in response to inflammation, allergy, tissue injury and pain (Bourdet *et al.*, 2010). The synthesis of kinin B_1 receptor agonists such as des [Arg9]-BK and Lys-des [Arg9]-BK are also upregulated during inflammation (McLean *et al.*, 2000a). It has also been demonstrated that the expression of B1 receptors is induced when B_2 receptors are absent or inactivated (Duka *et al.*, 2006a).

As a result of kinin-receptor interaction conformational changes within the receptor occur and this subsequently triggers several intracellular signal transduction pathways, which in turn stimulate phospholipase β phosphoinositide

kinetics, protein kinase C and calcium mobilisation (Montana and Sontheimer, 2011a) which lead to modulation of the cellular response. B_2 receptors are implicated in the activation of the mitogen-activated protein kinase (MAPK) pathway and play a crucial role in cross-talk between the RAS and the KKS by forming a complex with ACE on the plasma membrane (Chen *et al*., 2006b).

The existence of novel third type of high affinity receptors for BK has been proposed, with the report that the orphan GPCR named GPR100 may function as a supplementary BK receptor (Boels and Schaller, 2003, Leeb-Lundberg, 2004, Meini *et al.,* 2004). This proposal was based on the fact that BK, at nanomolar concentrations, enhances intracellular free Ca^{2+} via GPR100 (Leeb-Lundberg, 2004, Meini *et al*., 2004).

3.3.4 Assembly and Activation of KKS

Up until the last decade the assembly and activation of the KKS under "normal" physiological conditions had not been fully elucidated. Then, evidence of KKS activation in the intravascular compartment independent of FXII was demonstrated and it was suggested that the proteins of the KKS could be assembled on the endothelial cell membrane by binding to a multiprotein receptor complex presented by cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR (Rojkjaer *et al.*, 1998, Motta *et al.*, 2001, Schmaier, 2002) (Figure 3.7). Several researchers also demonstrated the involvement of the macrophage-1 (Mac-1) integrin receptor (CD11b) in activation of the KKS. The Mac-1 integrin receptor is a heparin sulphate receptor which is expressed on the surface of many leukocytes involved in the innate immune system; it mediates

inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity and chemotaxis. Mac-1 is involved in the complement system (Renne, 2012) and also activation of the KKS on the cell membrane of neutrophils, platelets (Sheng et al., 2000), macrophages (Barbasz *et al.*, 2008) and monocytes (Sainz *et al.*, 2007).

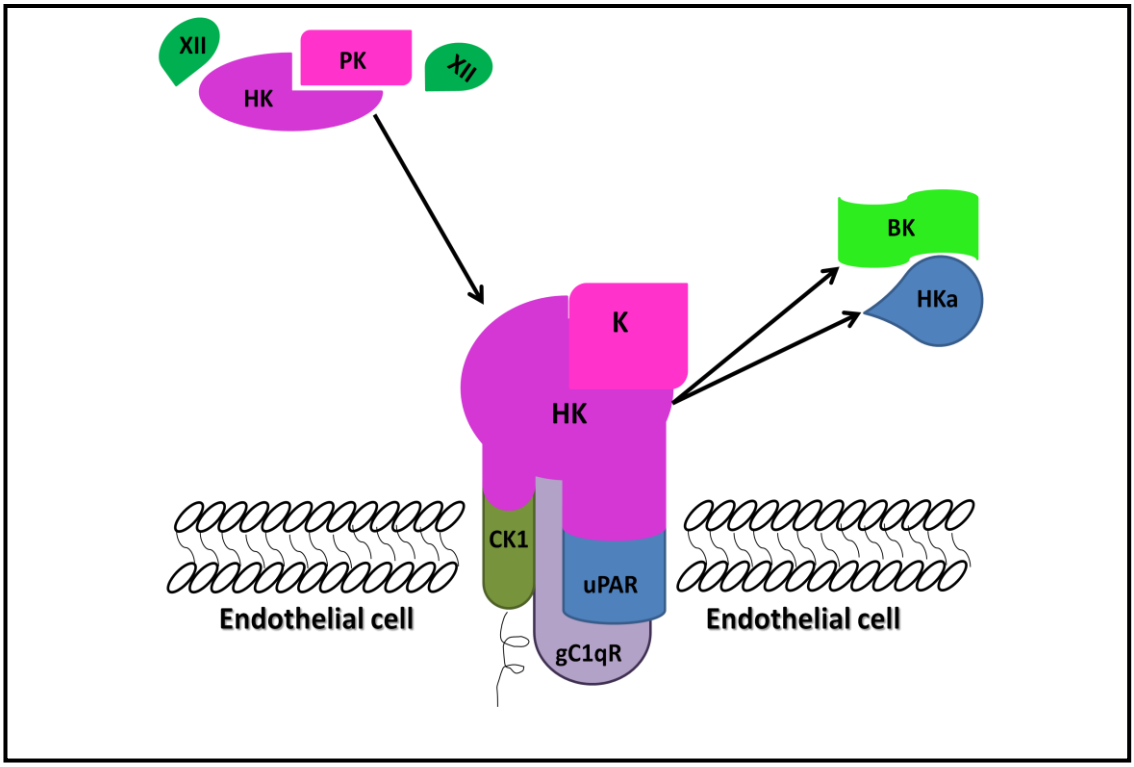


Figure 3-6 Assembly of KKS on Endothelial Cells

The HK-PK complex circulates in plasma and binds to endothelial cell membrane receptor complex consisting of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR resulting in transformation of PK into KLK and, as result of proteolytic cleavage, BK and HKa are liberate.

Zn^{2+} is an essential cofactor for KNG binding to cell membranes and contact activation *in vivo* is highly regulated by the plasma Zn^{2+} concentration (total plasma concentration is approximately 10 to 25 μM, however the concentration of free Zn^{2+} is 0.25 to 1 μM as the majority of Zn^{2+} circulates in a complex with albumin) (Rojkjaer *et al.*, 1998). Neutrophils, lymphocytes and platelets contain high total Zn^{2+} concentration (Whitehouse et al., 1982, Rojkjaer et al., 1998) and therefore the cells of the intravascular compartment such as platelets (Gustafson et al., 1986), neutrophils (Gustafson *et al*., 1986, Figueroa *et al*., 1992) monocytes (Barbasz *et al.,* 2008), macrophages (Barbasz and Kozik, 2009), astrocytes (Fernando *et al*., 2003) and vascular smooth muscle cells (Fernando et al., 2005) possess the KKS on their surface (Vergiliana *et al.*, 2010). Two high affinity HMWK receptors were identified to be involved in the activation of the KKS on platelets, namely glycoprotein Ib-IX-V (GPIb/IX/V), which is an abundant platelet glycoprotein receptor mediating platelet activation, adhesion to impaired blood vessels and vascular repair, and gC1qR, which is also expressed on the endothelial cells surface (Colman, 2006).

Recent studies have demonstrated that the interaction of HMWK with its endothelial cell-surface multiprotein receptor in a Zn^{2+} -dependant manner (Gustafson *et al*., 1986, Schmaier *et al.*, 1988) promotes the regulated activation of the PK/HMWK complex, which freely circulates in plasma (Schmaier, 2002). An active binding site encompassed in D3 of the HMWK heavy chain and D5 of the HMWK light chain interact with the N-terminal H1 subdomain of CK1 on the endothelial cell surface, whilst D2 and D3 of HMWK bind to the uPAR receptor and

D5 interacts with gC1qR (Shariat-Madar *et al.*, 2002). Activation of the PK/HMWK complex is suggested to be triggered by an interaction between PK and PRCP, which is constitutively expressed on endothelial cell membranes (Schmaier, 2002), or heat shock protein 90 (HSP90) (Joseph *et al*., 2002) and this results in conformational changes in PK leading to transformation to KLK. It is suggested that the activation of the KKS can be mediated independently of FXIIa, however the presence of FXIIa acts as a catalyst and augments this process (Vergiliana *et al.*, 2010). FXIIa, which is activated secondary to PK activation (Motta *et al.*, 2001) demonstrates high affinity to the same multiprotein receptor complex CK1/uPAR/gC1qR as HMWK (in the absence of HMWK). Although this interaction is highly regulated by Zn2+ concentration, requiring a 30-fold higher free Zn^{2+} concentration in comparison to HMWK. Newly generated KLK auto digests its receptor to release BK from substrate KNG (Shariat-Madar *et al.*, 2002).

3.4 KKS: Pathophysiology and Dysregulation

3.4.1 Physiology and Pathophysiology of KKS

The physiological function and pathophysiological implication of the KKS were intensively studied in recent decades however, since kinin receptor antagonists have become a new pharmacological target (Dendorfer *et al*., 1999), the attention to KKS has become more intense.

The activation of the KKS has been described to play a major role in several bio-physiological processes such as the proinflammatory response and tissue injury (Renne, 2012), control of vascular smooth muscle tone, regulation of arterial blood

pressure (Marcondes and Antunes, 2005), cardioprotection (Kolte *et al.,* 2011, Liesmaa *et al.*, 2009, Hall, 1992), enhancement of vascular permeability, contraction of intestinal smooth muscle, spasm of smooth muscle in airways, enhancement of airway resistance, stimulation of sensory neurons resulting in chronic hyperalgesia (Butt *et al.*, 1995), alteration of ion secretion by epithelial cells, production of nitric oxide, release of cytokines by leukocytes, release of eicosanoids such as prostaglandins from various cell types, anti-adhesion and regulation of cell migration .

Considering the broad range of activities displayed by the KKS, this system has been implicated in many pathophysiologies including acute attacks of hereditary angioedema, sepsis, diabetic retinopathy, induced arterial thrombosis, acute myocardial infarction (Schmaier, 2008), inflammatory bowel disease (Sartor *et al.*, 1996), arthritis (Dela Cadena *et al*., 1995), as well as pain, asthma, infection, allergy and symptoms associated with acute and chronic inflammation and cancer. Inflammation is a complex physiological response of vascular tissue to invading pathogens, tissue injury or irritants and is characterised by five classical signs of inflammation: pain, heat, redness, swelling, loss of function. Initial response or prolonged and persistent inflammations are recognised as acute or chronic inflammation respectively. At the onset of an inflammatory response, activation and interconnection of several metabolic cascades, encompassing the complement system, coagulation system, fibrinolysis system and KKS, take place in order to propagate and mature the inflammatory reaction.

During the onset and progression of inflammation the PK/HMWK complex, which is freely circulating in plasma, binds to the multiprotein complex on endothelial cells. As a result, conformational changes occur to convert PK to KLK, which in turn proteolytically cleaves HMWK to liberate BK and HKa. Under "normal" physiological conditions, BK has a very short half-life of 17/30s before this active molecule is metabolised by kininases, however this is long enough to trigger intracellular signalling initiated via the B_2 receptor (Ferreira and Vane, 1967, Colman, 2006). HKa has comparatively longer biological half-life of about 9 hours and is eventually deactivated by proteases. The activation of this BK-forming cascade on endothelial cells, neutrophils and also macrophages, in parallel to secretion of inflammatory mediators such as kinins, has been implicated in the pathogenesis of inflammation and vascular injury. The balance of evidence to date suggests that neutrophils and macrophages carry a significant amount of bound KNG, and possibly other contact system proteins, and deliver them to inflammatory foci to supplement the concentration of these proteins in the injured or infected tissues. Moreover, the activation of the KKS on the surface of neutrophils and macrophages enhances the tKLK-dependant kinin liberation and hence supports the development of an inflammatory response (Barbasz *et al*., 2008). pKLK has been shown to stimulate neutrophil chemotaxis aggregation (Wachtfogel et al., 1983) and acts as a potent aggregation agent for human blood polymorphonuclear leukocytes (Schapira *et al.,* 1982). Together with the ability of KLK to release neutrophil elastase, these factors contribute to tissue injury (Keith *et al.,* 2005). BK, released via B_2 receptors, and inflammatory cell adhesion molecules trigger

downstream intracellular signalling which initiates an acute inflammatory response, promotes the synthesis of other pain mediators via prostaglandin liberation and enhances vascular permeability via prostaglandin I2 and the production of nitric oxide (Sainz *et al.*, 2007).

Plasma CPN or tissue CPM cleaves the C-terminal Arg of the B_2 receptor ligands BK and Lys-BK to generate B_1 ligands such as des-Arg^9-BK and des-Arg^9-Lys^1-BK (Ignjatovic *et al.*, 2002). These ligands activate the downstream signalling pathway via B_1 and this facilitates the activation of further cell adhesion molecules, whilst the inhibition of migration promotes cell clustering in the inflammation site to develop the inflammatory reaction. The effects of released BK are initiated through B_1 and B_2 receptors, leading to activation of several intracellular signal transduction pathways including the phospholipase C (Leeb-Lundberg, 2004), phospholipase A_2, phospholipase D and MAPK cascades (Yang *et al*., 2003, Sainz *et al*., 2007). Several peptide receptors, including the B_2 receptor, are known to stimulate the MAPK cascade. The mitogenic potential of B_1 receptors is not completely established, although there are reports that the mediation of the MAPK signalling pathway is associated with B_1 receptors (Morbidelli *et al.*, 1998). Activation of the p42/p44 extracellular-signal-regulated kinase (ERK)/MAPK cascade plays a key role in the mediation of proliferative activity of receptor tyrosine kinases (RTKs), such as EGF receptor (EGFR), and GPCRs (Gutkind, 1998). Simultaneous and sustained activation of both Kinin receptors is known to be advantageous for immediate triggering of $ERK_{1/2}$ global phosphorylation and PLCγ2-dependent intracellular Ca^{2+} mobilisation. Several mechanisms of GPCR-induced MAPK

activation have been reported. EGFR-induced activation of MAPK is triggered by interaction of EGF with a monomeric receptor, which results in EGFR dimerisation, activation of Ras and downstream phosphorylation on tyrosine residues (Liebmann, 2001). Ras stimulates the MAPK pathway, comprising of Raf (a serine/threonine-protein kinase), mitogen-activated protein kinase (MEK) and ERK (Liebmann, 2001), thereby regulating the transcription of the genes involved in cell growth, differentiation and survival. The "transactivation" (or cross-communication between different signalling pathways) of RTKs has been reported as a principal mitogenic signal transduction cascade of GPCR (Daub *et al*., 1997). However, a mechanism, proposed by Prenzel *et al* shows that transactivation of the EGFR-dependent signalling pathways upon activation of GPCR by an extracellular ligand involves the interaction of membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) and metalloproteinase (Prenzel *et al.*, 1999). In conclusion, the cross-activation between the GPCR and MAPK pathways is extremely cell specific and may be dependent on the type of receptor, concentration and cellular localisation of the proteins, that play a major role in the signal transduction within the GPCR and MAPK signalling cascades (Liebmann, 2001).

The diverse effects of the bioactive BK peptide can produce the majority, but not all, of the features of the inflammatory reaction. Recently, the role of HMWK and its contribution to inflammation, particularly interactions of HKa with leukocytes and endothelial cells was emphasised. The histidine-rich D5 of the HMWK light chain, which possesses a binding site for vitronectin, is suggested to be associated with the mechanism of anti-cell spreading activities of HKa (Asakura *et al.*, 1998).

The immobilisation of vitronectin leads to the prevention of vitronectin-uPAR interaction on the surface of endothelial cells and results in inhibition of cell migration and adhesion (Asakura *et al*., 1998). The ability of HMWK to detach inflammatory cells such as neutrophils and monocytes from vitronectin by a mechanism of competitive binding to the Mac-1 (CD11a/CD18) receptor plays an imperative role in migration of the cells associated with inflammatory reaction (Sainz *et al.,* 2007). Moreover, HKa can inhibit intracellular changes that lead to the modulation of intracellular signals in endothelial cells and thus cell behaviour. It has been postulated that HKa stimulates monocyte activation by binding to uPAR, gC1qR and Mac-1 surface receptor complex. Both D3 and D5 of HKa exhibit high affinity to Mac-1, although only D5 (which is extremely exposed in two-chained HKa) is designed for binding to uPAR (Khan *et al.,* 2006). It is evidenced that upon this activation monocytes synthesise cytokines and chemokines such as TNF*α*, IL-1*β*, IL-6, IL-8 and monocyte chemotactic peptide 1 (MCP-1), which are reported to play a major role in the migration of monocytes (Khan *et al*., 2006).

BK can regulate Ca^{2+} kinetics in a dose-dependent manner by mediating the mobilisation of calcium from the thapsigargin-sensitive stores on inositol-1, 4, 5-trisphosphate and also by inducing extracellular Ca^{2+} release initiated via B_2 receptors (Wang *et al*., 2001). However, recently Kolte *et al* presented a study on role of HMWK in the absence of PK and provided evidence that HMWK (but not HKa) has the same capability as BK to bind to B_2 receptors (Kolte *et al.*, 2011). The study demonstrated that the interaction of HMWK with B_2 receptors is effective enough to trigger B_2 receptor-dependent downstream signalling, thereby mediating

a significant increase in intracellular Ca^{2+} with release of nitric oxide and PG_2 by endothelial cells providing two important mediators of inflammatory response (Kolte *et al*., 2011). At physiological concentrations HMWK displayed no effect on endothelial permeability but demonstrated cardio protective properties due to the ability to sustain normal endothelial function and cardiovascular circulation (Kolte *et al.*, 2011). These novel findings give a clearer understanding of the KKS however further investigation is required in order to identify the molecular events that contribute to dysregulation of the system and pathologic disorders.

3.4.2 Kinin-Kallikrein System: The Implications in Carcinogenesis

The role of the KKS, as a complex multifunctional cascade, is still poorly defined in cancer. The first studies which demonstrated the possible relevance of the KKS in cancer suggested that enhanced vascular permeability, nitric oxide synthase production and prostaglandin production in cancer tissue could be promoted by the activated KKS (Maeda *et al*., 1996, Czokało *et al.*, 1996). This lead to the suggestion that KLKs and possibly other members of the KKS could be novel cancer biomarkers (Rittenhouse *et al*., 1998, Yousef and Diamandis, 2002, Dlamini and Bhoola, 2005, Greco *et al.*, 2004). Recent evidence has demonstrated a convincing role of the KKS in the pathogenesis of cancer. Several proteins generated within the KKS have been confirmed to exhibit proinflammatory properties and to be involved in aetiology and genesis of inflammation. Chronic inflammation is known to be a major cause of cancer due to the liberation of powerful oxidants which serve as mutagens and also stimulate pathogenic cell multiplication. The KKS has become a target of intense investigation by scientific

researchers and the pharmaceutical industry in order to discover novel cancer biomarkers and anti-cancer therapy targets. Third generation kinin receptor inhibitors have been demonstrated to be cytotoxic to malignant cells (Dlamini and Bhoola, 2005).

3.4.2.1 *Kallikreins: The Role and Association with Carcinogenesis*

KLKs are a group of serine proteases. Tissue KLKs play an important role in a wide spectrum of normal physiological events such as kinin formation, blood pressure control, skin desquamation, electrolyte kinetics, tissue remodelling and prohormone processing (Borgono *et al*., 2004). The dysregulated expression of several KLK-related peptidases has been associated with various malignancies.

The serine protease properties of tKLKs may contribute to the malignant phenotype by promoting angiogenesis, invasiveness and metastasis of malignant cells due to dysregulation of extracellular matrix (ECM) components and activation of signal transduction cascades (Avgeris *et al*., 2011). In addition, multiple studies have demonstrated that KLKs may contribute to cancer development by the stimulation of malignant cell proliferation, enhancement of vascular permeability and malignant cells dissemination via mitogenic and cellular activities of the kinins (Dlamini and Bhoola, 2005). Tissue KLKs have been demonstrated to promote prostate cancer cell migration, invasion and proliferation via the activation of protease-activated receptor-1 (PAR1) and through crossactivation of EGFR, an effect which could be down regulated by EGFR inhibitors or ERK inhibitors (Gao *et al.*, 2010).

Several individual members of the KLK family have been identified as potential biomarkers for various malignancies. Human tKLK 1 (KLK1) was reported to play an important role in post-ischaemic neovascularisation (Stone *et al*., 2009) and was associated with progression and invasiveness in pancreatic carcinoma (Wolf *et al.*, 2001), oesophageal carcinoma (Dlamini and Bhoola, 2005), gastrointestinal stromal tumours (Dominek et al., 2010) and lung malignancies (Chee et al., 2008). Human KLK 3 (KLK3), which is also termed prostate-specific antigen (PSA), along with human tKLK 2 (KLK2) are biomarkers for prostate carcinoma (Rittenhouse *et al.*, 1998, Chan *et al*., 1987). Down regulation of tissue PSA was found to be associated with more aggressive forms of prostate cancer (Stege *et al*., 2000) and PSA can be utilised as a diagnostic marker in this malignancy (Partin *et al.,* 1990). The role of KLKs as prognostic biomarkers in prostate cancer became a catalyst for further research of KLK expression in other hormone-related malignancies including breast cancer. It was reported that PSA expression was significantly enhanced in more aggressive forms of breast cancer when compared with the indolent form or healthy breast tissue (Li *et al.,* 2011). However PSA-positive tumours were reported to be associated with favourable prognosis, earlier disease stage, longer median survival and reduced risk of disease relapse (Diamandis *et al.,* 1996). Other studies have reported KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14 and KLK15 up-regulation in ovarian carcinoma tissues, serum and/or cell lines (Borgono *et al.,* 2004).

In colorectal cancer, the over expression of KLK4, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK15 has been reported (Avgeris *et al*., 2010, Kontos and Scorilas, 2012). KLK5, KLK7 and KLK14 have been demonstrated to be independent prognostic factors (Talieri *et al.*, 2009). Recently KLK4 transcript expression has also been reported to be of clinical relevance in colorectal cancer and may predict poor disease free survival (Kontos *et al.*, 2013).

It has been reported that the quantification of KLK5 expression in breast tissue biopsies may represent a novel biomarker for the differential diagnosis between malignant and benign tumours of the mammary gland and may also be useful in the monitoring of breast cancer patients (Avgeris *et al*., 2011).

However, there are a few studies which have reported that KLKs can also inhibit carcinogenesis (Goyal *et al.,* 1998, Roman-Gomez *et al.*, 2004). For example, KLK10 (*NES1* gene) is a candidate tumour suppressor gene on chromosome 19q13.3-4 which was demonstrated to be down regulated in acute lymphoblastic leukaemia (Roman-Gomez *et al.*, 2004) and breast cancer (Dhar *et al*., 2001). Despite many reports which indicate a convincing association between dysregulation of KLK expression and carcinogenesis, several key questions still remain.

3.4.2.2 *Kininogens: Implications in Cancer*

It has been discussed earlier in this chapter that the PK/HMWK complex, which circulates freely in plasma, yields BK and HKa upon binding to the uPAR-CK1-gC1qR complex (Figure 3.8). HMWK expression was reported to be down regulated in the urine of patients with ovarian carcinoma in comparison to healthy

controls (Abdullah-Soheimi *et al*., 2010). However, it has also been demonstrated that two-chain HMWK (D_1, D2, D3-D5, and D6) promotes endothelial cell apoptosis and inhibits angiogenesis (Colman *et al*., 2000, Zhang *et al.*, 2000).

An underlying paradigm that the KKS plays an important role in promoting angiogenesis by proteolytic liberation of BK, the pro-angiogenic molecule, was contradicted by the report that HKa can serve as an angiogenic inhibitor (Browder *et al*., 2000). Since the interaction between urokinase and urokinase receptors was demonstrated to initiate intracellular signalling leading to stimulation of mitogenesis and HKa was reported to bind to uPAR with high affinity, the mechanism of HKa and uPAR/CK1/Gc1qR interaction has been intensively assessed *in vivo* and *in vitro*. uPAR has been shown to be involved in inhibition of cell adhesion, migration, proliferation, angiogenesis and tumour metastasis by inducing apoptosis and cell signalling failure (Bior *et al.*, 2007). uPAR is membrane-anchored receptor which is widely distributed in monocytes and endothelial cells but is upregulated in many cancer cells (Carroll and Binder, 1999). The inhibitory activity of HKa was found to be Zn^{2+}-dependant and the interaction between HKa and uPAR was mediated via HKa D5 (the high affinity endothelial cell binding region within D5 was allocated to amino acids sequence 479-498 of HMWK) with uPAR D2 and D3 (Zhang *et al.*, 2000, Colman *et al.*, 2000). D5 of HKa can block vascular endothelial growth factor (VEGF) and angiogenesis in the chick chorioallantoic membrane (Zhang *et al.,* 2000). Several studies were conducted in order to confirm D5-dependant inhibitory properties in various malignancies. HKa D5 caused dramatic inhibition of the growth of HT-1080 human fibrosarcoma cells (Colman *et al.*, 2000) and HCT-116

colorectal carcinoma cells (Bior *et al.,* 2007). It has been suggested that D5 possesses inhibitory properties against malignant cell migration and invasion of prostate cancer (Liu *et al*., 2009). It has been hypothesised that HKa disrupts the urokinase plasminogen activator-uPAR complex, inhibits ERK activation, and blocks the internalisation of uPAR, which lead to cell death and cell motility arrest (Colman *et al.,* 2010). However, the pathway/pathways by which D5-HMWK signals and regulates the cell cycle still remains the subject for future research.

3.4.2.3 *The Involvement of Kinins and Kinin Receptors in Tumourigenesis*

Under "normal"physiological conditions the active peptide and inflammatory mediator BK has a very short half-life before its enzymatic degradation, however over expression of BK and the B_1, and B_2 GPCRs have been demonstrated in various pathological conditions including carcinogenesis and cancer progression. B_1 receptors were reported to be overexpressed in prostate cancer (Taub *et al.,* 2003, Zhang *et al.*, 2008b) whilst B_2 receptors were expressed at elevated levels in brain, gastric, lung and liver cancer (Zhao *et al.,* 2005, Zhang *et al.,* 2008b). B_1 and B_2 receptors were overexpressed in human chondrosarcoma cells, in comparison to healthy cartilage cells (Yang *et al.*, 2010). Immunohistochemical detection of B_2 receptor overexpression in head and neck cancer samples was recently reported (Beck *et al.,* 2012a). The expression of BK receptors in human lung adenocarcinoma, gastric adenocarcinoma, lymphoma, squamous cell lung carcinoma and head and neck cancer suggests involvement in mediation of pathologic signal transduction, nitric oxide production and increased vascular

permeability (Wu *et al.*, 2002a) thus potentially leading to cancer growth, progression and tumour dissemination.

It has been reported that BK stimulates angiogenesis in endothelial cells due to B_2-dependant increase in vascular permeability in the early stages and B_2-dependant up-regulation of VEGF in the stromal fibroblasts in the later phases (Ishihara et al., 2002). Other researchers have demonstrated the role for BK-dependent increase of vascular permeability and elevated levels of VEGF in the development of solid tumours (Maeda *et al.*, 2003). The involvement of BK in angiogenesis via B_2-dependant stimulation of cell proliferation and cell migration has been reported (Seegers *et al.,* 2004). BK has been demonstrated to promote B_2-dependant stimulation of growth and migration of head and neck squamous cell carcinoma cells via EGFR transactivation and also to lead to the production of cyclooxygenase-2 and PGE2 through the MAPK signalling cascade in human airway cells (Zhang *et al.*, 2008b).

GPCR-dependant activation of the actin-cytoskeleton pathway is associated with cell migration/invasion. The initial migratory cell response requires a formation of cellular protrusions, such as actin microspikes, filopodia and lamellipodia, which promotes cell movements and invasion into surrounding tissue (Erices *et al.,* 2011). This process involves the polymerization of actin, induced by the actin-cytoskeleton signalling cascade, and is reported to be dependent on Cdc42 and Rac1 molecules. BK promotes the formation of Cdc42-dependent membrane protrusions in fibroblasts provoked by PGE2 secretion (Erices *et al.,* 2011). BK exhibits multiple activities which can modulate several cell signal transduction

pathways, initiated via B_1 and B_2 receptors, and therefore may play a significant role in the regulation of neovascularisation, tumourigenesis and tumour progression.

3.5 Conclusions

The KKS represents a complex network of interactions produced by multiple constituents within the system itself and the action of the KKS upon other important systems within the human body. We have described the KKS network and the implication of the KKS in carcinogenesis and cancer progression.

3.6 Project Aims and Objectives

Disease progression in CLL is characterised by a high degree of heterogeneity. Given the unmet need for research to explain the aetiology of clinical heterogeneity, the overall aim of this project is the identification of novel, reliable and affordable prognostic biomarkers in CLL. The hyper reactivity of the BCR to unknown external or/and self antigen stimulation and its association with malignant B-cell survival has been extensively studied in different experimental settings (Chapter 1).

A comparative proteomic approach was previously employed, by the Cancer Biology Proteomics Group supervised by Dr. Lynn Cawkwell and Dr. David Allsup, which utilising two-dimensional gel electrophoresis (2DE) with MALDI-TOF mass spectrometry (MS), offered the opportunity for comparative proteomic analysis of differentially expressed proteins (DEPs) associated with *in vitro* BCR activation

(Section 2.2.3.1). Three “high risk” CLL samples were selected based upon BCR responsiveness, demonstrated by up-regulation of phospho-ERK following in vitro stimulation. Proteomic analysis revealed several differentially expressed proteins, although from the generated list of the candidate biomarkers, Kininogen, a critical protein of Kinin-Kallikrein System, was shown to be upregulated in 3/3 clinical samples upon BCR stimulation. Moreover, Kininogen has never been linked to CLL or other B-malignancies previously. Therefore, the aim of the current project is to identify the possible clinical relevance of Kininogen and the Kinin-Kallikrein System in CLL.

As discussed in Chapter 3 there are two major forms of Kininogen: LMWK and HMWK, although, there is a paucity of information on the interrelation of HMWK and LMWK and their association with cancer. Although the peptides identified in the MALDI-TOF MS experiment described above best matched the sequence of LMWK isoform, both HMWK and LMWK were analysed in the current study.

Therefore the objectives of this project were:

- to confirm the differential expression of Kininogen in CLL samples previously used in proteomics using immunoblotting (Chapter 5);
- to validate the differential expression of Kininogen protein in the clinical context upon *in vitro* BCR stimulation and to analyse constitutive Kininogen protein expression using an increased number of B-CLL samples and to correlate Kininogen expression with clinical data and other biomarkers (Chapter 5);
- to investigate the Kininogen expression in normal B cells (Chapter 5);

- to evaluate the expression of corresponding Kininogen mRNA using standard Reverse transcription polymerase chain reaction (RT-PCR) (Chapter 6);
- to assess the expression of Kallikreins, which are known to digest Kininogen with subsequent liberation of bioactive Kinins (BK and Lys-BK), utilising immunoblotting in CLL and normal samples (Chapter 7);
- to evaluate the level of BK/Lys-BK in plasma samples from CLL patients using ELISA and to correlate BK/Lys-BK plasma concentration with clinical data and other biomarkers (Chapter 8);
- to employ immunoblotting and flow cytometry in order to analyse the expression of B_1 and B_2 kinin receptors on the surface of normal and CLL B cells (Chapter 9).

In order to answer this research question, the selection of appropriate research methodologies and identification of suitable clinical cases were necessary and considered as the key elements in successful research. The collection and storage of samples donated by CLL patients had already been initiated and would be continued. The quality-checked relevant entries specifying age, sex, diagnosis date, survival, time from diagnosis to first treatment, IgV_H status, and other prognostic markers would be accurately recorded, analysed and correlated with generated resultant data using bivariate statistical analysis.

This project sets out to test the hypotheses that CLL B-cells comprise the complete Kinin Kallikrein System, the activation of which can be associated with BCR ligation, leading to the synthesis and liberation of Kinins, which in turn

mediate the activation of Kinin B_1 and B_2 receptors possibly via an autocrine loop. It is hoped that this sequential investigative approach will generate data, which may improve our understanding of the functional role of the KKS in the biology and pathophysiology of CLL and give credible direction for further research in identifying novel biomarkers, which are both affordable and reliable.

CHAPTER 4

Materials and Methods

Chapter 4. Materials and Methods

4.1 Cell Culture

All experimental cell culture work was carried in a dedicated laboratory that has been adapted for cell culture purpose using a clean air down-draft hood Class II cabinet and appropriated good quality chemicals and reagents. Personal protective equipment was worn during the cell handling procedure for protection purpose.

4.2 Thawing of Cryopreserved Cells

The previously cryopreserved cells (Table 4-1) were thawed inside a sealed plastic bag, in a water bath at 37^0C. Then, the cells were immediately transferred into 30 mL screw-cup universal tube (Sterilin) using sterile technique in a laminar flow cell culture hood (ESCO Infinity Class II Biological Safety Cabinet (BSC)), and pre-warmed culture media was added dropwise (over 1-2 min) to achieve 1:10 dilution to minimise the toxic effects of cryopreservant. The cell suspension was spun using Sigma-Aldrich, 2-5 SciQuip centrifuge at 400 *x g* for 3 min and the supernatant discarded. The pellet then was re-suspended in the appropriate amount of fresh cell culture media, which was pre-heated to 37 ^{0}C prior to the cell culture, and transferred into T25 (25 cm^2 area on largest side) or T75 (75 cm^2 area on largest side) flasks (Sarstedt) according to the pellet size. The flask containing cells was placed in Scientific Laboratory Supplies Galaxy B CO_2 Incubator in a humid atmosphere at 37 ^{0}C with 5 % CO_2 for further cell growth and culture.

Table 4-1Cell Lines Utilised in the Current Study

Cell Line Name	Description	Morphology	Growth Mode	Source	Media
Raji	Human cell line from haematopoietic origin, which was first derived in 1963 from the left maxilla of a an 11 year old Nigerian patient with Burkitt's lymphoma *.	Lymphoblast	Suspension	European Collection of Cell Cultures (ECACC)	RPMI -1640 Culture Media
A549	Human lung adenocarcinoma epithelial cell line	Epithelial cell line	Adherent	Courtesy of Dr. Vijay Agarwal (CBPG, Castle Hill Hospital)	DMEM Culture Media
LNCaP	Human androgen-sensitive prostate adenocarcinoma cell line	Epithelial cell line	Adherent, single cells and loosely attached clusters	Courtesy of Dr. Laura Sadofsky (Respiratory Medicine, CCMR)	RPMI -1640 Culture Media
MCF-7	Human breast adenocarcinoma cell line	Epithelial cell line	Adherent	Courtesy of Dr. Lucy Scaife and Dr. Vicky Hodgkinson (both CBPG, Castle Hill Hospital).	DMEM Culture Media
T47D	Human ductal breast epithelial tumour cell line	Epithelial cell line	Adherent	Courtesy of Dr. Lucy Scaife and Dr. Vicky Hodgkinson (both CBPG, Castle Hill Hospital).	DMEM Culture Media
PJ49	Human oral squamous cell carcinoma cell line	Epithelial-like cell line	Adherent	Courtesy of Dr. Lucy Scaife and Dr. Vicky Hodgkinson (both CBPG, Castle Hill Hospital).	DMEM Culture Media
SW837	Human rectum adenocarcinoma cell line	Epithelial cell line	Adherent	Courtesy of Dr. Lucy Scaife and Dr. Vicky Hodgkinson (both CBPG, Castle Hill Hospital).	DMEM Culture Media
HUVEC	Human umbilical vein endothelial cells	Endothelial CELL LINE	Adherent	Courtesy of James Williamson (Respiratory Medicine, CCMR)	Endothelial Cell Basal Medium

**(Karpova et al., 2005)*
RPMI-1640 and DMEM (Dulbecco's Modified Eagles Medium) supplemented with fetal bovine serum (FBS), glutamine, penicillin / streptomycin and fungizone (Section 4.1.2) (Appendix A).
In the current project Raji cells were utilised only for optimisation purpose as despite its wide scale use in leukaemia and lymphoma experimental studies, the cells are not representative of CLL and no conclusions can be made based upon in vivo Raji cell behaviour.

4.2.1 Cells Growth and Culturing

Supplemented RPMI -1640 (#31870, Invitrogen)/DMEM (#D5546, Sigma Aldrich)/Endothelial Cell Basal (#C-22210 and #C-39210 (Supplement pack), PromoCell) (Appendix A) media was replaced 3 times per week to ensure good nourishment while cells are growing. Prior to cell transfer to a new flask, the media was pre-warmed in a water bath at 37ºC in order to avoid any additional stress during the transfer into a new environment. The suspension cells were gently removed from the flask and transferred into a sterile 30mL universal container within the laminar flow cell culture tissue hood; sterile techniques were adopted throughout the cell culture procedure. After the cell suspension was centrifuged at 400 *x g* for 3 min, the media was discarded. The cell pellet was re-suspended in the required amount of fresh media and transferred into a new flask. Then, providing that suspension cells achieved the confluence of 80% or above (Figure 4-1), the cells were passaged by dividing this cell suspension into two new flasks.

Adherent cells were transferred from the flasks by trypsinisation using pre-warmed TrypLE Select (#12563, Invitrogen). Trypsin (3 mL for T25 and T75 flasks) was added dropwise to the flask, gently agitated and incubated at 37ºC for 3-5 min. After incubation the flasks were lightly tapped to ensure the complete cells removal. The cell culture media (7 mL) was added to neutralise the action of the trypsin. The cell suspension was spun at 400 *x g* for 3 min and the cells were re-suspended in fresh media and transferred to a new cell culture flask.

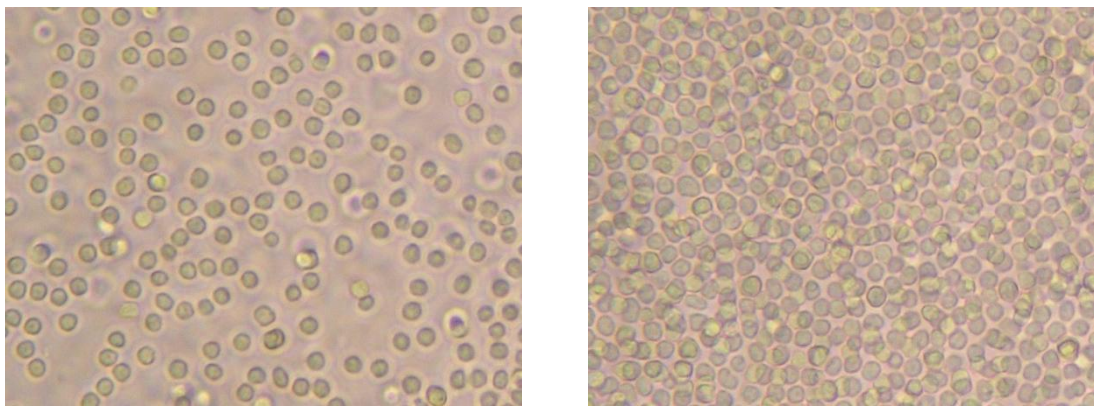


Figure 4-1 Cultured Raji Cells

Raji is a suspension cell line. Cells growing in exponential growth phase expected to look bright, round, single cells without attachment.
A. Raji cells reached approximately 30% confluence.
B. Raji cells reached approximately 80%-100% confluence.

4.2.2 Manual Cell Counting

For manual cell counting an Improved Neubauer Counting Haemocytometer (Hawksley) (Figure 4-2) was utilised. The counting chamber was prepared and the glass surface and cover slip were cleaned. The cover slip was positioned over the counting surface and the cell suspension was inserted into one of the V-shaped wells. The cell suspension was introduced by gently mixing 25 µl of media containing the cells and equal amount of 0.4% of Trypan blue dye. Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. Therefore the dead cells were shown as a distinctive blue colour under the microscope and live cells were excluded from staining. The area under cover slip is uniformly filled by capillary action. The counting chamber was lodged under the microscope and the

counting grid (Figure 4-2) was brought into focus and unstained viable cells were counted in each quadrant and the average number taken. The cells touching the right hand and lower borders of each quadrant were not included in the count. The average number of cells was multiplied X2 for dilution correction and the total count was calculated as the product of average count X 10^4.

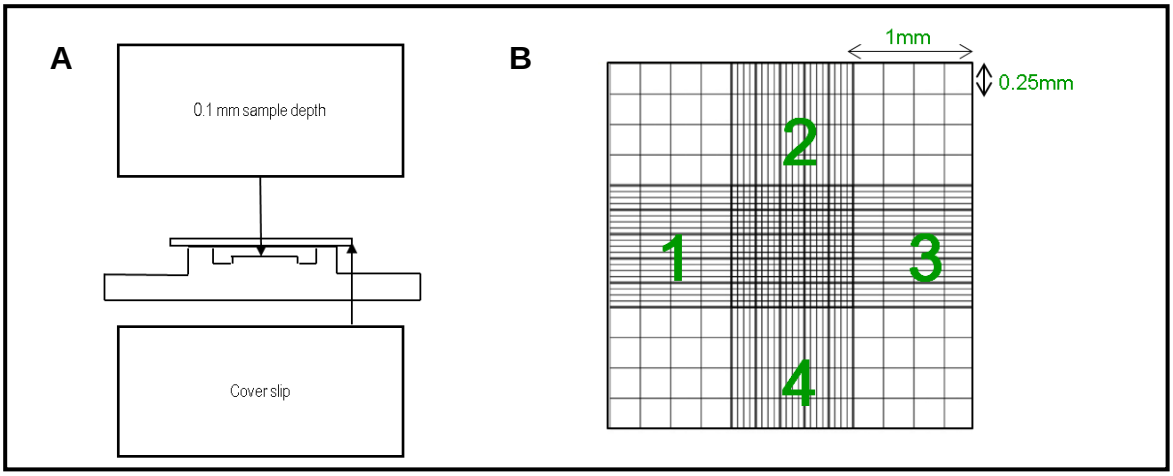


Figure 4-2 The Improved Neubauer Counting Chamber

This is a schematic representation of the Improved Neubauer Counting Chamber (A) and as seen under the microscope (B), which is used for determining cell concentration per unit volume of a suspension. The counting grid is presented by 9 large identical squares. Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Therefore the total counting grid has a volume of 0.9 mm³.

4.2.3 Cryopreservation of cultured cells

Cells in the log phase of growth and free from bacterial and fungal contaminants were harvested and pelleted by centrifugation at 1600 rpm for 3 min. Then the cell pellet was slowly re-suspended in freezing media containing dimethyl sulphoxide DMSO (#D2650, Sigma Aldrich) (Appendix A) allowing cell adaptation

to the new environment and avoiding rapid intracellular dehydration and fluid shift. The mixture was aliquoted into 1mL cryovials (Greiner) and contained approximately $1x10^7$ cells re-suspended in 10% DMSO and then stored for subsequent use in low sub-zero temperatures (-80^0C) or in liquid nitrogen.

4.3 Clinical Samples Collections and Characteristics

Informed consent from each patient involved in this study was obtained according to the Declaration of Helsinki. Peripheral blood (PB) samples (40 mL) were collected from 60 patients, who attended the Queen's Centre for Oncology and Haematology, Castle Hill Hospital and had been diagnosed with CLL according to standard international criteria (Hallek *et al.,* 2008a, Oscier *et al.,* 2012). All samples were drawn, used and stored according to both Research Ethics Committee approval (05/Q1104/33, Hull and East Riding Local Research Ethics Committee) and Hull and East Yorkshire NHS Trust R&D Approval (#08/H130435). The Binet stage was assessed by clinician at the time of sample collection. IgV_H mutation (carried out by Dr Paul Evans) and CD38 statuses were determined in the Haematological Malignancy Diagnostic Service (HMDS) in Leeds utilising gene sequencing and flow cytometry, respectively. Those CLL cells, which expressed IgV_H <98% homology with the corresponding germline, were regarded as mutated, and those with ≥98% were considered unmutated (Section 2.1.2.2.1). ZAP-70 analysis was performed by Miss Jo Pointon (University of Hull) utilising flow cytometry. A sample was regarded as ZAP-70 positive if the percentage of CLL cells expressing ZAP-70 was >20%. FISH was carried out in HMDS (Leeds)

according to published methods (Dohner et al., 2000a). ß2M was measured using fully automated nephelometric immunoassay. Additionally, 4 PB samples obtained from relatively healthy volunteers were utilised in this study (Appendix B).

The plasma samples, obtained from 36 CLL patients attending routine CLL clinic appointments during November and December 2011 in Queen's Centre for Oncology and Haematology (Castle Hill Hospital) along with commercially purchased Normal Donor Plasma Pool (Precision BioLogic CRYOcheck™, # CCNS-10) were collected and processed by an MSc student Paula Johnson (Haematology department, Coagulation section of Hull and East Yorkshire Hospitals NHS Trust), who carried out an associated study investigating the Prekallikrein and HMWK plasma concentrations in patients with CLL and evaluating their potential value as prognostic biomarkers (Appendix D).

All the clinical data, including clinical information regarding age, stage, date of diagnosis, full blood count was securely captured and stored in the database on the NHS server (Appendix B and D).

4.3.1 Isolation of Leukocytes from whole blood samples by density gradient centrifugation

The isolation of live leukocytes by density gradient centrifugation procedure is compatible with proteomics and other laboratory techniques employed in the current project. A sample containing 40 mL of whole blood was obtained by peripheral venepuncture from participating CLL patient and placed into 50 mL falcon tube (Sarstedt) containing 625 units of heparin (Leo Laboratories). At the time of collection, the total white cell count (WCC) was determined for each sample

utilising automated full blood count analyser (Sysmex). The tubes were stored at room temperature if they were not processed immediately after collection, however, cell degradation can occur if tubes are stored for more than four hours.

Four 50mL falcon tubes, each containing 6mL of ice cold Histopaque-1077 (#10771-500mL, Sigma Aldrich), were prepared for every blood sample. Ten mL of anticoagulated blood was layered onto 6 mL of Histopaque-1077 by gently sliding blood down side of the tube to prevent mixing. The tubes were centrifuged at 400 *x g* for 30 min at RT. During centrifugation the lymphocytes and other mononuclear cells which are collectively referred to as peripheral blood mononuclear cells (PBMCs) remained at the plasma/Histopaque-1077 interface which is also known as “buffy coat” (Figure 4-3). The opaque interface was carefully aspirated and transferred into 10mL of ice cold washing media (Appendix A). The cells were pelleted by centrifugation at 400 *x g* for 10 min before supernatant was discarded. At this point, the harvested cells can be immediately utilised for downstream application or cryopreserved.

4.3.1 Cryopreservation of Leukocytes

The required media volume for cell resuspension was calculated using the patient’s total white cell count (WCC) ensuring that a final minimum concentration of $1x10^7$ cells per 1 mL cryovial would be achieved. Initially the cell pellet was resuspended in 50% of total amount of media required. Then the remaining 50%, which is cryopreservation media containing 20% (v/v) DMSO (Appendix A), was added dropwise over 1-2 minutes (which resulted in final 10% (v/v) DMSO), it was then gently mixed and cell suspension was aliquoted into appropriately labelled

1mL cryovials (Greiner) and stored within 15 min after DMSO was added, in low sub-zero temperatures (at -80°C in a freezer or at -195.8°C in liquid nitrogen).

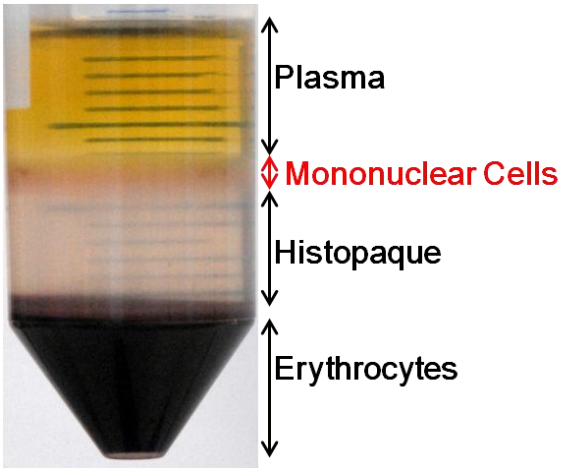


Figure 4-3 Separation of PBMCs from human peripheral blood using Histopaque-1077

During centrifugation, utilising Histopague-1077, the aggregated erythrocytes and increased density granulocytes become sediment at the bottom of the centrifuge tube. The lymphocytes and other mononuclear cells retained in the plasma/Histopaque-1077 interface and formed the cloudy mononuclear layer, which become visible in the tube after centrifugation.

4.3.2 Thawing of Cryopreserved Leukocytes

Inappropriate thawing of cryopreserved leukocytes can result in viability and cell recovery being compromised. Therefore cells were thawed quickly but diluted dropwise to remove DMSO following the same protocol as described in Section 4.1.1. In order to eliminate stress effects, the post-thawed leukocytes were subjected to extended acclimatisation by being transferred into a T25 or T75 flasks

and incubated in culture media for 3 hours at 37^0C with 5% CO_2 before being utilised in any subsequent assays.

4.4 Separation of B Lymphocytes from Peripheral Blood Mononuclear Cells Population

The fractionation of sub-populations of PBMCs is widely utilised in research, where the biochemical, genetic analysis as well cell culture of a particular cell population extracted from heterogeneous cell mixture is required. At the present time the commonly used techniques such as magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) offer the best possible outcome in B cell separation. In order for these separation techniques to be carried out successfully, both methods first had to be established and optimised for use in clinical samples. Unmanipulated clinical samples were used in the majority of the experiments within this project, although B cell separation was employed in association with other methods such as Western Blotting and flow cytometry.

Separation of B cells utilising either of these technologies is based upon the use of antibodies against differentially expressed cell-surface antigens as targets and provides purities exceeding 90%. For the isolation of the cell type of interest positive selection or negative selection (depletion) can be used. Positive selection offers the purification of a target cell population utilising an antibody that specifically binds to the lineage markers. Whereas, negative selection involves the depletion of all other cell populations present in the cell mixture except the cell type of interest. Although, the major challenge in B cell separation is to achieve a good

yield with high cell viability and recovery that are satisfactory for subsequent in vitro manipulation.

4.4.1 B-Lymphocytes Isolation Utilising Magnetic-Activated Cell Sorting Methodology

4.4.1.1 *Introduction to Magnetic-Activated Cell Sorting (MACS)*

The MACS technique enables the population of interest to be isolated by incubating with magnetic beads (magnetic labelling) coated with antibodies against a specific cell surface antigen (Figure 4-4) and in the current project was employed in conjunction with Western blotting (Section 4.4.3). The MACS magnetic beads (MicroBeads) were invented and registered trademark of the Miltenyi Biotec and are non-toxic and biodegradable nanaoparticles. MACS positive selection involves the process where the magnetically labelled population of interest retained within the column and eluted after magnetic field has been eliminated, whereas, unlabelled cells form a flow through fraction. Negative selection works in vice versa manner, during which the unwanted magnetically labelled cell populations retained within the column and unlabelled population of interest is collected in the flow-through fraction. On the basis of the findings conducted by Lyons *et al*, who have demonstrated that positive selection yields much higher purity of isolated cells and has no unfavourable effect on cellular transcription, positive B lymphocytes selection was prioritised over negative selection and utilised in this project (Lyons et al., 2007). The MicroBeads covered with CD19 antibody against CD19 antigen, which is known to be a specific B cell lineage marker were used in this study.

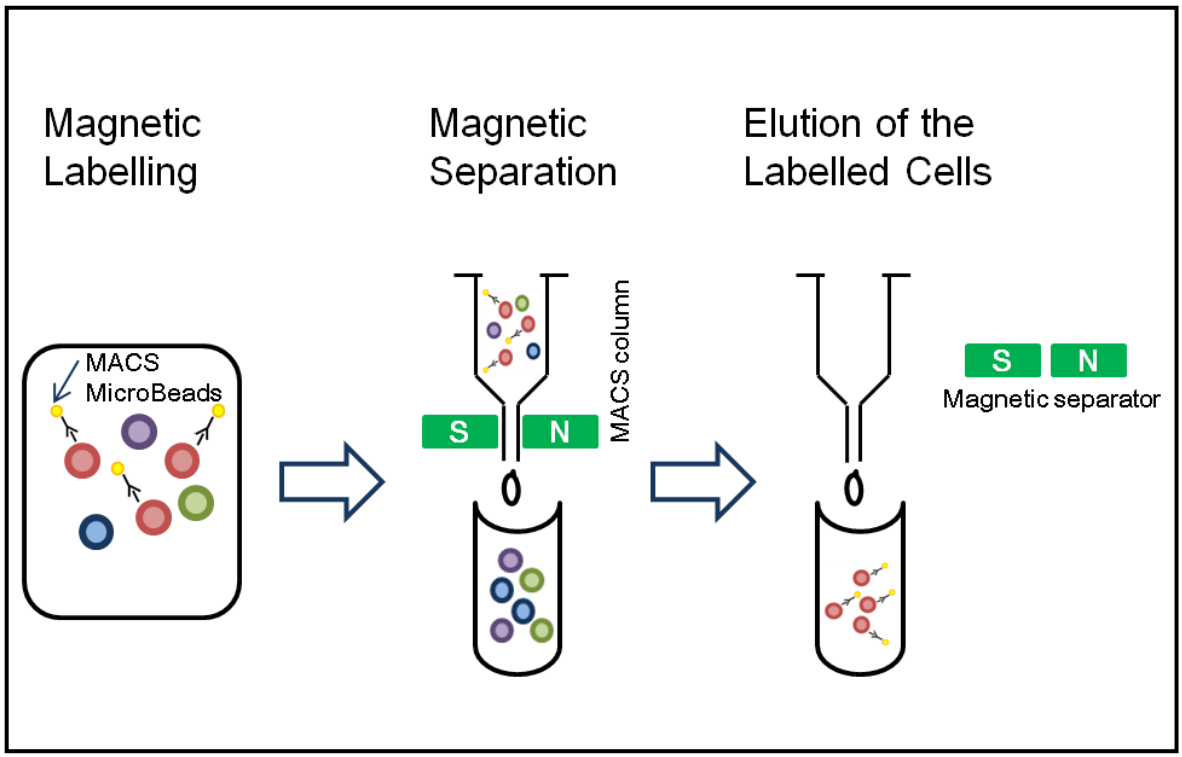


Figure 4-4 The Graphical Presentation of Basic Principles of MACS Positive Selection

This figure demonstrates the separation strategies involved in positive B cell purification. During magnetic labelling MACS MicroBeads specifically bind to antigens on the cell surface. Following this, the magnetic field is applied resulting in the magnetically labelled cells to be retained within the column and unlabeled cells flow through. After the column is removed from the magnetic field, the target cells are eluted from the column. Positive selection can be performed by direct or indirect magnetic labelling. Image adapter from (MACS MiltenyiBiotec, 2013).

4.4.1.2 *Sample Preparation*

PBMCs were isolated from blood samples by density-gradient sedimentation on Histopaque-1077 as described in Section 4.2.1. The cell viability was

determined using trypan blue exclusion as per Section 4.1.3 and cell concentration was adjusted to 10^7-10^8 of total cells.

4.4.1.3 *Magnetic Labelling*

In order to prevent non-specific cell labelling, the work was carried out quickly and efficiently utilising pre-cooled solutions and keeping cells on ice. Cells were harvested by centrifugation at 400 *x g* for 10 min and supernatant aspirated completely. Following this, cell pellet was resuspended in 80 µl of buffer containing phosphate-buffered saline (PBS) (Oxoid Limited), 0.5% BSA (Fisher Scientific, Thermo Scientific), 2mM ethylenediaminetetraacetic acid (EDTA) (#E6758, Sigma Aldrich). Then, 20 µl of CD19 MicroBeads (MiltenyiBiotec, #130-050-301) was added, mixed thoroughly and incubated at 2-8ºC for 15 min. The washing step was performed by adding 1-2 mL of buffer and centrifuging at 400 *x g* for 10 min, supernatant discarded. Harvested cells were resuspended in 500 µl of buffer and proceeded to magnetic separation (Figure 4-4).

4.4.1.4 *Magnetic Separation*

The appropriate MS, LS or XS column was chosen according to the number of cells in the sample (Table 4-2). When working with $1x10^7$ total cells LS column (MiltenyiBiotec, # 120-000-475) and MidiMACS^{TM} separator (MiltenyiBiotec, #130-042-302) (Figure 4-5) were used.

Table 4-2 MACS Columns and MACS Separators used for Positive Selection

The table is adapted from MiltenyiBiotec MACS reagent and instruments requirements protocol (MACS MiltenyiBiotec, 2013).

Column	Max. number of labelled cells	Max. number of total cells	Separator
MS	**$\leq 10^7$**	**$2x10^8$**	**MiniMACS**
LS	**$\leq 10^8$**	**$2x10^9$**	**MidiMACS**
XS	**$\leq 10^9$**	**$2x10^{10}$**	**SuperMACS**

After placing LS column in the magnetic field of a MidiMACS^{TM} separator (Figure 4-5), the column was prepared by rinsing with 3mL of buffer.

Then, cell suspension was applied onto the column. Unlabelled cell fraction passed through the column into the collecting tube. Residual unlabelled cells were washed off with 3 consecutive washes (3 × 3 mL of buffer each) and total effluent was collected in a 15 mL Falcon tube. To prevent overflow, every new wash was performed after the column reservoir was empty. In order to elute the population of interest, the column was removed from the magnetic field and placed on a collection tube (15 mL Falcon tube). Then, cells were washed, flushed and collected in the flow-through fraction by adding 5 mL of buffer onto the column and using plunger to push cells through (Figure 4-4). Isolated B cells were harvested by centrifugation at 400 *x g* for 3 min. The purity of isolated B cells can be assessed by phenotypic analysis utilising flow cytometry technique described in Section 4.4.1.

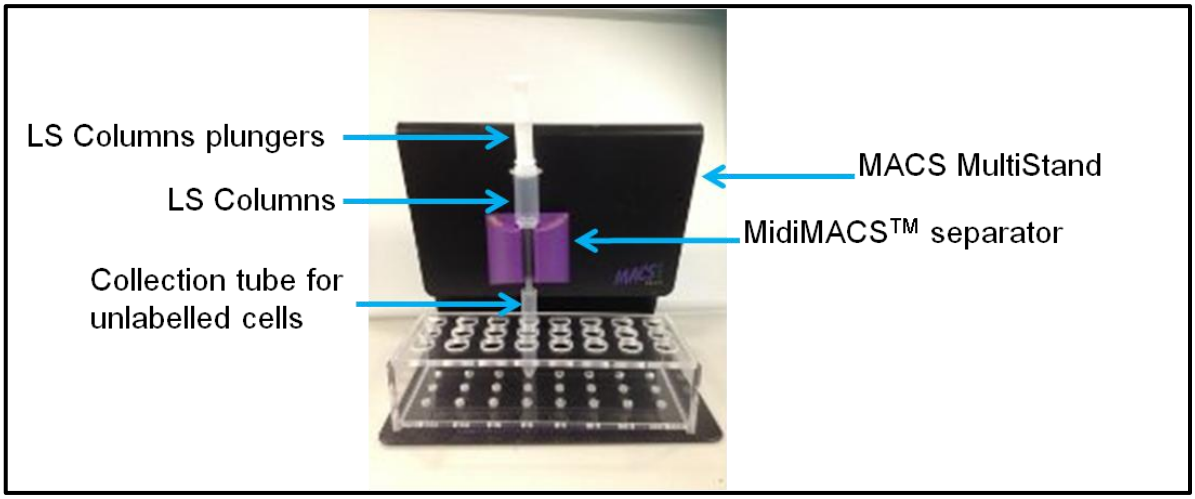


Figure 4-5 MidiMACS™ Separator Attached to a MultiStand, Holding an LS Column

The MidiMACS separator is positioned on a MACS MultiStand (MiltenyiBiotec, # 130-042-303) and holds an LS column. Magnetically labelled cells were added into the column. CD19+ cells were retained within the column and unlabelled cells were collected in the collection tube.

4.4.2 B-Lymphocytes Isolation utilising Fluorescence-Activated Cell Sorting (FACS) Technique

4.4.2.1 *Introduction to Fluorescence-Activated Cell Sorting*

Flow cytometry technique allows multi-parameter analysis of single cells, which is based upon the measurements of the fluorescence intensity generated by fluorescent-conjugated antibodies bound to a specific cell-associated antigen. FACS is a type of flow cytometry, which is used for separation of cell population of interest based upon the fluorescence emitted from stained cells (Figure 4-6).

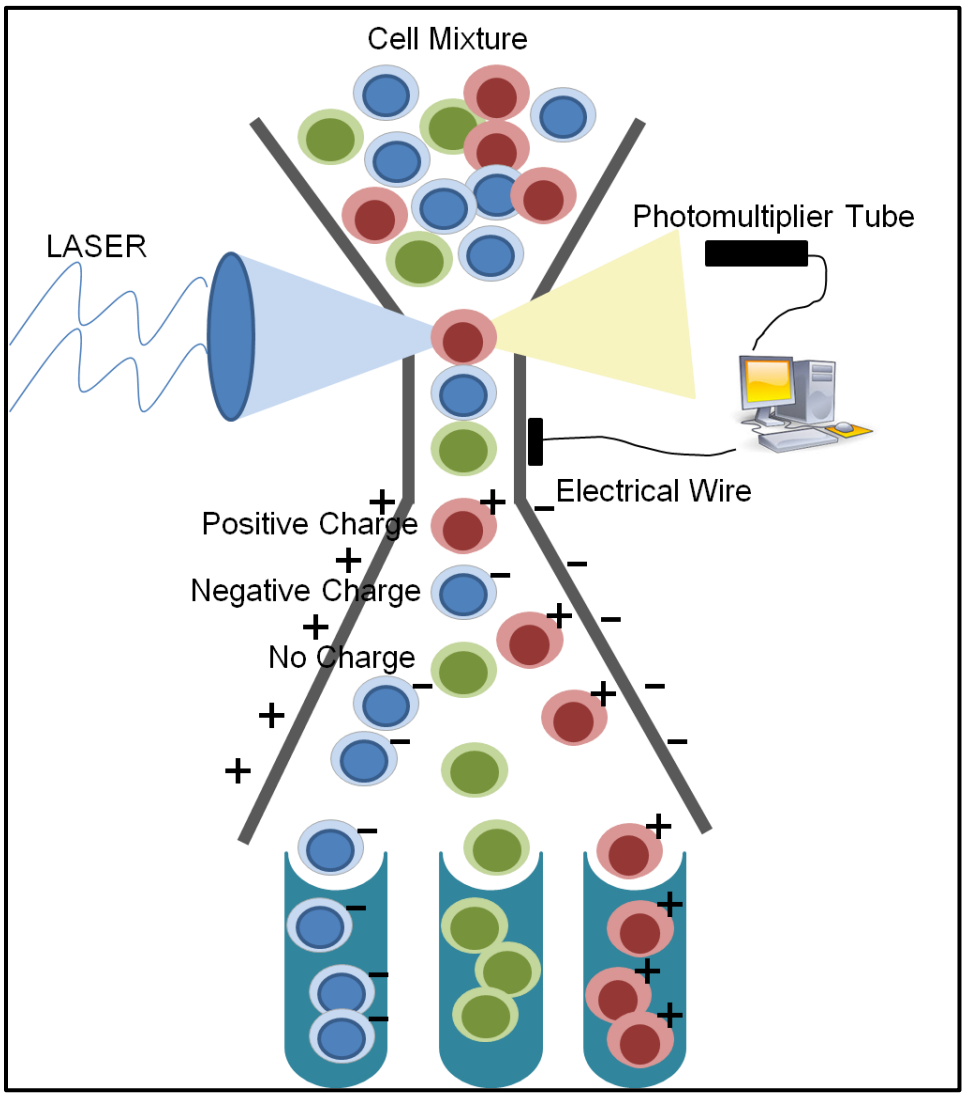


Figure 4-6 Diagram Explaining the Principles of FACS

After the cell suspension is applied into the center of the sheath stream of the flow cytometer, the fluidics system lines the cells up in the middle, in order to travel through as a single particle. After the cells of interest pass through the laser beam, they intercept and scatter the light. Light scattered in the forward direction (in line with the beam) referred as forward scatter (FSC) and is dependent on the cell size and viability. The side scatter (perpendicular to the beam) (SSC) is dependent on the content of the cell. An electrical charge is applied to the target cells at the level where the stream breaks into droplets. After moving through, the cells are directed based on their charge into the prescribed sample tubes. Image is adapted from (IGEM University of Aberdeen - ayeSwitch, 2010).

In the current project FACS was performed utilising BD FACSAria^{TM} II cell sorter (Becton-Dickinson Bioscience) and B cells were isolated according to the CD20 expression status.

4.4.2.2 *Sample Preparation*

4.4.2.2.1 From Peripheral Blood

PBMCs were extracted from peripheral blood samples by density-gradient centrifugation utilising Histopaque-1077 as described in Section 4.2.1.

4.4.2.2.2 From Cryopreserved Cells

The cryopreserved cells were removed from the minus 80 ºC freezer or liquid nitrogen and defrosted as outlined in Section 4.2.3. Then, 10 µl of cell suspension was removed for counting with a haemocytometer, cell viability was determined with trypan blue (Section 4.1.3).

4.4.2.3 *Antibody Incubation*

Cell number was adjusted to 1x10^7 cells per tube. The cell suspension was centrifuged at 400 *x g* for 10 min and the supernatant discarded. Pelleted cells were resuspended in 100 µl of PBS/BSA/Azide buffer (Appendix A) in appropriately labelled 5mL polystyrene tubes (BD Biosciences). Sample tubes were incubated: one with 10 µl of monoclonal CD20 antibody (1:11 dilution) (Miltenyi Biotec #130-091-109) conjugated to Phycoerythrin (PE) and other with corresponding concentration of anti-mouse IgG1 isotype control (BD Pharmingen, # 554680). Both sample tubes were incubated for 10 minutes in the dark at 2-8ºC. Following

incubation, the cells were washed by adding 1-2 mL of PBS and centrifuged at 400 *x g* for 10 min before supernatant was discarded. Finally, the cells were resuspended in 500 µl of PBS, mixed gently, filtered through a 35µm nylon mesh (BD Biosciences) and then immediately acquired and analysed on the BD FACSAria™ II (BD Biosciences, Oxford, UK) using the FACSDiva software (BD Biosciences).

4.4.2.4 *Fluorescence Activated Cell Sorter (FACS) AriaTM II Set Up and Operation*

4.4.2.4.1 Cytometer Startup

The BD FACSAria™ II was prepared for operation according to the Becton-Dickinson Reference Manual. Briefly, prior to switching the FACSAria™ II and computer, the fluid levels of the FACSFlow™ (BD Biosciences), ethanol (VWR International, Lutterworth, UK) were checked and replenished to required level. The waste container was emptied according to the standard laboratory regulations for biohazardous waste. An aseptic clean was undertaken and all surfaces were decontaminated by wiping down with ethanol wipes (Clinell). The FACSDiva software was opened and the fluidics start up procedure was performed following the on-screen instructions. The cytometer performance check was carried out by running the Cytometer Setup & Tracking beads (CS&T beads, BD Biosciences), which work with BD FACSDiva software to allow an integrated and consistent cytometer setup and performance. Four 5mL sorting collection tubes and a 70 micron nozzle were installed. Subsequent to turning the stream on, adjustments

were made to ensure that the stream was visible, centred and stabilised. Following the instructions from BD FACSAria II User's Guide, the drop pattern in the break off window was established, the amplitude range was calculated and the stream was adjusted so that the drop 1 value was between 200 and 300 pixels and the small satellite droplets joined the large droplets (Figure 4-7).

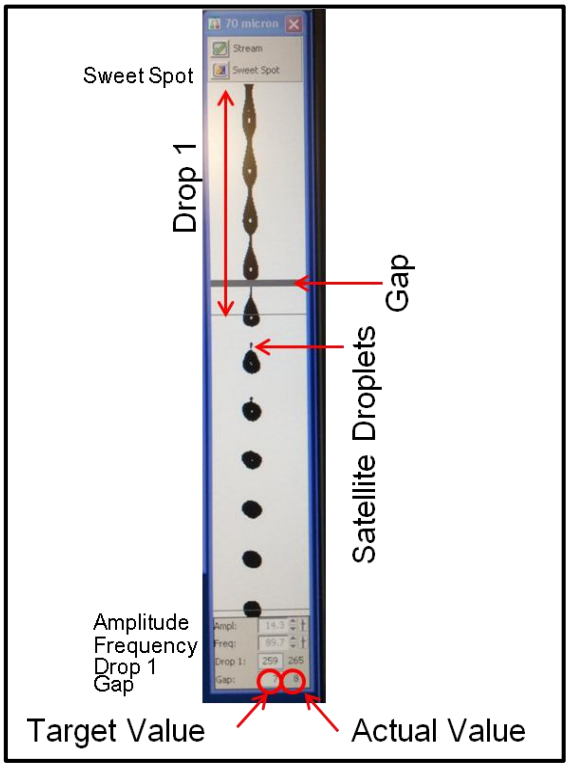


Figure 4-7 The Image of the Breakoff Window Controls

The upper camera transmits the image of the drop breakoff, which is transformed by the software and displayed in the Breakoff window as the numerical data. The drop brakoff has 2 main characteristics: ***Drop1*** *and* ***Gap****.* ***Drop 1*** *is the value representing the number of pixels from the top of the image to the line, which divides the first broken off drop.* ***Gap*** *is the value, which describes the number of pixels between the last attached droplet and the top of the first drop. The displayed Target Value is the pixel width of the gray line. The Actual Value as well as amplitude, frequency and drop1 fields are determined by user.*

After the drop patterns were completely settled and stable, the deflection plates were activated and the test sort function was checked by turning the voltage on. The camera position was altered to ensure that all four collection streams together with one waste stream were clearly visible. After that, the waste draw was opened to make certain that four collection streams were entering the four

collection tubes correctly and that the waste stream (positioned in the middle) was directed in to the waste.

Following this, BD FACS™ Accudrop Beads (BD Biosciences # 345249) were run to determine the optimal drop delay setting. The drop delay represents the travel time from the point where the particle is intercepted by the laser beam to the break-off point of the stream where it is charged and sorted; hence, this value was established and maintained in each sorting application. In short, the bead vial was vortexed briefly before use; 1 drop of beads was diluted in 0.5 mL of PBS and used as directed in the instrument user's guide.

4.4.2.4.2 Data Analysis and Sorting

The brief acquisition of the stained sample to be sorted was performed. Once the lymphocyte population had been identified, it was gated according to forward (FS) and side (SS) scatter of light (Figure 4-8, A). The voltages were adjusted to ensure that the target population was placed on scale. Amendments were made to acquire 10,000 events. Following this doublets were excluded using FSC-W (Figure 4-8, B) and SSC-W signals (Figure 4-8, C). Then finally, single cell population that fulfil the criteria was gated to include the cells positive for the expression of CD20 (Figure 4-8, D). A histogram was created so that the isotype control peak was fitted within the first segment of the grid.

The appropriately labelled sterile polypropylene 5 mL collection tubes (Sarstedt) were installed into the sorting collection device. The tubes were pre-coated with 0.2mL of the cell culture medium to ensure faster cell recovery. The sort layout was created to specify which gated population should be sorted into

each collection tube. Next, the yield and purity masks settings which determine how drops are charged when sorting conflicts takes place were appropriately selected. The Sweet Spot, which is designed to automatically maintain the sturdiness of the break off point, was turned on.

After ensuring that all of the above settings were completed, the sample to be sorted was loaded and the sort progress was monitored from the “Sort Layout” window. The settings were adjusted so the number of target events was set to “Continuous”. The number of events sorted into each sort location was displayed in the corresponding field. Finally, once the sorting was completed the isolated CD20 positive and negative cells can be subjected to phonotypic analysed using flow cytometry (Section 4.4.1) and subsequently stored in low sub-zero temperatures (at -80°C in a freezer or at -195.8°C in liquid nitrogen) or immediately utilised in downstream applications.

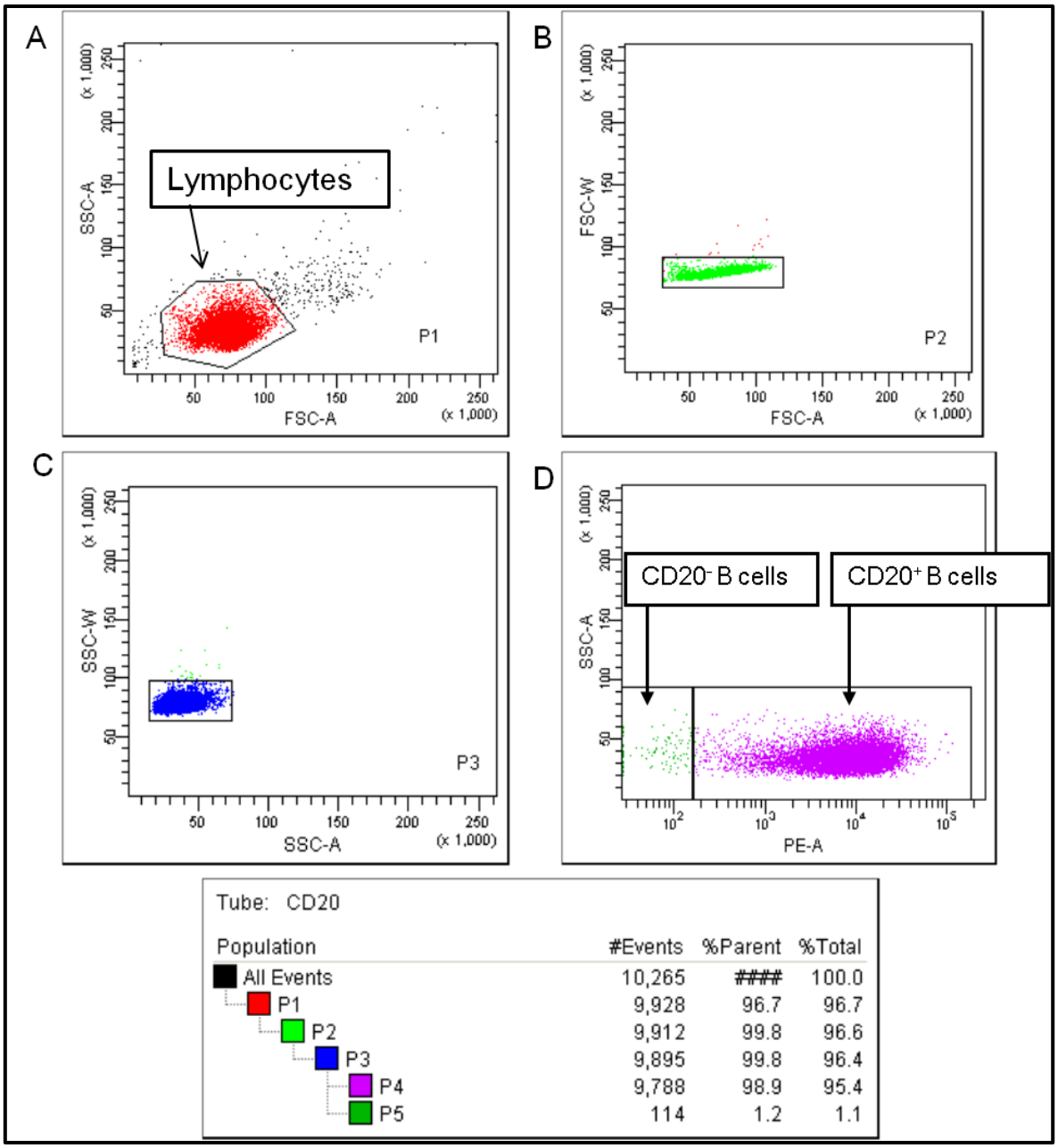


Figure 4-8 Example of Setting the Sort Gates During FACS Procedure

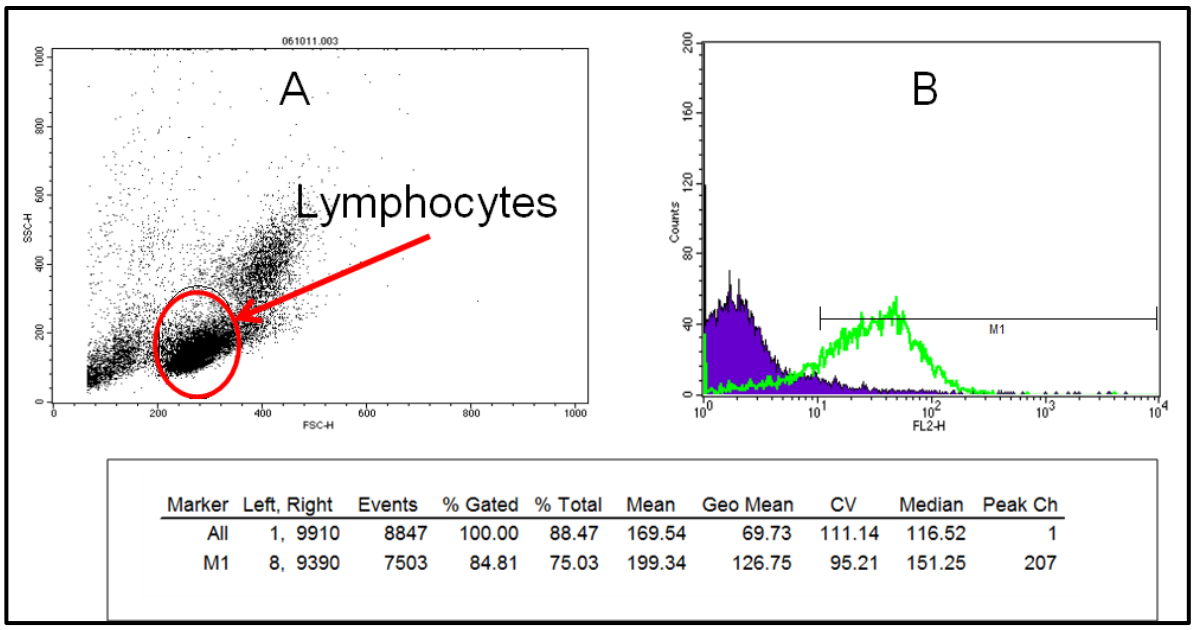
Dot plot used to isolate CD20 positive lymphocyte population in CLL sample 22. The cell population of interest was visualised by altering FSC & SSC voltage. Lymphocyte population was gated (Fig. A).The doublet discrimination was performed using FSC (Fig. B) and SSC channels (Fig. C), resulting in single cell population, which was assessed for CD20 expression using dot plot (Fig. D).

4.5 Protein Expression Assays

4.5.1 Phenotypic analysis of cell population utilising flow cytometry

4.5.1.1 *Flow Cytometry Extracellular Staining*

Cells isolated (Section 4.2.1) or thawed (Section 4.2.3) were counted, checked for viability and adjusted to $1x10^7$ cells/tube in 500µl of PBS/BSA/Azide buffer (Appendix A) before being filtered and aliquoted into appropriately labelled 5mL polystyrene tubes. Cells simultaneously were either incubated with primary antibody or corresponding concentration of a relevant isotype control. The concentration, time and condition of incubation for each used antibody are demonstrated in Table 4-3. Following this cells were washed twice by adding 1-2 mL of PBS/BSA/Azide buffer (Appendix A) and, then, cell suspension was centrifuged at 400 *x g* for 10 min before supernatant was discarded. When the primary antibody, which is not conjugated with a fluorochrome, was used, then a second incubation with a fluorochrome-conjugated secondary antibody was employed. Next, cells were washed twice with 1-2 mL of PBS/BSA/Azide buffer and harvested by centrifugation at 400 *x g* for 10 min. Finally, the cells were resuspended in 500 µl of PBS and immediately acquired and analysed on the FACSAriaTM II as outlined in Section 4.3.2.4.2. (Figure 4-9).The calculations were performed utilising the FACSDiva software and following instruction from BD FACSAria II User's Guide.



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8847	100.00	88.47	169.54	69.73	111.14	116.52	1
M1	8, 9390	7503	84.81	75.03	199.34	126.75	95.21	151.25	207

Figure 4-9 Gating the Positive Population

This figure is an example of phenotypic analysis of CD20 expression (a linage marker for B cells) in the CLL sample utilising flow cytometry. The population of interest was identified and gated on the dot plot (A). Gated cell population had the scatter properties of lymphocytes, whereas the cell population to the left is presumed to be the cellular fragments or dead cells and the cell population to the right is suggested to be the other mononuclear cells. The histogram (B) was created using the gated population (A). A marker was created on the isotype control histogram which enclosed ≤1% of the cells, which would subsequently be used to identify the positive populations in other samples. The histogram demonstrated that 88.47% of total cells are CD20 positive cells population.

4.5.1.2 *Flow Cytometry Intracellular Staining*

For detection of intracellular antigens, an intracellular staining protocol was employed. The cells isolated from peripheral blood were harvested by centrifugation as per Section 4.2.1 or defrosted as per Section 4.2.3 before being counted with trypan blue exclusion and adjusted to $1x10^6$ cells per tube. Following this, cells were fixed with 2% paraformaldehyde (Sigma Aldrich) (Appendix A) in 100 µl volume and incubated at room temperature for 10 min. The cells were then washed once with 1 mL of BSA/PBS blocking buffer (Appendix A) and 0.5 mL of FACS permeabilisation solution (BD FACS™ Permeabilizing Solution 2, # 340973) was added to each tube. The cell suspension was incubated at room temperature for 10 min before being washed once with 1 mL of blocking buffer. Cells were then incubated in 500 µl of blocking buffer for 30 min at room temperature. From this point forward the staining protocol described in Section 4.4.1.1 was followed.

Table 4-3 Table of Antibodies Utilised in Immunofluorescence Staining Application

Antibody	Usage	Dilution	Incubation Time
CD20 antibody (Miltenyi Biotec, # 130-091-109)	Primary PE-conjugated antibody	1:11	10 min, at 2-8 º C, protected from the light
Anti-mouse IgG1 (BD Pharmingen, # 554680)	Isotype control	1:11	10 min, at 2-8 º C, protected from the light
Anti-BDKRB2 (Abcam, # 134118)	Primary Rabbit monoclonal antibody	1:170	30 min, at RT, protected from the light
Rabbit IgG- ChIP Grade	Isotype control	1:170	30 min, at RT, protected from the light
Goat Anti-Rabbit IgG (Abcam, # 97068)	Secondary FITC-pre-absorbed antibody	1:170	30 min, at RT, protected from the light

4.5.2 In Vitro Stimulation of the B Cell Receptor

In order to mimic antigen-associated binding behaviour *in vitro*, BCR was stimulated by cross-linking with an anti-IgM antibody (Haas *et al*.2000, Allsup 2005), which has been shown to elicit prolonged activation of downstream intracellular signalling (Krysov *et al.*, 2012).

Raji cells and lymphocytes from CLL patients were taken from the freezer/liquid nitrogen, thawed following the protocol described in Section 4.1.1. Cell viability was assessed under the microscope by means of 0.4% trypan blue staining. Raji cells were counted as per Section 4.1.3 and directly subjected to the

downstream application, whereas CLL samples were transferred into a T25 or T75 flask and incubated in culture media for 3 hours at 37^0C with 5% CO_2 for acclimatisation. After allowing for full acclimatisation, cells were counted and adjusted to the required concentration. Approximate amount of $5X10^5$cells/mL of Raji cells and/or $5X10^6$cells/mL of CLL cells was re-suspended in culture media. The lymphocytes are much smaller in size thus a higher amount of cells was utilised. Each experiment included four flasks, which are:

- **BCR Stimulant**

  The BCR activation was achievable by incubating the cells with the antibody raised against the BCR complex itself using specific Goat anti-human IgM, Fc5μ specific fragment (#109-006-129, Jackson ImmunoResearch) antibody, which was added to the cell suspension at a final concentration of 10μg/mL. The antibody reacts specifically with Fc5μ portion of human IgM heavy chain avoiding reaction with IgA or IgG or light chain of Ig molecule.

- **Isotype Control**

  An isotype control was included. A use of Goat anti-human IgG $F(ab')_2$ fragments specific antibody (#109-006-006, Jackson ImmunoResearch) (final concentration 10μg/mL), which reacts with $F(ab')_2$ portion of human IgG and also with light chain of other Ig(s) ensures that the binding observed is not due to Fc receptors.

- **Positive Control**

  A positive control was attained by incubation with Phorbol 12-Miristate-13-Acetate (PMA) (#P1585, Sigma) at a final concentration of 100 nM. PMA synergistically enhances protein kinase C (PKC) activation and thus induces widespread intracellular phosphorylation.

- **Untreated Cells**

  Untreated Cells or Negative Control included cells without any treatment.

All four T75 flasks, each containing 5 mL of cell suspension were placed in a humidified environment at 37ºC for 5.5 hours. After each hour all four flasks were gently agitated to ensure that cell suspension would not clump and sediment to the bottom of the flask as this may reduce exposure to the stimulant. The preliminary work optimising the appropriate incubation time (carried out by Dr. Gina Eagle) investigated several time points, including 10 min, 1, 5.5, 6, 8 and 10-12 hours. The cells were observed under a microscope and stained with trypan blue to assess cell viability. ERK expression was examined after each time point utilising Western blotting to ensure that ERK was phosphorylated and the ERK pathway was activated. The experiments have shown that media was exhausted after 6hrs of stimulation and cell viability started to deteriorate. At 5.5 hrs cells were viable and the media retained its original colour.

Following this, the cell mixture was aspirated, transferred into a universal tube and centrifuged at 400 *x g* for 3 min. The pelleted cells were washed with 1 mL ice-cold PBS prior to proceeding to the downstream application.

4.5.3 Western Blotting

Western blotting also known as Immunoblotting is a widely utilised laboratory technique for detection of known proteins from the complex protein mixture according to their molecular weight with subsequent transfer on the nitrocellulose membrane before identification with monoclonal/polyclonal antibodies (Figure 4-10). The technique was first created in the laboratory of George Stark in 1979 in Stanford (Renart *et al*., 1979), modified by Harry Towbin (Towbin *et al*., 1979) and ratified in 1981 by Neal Burnette (Burnette, 1981). The ability of Western blot to analyse variable protein samples along with recombinant proteins synthesised in vitro has made this method one of the most powerful research techniques.

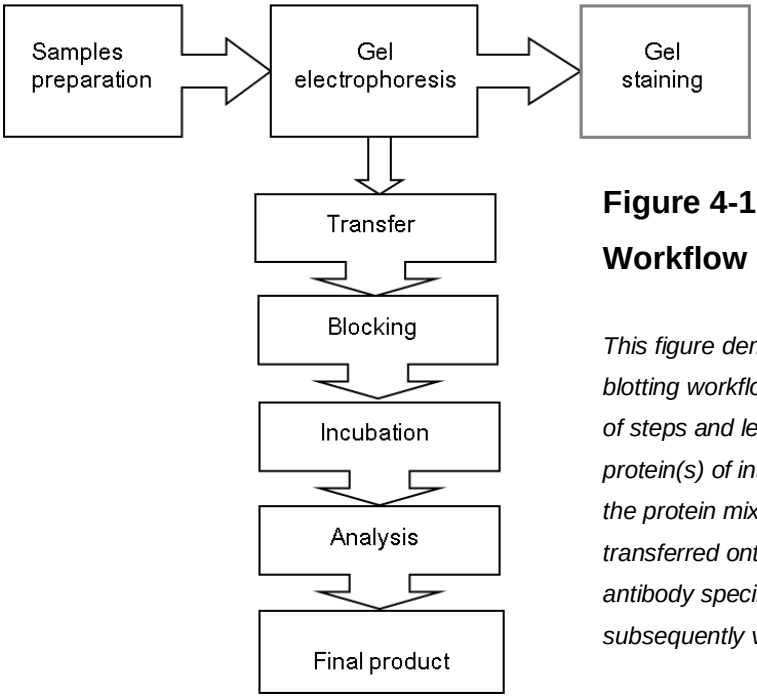


Figure 4-10 Western Blotting Workflow

This figure demonstrates the summary of western blotting workflow. Western blotting involves a number of steps and leads to the immunological detection of protein(s) of interest that have been separated from the protein mixture by gel electrophoresis, transferred onto a membrane, incubated with an antibody specific to the target protein and subsequently visualised.

4.5.3.1 *The Protein Extraction and Sample Preparation for Western Blotting*

The proteins from B lymphocytes and Raji cells were extracted in the same manner as follows. Cultured Raji cells with confluence ≥70-80% (Section 4.1.2), freshly isolated (Section 4.2.1) or post-thawed (Section 4.2.3) lymphocytes were harvested by centrifugation at 400 *x g* for 3 minutes before supernatant was discarded. The pelleted cells were re-suspended in 5mL of cold PBS and centrifuged at 400 *x g* for 3 minutes; this action was twice repeated, although, during the second time the cells were re-suspended in 1mL of cold PBS and transferred into microcentrifuge tubes. Next, the microcentrifuge tubes were spun at 400 *x g* for 3 minutes and inverted onto a paper towel to ensure a complete removal of any remaining culture medium.

Lysis buffer was prepared by mixing of 10 µl of fresh Phosphatase Inhibitor Cocktail 1 (#P2850, Sigma Aldrich), Phosphatase Inhibitor Cocktail 2 (#P5726, Sigma Aldrich) and Protease Inhibitor (#80-6501-23, Amersham Biosciences) together with 50 µl of 2-Mercaptoethanol (#M-7522, Sigma Aldrich) to every mL of Western blot (WB) extraction buffer (Appendix A). All ingredients were added to ensure quality protein purification without damaging of the target protein's integrity and activity. The pellet was then re-suspended in 250µl of Lysis buffer (Appendix A) and thoroughly mixed by vortexing for 5 min. The samples were pre-treated with 1µl of dilute Benzonase nuclease, which was prepared by mixing 1µl of Benzonase nuclease (#E8263 Sigma) and 9 µl of WB extraction buffer. Benzonase nuclease reduces the extract viscosity by digesting nucleic acids while having no proteolytic effect. The mixture was then placed on an end-over-end rotator overnight at 4^0C to

achieve a maximum protein lysis. Following this, the whole cell lysate was centrifuged at 10,000 *x g* for 15 min at 4^0C to remove insoluble cell debris. The supernatant was transferred into a fresh pre-chilled microcentrifuge tube and advanced to protein quantification step. The extracted proteins stored at −80°C until further analysis.

4.5.3.2 *Protein Quantification for Western Blotting*

The quantification of total protein concentration in solution was performed utilising the Reducing Agent Compatible Detergent Compatible Assay Kit (RCDC) (#500-0119 to -0122, Bio-Rad). This kit is a colorimetric-based assay, which offers an accurate and sensitive quantification of proteins as well as compatibility with components used in this experiment. Briefly, five dilution series of bovine serum albumin (BSA) ranging from 0.25mg/mL to 1.5 mg/mL were mixed according to the manufacturer's instructions. The protein mixture was diluted to 1:2, 1:5 and 1:10 dilutions to ensure that the determination of unknown will fall within the standard curve, which leads to a more accurate result.

Initially, 125 µl of Reducing Agent (RC) Reagent I was added to each microcentrifuge tube, then vortexed immediately and incubated for 1 minute. Following this, 125 µl of RC Reagent II was added to each mixture and vortexed. In order to precipitate the protein out of the solution, the samples were centrifuged at 15,000 x *g* for 5 min at room temperature. The supernatant was discarded and the microcentrifuge tubes were inverted on clean absorbent paper to achieve the maximum moisture removal. In the meantime, 'Working Reagent A' was prepared by mixing of 20 µl of Reagent S with every 1 mL of Reagent A. Then, 127 µl of the

prepared solution was added to each tube, vortexed for 5 min to enhance protein re-constitution. After this, 1 mL of Reagent B was added to each sample with immediate thorough mixing and incubation at room temperature for 15 min. After the incubation 200 µl of each standards and samples to be determined were transferred to a 96-well plate. The samples' absorbance was measured at 690 nm using a Multiscan plate reader (Labsystems). The total protein concentration of each sample was determined by mathematical calculation using a standard equation in Microsoft Excel 2006 (Figure 4-11).

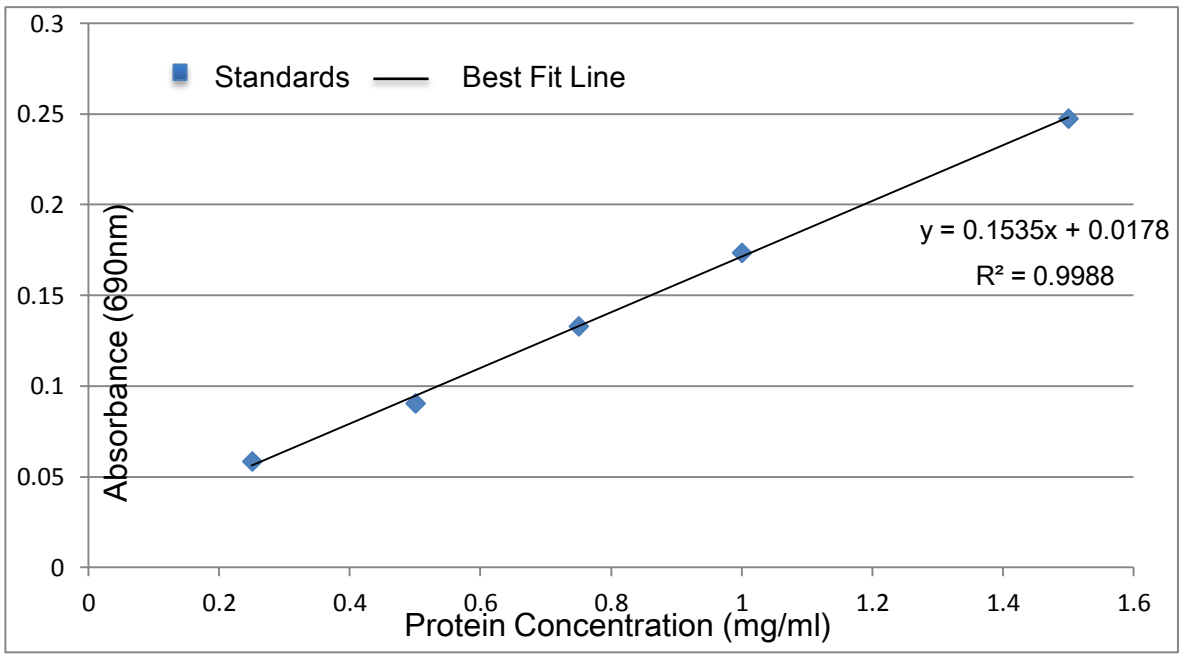


Figure 4-11 Example of a Standard Curve Using RCDC Assay Kit

It is essential to generate a standard curve with each experiment in order to maintain quality protein quantification. Any determined unknown that fell outside of the standard curve was declared invalid; it was necessary to make some alterations to the sample dilution before repeating the quantification. Regression analysis of the best fit line was employed and the protein concentration was calculated utilising an equation y=mx+c.

4.5.3.3 *One-dimensional gel electrophoresis*

The protein samples were prepared in 0.5mL eppendorf by dilution with WB extraction working buffer (Appendix A) containing 50 µl of 2-Mercaptoethanol (14.3M stock, #M-7522, Sigma Aldrich). WB extraction buffer contained Tris-HCl buffer; SDS, denatures proteins and confers a negative charge; glycerol retains the sample at the bottom of the wells and facilitates even gel loading; bromophenol blue makes visualisation of the proteins migration possible; beta-mercaptoethanol, which denatures proteins. The protein concentration was adjusted to 20 µg of protein lysate in 25µl of final loading volume. The samples were vortexed to ensure satisfactory mixing and heated to 95^0C in a thermocycler (Techne) for 5 min to facilitate protein denaturation. The immediate transfer of the heated samples into ice restricts the reversal of unfolded proteins back to their native conformation. After being on ice for at least 30 sec the samples were vortexed again and centrifuged at maximum speed of 12,000 *x g* 30 sec. During the intervening time the vertical dual electrophoresis tank was prepared with Tris-HEPES-SDS running buffer (#28368, Pierce Chemical) (Appendix A) and a cassette with commercially obtained 12 % Precise Protein Gel (#25222, Thermo Scientific) in 12-well formats was inserted. The gel was fully submerged in the running buffer. The protein samples were loaded into the gel in prescribed order. The empty wells were filled with 20 µl of Western blot working solution. In order to determine the protein molecular weight and also to enable monitoring of the electrophoretic run progress, 10 µl of Precision Plus Protein Western C Marker (#161-0376, Bio-Rad) (molecular weight range from 10 to 250 kDa) was loaded onto the gel alongside the samples.

After successful electrophoresis (Figure 4-12) the gel was carefully removed from the cassette. At this point the gel can be stained or prepared for protein transfer onto nitrocellulose membrane.

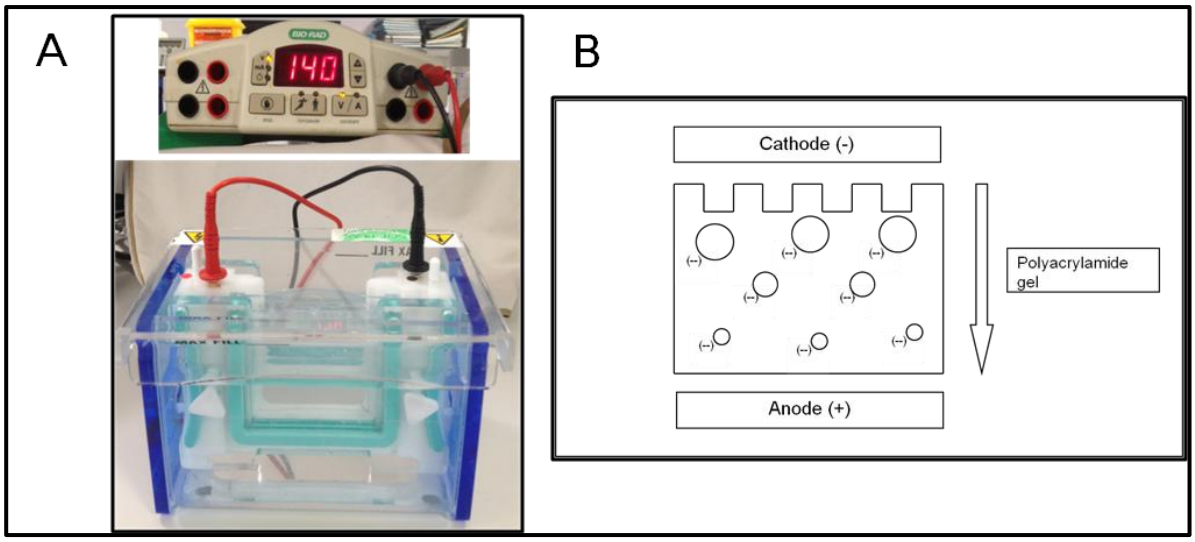


Figure 4-12 One-Dimensional Gel Electrophoresis

The running tank (A) can run up to two gels in commercially pre-formed cassettes, which are fully submerged into the running buffer (Appendix A). One Dimensional gel electrophoresis runs at a constant voltage of 140V from an electrophoretic power supply (Bio Rad) for 40-50 min at room temperature. The graph (B) explains how one-dimensional gel electrophoresis works: the proteins are migrating through the gel from cathode to anode at a rate determined by their size. Larger proteins migrate slowly, whereas smaller proteins move faster.

4.5.3.4 *Staining Protein Gels with Coomassie Dye*

For routine visualisation of proteins separated by gel electrophoresis Coomassie blue dye (Bio Rad, # 161-0787) staining method was utilised in the current study. After completion of one dimension electrophoresis the gel was removed from the casing and subsequently washed 3 times (5 min per wash) in nalgene staining pot in distilled water on an orbital shaker. The water was drained and the 20-30 mL of Coomassie stain was added into the pot to ensure that the gel

was fully submerged into the solution, followed by gentle agitation on the orbital shaker for 60 min. The gel was de-stained in 10 mL of distilled water, overnight on an orbital shaker. The gel was scanned using the setting options for gel scanning on GS800 calibrated densitometer with Quantity One software (Bio-Rad Laboratories) (Figure 4-13) and analysed.

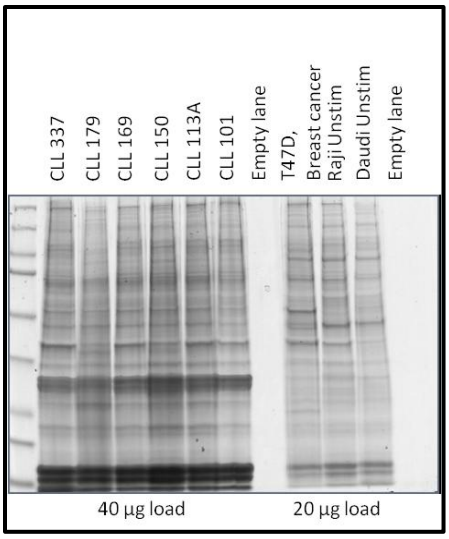


Figure 4-13 Example of Coomassie Blue Staining Showing Different Protein Load (20µg and 40 µg)

The total protein amounts of 40 µg and 20 µg per well along with molecular weight marker were loaded, electrophoresed and subsequently stained with Coomassie blue dye. The experiment aimed to confirm an equal protein load as well as establish the optimal protein load.

4.5.3.5 *Transfer of Proteins onto Nitrocellulose Membrane*

In order to allow for proteins to be accessible for antibodies, they were transferred from the polyacrylamide gel to a nitrocellulose membrane using the iBlot® 7-Minute Blotting System (Invitrogen) and following the manufacturers' instructions (Figure 4-14). The iBlot device consists of iBlot™ Gel Transfer Device and the commercially purchased disposable iBlot Gel Transfer Stack (#IB3010-01, Invitrogen) (Figure 4-15).

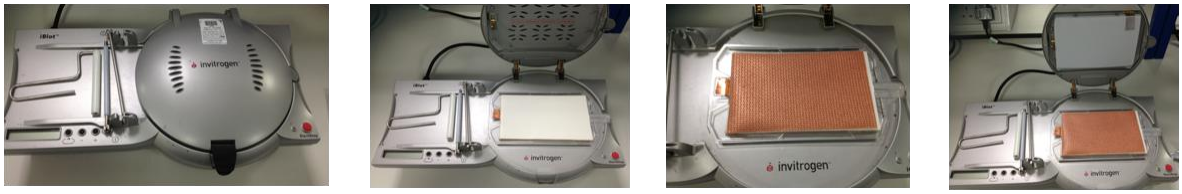


Figure 4-14 The iBlot Gel to Membrane Transfer Process

Initially, the bottom stack, containing the membrane was placed on the unit. The gel was then taken out of the case, carefully handled and positioned on the top of the membrane, so the marker was aligned with the left hand side. Next, the pre-soaked in distilled water filter paper was positioned on the top of the gel and any air bubbles were removed with the roller to ensure a good and uniform contact with the gel. Finally, the top stack and the sponge were placed. An appropriate 7 min pre-programmed cycles was selected, after which the membrane was accurately removed before the remaining layers were discarded.

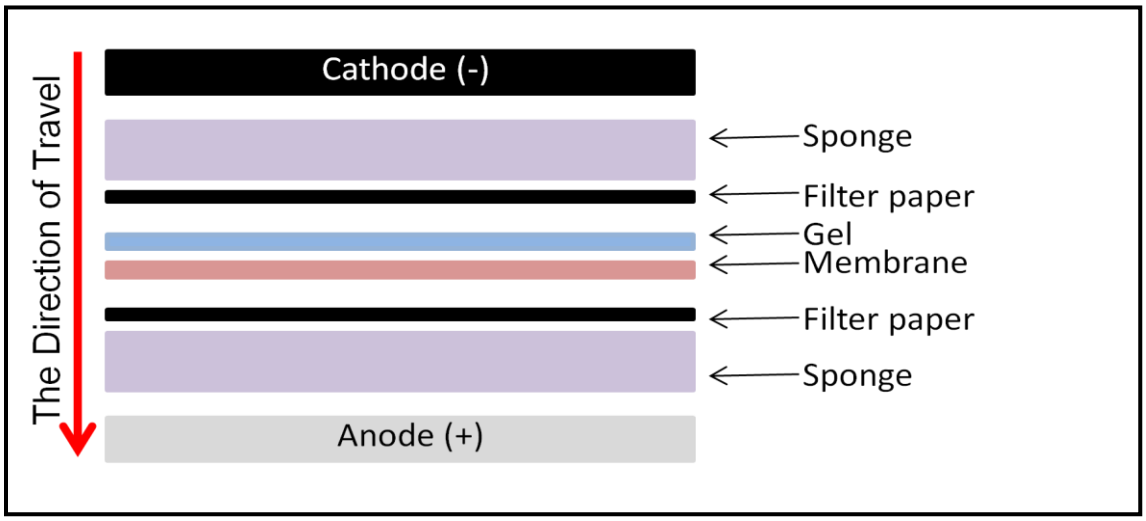


Figure 4-15 Diagram of iBlot Dry Transfer System Demonstrating the Direction of Current

Each iBlot™ Gel Transfer Stack consists of the Top and Bottom stacks, the disposable sponge and a filter paper. The gel is positioned in between the Bottom stack and filter paper followed by the Top stack. The Bottom stack includes anode and a nitrocellulose membrane, which are placed in a transparent plastic tray. The Top stack incorporates cathode and completes the sandwich. After electrical charge applied, the proteins are induced to migrate from the gel onto membrane and the red arrow indicates the direction of travel.

4.5.3.6 *Visualisation of Proteins on Membranes Utilising Ponceau Red Staining*

Ponceau stain is a fast and reversible staining method for protein visualisation on nitrocellulose membrane. Once the transfer was successfully completed the membrane was submerged into Ponceau S solution (Sigma, # P3504) for 5-10 min with gentle agitation. Light-red bands appeared on the blot, which were representative of the protein abundance (Figure 4-16). The stain can be effectively removed from the blot by continued washing.

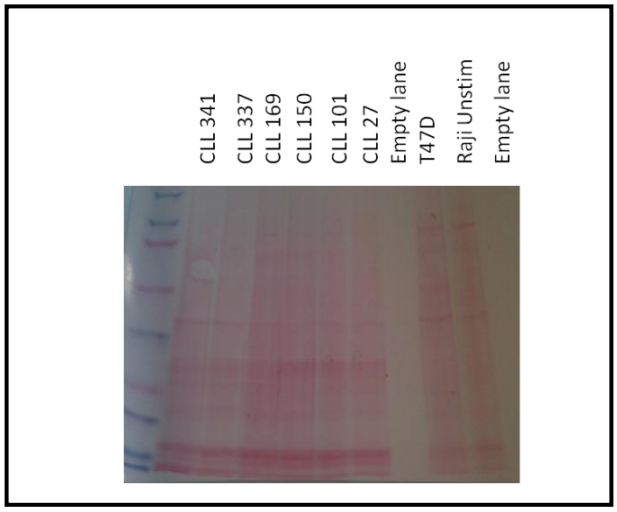


Figure 4-16 Example of Ponceau Red Staining

Ponceau Red is a sodium salt of a diazo dye, which is widely used for rapid reversible detection of protein bands on nitrocellulose or PVDFmembrane. The molecular weight marker-lane 1, CLL samples lysate -lane 2-7, cell lines lysate-lane 9-10.

4.5.3.7 *Blocking of binding sites on the membrane*

In order to counteract the non-specific antibody interactions, it is necessary to "block" the membrane with the blocking solution. There are several classic proteins used to block western blot membranes. Bovine serum albumin (BSA) is commonly used as a blocking agent in western blotting, however in this study 5 % non-fat milk powder (Marvel), diluted in TBS-Tween20 was used as blocking agents. The membrane was incubated in 5 % non-fat milk in 20 mL Nalgene

staining box on an orbital shaker for 1 hour at room temperature or overnight at 4^0C. Satisfactory blocking of the membrane diminishes unspecific binding and false positives, resulting in a clearer outcome.

4.5.3.8 *Immunoblotting*

Immunoblotting technique uses antibodies to identify the protein of interest among heterogeneous mixture of proteins. This technique was performed in two steps: first, an antigen-specific primary antibody was optimised and used to bind to the target-protein; second, the conjugated secondary antibody was utilised to cross-link with a species-specific portion of the primary antibody (Figure 4-17).

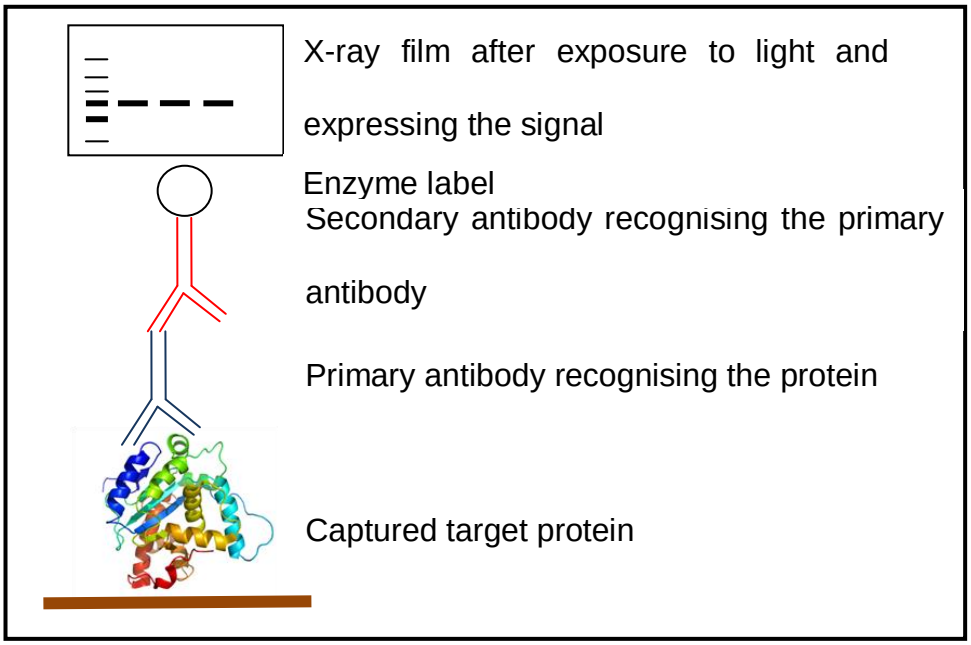


Figure 4-17 The Key Steps of Immunoblotting

The primary antibody can be monoclonal or polyclonal. Monoclonal antibodies interact with a single epitope on protein of interest with higher specificity of detection. Polyclonal antibodies recognise multiple epitopes. The secondary antibody is directed against the species of the primary antibody and usually carries a label for detection. Enzyme label is used with HRP antibody.

Following the blocking step, the membrane was incubated with the primary antibody diluted in 10mL of 5% non-fat milk. All utilised antibodies were subjected to preliminary optimisation work prior to the experiment. The membrane was incubated with diluted antibody and continuous gentle shaking according to Table 4-4. Afterwards the membrane was washed 3 times with TBS-Tween20 for 5min on an orbital shaker at room temperature to ensure the complete removal of unbound antibody.

Following probing with the primary antibody the membrane was incubated with a HRP-conjugated secondary antibody specific to the species the primary antibody was raised to such as anti-rabbit (#Sc2031) or an anti-mouse (#Sc 2030). The antibody was diluted to its optimised concentration (1:1000) in 5% non-fat milk and incubated for 1 hour at room temperature on an orbital shaker. One µl of Precision Protein StrepTactin-HRP conjugate (#161-0381, Bio-Rad) was also added to the secondary antibody aiding visualisation of the Precision Plus Protein Western C Standard molecular weight marker. Three washes of 5 min with TBS-Tween20 followed before proceeding to chemiluminescent detection and radiographic exposure.

4.5.3.9 *Loading Controls*

Loading control is important for validation of the result and necessary for confirmation of equal sample loading, particularly when a differential protein expression is compared between the alternative samples. Loading control is also known as ‘housekeeping proteins’.

Table 4-4 Optimised Primary Antibodies Used for Immunoblotting in the Current Project

Antibody	Company, Catalogue #	Clonality	Host species	Dilution	Temperature	Incubation time
ά-Tubulin (Loading control)	Abcam # -7291	Monoclonal	Mouse	1:2500-1:5000	RT	2 hours
β-Actin (Loading control)	Abcam # -8227	Polyclonal	Rabbit	1:2500	RT	2 hours
GAPDH (Loading control)	Abcam # -9485	Polyclonal	Rabbit	1:1000	RT	2 hours
HMWK	Abcam # -1004	Monoclonal	Mouse	1:200/1:300	4°C	overnight
LMWK	Abcam # -79650	Polyclonal	Rabbit	1:200/1:300	4°C	overnight
ERK	Santa Cruz # -153	Polyclonal	Rabbit	1:1000	RT	2 hours
ERK (C-14)	Santa Cruz # -154	Polyclonal	Rabbit	1:1000	RT	2 hours
p ERK	Santa Cruz # -7383	Monoclonal	Mouse	1:100	RT	2 hours
HMWK	Abcam # -1005	Monoclonal	Mouse	Not optimised	Not optimised	Not optimised
HMWK	Abcam # -79653	Polyclonal	Rabbit	Not optimised	Not optimised	Not optimised
Anti kininogen 1	Abcam # -97761	Polyclonal	Rabbit	Not optimised	Not optimised	Not optimised
Anti-Kallikrein 6 antibody- Kallikrein loop	Abcam # -28301	Polyclonal	Rabbit	1:500	RT	3 hours
Anti B_1 receptor antibody	Abcam # -75148	Polyclonal	Rabbit	1:500	RT	3 hours
Anti -B_2 receptor antibody	Abcam # -73625)	Polyclonal	Rabbit	1:500	RT	3 hours

4.5.3.10 *Detection of Protein of Interest*

The signal received on the blot corresponds to the amount of protein that the antibody attached to and the image is captured on photographic film. In order to detect the proteins the membrane was incubated with equal amounts Supersignal West Pico Stable Peroxide Solution and Supersignal West Pico Luminol Enhncer Solution from the Supersignal West Pico Chemiluminescent Substrate Kit (#34078, Thermo Scientific) for 5 min with restricted light and frequent gentle manual agitation. Afterwards the membrane was placed between transparent sheets in an intensifying cassette with CL-XPosure Film (#34090, Thermo Scientific) for 10 sec to 30 min depending on the strength of the signal. The film advances through the development process; firstly submerged into 250 mL of GBX Developer (#P7042, Sigma Aldrich) followed by gentle manual agitation until the signal becomes visible , secondly submerged in 250 mL 5 % Acetic Acid for 30 sec and finally into 250 mL GBX Fixer (#P7167, Sigma Aldrich) for 30 sec. The developed film was air-dried before analysed by densitometry.

4.5.3.11 *Densitometry*

GS800 Calibrated Densitometer (Bio-Rad) and Quantity One software (Bio-Rad) were utilised to quantify protein bands from scanner output. The photographic film was scanned and the bands on the image were detected in accordance with software instructions. The western blot band density was measured and normalised against the housekeeping protein. The obtained values were used to

calculate differential expression of protein of interest in used samples (Figure 4-18).

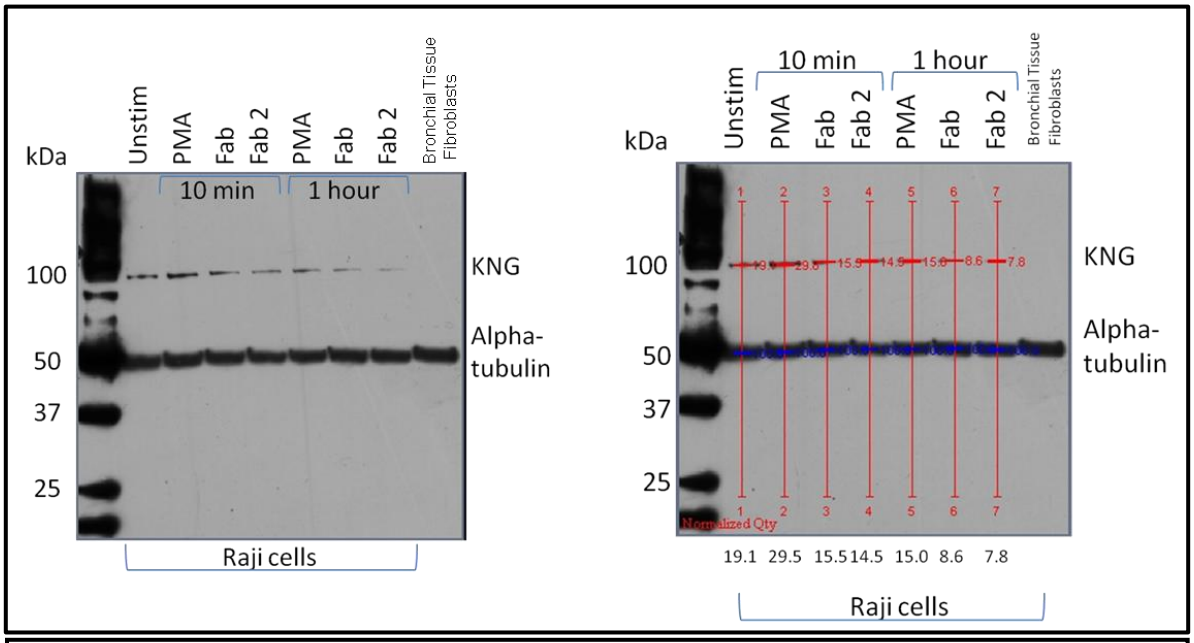


Figure 4-18 Example of Band Detection and Densitometry Analysis

The left hand image is a scanned western blot film. The detected upper bands are representative of a signal generated by Kininogen protein, whilst the lower bands are referred as loading control, α-tubulin. The vertical lanes were manually edited in the Quantity One software (BioRad) and then bands (horizontal lanes) were detected automatically. The calculations against the density of loading control were made by software. The differential expression (1.85 fold downregulation) of Kininogen in Raji cells can be observed after 60min in comparison to 10 min of stimulation (fab 2 (10 min) vs. fab 2 (1 hour).

4.5.3.12 *Membrane Stripping*

Membrane stripping is used for robust but careful removal of primary and secondary antibodies from a western blot membrane which was submitted to chemiluminescent substrates; thus this provides the opportunity to identify more than one protein on the same blot by re-probing with different antibody. The

stripping was performed with Restore Plus Western Blot Stripping buffer (Thermo Scientific #46430). If the membrane stripping is planned in advance, the probing for the low-abundant proteins is recommended first, before subsequent re-probing for higher molecular weight proteins. The membrane can be stored in PBS at 4ºC prior to the stripping procedure for up to 24 hours. Three washes (5 min each) with TBS-Tween 20 (Appendix A) are necessary for complete chemiluminescent substrate removal. The membrane was submerged in 20 mL of Restore Plus Western Blot Stripping buffer and incubated for 15 min at room temperature with constant gentle agitation on the orbital shaker. After that the buffer was discarded and 3 washes (5 min each) with TBS-Tween20 were performed. The blot was blocked with 20 mL of 5% non fat milk for 30-60 min at room temperature or overnight at 4 ºC. In order to test for total removal of the primary antibody , the membrane was incubated with the secondary anti-mouse or anti-rabbit antibodies (dependent upon the mammal origin of primary antibody) for 1 hour at room temperature, washed and incubated in fresh chemiluminescent substrate working agent for 5 min and exposed to radiographic film (see Section 2.8.10). The successful removal of primary antibody results in no signal on the film after 5-10 min of exposure. Once the membrane is completely stripped it was suitable for the immunoprobing against another antigen.

4.5.4 The Enzyme-Linked Immunosorbent Assay (ELISA)

4.5.4.1 *Introduction to ELISA Principles*

ELISA is a plate-based immunoassay, which is designed for detecting as well as quantifying the presence of the protein of interest (antigen) in the sample

with unknown protein abundance. Since 1971, when ELISA was first discovered by Peter Perlmann and Eva Engvall and Anton Schuurs and Bauke van Weemen (Engvall and Perlmann, 1971, Van Weemen and Schuurs, 1971), this technique has been widely utilised in research and diagnostic settings. The prime advantages of ELISA application: are the quantifiable result and high-throughput screening. ELISA methodology can be carried out in four different formats such as direct, indirect, sandwich and competitive or also known as inhibition ELISAs, which incorporated several modifications from the standard procedure. The key step of ELISA is the immobilisation of the target protein on the multiwell plate. This is achieved by pre-coating the plate with a sample containing the target protein or by target-specific capture by the antibody, which is directly pre-absorbed to the plate. The immobilised antigen is then detected directly with the primary enzyme-labelled antibody (Direct ELISA) or indirectly where antigen is crosslinked with unlabelled primary antibody before it is incubated with the enzyme-conjugated anti-species secondary antibody, which is bound to primary antibody (Indirect ELISA). The sandwich ELISA usually is employed for detection of low abundant protein of interest and involves the measurement of the target protein utilising matched antibody pairs (the capture antibody and the detection antibody), which are specific for a different non-overlapping antigenic sites.

Competitive Bradykinin ELISA (Enzo Life Sciences, # ADI-900-206) which cross reacts with Bradykinin, Lys-Bradykinin, 9-Bradykinin and 1-5 stable degradation products (Chapter 3) was utilised in the current project. The assay procedure was performed following the manufacturer's protocols (Figure 4-19).

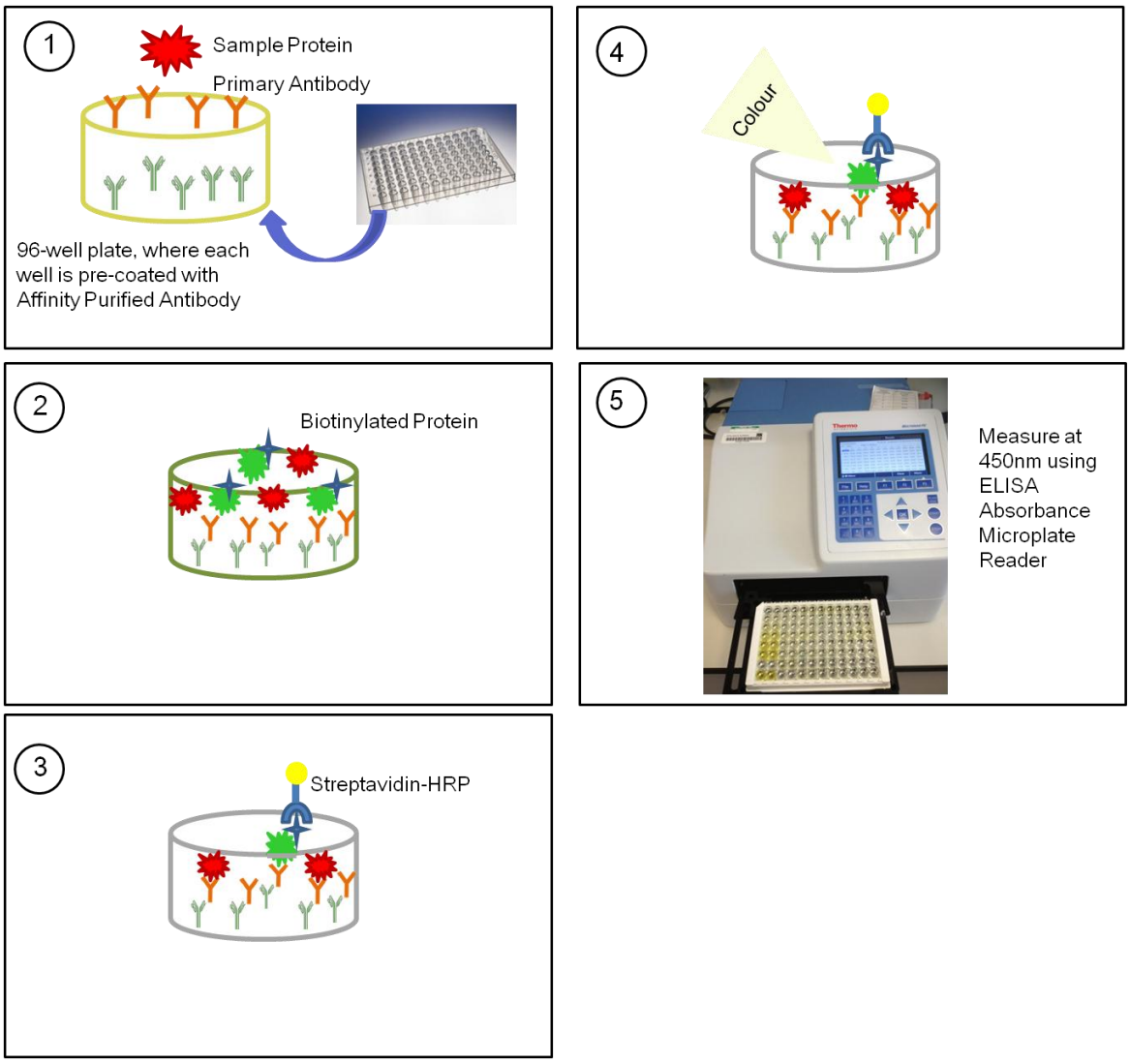


Figure 4-19 Principle of the Comparative Bradykinin ELISA Application

This is a schematic illustration of competitive Bradykinin ELISA application stages. Initially, standards and samples were added to the wells pre-coated with IgG antibody. Then, BK conjugated to biotin was applied, followed by primary antibody to BK (1). During incubation biotin-conjugated BK competes with BK, which is present in the samples, for binding to the capture antibody (2). After wash all unbound BK was removed and HRP conjugated streptavidin was added to bind the biotinylated BK (3). Following wash tetramethylbenzidine (TMB) was applied to generate a colour in the solution (4). Stop solution was added and the light absorbance of the final coloured solution is then measured on an ELISA Absorbance Microplate Reader (5). A decreased signal from purified BK confirms the presence of the BK antigen in the tested samples when compared to labelled BK antigen alone. Image adapted from (Enzo Life Sciences, 2014b)

4.5.4.2 *Sample Preparation and Handling*

Bradykinin level was measured in 36 plasma samples obtained from unselected CLL patients attending routine CLL clinic appointments in Hull and East Yorkshire Hospitals during November and December 2011. These samples were previously extracted by MSc student Paula Johnson (Appendix D).

4.5.4.3 *Bradykinin Standards Preparation*

Six assay-specific standard solutions were prepared by a series of dilutions to generate a six point concentration range following manufacturer's instructions (Appendix D) (Table 4-5).

Table 4-5 Concentration of Recombinant Bradykinin Used in ELISA

Standard N°	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
BK Concentration	30,000 pg/mL	3,000 pg/mL	750 pg/mL	187.5 pg/mL	46.9 pg/mL	11.7 pg/mL

4.5.4.4 *Assay Procedure*

Assay procedure was carried out following the recommended manufacturer's protocol. In brief, the 96-well plate was prepared and the sample layout was established before 150 µl of the assay buffer was added into the non-specific binding (NSB) well, 100 µl into 0 ng/mL standard well (B_0) and Blank well.

After this, 100 µl of Standards 1 through 6 were carefully applied to the bottom of the prescribed well. Then 50 µl of the conjugated Bradykinin was pipetted into each well except the blank and 50 µl of the primary antibody was added to each well with exception of the Blank and NSB wells. The plate was sealed and incubated for 2 hours on a plate shaker at RT. Following incubation the contents of the well were removed and plate was washed four times with 400 µl/well of wash buffer (supplied in the kit and prepared according to the manufacturer's protocol) using Wellwash 4 Mk 2 plate washer (Thermo Labsystems) (Figure 4-20).

Figure 4-20 Thermo Labsystems Wellwash 4 MK 2 Microplate Washer for 96-Well Plates Performing Washes

After the final wash, the plate was firmly tapped and inverted onto the lint free paper towel to ensure that any remaining fluid was removed. HRP-conjugated streptavidin (supplied in the kit and prepared according to the manufacturer's

protocol) was added in the amount of 200 µl to each well except the Blank before the plate was sealed and incubated for 30 min on a plate shaker at RT. The washing step was performed as above and 200 µl of the substrate solution was added into each well including the Blank. The solution was incubated for 30 min at RT without shaking. Finally, 50 µl of the stop solution was added to each well and multiwell plate (Figure 4-21) was inserted into Multiskan FC Absorbance Plate Reader (Thermo Labsystems) and absorbance was measured at 450nm.

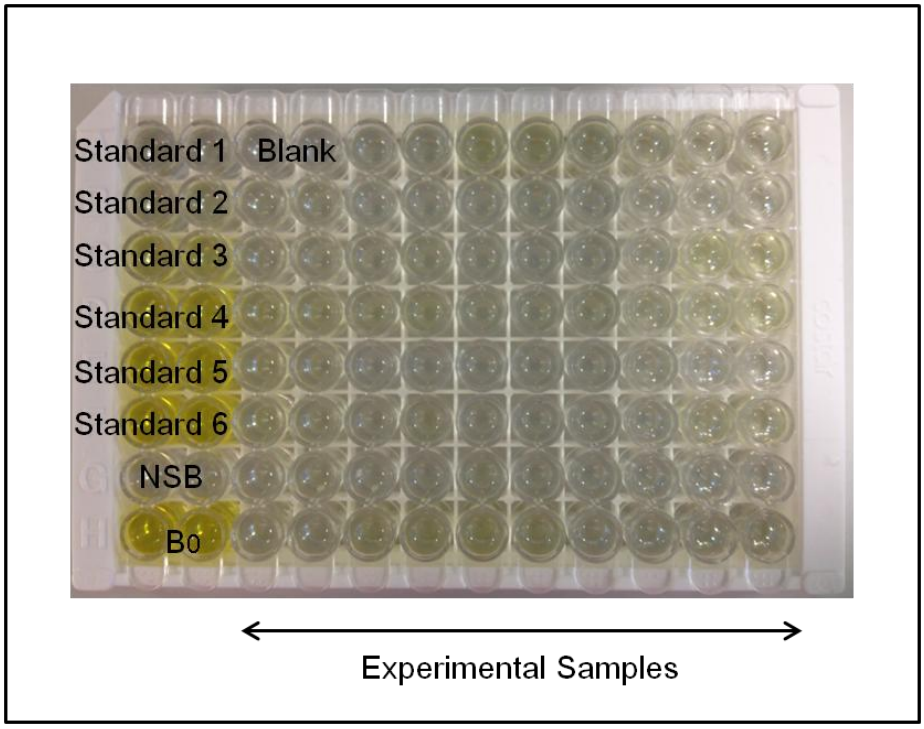


Figure 4-21 Completed Bradykinin ELISA Plate Prepared for Optical Density Reading

The BK ELISA application based upon the competitive binding of unlabeled BK from the samples and the labelled BK to the capture antibody. Thus, the greater the signal output from purified BK, the lower the concentration of the BK in the samples and vice versa. The optical density of the end-product is measured utilising a spectrophotometer.

4.5.4.5 *Result Calculations*

A quantitative measurement of Bradykinin concentrations was calculated using 4 parameter logistic (4PL) curve fitting program, Assay Blaster (Enzo Life Sciences, # ADI-28-0002), following the instructions from the software.

4.6 RNA Expression Assays

4.6.1 Standard Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is an *in vitro* laboratory technique which is named after a key component of the reaction – a deoxyribonucleic acid (DNA) polymerase. PCR relies on thermal cycling (comprised of repeated heating and cooling cycles, designed to regulate the thermo stable DNA polymerase activity and the primers binding), primers sequence and deoxynucleotide triphosphates (dNTPs). PCR leads to amplification of a particular segment of *DNA* sequence by extending an annealed primer using nucleotides, and this results in generation of millions of copies (up to ~10 kilo base pairs (kb)) referred as amplicons. In the current project PCR was employed for Kininogen RNA expression study.

4.6.1.1 *The key Stages of Polymerase Chain Reaction*

The PCR reaction involves three subsequent stages: denaturation, annealling and extension, which are repeated for many cycles, leading to the ***exponential amplification*** of the template DNA (Figure 4-22). In the first step, known as ***denaturation,*** the double-stranded gene region to be amplified is selected and subjected to a high temperature (94–98 °C) in a process called DNA

melting, which leads to the separation of the two strands of the DNA double helix. During the second step, named ***annealing*** (Figure4-21), the temperature is decreased (48–72 °C) and the two separated DNA strands form the DNA templates. This stage benefits from the use of primers. Primers or also known as oligonucleotides are short, single-stranded pieces of *DNA* that are synthesised *in vitro* and complimentary to the DNA sequence targeted for amplification. A primer set is required for standard PCR in order to enhance both strands, thus a primer for one strand, called the forward primer or sense, which is the beginning of target gene, and other primer called the reverse primer or antisense will begin the complementary strand (in the 5' end). The reduced temperature allows for primers to anneal to the single-stranded DNA template. Then, the heat- stable polymerase binds to the primer-template hybrid enabling the amplification reaction to begin. During the ***extension*** or also known as elongation step (Figure 4-21), the temperature and time are calculated depending on the type of DNA polymerase utilised in the experiment and the size of the gene sequence to be amplified. Taq polymerase, which is widely employed in the standard PCRs, requires temperature to be increased to 72°C, which is an optimal condition for synthesis of a new DNA strand complementary to the DNA template using building blocks such as dNTP. During the extension step the complementary 5'-phosphate end of the dNTPs is attached to the 3'-hydroxyl group of the DNA template in 5' to 3' direction. This process continues until no more amplicons are generated due to diminished polymerase activity or exhaustion of dNTPs and primers, which brings the reaction rate to a plateau.

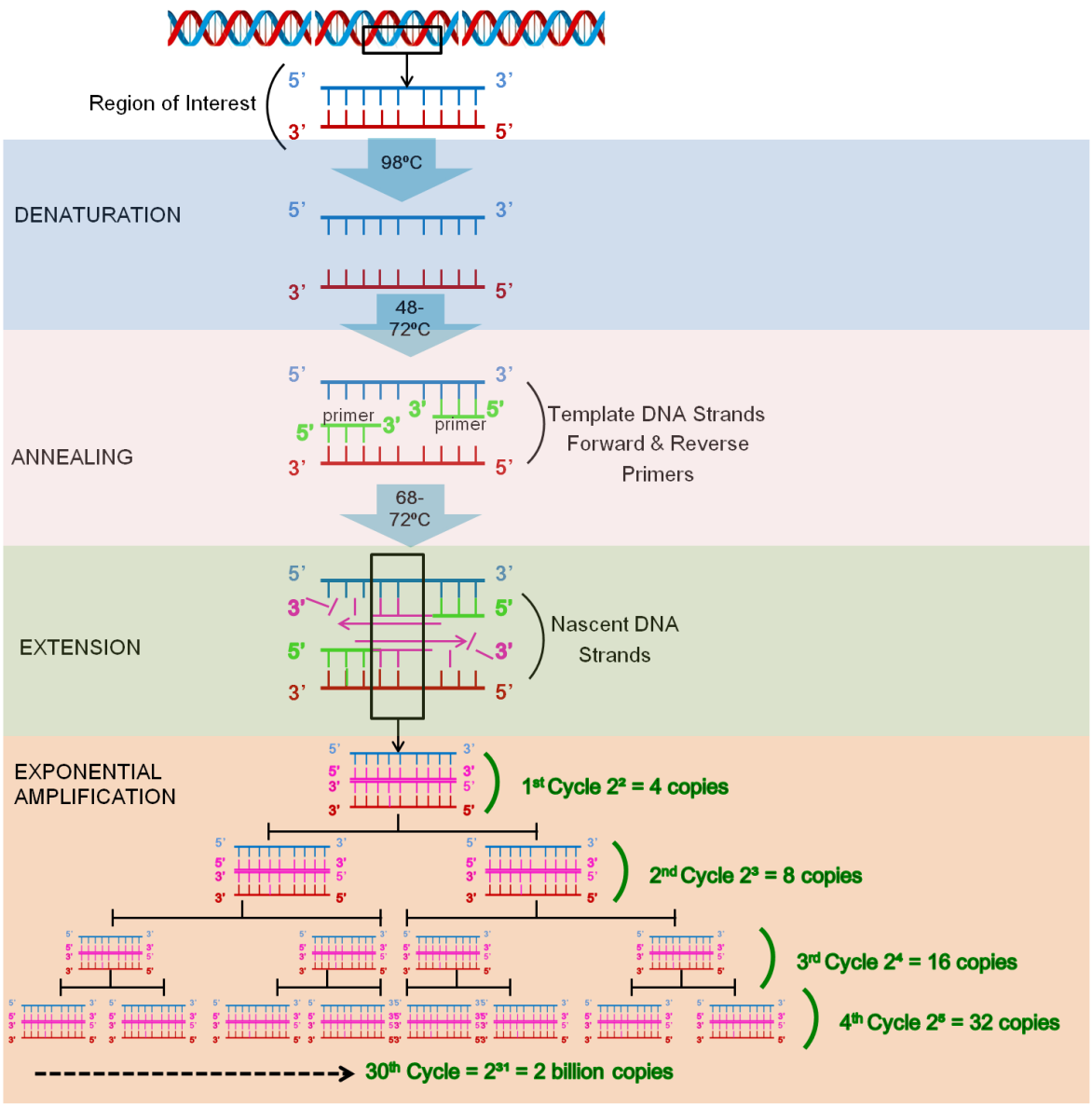


Figure 4-22 Schematic Presentation of the PCR Cycle

During a PCR, the changes in temperature are used to regulate the activity of the DNA polymerase enzyme and primers binding. Three steps: denaturation, annealing and extension will be repeated for many cycles and lead to the exponential amplification of the region of interest until all the reagents and enzyme will be fully consumed and/or polymerase loses its activity. At the end of the PCR, the gene sequence of interest in a mixed DNA sample will be amplified and accumulated in the billions of copies. This Figure was adapted from (New England Biolabs).

4.6.1.2 *Sample Preparation*

All Reverse Transcription Polymerase Chain Reaction work was performed with help of Dr. Laura R. Sadofsky and Dr. Kevin Morgan (Respiratory Medicine, CCMR and University of Hull). The fresh leukocytes isolated from peripheral blood were spun down as per Section 4.2.1 or defrosted as per Section 4.2.3. Cultured Raji, T47D, A549 and LNCaP cells were taken from the freezer/liquid nitrogen, defrosted as per Section 4.1.1 and cultured as per Section 4.1.2. Cells were counted with trypan blue exclusion. Cell number was adjusted to up to $1x10^6$ cells per tube.

4.6.1.3 *Total RNA Isolation*

Total cell RNA was extracted using a NucleoSpin RNA II (Macherey-Nigel, Fisher Scientific, # NZ74095550) extraction kit following manufacturer's instructions. The standard protocol enables isolation of up to 70 µg of total RNA per NucleoSpin RNA II column. Prior to commencing the experiment the wash buffer and DNase were prepared according to the user manual. In brief, wash buffer was prepared by adding 20 mL of 96-100% ethanol to 5 mL of buffer RA3 concentrate (provided). In order to prepare DNase, the lyophilized rDNase (RNase-free) was reconstituted with 230 µl RNase-free H_2O, mixed to ensure that RNase was completely dissolved and incubated for 1 min at RT. Then solution was aliquoted and stored at -20 º C for up to 6 months. Harvested cells were lysed by adding 350 µl of RA1 buffer (supplied) and 3, 5 µl of ß-mercaptoethanol (# M6250, Sigma Aldrich) directly to the cell pellet. In order to get a clear lysate and reduced

viscosity, cells were filtered through the NucleoSpin Filter (violet ring) into 2 mL collection tube and spun at 11,000 *x g* for 1 min before NucleoSpin Filter was discarded. Next, the RNA binding conditions were adjusted by adding 350 µl of 70 % ethanol to the homogenised lysate and mixed by reverse pipetting. Then, RNA binding was performed; for this the NucleoSpin RNA II column with light blue ring was placed in the collection tube (one column per preparation) The cell lysate was gently mixed by pipetting up and down (2-3 times), loaded into the column and spun at 11,000 *x g* for 30 sec. The column, then, was placed into new collection tube and silica membrane was desalted by adding 350 µl of the Membrane Desalting Buffer (MDB) and centrifuged at 11,000 *x g* for 1 min. In order to digest DNA, a DNase reaction mixture was prepared by adding 10 µl of reconstituted rDNase to 90 µl of reaction buffer for rDNase and mixed in a sterile 1.5 microcentrifuge tube. Then, 95 µl of nascent DNase reaction mixture was added directly onto the middle of the silica membrane of the column before it was incubated at RT for 15 min. Following the incubation the washing steps were carried out initially by adding 200 µl of Buffer RA2 to the NucleoSpin RNA II column and centrifuged at 11,000 *x g* for 30 sec. Then, the column was transferred into a new collection tube and the second wash was performed by applying 600 µl of Buffer RA3 and centrifugation at 11,000 *x g* for 30 sec. After that, the flow-through fraction was discarded and the column was placed back into collection tube. Third and final wash was achieved by adding 600 µl of Buffer RA3to the NucleoSpin RNA II column, spinning at 11,000 *x g* for 2 min to ensure that membrane is dried out fully. The column, then, was placed onto 1.5 mL nuclease-free collection tube

(supplied) and RNA was eluted in 60 µl RNase-free H_2O (supplied) by being centrifuged at 11,000 *x g* for 1 min.

4.6.1.4 *RNA quantification*

The total RNA was measured utilising Quant-iT ™ RNA Assay Kit (Invitrogen) with the Qubit®Fluorometer (Invitrogen) according to instruction manual. All the reagents were equilibrated to RT, mixed well and protected from light as much as possible In brief, the dye working solution was prepared in the plastic tube by adding 200 µl of buffer (Quant-iT™ RNA buffer (Component B)) and 1 µl of dye (Quant-iT™ RNA reagent (Component A)) (both supplied) for every sample and mixed by vortexing. Then, 190 µl of nascent working solution was aliquoted into two thin-walls, clear 0.5 mL PCR tubes (VWR, #10011-830) and labelled as Standard #1 and Standard #2. After this, 10 µl of each standard were added to an assay tube and mixed by vortexing. Each unknown sample to be quantified was prepared by adding 198 µl of working solution and 2 µl of RNA sample into thin-wall, clear 0.5 mL PCR tubes and mixed by vortexing. The tubes were incubated at RT for 2 min. Following the incubation, the Qubit®Fluorometer was calibrated using Standard #1 and Standard #2. The unknown RNA samples were measured by inserting into in the Qubit®Fluorometer. The readout from the Qubit®Fluorometer was multiplied by the value given by the dilution factor to determine the concentration of the original sample. If the concentration was too high, some alterations to the sample dilution were made before repeating the quantification.

4.6.1.5 *First-strand cDNA synthesis*

Following quantification, two micrograms of the DNase-treated total *RNA* were reverse transcribed into a first-strand c*DNA* prior to conventional PCR amplification, using RevertAid Premium Reverse Transcriptase (Thermo Scientific # EP0731) and following the protocol optimised for use in RT-PCR with the final volume of 14.5 µl.

All preparations were carried out on ice using sterile, nuclease-free tubes (1.7 mL, SmartTube, # CR-23-2052). All the components such as total RNA, Oligo $(dT)_{18}$ Primer, dNTP mix and DEPC-treated H_2O were added into each tube in the order as indicated in the Table 4-5 and mixed gently. Following this, the samples to be reverse transcribed were placed into PCR machine (Techne® TC-3000 thermal cycler), which was set to incubate the samples for 30 min at 50 ºC, after which the reaction was terminated by heating the samples at 85 ºC for 5 min. Once the program was completed, the reverse transcription reaction products were held at 4ºC and directly used in PCR or stored at -20 ºC (for up to one week) or -80 ºC (for longer storage).

Table 4-6 The Reaction Components for First Strand cDNA Synthesis

Reaction components	Amount
Template RNA (total RNA)	A volume containing 2 µg of total RNA
Oligo(dT)$_{18}$ Primer (Fermentas, #S0131)	1µl
dNTP mix (Fermentas, #R0191)	1µl
DEPC-treated H_2O(Fermentas, #R0601)	To bring the total volume up to the final 14.5 µl
	Total = 14.5 µl

Oligo(dT)18 Primer is a single-stranded 18-mer oligonucleotide with 5'-and 3' hydroxyl tails synthesised for use as a primer for first strand cDNA synthesis.
dNTP (Deoxynucleotide) mix is a premixed solution, which consists of four nucleotides dATP, dCTP, dGTP and dTTP.
DEPC-treated H_2O, which is therefore RNase-free H_2O reduces the risk of RNA degradation by RNases.

4.6.2 Polymerase Chain Reaction and Agarose Gel Electrophoresis

4.6.2.1 *Primer Selection*

Primers or oligonucleotides are synthetic, short, single-stranded pieces of DNA, usually 18-40 base pair (bp) (sometimes longer), that determine the product sequence for replication. A primer set is required for standard PCR in order to amplify both strand of the target, thus a primer for one strand, called the forward primer or sense at the (5' end) beginning of the target, and other primer called the

reverse primer or antisense will begin the complementary strand (in the 3' end). Accurate primer design or selection from a number of existing candidate primer sets is the first, and often the most challenging step in performing PCR with specific amplification and high yield. Moreover, poor primer design or selection results in generation of false PCR products. Primers must be designed to be specific to the target DNA; however, the sequence must be complex enough to enable a very low possibility of annealing to sequences other than the target DNA. Several key steps required to be taken into consideration when primers are designed or selected from a number of candidate primer sets (Appendix C). The sequence of primer sets utilised in the current project can be viewed in Table 4-7.

Table 4-7 Primers Used in PCR Amplifications to Study Gene Expression in Peripheral Blood Samples Obtained from CLL Patients

Oligo name	GenBank accession numbers	Primer	Sequence (5' -> 3')	Length	Location within mRNA sequence
KNG (homo sapiens)	NM_000893	Forward primer	*TGACCGAAAGCTG TGAGACC*	20	1007-1026
KNG (homo sapiens)	NM_000893	Reverse primer	*GTACTCGCAGGAC CTTAGGTG*	21	1227-1207
GAPDH (homo sapiens) "Housekeeping"	NM_002046	Forward primer	*AGCCGAGCCACAT CGCT*	17	150-166
GAPDH (homo sapiens) "Housekeeping"	NM_002046	Reverse primer	*TGGCAACAATATC CACTTTACCAGAG T*	27	245-271
GUSB (homo sapiens) "Housekeeping"	NM_000181	Forward primer	*GAAAATACGTGGT TGGAGAGCTCATT*	26	1864-1889
GUSB (homo sapiens) "Housekeeping"	NM_000181	Reverse primer	*CCGAGTGAAGATC CCCTTTTTA*	22	1943-1964
β-Actin (homo sapiens) "Housekeeping"	NM_001101.3	Reverse primer	*CGTGGGCCGCCCT AGGCACCA*	21	186-207
β-Actin (homo sapiens) "Housekeeping"	NM_001101.3	Reverse primer	*TTGGCCTTAGGGT TCAGGGGG*	21	407- 428
TRPM8 (homo sapiens)	NM_024080.4	Reverse primer	*TCAGCACGCTTGT GTACCGGA*	21	1542- 1563
TRPM8* (homo sapiens)	NM_024080.4	Reverse primer	*TTGCCTGGCCACG CCAAAGG*	20	2659- 2679

** TRPM8 -human transient receptor potential cation channel subfamily M member 8*

4.6.2.2 *PCR technique*

All amplifications were carried out in 50 µL reaction mixture containing 1 µL (138 ng) of c*DNA*, 38.75 µL of Diethylpyrocarbonate (DEPC)-treated water, 5 µL of ThermoPol reaction buffer (NewEngland Biolabs # B9004S), 1 µL of dNTP mix (Thermo Scientific, Fermentas), 1 µL of each forward and reverse primers (Table 6.1), 0.25 µL of *TaqDNA* Polymerase (Thermo Scientific, Fermentas) and including 2 µL of 50 mM $MgCl_2$ (which was determined to be optimal for generation of PCR product resulting in a final $MgCl_2$ concentration of 3mM). All ingredients were added into PCR tubes, mixed gently and placed in the PCR machine. The cycling conditions were programmed as indicated in the Table 4-8.

Table 4-8 The Cycling Conditions programmed for PCR

Cycles	Step	Temperature (°C)	Time
1	Initial Denaturation	94	3 min
35	Denaturing	94	15 sec
	Annealing	57	30 sec
	Extension	72	60 sec
1	Final Elongation/Extension	72	10 min
1	Final Hold	4	∞

Once the program was terminated, the PCR products were subjected to agarose gel electrophoresis. Two % agarose gel was prepared by mixing 4 g of agarose powder (Invitrogen, #15510-019) with 200 mL of TAE running buffer (Appendix A) and then heated in a microwave oven until it was completely melted. A 2% gel efficiently resolves size differences in the 50-500 bp range. After the solution was cooled down to 60 ºC, 8 µL of ethidium bromide (10mg/mL) (Sigma Aldrich, #E8751) was added and mixed gently to facilitate visualisation of the band after electrophoresis. The solution was mixed to ensure the uniform dispersion of the ethidium bromide molecules throughout the matrix and the solution was poured into a casting tray containing a sample comb and was left at RT until it was solidified. After the gel had solidified, the comb was carefully removed and the gel was horizontally submerged into TAE running buffer (Appendix A) in the electrophoresis chamber. While gel was solidifying, the samples were prepared by gently mixing 20 µL of each PCR reaction and 5 µL of loading (Appendix A) buffer. Then, samples were pipetted into the sample wells along with 100 bp DNA ladder (Invitrogen, #15628-019) (Figure 4-23). The lid and power leads were replaced and a current of 110 V for 50 min was applied. During electrophoresis the amplicons along with DNA ladder migrated towards the positive electrode and the position on the gel was determined by the amplicon's size. The pictures of the fluorescing fragments were captured using EpiChemi™ II Darkroom - UVP machine and LabWork software 4.0 following user's manual.

Figure 4-23 The Agarose Gel Inserted into Electrophoresis Chamber.

The percentage of agarose gel influences the size of DNA fragments that can be resolved. Greater concentrations of agarose promote separation of small DNAs, whereas low agarose concentrations facilitate resolution of larger DNA fragments. Once agarose gel is inserted into electrophoretic chamber and fully covered by running buffer, the samples along with the marker were loaded in the prescribed order.

4.7 Statistical Analysis

Survival analysis was performed with the help of Dr James Bailey (CBPG, University of Hull) using SPSS for Windows (v19, SPSS inc.) The survival analysis and time from diagnosis to first treatment was evaluated by Kaplan-Meier (KM) curves and the log-rank test. Bivariate analysis was performed utilising Fisher exact test and regression analysis in SPSS for Windows (v19, SPSS inc.). All *P* values were 2-sided and $p \leq 0.05$ was defined as statistically significant.

Receiver operated curve (ROC) analysis was performed with the help of Dr Rachel Crossland (Research Associate, Haematological Sciences at Newcastle University) using Sigma Plot (version 11). Overall survival values were estimated using Kaplan–Meier analysis with comparison between curves made using Mantel–Cox log-rank analysis. Correlations between data were studied using Pearson's correlation for continuous variables and Pearson Chi-square analysis for categorical variables.

CHAPTER 5

Result of Kininogen Protein Expression Analysis in CLL Study Cohort

KASHUBA E; EAGLE G;BAILEY G; EVANS P; WELHAM K; ALLSUP D; CAWKWELL L., *2013. Proteomic analysis of B-cell receptor signaling in chronic lymphocytic leukaemia reveals a possible role for kininogen. Journal of Proteomics, 18, 279-96.*

Chapter 5. Result of Kininogen Protein Expression Analysis in CLL Study Cohort

5.1 Proteomic Study

Detailed investigation of CLL is required in order to identify robust biomarkers which are suitable for routine use for the individualisation of patient management. The previous work carried out by Dr. Gina Eagle (CBPG, University of Hull) employed a strategy to artificially stimulate the BCR in BCR-responsive CLL cells and use comparative proteomics to investigate protein expression changes associated with BCR activation. The proteomics discovery phase identified several differentially expressed proteins (DEPs), which were associated with BCR activation (Table 5-1). A detailed description of this previous proteomics study, including sample selection criteria, BCR stimulation, two-dimensional polyacrylamide gel electrophoresis (2-DE) and matrix assisted laser desorption/ionisation with time of flight mass spectrometry (MALDI-TOF-MS) is presented in Appendix E. Following careful consideration of the DEPs identified by this proteomic analysis, the current project was designed to perform a detailed analysis of one DEP, which was identified by mass spectrometry as “Kininogen”. This DEP was selected for the following reasons:

- Kininogen was the only protein which was significantly upregulated (≥2 fold change) upon BCR stimulation in 3/3 “high risk” clinical samples. Other proteins were differentially expressed in only 1 or 2 of the 3 clinical samples analysed or exhibited discordant expression levels (Appendix E).

- Kininogen is an important constituent part of the Kinin-Kallikrein System, which to the best of our knowledge, had not been linked to CLL previously. The project was therefore designed to investigate this novel finding further and to establish a possible functional role for the Kinin-Kallikrein System in CLL.
- As Kininogen was also found to be associated with “high risk” CLL samples, the project was designed to investigate any possible clinical correlations. Kininogen, or related members of the Kinin-Kallikrein System, may have future potential utility as therapeutic targets, prognostic biomarkers or predictive biomarkers to aid in the management of patients with high risk CLL.

The overall aim of this chapter is to present the results of the confirmational phase of the proteomics biomarker discovery pipeline in respect of Kininogen. The data from this Chapter was published for the first time in (Kashuba *et al.*, 2013b).

Table 5-1 Differentially Expressed Proteins (at Least 2-Fold) Identified Using 2-DE/MS in CLL Samples Following Artificial Stimulation of the BCR

Sample	Protein identified	Gene ID (A-Z)	Expression change
078	Adenylate kinase isoenzyme 5 (recombinant)	AK5	↓
003	ASTN2 protein	ASTN2	↑
003	Astrotactin-2 isoform d	ASTN2	↓
003	Aspartyl-tRNA synthetase	DARS	↓
003	Fatty acid-binding protein	FABP5	↓
003	Glyoxalase domain-containing protein 4	GLOD4	↓
003	Interferon alpha-1/13 precursor	IFNA13	↓
089	Kazrin isoform A	KAZN	↑
003	**KNG1 protein**	**KNG1**	**↑**
078	**Kininogen 1**	**KNG1**	**↑**
089	**Kininogen 1**	**KNG1**	**↑**
003	Phosphatidylinositol transfer protein, beta, isoform CRA_b	PITPNB	↓
003	Tripartite motif protein TRIM19 zeta	PML	↑
089	FYVE-RING finger protein SAKURA	RFFL	↓
089	Mitochondrial Rho GTPase 1 isoform 1	RHOT1	↑
089	Thiopurine S-methyltransferase	TPMT	↓

KNG1 (highlighted in bold) was identified from 3/3 clinical samples. The data, included in this Table, was extracted from the work carried out by Dr. Gina Eagle (CBPG, University of Hull).

5.1.1 Confirmatory Immunoblotting of Kininogen in Stimulated CLL Samples

Kininogen was selected to verify its expression patterns with Immunoblotting. As previously discussed in Chapter 3, Kininogen can be present in 2 isoforms (HMWK and LMWK) as a result of alternative splicing within exon 10. Specific antibodies were selected for the analysis of each protein isoform. To confirm the differential expression of Kininogen protein upon BCR-stimulation in the 3 CLL samples analysed by 2-DE/MS (003, 078 and 089) were stimulated as per Section 4.4.2 for 10 minutes and 5.5 hours and compared with the corresponding isotype control sample using immunoblotting as per Section 4.4.3. The 2 isoforms of Kininogen, HMWK and LMWK, were analysed separately using isoform specific antibodies. Mouse monoclonal anti-HMWK antibody (ab1004, Abcam; 1:300, 4º overnight) (this antibody reacts with the unique light chain mw~46 kDa of human HMWK) and Rabbit polyclonal anti-LMWK antibody (ab79650, Abcam; 1:300, 4ºC overnight) (Human Kininogen (LMW) purified from human plasma was used as immunogen), or anti alpha-tubulin loading control (ab7291, Abcam; 1:2500, 2 hours) were applied to the membrane before visualisation and densitometry analysis. Significant up-regulation of LMWK upon prolonged BCR stimulation was confirmed in all 3 samples analysed using 2-DE/MS (Figure 5-1). LMWK expression was upregulated by 2.0-fold, 3.5-fold and 3.7-fold in samples 003, 078, 089 respectively, following prolonged stimulation of the BCR.

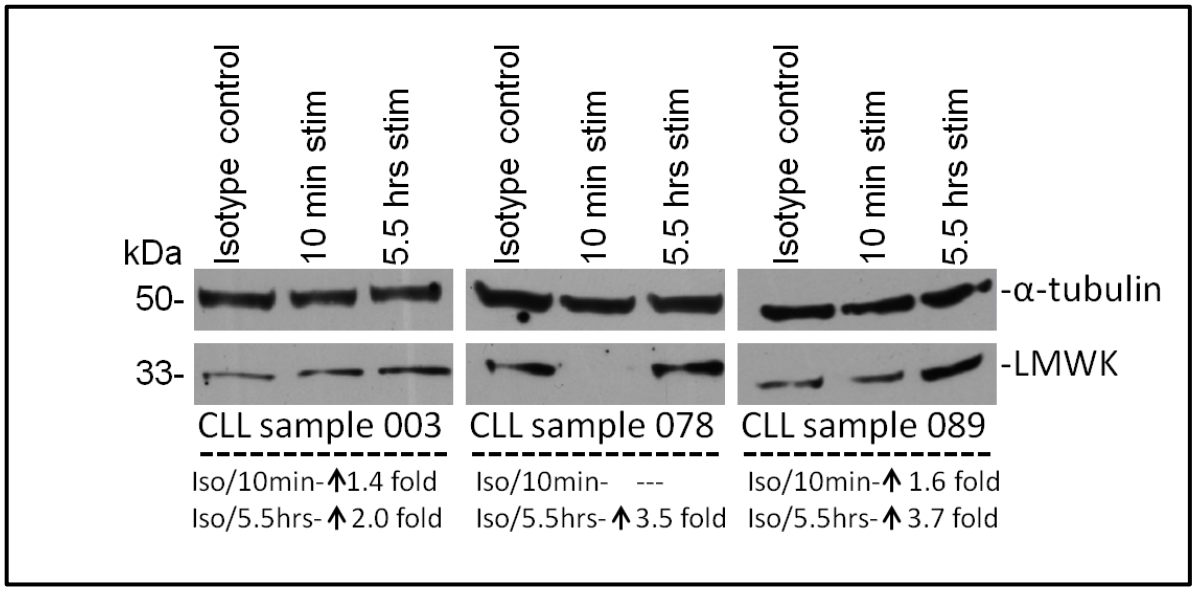


Figure 5-1 Immunoblotting of LMWK in 3 CLL Samples Following Artificial BCR Stimulation for 10 minutes and 5.5 hours

This is a Western blot image of LMWK (ab79650, Abcam) in 3 CLL samples (003, 078, 089) following in vitro BCR stimulation for 10 minutes and 5.5 hours. A single band of 33 kD was detectable, which may be a variant form of LMWK. Samples 003, 078 and 089 were previously analysed using 2-DE/MS, resulting in the identification of Kininogen upregulation upon prolonged (5.5 hours) artificial stimutation of the BCR. Alpha-tubulin was used as a loading control. Compared with untreated (isotype) control, all 3 samples demonstrated significant (≥2-fold) upregulation of LMWK (33 kDa form) at 5.5 hours. The image demonstrated that LMWK was not expressed after 10 min of stimulation in sample 78. Due to the dynamics of Kininogen it can only be suggested that the protein was not present in high enough abundance for detection in that sample at the 10 min time point, since the control was positive. The data shown is representative of a minimum of two analyses per sample.

The optimisation work was carried out using an anti-HMWK antibody; however, the robust assay was not established due to the inconsistent results or low protein abundance.

Anti-LMWK antibody (ab79650, Abcam) identified a 33kDa isoform of LMWK. The MW of this LMWK isoform (≈33 kDa) was different to expected MW of the protein of interest (≈48 kDa), however, it complimented the result from the

previous 2D/MS proteomic study, carried out by Dr. Gina Eagle, which identified a 33 kDa Kininogen isoform (NCBI, *gi116283732*, www.ncbi.nlm.nih.gov and UniProt *Q05CF8*, www.uniprot.org) (Figure). The 291 amino acids sequence had matched the protein structure of LMWK rather than HMWK.

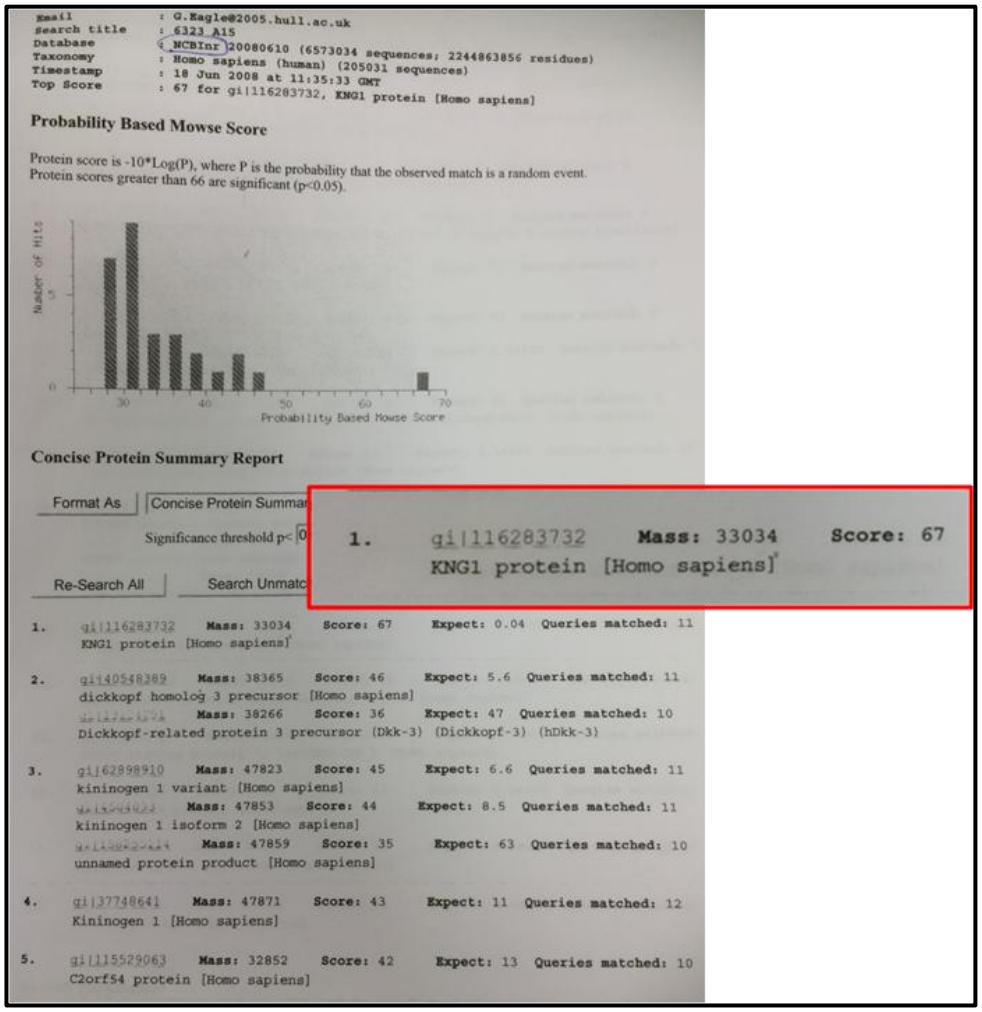


Figure 5-2 Representative Mascot Search Result from the Clinical CLL Sample 003

The Sequence Identification Numbers are assigned consecutively to each Sequence Record processed by NCBI .The top score has identified ***gi 116283732****, which was assigned to KNG1 protein (mass 33034) with Mowse scores ≥65 (p≤0.05).*

Additionally, the optimisation work was carried out using Rabbit polyclonal anti-KNG antibody (Ab 79653 and Ab 97761). Although a 33 kDa band was detected, additional bands were also observed, which made the analysis of the protein difficult and hence this reagent was no longer used in the current project (Figure 5.3).

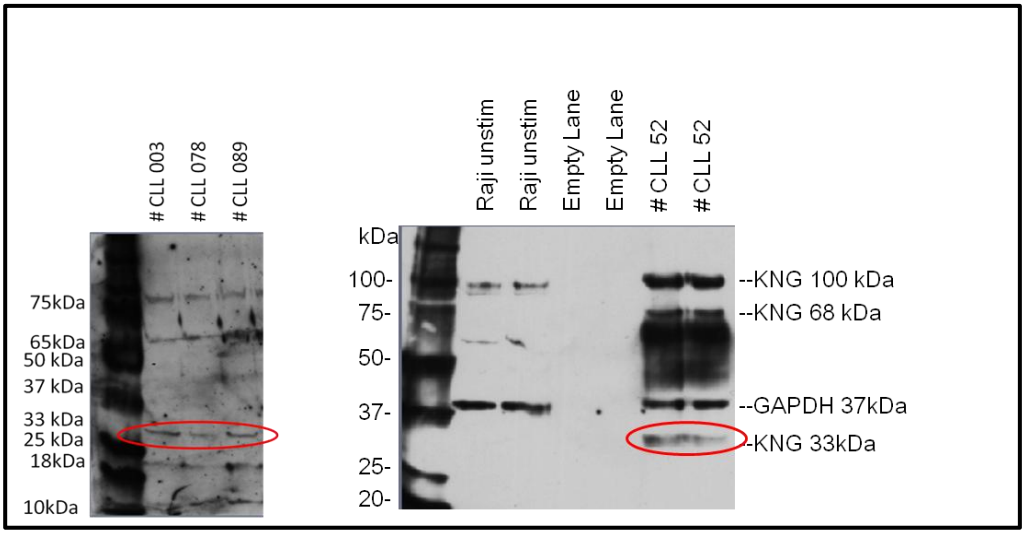


Figure 5.3 Representative Western Blot Utilising Rabbit Polyclonal Anti-KNG Antibodies (Ab 79653 and Ab 97761)

Immunoblotting of Kininogen (ab79653, Abcam left and ab 97761 Abcam right) in CLL clinical samples identified multiple bands with higher and lower molecular weights than the actual protein. The types of bands that are observed on the blot may represent true variants of the protein of interest (including 33kDa bands) or may occur as a result of non-specific bindings.

5.1.2 Immunoblotting of LMWK in Normal B-cells

PBMCs were obtained from 4 healthy volunteers (2 female, 2 male) (Appendix B) with an age range of 58-65 years (Section 4.2.1). B-cells were purified by positive depletion of $CD19^+$ cells as per Section 4.3.1. In brief, $CD19^+$ cells were magnetically labelled with CD19 microbeads and the cell suspension

was loaded onto a MACS LS column, which was placed into the magnetic field of a MACS separator. Magnetically labelled $CD19^+$ cells were retained on the column and subsequently eluted, giving a B-cell purity of approximately 98%. To assess the basal expression of LMWK protein in normal B-cells, immunoblotting was performed as described in Section 4.4.3. Full length native LMWK protein (tagged) (ab 91118, Abcam) (Figure 5-4) purified from human plasma (UniProt P01042) (Appendix G) was used as a positive control.

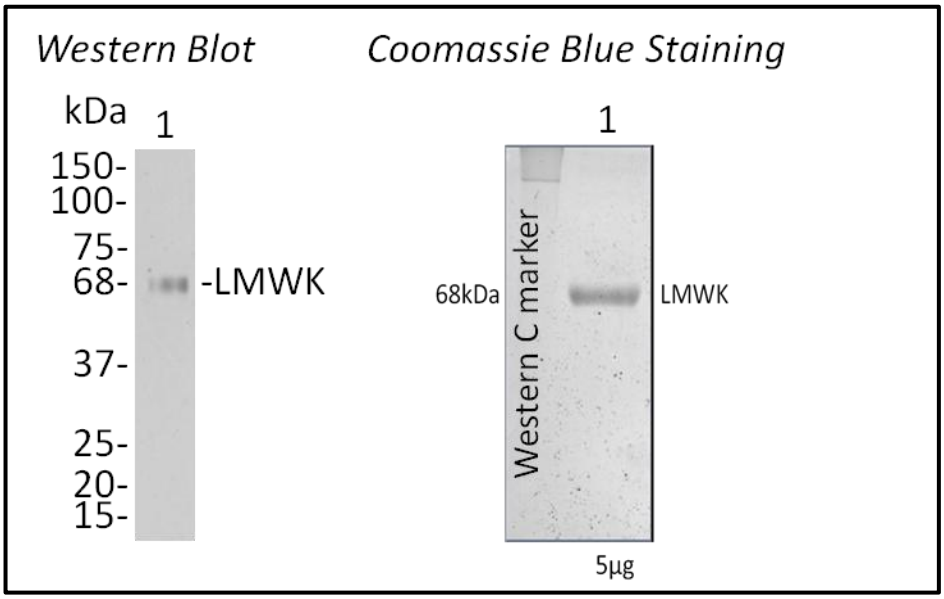


Figure 5.4 Full Length Native LMWK Protein

Full length native LMWK protein purified from human plasma (UniProt P01042) was commercially purchased from Abcam (ab 91118). Western blot image (left) of LMW Kininogen purified protein ab 91118. The loaded amount is 0.01 µg. The blot is incubated with ab79650 rabbit polyclonal Anti LMW Kininogen antibody (1:100 IN 5% non-fat milk, for 10-12 hours at 4º C).The specific 65-68 kDa band was identified. Coomassie Blue Staining (Section 4.4.3.4) of 1D gel was performed prior WB application (right). A 68 kDa band was visualised as recommended by manufacture data-sheet.

Purified B cells from 4 healthy volunteers were analysed for LMWK protein expression utilising anti-LMWK antibody (Ab 79650) and this was not detected in any sample (Figure 5-5).

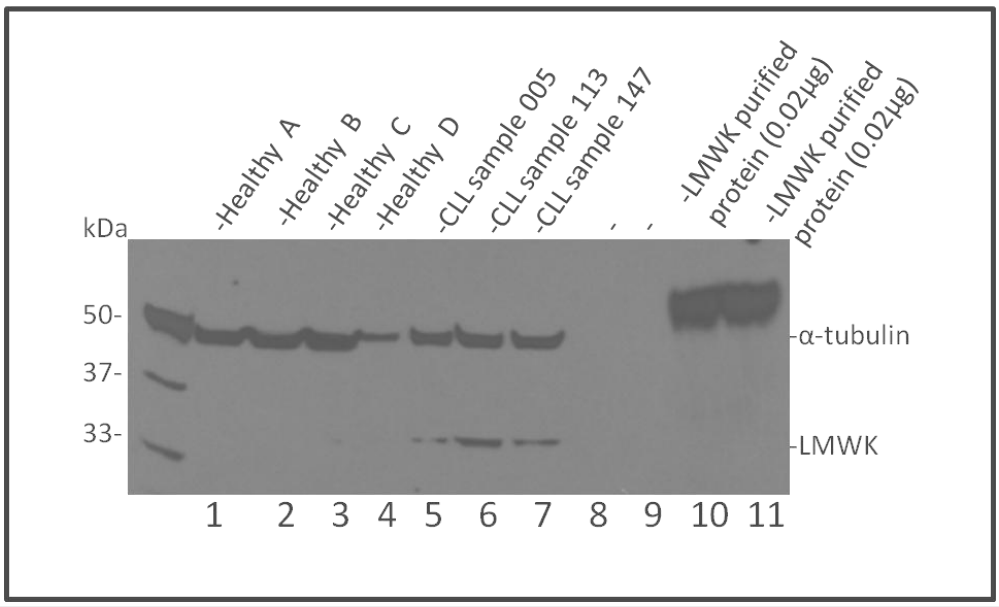


Figure 5.5 Immunoblotting of LMWK in Purified B Cells from 4 Healthy Volunteers and B-CLL samples

Immunoblotting of LMWK (ab79650, Abcam) in purified B cells from 4 healthy volunteers revealed no protein expression. In contrast, positive expression was identified in 3 CLL samples (33 kD form of LMWK) and when using purified LMWK protein (65-68 kD), which was used as appositive control. The data shown is representative of a minimum of two analyses per sample.

5.1.3 HMWK Expression

The expression of HMWK protein in normal and CLL B cells was assessed by immunoblotting following the protocol as per Section 4.4.3. The result demonstrated that no reliable signal was identified on the blot. With particular regards to plasma HMWK level, an associated study was conducted at the Haematology department, Coagulation section of Hull and East Riding by Paula

Johnson, which will be discussed in more details in Chapter 8 (Section 8.3) (Appendix I).

5.1.4 Immunoblotting of LMWK in Unstimulated CLL Samples

Neither LMWK nor HMWK isoforms have been previously associated with normal B-cells or CLL cells and therefore next were analysed for basal Kininogen expression. To assess the basal expression of LMWK protein in CLL samples, a pilot series of 52 randomly selected unstimulated samples (Table 5-2) were analysed using immunoblotting as described in Section 4.4.3

5.1.4.1 *Sample Characteristics*

The study cohort was composed of 28 males (53.8%) and 24 females (46.2%) who had a median age at diagnosis of 65 years (range, 41-87 years). The Binet stage was determined when the blood sample was collected and the series consisted of 41/52 (78%) patients with Binet stage A disease and 11/52 (21%) patients with Binet stage B or C disease. Binet stage was significantly associated with survival in this pilot series (median survival for stage A was 217 months; median survival for stage B/C was 75 months; p=0.007, log rank) (Figure 5-6). The death (if one has occurred from any causes) or the date of last follow-up (for those patients who have not progressed) and treatment status were updated prior to study closure in December 2012. At that time the series included 16 (30.8%) dead patients and 36 (69.2%) patients, which were last known to be alive. A total of 31 patients (59.6%) had received at least one course of treatment and 21 patients

(40.4%) were untreated, which was subsequent to the sample use for analysis (Appendix B).

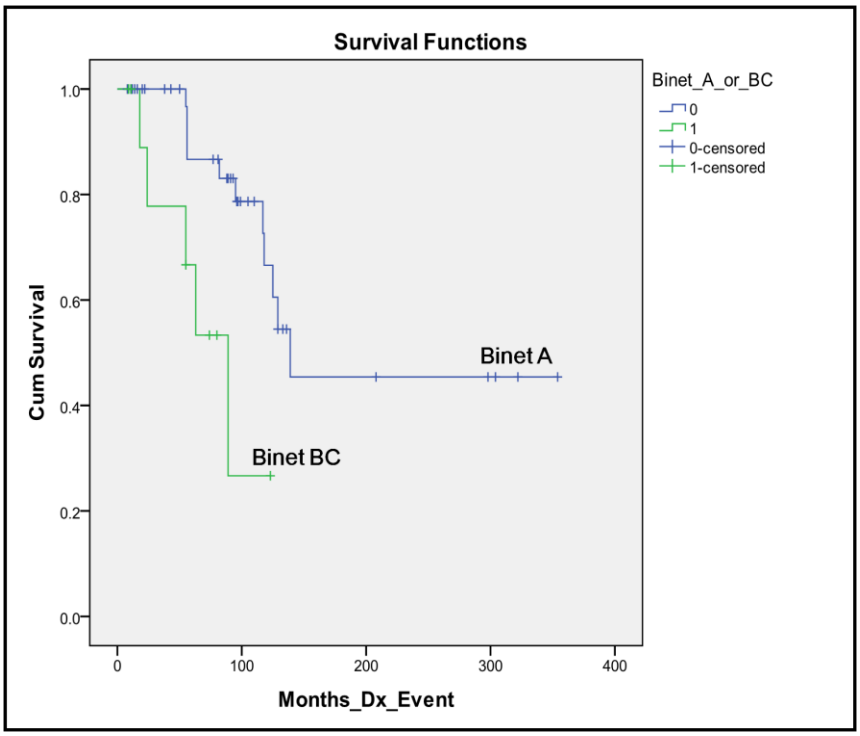


Figure 5-6 CLL Study Cohort Patients Survival by Binet Stage

Kaplan Meier survival curve for the study cohort of 52 CLL patients stratified by Binet Stage at diagnosis. Median survival was 217 and 75 months for stage A and stage B/C respectively (p=0.007, log rank). As Kaplan Meier plot shown Binet stage was significantly associated with survival in this pilot series. Censoring occurs when a case has reached its total follow-up time following the x-axis without an event, which in this case is death, had taken place.

An assessment of IgV_H status, ZAP70, CD38, B2M and genetic aberrations as prognostic markers in the dataset was also carried out. Although, perhaps due to the small size of the study cohort (n=52), which ultimately limited the statistical power; the result did not predict overall survival with statistical significance. However, the data from overall CLL cohort was previously published and all markers were shown to be predictable (Gupta *et al*., 2006).

5.1.4.2 *The Result of Immunoblotting Analysis of Unstimulated CLL samples*

A pilot series of 52 unselected CLL samples was screened for constitutive LMWK expression using specific anti-LMWK antibody (Figure 5-7, 5-8). The immunoblotting was carried out following the protocol described in Section 4.4.3.

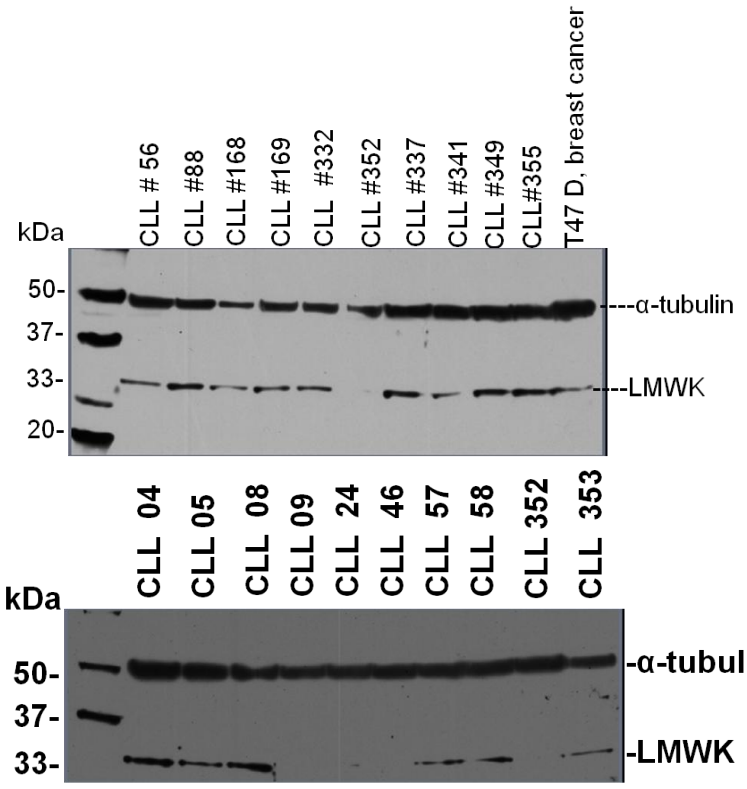


Figure 5.7 Representative Immunoblotting Images Analysing Constitutive LMWK expression in B-CLL samples

Representative Western Blots using ab79650 antibody (Abcam) to demonstrate differential constitutive LMWK (33 kDa) expression in unstimulated CLL samples. Raji (Burkitt lymphoma derived) cells and T47D (breast cancer) cells were used as positive control samples. Alpha tubulin was used as a loading control. The data shown is representative of a minimum of two analyses per sample.

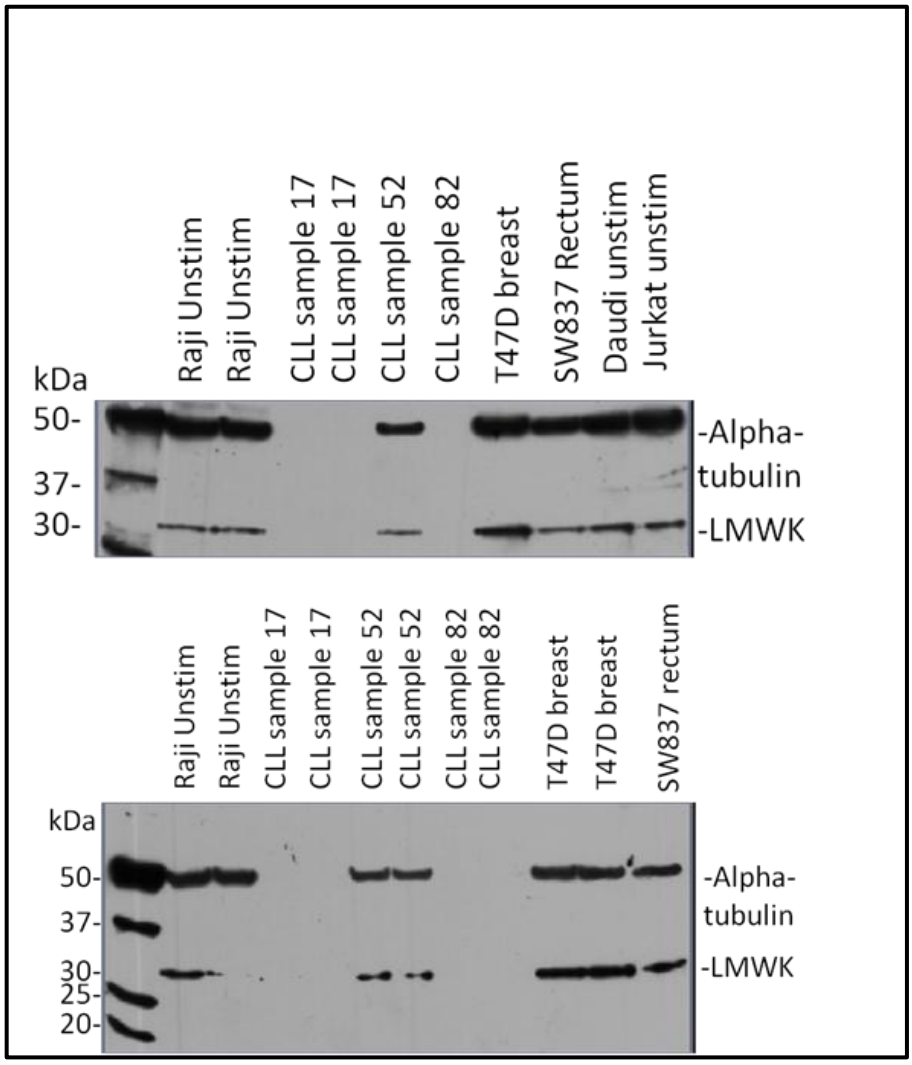


Figure 5.8 Representative Immunoblotting Images from Two Different Experiments Utilising Identical CLL Samples and Cell Lines.

Western Blots were carried out on two independent occasions to assess the reproducibility of the result obtain utilising anti-LMWK (ab79650) antibody. The result demonstrated that LMWK expression (33 kDa) was concordant in CLL samples 17, 52, 82, SW837 and Raji Cell lines in both experiments. Alpha tubulin was used as a loading control.

The expression level of LMWK differed between samples and was found to be positively expressed in 37/52 (71%) of CLL samples. LMWK expression status in unstimulated samples was correlated with clinical features and assessed for prognostic value using Kaplan Meier plots with log rank analysis (SPSS/PASW version 18). There was a trend towards shorter median survival in LMWK positive cases (147 months *versus* 253 months for LMWK negative cases; p=0.125, log rank; Figure 5-9).

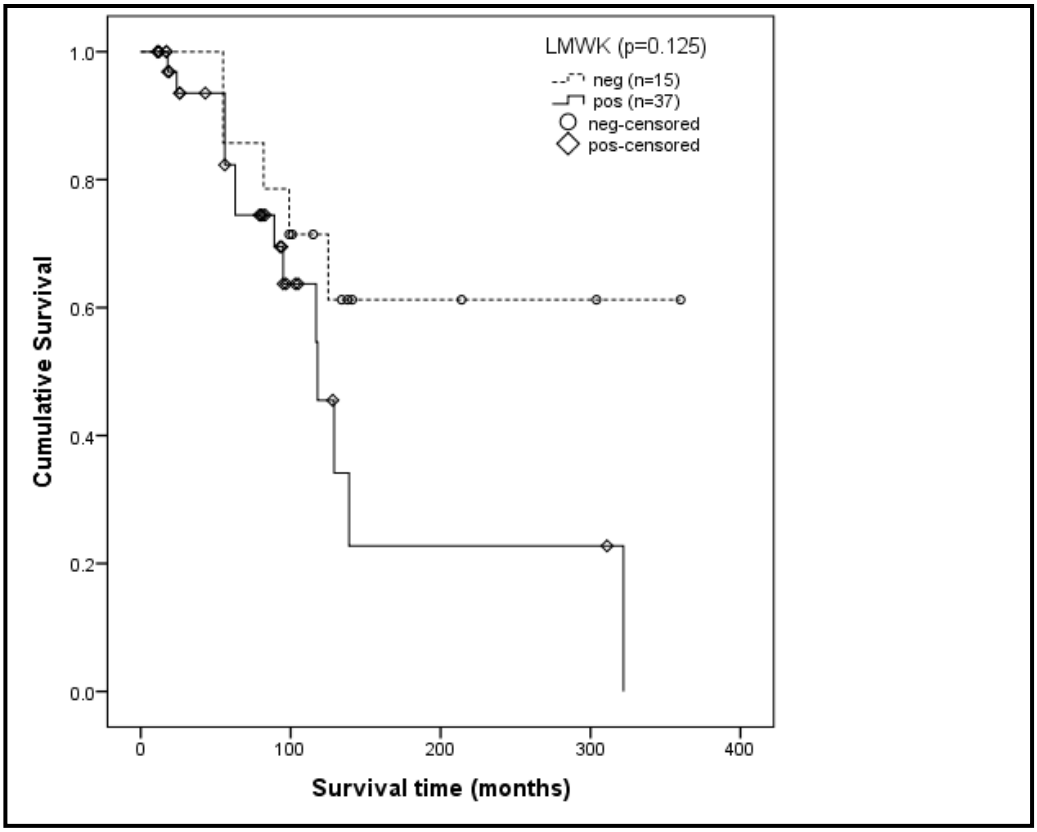


Figure 5.9 Kaplan Meier Plot Showing Overall Survival Analysis with LMWK Expression

Kaplan Meier plot demonstrates the Overall Survival analysis with LMWK expression (p=0.125, log rank). The median survival was 253 months in cases with negative expression of LMWK (broken line) versus 147 months for cases with positive expression (solid line).

There was no significant association with Time to First Treatment (TTFT; p=0.403, log rank; Figure 5-10). Moreover, there were no significant statistical correlations between LMWK and the data available for clinical characteristics or biomarkers (Table 5-1). The number of outcomes observed was too small to be statistically meaningful with this sized cohort of patients. The result for HMWK expression was inconsistent and hence was not analysed in the current project.

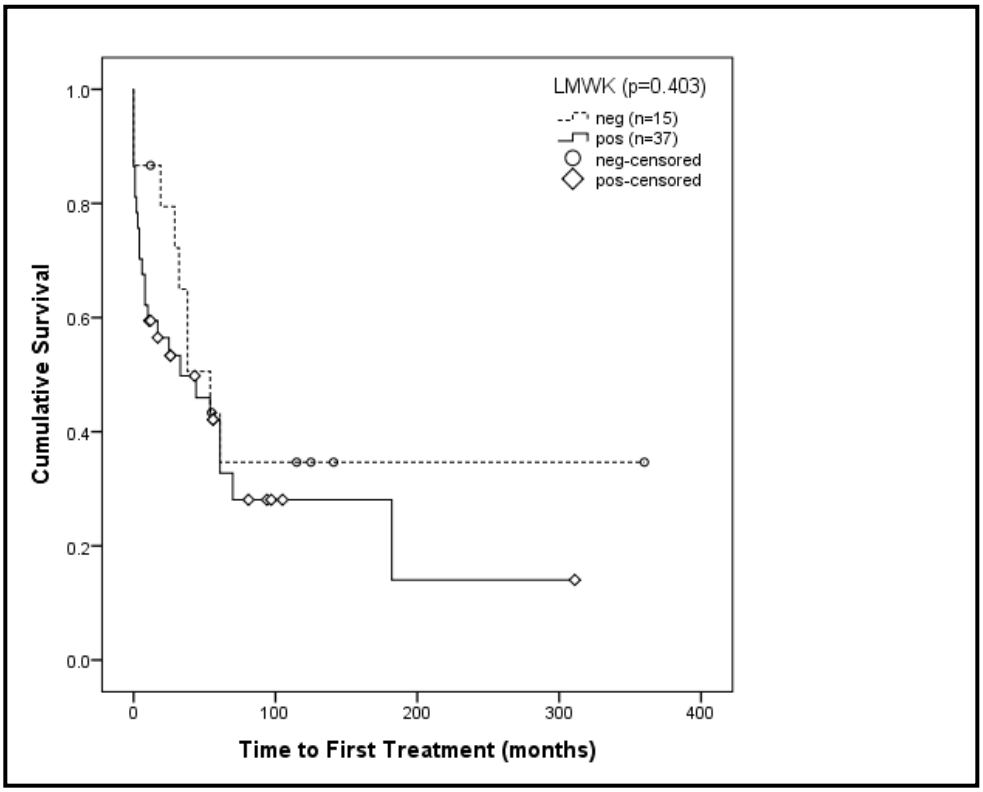


Figure 5.10 Kaplan Meier Plot Showing TTFT Analysis with LMWK Expression

Kaplan Meier plot demonstrates the TTFT analysis with LMWK expression (p=0.403, log rank). TTFT was 145 months in cases with negative expression of LMWK (broken line) versus 85 months for cases with positive expression (solid line).

		Sex	Binet Stage	IGVH (%) (m<98%)	CD 38	ZAP70 (%)	Treated	Genetic Aberration	B2M (mg/L
LMWK	Nº of CLL samples used in the analysis	52	52	39	47	33	52	33	42
	P-value	0.073	0.221	0.723	0.464	0.794	0.756	0.56	0.125

Table 5-2 The Result of Statistical Analysis Evaluating for Association between LMWK and Established Markers

This Table includes the result of the Statistical Calculation on the association between LMWK and available clinical characteristics or biomarkers using SPSS Version 22 and Pearson Chi-Square Test. Some clinical data was unavailable for the study cohort N=52. The primary disadvantage of this study design is the small size of the study cohort.

5.2 Discussion

Kininogen is a multifunctional protein and a critical regulator of the plasma KKS. Kininogens serve as a substrate from which serine proteases (plasma and tissue Kallikreins) liberate the physiologically active Bradykinin (BK) and Lys-Bradykinin (Lys-BK) peptides (Chapter 3). For some time it was believed that only the released bioactive peptides BK and Lys-BK displayed important bio-physiological properties and that HMWK and LMWK were simple precursors for proteolytic cleavage with no functional activity. However, there are a number of recent studies demonstrating that HMWK displays various biological activities (Hassan et al., 2007). The functional properties of LMWK have not been fully elucidated. Hyperactivity of the KKS and dysregulation of Kininogen levels have been previously reported in several pathological conditions, including human

inflammatory diseases and cancer (Colman et al., 1998, Barbasz et al., 2008). There have been no previous publications describing a potential role for Kininogen in CLL or normal B cells.

BCR-responsive protein expression is known to play a role in CLL (Kashuba et al., 2013a, Paterson et al., 2012), however basal protein expression may also be of clinical relevance (Duhren-von Minden et al., 2012). The current study, utilising Western Blotting, demonstrated for the first time that CLL B-lymphocytes express differing levels of basal 33 kDa protein, which may be of relevance in diagnosis.

Western blotting is an indispensable scientific method, which was employed in the current project, to quantify relative protein levels. However, as with any powerful analytic technique, western blotting has inherent limitations. The technique was found to be effective, however, very time-consuming, expensive and required the large sample quantities. Each sample processing requires a multiple steps of cell defrosting, acclimatisation, extraction and quantification of the protein before it will be proceeded to Western blot application.

Moreover, the MW of LMWK, reported in the literature, is approximately 68 kDa, whereas in the current study a LMWK protein product of 33 kDa was consistently identified with Rabbit polyclonal anti-LMWK antibody (ab79650, Abcam). The possible reasons could be that the target protein is generated through alternative splicing or the protein of interest is cleaved / degraded posttranslationally and that the utilised polyclonal antibody may recognise a lower molecular weight band. However, it is important to emphasise that there is a possibility that this band may be the result of non-specific antibody binding. The

key to a successful immunoblotting is to apply an antibody that specifically reacts with a single protein and has little cross-reactivity with other proteins. Therefore, other monoclonal and polyclonal antibodies were utilised in the current project such as Ab 1005, Ab 1004, Ab 79653, Ab 97761 (Abcam); however, a robust assay was not established due to the inconsistent results.

The data from the current project support the understanding that the identified band represents LMWK isoform: firstly, Western Blotting data, which detected a 33 kDa protein product, was consistent with the result from the previous 2D/MS proteomic study that also, identified a 33 kDa Kininogen isoform (Figure 5-2). Secondly, in order to ensure the integrity of the results, a full length native LMWK protein purified from human plasma was employed as a positive control; the results have shown that expected 68 kDa protein was detected with the same Rabbit polyclonal anti-LMWK antibody (ab79650, Abcam). Moreover, the sequence of 33 kDa LMWK (291 AA) (UniProt *Q05CF8*, www.uniprot.org) was blasted against a full sequence of canonical LMWK (427 AA). The sequence of 33 kDa isoform differs from the canonical sequence as follows:

1-17 AA position

MHGNRGEEEQYEILRGY → MKLITILFLCSRLLLSLTQESQSEEIDCNDKDLFKAV DAALKKYNSQNQSNNQFVLYRITEATKTVGSDTFYSFKYEIKEGDCPVQSGKTW QDCEYKDAAKAATGECTATVGKRSSTKFSVATQTCQITPAEGPVVTAQYDCLGC VHPIS TQSPDLEPIL (full sequence can be found in the Appendix E, Table III).

However, due to the time/cost restrictions and the technique limitations we were unable to sequence the detected 33 kDa protein band to confirm its identity.

Therefore further work will be required to establish the nature of the 33 kDa form of LMWK which has been demonstrated here in the CLL samples, and also to explain how 33 kDa protein products can be derived from the full length LMWK protein *in vivo.*

Immunoblotting demonstrated that the expression of 33 kDa LMWK isoform can be significantly increased after BCR ligation. Subsequently this suggests that a high concentration of LMWK, which is an endogenous source for proteolytic cleavage by Kallikreins and subsequent release of kinins, could be achieved and may potentially lead to the activation of kinin B1 and B2 receptors. The expression of HMWK was analysed using Western blotting, however, the result was inconsistent and unreliable. In a small number of B-cell samples from healthy volunteers, we were unable demonstrate either LMWK or HMWK expression in any sample using immunoblotting.

Furthermore, we have demonstrated in a pilot study that 71% of CLL B-lymphocytes express basal levels of 33 kDa LMWK protein, which may offer future therapeutic target options. In addition, this feature was associated with a trend towards shorter median survival. In order to fully evaluate the relationship between LMWK status and prognosis, an increased number of CLL cases should be examined. Although KNG expression is a novel finding and shown to be constitutively expressed in some CLL samples, the downstream molecular events that may lead to activation of kinin B_1 and B_2 receptors, and potentially to cell survival, need to be analysed.

Table 5-3 Clinical Data and LMWK Status in CLL Study Cohort

Sample ID	Sex	Age	Binet Stage	LMWK	IGVH (%) (m<98%)	CD38	ZAP70 (%)	Treated	Genetic Aberration	B2M (mg/L)
9	Male	68	A	*Negative*	94 m	Negative	n/a	Not treated	n/a	2.3
10	Female	79	B	*Negative*	99 um	Negative	19	Treated	17p,13q	n/a
17	Male	65	A	*Negative*	92 m	Negative	1	Treated	13q	2.0
20	Female	53	A	*Negative*	94 m	Negative	4	Treated	n/a	2.4
24	Male	65	A	*Negative*	99 um	Positive	60	Treated	t12,11q	2.1
25	Male	60	A	*Negative*	97 m	Negative	9	Not treated	n/a	2.4
27	Female	80	A	*Negative*	99 um	n/a	22	Treated	13q	4.1
28	Female	55	A	*Negative*	90 m	Negative	n/a	Treated	17p	n/a
30	Male	64	A	*Negative*	99 um	Positive	29	Treated	13q,11q	2.9
43	Female	60	A	*Negative*	96 m	Negative	5	Not treated	n/a	2.5
46	Female	53	A	*Negative*	92 m	n/a	14	Not treated	n/a	n/a
82	Female	80	A	*Negative*	94 m	Negative	6	Not treated	n/a	1.7
101	Female	74	A	*Negative*	100 um	Negative	34	Treated	n/a	2.7
191	Female	65	A	*Negative*	96 m	Negative	n/a	Treated	Normal	n/a
352	Female	65	A	*Negative*	n/a	Negative	0	Not treated	n/a	n/a
4	Male	63	A	*Positive*	93 m	Negative	1	Treated	13q	2.6
5	Male	80	A	*Positive*	98 um	Positive	17	Treated	Normal	3.9
8	Female	68	A	*Positive*	96 m	Positive	14	Treated	11q,13q	7.6
11	Male	56	A	*Positive*	98 um	Negative	6	Treated	13q	2.1
18	Female	62	A	*Positive*	92 m	Negative	5	Treated	13q	4.5
26	Female	58	A	*Positive*	91 m	Negative	11	Not treated	n/a	2.4
29	Male	76	A	*Positive*	87 m	Negative	1	Not treated	n/a	2.9
38	Female	55	A	*Positive*	99 um	Negative	63	Treated	13q	3.1
41	Female	72	B	*Positive*	89 m	Negative	2	Treated	13q	3.4
44	Male	59	B	*Positive*	96 m	Negative	36	Treated	13q,17p	3.2

45	Female	73	A	*Positive*	93 m	Negative	3	Not treated	n/a	3.1
52	Male	69	C	*Positive*	97 m	Negative	6	Treated	17p	7.2
55	Female	69	C	*Positive*	96 m	n/a	37	Treated	n/a	5.5
56	Male	66	A	*Positive*	100 um	Negative	87	Treated	13q	n/a
57	Male	63	B	*Positive*	96 m	Positive	n/a	Treated	Normal	2.5
58	Male	60	A	*Positive*	99 um	Positive	85	Treated	17p,t12	2.8
64	Male	48	A	*Positive*	94 m	Negative	5	Not treated	13q	n/a
88	Male	73	A	*Positive*	92 m	Negative	1	Treated	13q	2.6
89	Female	76	A	*Positive*	97 m	n/a	2	Treated	n/a	4.5
113	Male	63	A	*Positive*	96 m	Negative	70	Treated	13q	2.6
147	Male	87	C	*Positive*	100 um	Negative	6	Treated	Normal	9.3
150	Female	73	C	*Positive*	100 um	Positive	24	Treated	13q	n/a
166	Male	58	B	*Positive*	92 m	Positive	n/a	Treated	t12	2.8
168	Male	60	A	*Positive*	90 m	Negative	n/a	Not treated	n/a	1.6
169	Female	75	A	*Positive*	96 m	n/a	n/a	Treated	n/a	2.6
179	Male	66	A	*Positive*	n/a	Negative	n/a	Not treated	n/a	1.7
332	Male	65	A	*Positive*	n/a	Positive	n/a	Treated	13q	3.4
336	Female	41	A	*Positive*	n/a	Positive	n/a	Treated	t12	3.8
337	Female	44	A	*Positive*	n/a	Negative	n/a	Not treated	n/a	1.6
341	Male	78	A	*Positive*	n/a	Negative	n/a	Not treated	t12	2.7
342	Female	83	C	*Positive*	n/a	Negative	n/a	Treated	13q	n/a
346	Male	69	A	*Positive*	n/a	Negative	n/a	Not treated	n/a	3.7
349	Male	66	A	*Positive*	n/a	Positive	n/a	Not treated	0	1.9
351	Female	72	A	*Positive*	n/a	Negative	n/a	Not treated	Normal	4.3
353	Male	55	A	*Positive*	n/a	Positive	n/a	Not treated	t12	2.2
354	Male	67	A	*Positive*	n/a	Negative	n/a	Not treated	n/a	3.4
355	Male	62	B	*Positive*	n/a	Negative	n/a	Treated	Normal	n/a

CHAPTER 6

Kininogen Gene Expression Analysis at the RNA Level in CLL Study Cohort

KASHUBA E; EAGLE G;BAILEY G; EVANS P; WELHAM K; ALLSUP D; CAWKWELL L., *2013. Proteomic analysis of B-cell receptor signaling in chronic lymphocytic leukaemia reveals a possible role for kininogen. Journal of Proteomics, 18, 279-96.*

Chapter 6. Kininogen Gene Expression Analysis at the RNA Level in CLL Study Cohort

In this chapter the evaluation of the genetic expression of LMWK in CLL samples will be discussed. Primarily, Kininogen protein expression was identified during 2DE MS MALDI TOF experiments and was subsequently confirmed by Western blotting. Immunoblotting, using the specific antibodies, which were designed to discriminate between two Kininogen isoforms, have shown that Kininogen expressed in B Cells is presented by LMWK proteins rather than HMWK (Chapter 5). The result obtained from HMWK expression analysis was found to be weak and inconsistent, possibly owing to the relatively low protein abundance. This data complemented the result from previous 2DE MS MALDI TOF experiment. The primary objective of the RT-PCR analysis was to compare transcript level with protein expression level, and since the Western blot analysis failed to identify HMWK expression; LMWK was prioritised for further analysis of gene expression at the RNA level, whereas it was considered unnecessary to proceed to RT-PCR analysis of HMWK.

6.1 Introduction to Kininogen gene structure

The *KNG* gene is 27 kb long and is composed of 11 exons, which are interrupted by 10 introns (Kitamura *et al*., 1985). *KNG* maps to chromosome 3 (Fong *et al.*, 1991) at the 3q26-qter position (Cheung *et al*., 1992) (Genetic Home Reference) (Genetics Home Reference is the National Library of Medicine's Web site used for information about genetic conditions and the genes or chromosomes

associated with those conditions, available on the World Wide Web at http://ghr.nlm.nih.gov/). A human genome stores one *KNG* gene (Merkulov *et al*., 2008), which generates two gene products by alternative splicing after base 68 in exon 10; these are *HMWK* and *LMWK* messengers m*RNA*s. Exons 1-9 code for the signal peptide and heavy chain sequence representing D1-D3 in the corresponding proteins are typical for both HMWK and LMWK protein sequence (Figure 3-2 and 3-3). The gene consists of exons 2-9: 111bp, 85bp, 173bp, 108bp, 85bp, 173bp, 108bp, and 87 bp, respectively (Kitamura et al., 1985). There are two recognisable parts of Exon 10: one of which is located near the 5' end and known as exon 10b (where "b" stands for BK) and comprises of 78 bp and encodes the BK moiety sequence for both *HMW* and *LMW* prekininogens. The other is the 3' end part of exon 10, termed exon 10a, consists of 2015 bp and encodes for exclusive sequence for *HMW* prekininogen (Cheung *et al,* 1993). Downstream from exon 10a and following an interval of 90 bp originates exon 11, which is comprised of 248 bp, and responsible for unique *LMW* prekininogen sequence (Kitamura et al, 1985). In summary, the exclusive genomic sequences for *HMW* and *LMW* Kininogen mRNA are continuous, however, in the process of *LMW* prekininogen m*RNA* transcription an exon 10a and the following 90 bp region are utilised as "an intervening sequence (intron J)"(Kitamura *et al.*, 1985). A graphic representation of the structural organisation of the human *KNG* gene can be seen in the Figure 6-1. Transcript variant 1 or *HMW* prekininogen is translated into HMWK protein and transcript variant 2 or *LMW* prekininogen- into LMWK. HMWK and LMWK protein sequence was previously described in Figure 3-4 and 3-5, respectively.

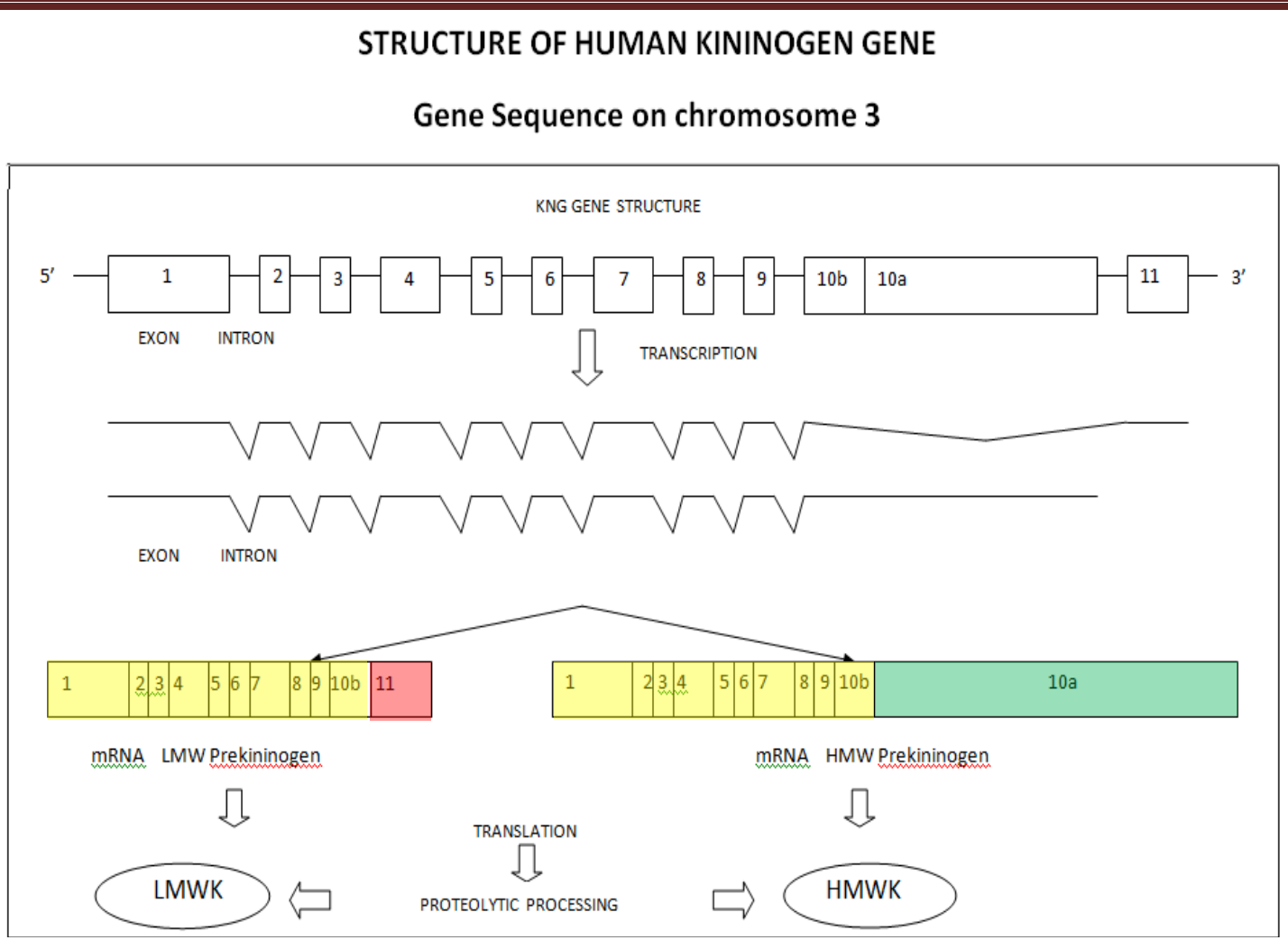


Figure 6-1 Structure of Human KNG Gene

This figure is adapted and modified from Structural organization of Human Kininogen Gene (Kitamura et al., 1985). HMWK and LMWK genes are generated by differential splicing. The boxes 1-11 represent the corresponding exons, connected by introns. After the following stages of structural rearrangement two final products are produced HMWK and LWWK.

Several factors were demonstrated to be involved in *KNG* gene regulation. Anderson and Lingrel analysed a rat *T-Kininogen* gene expression in response to glucocorticoid and estrogen treatment and shown that gene up-regulation can be hormone dependant (Anderson and Lingrel, 1989). The acute-phase reaction and aging can serve as the catalyst for augmentation of *KNG* gene transcription (Sierra *et al*., 1989). Chen and co-workers conducted a study in order to determine the regulation of rat *Kininogen* gene expression by female vs. male hormones in vivo. They demonstrated a differential estrogen vs. progesterone regulation of *LMWK* gene expression in the rat and the immunoreactive Kininogen levels was increased and decreased respectively. The outcome indicates that estrogen is one of the regulators of *LMWK* gene expression in vivo (Chen *et al.*, 1992). Zhao *et al* examined a role of the nuclear receptors for bile acids, known as farnesoid X receptor (FXR) and demonstrated that human Kininogen is a direct target for FXR and a robust induction of *KNG* gene expression was initiated by the agonists of FXR (Zhao *et al*., 2003).

6.2 Introduction to Kininogen Gene Expression Studies

For many years it was assumed that the origin of Kininogens was restricted to hepatic tissues (Okamoto *et al*., 1996). *T-Kininogen* (a rat analogue of human Kininogen) gene was found to be expressed in lung, renal, neural and cardio tissues (Mann and Lingrel, 1991). Kininogen immunoreactivity was identified in salivary and sweat glands, neutrophils, and brain tissue (Dendorfer *et al*., 1997).

However, based on the previous findings the only tissues where human *HMWK* m*RNA* expression were confirmed are liver, kidney (Iwai *et al*., 1988, Chao et al., 1993) and endothelial cells (Schmaier et al., 1988). More recent studies demonstrated LMWK expression in several other human cells and tissues such as neutrophils (Figueroa *et al*., 1992), human fibroblast (Takano *et al.*, 1996), kidney (Hermann *et al.*, 1996, Song et al., 1996), human hepatoma cells, human adrenal gland (Wang *et al.,* 1996). Okamoto and colleagues demonstrated Kininogen synthesis in fibroblasts upon stimulation by mediators of inflammation, such as prostaglandin-2 (PGE2), TNF and Interleukin-1 (IL-1) and simultaneously confirmed Kininogen expression in Rat tarsal bones in response to in vivo mediated inflammation (Okamoto *et al*., 1996, Takano *et al.*, 1995). Taking into consideration all of the above it can be hypothesised that Kininogen synthesis occurs in hepatic and various extrahepatic tissues and organs in humans and animals. However, it remains unknown whether or not CLL B-lymphocytes express the *Kininogen* gene. Standard reverse transcription polymerase chain reaction (RT-PCR) amplification was employed in order to analyse *LMWK* gene expression in untreated CLL samples.

6.3 The Polymerase Chain Reaction Application and the Primer Selection

As previously discussed, the focus of the gene expression study is specifically relative to *LMWK* due to greater abundance and reliability. Therefore, to facilitate optimum primer set effectiveness and specificity, which will exclusively

determine a target gene sequence for replication of *LMWK* and not *HMWK*, it was crucially important to understand the structural differences between *LMW* and *HMW Kininogen* genes, demonstrated in Figure 6-1. Primer set for *LMWK* amplification, which included the forward and reverse primers, was designed by Eurofins Scientific (www.eurofins.com) (PrimerBank ID: 4504893a3) (Table 6-2 and 6-3) and were selected utilising primer design guidelines (Appendix C). All primer pairs utilised in this study were selected to span an exon-intron boundary in order to eliminate amplification of genomic *DNA*.

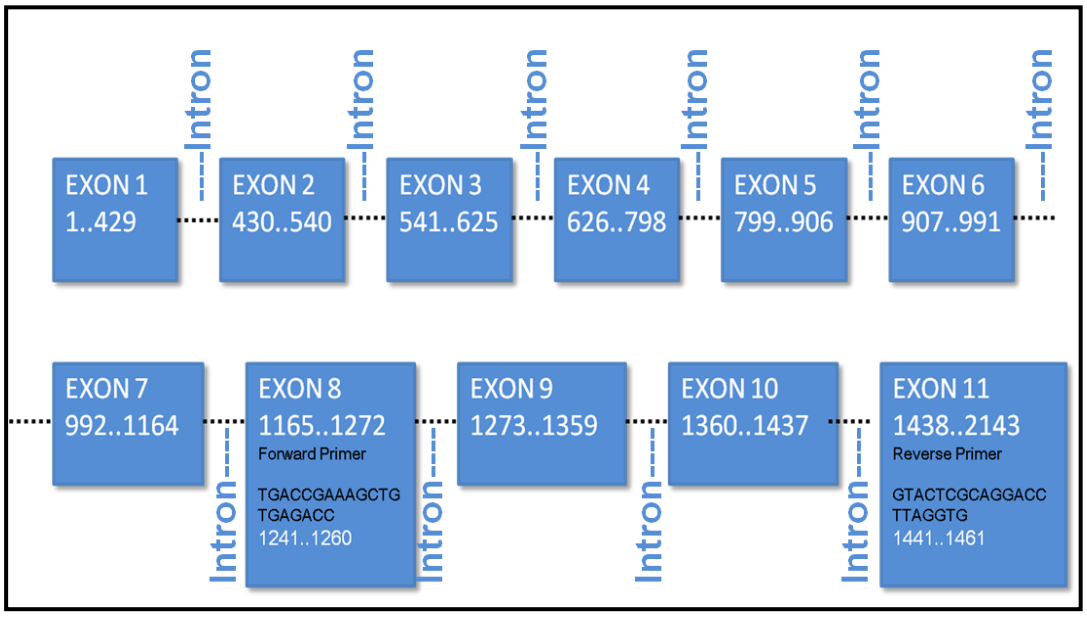


Figure 6-2 *LMWK* Exon Structure and *LMWK* Primer Sets Location Within

This graph demonstrates an exon structure of LMWK mRNA and the primers location within. Within each Exon, highlighted above, the position within mRNA sequence is stated below. The primer set is designed to discriminate between two transcribed variants LWMK and HMWK and specifically amplify targeted LMWK sequence. The expected amplicon size is 221 bp (1461-1240=221pb).

Figure 6-3 LMWK mRNA sequence and LMWK sequence detection primers location within.

```
   1 agtgttgcaa tgttctagct gtaaatatgt ttggataacc cagcagagcc atttaacctt
  61 tgggaacaag gcaactagcg tctggcagca ggaatccaac cagtgccctg agttctgagg
 121 cagagaggag gacagaagaa acaagaggct ggagattgtc aaattcagta tcccagttgg
 181 ctcttgattc ttggtgaaac catccctcag ctcctagagg gagattgtta gatcatgaaa
 241 ctaattacca tccttttcct ctgctccagg ctgctactaa gtttaaccca ggaatcacag
 301 tccgaggaaa ttgactgcaa tgacaaggat ttatttaaag ctgtggatgc tgctctgaag
 361 aaatataaca gtcaaaacca aagtaacaac cagtttgtat tgtaccgcat aactgaagcc
 421 actaagacgg ttggctctga cacgttttat tccttcaagt acgaaatcaa ggagggggat
 481 tgtcctgttc aaagtggcaa aacctggcag gactgtgagt acaaggatgc tgcaaaagca
 541 gccactggag aatgcacggc aaccgtgggg aagaggagca gtacgaaatt ctccgtggct
 601 acccagacct gccagattac tccagccgag ggccctgtgg tgacagccca gtacgactgc
 661 ctcggctgtg tgcatcctat atcaacgcag agcccagacc tggagcccat tctgagacac
 721 ggcattcagt actttaacaa caacactcaa cattcctccc tcttcatgct taatgaagta
 781 aaacgggccc aaagacaggt ggtggctgga ttgaactttc gaattaccta ctcaattgtg
 841 caaacgaatt gttccaaaga gaattttctg ttcttaactc cagactgcaa gtcctttggg
 901 aatggtgata ccggtgaatg tacagataat gcatacatcg atattcagct acgaattgct
 961 tccttctcac agaactgtga catttatcca gggaaggatt ttgtacaacc acctaccaag
1021 atttgcgtgg gctgccccag agatataccc accaacagcc cagagctgga ggagacactg
1081 actcacacca tcacaaagct taatgcagag aataacgcaa ctttctattt caagattgac
1141 aatgtgaaaa aagcaagagt acaggtggtg gctggcaaga aatattttat tgacttcgtg
1201 gccagggaaa ccacatgttc caaggaaagt aatgaagagt tgaccgaaag ctgtgagacc
1261 aaaaaacttg gccaaagcct agattgcaac gctgaagttt atgtggtacc ctgggagaaa
1321 aaaatttacc ctactgtcaa ctgtcaacca ctgggaatga tctcactgat gaaaaggcct
1381 ccaggttttt caccttccg atcatcacga ataggggaaa taaaagaaga aacaactagt
1441 cacctaaggt cctgcgagta caagggtcga cccccaaagg caggggcaga gccagcatct
1501 gagagggagg tctcttgacc aatgggcaga atcttcactc caggcacata gccccaacca
1561 cctctgccag caaccttgag aggaaggaca agaagaaaga tgggatagaa tttaaataga
1621 gaagaatgcc attttatcac tctgcctctg ggtgaaataa agatcagtct tgatgttcta
1681 actctaattc acagtggtct cctttcagcc ctacccattc tgcagcaaat tccagctggt
1741 cagagagtca gtgctgtggc tctgccatgg aggctcataa cccaacactg gaacattccc
1801 tagccaaggc agaagtcctt aggcgggact tccttaccac cacgggtgct aaaagaagag
1861 ttagtaggtc atgcttctac cagtaatcta aggactctct ccttctcttc ttcctctttc
1921 tccagatttc caagccttag ctaagagtaa tttggcttgt ttagtattgt tttcttatgg
1981 tccagttaat taccaaaaat atttttaaaa tcatctctgt taatagaatg tctaccaact
2041 tctcactatc agaaaatact caacctccaa acaatttgaa attatctttc tgatccaggt
2101 aaagaagcaa attgaaaatc aataaagtaa aaagtgacgg aga
```

This graph demonstrates the LMWK mRNA sequence, each exon is highlighted in a specific colour: agt-exon 1,ttg-exon 2, gcc-exon 3,ccg-exon 4,ggt-exon 5,ccg-exon 6,ttg-exon 7,gct-exon 8,aga-exon 9, tct-exon 10, cag-exon 11.

tga - primer set sequence, forward and reverse primers. The amplicon size is 221 bp (1461-1240=221pb)

6.4 Analysis of *LMWK* gene expression in human CLL B-lymphocytes utilising standard polymerase chain reaction

6.4.1 Identification of a suitable reference gene to compare *LMWK* gene expression in CLL samples

To ensure the reliability of PCR experiments and to obtain an accurate and biologically meaningful analysis of gene expression, an appropriate consistently expressed endogenous control (reference gene or "housekeeping gene") is required. GAPDH and Actin β (ACTB) are among the most often utilised reference genes in CLL gene expression studies. However, in order to identify the most suitable reference genes for genetic research in CLL, Valceckiene and colleagues analysed 9 potential genes for their consistent expression such as: β-actin (gene name ACTB), ß2M, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), glucuronidase-β (GUSB), hypoxanthine guanine phosphoribosyl transferase (HPRT1), hydroxymethylbilane synthase (HMBS), mitochondrial ribosomal protein L19 gene (MRPL19), TATA box binding protein gene (TBP) and ubiquitin C (UBC) in blood samples obtained from B-CLL patients (Valceckiene et al., 2010). Utilising three different descriptive statistics: geNorm, NormFinder and BestKeeper-1 this recent study has demonstrated that B2M, HPRT1 and GUSB were identified as the most invariantly expressed genes in CLL B-cells and therefore shown to be the appropriate reference genes in CLL gene expression analysis (Valceckiene *et al.*, 2010). Both *GAPDH* and / or *GUSB* genes were included as a control gene to ensure the quality and amount of the cDNA used in PCR experiments.

6.4.2 Implementation of the controls in Polymerase Chain Reaction

In order to obtain meaningful result positive and negative controls were selected. A positive control is essential for the verification of negative amplification result. A number of investigations have demonstrated that functional KKS is present in prostate cancer tissues and in an androgen-sensitive human prostate adenocarcinoma cell line (LNCaP) (Latham *et al*., 2000, Li *et al.*, 2011, Ylikoski *et al.,* 2001, Balk *et al*., 2003, Lawrence *et al.*, 2010). Following this, LNCaP cell line was used as a positive control in the PCR experiments for *LMWK* expression analysis.

Negative Control or no template control (NTC)/no cDNA control, which is essential for detecting contamination or non-specific amplification in the reaction was included in each PCR reaction. No cDNA control was processed exactly the same way as the samples and contained everything but the template DNA and enables surveillance for any contaminating amplicons.

6.4.3 Samples selection and preparation

A total of twenty samples, obtained from CLL patients, who were seen at the Queen's Centre for Oncology and Haematology, Castle Hill Hospital (the clinical diagnosis of CLL was based on standard morphologic and immunophenotypic criteria), from which 18 samples were from the study cohort (N=52) previously included in the proteomic study of KNG protein expression (Chapter 5) and 2 fresh samples were analysed in this experiment (Appendix B). In the present study the samples were selected on the basis of KNG protein expression status with 4/20 were LMWK negative and 16/20 LMWK positive. Three “high risk” samples

previously used in comparative proteomics (samples 003, 078, 089) (Chapter 5) were also included. The available laboratory data on a subset of these cases is shown in Appendix B. The LMWK protein expression status is shown in Appendix H. In addition, two peripheral blood samples, obtained from healthy volunteers were added to this study for subsequent analysis of *LMWK* gene expression in normal B-cells (Table 6-1). Raji, T47D and A549 cell lines (Table 1-1), which were applied as a control in the proteomic work and LNCaP were analysed in this gene expression study also.

Table 6-1 The Demographic Characteristic and LMWK Protein Expression Status of Healthy Samples Utilised in the Gene Expression Study

Sample N^0	Gender	Age	LMWK protein expression status
1	Male	61	Negative
2	Female	58	Negative

CLL B-lymphocytes were prepared as described in Section 4.5.1.2. For this study unselected CLL samples were used, the proportion of B-cells (estimated by Jo Pointon using flow cytometry with CD19 marker) was composed of a minimum of 80% B-cells. However, $CD19^+$ cells from healthy controls were isolated by positive immunomagnetic selection utilising MiltenyiBiotec separation kit according

to the vendor's recommendations as previously described in Section 4.3.1. The average B-cell purity obtained from the positive selection was approximately 98%.

Raji, T47D, A549 and LNCaP cells were cultured and harvested as described in Section 4.1 before proceeding to the experiment.

6.4.4 Total RNA preparations and quantifications of peripheral blood B cells obtained from CLL patients, participating healthy controls and cell lines

Total cellular RNA was extracted and quantity of total RNA was determined as described in Section 4.5.1.3 and 4.5.1.4 respectively.

6.4.5 First-strand cDNA synthesis

Two micrograms of the DNase-treated total *RNA* were reverse-transcribed into a first-strand c*DNA* prior to conventional PCR amplification following an optimised protocol outlined in Section 4.5.1.5.

6.4.6 Polymerase Chain Reaction and Agarose Gel Electrophoresis

Based on the information obtained from the genomic structure of *Kininogen* gene, two *LMWK* gene-specific primers (*LMWK*-F and *LMWK*-R) and control gene primers (*GAPDH*-F and *GAPDH*-R; *GUSB*-F and *GUSB*-R) were utilised (Table 4-7). All amplifications were carried out as described in Section 4.5.2.2.

6.4.7 Successful PCR Set Up Confirmation: Reaction Conditions and Components Optimisation Step

Notwithstanding the PCR application being widely employed in multiple studies and being known as a reliable, sensitive technique which facilitates a generation of a high yield of specific DNA target sequences, there is no single set of conditions that are applicable to all PCR amplifications. The reaction conditions and component concentrations, optimum time and temperature parameters, sample volumes have to be individually optimised for efficient amplification of specific targets. In addition, a successful PCR test validation experiment with anticipated DNA fragments of known sizes recommended ensuring the integrity of PCR technique. Several studies have reported that *TRPM8* is expressed in the prostate and in the prostate cancer-derived epithelial cell line, LNCaP (Zhang and Barritt, 2004, Thebault *et al.*, 2005). Therefore, RT-PCR experiment aiming to detect a known *TRPM8* and *β-actin* transcripts in LNCaP cells was initially performed prior to the initiation of *LMWK* gene expression study.

6.4.7.1 *Result of ancillary PCR experiment analysing* Actin *and TRPM8* gene expression in LNCaP cells to confirm the integrity of PCR methodology utilised

LNCaP cells were prepared and PCR reaction was carried out following the protocol outlined in Section 4.5.1. The sequence of *Actin* and *TRPM8* primers are shown in Table 4-7. The expected product size for *Actin* and *TRPM8* genes were 243 bp and 1157 bp, respectively.

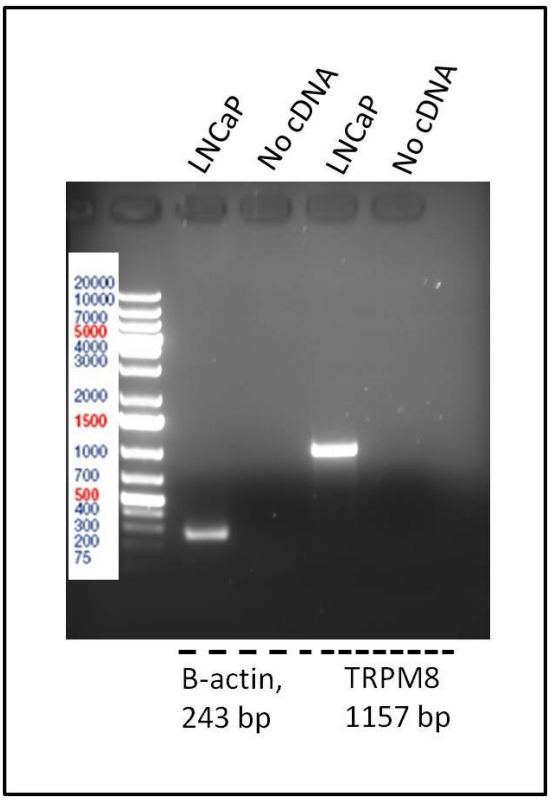


Figure 6-4 The Expression of the *TRPM8* and *Actin* Transcripts in LNCaP cells as determined by RT-PCR

The Actin PCR product is 243 bp. The band of 1157 bp is representative of a variant form of TRPM8.

The result of this RT-PCR experiment has indicated that the correct PCR conditions have been followed in order to optimise rationalisation of PCR in respect of application to further gene expression study.

6.4.8 Results of analysis of the *LMWK* gene expression

The result of *LMWK* transcript expression as determined by RT-PCR is presented in Appendix H. The expected 221 bp *LMWK* amplicon was detected as a clean single band in LNCaP cells, whereas no *LMWK* m*RNA* expression was seen in CLL samples (Figures 6-5 and 6-6). The control gene (*GAPDH* and *GUSB*), detected in all analysed samples, confirmed the presence of *RNA* in all samples, especially CLL and B cells.

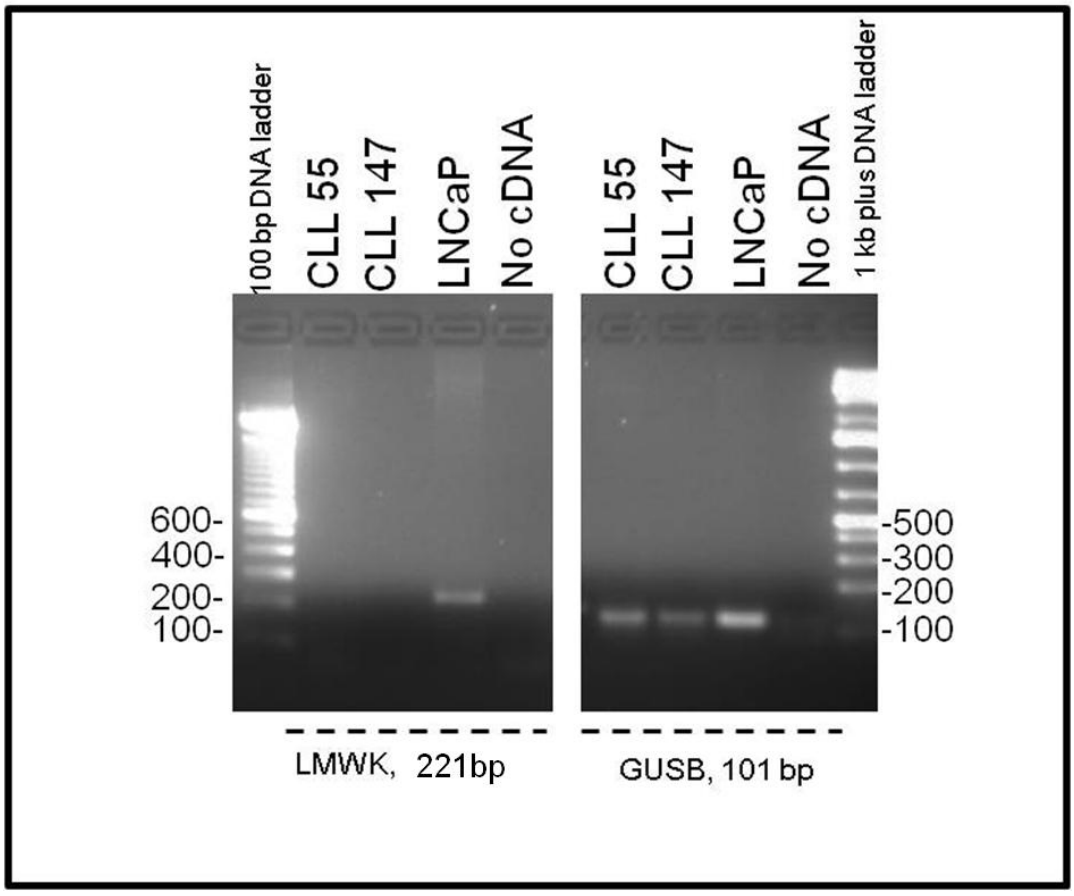


Figure 6-5 The Expression of *LMWK* Gene in CLL Samples and the Control Sample

As determined by RT-PCR, GUSB transcript (101 bp), which was applied as housekeeping gene was detected in LNCaP and two CLL samples. The band of 221bp, representing LMWK transcript is shown in LNCap cells, which serve as a positive control. The mRNA for LMWK was undetectable in CLL samples 55 and 147.

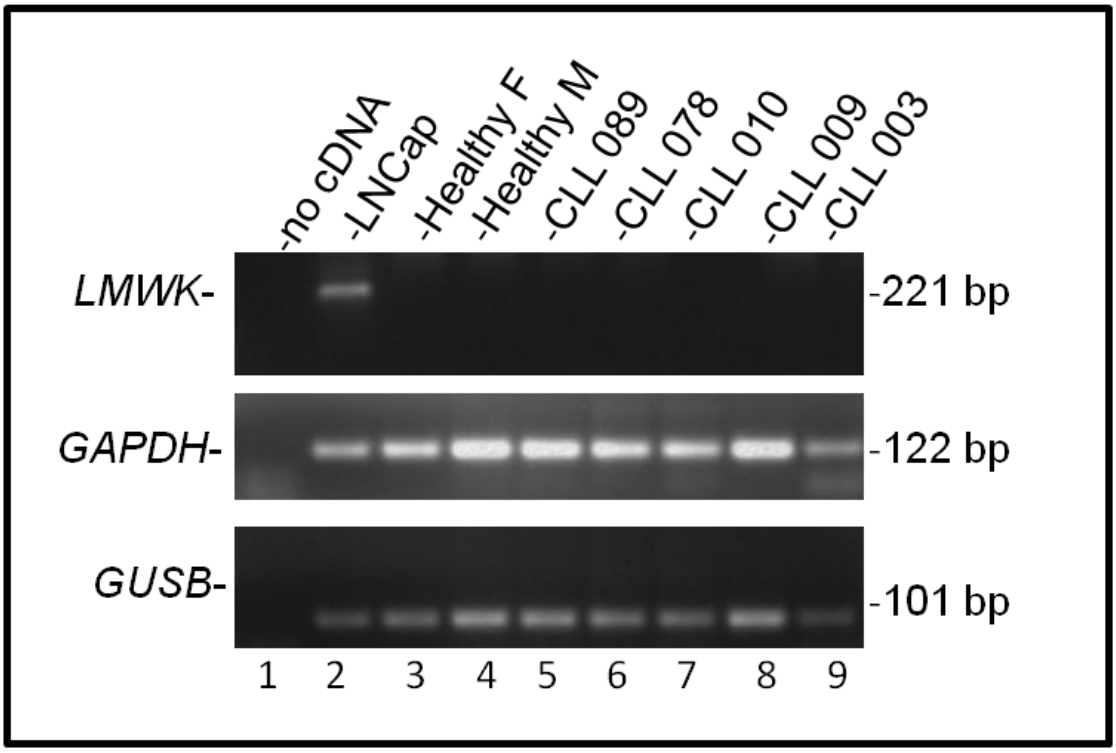


Figure 6-6 The Expression of *LMWK* Gene in CLL and Healthy Samples

LMWK gene expression in CLL (lanes 5-9) and healthy (lanes 3-4) samples as examined by RT-PCR. GAPDH and GUSB, the housekeeping genes, were used as a control gene and the product size of 122 bp and 101 bp respectively were identified.

The band of 221 bp, representing LMWK transcript was detected in positive control LNCaP cells, but not in CLL or healthy B-cells. However, taking into account that LMWK is known to be abundantly expressed in LNCaP cells and that intensity of the band is relative to the amount of product, a more intensive band was expected in the positive control cell line, suggesting that the assay may be relatively insensitive. PCR was performed for 35 cycles using the parameters indicated in Section 4.5.1. The addition of extra cycles (increased to 40 cycles) resulted in the opportunity for nonspecific amplification.

6.5 Discussion

The aim of this experimental work was to examine *LMWK* transcript level in CLL and normal B cells, which would progress our understanding about the origin of LMWK protein expression, previously identified using proteomics. In order to

investigate the expression of the *LMWK* gene, 20 CLL samples from our study cohort and 2 samples obtained from healthy individuals were reverse-transcribed and amplified using forward (exon 8) and reverse (exon 11) primers. The position of reverse primer to exon 11 has been exclusively determined by *LMWK* mRNA structure (Muller-Esterl *et al.,* 1986). Despite the existence of a number of techniques developed to study mRNA levels of target genes, RT-PCR, which amplifies the sequence of interest, was selected due to being the well-established, sensitive and reliable method.

The results demonstrated that, utilising a selective primer set specific for *LMWK* amplification, *LMWK* transcript was detected in the positive control cell line LNCap, however, it was undetectable in 20 unstimulated CLL samples, 2 normal controls and Raji, T47D, A549 cells.

Several studies have reported that due to the canonical mechanism of mRNA translation into protein, generally both mRNA and protein abundance should be complementary and positively correlated (Hegde *et al*., 2003, Mootha *et al.,* 2003b, Mootha *et al.*, 2003a, Alter and Golub, 2004, Nie *et al.*, 2006). However, a number of studies have demonstrated a weak relationship (Ideker *et al.*, 2001, Greenbaum *et al.*, 2003, Washburn *et al.*, 2003) or no correlation at all (Gygi *et al.,* 1999, Nie *et al.*, 2006) between protein and gene expression and thus challenged the interpretation that mRNA level can be predictive of the corresponding protein abundance. The controversy between gene and protein abundance was suggested to be possibly due to the mutations in the translational regulation, fast turnover, low protein abundance, the levels of experimental error, including unequal

experimental conditions being compared (Greenbaum *et al*., 2003, Beyer *et al*., 2004) and the sensitivity of the methods applied.

The interpretation of the result must be taken from several differing, but equally important perspectives. Firstly, the result obtained during gene expression study has emphasised the importance of proteomics approaches alongside the gene expression studies. It has clearly demonstrated that, mRNA expression analysis alone is not completely reflective of the corresponding protein expression translated from these mRNAs (Boisvert *et al*., 2012) and vice versa. Moreover, the data obtained from both gene or proteomics studies can be interpreted as independent sources of information, which provides us with greater understanding of a complex metabolic network within the human body. It is globally accepted that the number of proteins in a proteome is superior to the number of genes identified and therefore use of proteomics is crucial in the biomarker discovery field and without parallel use of proteomics the valuable information would remained undiscovered. Secondly, the lack of *LMWK* mRNA in CLL samples may suggest that proteomics data is detecting exogenous LMWK in CLL samples, which circulates in blood and may adhere to the surface of CLL cells resulting in detection by proteomics methods.

Finally, the results clearly demonstrate that further study in respect of *LMWK mRNA* expression is required to elucidate the complete understanding of the *LMWK*/LMWK and *HMWK*/HMWK gene-protein relationship and LMWK/HMWK protein origination. Despite the attempts to analyse *LMWK mRNA* expression with RT-PCR, one section of the work that could be critical is that the sensitivity of the

assay used was not fully determined. RT-PCR is generally accepted to be a sensitive technique, however, the level of sensitivity could have been enhanced by the use of fluorescent detection. Therefore, a more sensitive detection utilising fluorescence dyes and an optimisation of a competitive PCR method using standard curves to quantify transcripts would be beneficial for further study. The addition of a standard curve can determine the sensitivity of the assay and the signal can be compared to a standard curve established with a serial dilution of a standard consisting of purified PCR product. Based on standard curve linear regression analyses, copies for each target RNA can be calculated. In order to design standard curves, known amounts of target and competitor templates required to be co-amplified in PCR assays. After quantification of PCR products, the data can be plotted and evaluated. However, the time constraints in respect of this project have meant that further study into LMWK gene expression could not be undertaken (Alvares *et al*., 2003). However, the next step would be to carry out RT-PCR with different primer sets and to conduct an immunocytochemical (ICC) analysis with the aim to examine the cellular localisation and distribution of both HMWK and LMWK in CLL and healthy B-lymphocytes. The target of the above experiment was directed towards the identification of the specific *LMWK* transcript and therefore re-assessment of mRNA expression utilising different primer sets and/or primer sets targeted *HMWK* mRNA may provide a different insight then that currently presented.

CHAPTER 7

Analysis of Human Kallikrein Expression in CLL and Normal Cells

Chapter 7. Analysis of Human Kallikrein Expression in CLL and Normal B Cells

Following investigation into KKS signalling pathway, the expression of next downstream constituent such as KLK will be assessed in this chapter. As previously discussed in Section 3.4.1.1 and demonstrated in Figure 3-1 KLK (tissue and plasma) are the enzymes of the serine proteases family which form kinins by cleaving the peptide bonds between arginine (*-Arg*) and lysine (*-Lys*) residues in HMWK and LMWK. However, very little is known regarding KLK expression in human normal or CLL B-lymphocytes.

7.1 Introduction to Tissue KLK Expression Associated with Different Malignancies

During past decades, serine protease expression has attracted increasing interest in the cancer research field, due to the emerging evidence about the roles of these enzymes in carcinogenesis and metastasis, suggesting that serine proteases might be a possible target for therapeutic agents and could serve as a diagnostic and/or prognostic biomarker in several malignancies (Diamandis *et al*., 2004). Dysregulation of Kallikrein expression, Kallikrein abundance, extensive glycosylation and other posttranslational modifications have been shown to be associated with the tumour-associated antigens (Yousef and Diamandis, 1999). Tissue Kallikrein super family consists of 15 reference members (Yousef *et al.,*

1999) with their genes localised to chromosome 19q13 (Borgono *et al*., 2004) and according to the nomenclature, introduced in 2006, KLK2 to KLK15 are designated as Kallikrein-related peptidases and KLK1 has retained its original name, Kallikrein-1 due to its highly expressed kininogenase activity (He *et al.*, 2012). Furthermore, the data suggests that tissue-specificity and alternative splicing within the human Kallikrein gene can regulate generation of novel Kallikrein transcripts (Yousef *et al.,* 2000a). Kurlender *et al* reported the existence of 82 different Kallikrein splice variants (including 15 reference forms) (Liu *et al.*, 2011b). Recent studies suggested that reference or alternative Kallikrein transcripts are tightly associated with carcinogenesis. Therefore, it was necessary to conduct a detailed analysis of the physiological and pathological characteristic and clinical relevance of all 15 members of Kallikrein family in order to prioritise the Kallikrein member(s), which is (are) most likely to be expressed in CLL samples (Appendix F).

7.2 Investigation into KLKs Expression Utilising Immunoblotting

Following detailed research of KLKs expression and their association with CLL; it has become apparent that the role of KLKs in normal and/or CLL B cells has been studied only at a very superficial level.

When considering which of the 15 reference KLKs to focus upon for this ongoing research, several key criteria were influential in the decision making process:

- firstly, western blot was selected as an investigation technique for KLK study, however, with such a time- and resource-intensive technique, only relative KLK isoform(s) were considered for evaluation in the current project;
- secondly, known tissue specificity of a particular isoform;
- thirdly, the availability of previous research in the field of gene/protein Kallikrein expression in healthy B/T-lymphocytes or related lymphoproliferative disorders, and
- lastly, the availability of suitable and reliable antibodies.

Considering above criteria each of the 15 KLK isoforms were studied in detail and their characteristics, tissue specificity and associated pathologies are briefly presented in Appendix F. However, in terms of previous research, the only readily available study conducted of any significant relevance was a work performed by Scarisbrick *et al* “Dynamic role of kallikrein 6 in traumatic spinal cord injury (SCI)”, who studied the role of KLK6 in regulation of the immune T cell activation and survival (Scarisbrick *et al*., 2011). The author and colleagues investigated SCI and its association with inflammation, oedema, proteolysis, nitric oxide release, and apoptosis. In their previous work in order to identify participating serine proteases they used a degenerate primer cloning strategy, which was based on the serine protease catalytic triad and demonstrated a role for novel serine protease, kallikrein 6 (Singla *et al.*, 2006). In a recent study, by utilizing murine whole splenocyte preparations composed of both T- and B-lymphocytes and the human Jurkat T cell line, Scarisbrick *et al* studied the role for KLK6 and demonstrated that it significantly facilitates cell survival under normal conditions and/or pre-treated

with camptothecin, dexamethasone, staurosporine and Fas-ligand via overexpression of pro-survival molecule such as B-cell lymphoma-extra large (Bcl-XL), and downregulation of pro-apoptotic molecule such as Bcl-2 interacting mediator of death (Bim) (Scarisbrick *et al*., 2011). Given the role of KLK6 in cell survival considerations, the aim herein was to determine the possible expression of KLK6 in normal and CLL B Cells utilising immunoblotting.

7.2.1 Antibody and Positive Control Selection and Optimisation

In order to analyse protein expression utilising western blotting, a suitable and reliable antibody is required. As indicated previously, all 15 members of the Kallikrein family share a considerable structural homology at the gene and protein level (Li *et al*., 2011). However, there are identifiable loop regions that demonstrate sequencing heterogeneity (Bernett *et al*., 2002). There are three isoforms of KLK 6 produced by alternative splicing with MW= 27 kDa, 15 kDa and 4 kDa respectively (UniProt, 2014). Therefore, the selected antibody is required to be specifically designed to react with the discriminating epitope(s). Anti-Kallikrein 6 antibody - Kallikrein loop (Abcam 28301) (a polyclonal antibody, which has specificity to react with human KLK 6, and will not interact with the human KLK1-15) was optimised for subsequent application in the Western blotting protocol.

In order to validate the result obtained from Western blotting the positive controls were selected. Several studies have repeatedly reported an upregulated expression of KLK6 mRNA in colorectal cancer (Ogawa *et al.*, 2005, Yousef *et al.,* 2004, Ohlsson *et al.*, 2012), hence a rectal cancer cell line SW837, known to be positive for KLK expression, was utilised as a positive control in Western blot

experiment. In addition to this, MCF7 and PJ49 cell lines and the recombinant full length KLK6 protein, consisting of amino acids 17-244 (Swiss-Prot Q92876) (Appendix G) commercially purchased from Abcam (# Ab 82792) were also employed as a positive control. A proprietary glutathione-S-transferase (GST) tag, which was implanted to the N-terminus of a recombinant KLK6 protein, served as an affinity and/or solubilisation tag that facilitated the complete protein folding and prevented protein precipitation. GST tag is a genetically sequenced 211 amino acid peptide with MW of 26 kDa. Hence, a recombinant full length KLK 6 with proprietary GST tag (Abcam 82792) had a predicted Molecular Weight of approximately 51.8 kDa.

7.2.2 Samples Characteristic and Preparation

In order to assess KLK6 expression, a total of 27 cases diagnosed with B-CLL were analysed. A total of 25/27 samples were selected from the research cohort previously used in proteomics (N=52) (Chapter 5). CLL cells were defrosted and rested for 3 hours prior to proceeding to Western Blotting (Section 4.4.3). Two of the selected 27 samples (samples 35 and 292) were obtained from CLL patients, B cells were isolated (Section 4.2.1) and immediately utilised in Western blotting (4.4.3) without being subjected to storage in low sub-zero temperatures (-80^{0}C) or in liquid nitrogen. The combination of the fresh/frozen samples was included due to the limited availability of the frozen samples (N=52). In addition, the outcome of this experiment may offer a subjective assessment of whether freezing the samples would affect western blot quality. The clinical characteristics of the selected samples are available in Appendix B. In addition, four healthy

controls (two female and two male samples) were included (Appendix B). Normal B cells were isolated using MACS method following the protocol described in Section 4.3.1.

7.2.1 Result of Assessment of KLK 6 Isoform Expression in CLL and Normal B Cells Utilising Western Blotting

Twenty µg of CLL (n=27) and normal B-cells lysate per well along with 20 µg of cell line lysate per well were loaded and one dimensional gel electrophoresis was performed (Section 4.4.3.3). Subsequently, the proteins were transferred onto a nitrocellulose membrane using the iBlot system, then blocked for 1 hour with 5% non-fat milk and incubated with Anti-Kallikrein 6-Kallikrein loop antibody (Abcam 28301) (1:500 in 5% non-fat milk) for 3 hours at RT.

The result of this experiment demonstrated that an approximately 65/70 kDa bands were observed in the clinical samples, MCF7, PJ49 and SW837 (Figure 7-1).

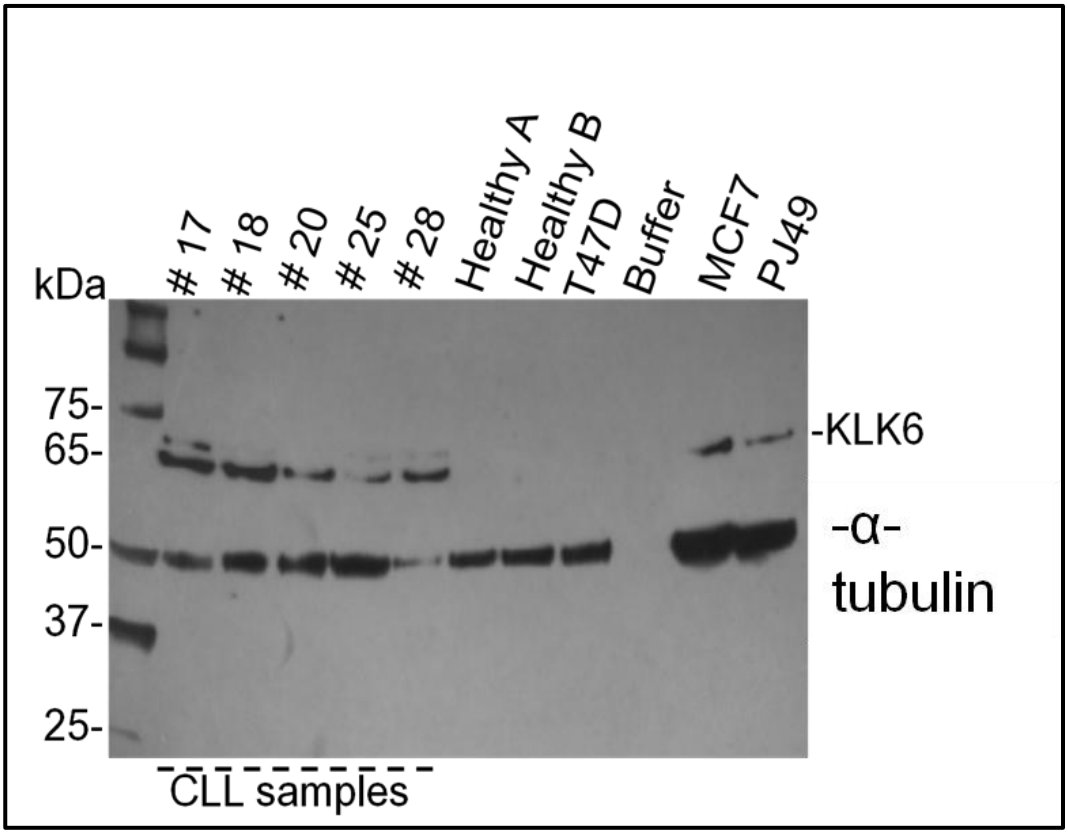


Figure 7-1 Western Blot Analysis of KLK6 Expression in CLL and Normal B Cells

This figure demonstrates a Western blot analysis of KLK 6 expression in CLL samples, healthy controls and cancer cell T47D, MCF-7 and PJ49 lines.
An approximately 65/70 kDa bands can be observed in the CLL clinical samples, MCF7 and PJ49 cells. No signal is detected in 2 healthy control samples. Alpha-tubulin was used as loading control (50 kDa). The antibodies were used at optimised concentrations, which are shown in Table 4-4.The expected molecular weight for KLK 6 (Ab28301) is 27 kDa. The observed bands are significantly higher than the predicted MW and no bands appear in the expected position. The reason for this molecular mass shifts cannot be explained at this stage. The data shown is representative of a minimum of two analyses per sample.

Western blotting experiment included the combination of fresh/ frozen CLL samples, which were not matched. Although it was only a subjective evaluation of whether freezing the samples affected the quality of the experiment, the outcome

was noted and recorded. The result indicated that no difference in the protein expression, associated with previous freezing, was identified between fresh (n=2) and frozen (n=25) CLL samples.

The bands representing KLK 6 isoform were identified at a higher molecular weight (MW=65-70 kDa) than expected (27 kDa). This molecular mass shift may occur as outcomes of protein/protein interactions due to the samples having not been fully reduced / denatured. However, these conclusions can be contested as all Western Blots were run under denaturing/reducing conditions, hence, protein: protein interactions should not be retained. Therefore, in order to further analyse whether observed bands are due to technical artifacts or they represent true variants of KLK 6, a recombinant full length KLK 6 protein was included as a positive control.

7.2.1.1 *Recombinant KLK 6 Protein Staining and Visualisation*

Initially, the recombinant full length KLK 6 (Appendix G) was visualised using Coomassie Blue staining technique and detected on the membrane utilising Ponceau S staining as per Section (4.4.3.4 and 4.4.3.6 respectively) (Figure 7-2).

Te recombinant full length KLK 6, which was presented in lyophilised form (500 µg), was reconstituted by adding 450 µl of distilled sterile water and 50 µl glycerol (Sigma Aldrich, # G5516-100ML) to the vial. This resulted in the final concentration of 1 µg/µl solution, which was aliquoted and subsequently utilised for downstream application or kept at -80°C for long term storage.

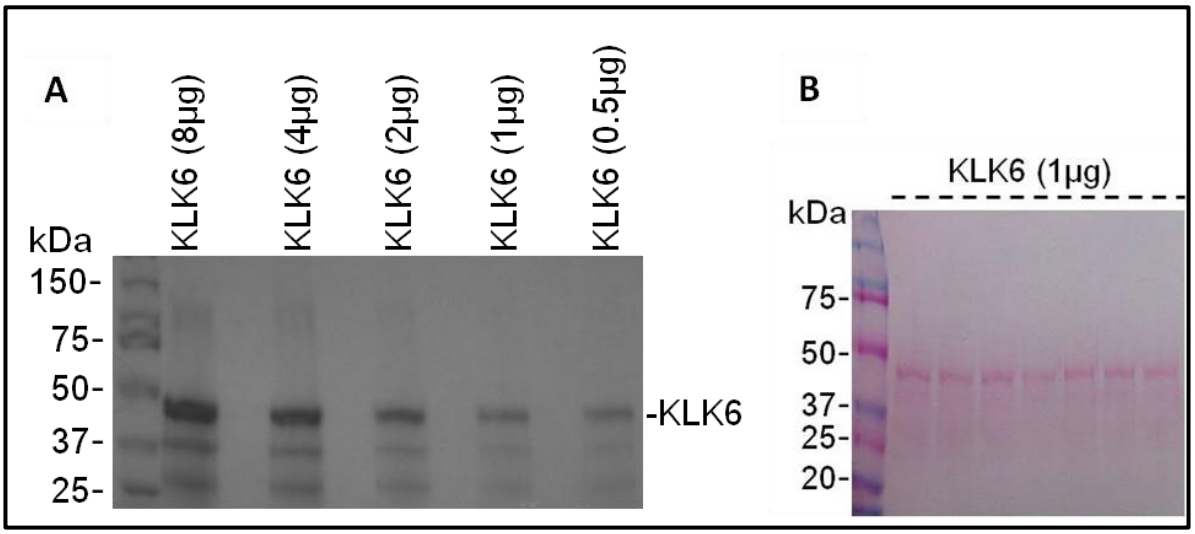


Figure 7-2 Full Length Recombinant KLK6 Staining and Visualisation

A recombinant full length KLK6 (Ab 82792) was loaded and co-electrophoreses with a Western C marker (Bio-Rad #161-0376) at 140V for 50 min and then the gel was stained with Coomassie Blue Staining (A) and the membrane was stained with Ponceau S staining (B) in order to visualise KLK6 protein. An expected molecular weight of a recombinant KLK6 protein (tagged) (ab82792) is approximately 51.8 kDa. The bands are of approximately 49-50 kDa were observed on both the gel and the membrane.

7.2.2 Western Blotting Analysis Utilising Full Recombinant KLK6 protein

In order to utilise the recombinant KLK6 protein in Western blotting application, the loading amount of the protein required to be optimised. For this purpose, several loading options ranging from 1µg, 0.5µg, 0.1µg, 0.05µg, 0.01µg, 0.005 µg and 0.001µg and different dilutions were performed. As a result, the protein load of 0.001-0.005 µg per well was defined as optimal (Figure 7-3). The outcome of these experiments have demonstrated that anti-KLK 6 antibody strongly reacted with a recombinant KLK 6 protein (MW of 49-50kDa) and the

protein of about 65/70 kDa in CLL cells extracts, whereas expected 27 kDa KLK 6 was identified in the positive control SW837 cell line.

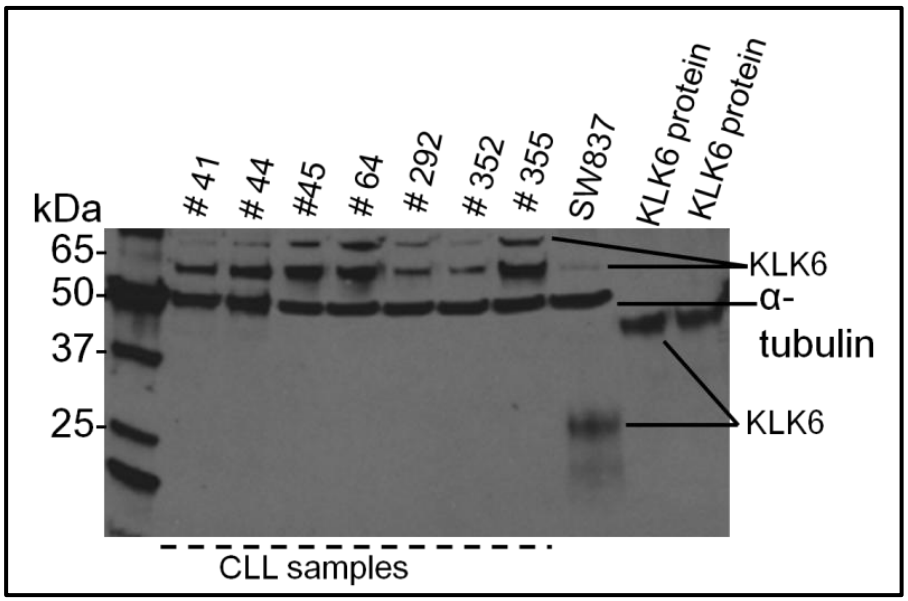


Figure 7-3 Western Blot Analysis of KLK6 Expression in CLL Samples and Other Cancer Cell Line

*A Western blot analysis of KLK6 expression in **CLL samples**, **SW837.** A recombinant KLK6 (Ab82792) (tagged) was utilised as a control. A 65/70 kDa protein is observed in the clinical samples; 27 and 65 kDa proteins are detected in SW837, which were used as a positive control and approximately 49 kDa band is identified for a recombinant KLK6 protein. The expected molecular weight for KLK6 (Ab28301) is 27 kDa, which is observed in the positive control cell line. The bands that are observed in CLL samples are significantly higher than the predicted MW and no bands appear in the expected position. The reason for this molecular mass shifts cannot be explained at this stage. Alpha-tubulin (Ab7291) was used as loading control (50 kDa). The data shown is representative of a minimum of two analyses per sample.*

7.2.3 KLK 6 Protein Expression in CLL Samples vs. Normal Samples

Western blot was carried out (Section 4.4.3) utilising two CLL samples and two healthy volunteer samples. The differential KLK 6 expression, determined by Western Blot in two CLL vs. two healthy samples, was assessed utilising densitometry (Section 4.4.3.11) (Figure 7-4). If it is accepted that the 65/70 kDa

protein product is representative of KLK 6 isoform, then the result demonstrated that the level of KLK 6 isoform expression is significantly (≥2 fold change) upregulated in two CLL in comparison to two normal B cells.

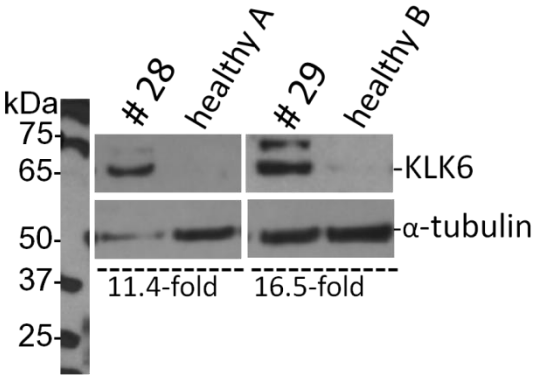


Figure 7-5 Western Blot Analysis of KLK6 Expression in CLL and Normal B Cells

*This figure demonstrates a Western blot analysis of KLK 6 expression in **CLL samples** vs. healthy controls. KLK6 expression is shown to be significantly (≥ 2-fold change) upregulated in CLL samples in comparison to healthy controls. Alpha-tubulin (Ab7291) was used as loading control. The antibodies were used at optimised concentrations, which are shown in Table 4-4. The bands of 65/70 kDa may represent KLK6 isoform or are the result of non-specific binding. The data shown is representative of a minimum of two analyses per sample.*

7.3 Plasma KLK Associated Study

Plasma KLK cleaves HMWK and generates BK and Bradykinin-free Kininogen (HKa), whereas studies have shown that tissue KLKs digest LMWK to release Lys-BK and unbound heavy and light chains. With particular regards to plasma KLK level, an associated study was conducted at the Haematology department, Coagulation section of Hull and East Riding by Paula Johnson, which will be discussed in more details in Chapter 8 (Section 8.3).

7.4 Discussion

The overall aim of this chapter was to discern whether normal and CLL B Cells express tissue KLK particularly in respect of tissue KLK 6 protein. The determination of KLK expression in CLL samples can facilitate further progress by conceivably hypothesising that CLL B-cells possess all the constituents for potential activation of Kinin-Kallikrein System. The assembly of KKS, which is triggered on the cell surfaces, has been described in several cell types including platelets (Greengard and Griffin, 1984, Gustafson *et al.,* 1989), astrocytes (O'Farrell *et al*., 2001), neutrophils (Gustafson et al., 1989, Henderson et al., 1994), smooth muscle (Imami *et al.,* 2001) and endothelial cells (Colman and Schmaier, 1997, Joseph and Kaplan, 2005). As it was demonstrated in Chapter 5, CLL B-lymphocytes express differing levels of KNG protein, which can be significantly upregulated upon BCR ligation. This suggests the presence of a greater substrate for KLK to act upon, which can potentially lead to raised levels of liberated kinins.

Primarily all 15 KLK isoforms were studied in detail and their characteristics, tissue specificity and associated pathologies are briefly presented in Appendix F. When considering which of the 15 reference KLKs to focus upon for this research, several criteria were considered and based upon the research results and probability of having the most relevance. The current research was focused upon the most relevant target protein only, KLK 6, with an attempt to dichotomise the outcome (positive/negative expression). This relevance however was not wholly conclusive and further study of other forms of Kallikrein is recommended. Although the quantification of the KLK 6 expression at the protein level would improve our

understanding, it was not the intention of this study to research this particular facet due to time and cost constraints.

In respect of the results obtained from KLK 6 protein expression study, the experiments have shown that 27/27 unstimulated CLL samples expressed a 65/70 kDa protein band, which presumably corresponded to KLK 6, whereas no expression was identified in 4/4 normal B cells.

However, the question is posed regarding apparent discrepancies between determined (65/70 kDa) and expected (27 kDa) protein sizes. Western blotting is an extremely powerful method and provides required information about a target protein identity. As previously discussed in Section 7.2.1, significantly higher molecular weight protein bands can be seen when protein: protein interactions occur due to samples not being fully reduced / denatured. However, all Western Blots in the current project were run under denaturing/reducing conditions; hence, protein:protein interactions should not be retained. In order to resolve the issue several steps were undertaken, including fresh β-ME being added, samples were also reheated before repeating a new experiment and samples were also prepared with fresh loading buffer. Additionally, in order to ensure the quality of the antibody, a full length recombinant KLK 6 protein was employed as a positive control. Despite this, a 65/70 kDa band was consistently expressed in CLL clinical samples, whereas expected 27 kDa and 49-50 kDa signals were detected with the same Rabbit polyclonal anti-KLK 6 antibody-Kallikrein loop (Abcam 28301) in the positive control cell line and recombinant KLK 6 protein, respectively.

It can be anticipated that 65/70 kDa protein band is representative of KLK 6, however, the possibility of the result is being false-positive and the bands appear as a result of non-specific antibody binding cannot be excluded.

There is an increasing amount of evidence suggesting that KLK6 is associated with cancer growth, progression, more aggressive types of cancer, chemoresistance and, hence, recently KLK 6 was utilised as a biomarker for screening, diagnosis and prognosis (Bayani and Diamandis, 2012). To the best of recent knowledge, the expression of KLK has never been linked to CLL previously and the result demonstrated, for the first time, that CLL B-lymphocytes may express tissue KLK (human KLK6 isoform) and, therefore, contribute to KKS organisation and downstream signalling.

KLK 6 has been reported to be posttranslationally modified, especially N-glycosylated at several sites (Kuzmanov *et al*., 2009). Lina Seiz *et al* observed glycosylated KLK6 in the brain tissue of around 43 kDa in comparison to non-glycosylated of about 27 kDa (Seiz *et al.*, 2012).

The observation of 65/70 kDa band in the current study requires further investigation to verify the identity of the band. Protein sequencing, which is designed to determine the identity of the protein of interest, may confirm the hypothesis. Several other steps such as use of the different antibodies or blocking peptide would help to determine whether the antibodies are detecting the correct protein target. At that point in time, after detailed research on the available antibodies, methodology, and considering that the confirmation may take significant time and cost, it was decided to focus the project on testing clinical samples.

CHAPTER 8

Plasma Bradykinin Level Determination and Evaluation of Possible Clinical Correlations in CLL Samples

Chapter 8. Plasma Bradykinin Level Determination and Evaluation of Possible Clinical Correlations in CLL Samples

In this chapter the quantitative measurement of Bradykinin concentrations in a small cohort of B-CLL and Normal Donor Plasma Pool samples utilising Bradykinin ELISA kit (Enzo Life Science, #ADI-900-206) will be discussed. The Bradykinin plasma concentration data will be further analysed in order to determine any possible correlations with clinical features and/or other putative biomarkers that may be involved in the pathophysiology of a disease. As previously outlined in Chapter 3 (Figure 3-1), Kininogens serve as a substrate from which serine proteases such as plasma and tissue Kallikreins liberate the physiologically active Bradykinin (BK) and Lys-Bradykinin (Lys-BK) peptides. BK and Lys-BK are very small proteins consisting of 9 and 10 amino acids respectively. Additionally, *in vivo,* human BK and Lys-BK immediately metabolised by enzymes such as ACE (kininase II, EC 3.4.15.1), aminopeptidase P (EC 3.4.13.19), neutral endopeptidase (EC3.4.24.11) and carboxypeptidase N (kininase I, EC 3.4.17.3) (Murphey *et al.*, 2000) to a smaller and more stable 1-to-5 amino acid fragment (BK1-5 [Arg-Pro-Pro-Gly-Phe]). BK's half-life was reported to be only 17s (Ferreira and Vane, 1967), whereas BK1-5 was shown to have a terminal half-life of 86 to 101 min (Murphey *et al*., 2000). BK-related peptides act upon the kinin B_1 and B_2 receptors to modulate cell migration, proliferation, vascular permeability and inflammation (Liu *et al.*, 2009, Kolte *et al.*, 2011). Therefore in the present study BK levels were

evaluated utilising Bradykinin ELISA, which was designed and manufactured to cross-react with a number of BK-related compounds due to the BK short half life (Table 8-1). These BK-related compounds were determined by diluting and measuring the cross reactant to concentration in the range of 0.1 pM to 500 nM. The sensitivity of the ELISA kit was determined by the manufacturer and shown to be 24.8 pg/mL.

Table 8-1 Performance Characteristics: Specificity of BK ELISA

This table has been extracted and can be found at Enzo Life Science (#ADI-900-206) user manual (Enzo Life Sciences, 2014a)

Analyte	Amino acid Sequence	% Cross-Reactivities in the range of 0.1 pM- 500 nM
BK	*RPPGFSPFR*	100%
Lys-BK	*KRPPGFSPFR*	100%
Les-Des-Arg_9-BK	*KRPPGFSPF*	<1%
BK1-5 Metabolite	*RPPGF*	<0.1%

8.1 The Preliminary Study and Optimisation Steps for Bradykinin ELISA Technique

Serial dilution titration analyses were carried out to ensure that the dilution for analyte levels fell within the range of the assay. All plasma samples and

standards were made as described in Section 4.4.4.2 and Section 4.4.4.3 respectively. Expecting that some samples might be above the standard curve, all samples were run neat and also diluted 1:2 and 1:5 in assay buffer (Enzo Life Science, #80-1628). Samples with concentration outside of the standard curve range were re-analysed using a different dilution. ELISA assay was carried out as outlined in Section 4.4.4.4. The level of BK-related compounds, which hereafter is referred to as BK, was measured at 450nm using a Multiskan FC Absorbance Plate Reader and calculated utilising Assay Blaster. The results demonstrated that 1:5 dilutions were optimum for this particular ELISA experiment and samples conditioning.

8.2 Determination of Bradykinin Level in CLL and Healthy Control plasma samples and ELISA Data Analysis

8.2.1 Sample Selection and Characteristics

The study cohort was composed of a total 36 plasma samples (Section 4-2): 27 males (75%) and 9 females (25%) who had median age at diagnosis of 71 years (range, 53-87years). The Binet stage was assessed at the time of diagnosis and available for 35/36 patients (stage data for one patient was unavailable due to being primarily diagnosed at another centre, outside of the region). The distribution of patients with Binet A, B, C stages were 25/35 (71.4%), 5/35 (14.3%) and 4/35 (11.4%), respectively. At the time of sample collection the cohort consisted of 26/36 (72.2%) treated and 10/27 (27.8%) untreated CLL patients. The patients' demographic data is presented in Table 8-2 and Appendix I. The Normal Donor

Plasma Pool comprised of pooled platelet-poor plasma from 25 carefully screened mixed sex donors ranged between 18 to 66 years old.

Table 8-2 Demographic Data on the Study Cohort (December 2011)

CLL patients were grouped into two clinical groups: patients with stage A (low risk) and stage B/C (moderate and high risk). One sample had its stage value missing; therefore the stage characteristic for 35 samples (97.2%) was included

Sex	Binet Stage (Total Cases)		Treatment Status (Total Cases)		Vital Status (Total Cases)	
	A	BC	Treated	Untreated	Alive	Dead
Female	8	0	6	3	6	3
Male	18	9	20	7	18	9
Total	26	9	26	10	24	12

8.2.2 Bradykinin ELISA Assay

CLL plasma samples and standards were prepared as described in Section 4.4.4.2 and Section 4.4.4.3 respectively. The normal Donor Plasma Pool was included as a normal control. Each sample, including control was diluted (1:5) in assay buffer and run in duplicate to ensure that enough data was provided for statistical validation of the results. ELISA assay was carried out following the protocol described in Section 4.4.4.4 and calculations were performed using Assay Blaster software.

8.2.3 Generating a Standard Curve

A standard curve was generated by plotting the mean absorbance (also known as optical density [OD]) for each standard concentration (*x axis*) against the concentration of target protein (*y axis*). A best fit curve was created through the points in the graph utilising Assay Blaster software. A representative standard curve is shown in the Figure 8-1.

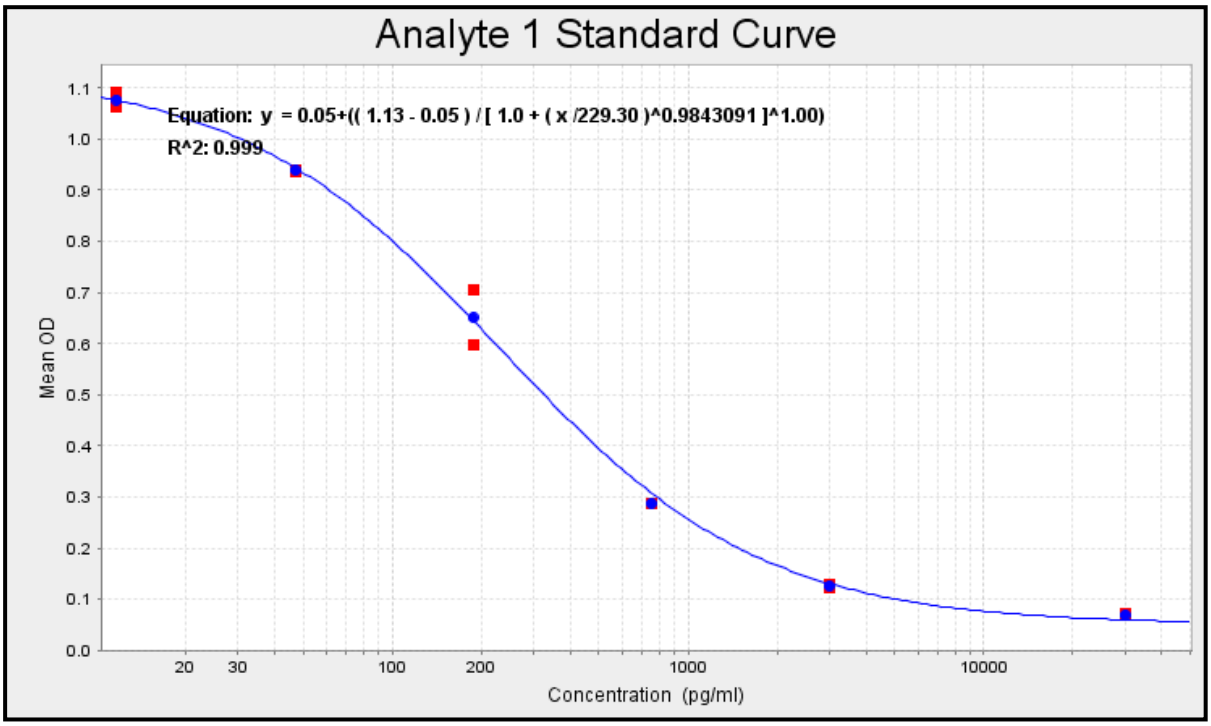


Figure 8-1 Bradykinin Standard Curve

In order to obtain valid and accurate result a standard curve was created for each ELISA plate. Mean OD was plotted on x axis and BK concentration in pg/mL was plotted on y axis. The standards concentration was measured and graphed, which then allowed the determination of the BK concentration in unknown samples by interpolation on the graph. The R^2 value (or the Pearson Coefficient of Determination) =0.999 indicating that data fits the model. .

8.2.4 Result of Quantitative Measurement of Bradykinin Concentrations

In order to determine the concentration of the target protein in each unknown sample, the mean OD (optical density) value of the sample (*X axis* of the standard curve graph) was intersected with standard curve, where the corresponding concentration of BK was read from Y axis. The calculation & evaluation of BK concentration was performed utilising Assay Blaster software (Section 4.4.4.4).

8.2.5 Measurement of Bradykinin Concentration in Normal Donor Plasma Pool and CLL samples

The normal plasma Bradykinin concentration has been investigated by a number of studies. Using radioimmunoassay (RIA) and ELISA from Phoenix Pharmaceuticals, Bradykinin levels in healthy control plasma was reported to be 0.3pg/mL - 8.7pg/mL (an average of 2.76pg/mL) and 230pg/mL -810pg/mL (an average of 430pg/mL), respectively (Cugno et al., 2000, Mason et al., 2009). It is difficult to compare these results with the results obtained during the current study due to the different methodologies and assay sensitivities being applied. The Bradykinin ELISA utilised in the current project may lead to slightly higher concentrations than those described in current literature due to its sensitivity. Hence, the Normal Donor Plasma Pool comprising of pooled plasma from 25 donors was included for comparison. The results for BK concentration in Normal Donor Plasma Pool from 2 (x2) experiments are included in the Table 8-3.

Sample	BK concentration
Normal Donor Plasma	7867.78 pg/ml
Normal Donor Plasma	10432.8 pg/ml
Normal Donor Plasma	10326.55 pg/ml
Normal Donor Plasma	9642.39 pg/ml
Average Concentration	**9567.38 pg/ml**

Table 8-3 Bradykinin Concentration in Normal Donor Plasma Identified by ELISA

The Bradykinin concentration differs between B-CLL samples ranging between 7607.66 pg/mL to 250170.28 pg/mL (Table 8-4), whereas an average concentration for BK in Normal Donor Plasma Pool is 9567.38pg/mL.

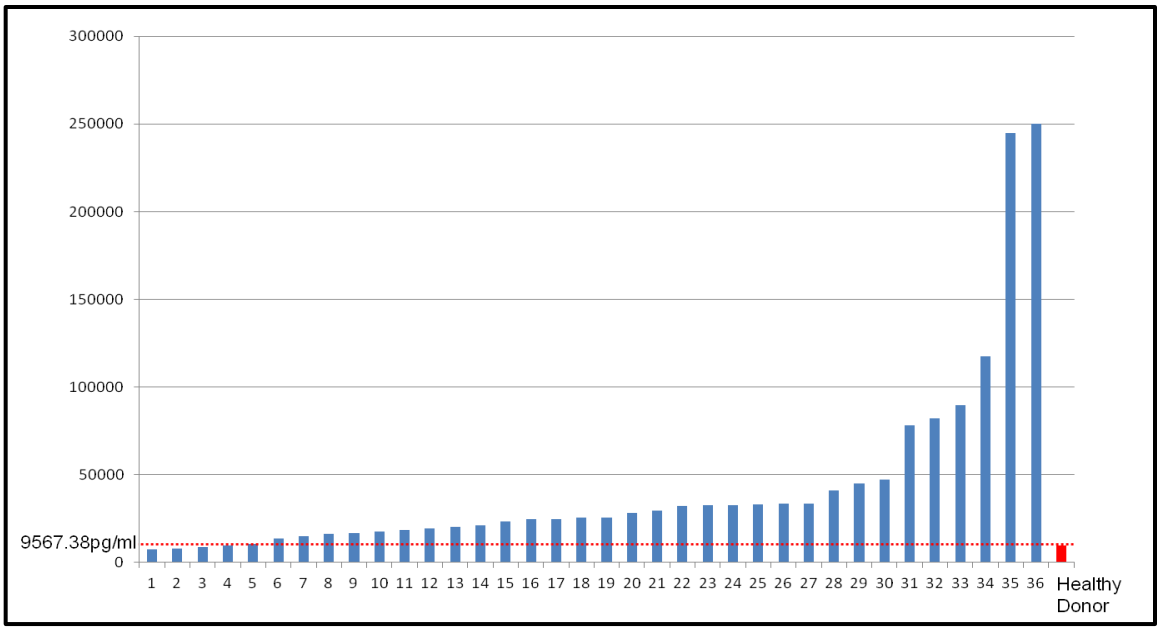


Figure 8-2 Bar Chart representing Bradykinin Level in Clinical Samples

This Figure demonstrates the Bar Chart in which each bar represents the concentration of BK in CLL and Normal Donor Plasma Pool samples. The level of BK varies between CLL samples, although 9/36 samples have shown a significantly higher level of BK in comparison to 27/36 samples, where BK ≥33000pg/ml.

8.3 An Investigation into the Plasma Level of HMWK and PK in CLL Clinical Samples

8.3.1 Methodology

The evaluation of HMWK and Prekallikrein (PK) concentration in the plasma samples obtained from 36 CLL patients, previously analysed by ELISA, and relatively healthy controls were conducted by an MSc student, Paula Johnson, under supervision of Alex Clubley at the Haematology department, Coagulation section of Hull and East Yorkshire Hospitals NHS Trust utilising an automated one-stage factor assay on the Instrumentation Laboratory (IL) TOP 700 CTS analyser (Figure 8-4). The one stage activated partial thromboplastin time (APTT) -based factor assay is widely used for the measurement of coagulation factors including Prekallikrein and HMWK. This assay is designed to utilise an estimated ability of dilutions of a known standard vs. factor deficient plasma to adjust the time that takes for a sample of blood to clot. The HMWK and PK assays were run using a commercial calibrator with known levels of both analytes, supplied by Technoclone (Technoclone Coagulation Reference plasma). The clotting time of different dilutions of this plasma was determined by running APTTs after dilution in plasma deficient in the analyte of interest (PK deficient plasma was Technoclone Fletcher Trait, HMWK deficient plasma was Technoclone Fitzgerald Trait) but included all other factors required for normal blood clotting. Results were used to plot the curve (time in seconds versus % of analyte). Controls used were: normal – the

Reference plasma used to produce the curve, abnormal: Technoclone Coagulation A. Controls were run with each batch of tests. The patients, controls and calibrators

were diluted 50:50 in factor diluents (buffer solution provided by IL) and then diluted 1:10 in the appropriate factor deficient plasma. An APTT using IL synthasil and calcium reagents was then performed and the clotting time in seconds was read of the curve to give a % value for the analyte.

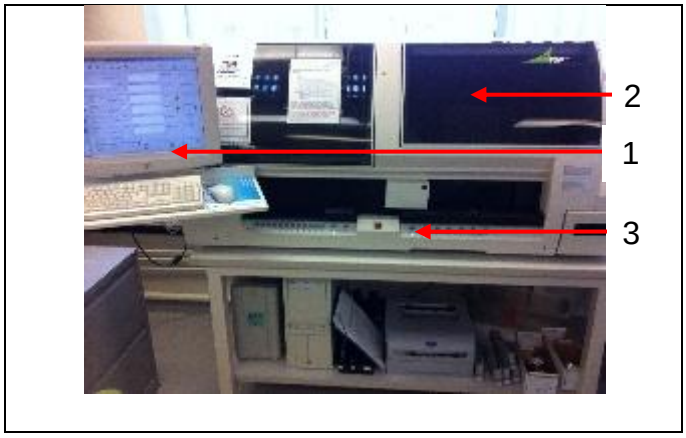


Figure 8-3 ACL-TOP Coagulation Analyser Marketed by Instrumentation Laboratory

ACL-TOP is a fully automated coagulation analyser, that consists of two major parts, which are: a personal computer (1) and an analyser (2). ACL-TOP analyser allows running multiple assays with high throughput holding up to 120 samples and maintaining 60 reagents (3).

8.3.2 Result of HMWK and Prekallikrein Plasma Levels Determination Utilising an Automated One-Stage Factor Assay

The values for HMWK and Prekallikrein parameters in Normal Donor Plasma Pool and CLL samples demonstrated normal distribution profiles and are presented in the Table 8-4.

8.4 Determination of the Cut-off Values for HMWK, PK and BK and their prognostic impact

In order to examine the prognostic value of the assays and subsequently to discriminate CLL samples following two-group classification: with high and low factor levels, particularly with respect to HMWK, PK and BK, the cut-off points were determined using statistical analysis. The cut-off value for a candidate biomarker can be estimated by utilising a variety of statistical methods. Two standard deviations of the mean (Mean ± 2SD) (the utilisation of 95% confidence interval (CI) of mean for determination of cut-off value), receiver operating characteristic (ROC) analysis (this method will be discussed in detail in a subsequent section) and discriminant function analysis (when a discriminant score is assigned for each case, dichotomising cases corresponding to the observed test value) were undertaken. In particular, ROC analysis has been widely utilised to investigate the thresholds for several potential biomarkers in the field of CLL (Trojani *et al.*, 2010, Mazumdar *et al*., 2013). Therefore, in the current project ROC analysis has been applied to investigate the most significant cut-off values for HMWK, PK and BK and their prognostic impact as the potential biomarkers.

The conventional ROC curve method was carried out on the available dataset of thirty six CLL cases by Dr. Rachel Crossland (Research Associate, Haematological Sciences at Newcastle University) using Sigma Plot (version 11) (Overall survival (OS) was utilised as the endpoint in this analysis). OS was defined as the time between the date of sampling and the date of death or last follow-up.

8.4.1 ROC Analysis

The ROC Analysis was originally developed by engineers during World War II for RADAR detection. Since then, it was promptly expanded and widely applied in the various fields including biomedical research and medical diagnostic (Zhou *et al.*, 2011). 'ROC analysis is widely used for evaluating the discriminatory performance of a continuous variable representing a diagnostic test, a marker, or a classifier' (Goncalves *et al* (2014:3). ROC utilises a curve, which allows the graphical representation of the relationship between true/false, positive/negative rates or the specificity (true negative rate) vs. sensitivity (true positive rate). ROC is a statistical tool, which is widely employed for assessment of the discriminatory power of a continuous marker for rationale separation into a two-group classification; for identification of an optimal threshold; for comparative analysis of two (or more) diagnostic tests/markers values; and also for estimation of the variability when two (or more) of the same continuous variables are determined (Goncalves *et al.*, 2014).

The ROC curve was generated by plotting the sensitivity (y-axis) vs. 1-specificity (x-axis) at different threshold settings. Each dot on the ROC curve was representative of a true positive rate / true negative rate pair, according to a particular decision threshold. The area under the ROC curve (AUC) measured the quality of a parameter ability to discriminate between two diagnostic groups (high/low).

Each factor (HMWK, PK, BK and lymphocyte count) was assessed utilising ROC curves, and was followed with a survival Kaplan-Meier Analysis using the

optimal thresholds identified by ROC as the cut-off to dichotomise the expression as high or low.

8.4.2 The Result of ROC and Kaplan-Meier Survival Analyses

The ROC analyses have shown that although HMWK, PK and BK are not prognostic for OS by ROC (p=0.81, p=0.16, p=0.67; Figures 8-4 A, 8-5 A, 8-6 A, respectively), the lymphocyte count was found to be significant for OS by ROC (p =0.04; Figure 8-7, A). The result offered the most useful validation of the patient cohort (n=36). The data were in line with the recent research conducted in the field of CLL, reporting that lymphocyte count is a valuable predictor of patient outcome, OS and TFT irrespectively of IgVH mutation, ZAP70, CD38 or FISH status (Shanafelt *et al*., 2009).

The ROC curves were generated to determine the HMWK, PK and BK expression cut-off values that best distinguished high vs. low cases. All optimal thresholds were calculated using ROC curve analysis (Figures 8-4, 8-5, 8-6, 8-7) and are provided in Table 8-3. Utilising cut-offs for dichotomising expression based on ROC, elevated plasma HMWK, PK and BK were identified in 64% (23/36), 72% (26/36) and 83% (30/36) of CLL patients, respectively.

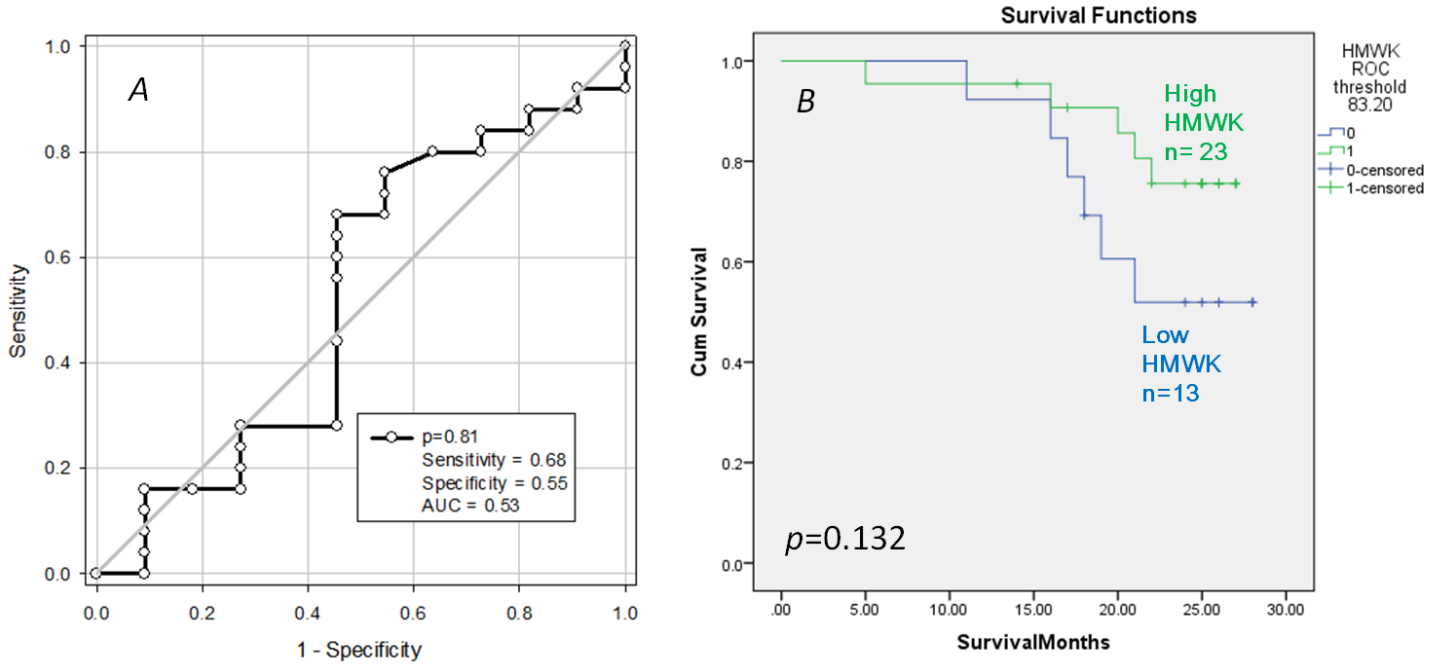
Table 8-4 Table of Factors / Markers Subjected to ROC and Kaplan Meier-based Survival Analyses

HMWK, PK, BK and lymphocyte count were analysed using ROC and the Kaplan–Meier methods. CLL samples were discriminated using thresholds for dichotomising expression based on ROC into two groups (High vs. Low). Lymphocyte Count Data was available on 35/36 samples. HMWK, PK data was extracted from the automated one-stage factor assay study, the methodology of which is explained in Section 8.3.1. During the automated one-stage factor assay, clotting time in seconds was read of the curve to give a % value for the analyte (HMWK and PK).

Factor/Marker	HMWK		PK		BK		Lymphocyte Count	
Two-Groups Classification	High	Low	High	Low	High	Low	High	Low
N° CLL Cases	23	13	26	10	30	6	15	20
Threshold	83.20%		96.70%		14225 pg/mL		14.85x10⁹/L	
p-value OS by Factor-using KM Plot	0.132		0.005		0.009		0.004	

Notwithstanding the statistically valid results obtained from the analysis, it is important to emphasise that the study cohort only consisted of thirty six CLL cases, which can be considered as a small sample size. The main influence of a small sample size is the effect it has on statistical power. The limitations of this analysis will be further discussed in the Chapter conclusion. Treatment and survival data (from all causes) was last updated in December 2012. In the study cohort, at the time of writing, the patients' follow-ups ranged from 16 months to 310 months with a median of 67 months.

Figure 8-4 ROC and Kaplan Meier-Based Survival Analyses of HMWK

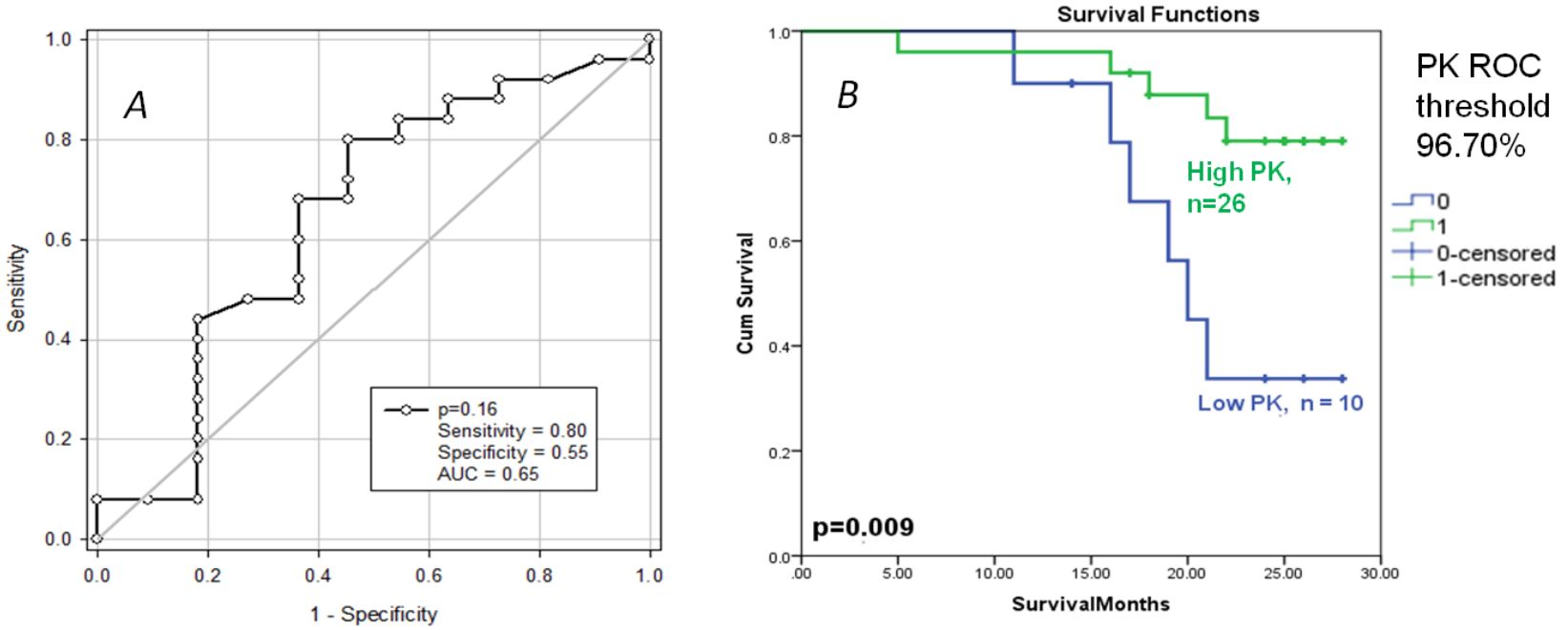


This is a ROC analysis of HMWK and OS generated by SigmaPlot (A) and Kaplan–Meier HMWK-specific survival of the 36 CLL patients (B).

A. Each point on the ROC curve represents the true-positive rate and false-positive rate associated with a particular test value. ***Sensitivity:*** *the fraction of CLL cases with the disease that the test correctly identifies as positive, whilst* ***Specificity:*** *the fraction of CLL cases without the disease that the test correctly identifies as negative. The AUC value provides a useful parameter to compare different tests. An AUC value close to 1 indicates an excellent diagnostic test; a curve that lies close to the diagonal (AUC = 0.5) has a lower information content and diagnostic utility, whereas, when it is near 0-there is higher chance of being incorrectly assigned into opposite group.*

B. OS was calculated using the Kaplan–Meier method and survival curves were compared using the log-rank test. The cut-off for dichotomising expression as high (n=23) vs. low (n=13) was based on ROC: 83.20 %. No significant correlation between HMWK and OS was found (p=0.132). Censoring occurs when an individual case has achieved its total available follow-up time without an event (death) had occurred. Deaths not attributable to B-CLL were censored. Median survival by HMWK plasma level was not reached for high HMWK level, for low level it was 21 months. Mean survival was calculated to be 24 and 22 months for high and low HMWK, respectively.

Figure 8-5 ROC and Kaplan Meier-Based Survival Analyses of PK

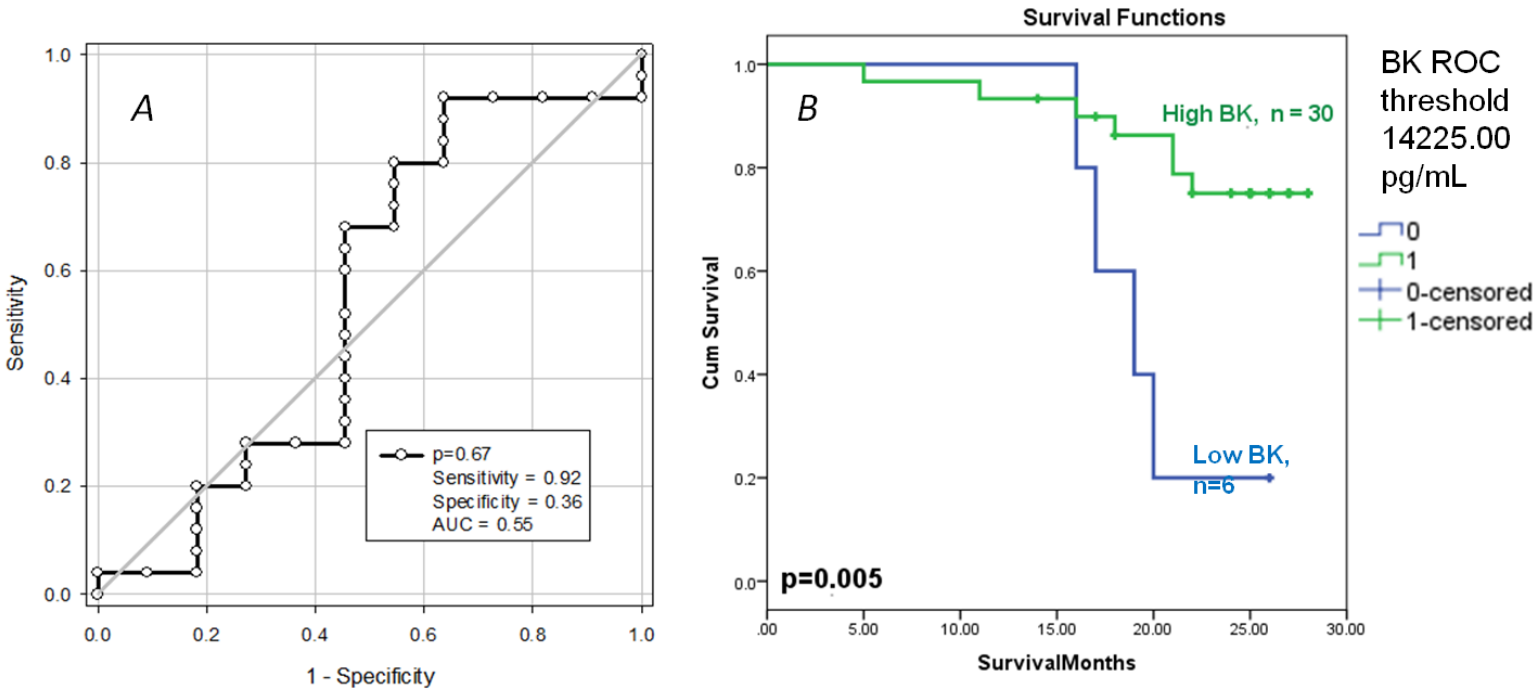


This is a ROC analysis of PK and OS generated by SigmaPlot (A) and Kaplan–Meier PK-specific survival of the 36 CLL patients (B).

A. The ROC curve has demonstrated that PK is not prognostic for OS by ROC (p=0.16). The ROC curve-identified threshold is 96.70%. Utilising ROC-identified threshold high PK was identified in 26/36 CLL cases.

B. Kaplan Meier plot is showing survival of thirty six CLL samples by PK. PK and OS were found significantly correlated (p=0.009, Mantel-Cox log rank). Median survival by PK level was not reached for high PK level, for low level it was 20 months. Mean survival was calculated to be 25 months and 20 months for high and low PK respectively.

Figure 8-6 ROC and Kaplan Meier-Based Survival Analyses of BK in CLL Study Cohort

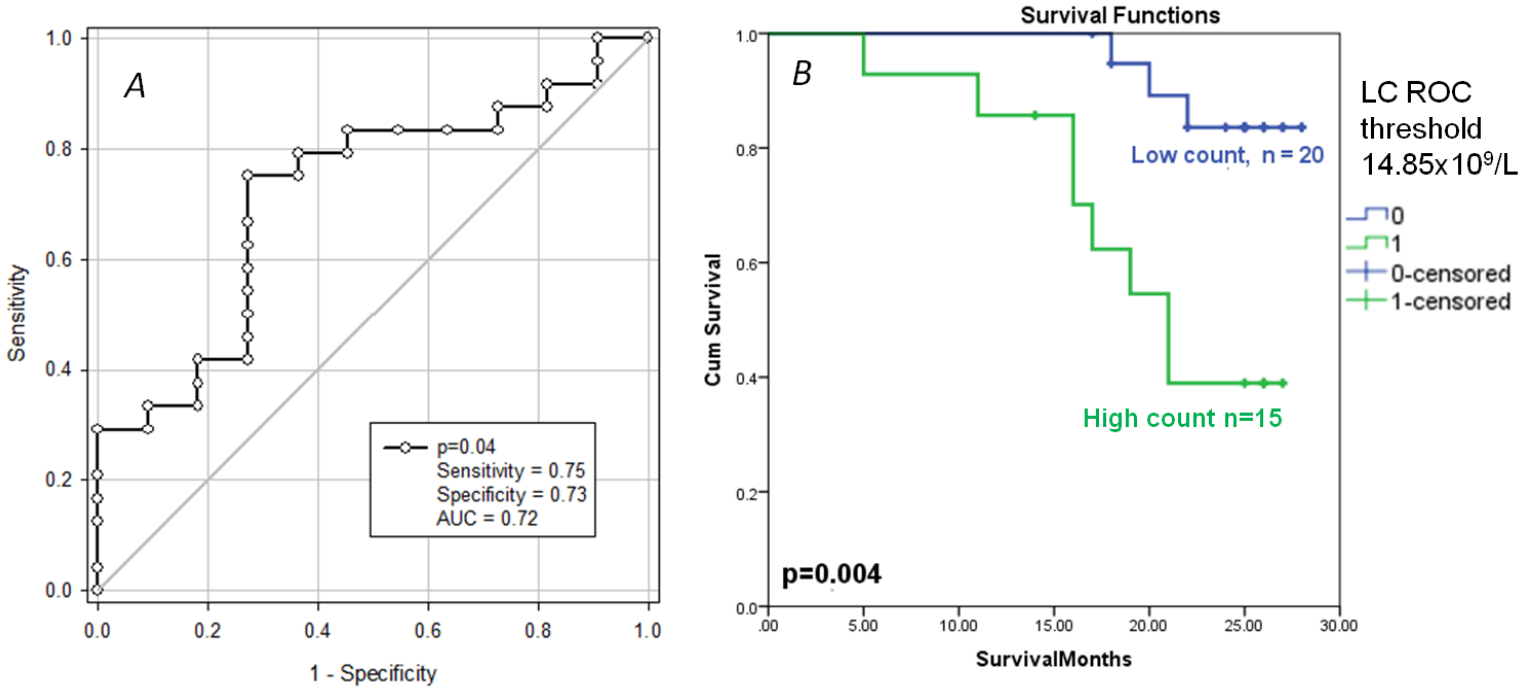


This is a ROC analysis of BK and OS generated by SigmaPlot (A) and Kaplan–Meier BK-specific survival of the 36 CLL patients (B).

A. The ROC curve has demonstrated that BK is not prognostic for OS by ROC (p=0.67). Using threshold for dichotomising expression based on ROC: 14225.00 pg/mL, high BK was identified in 30/36 CLL cases.

B. Kaplan Meier plot is showing survival of thirty six CLL samples by PK. PK and OS were found significantly correlated (p=0.005, Mantel-Cox log rank). Median survival by BK was not reached for high BK, for low level it was 19 months. Mean survival was calculated to be 25 months and 19 months for high and low BK respectively.

Figure 8-7 ROC and Kaplan Meier-Based Survival Analyses of Lymphocyte Count in CLL Study Cohort



This is a ROC analysis of lymphocyte count (LC)and OS generated by SigmaPlot (A) and Kaplan–Meier LC-specific survival of the 36 CLL patients (B).

A. The ROC analysis has demonstrated that LC is found to be significant for OS by ROC (p =0.04).). Recently, several studies have reported the association of Lymphocyte Count and CLL OS. Lymphocyte Count remains to be a valuable independent predicting marker for TFS after IgVh, ZAP70, CD38 and FISH (all p=0.001) (Shanafelt et al., 2009). This data offers the most useful validation of the patient cohort (N=36). Using threshold for dichotomising expression based on ROC: 14.85x10^9/L, high LC was identified in 15/35 CLL cases. LC data was only available on 35 CLL cases.

B.Kaplan Meier plot showing survival of CLL samples by Lymphocyte Count. Using threshold for dichotomising expression based on ROC: 14.85x10^9/L. Lymph Count and OS were found significantly correlated (p=0.004). Median survival by Lymphocyte count was not reached for low Lymphocyte count, for high it was 21 months. Mean survival was calculated to be 20 months and 26 months for high and low Lymphocyte count respectively.

Table 8-5 Clinical Data and Concentrations of Plasma HMWK, Prekallikrein and BK in CLL Study Cohort

CLL cases with high level of HMWK, PK and BK are highlighted in red

SAMPLE N^0	AGE	GENDER	BINET	HMWK	PK	BK pg/mL	Lymphocyte Count x10^9/L
4	63	M	A	**83.60%**	82.40%	**16519.86**	39.7
13	85	M	**C**	74.00%	85.80%	**21366.68**	113.9
19	67	M	A	**93.70%**	**128.20%**	**32480.12**	73.9
22	61	M	**B**	79.40%	**122.80%**	**40906.25**	13.1
30	64	M	A	82.80%	**110.40%**	**33430.03**	1.2
31	72	M	A	81.90%	**97.20%**	**24642.5**	0.4
44	59	M	**B**	79.40%	77.50%	8872.07	45.7
57	63	M	**B**	**102.90%**	96.20%	13537.95	3.2
58	67	M	A	72.60%	91.30%	**33239**	202
73	78	F	A	**100.80%**	**100.30%**	**17761.07**	15.1
100	82	F	A	70.40%	80.70%	9687.74	55.8
116	57	M	A	**109.70%**	**136.80%**	**78368.35**	43.2
125	75	M	A	**115.80%**	**124.10%**	**32587.74**	2.9
152	74	M	A	**90.80%**	**102.50%**	**24789.25**	14.6
155	74	M	A	81.10%	**125.50%**	**82007.71**	1.1
164	86	F	A	**89.80%**	**101.40%**	**23203.74**	0.4
173	53	M	A	**127.70%**	**138.30%**	**244664.7**	89
185	85	M	**B**	77.90%	81.50%	10734.65	18.6
188	54	F	A	**108.60%**	**172.50%**	**250170.3**	0.8
223	54	F	A	**105.23%**	**131.00%**	**47223.91**	8.9
245	71	M	**B**	**90.80%**	**99.30%**	**20495.04**	105.6
263	57	M	A	77.10%	**97.20%**	**14912.18**	5.1
272	84	F	A	69.70%	80.70%	7607.66	133.7
273	64	M	A	**90.80%**	**104.70%**	**16983**	171.7
283	75	M	**A**	**89.80%**	**101.40%**	**25507.24**	17.1
285	76	M	A	**89.80%**	**101.40%**	**25500.20**	
311	69	M	**B**	**92.70%**	**151.00%**	**117406.3**	1.5
314	77	F	A	**96.60%**	**116.40%**	**19273.4**	9.0
323	53	M	**C**	71.10%	89.50%	8129.59	0.3
329	69	M	A	**84.50%**	**172.50%**	**89577.33**	1.0
330	58	M	A	**88.90%**	**117.70%**	**32205.33**	1.3
344	57	M	A	**89.80%**	**110.40%**	**33703.18**	1.8
345	87	F	NO	**92.70%**	**109.20%**	**44883.12**	44.8
347	73	M	A	73.30%	94.20%	**18495.07**	5.3
351	72	F	A	**113.30%**	**131.00%**	**29553.69**	1.1
361	79	M	A	**98.70%**	**120.20%**	**28478**	8.4

8.5 Assessment of the Relationship between Plasma PK/BK Levels and Clinical Features in CLL Study Cohort

In order to determine the significance of associations between PK and BK and their association with available clinical data such as Lymphocyte count, clinical stage, treatment and vital statuses, Spearman's rank correlation test (with help of Dr. Rachel Crossland, Research Associate, Haematological Sciences at Newcastle University) and Pearson's Chi-Square Test (SPSS version 19) were used. Data concerning other clinical markers such as IgVH mutation status, ZAP70, CD38 or FISH was unavailable for this study cohort.

The result revealed a strong and highly significant association between BK and PK plasma levels (Spearman's rank correlation test, p=0.001) as well as a weak-moderate inverse correlation between PK and Lymphocyte Count ($p=0.018$) among the whole patient population (N-35) (Table 8-6).

Table 8-6 Correlation Analysis of BK/PK and Lymphocyte Count

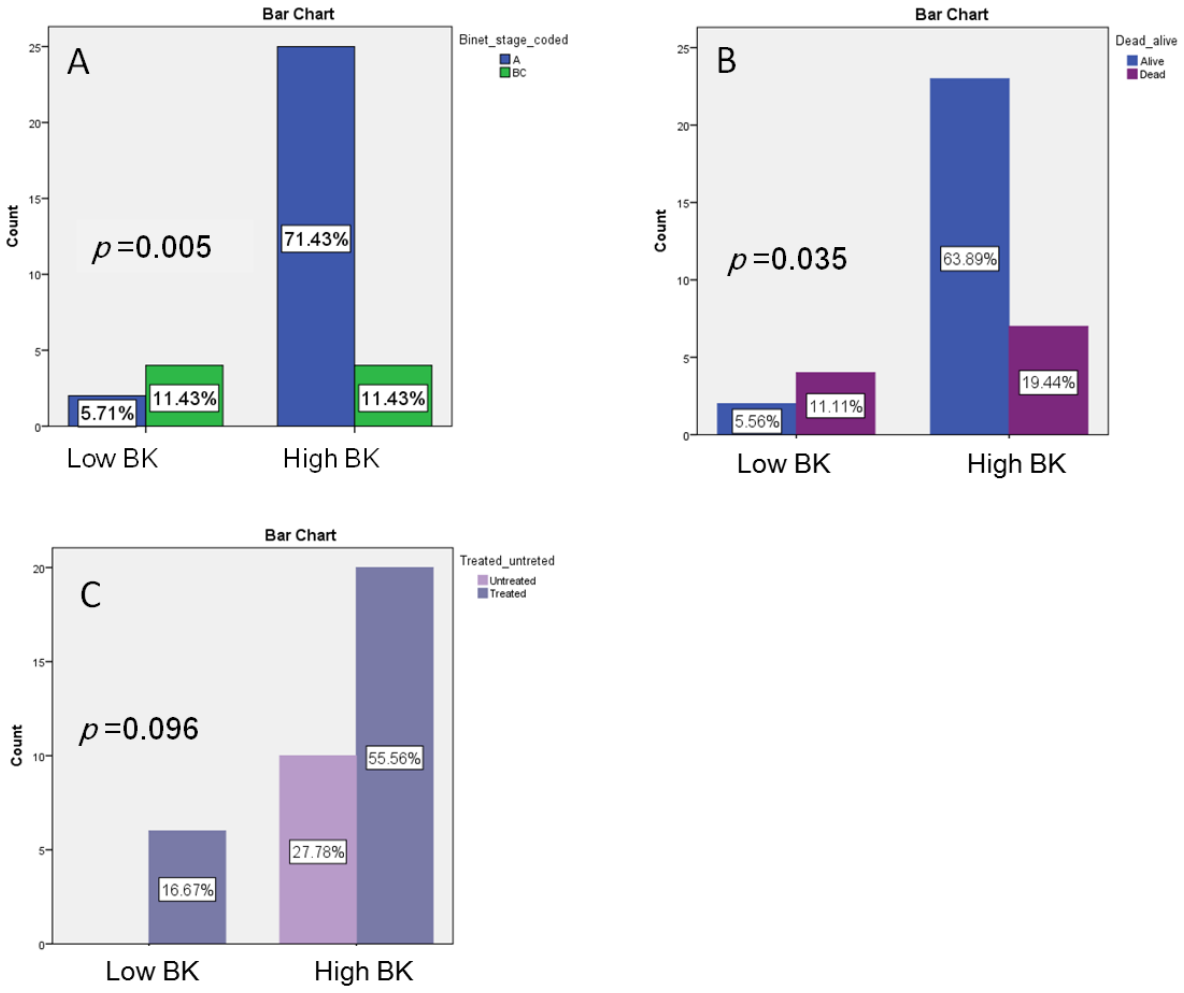
This Table represents the result obtained from the correlation analysis of PK and BK as well as association study of PK/BK and lymphocyte Count utilising Spearman's rank correlation coefficient. The data indicated that there is a significant weak-moderate inverse correlation between PK and Lymphocyte Count (p=0.018). Additionally, an inverse trend between BK and Lymphocyte Count can be observed but this is not to a significant level (p=0.107). Moreover, the correlation between PK and BK expression is shown to be strong and highly significant (p=0.001).

			PK	BK	LymphCount
Spearman's rho	PK	Correlation Coefficient	1.000	.747**	-.398*
		Sig. (2-tailed)	.	.000	.018
		N	36	36	35
	BK	Correlation Coefficient	.747**	1.000	-.277
		Sig. (2-tailed)	.000	.	.107
		N	36	36	35
	LymphCount	Correlation Coefficient	-.398*	-.277	1.000
		Sig. (2-tailed)	.018	.107	.
		N	35	35	35

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

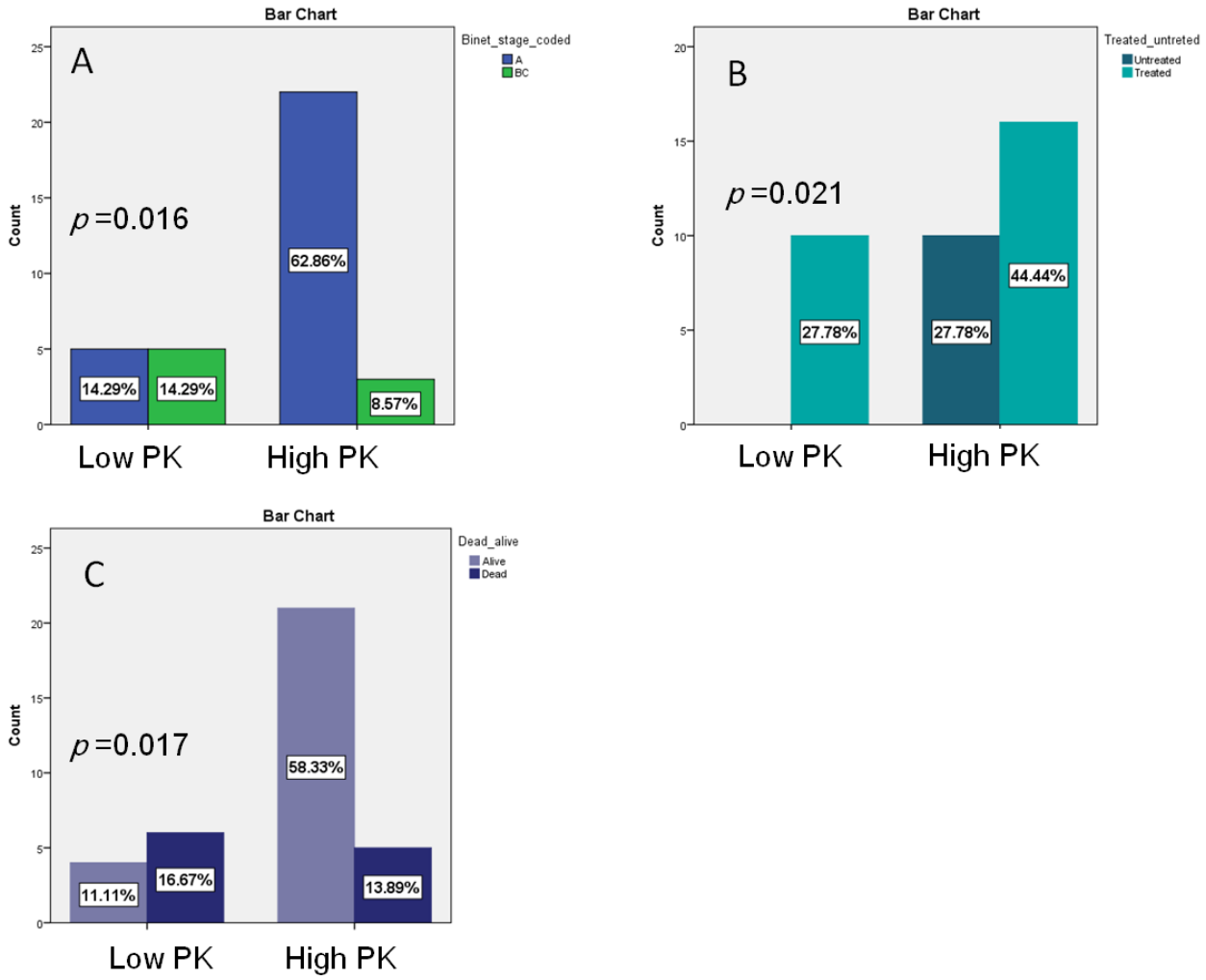
In respect of determination of possible correlation between BK/PK and CLL clinical characteristics, Pearson's Chi-Square Test was employed and the following result was found: the high plasma BK concentration was more often associated with stage A (*p=0.005*) and live CLL patients (*p=0.035*). Additionally, there was a trend towards association between high BK level and untreated CLL patients, although this is not to a significant level (*p=0.096*) (Figure 8-9). A high level of plasma PK was shown to be significantly associated with Stage A (*p*=0.016), alive (*p*=0.017) and untreated (*p =0.021*) CLL cases (Figure 8-10).

Figure 8-8 Bar Charts Representing Correlation between Plasma Bradykinin Concentration and Sample Clinical Characteristics



Bar charts representing relationship between BK and Binet stage A/BC (A), Alive/Dead (B) and treated/untreated (C) statuses. Elevated level of BK is shown to be associated with Alive (p =0.0035) Binet Stage A (p=0.005) CLL patients. All statistical calculations were performed using Pearson Chi-Square Test (SPSS, version 19).

Figure 8-2 Representative Bar Charts of the Correlation Analysis between PK and Clinical Data



Bar charts representing relationship between PK and Binet stage A/BC (A), Treated/untreated (B) and Alive/Dead (C) statuses. High Plasma level of PK is shown to be associated with Alive (p =0.017) Untreated (p =0.021) Binet Stage A (p=0.016) CLL patients. All statistical calculations were performed using Pearson Chi-Square Test (SPSS, version 19).

8.6 Discussion

Plasma level of Bradykinin has been extensively studied in several clinical conditions due to being associated with cell migration, proliferation, vascular

permeability and sustaining the inflammatory response (Renne, 2012, Yu *et al.*, 2013). Recently the number of studies investigating Bradykinin levels in different malignancies have increased due to the reported carcinogenic role of Bradykinin in prostate and lung cancer (Stewart *et al.*, 2002, Stewart *et al.*, 2005) . However, to the best of our knowledge, no Bradykinin-associated research was available in the field of CLL. Utilising previous findings (Chapter 5) it is hypothesised that CLL B-lymphocytes express differing levels of Kininogen, the expression of which was significantly increased upon BCR activation. Therefore, one of the main aims of this Chapter was to specifically measure the plasma levels of Bradykinin (the downstream molecule of the KKS pathway) in CLL samples. Additionally, the data acquired from the associated study of HMWK and PK plasma levels were analysed in this Chapter to simultaneously evaluate the prognostic impact of HMWK, PK and BK as putative biomarkers.

In order to achieve this aim the Bradykinin ELISA assay was employed and the results indicated that the calculated average Bradykinin concentration in Normal Donor Plasma Pool was 9567.38pg/mL. No studies were readily available to benchmark the known level of Bradykinin in normal plasma samples utilising the same ELISA kit. The level of plasma Bradykinin in B-CLL patients was found to range between 7607.66 pg/mL to 250170.28 pg/mL.

The ROC curve was used to determine the HMWK, PK and BK threshold values by OS, best discriminating between high and low levels, potentially suggesting prognostic significance of the parameters investigated in the present study.

Given that BK is the downstream molecule in the KKS pathway (Figure 3-1), it is hypothesised that there may be an association between BK and PK plasma levels. The results confirmed that there was a highly significant association (Spearman's rank correlation test, *p=0.001*). Both BK and PK are important constituent parts of KKS and have demonstrated a similar association between high plasma level and favourable patient outcomes. Additionally, a weak-moderate inverse correlation was found between PK and Lymphocyte Count (*p=0.018*) and inverse correlation between BK and Lymphocyte count but not to a significant level (*p =0.107*).

As outlined in Chapter 3 (Figure 3-1) human plasma Prekallikrein is a zymogen precursor for plasma Kallikrein, which is mainly secreted by hepatocytes but has been reported to be synthesised extrahepatically. Prekallikrein is secreted into plasma as an inactive zymogen, which in 75% activates *in vivo* and becomes plasma Kallikrein by docking to HMWK and in the remaining 25% it circulates freely in plasma (Moreau *et al.*, 2005, Colman, 2006). Following the results from Chapter 7, it can be suggested that both plasma and tissue KLK are present in CLL, which therefore may contribute to the continuous activation of KKS and increased level of liberated Bradykinin.

Following this, CLL samples were discriminated, based on two-group classification into: high (BK≥14225 pg/mL) and low BK plasma levels (BK<14225 pg/mL). A high level of BK was identified in 30/36 (83%), whereas 6/36 (17%) of samples from the study cohort have shown a low BK level. The statistical analyses demonstrated that high BK is significantly associated with stage A (Pearson Chi-Square Test, *p=0.03*) live CLL cases (*p=0.035*). Moreover, there was a trend

towards an association of Bradykinin levels with untreated status (*p=0.096*). In respect of plasma PK, the data have indicated a significant association of high PK with Stage A (Pearson Chi-Square Test*, p=0.016*), alive (*p=0.017*) and untreated (*p =0.021*) CLL patients.

Although, some of the statistical analyses carried out on the available dataset demonstrated significance, there is always likelihood for sampling error due to the small sample size. Sample collection was successful and resulted in a blood donation from almost every suitable CLL patient, who attended the clinic. However, this was a pilot study of only thirty six CLL cases and this cohort was limited in number partly due to cost and time limitations. A larger study should follow due to its potential value and if the results thereon were statistically aligned, then the results obtained from this study would be compelling.

In summary, the data obtained from this work is shown to be statistically compelling, which highlights the need for further investigation into PK and BK as clinically relevant biomarkers by utilising a larger sample cohort of relevant CLL patients to challenge or support the overall hypothesis. The identification and development of putative biomarkers would help to determine the clinical heterogeneity of CLL, support patient management, prognosis and help to predict CLL outcomes.

CHAPTER 9

Evaluation of Kinin Receptors Surface Expression in CLL and Normal B Cells

Chapter 9. Evaluation of Kinin Receptors Surface Expression in CLL and Normal B Cells

The previous Chapter demonstrated that plasma Bradykinin was found to be elevated in 27/36 (75%) CLL cases which also was associated with untreated stage A CLL. The main objective of this chapter is to investigate whether Kinin-specific receptors are expressed on the surface of CLL vs. normal B cells utilising the scientific techniques such as immunoblotting and flow cytometry.

Bradykinin and Lys-Bradykinin act upon two transmembrane receptors from G-Protein-Coupled Receptor super family (GPCR) known as Kinin B_1 and B_2 receptors as discussed in detail in Chapter 3 (Figure 3-1). The depth of knowledge to date is superficial in the context of the expression of either B_1 or B_2 receptors on the surface of CLL or healthy B-cells. The potential identification of the latent possibility of Kinin B_1 and/or B_2 receptors expression on the surface of CLL B-cells may validate the hypothesis that CLL B-cells posses all the components for Kininogen Kinin-Kallikrein System signalling.

9.1 Introduction to the Structural Aspects, Subtype Specificity, Activation and Signalling of the Kinin B_1 and B_2 Receptors

Kinin B_1 and B_2 receptors are composed of seven hydrophobic domains that span the membrane from one side of the phospholipid bilayer to another (Leeb et al., 1997) (Figure 9-1). Kinin B_1 and B_2 receptors (353 and 391 amino acids, respectively) differ in their primary structures and only share 36% of overall structural homology (Menke *et al*., 1994). These structural differences between B_1 and B_2 receptors may serve as a catalyst for their ligand heterogeneity, which, however, only differentiate in the carboxy-terminus amino acid (Leeb *et al*., 1997) (Section 3.4.3.2). The study has shown that specific B_1 and B_2 receptors binding activity is likely to be regulated by the sequence heterogeneity in a transmembrane domain 6, which was also found to be responsible for the subtype specificity (Leeb *et al*., 1997). Despite the structural and ligand heterogeneity, which is transcribed into different physiological effects of kinins (Kuhr *et al*., 2010), both receptors can trigger similar downstream signalling cascades (Tropea *et al*., 1993). Thereupon, several conducted studies have indicated that B_1 receptors can be upregulated under conditions when B_2 receptors are absent or inactive (Duka et al., 2006b). Other researchers have shown that persistent stimulation of B_2 receptors may lead to crossactivation of B_1 receptors (Calixto *et al*., 2004) with subsequent triggering of the downstream signalling pathways. Moreover, evidence indicated that under the condition of chronic inflammation the kinin B_1 receptors can augment or substitute kinin B_2 receptors (Ahluwalia and Perretti, 1999, Phagoo *et al*., 1999,

Bockmann and Paegelow, 2000). In conclusion, data suggests that B_1 and B_2 receptors crosstalk (Calixto *et al*., 2004) via complex molecular signal transmission pathways.

Upon an antigen binding with the large extracellular ligand-binding domain of B_1 and/or B_2 receptor, receptor undergoes conformational changes and activates heterotrimeric G-proteins, which transmit the extracellular signals to the second messengers (Ferguson, 2001), and then downstream into the cell, evoking a physiological response. G-protein composed of *alpha* (α), *beta* (β) and *gamma* (γ) subunits (Hurowitz *et al.*, 2000). An activated G-proteins regulate the molecular mechanisms for desensitisation and re-sensitisation (Ferguson, 2001) via heterotrimeric G protein uncoupling and coupling, respectively. When physiological conditions are not impaired kinin receptors undergo receptor desensitisation in time-dependant manner, which prevents acute and/or chronic receptor overstimulation (Ferguson, 2001).

9.1.1 Classical Signalling Pathways Mediated by B_1 and B_2 Kinin Receptors

Upon extracellular agonist engagement with co-expressed B_1 and/or B_2 kinin receptors, the signal transduction occurs, which results in the simultaneous activation of several downstream signalling pathways and subsequent cellular responses (Figure 9-1). Several studies have indicated that certain Kinin receptor signalling cascades are cell/tissue -specific and are prioritised for the activation according to participating cell or tissue type (Ehrenfeld *et al.,* 2012). Ultimately, the receptor-associated signal triggers an activation of a membrane-bound enzyme,

phospholipase C (PLC) (Madeddu *et al.*, 2007). Subsequently, PLC digests phosphatidylinositol 4, 5-bisphosphate (PIP2), a phospholipid that is localised in the plasma membrane, and generates two classic second messengers such as triphosphoinositol (IP_3) and diacylglycerol (DAG). IP_3 and DAG, mediates calcium signalling resulting in release of intracellular calcium ions (Ca^{2+}) from smooth endoplasmic reticulum (ER) and turns on downstream protein kinase C (PKC), respectively. Calcium signalling plays an essential physiological role for cellular motility, cell growth and proliferation and signal transmission. PKC in turn is widely involved in the intracellular signalling network and regulates phosphorylation of other intracellular proteins and enzymes. The agonist-mediated activation of the kinin receptors simultaneously induces an immediate and sustained activation of the mitogen-activated protein kinase (MAPK) / nuclear factor-kappa B (NF-κB) pathways (Vidal *et al.*, 2005) and as a result a MAPK-dependent phosphorylation of phospholipase A2 (PLA2). At this point, it is important to emphasise that Kinin receptor-dependant 42/44 MAPK pathway phosphorylation plays an important role in cell cycle modulation (Ehrenfeld *et al*., 2012), including cell longevity, differentiation (Vidal *et al.*, 2005) and proliferation. The MAPK-MEK-1/2 ERK-dependant proliferative effect has been shown in several cell/tissue types including vascular smooth muscle cells, estrogen-sensitive breast cancer cells (Christopher *et al*., 2001, Molina *et al*., 2009) keratinocytes (Vidal *et al*., 2005) and fibroblasts. However, some of these studies have demonstrated that this response was associated with epidermal growth factor receptor (EGFR) transactivation (Ehrenfeld *et al.,* 2012). Furthermore, several of the GPCR ligands initiate the activation of cellular proto-oncogene tyrosine-protein kinase Src, which is largely

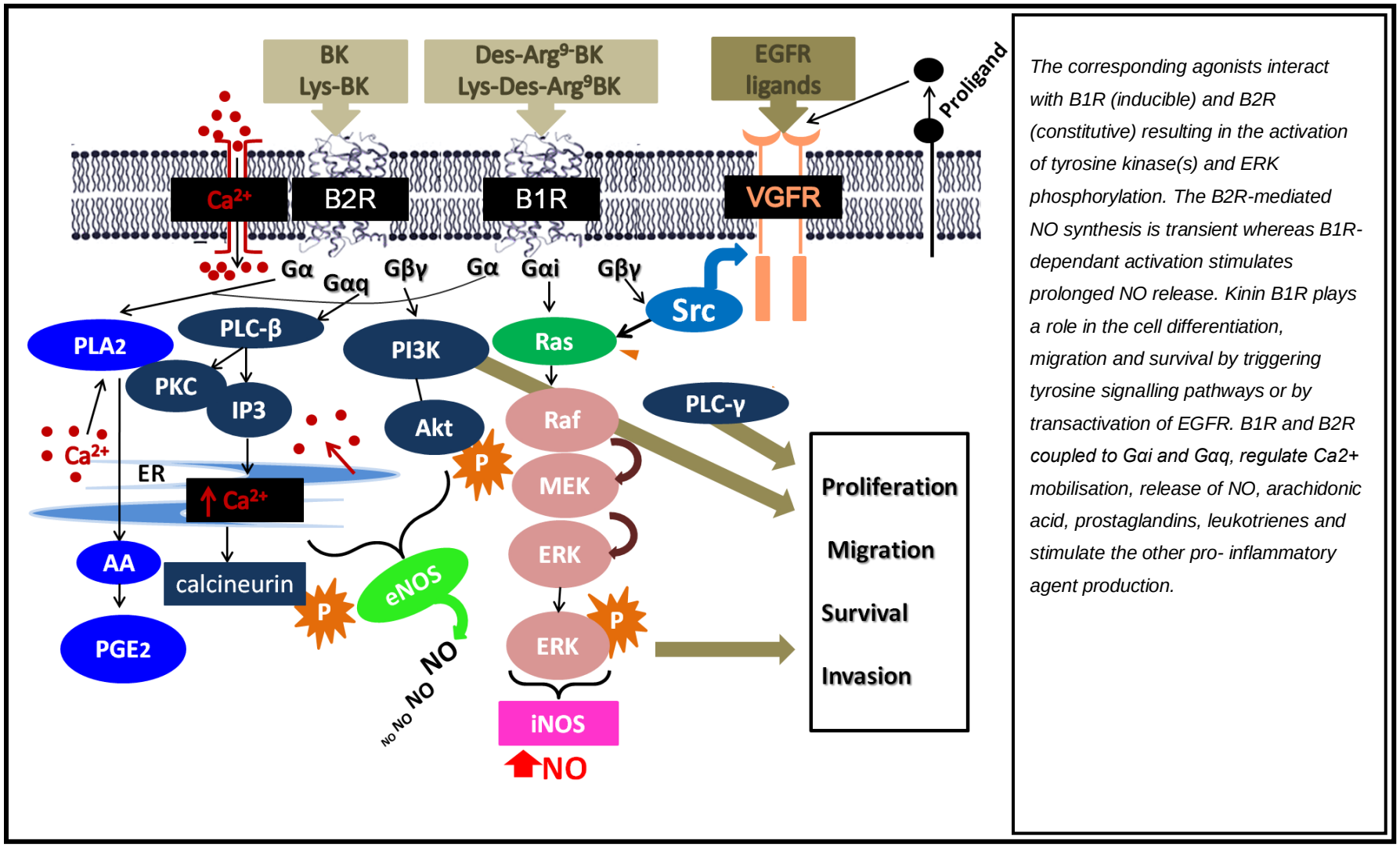
known to be involved in phosphorylation of EGFR (Ehrenfeld *et al*., 2012). Activated EGFR serves as the signal transducer for Kinin receptors with subsequent triggering of the downstream signalling cascades, including MAPK pathway (Tice *et al.*, 1999). Moreover, multiple evidences have indicated that cellular Src and EGFR up-regulation are associated with carcinogenesis and tumour progression (Tice *et al.*, 1999, Irby and Yeatman, 2000, Sen and Johnson, 2011).

The Kinin receptor-associated influx of intracellular Ca^{2+}, which in turn prompts an entry of extracellular Ca^{2+}, leads to both stimulating phospholipase A_2-dependant (PLA_2) cascade and subsequent calcineurin-dependent mediation of endothelial nitric oxide (eNOS) (Madeddu *et al*., 2007). Upon downstream signalling, PLA_2 stimulates liberation of arachidonic acid (AA), which is metabolised by cyclooxygenases (COX) to generate prostaglandins (Bhoola *et al.*, 1992, Bockmann and Paegelow, 2000, Bhoola *et al*., 2001, Leeb-Lundberg *et al.*, 2005). The time and dose dependent B_2 receptor-mediated arachidonic acid release and following prostanoid formation was shown in gingival fibroblasts (Modeer *et al.*, 1990). Additionally, activated Kinin receptors trigger the phosphatidylinositol 3 kinase (PI3K) pathway (Madeddu *et al*., 2007). Accumulating evidences suggest that PI3K enzyme and PI3K / Akt (serine-threonine kinase)-downstream pathways are crucial elements in cell senescence, proliferation, differentiation, cell migration and therefore known to be associated with carcinogenesis and neoplasia (Fresno Vara *et al.*, 2004). Phosphorylated Akt triggers a series of biochemical reactions leading to significant increase in the endothelial nitric oxide synthase (eNOS), which has been associated with kinin B_2 receptor activation (Madeddu *et al*., 2007).

Whereas, B_1 receptor stimulate NO synthesis (Bader, 2009). The data demonstrates that Kinin receptor-associated elevated concentration of nitric oxide, VGFR crossactivation and prostaglandin liberation play a critical role for induction of neovascularisation and angiogenesis (Bader, 2009).

The ability of B_1 receptors to initiate the intracellular signalling pathways is reciprocal to that of B_2 receptors (Leeb-Lundberg *et al*., 2005), however, the difference in the downstream effects is prompted by the distinctive patterns of the receptor recycling upon its ligand engagement, not the sequence of interaction (Ehrenfeld *et al.,* 2006).

Figure 9-1Schematic Diagram Showing the Kinin B1 and B2 Signal Transduction Pathways



The corresponding agonists interact with B1R (inducible) and B2R (constitutive) resulting in the activation of tyrosine kinase(s) and ERK phosphorylation. The B2R-mediated NO synthesis is transient whereas B1R-dependant activation stimulates prolonged NO release. Kinin B1R plays a role in the cell differentiation, migration and survival by triggering tyrosine signalling pathways or by transactivation of EGFR. B1R and B2R coupled to Gαi and Gαq, regulate Ca2+ mobilisation, release of NO, arachidonic acid, prostaglandins, leukotrienes and stimulate the other pro- inflammatory agent production.

9.1.2 Mechanisms for Kinin B_1 and B_2 Receptors Regulation and Cellular Response

9.1.2.1 *The Recycling and Degradation of Kinin B_1 Receptors*

As previously discussed in Section 3.4.3.2 Kinin B_1 receptors are expressed in low abundance in the vast majority of healthy tissues and can be immediately induced by the action of secondary generated kinins, such as Des-Arg9-BK, *Lys*-Des-Arg9BK, bacterial endotoxins such as lypopolysaccharide, several cytokines (Duchene *et al*., 2007), like interleukin-1 and interleukin -8, tumour necrosis factor and other pro-inflammatory mediators such as epidermal growth factor and endothelial growth factor (Marceau and Bachvarov, 1998, Yang *et al*., 2001). The mechanisms underlying the unique expression profile of B_1 receptors were extensively studied during the past decades. It has been demonstrated that, the B_1 receptor susceptibility to desensitisation (which is significantly different from B_2 receptors and other GPCRs), kinin receptor-mediated generation of pro-inflammatory molecules (Calixto *et al.*, 2004) and activation of several distinct intracellular signalling pathways such as stress mitogen-activated protein and NF-κβ signalling cascades are associated with B_1 receptor abundance (Ehrenfeld *et al.*, 2006). Interestingly, kinin B_1 receptors are predisposed to internalisation even under basal conditions through clathrin-coated pits and subsequently taken up via endocytosis to the lysosome (Ehrenfeld *et al.,* 2012) (Figure 9-2). The ligand-associated receptor activation diminishes constitutive rate of B_1 receptor internalisation (Ehrenfeld *et al.,* 2012) during which B_1 receptor translocates and

aggregates to caveolae-related rafts (Sabourin *et al.*, 2002), which in turn decreases the rate of endocytosed receptors. This receptor behaviour, firstly, assists in B_1 receptor-mediated signals amplification, secondly, it delays degradation of a participating receptor and, lastly, in combination with cytokines and other pro-inflammatory molecules, leads to the dramatic receptor overexpression (Ehrenfeld *et al.,* 2012).

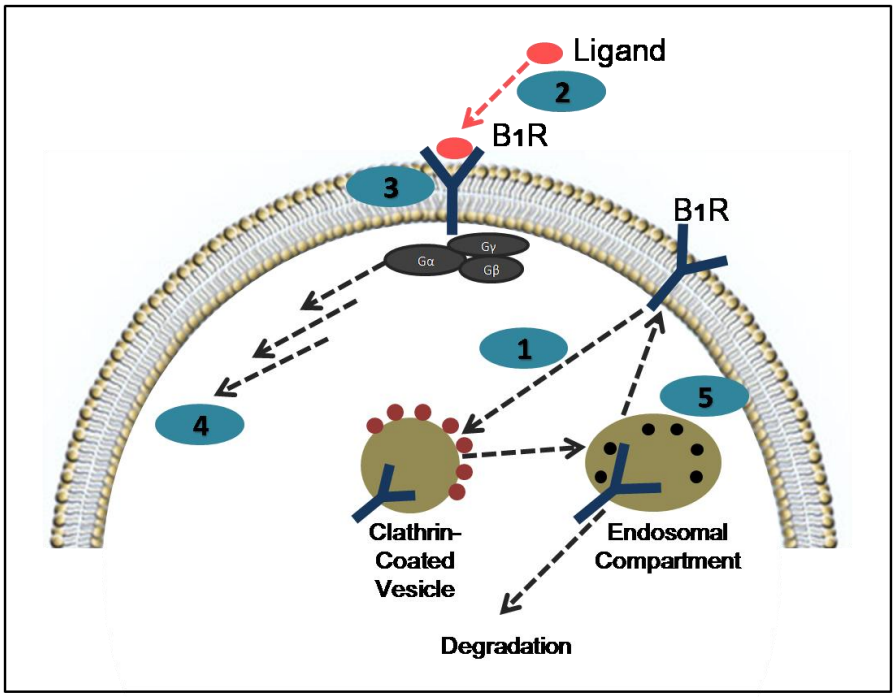


Figure 9-2 The Kinin B_1 Receptor Regulation.

The Kinin B_1 Receptors are constitutively internalised (1). Upon ligand interaction (2), the ligand encapsulates receptor in the membrane (3). Subsequently, the external signal is transmitted into the cell allowing to activate G-proteins and downstream signalling (4). Once receptor is in clathrin-coated vesicle, it is taken to endosomal compartment. Progressing from this the receptors can either be recycled back to the plasma membrane (5), which is defined as sequestration, or can be proceeded to lysosomes for subsequent degradation. Ligand binding induces Kinin B_1 Receptor upregulation by increasing the number of Kinin B_1 Receptors on the cell membrane. This diagram is adapted from (Ehrenfeld et al., 2012).

Nevertheless, in contrast to B_2 receptors, which initiate acute response and undergo immediate agonist-mediated sequestration, B_1 receptors escape endocytosis and sustain the signal and hence are likely to be responsible for prolonged and chronic activation (McLean et al., 2000b).

B_1 receptor-mediated stimulation is known to play an important role in:

- sub-acute and chronic inflammatory response and hyperalgesia,
- increase of cytosolic Ca^{2+} concentration,
- cell differentiation and migration via tyrosine signalling pathways mediation or by EGFR transactivation (Matus *et al.*, 2008).
- regulation of cell growth and protein phosphorylation,
- involved in leukocyte trafficking (Ehrenfeld *et al.*, 2006, Duchene *et al*., 2007).

9.1.2.2 *Pathways for internalisation and recycling of B_2 Receptor*

The study suggests that B_2 receptor activation is tyrosine kinase(s)-dependant and is inferior to receptor phosphorylation (Reyes-Cruz *et al.*, 2000). Following antigen-induced stimulation, B_2 receptor couples with G proteins (Haasemann *et al.*, 1998) and mediates the downstream intracellular signalling pathways, such as PLCβ / IP3 cascade, which results in the transient increase in intracellular Ca2+ activity, intermediate release of NO and immediate receptor desensitisation, which is characterised by transitory receptor downregulation (about 10-20 min). During this time the cell surface may remain refractory to any present ligands (Roscher *et al.*, 1984, Haasemann *et al.,* 1998). Activated B_2 receptors undergo rapid internalisation by endocytosis and B_2 receptor-mediated

signalling induces an instantaneous and potent cellular response. Whereas B_1 receptor activation triggers the robust but prolonged kinin effect and as such (about 90 min) NO synthesis, that results in post-translational activation of inducible nitric oxide synthase (iNOS) (Kuhr *et al.*, 2010) via extracellular signal regulated kinase (ERK)-induced phosphorylation (Brovkovych *et al.*, 2011).

The activation of B_2 receptor is known to be involved in the following processes:

- an acute inflammatory response to antigenic stimulus (Chen *et al*., 2006a) or tissue insult,
- hyperalgesia,
- regulates cellular response to hypoxia,
- mediates bronchoconstriction, vasoconstriction and smooth muscle contraction, vascular permeability,
- regulation of cytosolic Ca2+mobilization (Reyes-Cruz *et al*., 2000),
- regulation of apoptotic process and cell proliferation.

9.2 Hierarchical structure for Kinin B_1 and B_2 Receptors Identification Analysis

The data from the previous chapters have shown that CLL cells posses the components for KKS activation: the Kininogen, which serves as a substrate, the enzymes: Kallikreins plasma and tissue and the product of the Kallikrein digestion upon Kininogen such as Bradykinin. The Bradykinin, however, exerts its effect which is known to play an important role in sustaining inflammatory response, vascular permeability, neovascularisation (Bader, 2009), migration, proliferation, survival and invasion, via kinin B_1 and B_2 receptors, the expression of which has remained to be determined. Therefore, the aim of this chapter was to independently evaluate the kinin B_1 and B_2 receptors expression with respect to CLL and normal B cells utilising selected B-cells and unselected B-cells. In the context of unselected B-cells Western blot was directly employed. The previous localised work did not establish an optimised technique for B-cells isolation, therefore both methods MACS and FACS were utilised to ascertain a primary method for B-cells isolation. B-lymphocytes isolated by MACS and/or FACS were simultaneously examined for kinin B_1 and B_2 receptors expression using Western blot and flow cytometry techniques (Figure 9-3).

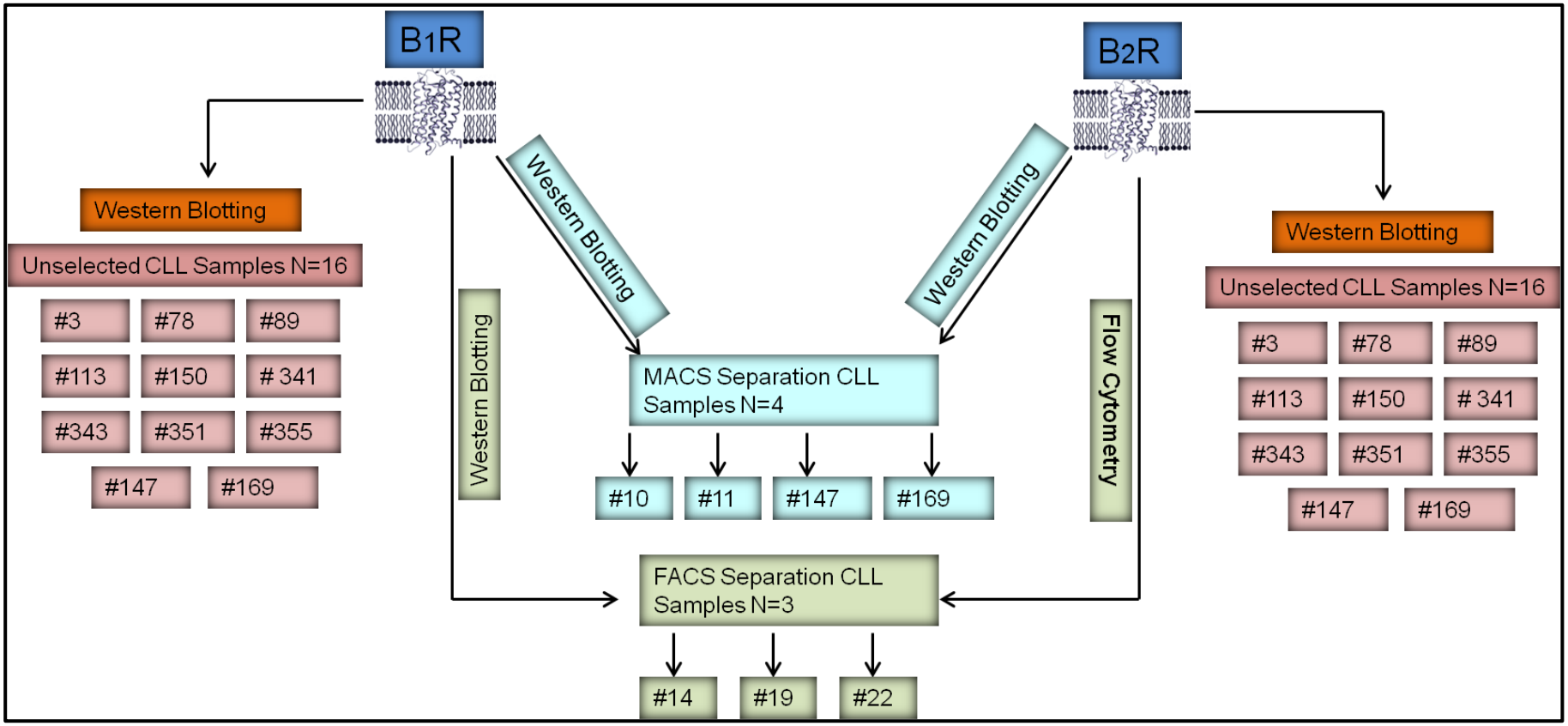


Figure 9-3 Schematic Diagram of Hierarchical Structure of kinin B_1 and B_2 Receptors Expression Analysis

This Figure explains a hierarchical structure of the steps undertaken for receptor expression analysis. The study will be conducted for B_1Receptor (B_1R) and B_2Receptor (B_2R) independently, utilising selected and unselected samples. MACS and FACS techniques were employed for B-cell isolation. Selected and unselected samples were subjected to Western blotting and flow cytometry to ascertain the kinin B_1Receptor and B_2Receptor expression.

9.3 Separation of Human B-lymphocyte subset

Although the previous flow cytometry analysis of CLL samples (carried out by Jo Pointon using CD19 marker) shown that the proportion of B-cells in CLL samples isolated utilising Histopaque-1077, was composed of a minimum of 80%, there is a minor percentage of cells within CLL samples which may be presented by T-lymphocytes, NK cells, monocytes or macrophages. In order to omit unnecessary manipulation of B-cells, which would have been an outcome from B-cells purification, unselected CLL samples were used in the major part of this project. In particular respect to kinin B_1 and B_2 receptors expression analysis, B-cells purification was employed due to the published data showing that other PBMCs may express kinin B_1 and/or B_2 receptors on their surface. In particular, expression of kinin B_1 and B_2 receptors was studied in several types of white blood cells. Recently, it has been demonstrated that neutrophils possess a complete KKS on their surface and may regulate vascular permeability via kinin production (Bockmann and Paegelow, 2000). Other studies on human PBMCs have indicated that human monocytes express a low number of kinin B_2 receptors and that kinin B_2 receptors are constitutively expressed or in coexistence with the kinin B_1 receptor on all macrophages (Bockmann and Paegelow, 2000). Therefore in this study, the expression status of kinin B_1 and B_2 receptors will be simultaneously analysed in selected and unselected samples (Figure 9-3).

9.3.1 Magnetic-Activated Cell Sorting Separation of CLL B-lymphocytes

Four CLL samples from the study cohort previously used in proteomics (N=52) were subjected to MACS isolation of B-lymphocytes and their purity was determined by flow cytometry analysis as described in Section 4.3.1 (Table 9-1) (Figure 9-3). Generally, MACS MicroBeads designed to couple to approximately 20% of the cell surface epitopes and successfully isolate populations with excellent purities and recoveries. However, in the occasional exceptions, such as CD19 MicroBeads (MiltenyiBiotec), beads were designed to bind to a higher percentage of the epitopes on the cell surface; therefore, it was not advisable to utilise a CD19 antibody for downstream application due to the possibility of surface CD19 antigen being occupied with MicroBeads. For that reason, a CD20 marker, which is widely used in CLL research (Delgado *et al.,* 2003) was employed in the current research.

Table 9-1 The B-cell Percentage in CLL and Healthy Samples

The data show the purity of CLL and healthy samples after B Cell purification

Sample ID	B Lymphocytes count (%)
Sample 10	98
Sample 11	96
Sample 147	92
Sample 169	94
Healthy Sample	96

9.3.2 B-Lymphocytes Isolation Utilising Fluorescence-Activated Cell Sorting Methodology (FACS)

FACS methodology was chosen as an alternative method to MACS for B-cell isolation, which was conducted following the protocol outlined in Section 4.3.2. Three peripheral blood samples obtained from CLL patients were subjected to B cell isolation and result is shown in Table 9-2 (Figure 9-3).

Table 9-2 Fluorescence-Activated Cell Sorting Analysis of CLL samples 14, 19, 22 with the Total B-lymphocytes Count

An example of FACS isolation and gating was previously shown in Figure 4-8 (CLL sample #22)

Sample ID	B-lymphocytes count (%)
Sample 14	97
Sample 19	96
Sample 22	98
Healthy Sample	98

9.4 The Kinin B_1 Receptors Expression, Determined by Western Blotting, on the Surface of CLL and Normal B-cells

9.4.1 Analysis of Kinin B_1 Receptors Expression in Unselected B-cells Utilising Western Blotting

A total of 16 CLL samples, from which 12 CLL samples were selected from the study cohort previously used in proteomics (N=52) (Chapter 5) were analysed (Appendix B). CLL cells were defrosted and rested for 3 hours prior to proceeding to Western Blotting (Section 4.4.3). Four of the selected 16 samples were obtained from CLL patients, B cells were isolated (Section 4.2.1) and immediately utilised in Western blotting (4.4.3) without being subjected to storage in low sub-zero temperatures (-80^{0}C) or in liquid nitrogen. Normal B cells were isolated utilising MACS technique (Section 4.3.1) (Appendix B) and were also included in this experiment. SW837 rectum cancer cell line was used as a positive control. Western blotting was performed as outlined in Section 4.4.3. To detect the protein of interest, a rabbit polyclonal anti-B_1 receptor antibody (Abcam # 75148), which is designed to react with the internal region of human B_1 receptor was utilised. The aim of this experiment was to detect the presence of kinin B_1 Receptors in CLL and Normal B cell samples (Figure 9-4).

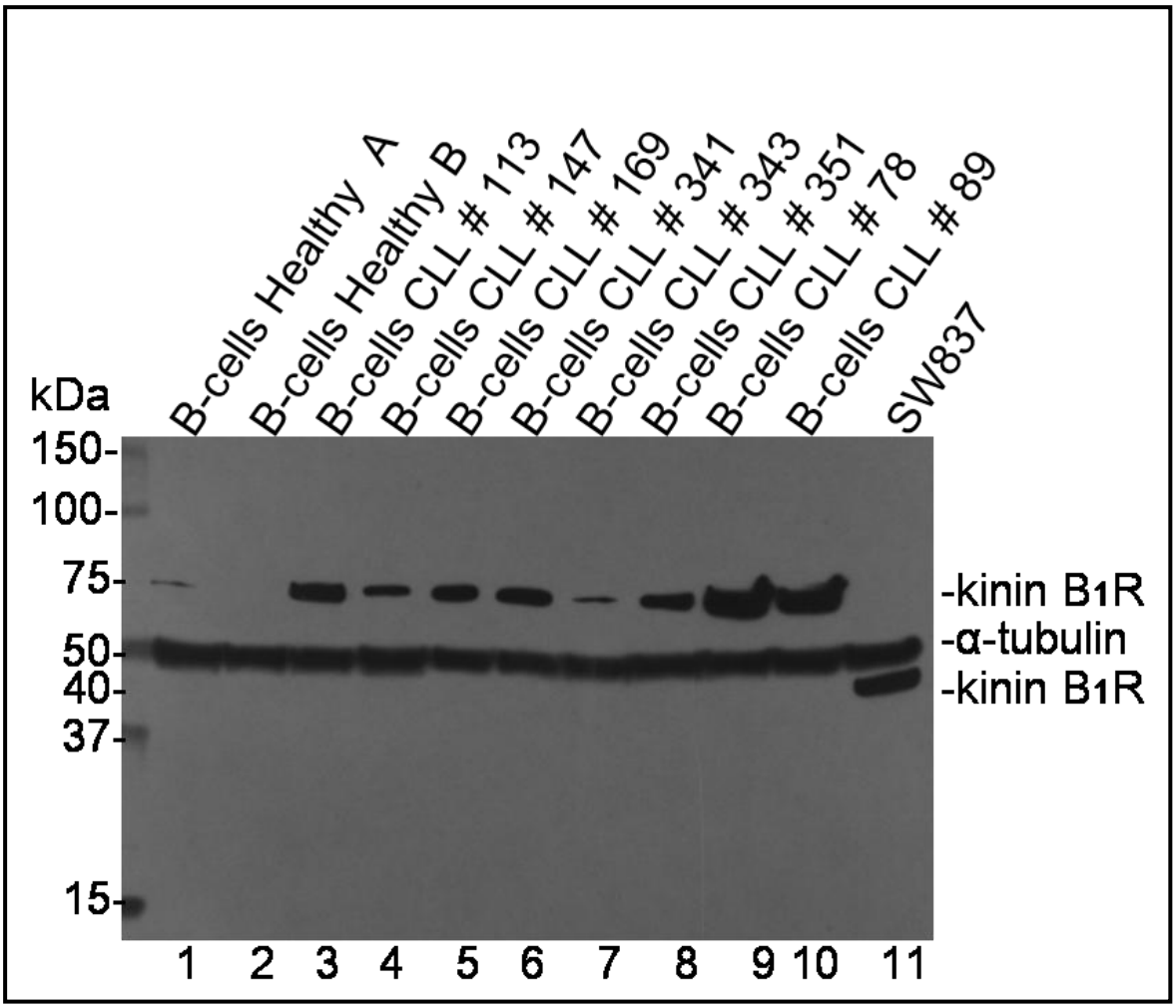


Figure 9-4 Western Blot Analysis of the Kinin B_1 Receptor Expression

Western blot analysis of the baseline kinin B_1 receptor expression in CLL and Normal B-cell subsets. SW837 cells were utilised as a positive control. Cell lysate in the amount of 20 µg was loaded to well 1-11 and co-electrophoresed with the Western molecular weight Marker. The primary antibody (Ab75148) was applied at concentration 1:500 for 3 hours. The secondary antibody (Santa Cruz Biotechnology, #F2909) for 1 hour and the film exposed for 30 min. The predicted molecular weight for B_1 receptor is 40 kDa and the band is observed of approximately this size in SW837 cells. A single clear band of about 75 kDa is detected in B-cells. The observed bands are significantly higher than the predicted MW and no bands appear in the expected position. The identities of the 75 kDa bands are unknown. The possibility that the detected bands appear as a result of non-specific antibody binding cannot be excluded. Alpha-tubulin was used as loading control (molecular weight is 50 kDa). The antibodies were used at optimised concentrations, which are shown in Table 4-4. The data shown is representative of a minimum of two analyses per sample.

The result demonstrated that a single protein bands of approximately 75 kDa, which may represent a variants of B_1 Receptors, are expressed in 16/16 CLL B-lymphocytes and 1/2 normal B cell samples. The expected 40 kDa band is detected in the positive control SW837 cells. The molecular weight of the bands, observed in B cells, is higher than the predicted molecular weight of the target protein. The identities of the 75 kDa bands are not confirmed. The possibility that the detected bands appear as a result of non-specific antibody binding cannot be excluded. Additionally, the result indicated that kinin B_1 Receptors expression is more abundant in CLL cells in comparison to normal B cells (Figure 9-4), although its expression differs from samples to sample.

9.4.2 Analysis of Kinin B_1 Receptors Expression in Selected vs. Unselected CLL B Cells

Four CLL samples from the study cohort previously used in proteomics (N=52) were subjected to B-lymphocyte isolation utilising MACS (Table 9-1) and FACS (Table 9-2) techniques. Western blotting (Section 4.3.1) utilising rabbit polyclonal anti-B_1 receptor antibody (Abcam # 75148) has been employed for subsequent analysis of kinin B_1 expression on the surface of selected vs. unselected (pre-sort) CLL B-lymphocytes. The result is shown in Figures 9-5 and 9-6 (Appendix B).

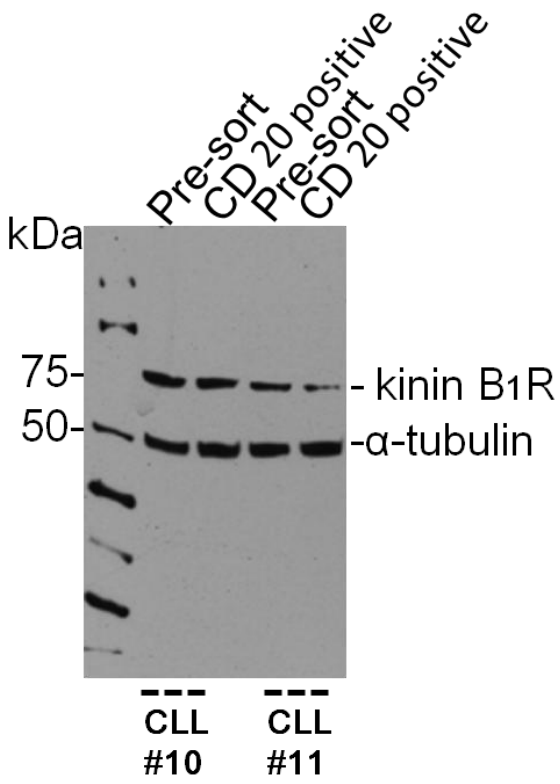


Figure 9-5 Western Blot Analysis of the Kinin B_1 Receptor on the Surface of B-lymphocytes, Selected Utilising MACS

Western blot analysis of the baseline kinin B_1 receptor expression in CLL samples (pre-sort) and B-cell subsets (CD20 positive), isolated utilising MACS methodology. Cell lysate in the amount of 40 µg was loaded to well 1-4 and co-electrophoresed with the Western molecular weight Marker. The primary antibody (Ab75148) was applied at concentration 1:500 for 3 hours. The secondary antibody (Santa Cruz Biotechnology, #F2909) for 1 hour and the film exposed for 30 min. The bands of about 75 kDa are observed in both selected and unselected CLL samples. Alpha-tubulin was used as loading control (molecular weight is 50 kDa). The antibodies were used at optimised concentrations, which are shown in Table 4-4. The data shown is representative of a minimum of two analyses per sample.

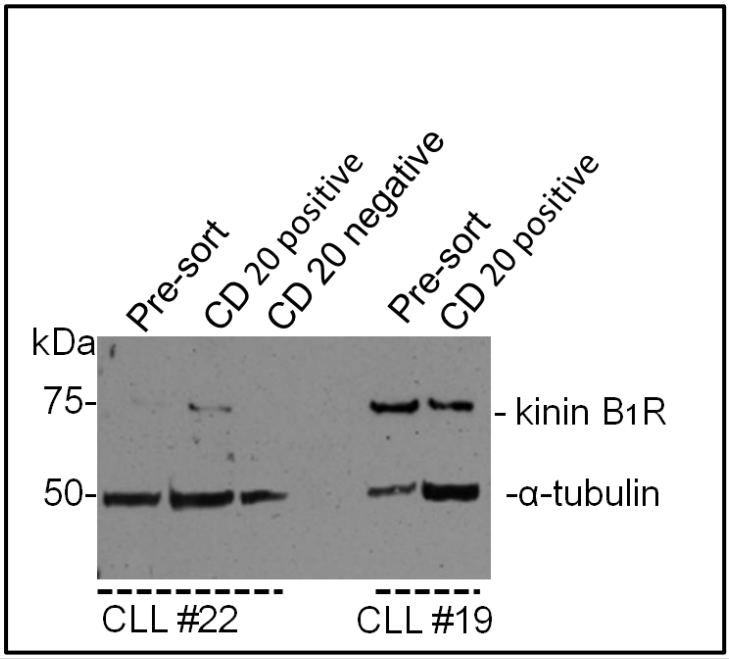


Figure 9-6 Western Blot Analysis of the Kinin B_1 Receptor on the Surface of B-lymphocytes, Selected Utilising FACS

Western blot analysis of the baseline kinin B_1 receptor expression in CLL samples (pre-sort), B-cell subsets (CD20 positive) and CD20 negative cells, isolated utilising FACS technology. Due to not having the requisite number of CD20 negative cells in sample 19, western blot analysis was not achievable in the context of kinin B_1 receptor expression. Sample 14 had an inadequate number of cells allowing performance of western blotting on pre-sort portion. Cell lysate in the amount of 40 µg was loaded and co-electrophoresed with the Western molecular weight Marker. The primary antibody (Ab75148) was applied at concentration 1:500 for 3 hours. The secondary antibody (Santa Cruz Biotechnology, #F2909) for 1 hour and the film exposed for 30 min. The observed bands are significantly higher than the predicted MW and no bands appear in the expected position. The identities of the 75 kDa bands are unknown. The possibility that the detected bands appear as a result of non-specific antibody binding cannot be excluded. Alpha-tubulin was used as loading control (molecular weight is 50 kDa) this indicates that the wells were loaded approximately equally. The data shown is representative of a minimum of two analyses per sample.

Western blots demonstrated that the result obtained from selected B-cells is completely reflective of unselected B-cells in the context of Kinin B_1 expression.

9.5 Analysis of Kinin B_2 Receptors Expression in Normal and CLL B Cells

9.5.1 Western Blot Analysis of Kinin B_2 Receptors Expression in Unselected B-lymphocytes

The analysis of the Kinin B_2 Receptors expression was carried utilising a total of 16 CLL samples, from which 12 CLL samples were selected from the study cohort previously used in proteomics (N=52) (Chapter 5). CLL cells were defrosted and rested for 3 hours prior to proceeding to Western Blotting (Section 4.4.3). Four of the selected 16 samples were obtained from CLL patients, B cells were isolated (Section 4.2.1) and immediately utilised in Western blotting (4.4.3) without being subjected to storage in low sub-zero temperatures (-80^{0}C) or in liquid nitrogen (Appendix B). A rabbit polyclonal anti-kinin B_2 antibody, which is designed to interact with immunogen derived from within residues 350 to the C-terminus of human kinin B_2 receptor (Abcam # 73625) was used. Human umbilical vein endothelial cells (HUVECs), derived from the endothelium of veins from the umbilical cord, which are known to express kinin B_2 receptor (Wohlfart *et al*., 1997) were used as a positive control (Figure 9-7).

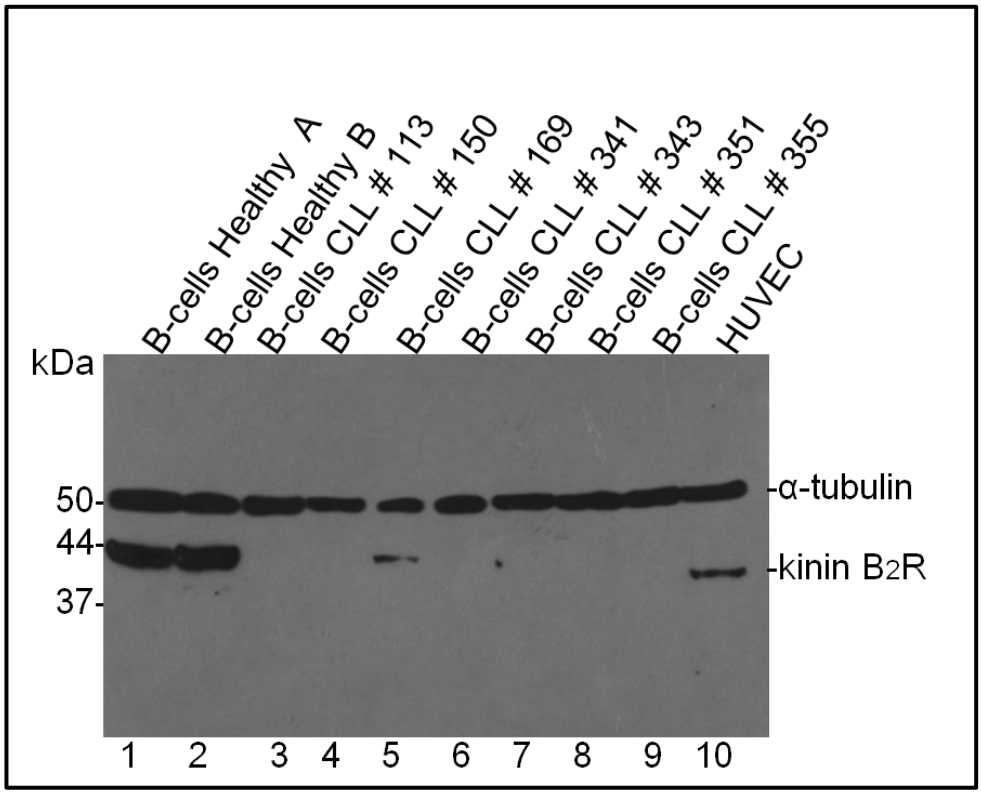


Figure 9-7 Western Blot Analysis of the Kinin B_2 Receptor

Western blot analysis of the constitutive kinin B_2 receptor expression in B-cells, derived from healthy controls and CLL patients. HUVEC cells were used as a positive control. Cell lysate in the amount of 40 µg was loaded to well 1-10 and co-electrophoresed with the Western molecular weight Marker. The primary antibody (Ab73625) was applied at concentration 1:500 for 3 hours. The secondary antibody (Santa Cruz Biotechnology, #F2909) for 1 hour and the film exposed for 30 min. The predicted molecular weight for B_2 receptor is 44 kDa and the band is observed of approximately this size. Alpha-tubulin was used as loading control (molecular weight is 50 kDa). The data shown is representative of a minimum of two analyses per sample.

The result has demonstrated that expected 44 kDa kinin B_2 receptors are expressed in positive control HUVEC cell line, 4/16 CLL samples and 2/2 normal selected B cells. As Western blot indicates, the abundance of kinin B_2 receptors is significantly lower in CLL B-lymphocytes when compared to B-lymphocytes, derived from healthy individuals.

9.5.2 Western Blot Analysis of Kinin B_2 Receptors Expression in CLL B-Cells, Isolated Utilising MACS Purification

Kinin B_2 Receptors Expression was also assessed in 3 selected CLL B-Cell samples vs. unselected (pre-sort) (Appendix B). B cell isolation was performed utilising MACS protocol (Section 4.3.1). Western blot was carried out with anti-kinin B_2 receptor antibody (Abcam 73625) as per Section 4.4.3.

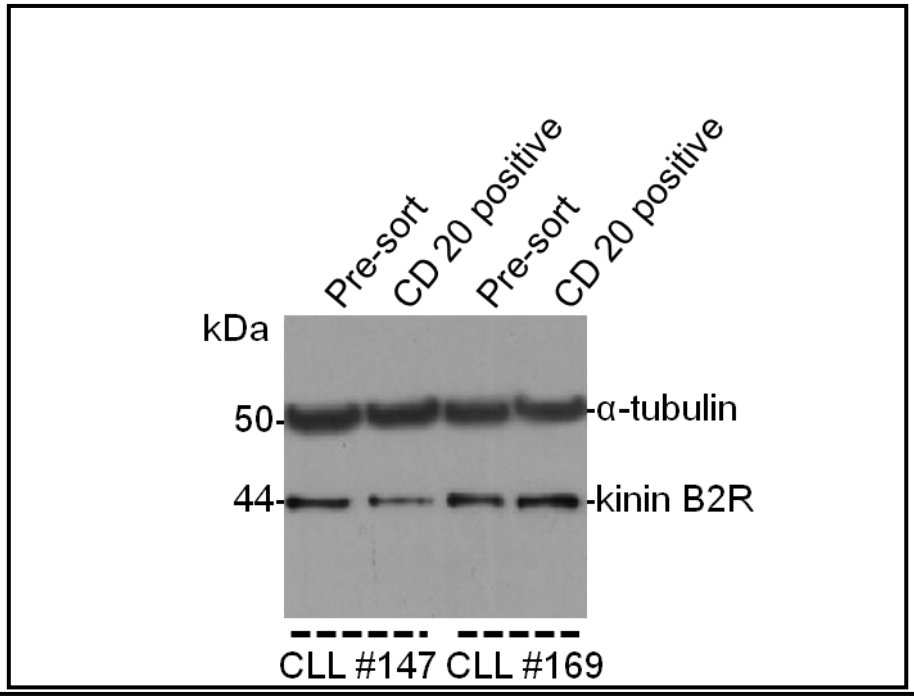


Figure 9-8 Kinin B_2 Receptor Expression in Selected vs. Unselected CLL B Cells Determined by Western Blot

Western blot analysis of the basal kinin B_2 receptor expression in selected vs. unselected CLL cells. The primary antibody (Ab73625) was applied at concentration 1:500 for 3 hours. The secondary antibody (Santa Cruz Biotechnology, #F2909) for 1 hour and the film exposed for 30 min. The predicted molecular weight for B_2 receptor is 44 kDa and the band is observed of approximately this size. Alpha-tubulin (50 kDa) was used as loading control. The data shown is representative of a minimum of two analyses per sample.

As Western blot demonstrates (Figure 9-8), the Kinin B_2 receptors are expressed in B cell samples irrespectively of B cell selection as the protein abundance in selected B cells is not shown to be different to unselected B cells.

9.5.3 Assessment of the Kinin B_2 Receptors Expression in CLL and Normal B-lymphocytes, Utilising Flow Cytometry

Three CLL samples and 1 healthy sample (Table 9-2) (Appendix B) were subjected to FACS (Section 9.3.2) and assessed for B_2 receptor expression utilising flow cytometry following the protocol outlined in Section 4.4.1. Due to the design of the selected rabbit monoclonal anti-Kinin B_2 Receptors antibody, to bind to a region within the C terminal end in the intracellular domain of human Kinin B_2 Receptor (Abcam # 134118), a free access of the antibody to its antigen had to be ensured. Therefore, fixation and permeabilisation as per protocol described in Section 4.4.1.2 was conducted.

The flow cytometry analysis of 1/1 healthy sample demonstrated that 79 % of normal B cells express kinin B_2 receptor (Figure 9-9 A). In respect of B_2 receptor expression in CLL samples, 1/3 sample was shown to be positive for B_2 receptor (71% of cells are B_2 receptor positive), whereas 2/3 CLL samples were negative as determined by flow cytometry (Figure 9-9 B).

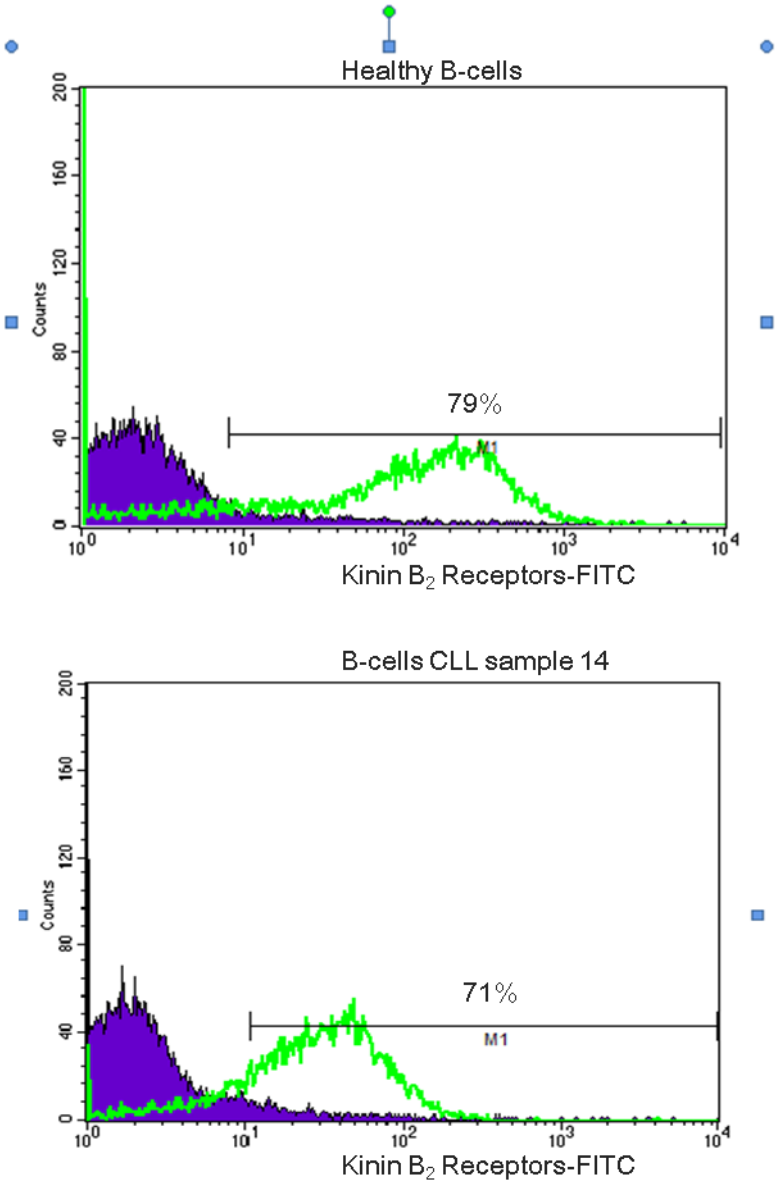


Figure 9-9 Flow Cytometric Analysis of Kinin B_2 Receptors Expression on B-cells

Following FACS and labelling with an Anti-Kinin B_2 Receptors antibody (ab134118), CLL sample 14 and healthy B-cell sample were analysed. The dot plot was created and the isolated B-cell population was gated based on forward and side scatter properties. Histogram was plotted for CLL B-cells (sample 14) (B) and healthy B-cells (A) using the gated population and demonstrated that 71 % and 79 % of total population are Kinin B_2 Receptors positive, respectively.

9.6 Discussion

The aim of this study was to investigate the expression of Kinin B_1 and B_2 receptors on the surface of CLL and healthy B-lymphocytes. B_2 receptors are widely distributed throughout the human body and ubiquitously constitutively expressed under “normal” physiological conditions, whereas B_1 receptors are often undetectable in healthy human cells and tissues but demonstrated to be overexpressed under several pathological conditions including an inflammation, allergy, tissue injury, pain and cancer. Recently, Kinin B_1 and B_2 receptors as a part of Kinin-Kallikrein System, were extensively studied in different malignancies and have been shown to play an important role in increasing of vascular permeability, cancer cell survival and proliferation, invasion and migration of the neoplastic cells into the normal tissue adjacent the tumour (Beck *et al.*, 2012b, Figueroa *et al.*, 2012). The up-regulation of Kinin B_1 receptors was abundantly reported in prostate cancer (Zhang *et al.*, 2008a, Beck *et al.*, 2012b), whereas increased expression of Kinin B_2 receptors was shown in human gastric, colon, lung, liver cancer and human gliomas (Zhang *et al.*, 2008a, Beck *et al.*, 2012b). Several studies have advanced the concept that Kinin B_1 and B_2 receptors can be considered as a target for cancer therapy (Figueroa *et al*., 2012).

In order to examine Kinin B_1 and B_2 receptors expression status on the surface of CLL and healthy B-lymphocytes, different scientific techniques such as Western blot and flow cytometry were employed. In parallel, the expression of both receptors was independently evaluated in selected and unselected samples, derived from CLL patients and apparently healthy controls. In terms of B-cells isolations, FACS and MACS were two competitive methods employed in this

project as no relevant studies, comparing FACS vs. MACS for separation of B-lymphocytes were readily available. Both of these methods have their advantages and disadvantages, which had to be considered in the context of B-cell isolation.

FACS advantages:

- *Isolates subpopulations out of complex cell mixture*
- *Isolates the target cell population with a very low level of the identifying marker*
- *Advance cell sorting, no restriction to the single parameter isolation*
- *Offers wide range of available markers*
- *Accommodates high purity*
- *Allows cell isolation based on intracellular protein expression (Basu et al., 2010)*
- *FACS data can be quantitative and can differentiate with respect to a level of antigen expressed*

FACS disadvantages:

- *Requirements for an expensive instrument with high maintenance*
- *Low cell throughput rate (approximately 10^7 particles per hour (Miltenyi et al., 1990))*
- *High skills to achieve an acceptable levels of performance*

MACS advantages:

- *Yields Relatively High Purity Cell Populations*
- *Offers fast, gentle and sufficient separation (Thiel, 1998, immunomagnetic)*

- *The small size of microbeads permits prevention from cell activation and cell surface epitope saturation*
- *Provides high-throughput screening with rich data outputs*

MACS disadvantages:

- *Primary equipment and set up expensive*
- *Offers a narrow range of markers*
- *Limited to the cell isolation according to the expression of a surface molecule*
- *Unable to discriminate between cells with low and high expression level.*

Both FACS and MACS separation were conducted successfully in this project, the average percentage of $CD20^+$ B-cells in samples was achieved 98% and 95.2% respectively. .

This study shown that although FACS can offer more superior purity, MACS, however, is simpler and significantly less time consuming, labour intensive and also yields high purity isolation. Moreover, if Western blotting was utilised downstream it would require a very large number of cells. This means that, due to limited separation capacity of FACS machine (approximately 10^7 particles per hour Miltenyi), the sorter requires to run for an extended period, this is not only expensive, but also can reduce cell viability and question the quality of cell output because the sorting from such long runs may need extra care and interim incubation. The results have shown that the sorting time of the FACS application was almost twice that of MACS despite achieving only the same output. However, if a small amount of cells are required for a specific experiment, such as flow

cytometry, then both MACS and FACS could be considered and evaluated in those instances.

This concludes, firstly, that both FACS and MACS techniques are suitable for B-lymphocyte selection, however, additional care has to be taken with FACS application, particularly if utilising proteomics downstream. Secondly, the result obtained from selected B-cells is completely reflective of unselected B-cells in the context of Kinin B_1 and B_2 receptor expression. Lastly, as the results from cell selection versus unselected does not indicate a significant improvement in terms of B-cell enrichment, therefore, the additional manipulation during cell isolation is not merited.

Furthermore, when interpreting the results gained from investigation of Kinin B_1 and B_2 receptors expression in CLL and healthy B-cells, it is important to emphasise that western blotting was selected for kinin B_1 receptor analysis and a combination of western blotting and flow cytometry approaches were employed for kinin B_2 receptor study. When choosing an ideal study tool it is crucial to ensure the appropriateness and the feasibility of selected method, manufacturing consistency, recommendations and comparability studies. Western blotting has been well-established as a straightforward technique for protein expression level evaluation (Germano and O'Driscoll, 2011) and therefore was prioritised for this project. Western blotting for kinin B_1 receptor was determined in CLL samples using a selective polyclonal anti-kinin B_1 receptor antibody (Abcam # 75148). A polyclonal anti-kinin B_1 receptor antibody is designed to crosslink with an epitope within an internal region of human kinin B_1 receptor and generates a signal with predicted molecular weight of approximately 40 kDa, which was identified in

Human rectum adenocarcinoma SW837 cell line. Interestingly, the single band that was repeatedly identified in CLL samples was of approximately 75 kDa, which is significantly higher than apparent. This increased molecular weight is strongly reminiscent of the ability of kinin B_1 receptor protein target to form multimers. This was supported by a study which challenged the concept of GPCRs, including kinin B_1 receptor, acting as monomeric proteins, indicating that these receptors may fold as constitutive dimer/oligomer or progress to ligand-promoted dimerisation at the cell surface (Angers *et al.*, 2002) or even form the dimer, oligomer or heterodimer with kinin B_2 receptors or other molecules such as CPM (Zhang *et al*., 2008c).

Ultimately, western blotting result demonstrated that kinin B_1 receptor is upregulated in 100% (16/16) of CLL samples as opposed to low abundance in healthy B-lymphocytes. The result, therefore, demonstrates that the up-regulation of kinin B_1 receptor in CLL is representative of the inducible status of this receptor which reflects research undertaken in several other cancers and low abundance of kinin B_1 receptor generally expressed in healthy cells and tissues is also reflective of the result. Recent studies, utilising Human Embryonic Kidney 293 cells, have shown that under normal physiological condition the abundant portion of kinin B_1 receptors is restricted to the intracellular location, with relatively large amount of the receptors to be found in the endoplasmatic reticulum (ER) and only a limited number of the receptors localised on the cell surface (Sanden and Leeb-Lundberg, 2013).

Unfortunately, due to experimental limitations of Western blotting, the origin of the detected 75 kDa band cannot be reported with certainty at this time.

Considering the time and cost constraints, it was not possible to extend analysis towards definitive identification of the 75 kDa band. As previously discussed in Section 7.4 protein sequencing, which is designed to determine the identity of the protein of interest may confirm the hypothesis. Therefore, the next steps would have been to continue investigations on gene (RT-PCR) and protein levels. In parallel, focusing on the proteomics, other methods such as immunocytochemistry, further western blotting using blocking peptide could assist in definition of the identified protein structure. Quantitative confocal microscopy would be another option for detailed analysis; however, this facility was not available for this project.

Flow cytometry was not successfully carried out on kinin B_1 receptor expression due to the lack of availability of suitable directly or indirectly conjugated antibodies. The indirectly conjugated primary antibody utilised in western blotting (Abcam, #75148) failed to generate the signal, despite, the multiple dilution optimisations, cell fixation and permeabilisation.

In the context of Kinin B_2 receptor expression analysis, the result can be summarised as follow. Firstly, western blotting analysis of sixteen CLL and two healthy samples, utilising a primary polyclonal anti-kinin B_2 receptor antibody (Abcam, 73625) identified a single band with molecular weight of approximately 44 kDa. The observed band was of the same molecular weight as the band detected in the positive control, HUVEC cell line, and as predicted by the antibody manufacture. The result indicated that kinin B_2 receptor was found to be expressed 25% (4/16) of CLL and 2/2 normal samples. Secondly, the expression of kinin B_2 receptor was shown to be significantly downregulated in CLL samples when examined in contrast to healthy controls. Lastly, flow cytometry was carried out on

three CLL and one healthy sample, utilising an indirectly conjugated monoclonal anti-Kinin B_2 Receptors antibody, designed to bind to an intracellular region of human Kinin B_2 Receptor (Abcam, 134118). The result obtained during flow cytometry study was complementary to that of western blotting, which confirmed the baseline expression of kinin B_2 receptors on a proportion of untreated CLL samples (1/3) and healthy B-lymphocytes (1/1).

In conclusion, Kinin B_1 and B_2 receptors expression was studied in monocytes and macrophages (Rajasekariah et al., 1997, Bockmann and Paegelow, 2000); however, there is complete lack of availability of specific studies that focus solely upon CLL and normal B-cells in the context of Kinin B_1 and B_2 receptors expression. Therefore, the significance of this study can be rationalised in the context of the full cascade starting from the identification of Kininogen, downstream via liberation of Bradykinin to understanding of the dynamics of Bradykinin through the activation of Kinin B_1 and B_2 receptors. Although, it is noteworthy that, the analysis of larger series of CLL samples will be advantageous in this project as this may provide us with the opportunity to address the relevance of kinin receptors in the patient cohort and correlate it to other prognostic and/or diagnostic biomarkers in CLL.

CHAPTER 10

Discussion

Chapter 10. Discussion

10.1 The Aims and Objectives Overview

Despite an advanced understanding of the pathology of CLL, the disease still remains incurable. There is still no unequivocal definition of the catalyst for aggressive disease progression and relapse in CLL. Therefore the demand for continued research, leading to the development of novel therapeutic agents, is paramount to achieving more effective disease control and true complete remission in patients with CLL. Therefore, the principal aim of the current project was to investigate novel proteins which may have clinical relevance in CLL and in conjunction with this aim the seven following objectives were outlined:

1. ***to validate the differential expression of Kininogen protein in the clinical context upon in vitro BCR stimulation,***
2. ***to analyse constitutive Kininogen protein expression using an increased number of CLL samples and to correlate Kininogen expression with clinical data and other biomarkers,***
3. ***to investigate the Kininogen expression in normal B cells***.

In order to meet these aims it was necessary to collect both *in vivo* samples and high quality clinical data from a large number of CLL patients. BCR-responsive protein expression is known to play a role in CLL; however, basal protein expression may also be of clinical relevance. The data from this project hypothesise for the first time that CLL B-lymphocytes express differing levels of a

basal 33 kDa protein product, which is suggested to be a mutated or truncated isoform of LMWK. The expression of this protein can be significantly increased after BCR ligation in CLL B-lymphocytes, whilst no expression was detected in normal B-cells (n=4). This project has demonstrated in a pilot study (n=52) that 71% of CLL B-lymphocytes express basal levels of this 33 kDa protein, which was associated with a trend towards shorter median survival (Chapter 5).

Due to time and cost limitations, complexity, and availability of the required techniques we were unable to determine with a significant level of certainty the identity of 33 kDa band, the MW of which differs from that of conventional LMWK (reported to be approximately 65-68 kDa). The utilisation of advanced sequencing techniques would provide the peptide sequence of the target protein and, thus, would confirm its origin. The 33 kDa protein detected by immonoblotting had matched the MW of the KNG1 protein identified during previous 2-DE and MS analysis. The detailed analysis of NCBI and UniProt databases revealed that the National Institutes of Health Mammalian Gene Collection (MGC) Program (designed to identify and sequence a cDNA clone for a number of human and mouse genes) reported a transcript for a 33 kDa LMWK protein (291 amino acids) (NCBI, ***gi116283732***, www.ncbi.nlm.nih.gov and UniProt ***Q05CF8***, www.uniprot.org) (Strausberg *et al.*, 2002). However, expression of the 33kDa LMWK isoform had not been fully determined or reported previously. This study is the first to report the LMWK splice variant of 33 kDa in this context. Nevertheless the question remains and further investigation is required to establish whether the

detected band is a true variant of LMWK or may have occurred as a result of non-specific antibody binding.

In this project, the expression of 33 kDa protein was investigated in 52 clinical samples. A limitation of this study is the number of studied cases, which can be regarded as a small sample size. If statistical analysis is applied to a greater sample cohort by continued research in this field, and the results are consistent with this study then the hypothesis would be strengthened. Therefore in order to fully evaluate the relationship between LMWK expression status and prognosis, an increased number of CLL cases should be examined. Subsequently the statistical output of this research aligned to that of this study is necessary to challenge or support the hypothesis.

The next key research question was:

4. ***to evaluate the expression of corresponding Kininogen mRNA using standard reverse transcription polymerase chain reaction***.

The basal level of the *LMWK* transcript, utilising a selected primer set, was undetectable in 20 CLL samples, which may be due to low abundance or rapid turnover of the transcript (Chapter 6). As no signal was detected for LMWK gene expression during RT-PCR, despite multiple optimisation steps, it seemed unnecessary to progress to quantitative Real-Time PCR. However, the specific sensitivity of the LMWK assay was not determined. A more sensitive detection, utilising fluorescence dye and an optimisation of a competitive PCR method using standard curves for transcript quantification would assist to contextualize the results and direct further study.

Although KNG expression is a novel finding and is shown to be constitutively expressed in some CLL samples, the downstream molecular events that may lead to the activation of kinin B_1 and B_2 receptors, and potentially to cell survival, required investigation. Therefore, the next objectives were:

5. ***to assess the expression of Kallikreins, which are known to digest Kininogen with subsequent liberation of bioactive Kinins (BK and Lys-BK), utilising immunoblotting in CLL and normal samples and***
6. ***to evaluate the level of BK/Lys-BK in plasma samples from CLL patients using ELISA and to correlate BK/Lys-BK plasma concentration with clinical data and other biomarkers***.

In order to strengthen or challenge the overall hypothesis, the results obtained from an associated study of plasma PK were also discussed in this project.

The tissue KLK 6 expression was investigated utilising immunoblotting. The MW of the experimental protein (65-70 kDa when utilising anti-Kallikrein 6-Kallikrein loop antibody) was discrepant from the expected MW of 27 kDa. Several studies have reported an increase in the apparent MW for KLKs, which was associated with PTMs such as phosphorylation and/or ubiquitin (Ub) modifications (Kuzmanov *et al.*, 2009, Seiz *et al*., 2012). The work performed by other researchers has shown that when compared with the MW of the unmodified form, mono-Ub-conjugates display an increase of approximately 8 kDa after mono-ubiquitination and even larger shift after poly- ubiquitination (Seyfried *et al.*, 2008). However, no previous reports regarding a 65-70 kDa KLK 6 protein product were

found in the literature. Interestingly, the dramatic increase in the experimental MW was only detected in CLL samples, whereas expected 27 kDa bands were identified in a positive control cell line. Protein sequencing would support the confirmation of the protein identity and subsequently strengthen our hypothesis; however, the possibility that the visualised bands may appear as a result of non-specific antibody binding cannot be excluded. A number of recent studies have shown a potential role of KLKs as novel prognostic biomarkers and therapeutic targets (Section 3.4.2.1). To the best of the author's knowledge, the expression of KLK has not been linked to CLL previously in this novel context; therefore, detailed study of KLK may unravel the role of KKS in CLL.

Utilising a one stage coagulation assay, plasma PK was found to be increased in more than 50% of CLL patients. BK plasma concentration was subsequently examined using ELISA and correlated with PK, clinical data and other biomarkers. PK and BK are the key molecules in the KKS pathway, therefore, the finding that the association between the expression of these two molecules was shown to be highly significant ($p=0.001$), was very compelling. BK as well as PK were found to be significantly associated with stage A ($p=0.03$ and $p=0.016$, respectively) live CLL cases ($p=0.035$ and $p=0.017$ respectively). Moreover, PK was shown to be associated with untreated ($p=0.021$) CLL cases and there was a trend towards association of BK level and untreated status ($p=0.096$).

Utilising these novel findings it can be suggested that upon BCR ligation the upregulation of LMWK expression can be achieved, therefore, as it is an endogenous source for proteolytic cleavage by KLKs, it is likely to activate the

subsequent release of BK. BK is one of the most powerful vasodilators that causes peripheral blood vessel dilation and increases vascular permeability. Thus, the elevated level of BK in the systemic circulation could potentially be the catalyst for local vasodilatation in nodal sites leading to increase in the nodal volume. Additionally, there is a possible role for high BK in the manifestations of the systemic B-CLL symptoms such as tiredness, night sweats, angioedema (Mohyuddin and Rabinowitz, 2013), potentially providing the suggestions as to the origin of these symptoms, as the exact causes are still unknown.

Consequently, further analysis of a larger number of CLL samples may advance BK as an attractive screening, diagnostic or monitoring biomarker for routine detection of CLL patients who have early stages disease but poor risk biomarker profile. These patients may benefit from closer monitoring of their symptoms for early treatment intervention. BK can be assessed in blood fluids as well as plasma using a cost-effective method such ELISA (Chapter 8).

The increased level of BK in near proximity to its Kinin B_1 and B_2 receptors, may potentially lead to their hyper activation and signalling. Therefore, the pertinent research activity *was:*

7. ***to employ immunoblotting and flow cytometry in order to analyse the expression of B_1 and B_2 kinin receptors on the surface of normal and CLL B cells.***

Utilising immunoblotting, kinin B_2R was found to be expressed on the surface of CLL B Cells. However, the question still remains unclear regarding the identity of the band detected by anti-kinin B_1R antibody, which could be confirmed

utilising protein sequencing techniques. In the past decade, GPCRs including B_1 and B_2 receptors have become desirable targets for drug development due to the availability of specific targeting agents directed against these receptors and their overexpression in multiple cancer types. Therefore, the evidence suggests that the cell surface expression of B_1R and B_2R could be a novel finding. This preliminary study of the identification of B_1R and B_2R expression on the surface of B Cells may help to progress the understanding toward the downstream signalling mediated via these receptors and their role in CLL, which could potentially offer future therapeutic target options.

10.2 Further Work

The evidence produced from the current project research has clearly identified many differing directions for research progression and therapeutic solutions.

The previous work revealed that BCR stimulation resulted in consistent up-regulation of only one protein KNG, although such complex process as BCR stimulation is expected to be associated with the significantly larger number of DEPs. This may be due to the technical limitations of this proteomic approach, which has not generated the full array of protein expression changes. Additional discovery-phase experiments, using complementary methods such as gel-free MS and microarray-based proteomic methods would undoubtedly improve the discovery phase results.

The investigation into the nature of the 33 kD form of LMWK, 65-70 kDa tissue KLK 6 and 65 kDa Kinin B1 Receptors, which were found to be consistently expressed in the CLL samples would greatly improve our understanding about the role of the KKS in CLL. Proteins can be precipitated from whole cell lysates and subsequently sequenced utilising MS. PCR and Real-Time PCR can be employed to evaluate the gene expression status for these proteins. Additionally, a study of a larger number of both stimulated and unstimulated samples may highlight and direct further investigation into the possible association with CLL prognosis.

Utilising a selected primer set, RT-PCR was unable to detect LMWK transcript in any of 20 CLL samples. The use of Real Time PCR, which is more sensitive method, may offer the opportunity to compare LMWK transcript expression in pre- and post-stimulated samples.

In order to examine the cellular localisation of LMWK/KLK and the subcellular distribution, the peripheral blood CLL cells and LN tissue can be analysed utilising immunocytochemistry (ICC) and immunohistochemistry (IHC) respectively.

10.3 Concluding Remarks and Future Directions

This project has demonstrated for the first time that CLL cells express the components for the KKS signalling pathway. Future work in this area is necessary to confirm and quantify statistically the potential of several key molecules from the KKS pathway as novel biomarkers in CLL. This may offer both new insights into the pathogenesis of CLL, as well as new therapeutic targets for future treatments.

As previously discussed the KKS and its downstream signalling pathway play an important role in sustaining the inflammatory response and in the synthesis of proteins associated with stimulation of nitric oxide formation, liberation of prostacyclin, transduction of pathologic signals, vasodilation, increased vascular permeability and potentiating of calcium signalling, which results in cell growth, survival and proliferation. It has also been suggested that Kinins influence cells of the immune system, such as T-cells and B-cells, by modulating the activation, proliferation, migration and effect or functions of these cells. Hyperactivity of the KKS and dysregulation of KNG levels have been previously reported in several pathological conditions, including human inflammatory diseases and cancer. The increased understanding of the role of the KKS in the chronic inflammatory response has heightened the interest of the pharmaceutical industry in KKS proteins and their potential therapeutic application in inflammatory diseases and cancer.

10.3.1 Kininogen Discovery

There have been no previous publications describing a potential role for KNG in CLL or normal B Cells. Therefore this study supports the hypothesis that this is a critical area for further research. Should the result of further research follow in this context and validate the early data produced by this study, this will therefore offer the potential for development of novel biomarkers for patients assessment, to improve patient diagnosis/prognosis and even support the development of new drugs to help combat currently incurable CLL and lead to alignment of the role of KKS in other cancers.

If the 33 kDa protein consistently expressed in CLL is confirmed to be a variant of LMWK; and subsequently reconfirmed utilising a larger sample cohort to be associated with CLL outcomes, then this will potentially attract investment to target the KKS as a reliable affordable prognostic biomarker for early identification of patients who potentially can develop aggressive CLL. This could lead to a simple test without having to undergo an expensive and time consuming gene sequencing technique. A positive KNG expression may indicate a higher risk for progression and the development of CLL. Therefore those high-risk CLL patients should be managed accordingly and possibly could have their preventative cancer treatment initiated earlier without the added risk of aggressive disease progression.

KNG is an important constituent of the KKS pathway; however, the data from the current project has shown that other downstream molecules from this pathway could be of potential interest. These could be molecules such as, but not limited to Kallikreins, Bradykinin and Kinin receptors.

10.3.2 Prediction for the Future of KLKs in CLL

As an example, KLK 3 (PSA) is the best prostate cancer screening marker available to date (De Angelis *et al*., 2007, Balk *et al.*, 2003). Although not in clinical use, it has been shown that other KLKs can serve as new screening, diagnostic, prognostic, and monitoring biomarkers for prostate, ovarian, breast, testicular, and lung cancers. Therefore, the understanding of their regulation and the investigation of their association with cancer are also of high priority (Carlsson *et al*., 2013). At the present time, two well-established technologies are in use to quantify KLK expression, these are RT-PCR and ELISA assays. RT-PCR can detect the

presence of any individual KLK transcript in a tissue source. Additionally, as KLK is secreted into the extracellular space and fluids, ELISA assays can measure protein concentration from a wide variety of biological samples. The development of more sensitive ELISA assays and multiparametric analysis with subsequent confirmation of KLK role in CLL outcomes could progress KLK from the investigational phase into routine clinical practice as a fast, efficient and cost-effective test with no express need for expensive techniques and highly skilled personnel.

10.3.3 Bradykinin as a Rich Source of Novel CLL Biomarker

Currently, in general practice, newly diagnosed patients with asymptomatic early-stage CLL (Rai 0, Binet A) are monitored and therapy is withheld until symptoms manifestation or the disease progresses to an essentially measurable diagnosis. A meta-analysis of recently conducted CLL trials, utilising exclusively alkylating agents, has shown no survival advantages for immediate vs. deferred treatment in asymptomatic early-stage CLL patients. However, there is still uncertainty regarding the therapeutic approach in asymptomatic high-risk patients and establishing whether these CLL patients should be treated earlier in their disease course (Gribben, 2010). Therefore, in order to support discrimination of high-risk CLL patients, there is a great value in identifying new intermediate biomarkers, which can be predictive of disease outcome and that can be efficiently incorporated into routine clinical practice and become preventative measures or at the very least extend life expectancy. Generally, asymptomatic CLL patients, who exhibit a stable clinical course, are followed every 6 months. One of the key measures of disease activation is progressive enlargement of lymph nodes and

splenomegaly. In association with this, the data from the current project heightens the potential interest in BK, which acting as a vasodilator, potentially increases vascular leakage and this can result in the node bulkiness. Therefore further investigation into association of BK and CLL clinical presentation including assessment of lymph nodes and spleen, may lead to development of potential screening methods and cost effective tests for early stage untreated CLL patients who require early treatment intervention without waiting for disease to progress to an arbitrary measure of burden. Herewith, along with the physical examination, blood count and disease history, CLL patients can be tested for plasma BK level, thus identifying CLL patients with high BK level, who may become candidates for more frequent follow-up and possibly early maintenance or cancer therapy.

10.3.4 B_1R and B_2R as New Therapeutic Options

New data shows that BCR-inhibiting agents are very successful for the treatment of relapsed CLL (Jones and Byrd, 2014). Pharmacologic inhibition of downstream BCR-associated molecules results in apoptosis of CLL cells *in vitro*, with immediate decrease in nodal mass, CLL-related symptoms, and cytopenias (Woyach *et al*., 2012). However relapse is inevitable, suggesting that the malignant CLL Cells develop survival signals which are generated independently from those emanating from the BCR. Therefore the investigations of other associated signalling pathways are also of high priority. If B_1R and B_2R signalling (as a part of the KKS) are confirmed to play a role in the cross-activation of the BCR-associated molecules, the simultaneous inhibition of these receptors may become a target for CLL therapies. The role of the KKS signalling, mediated via Kinin B_1 and B_2

receptors, in tumour cell survival, proliferation and migration was intensively studied in a number of cancers especially lung and prostate tumours (Montana and Sontheimer, 2011b). Recently, based upon the findings that BK promotes angiogenesis and neovascularisation, several experimental studies were undertaken investigating the potential anticancer effects of BK antagonists in different cell lines (Worden and Kalemkerian, 2000, Stewart *et al.*, 2005). Multiple studies have demonstrated that BK effects were abolished by the B_1 receptor antagonist des-Arg (9)-[Leu (8)]-Bradykinin (DALBK) or/and B_2 receptors antagonist Icatibant (HOE 140) (Leeb-Lundberg *et al.*, 2005). Therefore, further investigation of B_1R and B_2R signalling in CLL B Cells with subsequent inhibition of these receptors is of potential interest in CLL.

In summary, four potential novel biomarkers have been identified and explored using various techniques. A prospective study should be designed for simultaneous evaluation of these biomarkers in both extracted PBMC as well as plasma and tissue samples, utilising the same study cohort. Subject to successful confirmatory investigation, these biomarkers may offer both novel insights into the biology of CLL, as well as new therapeutic targets for future treatments.

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APPENDICES

Appendix A Buffers and Reagents

Cell/Tissue Culture Media

RPMI 1640 (#31870, Invitrogen)	500 mL
Fetal Bovine Serum (#10106, Invitrogen)	50 mL
Fungizone (#15290, Invitrogen)	5 mL
Penicillin / Streptomycin (#15140, Invitrogen)	5 mL
L-Glutamine (#25030, Invitrogen)	5 Ml

DMEM (#D5546, Sigma Aldrich)	500 mL
Fetal Bovine Serum (#10106, Invitrogen)	50 mL
Fungizone (#15290, Invitrogen)	5 mL
Penicillin / Streptomycin (#15140, Invitrogen)	5 mL
L-Glutamine (#25030, Invitrogen)	5 mL

20% (v/v) DMSO Cell Cryopreservation Media

RPMI 1640 (#31870, Invitrogen)	400 mL
DMSO (#D2650, Sigma Aldrich)	100 mL

Washing Media for PBMCs Extraction

RPMI 1640 (#31870, Invitrogen)	500 mL
Fetal Bovine Serum (#10106, Invitrogen)	50 mL
Fungizone (#15290, Invitrogen)	5 mL
Penicillin / Streptomycin (#15140, Invitrogen)	5 mL
L-Glutamine (#25030, Invitrogen)	5 Ml

Phosphate Buffered Saline (PBS)

PBS tablets (#18912-014, Gibco)	1 tablet
dH_2O	500 Ml

PBS/BSA/Azide Buffer

PBS (as above)	1 L
BSA	2.5 g
Sodium Azide (10% Solution)	6.24 Ml

2% Paraformaldehyde Solution

PBS (as above)	100 mL
Paraformaldehyde (#P6148, Sigma Aldrich)	2 g

BSA/PBS Blocking Buffer

PBS (as above)	200 mL
BSA	1 g

Western Blotting (WB) Extraction Buffer

dH_2O	4 mL
Glycerol (#G8773, Sigma Aldrich)	0.8 mL
0.5 M Tris: HCL pH=6.8	1 mL
10% SDS (L3771, Sigma Aldrich)	1.6 mL
0.05 % Bromophenyl Blue (#B0126, Sigma Aldrich)	200 µl

TBSTWEEN-20 TBS Stock (Concentrated)

Trizma Base (#93304, Fluka)	121 g
Sodium Chloride (#S3014, Sigma Aldrich)	170 g

Made to 1 L with dH_2O and Adjusted to pH=7.6 with Concentrated HCl

Lysis Buffer / Protein Extraction Buffer-Working Solution

WB Extraction Buffer (as above)	1 ml
β Mercaptoethanol (#M7522, Sigma Aldrich)	50 µl
Protease Inhibitor (#80-6501-23, Amersham)	10 µl
Phosphatase Inhibitor 1 (#P2850, Sigma Aldrich)	10 µl
Phosphatase Inhibitor 2 (#P5726, Sigma Aldrich)	10 µl

Tris-HEPES-SDS Running Buffer

20x Tris-HEPES (#28368, Thermo-Scientific)	500 mL
dH_20	9,500 mL

TBS/TWEEN

Concentrated TBS	250 mL
dH_2O	4,750 mL
TWEEN-20 (#P5927, Sigma Aldrich)	2.5 mL

Western Blotting Blocking Solution (5% Non Fat Milk)

Non-fat dried milk powder (Marvel)	2 g
TBS / TWEEN 0.05% (as above)	40 mL

Western Blotting Blocking Solution (5% BSA)

BSA (#A6283, Sigma Aldrich)	2 g
TBS / TWEEN 0.05% (as above)	40 mL

TAE Running Buffer (50X Concentrated)

Trizma Base (#T1503, Sigma Aldrich)	242 g
Acetic Acid, Glacial ≥99.85%	57.1 mL
EDTA (#E6758, Sigma Aldrich) 0.5M	100 mL
Made to 1 L with dH_2O and Adjusted to pH=8	

Loading Buffer

dH_2O	7 mL
Glycerol (#G8773, Sigma Aldrich)	3 mL
Bromophenyl Blue (#B0126, Sigma Aldrich)	25 mg

Appendix B Demographic and Clinical Data on the CLL Study Cohort

Sample ID	Sex	Age	Binet Stage	IGVH (%) (m<98%)	CD38	ZAP70 (%)	Treated	Genetic Aberration	CLL Samples Discovery Proteomics LMWK Expression	LMWK protein expression (Chapter 5)	*LMWK* transcript expression (Chapter 6)	KLK expression (Chapter 7)	MACS (% of B cells)	FACS (% of B cells)	Kinin B1 Receptor expression (Chapter 9)	Kinin B2 Receptor expression (Chapter 9)
4	Male	63	A	93 m	Negative	1	Treated	13q		*Positive*	*Negative*					
5	Male	80	A	98 um	Positive	17	Treated	Normal		*Positive*	*Negative*					
8	Female	68	A	96 m	Positive	14	Treated	11q,13q		*Positive*	*Negative*	*Positive*				
9	Male	68	A	94 m	Negative	n/a	Not treated	n/a		*Negative*	*Negative*	*Positive*				
10	Female	79	B	99 um	Negative	19	Treated	17p,13q		*Negative*	*Negative*	*Positive*	98		*Positive*	*Positive*
11	Male	56	A	98 um	Negative	6	Treated	13q		*Positive*	*Negative*	*Positive*	96		*Positive*	*Negative*
17	Male	65	A	92 m	Negative	1	Treated	13q		*Negative*		*Positive*				
18	Female	62	A	92 m	Negative	5	Treated	13q		*Positive*	*Negative*	*Positive*				
20	Female	53	A	94 m	Negative	4	Treated	n/a		*Negative*						
24	Male	65	A	99 um	Positive	60	Treated	t12,11q		*Negative*						
25	Male	60	A	97 m	Negative	9	Not treated	n/a		*Negative*		*Positive*				
26	Female	58	A	91 m	Negative	11	Not treated	n/a		*Positive*	*Negative*	*Positive*				
27	Female	80	A	99 um	n/a	22	Treated	13q		*Negative*						
28	Female	55	A	90 m	Negative	n/a	Treated	17p		*Negative*		*Positive*				
29	Male	76	A	87 m	Negative	1	Not treated	n/a		*Positive*		*Positive*				
30	Male	64	A	99 um	Positive	29	Treated	13q,11q		*Negative*		*Positive*				
38	Female	55	A	99 um	Negative	63	Treated	13q		*Positive*						

41	Female	72	B	89 m	Negative	2	Treated	13q		Positive		Positive				
43	Female	60	A	96 m	Negative	5	Not treated	n/a		Negative						
44	Male	59	B	96 m	Negative	36	Treated	13q,17p		Positive		Positive				
45	Female	73	A	93 m	Negative	3	Not treated	n/a		Positive		Positive				
46	Female	53	A	92 m	n/a	14	Not treated	n/a		Negative	Negative					
52	Male	69	C	97 m	Negative	6	Treated	17p		Positive						
55	Female	69	C	96 m	n/a	37	Treated	n/a		Positive	Negative					
56	Male	66	A	100 um	Negative	87	Treated	13q		Positive						
57	Male	63	B	96 m	Positive	n/a	Treated	Normal		Positive						
58	Male	60	A	99 um	Positive	85	Treated	17p,t12		Positive						
64	Male	48	A	94 m	Negative	5	Not treated	13q		Positive		Positive				
82	Female	80	A	94 m	Negative	6	Not treated	n/a		Negative	Negative					
88	Male	73	A	92 m	Negative	1	Treated	13q		Positive	Negative					
89	Female	76	A	97 m	n/a	2	Treated	n/a	Positive	Positive	Negative	Positive			Positive	Negative
101	Female	74	A	100 um	Negative	34	Treated	n/a		Negative						
113	Male	63	A	96 m	Negative	70	Treated	13q		Positive	Negative	Positive			Positive	Negative
147	Male	87	C	100 um	Negative	6	Treated	Normal		Positive	Negative	Positive	94		Positive	Positive
150	Female	73	C	100 um	Positive	24	Treated	13q		Positive	Negative	Positive			Positive	Negative
166	Male	58	B	92 m	Positive	n/a	Treated	t12		Positive	Negative					
168	Male	60	A	90 m	Negative	n/a	Not treated	n/a		Positive						
169	Female	75	A	96 m	n/a	n/a	Treated	n/a		Positive	Negative	Positive	94		Positive	Positive
179	Male	66	A	n/a	Negative	n/a	Not treated	n/a		Positive						
191	Female	65	A	96 m	Negative	n/a	Treated	Normal		Negative						
332	Male	65	A	n/a	Positive	n/a	Treated	13q		Positive						
336	Female	41	A	n/a	Positive	n/a	Treated	t12		Positive						
337	Female	44	A	n/a	Negative	n/a	Not treated	n/a		Positive						
341	Male	78	A	n/a	Negative	n/a	Not treated	t12		Positive					Positive	Negative

342	Female	83	C	n/a	Negative	n/a	Treated	13q		*Positive*						
346	Male	69	A	n/a	Negative	n/a	Not treated	n/a		*Positive*						
349	Male	66	A	n/a	Positive	n/a	Not treated	0		*Positive*						
351	Female	72	A	n/a	Negative	n/a	Not treated	Normal		*Positive*					*Positive*	*Negative*
352	Female	65	A	n/a	Negative	0	Not treated	n/a		*Negative*		*Positive*				
353	Male	55	A	n/a	Positive	n/a	Not treated	t12		*Positive*						
354	Male	67	A	n/a	Negative	n/a	Not treated	n/a		*Positive*		*Positive*				
355	Male	62	B	n/a	Negative	n/a	Treated	Normal		*Positive*		*Positive*			*Positive*	*Negative*
3	Male	86	A	100 um	Positive	0	Treated		*Positive*	*Positive*	*Negative*	*Positive*			*Positive*	*Negative*
78	Male	85	A	100 um	Positive	20	Treated		*Positive*	*Positive*	*Negative*	*Positive*			*Positive*	*Negative*
35	Male	88	A	92 um	n/a	9	Treated					*Positive*				
292	Male	75	A	n/a	n/a							*Positive*				
14	Female	67	A	93 um	Negative	1	Treated							97	*Positive*	*Positive*
19	Male	70	A	92 um	Negative	9	Not treated							96	*Positive*	*Negative*
22	Male	64	A	90 um	Negative	7	Treated							94	*Positive*	*Negative*
343	Male	61	A	n/a	n/a										*Positive*	*Negative*
TOTAL Samples									**3**	**52**	**20**	**27**	**4**	**3**	**16**	**16**

Demographic Data on the Samples Obtained from the Healthy Volunteers

Sample N^0	Gender	Age
1	Male	61
2	Female	58
3	Male	59
4	Female	65

Appendix C Primer Design Guidelines

Primer Design Guidelines was adapted from (Premier Biosoft International, 1994-2014).

- ***Primer Length:*** The recommended optimal length of PCR primers is between 18-22 bp, which enables adequate specificity and easy primer binding to the template at the annealing temperature.
- ***Primer Annealing Temperature*:** Primer annealing/melting Temperature (T_m) is characterised as a temperature that indicates the stability of a primer-template *DNA* duplex and enables 50% of *DNA* duplex molecule to detach and to form the single strand *DNA* molecules. T_m generally corresponds to a nucleotide length and base content and the simplest equation for T_m calculation is the Wallace rule formula, where +2°C or +4°C are added for each Adenine / Thymine (A/T) or Guanine / Cytosine (G/C) nucleotides respectively. However, this equation was developed for short primer sequences not exceeding a length of 20-22 nucleotides and it is only a rough estimate. For longer primer sequences and precise calculation of the primers T_m a more superior method based on thermodynamic theory for nearest neighbour interactions can be utilised (Freier et al., 1986). The selected primer pair is recommended to have as closely matched melting temperatures as possible. A sub or super optimal T_m can stimulate a decrease of specificity or a greater likelihood of mispriming respectively.
  Generally, T_m values should range between 52-62^oC for 18-22 bases long PCR primers with about a 50% of the GC content. Therefore, the GC content is the next important prime consideration in the primer design / selection.
- ***GC Content**:* The preferred total percentage of G's and C's nucleotides in the primer should be between 40-60%.
- ***Primer Annealing Temperature:*** The annealing temperature (T_a) depends directly on the primer(s) sequence and usually about 5°C below the lowest T_m of the chosen primers pair. Too high T_a may possibly lead to low PCR amplicon yield, when too low T_a may result in an increase of base pair mismatches resulting in generation of non-specific binding. When long products are synthesised an equation can be used for precise primers T_a calculation (Rychlik *et al.*, 1990).
- ***GC Clamp:*** Addition of G or C nucleotides within the last five bases from the 3' end of primers, named GC clamp, facilitates a formation of stable complex with the target *DNA* due to the prevailing ability of G and C bases to arrange a stronger specific binding at the 3' end. However, it is recommended to avoid a presence of more than 3 G's or C's in the last 5 bases at the 3' end of the primer.

- ***Primer and Template Secondary Structures****:* The presence of the secondary structures (the folded, helical structure of double-stranded DNA)in PCR primers adversely affect amplification leading to significant reduction of the yield of PCR products due to the high stability of the secondary structures even above the optimised T_a and thus impairing primer binding to the template DNA. However, it is equally vital to establish the template regions that potentially can fold into conformations during the PCR reaction and to design primers avoiding secondary structures of the template, which can lead to additional, unexpected priming and generating of unwanted PCR products.
- ***Repeats and Runs:*** It is known with greater confidence and therefore preferred most frequently to avoid the frequent di-nucleotide repeats and a long single base run. Hence, in order to prevent mispriming a maximum of 4 di-nucleotide and a maximum of 4 bp of a single base run can be accepted in a primer sequence.
- ***Primers Cross Homology:*** The identification of the relevant cross homology regions and their bypass is a crucial step for primer design and selection.
- ***Primer Pair Design:*** It is advantageous for successful standard PCR reaction to design / select a primer set so that the PCR amplicon length is in the range between 100-500 bp. The product size can be calculated as follows:

Product size = (Position of antisense primer-Position of sense primer) + 1

In order to maximise PCR amplicon yield the T_m of forward and reverse primers should be similar or closely matched, however the difference in T_m values should not exceed 5^oC.

Several primer design software such as AlleleID, Beacon Designer and others are available for assisting in PCR primer design. Nevertheless in this study the primer sets were selected from a list of already pre-designed and synthesised oligonucleotide pairs within a sequence of interest. However, all of the above key characteristics were considered when selecting a primer sets.

Appendix D Plasma Samples and ELISA Standards Preparations

Plasma Sample Preparation Protocol (Performed by Paula Johnson)

Whole peripheral blood was collected in 2.7 mL -treated vacutainer tubes filled with buffered tri-sodium citrate solution with concentration of 0.109M (3.2%) (Greiner Bio-One, # 454334). The samples were subjected to centrifugation for 15 minutes at 1,000 *x g* using StatSpin Express (Iris Sample Processing). Following centrifugation whole blood was fractioned into three layers: sedimented erythrocytes at the bottom of the tube, “buffy coat” and plasma on the top. The resulting supernatant was immediately removed for up to 1 cm above the “buffy coat” and subsequently transferred into a clean polypropylene tube using a Pasteur pipette. The samples then were spun down again for 15 minutes at 1,000 *x g* before supernatant was transferred into 1 mL microcentrifuge tubes. The resulting plasma samples were stored at –80°C before they were safely transported to the Daisy building (Castle Hill Hospital) research laboratory. CLL plasma samples were thawed to acclimate to room temperature (18 to 25°C) and inverted gently prior to use.

ELISA Standards Preparation

A vial of Bradykinin standard was reconstituted with 1 mL of assay buffer and vortexed vigorously. This vial was labelled as Standard 1. After appropriate labelling, 900 µl of assay buffer was added to Standard 2, 750 µl of the assay buffer were added into Standards 3 to 6. Next 100 µl were removed from the reconstituted standard vial 1 and applied to Standard 2 and vortexed thoroughly. Then, 250 µl were taken out from Standard 2 and added to Standard 3 before

being vortexed to ensure good mixing. This procedure continued for Standards 4 through 6. Diluted Standards were used immediately within a maximum of 30 min of preparation.

Appendix E Comparative Proteomic Analysis Conducted by **Dr. Gina Eagle** (CBPG, University of Hull)

The data from this Appendix was previously published in (Kashuba et al., 2013b)

Samples Preparation and BCR Stimulation

CLL samples were collected at Queen's Centre for Oncology and Haematology, Castle Hill Hospital, prepared and stimulated as according to published methods (Kashuba et al., 2013b). CLL samples expressed a high proportion of B-cells with the mean percentage of cells expressing $CD19^+$ being min 80 %.

BCR Responsiveness Assay

BCR responsiveness assay was performed by Dr. James Bailey (the University of Hull). Immunoblotting was used to assess BCR responsiveness. Following a 10 minute incubation, stimulated, isotype control, positive control and untreated cells were lysed in LaemmLi buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 1% protease inhibitor mix, 0.00125% bromophenol blue) and quantified using the RC-DC Protein Assay Kit (500-0122, Bio-Rad). Equal amounts of denatured proteins (20 µg/lane) were electrophoresed on a homogenous Novex 12% tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. Following blocking (5% non-fat dry milk, 0.05% Tween 20 in TBS), the primary antibody was applied (1:100 anti phospho-ERK, sc-7383, Santa Cruz or 1:10000 anti-GAPDH loading control, ab9485, Abcam) for 2 hours. The appropriate horseradish peroxidase conjugated secondary antibody (1:1000

sc-2031 or sc-2030, Santa-Cruz) was applied prior to protein detection with the SuperSignal West Pico Chemiluminescence Substrate (Pierce). Films were scanned using a GS-800 laser densitometer and differential protein expression was measured using Quantity One software (version 4.6.1, Bio-Rad) following normalisation against the loading control. A sample categorised as BCR responsive was defined by ≥ 2-fold change in phospho-ERK expression upon BCR stimulation, with little or no constitutive phospho-ERK expression in untreated control cells and pERK ELISA assay. All three samples for proteomics upregulated pERK in both assays (Kashuba et al., 2013b).

The Discovery Phase

2-Dimensional Gel Electrophoresis with MALDI-TOF Mass Spectrometry

The study, conducted by Dr. G. Eagle aimed to construct a small-scale comparative proteome profile of B-CLL cells in BCR-stimulated and unstimulated samples using 2-DE and peptide mass fingerprinting by MALDI-TOF-MS. Three CLL samples (003, 078, 089) were selected for proteomic investigation based upon BCR responsiveness (≥ 2-fold increase in phospho-ERK expression upon in vitro crosslinking of the BCR). Following a 5.5 hour incubation, protein expression upon stimulation was compared with the corresponding isotype control. Briefly, total protein was extracted in isoelectric focusing (IEF) buffer in triplicate from each stimulated and unstimulated sample, quantified using the 2D Quant Kit (GE Health care) and desalted using the 2D Clean-Up Kit (Bio-Rad). Separation in the 1st dimension was carried out using 11 cm pH 4-7 ReadyPrep immobilised pH

gradient (IPG) strips (Bio-Rad). Following 2nd dimension electrophoresis on Criterion gels (Bio-Rad), proteins were visualised with Bio-Safe coomassie blue stain (Bio-Rad).The 3 gels from each sample were digitised with a GS-800 laser densitometer (Bio-Rad) and image analysis was performed using PDQuest (version 8.0, Bio-Rad). Normalisation was performed using the Total Quantity in Valid Spots method and the Students t-test ($p < 0.05$) was used to identify proteins which had change in expression by ≥2-fold between the stimulated sample and corresponding isotype control. The representative 2D gel and histogram of a clinical sample 3 showing proteins which have changed in expression are presented in Figures E I, II, III and Table E-I,II).

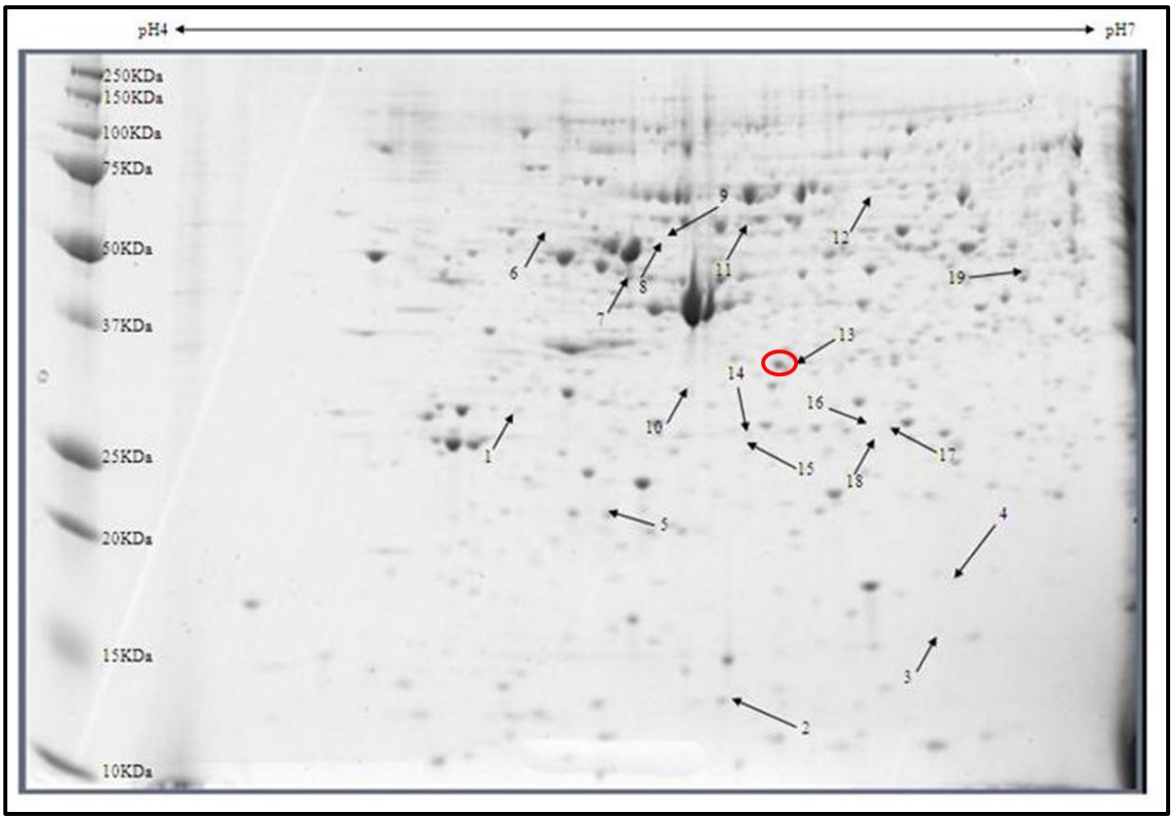

Figure E-I 2D Gel Showing Differentially Expressed Proteins Between Stimulated and Unstimulated Cells from CLL Clinical Sample 003

Presentation of a Sample 003 2D gel after successful IEF and electrophoresis. The spots represent individual proteins or clusters of proteins separated by pH in x- axis and molecular weight in the y- axis. The BioRad Precision Plus Protein Standards are visible along the left border of the gel and an approximation of the pH gradient is marked above. PDQuest highlighted 19 proteins spots which were differentially expressed between stimulated and unstimulated cells by over 2-fold with p<0.05. All 19 protein spots were excisable and were analysed by MALDI-OF-MS. Ten were identified with a significant score (score >66) (Table E-I). The picture shows the 19 proteins spots (arrows) which were identified by PDQuest with differential expression between stimulated and unstimulated cells by over 2-fold (p<0.05). The spot 13 (highlighted in red) is representative of ***KNG1****.*

Table E-I Table Showing Identifications of Differentially Expressed Proteins Between Stimulated and Unstimulated Cells from CLL Clinical Sample 003. The spot 13 identifies Kininogen 1 (~33 kDa).

Spot	PDQuest Spot Number	MW (KDa)	Estimated pI	Stimulated Cells	Protein Identified	Theoretical MW (KDa)	Theoretical pI	Score	Sequence Coverage
1	2222	27.3	~5.0	↓	Unnamed Protein Product	28.101	5.65	75	64%
2	5017	13	<6	↓	Not Identified	N/A	N/A	N/A	N/A
3	7014	15.6	>6.5	↓	Not Identified	N/A	N/A	N/A	N/A
4	7114	17.6	>6.5	↓	Fatty Acid Binding Protein 5	15.155	6.6	68	100%
5	3111	20.9	~5.5	↓	Interferon Alpha-1/13 Precursor	21.711	5.32	66	52%
6	2512	56.1	~5.0	↑	Tripartite Motif Protein (TRIM19 Zeta)	69.953	5.17	73	39%
7	3418	43.9	~5.5	↓	Not Identified	N/A	N/A	N/A	N/A
8	4504	52.5	~5.5	↑	Astrotactin 2 (ASTN2 Protein)	49.557	5.53	73	56%
9	4517	54.5	~5.5	↓	Astrotactin 2 Isoform d	44.532	5.57	68	60%
10	4210	29.9	~5.5	↓	Glyoxalase Domain Containing 4	33.228	5.4	70	55%
11	5531	56.4	~6	↓	Aspartyl-tRNA Synthetase (DARS)	57.088	6.11	68	51%
12	6617	64.4	<6.5	↑	Erythrocyte Membrane Protein Band 4.1 (EPB41)	69.847	6.32	59	43%
13	**6323**	**32.4**	**<6.5**	↑	**KNG1 Protein (Kininogen 1)**	**33.034**	**6.27**	**67**	**54%**
14	5116	26.2	~6	↓	Not Identified	N/A	N/A	N/A	N/A
15	5104	25.5	~6	↓	Phosphatidylinositol Transfer Protein Beta	22.23	5.89	70	64%
16	6218	26.6	<6.5	↓	Coenzyme Q7 Homolog Ubiquinone (Yeast)	22.839	6.45	61	62%
17	6216	26.6	<6.5	↓	Not Identified	N/A	N/A	N/A	N/A
18	6116	24.9	<6.5	↓	TatD Dnase Containing 3 (TATDN3 Protein)	30.314	6.67	47	37%
19	8408	44.2	>6.5	↓	DnaJ (HSP40) Homolg Subfamily C Member 7	56.405	6.565	58	44%

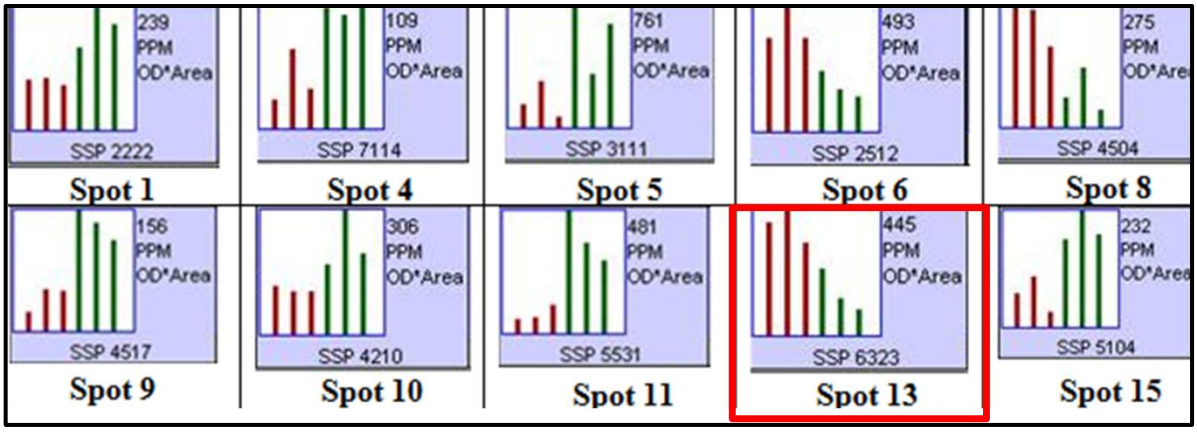

Figure E-II PDQuest histograms of the 10 proteins identified by MADLI-TOF-MS showing differential expression between stimulated and unstimulated cells in CLL clinical sample 003

The histograms show protein expression in the three test replicates (stimulated cells, red) and three control replicates (unstimulated cells, green). The protein spots had a >2-fold change in expression between stimulated and unstimulated cells ($p<0.05$) and were identified by MALDI-TOF-MS (Table 9.1). Protein spots 6, 8 and 13 were over expressed in stimulated cells and spots 1, 4, 5, 9, 10, 11 and 15 were over expressed in unstimulated cells. The spot 13 (highlighted in red) is representative of KNG1.

Differentially expressed proteins were excised, de-stained, reduced and alkylated prior to trypsin digestion (Trypsin Gold, Promega). Peptides were spotted onto an anchorchip MALDI target (Bruker Daltonics) in a saturated 2,5dihydroxybenzoic acid matrix solution and mass spectra were recorded in the reflectron mode on a Bruker Daltonics Reflex IV MALDI-TOF MS equipped with a 337 nm nitrogen laser. Ions were accelerated through a potential of 20 Kv into the TOF mass analyser and ions within the mass to charge (m/z) range of 950-2500 were detected. Data were acquired using the Flex Control (version 2.4) programme

and processed with Flex Analysis (version 2.4) and Biotools (version 3.0). Peaks were de-isotoped and monoisotopic peptide masses were assigned. Contaminating ions from the autolysis of trypsin and keratin were removed. The resultant peptide mass fingerprints were searched against the NCBInr database using the MASCOT search engine (www.matrixscience.com) for protein identification. The search was restricted to human taxonomy. Fixed carbamidomethyl modifications and variable proprionamide and methionine oxidation modifications were considered. A maximum of 2 missed tryptic cleavage was considered and the mass tolerance for the monoisotopic peptide masses was set at ± 0.1%. Mowse scores were used to measure the level of significance of each match ($p < 0.05$) and in these experiments a minimum significant score was 65. In addition, a minimum of 30% sequence coverage was required for each significant match and the estimated molecular weight and pI information (from gel positioning) was compared with each match to further ensure correct identifications were assigned.

The Result of 2-Dimensional Gel Electrophoresis with MALDI-TOF Mass Spectrometry

Samples 003, 078, 089 demonstrated 10.4-fold, 15.9-fold and 216.1-fold and ELISA up-regulation of phospho-ERK upon stimulation and were classified as BCR responsive samples. These samples were successfully analysed using 2-DE/MS analysis. A total of 48 DEP spots were excised from the gels and 16 of these were identified using MS (Table A and Table B). The differential expression of kininogen (up-regulation) upon artificial stimulation of the BCR was identified in

3/3 clinical samples. Due to this observation, the expression of Kininogen was further investigated in immunoblotting.

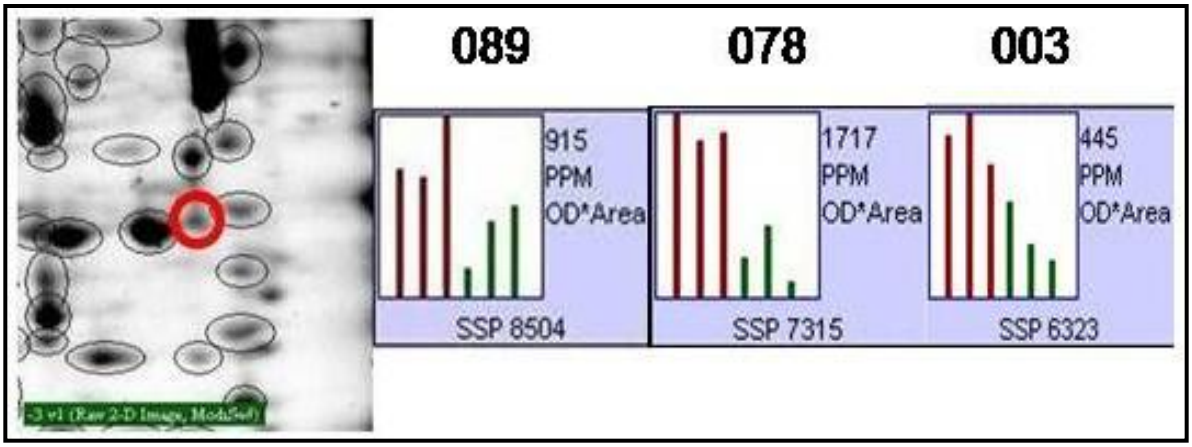

Figure E-III Representative gel and histograms from 3 clinical CLL samples (003, 078, 089) showing a protein spot (KNG) which has increased by over 2 fold with $p<0.05$ in CLL cells upon BCR stimulation.

Histograms shows 3 technical replicates from each group; cells with stimulated BCR (red) and control cells (green).

Table E-II DEPs identified using 2-DE/MS in CLL samples following artificial stimulation of the BCR.

Sample	PD Quest Ref	Estimated MW (KDa)	Estimated pI	Expression change	Protein Identified	Gene ID (A-Z)	gi ref
78	1323	30.3	5.5	↓	Adenylate kinase isoenzyme 5 (recombinant)	AK5	gi|9296996
3	4504	52.5	~5.5	↑	ASTN2 protein	ASTN2	gi|20809687
3	4517	54.5	~5.5	↓	Astrotactin-2 isoform d	ASTN2	gi|38016949
3	5531	56.4	~6	↓	Aspartyl-tRNA synthetase	DARS	gi|78394948
3	7114	17.6	>6.5	↓	Fatty acid-binding protein	FABP5	gi|4557581
3	4210	29.9	~5.5	↓	Glyoxalase domain-containing protein 4	GLOD4	gi|34850074
3	3111	20.9	~5.5	↓	Interferon alpha-1/13 precursor	IFNA13	gi|124455
89	5713	51.2	~6	↑	Kazrin isoform A	KAZN	gi|63147424
3	6323	32.4	<6.5	↑	KNG1 protein	**KNG1**	gi|116283732
78	7315	28.7	6.7	↑	Kininogen 1	**KNG1**	gi|37748641
89	8504	49.6	<6.5	↑	Kininogen 1	**KNG1**	gi|37748641
3	5104	25.5	~6	↓	Phosphatidylinositol transfer protein, beta, isoform CRA_b	PITPNB	gi|119580148
3	2512	56.1	~5.0	↑	Tripartite motif protein TRIM19 zeta	PML	gi|12275901
89	1615	43.8	<6	↓	FYVE-RING finger protein SAKURA	RFFL	gi|21064943
89	7725	67.7	<6.5	↑	Mitochondrial Rho GTPase 1 isoform 1	RHOT1	gi|75750480
89	4118	22.3	~6	↓	Thiopurine S-methyltransferase	TPMT	gi|13529134

Table E-III LMWK and 33 kDa LMWK Isoform AA Sequence

33 kDa LMWK Isoform

```
10         20         30         40         50
MHGNRGEEEQ YEILRGYPDL EPILRHGIQYFNNNTQHSSLFTLNEVKRAQ
        60         70         80         90        100
RQVVAGLNFR ITYSIVQTNC SKENFLFLTP DCKSLWNGDT GECTDNAYID
       110        120        130        140        150
IQLRIASFSQ NCDIYPGKDF VQPPTKICVG CPRDIPTNSP ELEETLTHTI
       160        170        180        190        200
TKLNAENNAT FYFKIDNVKK ARVQVVAGKK YFIDFVARET TCSKESNEEL
       210        220        230        240        250
TESCETKKLG QSLDCNAEVY VVPWEKKIYP TVNCQPLGMI SLMKRPPGFS
       260        270        280        290
PFRSSRIGEI KEETTSHLRS CEYKGRPPKA GAEPASEREV S
```

Full Length LMWK Isoform

```
        10         20         30         40         50         60
MKLITILFLC SRLLLSLTQE SQSEEIDCND KDLFKAVDAA LKKYNSQNQS NNQFVLYRIT

        70         80         90        100        110        120
EATKTVGSDT FYSFKYEIKE GDCPVQSGKT WQDCEYKDAA KAATGECTAT VGKRSSTKFS

       130        140        150        160        170        180
VATQTCQITP AEGPVVTAQY DCLGCVHPIS TQSPDLEPIL RHGIQYFNNN TQHSSLFMLN

       190        200        210        220        230        240
EVKRAQRQVV AGLNFRITYS IVQTNCSKEN FLFLTPDCKS LWNGDTGECT DNAYIDIQLR

       250        260        270        280        290        300
IASFSQNCDI YPGKDFVQPP TKICVGCPRD IPTNSPELEE TLTHTITKLN AENNATFYFK

       310        320        330        340        350        360
IDNVKKARVQ VVAGKKYFID FVARETTCSK ESNEELTESC ETKKLGQSLD CNAEVYVVPW

       370        380        390        400        410        420
EKKIYPTVNC QPLGMISLMK RPPGFSPFRS SRIGEIKEET TSHLRSCEYK GRPPKAGAEP


ASEREVS
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AA Sequence for both proteins was extracted from http://www.uniprot.org/uniprot. The sequence of 33 kDa isoform differs from the canonical sequence LMWK at position 1-17. The AA Sequence from 18-291 is identical for both proteins.

Appendix F KLKs Gene/Protein Nomenclature, Characteristics and Implications in Physiology and Cancer

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
*KLK*1	Tissue kallikrein1 (hK1), KLK1 Pancreatic/ renal/urinary Kallikrein.	Trypsin-like enzymatic or kininogenase activity (proteolysis of LMWK at *Arg–Ser* and *Met–Lys* residues to liberate Lys-BK (Scarisbrick *et al.,* 2006)). Chymotrypsin-like activity: lysis of kallistatin (human kallikrein-binding protein) and somatostatin (Debela *et al*., 2007). Growth factors and peptide hormones regulation (Cho *et al.*, 2003).	Not known	Abundantly expressed in healthy tissues such as pancreas, salivary glands, kidney, colon, prostate gland, testis and spleen.
*KLK*2	Tissue Kallikrein 2 (hK2), KLK2, Human glandular Kallikrein 1	Shares 80% sequence homology with KLK3 (Saigusa *et al.*, 2011). Trypsin-like enzymatic activities: lower kininogenase activity in comparison to KLK1 (Dong *et al*., 2003, She *et al*., 2003). Seminogelins hydrolysis. Regulation of growth factor through insulin-like growth factor binding protein. Urokinase-type plasminogen (uPA) activation (Magklara *et al.,* 2003), which has been associated with metastatic progression in prostate cancer (Yousef *et al.*, 1999).	Prognostic marker. Diagnosis, and monitoring	Abundant expression in prostate (Saigusa *et al*., 2011) and breast (Nomura *et al*., 2010) cancers.

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
*KLK*3	Tissue Kallikrein 3 (hK3), KLK3, Prostate-specific antigen (PSA), seminin, Kallikrein-3, P-30 antigen, Semenogelase	Chymotrypsin-like substrate specificity. Activity is strongly regulated by Zn^{2+}. Post-ejaculation liquefaction of the seminal plasma clot (Yousef *et al*., 1999), growth factors and hormone-related peptides regulation.	Prognostic marker. Diagnosis, and monitoring.	Abundant expression in prostate, (Rajapakse *et al*., 2005, Kishi *et al.,* 2003b) and breast (Pampalakis and Sotiropoulou, 2007) cancers.
*KLK*4	Tissue Kallikrein 4 (hK4), KLK4, Prostase, KLK-L1 protein, EMSP1*, PSP17*.	Trypsin-like specificity. This protein is regulated by androgens and known to be involved in enamel formation. KLK4 has been shown to be a urokinase-type plasminogen modulator (uPA) due to the ability to cleave after *Pro-Arg-Phe-Lys* (Diamandis *et al*., 2000) and hydrolyses the uPA receptor (uPAR) thus serving as the tumour-associated uPA/uPAR-pathway activity regulator (Yousef *et al*., 2000c, Obiezu *et al*., 2000).	Prognostic marker. Resistance to chemotherapy and decreased OS* in Ovarian cancer.	Highly expressed in healthy prostate tissue. Overexpression in ovarian (Shvartsman *et al.*, 2003) and prostate (Diamandis *et al*., 2004, Patsis *et al.*, 2012) cancers.

* EMSP1- Enamel matrix serine proteinase 1; * PSP17-Prostase Serine protease 17; *OS-overall survival .

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
*KLK*5	Tissue Kallikrein 5 (hK5), KLK5, KLK-L2 protein, Human stratum corneum tryptic enzyme (HSCTE)	Trypsin-like activity with preferential selection to cleave *-Arg-* over *-Lys-* as. Autoactivation in conjunction with activation of KLK7 and KLK14. Serpins α_2-antiplasmin and α_1-antithrombin can inhibit the proteolytic activity of KLK5 (Michael *et al*., 2005). KLK5 can serve as an enzyme for extracellular matrix digestion especially for the components such as type I-IV collagen, fibronectin, and laminin. KLK5 known to liberate angiostatin from plasminogen, to cleave a "cystatin-like domain 3" from LMWK, and also to degrade fibrinogen (Michael *et al.*, 2005). Several findings shown the role for KLK5 in tumour invasion and angiogenesis, which is associated with tumour progression (Paliouras *et al.*, 2007, Michael *et al*., 2005).	A potential cancer biomarker for endocrine-related malignancies. An over-expression is an indicator for unfavourable prognosis (reduced survival) in ovarian cancer.	Widely expressed in diverse human tissues but found at high levels in skin, breast, brain and testis (Yousef and Diamandis, 1999). Upregulated mRNA expression in endocrine-related malignancies, including ovarian, breast (Dong *et al*., 2003) and testicular cancer.

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
*KLK*6	Kallikrein 6 (hK6), KLK6, Zyme, Protease M, Neurosin, Serine protease-18, Serine protease-9.	Trypsin-like serine protease properties, cleaving preferentially after -*Lys*- or -*Arg*- residue. Shown to play role in pathogenesis of Alzheimer's disease, Parkinson disease and other synucleinopathies (Iwata *et al.*, 2003) and traumatic spinal cord injury (Scarisbrick *et al.,* 2006). Upregulated expression in autoimmune inflammatory disease such as multiple sclerosis and various malignancies, specifically in ovarian cancer. KLK6 was demonstrated to play an important role in tumour invasion and metastasis through degradation of high-molecular-weight extracellular matrix proteins such as vitronectin, fibronectin, laminin, and collagen and that the malignant cells pre-treated with a neutralizing KLK6 antibody have shown reduced cell migration in vitro in comparison to control cells (Ghosh *et al.,* 2004). KLK6 expression is implicated in the immune (T- and B-lymphocytes) cell survival via overexpression of B-cell lymphoma-extra large (Bcl-XL) (pro-survival molecule), and downregulation of Bcl-2 interacting mediator of death (Bim) (the pro-apoptotic molecule) (Scarisbrick *et al*., 2011).	Elevation of KLK6 level in serum is a diagnostic and prognostic marker in ovarian cancer. A progression marker for colorectal cancer (Yousef *et al.*, 2004, Ohlsson et al., 2012).	Physiological expression in neuronal, renal, endometrial, mammary, pancreatic and prostatic tissues (Magklara *et al.*, 2003); upregulated protein expression in ovarian cancer.

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
KLK7	Kallikrein 7 (hK7), KLK7, Serine protease 6, Stratum corneum chymotryptic enzyme	Chymotrypsin-like serine property. Several endogenous protein, Zn^{2+} and Cu^{2+} ions are regulators of KLK7activity (Debela *et al*., 2007). KLK7 is important in maintaining of skin homeostasis by playing a role in the regulation of skin desquamation via degradation of desmosomal components, such as desmogleins, which serves as the cell adhesion components. KLK7 dysregulation in pancreatic cancer diminishes desmogleins level, resulting in cell adhesion depression and malignant cell invasion facilitation (Ramani *et al.*, 2008).	Over-expression of mRNA is correlated with poor prognosis in colon cancer (Inoue et al., 2010).	Expressed in brain, mammary gland, cerebellum, spinal cord, kidney and keratinocytes. Differentially expressed in colon, pancreatic, ovarian and breast cancer.
KLK8	Kallikrein 8 (hK8), KLK8, Neuropsin, Ovasin, Tumour-associated differentially expressed gene 14 protein.	Trypsin-like activity and can proteolytically hydrolyse several proteins such as kininogen, fibronectin, fibrinogen, gelatin, collagen type IV and casein and therefore associated with extracellular matrix protein degradation (Rajapakse et al., 2005). Elevated serum concentration may be an indicator of ovarian cancer progression (Kishi et al., 2003a).	A potential biomarker of ovarian (Kishi et al., 2003a) and breast cancer.	Expressed in breast, kidney, testical, skin, oesophagus and salivary gland (Darling *et al.,* 2008). Aberrantly expressed in squamous cell carcinoma, prostate cancer (Pampalakis and Sotiropoulou, 2007).

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
KLK9	Kallikrein9 (hK10),KLK9,Kallikrein-like protein 3.	Chymotrypsin-like property.	A potential prognostic biomarker in ovarianand breast cancer (Yousef *et al*., 2003b, Yousef *et al.*, 2003a) (KLK9 positivityis associated with favourable prognosis and increased PFS and OS comparing to KLK9 negativity).	Expressed in skin, trachea, cerebellum, thymus, and spinal cord. Ovarian and breast cancer.
KLK10	Kallikrein 10 (hK10), KLK10, Normal epithelial cell-specific 1, Protease serine-like 1.	Trypsin-like serine proteinase distribution of which is hormone-regulated and tissue specific. Upregulation observed in pancreatic and ovarian cancer, however it is downregulated in prostate and breast cancer and shown to express the tumour-suppressor properties (Luo *et al*., 2001, Bayani *et al.*, 2008).	Prognostic value in breast, pancreatic and ovarian cancer (Shvartsman *et al.,* 2003).	Expressed in skin pancreas, tonsils, oesophagus, brain, and testis and ovary tissue. Aberrant expression in hormone-dependant malignancies and pancreatic cancer.

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
KLK11	Kallikrein 11 (hK11), KLK11, Hippostasin, Serine protease 20, Trypsin-like protease.	Trypsin-like serine protease, which is regulated by steroid hormones, particularly estrogens. Aberrant expression in various malignancies. KLK11 overexpression in human breast cancer shown to be associated with liberation of insulin-like growth factor (IGF) resulting in enhanced bio viability of IGF and tumour progression (Sano *et al.*, 2007).	Potential diagnostic and prognostic marker in ovarian cancer (Diamandis *et al.*, 2004), laryngeal cancer (Patsis *et al.*, 2012), may serve as serological marker for prostate and ovarian cancer(Borgono et al., 2003a).	Expressed in brain, skin and prostate. Dysregulated expression in ovarian, prostate and laryngeal cancer.
KLK12	Kallikrein 12 (hK12), KLK12, Kallikrein-like5 (KLK-L5)	KLK12 is a trypsin-like serine proteinase. Liberated as an inactive enzyme which activates in vivo following proteolytic hydrolysis. KLK12 expression is steroid hormones-regulated.	Potential diagnostic biomarker for breast, prostate (Yousef *et al*., 2000b), gastric cancer(Zhao *et al.,* 2012), lung(Planque *et al.*, 2008).	Expressed in pancreas, salivary gland, breast, prostate, uterus, lung, thymus, colon, thyroid, brain and stomach tissues. Differential expression in breast, prostate and gastric cancers.

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
KLK13	Kallikrein 13 (hK13),KLK13, Kallikrein-like protein 4	Trypsin-like enzymatic activity. Prevalent localisation within a cell structure is the glandular epithelia, including prostate, breast and ovary tissues (Scorilas *et al*., 2004). Breast cancer cell line pre-treated with a KLK13 neutralizing antibody demonstrated diminishing migration in comparison to untreated control leading to suggestion that KLK13 may play a role in tissue remodelling and/or tumour invasion and metastasis (Kapadia *et al*., 2004).	Potential independent marker of favourable prognosis in ovarian (Scorilas *et al.*, 2004)and breast malignancies (KLK13 positive cases exhibit an increased PFS and OS)(Chang *et al*., 2002).	Expressed in endocrine tissue such as prostate, breast, testis and salivary gland. Differentially expressed in breast, ovarian cancer.
KLK14	Kallikrein 14 (hK14),KLK14, Kallikrein-like protein 6	This steroid hormone-regulated serine-type enzyme expresses both trypsin- and chymotrypsin -like activity; regulates the activation/inactivation of other kallikreins such as KLK1, KLK3, KLK5 and KLK11 and several receptors. Shown to be implicated in clot liquefaction, epidermal desquamation, cancer growth, invasion and angiogenesis (Oikonomopoulou *et al*., 2006, Borgono *et al.*, 2007).	Potential diagnostic breast tumour marker (abundant expression is associated with tumour aggression (Borgono *et al.,* 2003b, Fritzsche *et al*., 2006)**,** and ovarian cancer.	Expressed in CNS*, bone marrow, colon, breast, kidney, small intestine, uterus, thyroid, pancreas, spleen, prostate, and skeletal muscle. Aberrant expression in breast and ovarian cancer.

*CNS-central nervous system

Gene Name	***Known Protein Name***	***Physiology/Pathology***	***Clinical Relevance***	***Healthy/Tumour Tissue Specificity***
KLK15	Kallikrein 15 (hK15),KLK15, ACO protease.	KLK15is a steroid hormone-regulated trypsin-like serine protease with preferential cleavage site after *Arg*- and/or *Lys*- residues. KLK15 exhibits significant structural and positional similarities with hK3(Batra *et al.*, 2011).The physiological substrate for this proteases is not yet fully elucidated, however it has been shownthatKLK15 has the potential to activate/inactivate hK3 (Shaw *et al*., 2007).	Potential favourable diagnostic marker for breast cancer(Yousef *et al.*, 2002), a prognostic predictor of ovarian cancer progression (reduced PFS and OS)(Batra *et al*., 2011)and diagnostic and/or prognostic marker for prostate cancer(Liu *et al*., 2011a).	Expressed in prostate, thyroid, salivary, and adrenal glands, colon, testis and kidney(Kuzmanov *et al*., 2012). Differential mRNA expression in prostate, ovarian and breast cancer.

Appendix G Full Length Native Human Proteins

Protein Name	Company / Catalogue #	Molecular Weight (kDa)	UniProt Accession
Full length native LMWK protein	Abcam, #91118	65	P01042
Full length Human Kallikrein 6 protein	Abcam, #82792	51.8	Q92876

Appendix H The Result of *LMWK* Transcript Expression Analysis as Determined by RT-PCR

N°	Sample ID	LMWK protein expression	*LMWK* gene expression	*GAPDH* gene expression	*GUSB* gene expression
1	3	Positive	Negative	Positive	Positive
2	4	Positive	Negative	Positive	Positive
3	5	Positive	Negative	Positive	Positive
4	8	Positive	Negative	Positive	Positive
5	9	Negative	Negative	Positive	Positive
6	10	Negative	Negative	Positive	Positive
7	11	Positive	Negative	Positive	Positive
8	18	Positive	Negative	Positive	Positive
9	26	Positive	Negative	Positive	Positive
10	46	Negative	Negative	Positive	Positive
11	55	Positive	Negative	Positive	Positive
12	78	Positive	Negative	Positive	Positive
13	82	Negative	Negative	Positive	Positive
14	88	Positive	Negative	Positive	Positive
15	89	Positive	Negative	Positive	Positive
16	113	Positive	Negative	Positive	Positive
17	150	Positive	Negative	Positive	Positive
18	147	Positive	Negative	Positive	Positive
19	166	Positive	Negative	Positive	Positive
20	169	Positive	Negative	Positive	Positive
21	Healthy M	Negative	Negative	Positive	Positive
22	Healthy F	Negative	Negative	Positive	Positive
23	LNCaP	Positive	**Positive**	Positive	Positive
24	Raji	Positive	Negative	Positive	Positive
25	T47D	Positive	Negative	Positive	Positive
26	A549	Positive	Negative	Positive	Positive
27	SW837	Positive	Negative	Positive	Positive

Appendix I Published Articles Associated with the Current Project

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Proteomic analysis of B-cell receptor signaling in chronic lymphocytic leukaemia reveals a possible role for kininogen

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ABSTRACT

CLL is an incurable disease with variable prognosis. The hyper reactivity of the B-cell receptor (BCR) to unknown antigen ligation plays a pivotal role in CLL-cell survival. We aimed to investigate the BCR signalling pathway using proteomics to identify novel proteins which may have clinical relevance in this disease.

Three CLL samples were selected based upon BCR responsiveness, demonstrated by upregulation of phospho-ERK following in vitro stimulation. The differential expression of proteins, upon artificial stimulation of the BCR, was examined in these samples using two-dimensional gel electrophoresis in combination with mass spectrometry. Proteins of interest were subsequently examined using immunoblotting. Proteomic analysis revealed that kininogen, a critical protein of kinin–kallikrein system, was upregulated in all 3 clinical samples upon BCR stimulation. There are 2 forms of kininogen: HMWK and LMWK. The upregulation of LMWK upon BCR stimulation was confirmed by immunoblotting in all 3 of these samples. In a pilot series of 52 unselected CLL samples, 71% demonstrated basal LMWK expression. There was a trend towards shorter median survival in LMWK positive cases (147 months versus 253 months for LMWK negative cases; $p = 0.125$). Kininogen may be a novel therapeutic target in CLL and the possible association with prognosis warrants further investigation.

Biological significance

We have identified the upregulation of LMWK upon BCR stimulation of CLL samples. There is no previous published research to suggest a link between kininogen and normal B-cells or CLL cells. In 52 unselected CLL samples, 71% demonstrated basal LMWK expression. There was a trend towards shorter median survival in LMWK positive cases. The absence of LMWK protein expression on normal B-cells suggests that this could be a biomarker for CLL and further research should be undertaken.

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1. Introduction

Chronic lymphocytic leukaemia (CLL) is the most common adult leukaemia and remains an incurable disease despite recent advances in diagnosis and treatment [1]. The clinical course of patients with CLL is heterogeneous; some patients survive for years without treatment whilst others die of a drug-resistant disease within 1 to 2 years of presentation [2,3]. Treatment is recommended if the disease progresses or in the presence of disease-related symptoms. At present there is no reliable clinical test to predict the probability of disease progression. CLL B-cell expression of CD38, zeta-associated protein 70 (ZAP-70), serum beta 2-microglobulin (B2M), the presence of somatically unmutated immunoglobulin V_H (IgV_H) genes and cytogenetic abnormalities such as deletions on 11q or 17p have been reported to indicate poor prognosis in CLL patients [4–7]. Cytogenetic analysis and sequencing analysis of IgV_H may be the most robust current methods for predicting disease outcome [8–10]; however these are not routinely performed in the clinic. Therefore further investigation of CLL is required in order to identify robust biomarkers which are suitable for routine use for the individualisation of patient management.

CLL is defined as a lymphoproliferative disorder characterised by the progressive accumulation of antigen-experienced malignant CD5+/CD23+ CLL-cells which genetically and immunophenotypically resemble memory B cells in the peripheral blood, bone marrow, lymphoid tissue and spleen [11–13]. Historically, CLL was described as a B-cell malignancy composed of non-proliferating cells which were resistant to senescence and apoptosis; however more recent studies describe CLL as a disease of both cell proliferation and cell accumulation [13–20]. The BCR plays an important role in CLL pathogenesis. Microenvironment-derived antigen stimulation of the BCR can prevent apoptosis of CLL B-cells and cell behaviour may therefore be related to the differential ability of the BCR to respond to antigen [15,17,21–24]. Activation of the BCR pathway initiates cascades of cellular signalling pathways, including the ERK pathway, supporting the survival of CLL B-cells [17,25]. The BCR can be artificially stimulated using an anti-IgM antibody *in vitro* [17,26] in order to investigate protein expression, which may be associated with the survival of CLL B-cells, induced by antigen-receptor signalling. We aimed to artificially stimulate the BCR and use comparative proteomics to investigate protein expression changes associated with BCR activation in BCR-responsive CLL cells. Proteins of interest would be further analysed using immunoblotting in order to determine any possible clinical correlations.

2. Materials and methods

2.1. CLL Samples

Following Research Ethics Committee approval (05/Q1104/33) and informed consent, heparinised blood samples were collected at Castle Hill Hospital from newly diagnosed or existing CLL patients who had not received treatment within 3 months. Peripheral blood mononuclear cells (PBMCs) were separated using Histopaque (H8889, Sigma-Aldrich, UK) and cells were stored at minus 80 °C in aliquots at 1×10^7 cells/ml in RPMI 1640 media supplemented with 10% v/v foetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% DMSO. Standard clinical information was collected for all patients. IgV_H gene analysis and ZAP-70 flow cytometric analysis were performed as described previously [27]. The proportion of B-cells was estimated using CD-19 in flow cytometry as described previously [27] to ensure all samples were composed of a minimum of 80% B-cells.

2.2. BCR stimulation of CLL cells

CLL cells were rested for three hours at 37 °C with 5% CO_2 to acclimatise after thawing. Cells were resuspended at a final concentration of 5×10^6/ml and stimulated using 10 μg/ml AffiniPure F(ab')$_2$ fragment goat anti-human IgM, $Fc_{5\mu}$ fragment specific (109-006-129, Jacksons Immuno Research Laboratories Inc), which crosslinks the BCR [28,29]. To produce an isotype control [30,31], cells were resuspended at a final concentration of 5×10^6/ml and exposed to 10 μg/ml ChromPure goat IgG, F(ab')$_2$ fragment (005-000-006, Jacksons Immuno Research Laboratories Inc., UK). To serve as a positive control, cells resuspended at a final concentration of 5×10^6/ml were treated with 100 nM phorbol 12-myristate 13-acetate (PMA; P1585, Sigma-Aldrich, UK). PMA activates protein kinase C, which will subsequently lead to the activation of the ERK pathway. Cells were incubated at 37 °C with 5% CO_2 for 10 minutes or 5.5 hours.

2.3. BCR responsiveness assays

2.3.1. *Semi quantitative immunoblotting*

Immunoblotting was used to assess BCR responsiveness. Following a 10 minute incubation, stimulated, isotype control, positive control and untreated cells were lysed in Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 1% protease inhibitor mix, 0.00125% bromophenol blue) and quantified using the RC-DC Protein Assay Kit (500-0122, Bio-Rad). Equal amounts of denatured proteins (20 μg/lane) were electrophoresed on a homogenous Novex 12% tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. Following blocking (5% non-fat dry milk, 0.05% Tween 20 in TBS), the primary antibody was applied (1:100 anti phospho-ERK, sc-7383, Santa Cruz or 1:10000 anti GAPDH loading control, ab9485, Abcam) for 2 hours. The appropriate horseradish peroxidase conjugated secondary antibody (1:1000 sc-2031 or sc-2030, Santa-Cruz) was applied prior to protein detection with the SuperSignal West Pico Chemiluminescence Substrate (Pierce). Films were scanned using a GS-800 laser densitometer and differential protein expression was measured using Quantity One software (version 4.6.1, Bio-Rad) following normalisation against the loading control. A sample categorised as BCR responsive was defined by ≥2-fold change in phospho-ERK expression upon BCR stimulation, with little or no constitutive phospho-ERK expression in untreated control cells.

2.3.2. *Quantitative ELISA*

CLL cells were counted using an automated laboratory analyser (Sysmex) and aliquots of 2×10^6 cells were prepared. Following a 10 minute incubation, stimulated, isotype control,

positive control and untreated cells were washed once with ice-cold PBS and lysed according to the manufacturer's instructions. Quantitative ELISA was employed to assay phospho-ERK1 (T202/Y204) and phospho-ERK2 (T185/Y187) using the Surveyor IC kit (SUV1018, R&D Systems) according to the manufacturer's instructions. The recombinant protein provided was used to produce a 7 point standard curve and the ELISA results were normalised using a natural log transformation.

2.4. 2-dimensional gel electrophoresis (2-DE) with MALDI-TOF mass spectrometry (MS)

Three CLL samples (003, 078, 089) were selected for proteomic investigation based upon BCR responsiveness (≥2-fold increase in phospho-ERK expression upon *in vitro* crosslinking of the BCR). Following a 5.5 hour incubation, protein expression upon stimulation was compared with the corresponding isotype control using 2-DE and peptide mass fingerprinting by MALDI-TOF-MS as described previously [32]. Briefly, total protein was extracted in isoelectric focusing (IEF) buffer in triplicate from each stimulated and unstimulated sample, quantified using the 2D Quant Kit (GE Healthcare) and desalted using the 2D Clean-Up Kit (Bio-Rad). Separation in the 1st dimension was carried out using 11 cm pH 4–7 ReadyPrep immobilised pH gradient (IPG) strips (Bio-Rad). Following 2nd dimension electrophoresis on Criterion gels (Bio-Rad), proteins were visualised with Bio-Safe Coomassie blue stain (Bio-Rad). The 3 gels from each sample were digitised with a GS-800 laser densitometer (Bio-Rad) and image analysis was performed using PDQuest (version 8.0, Bio-Rad). Normalisation was performed using the Total Quantity in Valid Spots method and the Students t-test ($p < 0.05$) was used to identify proteins which had change in expression by ≥2-fold between the stimulated sample and corresponding isotype control. Differentially expressed proteins (DEPs) were excised, de-stained, reduced and alkylated prior to trypsin digestion (Trypsin Gold, Promega). Peptides were spotted onto an anchorchip MALDI target (Bruker Daltonics) in a saturated 2,5-dihydroxybenzoic acid matrix solution and mass spectra were recorded in the reflectron mode on a Bruker Daltonics Reflex IV MALDI-TOF MS equipped with a 337 nm nitrogen laser. Ions were accelerated through a potential of 20 kV into the TOF mass analyser and ions within the mass to charge (m/z) range of 950–2500 were detected. Data were acquired using the Flex Control (version 2.4) programme and processed with Flex Analysis (version 2.4) and Biotools (version 3.0). Peaks were de-isotoped and monoisotopic peptide masses were assigned. Contaminating ions from the autolysis of trypsin and keratin were removed. The resultant peptide mass fingerprints were searched against the NCBInr database using the MASCOT search engine (www.matrixscience.com) for protein identification. The search was restricted to human taxonomy. Fixed carbamidomethyl modifications and variable proprionamide and methionine oxidation modifications were considered. A maximum of 2 missed tryptic cleavage was considered and the mass tolerance for the monoisotopic peptide masses was set at ±0.1%. Mowse scores were used to measure the level of significance of each match ($p < 0.05$) and in these experiments a minimum significant score was 65. In addition, a minimum of 30% sequence coverage was required for each significant match and the estimated molecular weight and pI information (from gel positioning) was compared with each match to further ensure correct identifications were assigned.

2.5. Confirmatory immunoblotting of kininogen in stimulated CLL samples

To confirm the differential expression of kininogen protein upon BCR-stimulation in the 3 CLL samples analysed by 2-DE/MS (003, 078 and 089) were stimulated as above for 10 minutes and 5.5 hours and compared with the corresponding isotype control sample using immunoblotting. The 2 isoforms of kininogen, high molecular weight (HMWK) and low molecular weight (LMWK), were analysed separately using isoform specific antibodies. Anti HMWK antibody (ab1004, Abcam; 1:300, 4 °C overnight), anti LMWK antibody (ab79650, Abcam; 1:300, 4 °C overnight), or anti α-tubulin loading control (ab7291, Abcam; 1:2500, 2 hours) was applied to the membrane before visualisation and densitometric analysis as described above.

2.6. Immunoblotting of LMWK in normal B-cells

PBMCs were obtained from 4 healthy volunteers (2 female, 2 male) with an age range of 55–75 years. B-cells were purified by positive depletion of $CD19^+$ cells (130-050-131, Miltenyi Biotec). $CD19^+$ cells were magnetically labelled with CD19 microbeads and the cell suspension was loaded onto a MACS LS column (130-042-401, Miltenyi Biotec), which was placed into the magnetic field of a MACS separator. Magnetically labelled $CD19^+$ cells were retained on the column and subsequently eluted, giving a B-cell purity of approximately 98%. To assess the basal expression of LMWK protein in normal B-cells, immunoblotting was performed as described above. Full length native LMWK protein (ab91118, Abcam), purified from human plasma (MW = 65 kDa, UniProt P01042) was used as a positive control.

2.7. Immunoblotting of LMWK in unstimulated CLL samples

To assess the basal expression of LMWK protein in CLL samples, a pilot series of 52 randomly selected, unstimulated samples was analysed using immunoblotting as described above. LMWK expression status in unstimulated samples was correlated with clinical features and assessed for prognostic value using Kaplan Meier plots with log rank analysis (SPSS/PASW version 18).

2.8. RT-PCR of LMWK transcript in unstimulated CLL samples

To assess the basal expression of the *LMWK* transcript in CLL samples, a pilot series of 20 randomly selected, unstimulated samples was analysed using reverse transcriptase PCR (RT-PCR). Total RNA was extracted from these 20 CLL samples and a positive control prostate cancer cell line (LNCaP) using a NucleoSpin RNA II extraction kit (NZ74095550 Fisher Scientific) according to manufacturer's instructions and quantified using a Qubit® fluorometer (Invitrogen). First-strand cDNA was synthesised using 2 μg of total RNA, oligo dT and the RevertAid Premium Reverse Transcriptase (Thermo Scientific

EP0731) in a Techne TC-3000 thermal cycler. Primers used for LMWK-specific RT-PCR, and associated control genes, are detailed in Supplementary Table 1. These included 2 control gene primer sets (*GAPDH* and *GUSB*) previously assessed in CLL [33]. All primer pairs were selected to span an exon–intron boundary to eliminate amplification of genomic DNA. PCR reactions were performed using 1 μl of cDNA and an annealing temperature of 57 °C (35 cycles).

3. Results

3.1. 2-DE/MS

Sample 003 demonstrated significant upregulation of phospho-ERK upon stimulation (10.4 fold increase by immunoblotting; 2.8 fold increase by ELISA). Sample 078 demonstrated significant upregulation of phospho-ERK upon stimulation (15.9 fold increase by immunoblotting; 5.1 fold increase by ELISA). Sample 089 demonstrated significant upregulation of phospho-ERK upon stimulation (216.1 fold increase by immunoblotting; 6.3 fold increase by ELISA). These 3 samples were therefore classified as BCR-responsive samples and were subsequently successfully analysed using 2-DE/MS analysis. A total of 48 DEP spots were excised from the gels and 16 of these were identified using MS (Table 1 and Supplementary Table 2). The differential expression of kininogen (upregulation) upon artificial stimulation of the BCR was identified in 3/3 clinical samples. Due to this observation, the expression of kininogen was further investigated in immunoblotting. Kininogen, which is a critical component of the kinin–kallikrein system (KKS), can be present in 2 isoforms (HMWK and LMWK) as a result of alternative splicing within exon 10 [34]. Specific antibodies were selected for the analysis of each protein isoform.

Table 1 – Differentially expressed proteins (at least 2-fold) identified using 2-DE/MS in CLL samples following artificial stimulation of the BCR. KNG1 (highlighted in bold) was identified from 3/3 clinical samples. Full details are given in Supplementary Table 2.

Sample	Protein identified	Gene ID (A–Z)	Expression change
078	Adenylate kinase isoenzyme 5 (recombinant)	AK5	↓
003	ASTN2 protein	ASTN2	↑
003	Astrotactin-2 isoform d	ASTN2	↓
003	Aspartyl-tRNA synthetase	DARS	↓
003	Fatty acid-binding protein	FABP5	↓
003	Glyoxalase domain-containing protein 4	GLOD4	↓
003	Interferon α-1/13 precursor	IFNA13	↓
089	Kazrin isoform A	KAZN	↑
003	KNG1 protein	KNG1	↑
078	Kininogen 1	KNG1	↑
089	Kininogen 1	KNG1	↑
003	Phosphatidylinositol transfer protein, beta, isoform CRA_b	PITPNB	↓
003	Tripartite motif protein TRIM19 zeta	PML	↑
089	FYVE-RING finger protein SAKURA	RFFL	↓
089	Mitochondrial Rho GTPase 1 isoform 1	RHOT1	↑
089	Thiopurine S-methyltransferase	TPMT	↓

3.2. Confirmatory immunoblotting of kininogen in stimulated CLL samples

Significant upregulation of LMWK (33 kDa form) upon prolonged BCR stimulation was confirmed in all 3 samples analysed using 2-DE/MS (Fig. 1). LMWK expression was upregulated by 2.0-fold, 3.5-fold and 3.7-fold in samples 003, 078, 089 respectively, following prolonged (5.5 hours) stimulation of the BCR. We were unable to optimise a robust assay using the anti-HMWK antibody.

3.3. Immunoblotting of LMWK in normal B-cells

Purified B cells from 4 healthy volunteers were analysed for LMWK protein expression and this was not detected in any sample (Fig. 2).

3.4. Immunoblotting of kininogen in unstimulated CLL samples

A pilot series of 52 unselected CLL samples (Supplementary Table 3) was screened for constitutive LMWK expression using immunoblotting. The expression level of LMWK differed between samples and was found to be positively expressed in 37/52 (71%) of CLL samples (Fig. 3). The series consisted of 41/52 (78%) patients with Binet stage A disease and 11/52 (21%) patients with Binet stage B or C disease. Binet stage was significantly associated with survival in this pilot series (median survival for stage A was 217 months; median survival for stage B/C was 75 months; $p = 0.007$, log rank). There was a trend towards shorter median survival in LMWK positive cases (147 months *versus* 253 months for LMWK negative cases; $p = 0.125$, log rank; Fig. 4). There was no significant association with time to first treatment (TTFT; $p = 0.403$, log rank; Fig. 5). There were no significant statistical correlations between LMWK and the data available for clinical characteristics or biomarkers.

3.5. RT-PCR of LMWK transcript in unstimulated CLL samples

Twenty CLL samples (16 of which demonstrated expression of 33 kD LMWK protein) were analysed using RT-PCR. Positive expression of both *GAPDH* and *GUSB* transcripts, at the expected size, was detected in 20/20 CLL samples and the LNCaP positive control. The LMWK transcript was detected at the correct size in LNCaP cells; however we were unable to demonstrate expression of LMWK in any of the 20 CLL samples.

4. Discussion

Kininogen is a multifunctional protein and a critical regulator of the plasma KKS [35]. Kininogens serve as a substrate from

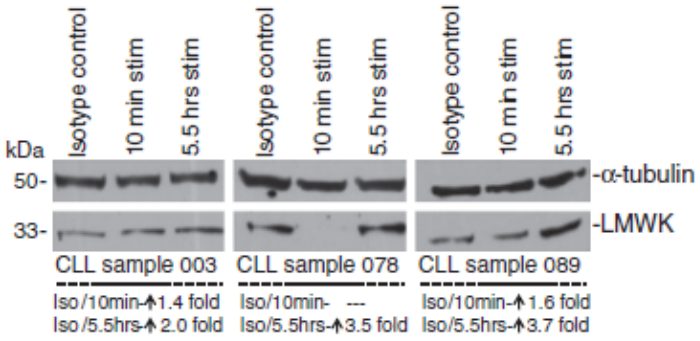

Fig. 1 – Immunoblotting of LMWK (ab79650, Abcam) in 3 CLL samples following artificial BCR stimulation for 10 minutes and 5.5 hours. A single band of 33 kD was detectable, which may be a variant form of LMWK. Samples 003, 078 and 089 were previously analysed using 2-DE/MS, resulting in the identification of kininogen upregulation upon prolonged (5.5 hours) artificial stimulation of the BCR. α-Tubulin was used as a loading control. Compared with untreated (isotype) control, all 3 samples demonstrated significant (≥2-fold) upregulation of LMWK (33 kDa form) at 5.5 hours.

which serine proteases (plasma and tissue kallikreins) liberate the physiologically active bradykinin (BK) and Lys-bradykinin (Lys-BK) peptides [36]. These peptides act upon the kinin B1 and B2 receptors to modulate cell migration, proliferation, vascular permeability and inflammation [37–39]. HMWK and LMWK, the two predominant kininogen isoforms, are comprised of a heavy chain, a BK moiety and a light chain. The light chain amino acid sequence discriminates these two isoforms. The proteolytic cleavage of HMWK by plasma kallikrein produces BK and bradykinin-free kininogen (HKa). The proteolytic cleavage of LMWK by tissue kallikrein produces Lys-BK and unbound heavy and light chains. For some time it was believed that only the released bioactive peptides BK and Lys-BK displayed important bio-physiological properties and that HMWK and LMWK were simple precursors for proteolytic cleavage with no functional activity. However, further studies demonstrated that HMWK displays various biological activities [40,41]. The functional properties of LMWK have not been fully elucidated. BK and Lys-BK, which act upon constitutive B2 and inducible B1 receptors, play a crucial role in sustaining

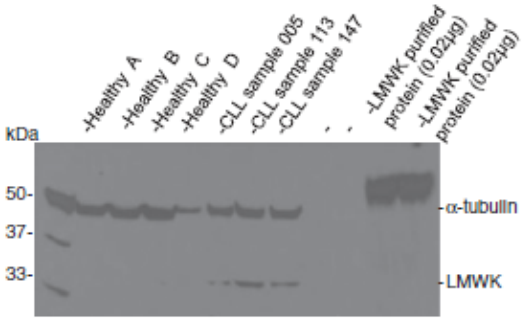

Fig. 2 – Immunoblotting of LMWK (ab79650, Abcam) in purified B cells from 4 healthy volunteers revealed no protein expression. In contrast, positive expression was identified in 3 CLL samples (33 kD form of LMWK) and when using purified LMWK protein, which is 65 kD.

the inflammatory response and in the synthesis of proteins associated with stimulation of nitric oxide formation, liberation of prostacyclin, transduction of pathologic signals, vasodilation, increased vascular permeability and potentiation of calcium signalling, which result in cell growth and proliferation [36,42,43]. It has also been suggested that kinins influence cells of the immune system, such as T-cells and B-cells, by modulating the activation, proliferation, migration and effector functions of these cells [44]. Hyperactivity of the KKS and dysregulation of kininogen levels have been previously reported in several pathological conditions, including human inflammatory diseases and cancer [45,46]. The increased understanding of the role of the KKS in the chronic inflammatory response has heightened the interest of the pharmaceutical industry in KKS proteins and their potential therapeutic application in inflammatory diseases and cancer.

There have been no previous publications describing a potential role for kininogen in CLL or normal B cells. Despite an advanced understanding of the pathology of CLL the disease still remains incurable. There is still no unequivocal definition of the catalyst for aggressive disease progression and relapsed CLL. Therefore the demand for continued research leading to the development of novel therapeutic agents is paramount to achieving a more effective disease control and true complete remission in patients with CLL. BCR-responsive protein expression is known to play a role in CLL [23,24,47]; however basal protein expression may also be of clinical relevance [48]. Our study demonstrates for the first time that CLL B-lymphocytes express differing levels of basal LMWK (33 kD form) and that the expression can be significantly increased after BCR ligation. Consequently a high concentration of LMWK, which is an endogenous source for proteolytic cleavage by kallikreins and subsequent release of kinins, could be achieved and may potentially lead to the activation of kinin B1 and B2 receptors. These are known to be advantageous for immediate triggering of ERK, global phosphorylation and PLCγ2-dependent intracellular Ca^{2+} mobilisation [49–51] and may play an important role in the induction of pathologic signal transduction leading to apoptotic resistance in CLL B-cells [52].

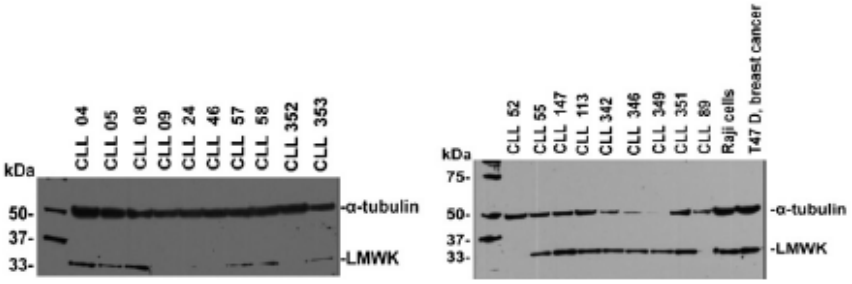

Fig. 3 – Representative immunoblotting using ab79650 antibody (Abcam) to demonstrate differential constitutive LMWK (33 kDa) expression in unstimulated CLL samples. Raji (Burkitt lymphoma derived) cells and T47D (breast cancer) cells were used as positive control samples. α-Tubulin was used as a loading control.

The molecular weight of LMWK is predicted to be 65 kD; therefore further work will be required to establish the nature of the 33 kD form of LMWK which has been demonstrated in the CLL samples in this study.

In a small number of B-cell samples from healthy volunteers, we were unable to demonstrate LMWK expression in any sample using immunoblotting. Further work in this area may confirm the relevance of LMWK as a novel biomarker in CLL, which may be of relevance in diagnosis. Furthermore, we have demonstrated in a pilot study that 71% of CLL B-lymphocytes express basal levels of LMWK protein, which may offer future therapeutic target options. In addition, this feature was associated with a trend towards shorter median survival. We were unable to detect basal levels of the LMWK transcript in any of 20 CLL samples. This may be due to low abundance or rapid turnover of the transcript.

In order to fully evaluate the relationship between LMWK status and prognosis, an increased number of CLL cases will now be examined. Although kininogen expression is a novel finding and shown to be constitutively expressed in some CLL samples, the downstream molecular events that may lead to activation of kinin B1 and B2 receptors, and potentially to cell survival, remain to be determined.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.08.002.

Conflicts of interest

None declared.

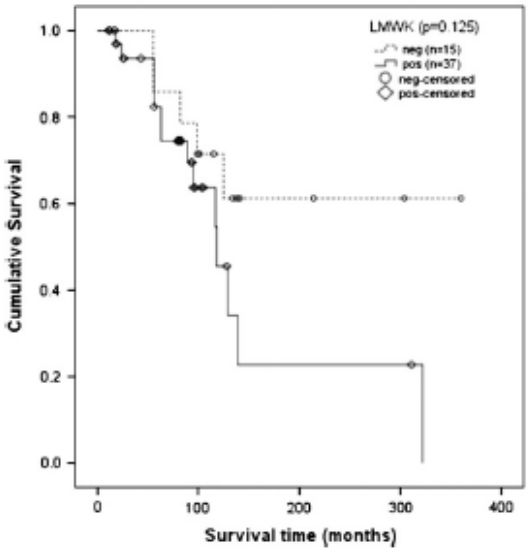

Fig. 4 – Kaplan Meier plot showing overall survival analysis with LMWK expression (p = 0.125, log rank). The median survival was 253 months in cases with negative expression of LMWK (broken line) versus 147 months for cases with positive expression (solid line).

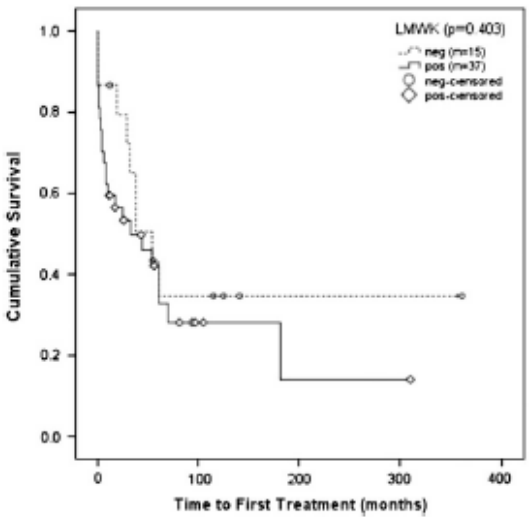

Fig. 5 – Kaplan Meier plot showing TTFT analysis with LMWK expression (p = 0.403, log rank). TTFT was 145 months in cases with negative expression of LMWK (broken line) versus 85 months for cases with positive expression (solid line).

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None.

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REVIEW ARTICLE

The kinin–kallikrein system: physiological roles, pathophysiology and its relationship to cancer biomarkers

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Abstract

The kinin–kallikrein system (KKS) is an endogenous multiprotein cascade, the activation of which leads to triggering of the intrinsic coagulation pathway and enzymatic hydrolysis of kininogens with the consequent release of bradykinin-related peptides. This system plays a crucial role in inflammation, vasodilation, smooth muscle contraction, cardioprotection, vascular permeability, blood pressure control, coagulation and pain. In this review, we will outline the physiology and pathophysiology of the KKS and also highlight the association of this system with carcinogenesis and cancer progression.

Keywords

Bradykinin, carcinogenesis, kinin–kallikrein system, kininogen, kinins

History

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Introduction

The kinin–kallikrein system (KKS) is an endogenous multiprotein cascade, the activation of which leads to triggering of the intrinsic coagulation pathway and enzymatic hydrolysis of kininogens (KNGs) with the consequent release of bradykinin (BK)-related peptides (Figure 1). Synthesis of BK is known to be a predominant bio-physiological function of the KKS. BK/Lys-BK are generated by proteolytic cleavage of KNGs including high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK) by a family of serine proteases consisting of kallikreins (KLKs) originating from plasma (pKLK) and tissue (tKLK) (Bourdet et al., 2010). The liberated BK/lys-BK molecules are the potent vasoactive peptides and display a high affinity to B_1 and B_2 receptors, which are members of the G-protein coupled receptors (GPCRs) family and are initiators for the complex intracellular signalling network. For the past five decades the complexity of the KKS, and the multiple interactions with other endogenous metabolic cascades such as renin–angiotensin system (RAS) and coagulation, has been intensively studied (Schmaier, 2003; Shariat-Madar & Schmaier, 2004). The KKS plays a crucial role in inflammation, vasodilation, smooth muscle contraction, cardioprotection, vascular permeability, blood pressure control, coagulation and pain (Bossi et al., 2011; Colman, 2006; Colman & Schmaier, 1997; Marcondes & Antunes, 2005). In this review, we will outline the physiology and pathophysiology of the KKS and highlight the association of this system with carcinogenesis and cancer progression.

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Historical aspects

The first recognition of the KKS and its hypotensive properties came from the study of human urine by Abelous and Bardier at the beginning of the twentieth century, when researchers discovered signs of hypotension in dogs following the injection of human urine (Moreau et al., 2005). A few decades later the German surgeon and scientist Emil Karl Frey, in collaboration with his associates Heinrich Kraut and Eugen Werle, observed that a hypertensive substance isolated from human urine was formed in the pancreas; this they termed KLK (Hillmeister & Persson, 2012). In 1949, the Brazilian physician and pharmacologist Rocha e Silva and colleagues described a peptide isolated from snake venom which displayed hypotensive properties; this peptide was termed BK (Rocha e Silva et al., 1949). Independently in 1950, Eugen Werle elucidated the mechanism of KLK activation and suggested that KLK facilitated the liberation of the active hypotensive peptide from the precursor molecule and subsequently a role for Factor XII (FXII; Hageman factor) was suggested in the activation of KLK (Derkx et al., 1979). FXII was first discovered in 1955 when Dr Oscar Ratnoff found that a blood sample from 37-year-old John Hageman lacked a previously unknown clotting factor, which promoted an increased clotting time but without any symptoms of haemorrhagic disorder (Ratnoff & Margolius, 1955). Multiple clinical studies were then conducted in order to understand the mechanism of KKS assembly and the relevance of FXII in KKS activation. Around that time the activated partial thromboplastin time (APTT) test was performed and the

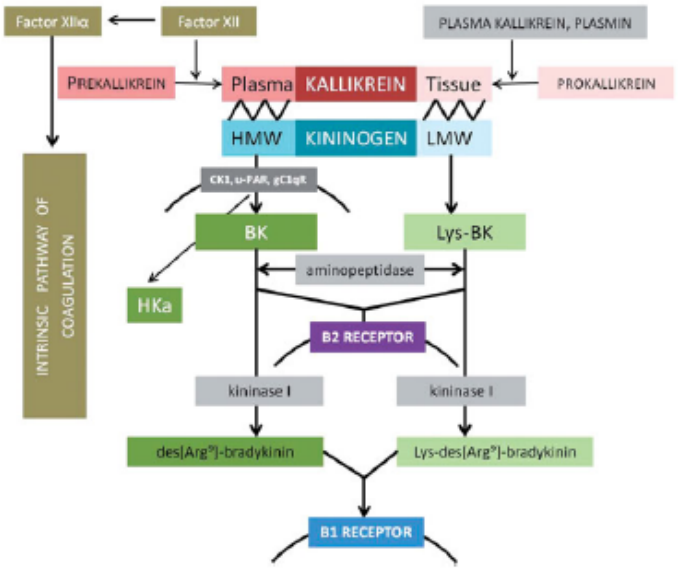

Figure 1. KKS organisation.
KKS is a multiprotein metabolic pathway. The primary biologic feature of this system is hydrolysis of KNG with subsequent liberation of HKa and vasoactive BK and Lys-BK (Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^9 and Lys^1–Arg^2–Pro^3–Pro^4–Gly^5–Phe^6–Ser^7–Pro^8–Phe^9–Arg^{10}, respectively), which are the B_1 receptor ligands and des-Arg-BK and Lys-des-Arg-BK (Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8 and Lys^1–Arg^2–Pro^3–Pro^4–Gly^5–Phe^6–Ser^7–Pro^8-Phe, respectively), which exert their effect via the B_2 receptor.

autoactivation of FXII upon contact to a glass surface, which leads to thrombin formation, was observed (Schmaier, 2002). FXII was identified as a key factor in the intrinsic pathway of the coagulation cascade through the activation of Factor XI (FXI) and also as an initiator for activation of the KKS and from then the KKS system was termed a contact system (Colman & Schmaier, 1997). However, this hypothesis remained speculative due to the failure to identify and present an *in vivo* surface which was equivalent to glass, and also the absence of bleeding disorders in FXII- and KNG-deficient patients (Schmaier, 2002). In the late 1970s, it was suggested that prekallikrein (PK) and KNG circulate in plasma as a complex (Scott & Colman, 1980). Subsequently it was suggested that endogenous activation of KKS could be triggered through binding of the HMWK–PK (HK–PK) complex to a multiprotein receptor complex consisting of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR (Schmaier, 2002).

The contact system and the mechanism of activation

KKS in multiple scientific sources is termed as the contact system. The contact system of plasma is also called the plasma kinin-forming system which is initiated by intravascular activation of FXII and it has been postulated to be composed of three serine endopeptidase family proteins, namely FXII (Hageman factor), FX1 (plasma thromboplastin antecedent) and PK (Fletcher factor), with the multifunctional plasma and tissue protein KNG, which is also a kinin precursor. The interaction of all four of these proteins leads to activation of the intrinsic clotting pathway, and hence to a fibrin clot organisation, and is known as ''contact activation'' (Kaplan et al., 1997). The activation of the contact system has two principal effects: mediation of the intrinsic pathway of coagulation which proceeds to fibrin formation (with associated procoagulant properties) and proteolytic cleavage of KNG leading to the release of BK-related kinins (with proinflammatory properties) (Colman & Schmaier, 1997).

Factor XII and Factor XI

FXII (Hageman factor) is a 596 amino acids single-chain plasma protein with a molecular weight of 80 kDa and a plasma concentration of 31 ± 8 µg/ml (0.375 µM) (Colman, 2006; Marcondes & Antunes, 2005; Renné et al., 2012; Saito et al., 1976). FXII possesses the properties of a zymogen (an inactive enzyme precursor). FXII is encoded by a 12 kb gene on chromosome 5 composed of 13 introns and 14 exons. FXII mRNA was reported to be expressed by human hepatocytes (Gordon et al., 1990). The gene and protein domain organisation of FXII exhibit structural homology with the serine protease family of tissue-type plasminogen activators and urokinase-type plasminogen, suggesting the allocation of FXII into the protease subfamily (Colman, 2006; McMullen & Fujikawa, 1985). FXII has a multidomain structure and possesses an epidermal growth factor (EGF)-like domain and a catalytic region that interacts to activate the functional site of this protein on its surface (Schmaier, 2008). The biochemistry of *in vitro* activation of factor XII is well studied and it was confirmed that after contact of FXII with an anionic surface (surfaces which are negatively charged due to prevalence of electrons on the surface), such as glass or silicates, dextran sulphate, heparin (Marcondes & Antunes, 2005) and kaolin, carrageenan (Moreau et al., 2005), FXII undergoes conformational changes and is converted into its active form FXIIa and demonstrates procoagulant protease activity. The physiologic mechanism of FXII activation *in vivo* is still obscure and has continued to be a subject of speculation. Several assumptions were made in order to identify an *in vivo* physiologic surface for FXII activation in the intravascular department, and these included diverse cell membranes, platelets, endothelial cells and also neutrophils

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(Rojkjaer et al., 1998). However there are studies which hypothesise that several pathogens can affect the KKS pathway via FXII activation. Morrison and Cochrane in 1974 demonstrated that injection or release of endotoxins or bacterial lipopolysaccharides (LPS) have a direct influence on the activation of FXII (Morrison & Cochrane, 1974). In 1997, Brunnée et al. (1997) demonstrated that mast cell heparin proteoglycan isolated from a Furth mouse mastocytoma-derived cell line, which was a replica of human tissue-type mast cell HepPG, expressed the unique competence to reciprocally activate FXII and PK and could also autoactivate FXII. In 2006, Frick et al. (2006) described the contact system during defence against invasive bacterial infection and suggested that the contact system can be activated on the surface of bacterial pathogens, resulting in the promotion of anti-bacterial activity and inhibition of bacterial dissemination.

Once the activation of FXII and transformation into an active FXIIa form in the intravascular department occurs, FXIIa initiates the reaction of plasma PK modification to KLK. As a result, KLK digests KNG to release BK. Although this hypothesis was universally recognised there are also substantial studies which challenge the involvement of FXII in mediation of PK to KLK conversion and also in FXI activation due to (1) failure to clearly identify the endogenous negatively charged surface which triggers KKS activation; (2) existing evidence which indicates that individuals deficient in FXII, PK or HMWK do not exhibit bleeding disorders and (3) evidence that activation of FXI (deficiency of FXI is more associated with haemophilia) can be initiated by thrombin, which is an alternative mechanism for this activation (Schmaier, 2002). Rojkjaer et al. (1998) and Motta et al. (2001) convincingly demonstrated that an *in vivo* physiologic negatively charged surface, mediating the activation of the KKS, is presented by the surface receptor complex positioned on endothelial cells and that this complex facilitates HMWK binding, allowing PK to be converted into KLK. This activation mechanism on the endothelial cells will be discussed in detail later in this review. The involvement of FXII in the chain of bio-physiological reactions which leads to the activation of the KKS has not been completely elucidated. FXII is capable of mediation of the structural transformation of PK into its active form (KLK) in the presence of KNG (Schmaier, 2008). However, there are other studies which confirm that the conversion of PK into KLK is still possible on endothelial cell membranes cultured without serum, or in human serum lacking FXII, which suggest that FXII may not be essential for PK activation (Motta et al., 2001). KLK has an ability to reciprocally activate FXII, and thus supplements the KKS activation in the intravascular compartment (Rojkjaer et al., 1998) and also it activates the structural transformation of a single-chained FXII into an active form called α-factor XII (XIIα) consisting of two chains (amino terminal heavy chain connected by a disulphide bridge to a carboxy terminal light chain with molecular weight of 50 kDa and 28 kDa, respectively) (McMullen & Fujikawa, 1985). Subsequently α-factor XIIα activates the transformation of FXI to Factor XIa, which in turn initiates the intrinsic pathway of coagulation (Schmaier, 2008) and the downstream activation of Factor IX leading to thrombin formation. Digestion of α-FXIIα by trypsin (McMullen & Fujikawa, 1985) or pKLK (Marcondes & Antunes, 2005) results in formation of β-factor XIIα, which is a heterodimeric molecule presented by a 28 kDa light chain connected by an S–S bond to a nonapeptide (McMullen & Fujikawa, 1985).

FXII can be autoactived upon contact with negatively charged surfaces and therefore the downstream coagulation signalling cascade in PK-deficient plasma occurs in a normal physiologic manner (Marcondes & Antunes, 2005). However, it is important to highlight that, despite the fact that multiple endogenous physiologic surfaces are available for FXII autoactivation, generally the reaction with this zymogen does not occur constitutively within normal physiological or non-pathological conditions unless it is regulated by the plasma concentration of HK and Zn^{2+} (Colman & Schmaier, 1997). In the intravascular compartment, FXII binds to the endothelial cell receptor complex composed of uPAR, CK1 and gC1qR (Schmaier, 2008).

Activated FXII can mediate the activation of downstream FXI. However, several studies have demonstrated that FXI can be activated in an FXIIa-deficient model system, emphasising the role for thrombin in FXI activation and the subsequent coagulation cascade (Oliver et al., 1999). The absence of a bleeding disorder in FXII-deficient patients leads to further investigation of the role of FXII in the process of FXI activation (Choi et al., 2011). HMWK can form a complex with FXIIa or FXI, which may promote the activation of FXI and subsequent triggering of the intrinsic coagulation cascade (Mandle et al., 1976; Thompson et al., 1977). A new hypothesis was therefore postulated where PK and FXI become tightly bound to KNG and ligate with the multiprotein receptor complex comprising CKI, gC1qR and uPAR on the surface of endothelial cells. This would facilitate the digestion of PK into KLK by prolylcarboxypeptidase (PRCP) (Shariat-Madar et al., 2002).

The structure and organisation of the KKS

The KKS is an endogenous metabolic activation pathway with multiple physiological and pathophysiological effects but the primary biological feature of the system is synthesis of the vasoactive peptides kinins by enzymatic hydrolysis of KNGs (Moreau et al., 2005). In order to understand the complexity of the system (with multiple cross-talking pathways, autocrine and paracrine activities, and multiple modes of activation and regulation) within this biologically and physiologically important system it is essential to understand the function and the properties of each constituent of the KKS and their interactions within the system. The system consists of a number of enzymes, large proteins and polypeptides which interact (intra- and inter-system) in order to activate or deactivate the bio-chemical reactions within this metabolic cascade.

Enzymes

Kinin-forming enzymes

KLKs (also known as kinin-forming enzymes) are serine proteases that liberate kinins from the KNGs. Two types of KLKs are described: pKLK and tKLK. Human plasma PK

(PPK) is a zymogen precursor for pKLK. It is a single-chain polypeptide, the production of which is primarily located in hepatocytes with subsequent release into blood stream. Although the highest PPK mRNA expression levels are reported in hepatocytes, recent studies have confirmed the existence of extrahepatic PPK synthesis. PPK protein expression has been described in liver (epithelial cells), pancreas (cells of the islet of Langerhans), testicles (interstitial cells of Leydig), ovaries (follicular and thecal granulosa cells), parotid gland, oesophagus, skin, respiratory tract, prostate and breast (Fink et al., 2007). PPK has been demonstrated to play a marked role in biological processes including the surface-dependent activation of blood coagulation (intrinsic pathway), the activation of FXII, kinin generation, inflammatory response, plasminogen activation and positive regulation of fibrinolysis. PPK is secreted into plasma as an inactive molecule, either (75%) circulating as a heterodimer complex bound to HMWK or (25%) as a freely circulating protein (Colman, 2006; Moreau et al., 2005). PPK is a serine protease glycoprotein comprised of 619 amino acids. It exists in two molecular weight variants that circulate in plasma and vary between 85 kDa and 88 kDa (Marcondes & Antunes, 2005) and with a plasma concentration of 42 ± 3 µg/ml (0.49 µM) (Fisher et al., 1982). PPK is encoded by the *KLKB1* gene (Moreau et al., 2005).

Upon activation of PPK, a chain of proteolytic reactions cleaves PPK within its disulphide bond to generate the double-chain variant pKLK (Yu et al., 2000). pKLK consists of a 53 k Da heavy chain (371 amino acids) and a 33–36 kDa light chain (248 amino acids) connected by a disulphide bond. The pKLK heavy chain originates from the N-terminus and is presented by four tandem repeats that are 90–91 amino acids long in their sequence and displays a structural resemblance to human FXI. The pKLK light chain exhibits structural homology with the trypsin family of serine proteases (Chung et al., 1986). The heavy chain of pKLK interacts with the negatively charged surface and possesses a high affinity binding site for HMWK, while the light chain serves as a catalyst (Marcondes & Antunes, 2005). HMWK is a substrate for pKLK and, as a result of this interaction, the nonapeptide BK is generated. pKLK and FXII (but at a much lower rate) are both capable of proteolytic digestion of HMWK with the subsequent release of BK and residual, cleaved (BK-free) KNG (termed HKa) (Colman, 2006). The pKLK gene and protein are homologous to human FXI and both of these genes are located on chromosome 4 (Colman, 2006). The structural organisation of the pKLK gene was reported by Yu et al. in 2000 and comprises 15 exons and 14 introns (Yu et al., 2000).

Human tKLK or glandular KLK is a member of the S1 serine protease superfamily and a secreted acid glycoprotein with molecular weight between 24 k Da and 45 k Da. The enzyme activity of tKLK differs from pKLK and is distinguished by the catalytic mechanism and the proteins targeted for cleavage. In the human genome tKLK is encoded by 15 steroid hormone-regulated genes that co-localise on chromosome band 19q13.4 (Borgono et al., 2004). tKLK is synthesised as an inactive preproenzyme (termed proKLK) and is widely expressed in diverse human tissues such as kidney, pancreas, colon, pituitary gland, erythrocytes (MacDonald et al., 1988), central nervous system, spleen, adrenal and neutrophils (Marcondes & Antunes, 2005). The activation of proKLK *in vitro* can be achieved by trypsin-type enzymes (Frey, 1962); however, *in vivo* plasmin (Yamada & Erdos, 1982) and pKLK (Takada et al., 1985) are reported to be responsible for bio-chemical conversion of proKLK into tKLK. There is a close resemblance between tKLK and trypsin; however, tKLK expresses a higher specificity for interaction with cleavage sites in synthetic peptides (MacDonald et al., 1988). KNGs are the principal physiological substrates for proteolytic digestion by tKLK. tKLK cleaves KNG at Met-Lys and carboxy-terminus Arg-Ser sites, resulting in the subsequent liberation of the decapeptide Lys-BK (Kallidin) (MacDonald et al., 1988). tKLK (as opposed to pKLK which preferentially acts upon HMWK) can generate Kallidin from either HMWK or LMWK. *In vivo*, the decapeptide Lys-BK can be easily converted into the nonapeptide BK by cleavage at the Lys–Arg bond (Erdos, 1979).

Carboxypeptidases

Carboxypeptidases (kininases) play the predominant role in kinin degradation. Once kinins are liberated into the circulatory system they are catalysed by kininases, which cleave and release Arg from the carboxy-terminus of BK and Lys-BK. Kininase I-type enzymes are present in two forms, namely carboxypeptidase N (CPN) in plasma (Erdos, 1979) and carboxypeptidase M (CPM) in cell membranes(Johnson et al., 1984; Skidgel et al., 1984; Skidgel et al., 1986). Although these enzymes share some properties, they are structurally and catalytically distinct from each other and from other carboxypeptidases. Human CPM is a single-chain glycoprotein (molecular weight of 62 kDa) with maximal enzyme activity at neutral pH and is named due to its localisation on the plasma membrane. CPM expresses some homology in enzyme properties but is structurally, catalytically and immunologically distinct from other carboxypeptidase B-like enzymes (Skidgel et al., 1989). The function of CPM is the selected cleavage of peptides containing carboxy-terminal Arg residues (wheras CPN preferentially acts on peptides containing carboxy-terminal Lys residues) (Skidgel & Erdos, 1998). CPM regulates the interaction between BK and its receptors by converting BK to des-Arg-BK and thus abrogating the unique specificity required to interact with B_2 receptors, whilst producing agonism against B_1 receptors (Regoli & Barabe, 1980). Zhang et al. (2011) recently reported that the kinin B_1 receptor possesses the ability to form a complex with CPM which potentiates B_1-mediated signal transduction and that the disruption of this heterodimer can lead to inhibition of B_1-dependent nitric oxide production in cytokine-treated human lung microvascular endothelial cells. CPM is widely distributed in diverse human tissues. Primarily CPM was purified from human placenta but lately it has been identified in human kidney, lung, pulmonary arterial endothelial cells, fibroblasts, amniotic fluid (Skidgel et al., 1984), porcine blood vessels and aortic endothelial cells (Palmieri et al., 1986).

Human CPN (plasma zinc metalloprotease) is a 280 kDa extracellular glycoprotein (Levin et al., 1982) which is

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synthesised and secreted into the intravascular compartment by hepatocytes as a hetero-tetramer (Keil et al., 2007). CPN is composed of two large domains (approximately 83 kDa each), which are responsible for protecting the protein from degradation, and two small domains (approximately 48–55 kDa) which exhibit enzymatic properties (Matthews & Wetsel, 2001). CPN selectively removes basic residues at the carboxy-terminus and thus regulates the activity of kinins, growth factors, cytokines (Keil et al., 2007) and anaphylatoxins within the blood circulation (Levin et al., 1982). Several endogenous enzymes, such as trypsin, plasmin and pKLK or urinary KLK can fragment CPN; however, these newly generated active fragments are less stable at 37 °C in comparison to the original tetrameric protein (Levin et al., 1982). CPN is a constitutively active enzyme and due to its ability to metabolise, and hence to protect the human body from the systemic dissemination of peptides such as vasoactive kinins, anaphylatoxins and fibrinopeptides, it is considered to be essential for sustaining life (Keil et al., 2007).

Angiotensin converting enzyme

Angiotensin converting enzyme (ACE) (kininase II) is primarily synthesised as a membrane-bound protein. Due to post-translational proteolytic cleavage of the membrane anchor the ACE molecule is detached from the cell surface and liberated into the circulation as a freely circulated enzyme (Costerousse et al., 1992). There are two known forms of ACE: somatic (150–180 kDa) and low molecular weight (90–100 kDa). The amino- and carboxy-domains of ACE each possess an active site (Danilov et al., 1994) and both of these domains exhibit similar enzymatic, but different immunologic activities. ACE is widely distributed within diverse human tissues and found to be expressed in endothelial cells, epithelial cells, germinal cells, neurons, macrophages, T-lymphocytes (Costerousse et al., 1993) and also in body fluids such as urine, cerebrospinal and amniotic fluids. The role of ACE is, firstly, to convert the inactive hormone peptide angiotensin I into the potent vasopressor angiotensin II, which is an important biologically active constituent of the RAS, which controls blood pressure and modulates fluid–electrolyte homeostasis within a human body. Secondly, ACE is involved in BK inactivation pathway (Nowak et al., 2011), where ACE metabolises the vasoactive nonapeptide BK. Finally, ACE is reported to cleave Substance P, which is a protein from the tachykinin family which expresses a broad spectrum of enzymatic properties. It is widely expressed in cells and tissues of the human peripheral and central nervous systems but is also found in extraneuronal cells and innervated tissues.

Neutral endopeptidase

Neutral endopeptidase (NEP) is a zinc-dependent metalloprotease enzyme, which is synthesised as a membrane-bound protein with a molecular weight of 89–96 kDa. NEP is reported to be involved in the metabolism of several biologically active secreted peptides and has a wide distribution within various human cells. Substrates for NEP activation include Angiotensin I, Angiotensin II, substance P, several vasodilator peptides (BK, Adrenomedullin) and vasoconstrictor peptides (Endothelin), the regulators of nociceptive function (enkephalins), a protein associated with Alzheimer's disease (amyloid β peptide) and chemotactic peptide (Campbell, 2003). Graf et al. (1992) utilised immunostaining to demonstrate the role of NEP in the degradation of the endogenous vasoactive peptide BK by the vascular endothelium, which is increased markedly upon inhibition of ACE.

Large proteins

Kininogens

KNGs are also known as the Williams–Fitzgerald–Flaujeac factor, the Fitzgerald factor and the KLK factor. KNGs are large proteins and the substrate from which the serine proteases pKLK and tKLK, respectively, liberate the physiologically active peptides BK and Lys-BK (Sainz et al., 2007). Currently, there are several types of human KNG which are well described: HMWK, LMWK and HKa. T-KNG is the analogue of human KNG reported in rats (Anderson et al., 1989).

For a significant time only the released bioactive peptides BK and Lys-BK were reported to express diverse biophysiological properties, whilst KNGs were described as precursors for proteolytic cleavage only and lacked specific activity. However, convincing studies have demonstrated that KNGs also display various biological activities. HNWK exhibits anti-thrombotic activity (Colman et al., 1999; Hassan et al., 2007) and participates in cell-adhesive interactions (Motta et al., 2001). HKa is an anti-adhesive molecule which prevents cell interaction with vitronectin and also expresses anti-angiogenic properties (Motta et al., 2001).

KNGs are the multifunctional proteins of KKS and, up until the past two decades, it was assumed that the origin of KNGs was restricted to hepatic tissues (Okamoto et al., 1996). Rat T-KNG was found to be expressed in lung, renal, neural and cardiac tissues (Mann & Lingrel, 1991). Human KNG mRNA expression was confirmed in liver, kidney (Chao et al., 1993; Iwai et al., 1988) and endothelial cells (Schmaier et al., 1988). KNG synthesis was demonstrated in fibroblasts upon stimulation by mediators of inflammation such as prostaglandin-2 (PGE2), tissue necrosis factor (TNF) and Interleukin (IL)-1 and KNG expression was demonstrated in rat tarsal bones in response to *in vivo* mediated inflammation (Okamoto et al., 1996; Takano et al., 1995). Taking into consideration all of the above it can be concluded that autonomous KNG synthesis is taking place in hepatic and various extrahepatic tissues and organs in humans and animals.

Protein structure and function. There are two different human protein isoforms of KNG which are recognised: HMWK (Figure 2) and LMWK (Figure 3). HK is a β-globulin with an approximate molecular weight of 120 kDa and human plasma concentration of approximately 80 mg/ml (670 nM) (Sainz et al., 2007). However, there is a tendency for the increase in plasma level of KNGs with advancing age in both humans and animal models (Acuna-Castillo et al., 2005). LMWK is a polypeptide with a reported molecular weight of

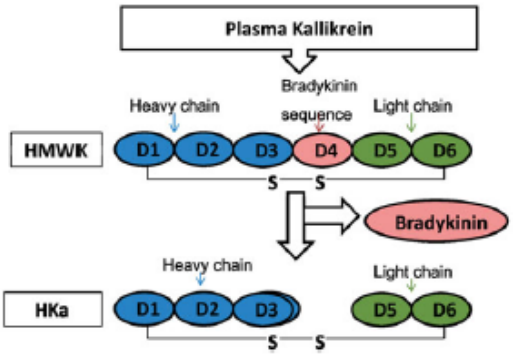

Figure 2. HMWK formed by six single-chain domains (D1–D6), represented by a heavy chain (D1–D3) and a light chain (D5h–D6). The heavy and the light chain are connected by D4, which contains the sequence of BK. After activation by KLK and the release of BK, the cleaved form HKa consists of a heavy chain and a light chain linked by a disulphide bridge.

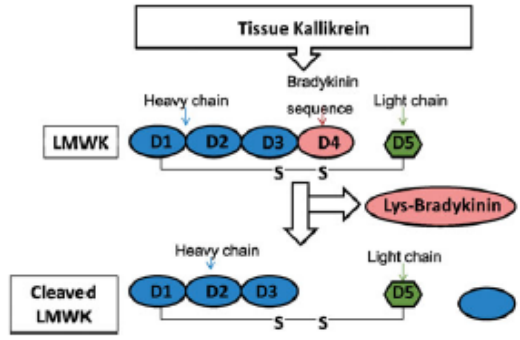

Figure 3. LMWK consists of five single-chain domains (D1–D5), these are presented by a heavy chain (D1–D3) and a small unique light chain D5. Two chains are linked by D4, which includes the sequence of Lys-BK. After proteolytic cleavage of tissue KNG by KLK, Lys-BK is released, the cleaved form of LMWK consists of a heavy chain and a light chain linked by an S–S bond.

approximately 68–75 kDa and a human plasma concentration of approximately 60 mg/ml (Sainz et al., 2007).

The sequence of HMWK and LMWK consists of 626 and 427 amino acids, respectively (Figures 4 and 5). The two KNG isoforms share a common signal peptide (18 amino acids), the heavy chain (362 amino acids) and the BK sequence (9 amino acids). The unique light chain differentiates HMWK and LMWK. This consists of 255 amino acids (approximately 48–56 kDa) in HMWK and 38 amino acids (approximately 4 kDa) in LMWK (Colman, 1996). The shared heavy chain has an approximate molecular weight of 64–75 kDa (Colman, 1996).

KNGs are large multidomain molecules and each domain (D) exhibits specific and distinctive properties (Colman, 2006). There are studies which report the contrasting functions of the heavy and light chains of HMWK, represented by cysteine proteinase inhibitory activities and cofactor properties, respectively (Schmaier et al., 1986).

HMWK D6 possesses a high affinity site for binding PK and interaction of these proteins results in the formation of an HMWK/PK complex which freely circulates in plasma (Colman, 2006). D6 has the ability to downregulate urokinase-dependent plasminogen activator and therefore inhibits HMWK and PK interaction (Colman et al., 2010). Additionally, D6 is reported to express a binding site for FXII (Moreau et al., 2005).

D5 of HMWK was demonstrated to be responsible for HMWK binding to hydrophilic anionic surfaces (Colman, 1996); however, the unique structure of LMWK D5 is inadequate for contact activation and lacks PK binding sites (Moreau et al., 2005). The function of LMWK D5 is still undefined.

D4, which is shared by HMWK and LMWK, contains the sequence of BK and Lys-BK, respectively, and additionally the first five amino acid residues provide a low affinity binding site for platelets (Hasan et al., 1996).

D3 and D2 represent the heavy chain of both HMWK and LMWK which is responsible for inhibition of cathepsin L (Colman, 2006) and platelet calpain (Schmaier et al., 1986) leading to inhibition of platelet aggregation and the suppression of platelet-thrombin binding, respectively (Puri et al., 1991). D3 has the ability to inhibit cathepsin L, B and H but not calpain (Colman, 2006). The specific amino acid residues Gln–Val–Val–Ala–Gly within D3 and D2 are responsible for protease inhibition activity (Salvesen et al., 1986).

D3 and D5 also contain neutrophil binding sites (Colman, 1996); however, D3 and D5 interact with different receptors or with different sites within the same receptors (Colman, 2006). D3 binds with high affinity to monocytes, platelets and endothelial cells.

D1 is reported to exhibit an attenuated binding site for calcium (Cheung et al., 1993).

HMWK is characterised as a precursor for both BK and HKa. HKa is an active protein produced as a result of the proteolytic cleavage of KNG with subsequent liberation of BK. It plays a prominent role in several intracellular, molecular and signalling events. HKa suppresses angiogenesis with the anti-adhesive activity of D5 (kininostatin) leading to subsequent inhibition of endothelial cell proliferation and the facilitation of endothelial cell apoptosis (Colman et al., 2010). HKa promotes cell detachment by binding to the amino-terminus of vitronectin (a cell adhesion and spreading protein found in plasma and the extracellular matrix (ECM), which serves to regulate proteolysis directly mediated by plasminogen activation) and thus obstructing vitronectin interaction with uPAR, all of which takes place on the surface of endothelial cells in Zn^{2+}-dependent manner (Sainz et al., 2007). HKa freely circulates in plasma; however, the persistence of HKa supplements some pathophysiological conditions such as the inflammatory response (Sainz et al., 2007).

KNG gene structure. The *KNG* gene is 27 kb long and composed of 11 exons and 10 introns (Kitamura et al., 1985). This gene produces both HMWK and LMWK by alternative splicing after base 68 in exon 10 (Figure 6). LMWK mRNA additionally comprises an exon 11 (1.7 kb) and HMWK mRNA contains the remainder of exon 10 (3.5 kb)

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     10         20         30         40         50         60
MKLITILFLC SRLLLSLTQE SQSEEIDCND KDLFKAVDAA LKKYNSQNQS NNQFVLYRIT

     70         80         90        100        110        120
EATKTVGSDT FYSFKYEIKE GDCPVQSGKT WQDCEYKDAA KAATGECTAT VGKRSSTKFS

    130        140        150        160        170        180
VATQTCQITP AEGPVVTAQY DCLGCVHPIS TQSPDLEPIL RHGIQYFNNN TQHSSLFMLN

    190        200        210        220        230        240
EVKRAQRQVV AGLNFRITYS IVQTNCSKEN FLFLTPDCKS LWNGDTGECT DNAYIDIQLR

    250        260        270        280        290        300
IASFSQNCDI YPGKDFVQPP TKICVGCPRD IPTNSPELEE TLTHTITKLN AENNATFYFK

    310        320        330        340        350        360
IDNVKKARVQ VVAGKKYFID FVARETTCSK ESNEELTESC ETKKLGQSLD CNAEVYVVPW

    370        380        390        400        410        420
EKKIYPTVNC QPLGMISLMK RPPGFSPFRS SRIGEIKEET TVSPPHTSMA PAQDEERDSG

    430        440        450        460        470        480
KEQGHTRRHD WGHEKQRKHN LGHGHKHERD QGHGHQRGHG LGHGHEQQHG LGHGHKFKLD

    490        500        510        520        530        540
DDLEHQGGHV LDHGHKHKHG HGHGKHKNKG KKNGKHNGWK TEHLASSSED STTPSAQTQE

    550        560        570        580        590        600
KTEGPTPIPS LAKPGVTVTF SDFQDSDLIA TMMPPISPAP IQSDDDWIPD IQIDPNGLSF

    610        620        630        640
NPISDFPDTT SPKCPGRPWK SVSEINPTTQ MKESYYFDLT DGLS
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Subsections	Position in the chain	Length (aminoacids)	*Graphical view*
Signal peptide	*1-18*	*18*	
Peptide/Kininogen-1	*19-644*	*626*	
KNG Heavy chain	*19-380*	*362*	
Peptide/T-kinin	*376-389*	*14*	
Peptide/Lysyl-Bradykinin	*380-389*	*10*	
Peptide/Bradykinin	*381-389*	*9*	
KNG light chain	*390-644*	*255*	
Low molecular weight growth-promoting factor	*431-434*	*4*	

Figure 4. The protein structure of HMWK isoform. Information based on the Uniprot database.

(Cheung et al., 1993). Exons 1–9, which code for the signal peptide and heavy chain sequences, are common to both KNG isoforms. Exon 10 encodes the BK moiety sequence and exclusive sequences for HMWK. Exon 11 encodes the LMWK specific sequence (Kitamura et al., 1985) *KNG* maps to chromosome 3q26-qter and is not co-localised with other members of the cystatin superfamily (Cheung et al., 1993; Fong et al., 1991).

Polypeptides

Kinins

In humans, the term ''kinins'' refers to BK-related polypeptides such as BK and Lys-BK and their carboxy-terminal des-Arg metabolites; other kinins such as T-kinin (Ile-Ser-BK) and Met-T-kinin generally known to be expressed in rat (Moreau et al., 2005). Human Ile-Ser-BK with the amino

Subsections	Position in the chain	Length	Graphical view
Signal peptide	*1-18*	*18*	
Peptide/LMWK	*19-427*	*408*	
LMWK Heavy chain	*19-380*	*362*	
Peptide/T-kinin	*376-389*	*14*	
Peptide/Lysyl-Bradykinin	*380-389*	*10*	
Peptide/Bradykinin	*381-389*	*9*	
LMWK light chain	*390-427*	*37*	

Figure 5. The protein structure of LMWK isoform. Information based on the Uniprot database.

acid sequence:

$$Ile^1 - Ser^2 - Arg^3 - Pro^4 - Pro^5 - Gly^6 - Phe^7 - Ser^8 - Pro^9 - Phe^{10} - Arg^{11}$$

was isolated from ovarian carcinoma ascites and showed strong similarity to T-kinin, which had been previously reported in rats (Wunderer et al., 1986).

HMWK yields BK upon pKLK lysis. BK is a physiologically and pharmacologically active member of the kinin group of proteins and consists of nine amino acids with the following amino acid sequence:

$$Arg^1 - Pro^2 - Pro^3 - Gly^4 - Phe^5 - Ser^6 - Pro^7 - Phe^8 - Arg^9.$$

Once released locally or in plasma BK has a very short half-life and under normal physiologic conditions is rapidly degraded by plasma. There are several enzymes known to be involved in the plasma degradation of BK including CPN (kininase I), ACE (kininase II) and NEP which cleave BK at Phe^8–Arg^9, Phe^5–Ser^6 and Pro^7–Phe^8 positions, respectively, and convert it to an inactive peptide (Kuoppala et al., 2000). Lys-BK (Kallidin), the decapeptide from the kinin-related group, is released from LMWK upon tKLK lysis (Sheikh & Kaplan, 1986a, b). Lys-BK consists of the same amino acid sequence as BK but with an additional lysine amino acid at the N-terminus:

$$Lys^1 - Arg^2 - Pro^3 - Pro^4 - Gly^5 - Phe^6 - Ser^7 - Pro^8 - Phe^9 - Arg^{10}.$$

Kinins are implicated in many physiological and pathological events and will be discussed later in this review.

Kinin receptors

The physiological and pathophysiological effects of kinins such as BK and Lys-BK are initiated via two distinct transmembrane receptors from the GPCR superfamily which are named B_1 and B_2 kinin receptors. BK and Lys-BK are

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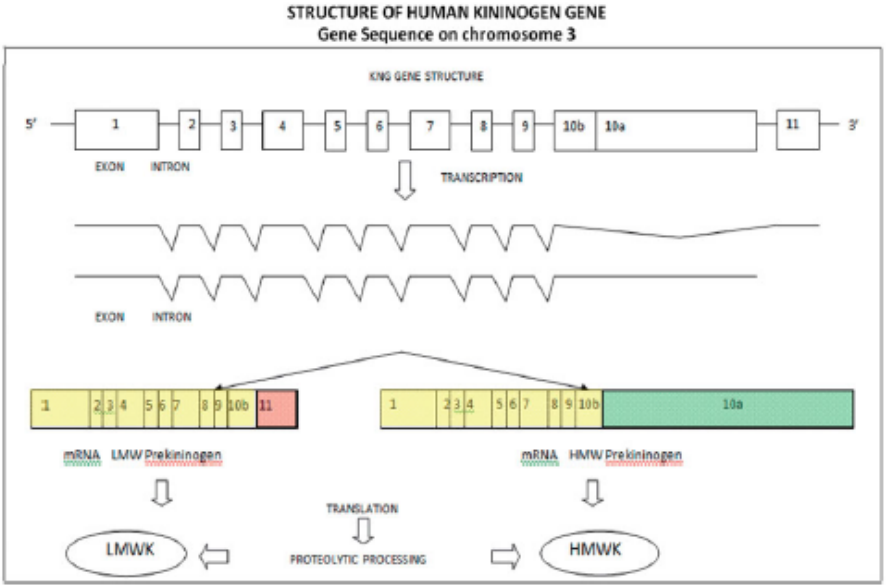

Figure 6. HMWK and LMWK genes are generated by alternative splicing. Boxes 1–11 represent the corresponding exons, connected by introns. Exons 1–9 encode signal peptide and heavy chain for both HMWK and LMWK, Exon 10b encodes the BK sequence and the following 10a specifies an HMWK light sequence. Exon 11 consists of sequence unique to the LMWK light chain.

specific antagonist for B_2 receptors, whereas endogenous secondary generated kinin metabolites such as des[Arg^9]–BK and Lys–des[Arg^9]–BK (these fragments are the product of kinin degradation by kininases and are generated without the Arg residue at the carboxy-terminus of BK and Lys–BK (Drapeau et al., 1991)) exert their effect through kinin B_1 receptors (Leeb-Lundberg, 2004). In humans, the B_1 and B_2 receptors are encoded by the *BDKRB1* gene. B_2 receptors are widely distributed and ubiquitously constitutively expressed in various human tissues except liver and spleen (Kakoki et al., 2007) and usually are identified under ''normal'' physiological conditions. B_1 receptors however are very difficult to identify under ''normal'' physiological conditions but are found to be significantly upregulated by proinflammatory cytokines such as IL-1β (Marceau et al., 1998) and growth factors such as EGF in several pathophysiological conditions such as inflammation, allergy, tissue injury and pain (Bourdet et al., 2010). The synthesis of kinin B_1 receptor agonists such as des[Arg^9]–BK and Lys–des[Arg^9]–BK is significantly upregulated during inflammation (McLean et al., 2000). It has also been demonstrated that the expression of B_1 receptors is induced when B_2 receptors are absent or inactivated (Duka et al., 2006). As a result of kinin–receptor interaction conformational changes within the receptor occur and this subsequently triggers several intracellular signal transduction pathways, which in turn stimulate phospholipase β phosphoinositide kinetics, protein kinase C and calcium mobilisation (Montana & Sontheimer, 2011) which lead to modulation of the cellular response. B_2 receptors are implicated in the activation of the mitogen-activated protein kinase (MAPK) pathway and play a crucial role in cross-talk between the RAS and the KKS by forming a complex with ACE on the plasma membrane (Chen et al., 2006).

The existence of novel third type of high affinity receptors for BK has been proposed, with the report that the orphan GPCR named GPR100 may function as a supplementary BK receptor (Boels & Schaller, 2003; Leeb-Lundberg, 2004; Meini et al., 2004). This proposal was based on the fact that BK, at nanomolar concentrations, enhances intracellular free Ca^{2+} via GPR100 (Leeb-Lundberg, 2004; Meini et al., 2004).

Assembly and activation of KKS

Up until the past decade the assembly and activation of the KKS under ''normal'' physiological conditions had not been fully elucidated. Then, evidence of KKS activation in the intravascular compartment independent of FXII was demonstrated and it was suggested that the proteins of the KKS could be assembled on the endothelial cell membrane by binding to a multiprotein receptor complex presented by CK1, uPAR and gC1qR (Motta et al., 2001; Rojkjaer et al., 1998; Schmaier, 2002) (Figure 7). Several researchers also demonstrated the involvement of the macrophage-1 (Mac-1) integrin receptor (CD11b) in activation of the KKS. The Mac-1 integrin receptor is a heparin sulphate receptor which is expressed on the surface of many leukocytes involved in the innate immune system; it mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity and chemotaxis. Mac-1 is involved in the complement system (Renne, 2012) and also activation of the KKS on the cell membrane of neutrophils, platelets (Sheng et al., 2000), macrophages (Barbasz et al., 2008) and monocytes (Sainz et al., 2007).

Zn^{2+} is an essential cofactor for KNG binding to cell membranes and contact activation *in vivo* is highly regulated

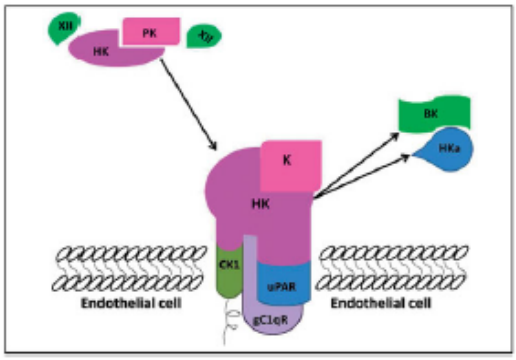

Figure 7. Assembly of KKS on endothelial cells. The HK–PK complex circulates in plasma and binds to endothelial cell membrane receptor complex consisting of CK1, uPAR, gC1qR resulting in transformation of PK into KLK and, as result of proteolytic cleavage, BK and HKa are liberated.

by the plasma Zn^{2+} concentration (total plasma concentration is approximately 10–25 μM, however the concentration of free Zn^{2+} is 0.25–1 μM as the majority of Zn^{2+} circulates in a complex with albumin) (Rojkjaer et al., 1998). Neutrophils, lymphocytes and platelets contain high total Zn^{2+} concentration (Rojkjaer et al., 1998; Whitehouse et al., 1982) and therefore the cells of the intravascular compartment such as platelets (Gustafson et al., 1986), neutrophils (Figueroa et al., 1992; Gustafson et al., 1986) monocytes (Barbasz et al., 2008), macrophages (Barbasz & Kozik, 2009), astrocytes (Fernando et al., 2003) and vascular smooth muscle cells (Fernando et al., 2005) possess the KKS on their surface (Vergiliana et al., 2010). Two high affinity HMWK receptors were identified to be involved in the activation of the KKS on platelets, namely glycoprotein Ib-IX-V (GPIb/IX/V), which is an abundant platelet glycoprotein receptor mediating platelet activation, adhesion to impaired blood vessels and vascular repair, and gC1qR, which is also expressed on the endothelial cells surface (Colman, 2006).

Recent studies have demonstrated that the interaction of HMWK with its endothelial cell–surface multiprotein receptor in a Zn^{2+}-dependent manner (Gustafson et al., 1986; Schmaier et al., 1988) promotes the regulated activation of the PK/HMWK complex, which freely circulates in plasma (Schmaier, 2002). An active binding site encompassed in D3 of the HMWK heavy chain and D5 of the HMWK light chain interact with the N-terminal H1 subdomain of CK1 on the endothelial cell surface, whilst D2 and D3 of HMWK bind to the uPAR receptor and D5 interacts with gC1qR (Shariat-Madar et al., 2002). Activation of the PK/HMWK complex is suggested to be triggered by an interaction between PK and PRCP, which is constitutively expressed on endothelial cell membranes (Schmaier, 2002), or heat shock protein 90 (HSP90) (Joseph et al., 2002) and this results in conformational changes in PK leading to transformation to KLK. It is suggested that the activation of the KKS can be mediated independently of FXIIa; however, the presence of FXIIa acts as a catalyst and augments this process (Vergiliana et al., 2010). FXIIa is activated secondary to PK activation (Motta et al., 2001) and demonstrates high affinity to the same multiprotein receptor complex as HMKK (in the absence of HMWK), but this interaction is highly regulated by Zn^{2+} concentration, requiring a 30-fold higher free Zn^{2+} concentration in comparison to HMWK. Newly generated KLK auto digests its receptor to release BK from substrate KNG (Shariat-Madar et al., 2002).

The functional role of HMWK in the absence of PK on vascular endothelial cells is not fully defined. Therefore, an investigation into the effect of HMWK in the absence of PK was performed. Kolte et al. (2011) demonstrated that HMWK regulates endothelial cell function in human pulmonary artery endothelial cells in PK-deficient conditions by stimulating a considerable and dose-dependent increase in the intracellular calcium level, which in turn stimulates the production of endothelial nitric oxide and prostacyclin (PGI_2).

The KKS: pathophysiology and dysregulation

Genetic deficiency

FXII deficiency (Hageman trait) was first discovered in 1955 and named after John Hageman, the first patient diagnosed with this condition (Stavrou & Schmaier, 2010). FXII deficiency is known predominantly to be a hereditary defect, which is inherited in an autosomal recessive manner. Despite some understanding of the function of FXII in the intrinsic coagulation pathway and the KKS, the definitive role of FXII is still unclear. In recorded cases where severe FXII deficiency was identified thrombotic events were observed instead of bleeding disorders, whilst a decreased level of FXII has low to no clinical significance (Kanjanapongkul, 2011; Renné et al., 2012).

PK deficiency (Fletcher trait) is also a rare autosomal recessive condition and not considered to be associated with a tendency for bleeding, despite marked prolongation of APTT (Nakao et al., 2011).

KNG deficiency is a rare genetic event with no tendency for bleeding but is associated with abnormal coagulation tests (Giangrande, 2003). Williams trait is characterised by a plasma deficiency of both KNG isoforms. Williams trait is associated with a mutation at nucleotide 586 of *KNG* which results in a premature stop codon in exon 5. This gives rise to complete termination of HMWK biosynthesis after amino acid position 195. Fitzgerald trait is characterised by the absence of functional HMWK accompanied by reduced levels of LMWK; however, plasma BK levels are within the normal clinical concentration range (Krijanovski et al., 2003). A study of 12 patients with HMWK or HMWK/LMWK deficiencies demonstrated a prolonged APTT but no clinical haemostatic abnormalities (Cheung et al., 1993).

Inherited excessive activation of the contact system causes a life-threatening swelling disorder, termed hereditary angioedema (Renne, 2012). This is a genetic disorder characterised by significant diffuse mucosal oedema that, in extreme cases can affect the airway leading to asphyxiation (Parish, 2011). The primary cause is a deficiency or dysfunction of the C1 esterase inhibitor (a member of the serpin superfamily), which is a protease inhibitor involved in the regulation of the complement system.

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Physiology and pathophysiology of KKS

The physiological function and pathophysiological implication of the KKS were intensively studied in recent decades; however, since ACE inhibitors and kinin receptor antagonists have become a target for pharmacological intervention (Dendorfer et al., 1999), the molecular analysis of KKS has become more intense.

The activation of the KKS has been described to play a major role in several bio-physiological processes such as the proinflammatory response and tissue injury (Renne, 2012), control of vascular smooth muscle tone, regulation of arterial blood pressure (Marcondes & Antunes, 2005), cardioprotection (Hall, 1992; Kolte et al., 2011; Liesmaa et al., 2009), enhancement of vascular permeability, contraction of intestinal smooth muscle, spasm of smooth muscle in airways, enhancement of airway resistance, stimulation of sensory neurons resulting in chronic hyperalgesia (Butt et al., 1995), alteration of ion secretion by epithelial cells, production of nitric oxide, release of cytokines by leukocytes and release of eicosanoids such as prostaglandins from various cell types, anti-adhesion and regulation of cell migration.

Considering the broad range of activities displayed by the KKS, this system has been implicated in many pathophysiologies including acute attacks of hereditary angioedema, sepsis, diabetic retinopathy, induced arterial thrombosis, acute myocardial infarction (Schmaier, 2008), inflammatory bowel disease (Sartor et al., 1996), arthritis (Dela Cadena et al., 1995), as well as pain, asthma, infection, allergy and symptoms associated with acute and chronic inflammation and cancer.

Inflammation is a complex physiological response of vascular tissue to invading pathogens, tissue injury or irritants and is characterised by five classical signs of inflammation: pain, heat, redness, swelling and loss of function. Initial response or prolonged and persistent inflammation is recognised as acute or chronic inflammation, respectively. At the onset of an inflammatory response, activation and interconnection of several metabolic cascades, encompassing the complement system, coagulation system, fibrinolysis system and KKS, take place in order to propagate and mature the inflammatory reaction. The key function of the KKS in sustaining an inflammatory response is the synthesis of proteins associated with vasodilation, increased vascular permeability, smooth muscle contraction and pain (Dendorfer et al., 1999).

During the onset and progression of inflammation the PK/HMWK complex, which is freely circulating in plasma, binds to the multiprotein complex on endothelial cells. As a result, conformational changes occur to convert PK to KLK, which in turn proteolytically cleaves HMWK to liberate BK and HKa. Under ''normal'' physiological conditions, BK has a very short half-life of 17/30 s before this active molecule is metabolised by kininases; however, this is long enough to trigger intracellular signalling initiated via the B_2 receptor (Colman, 2006; Ferreira & Vane, 1967). HKa has comparatively longer biological half-life of about 9 hand is eventually deactivated by proteases. The activation of this BK-forming cascade on endothelial cells, neutrophils and also macrophages (one of the major types of defence cells engaged at the site of inflammation), in parallel to secretion of inflammatory mediators such as kinins, has been implicated in the pathogenesis of inflammation and vascular injury. The balance of evidence to date suggests that neutrophils and macrophages carry a significant amount of bound KNG, and possibly other contact system proteins, and deliver them to inflammatory foci to supplement the concentration of these proteins in the injured or infected tissues. Moreover, the activation of the KKS on the surface of neutrophils and macrophages enhances the tKLK-dependent kinin liberation and hence supports the development of an inflammatory response (Barbasz et al., 2008). pKLK has been shown to stimulate neutrophil chemotaxis aggregation, oxygen consumption (Wachtfogel et al., 1983) and acts as a potent aggregation agent for human blood polymorphonuclear leukocytes (Schapira et al., 1982). Together with the ability of KLK to release neutrophil elastase, these factors contribute to tissue injury (Keith et al., 2005). BK, released via B_2 receptors, and inflammatory cell adhesion molecules trigger downstream intracellular signalling which initiates an acute inflammatory response, promotes the synthesis of other pain mediators via prostaglandin liberation and enhances vascular permeability via prostaglandin I2 and the production of nitric oxide (Sainz et al., 2007).

Plasma CPN or tissue CPM cleaves the C-terminal Arg of the B_2 receptor ligands BK and Lys–BK to generate B_1 ligands such as des–Arg^9–BK and des–Arg^9–Lys^1–BK (Ignjatovic et al., 2002). These ligands activate the downstream signalling pathway via B_1 and this facilitates the activation of further cell adhesion molecules, whilst the inhibition of migration promotes cell clustering in the inflammation site to develop the inflammatory reaction. The effects of released BK are initiated through B_1 and B_2 receptors, leading to activation of several intracellular signal transduction pathways including the phospholipase C (Leeb-Lundberg, 2004), phospholipase A_2, phospholipase D and MAPK cascades (Sainz et al., 2007; Yang et al., 2003). Several peptide receptors, including the B_2 receptor, are known to stimulate the MAPK cascade. The mitogenic potential of B_1 receptors is not completely established, although there are reports that the mediation of the MAPK signalling pathway is associated with B_1 receptors (Morbidelli et al., 1998). Activation of the p42/p44 extracellular-signal-regulated kinase (ERK)/MAPK cascade plays a key role in the mediation of proliferative activity of receptor tyrosine kinases (RTKs), such as EGF receptor (EGFR) and GPCRs (Gutkind, 1998). Simultaneous and sustained activation of both kinin receptors is known to be advantageous for immediate triggering of $ERK_{1/2}$ global phosphorylation and PLCγ2-dependent intracellular Ca^{2+} mobilisation. Several mechanisms of GPCR-induced MAPK activation have been reported. EGFR-induced activation of MAPK is triggered by interaction of EGF with a monomeric receptor, which results in EGFR dimerisation, activation of Ras and downstream phosphorylation on tyrosine residues (Liebmann, 2001). Ras stimulates the MAPK pathway, comprising Raf (a serine/threonine-protein kinase), mitogen-activated protein kinase (MEK) and ERK (Liebmann, 2001), thereby regulating the transcription of the genes involved in cell growth, differentiation and survival. The ''transactivation''

(or cross-communication between different signalling pathways) of RTKs has been reported as a principal mitogenic signal transduction cascade of GPCR (Daub et al., 1997). However, a mechanism, proposed by Prenzel et al. (1999) shows that transactivation of the EGFR-dependent signalling pathways upon activation of GPCR by an extracellular ligand involves the interaction of membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) and metalloproteinase. In conclusion, the cross-activation between the GPCR and MAPK pathways is extremely cell specific and may be dependent on the type of receptor, concentration and cellular localisation of the proteins that play a major role in the signal transduction within the GPCR and MAPK signalling cascades (Liebmann, 2001).

The diverse effects of the bioactive BK peptide can produce the majority, but not all, of the features of the inflammatory reaction. Recently, the role of HMWK and its contribution to inflammation, particularly interactions of HKa with leukocytes and endothelial cells was emphasised. The histidine-rich D5 of the HMWK light chain, which possesses a binding site for vitronectin, is suggested to be associated with the mechanism of anti-cell spreading activities of HKa (Asakura et al., 1998). The immobilisation of vitronectin leads to the prevention of vitronectin-uPAR interaction on the surface of endothelial cells and results in inhibition of cell migration and adhesion (Asakura et al., 1998). The ability of HMWK to detach inflammatory cells such as neutrophils and monocytes from vitronectin by a mechanism of competitive binding to the Mac-1 (CD11a/CD18) receptor plays an imperative role in migration of the cells associated with inflammatory reaction (Sainz et al., 2007). Moreover, HKa can inhibit intracellular changes that lead to the modulation of intracellular signals in endothelial cells and thus cell behaviour. It has been postulated that HKa stimulates monocyte activation by binding to uPAR, gC1qR and Mac-1 surface receptor complex. Both D3 and D5 of HKa exhibit high affinity to Mac-1, although only D5 (which is extremely exposed in two-chained HKa) is designed for binding to uPAR (Khan et al., 2006). It is evidenced that upon this activation monocytes synthesize cytokines and chemokines such as TNFα, IL-1β, IL-6, IL-8 and monocyte chemotactic peptide 1 (MCP-1), which are reported to play a major role in the migration of monocytes (Khan et al., 2006).

BK can regulate Ca^{2+} kinetics in a dose-dependent manner by mediating the mobilisation of calcium from the thapsigargin-sensitive stores on inositol-1,4,5-trisphosphate and also by inducing extracellular Ca^{2+} release initiated via B_2 receptors (Wang et al., 2001). However, recently Kolte et al. (2011) presented a study on role of HMWK in the absence of PK and provided evidence that HMWK (but not HKa) has the same capability as BK to bind to B_2 receptors. The study demonstrated that the interaction of HMWK with B_2 receptors is effective enough to trigger B_2 receptor-dependent downstream signalling, thereby mediating a significant increase in intracellular Ca^{2+} with release of nitric oxide and PG_2 by endothelial cells providing two important mediators of inflammatory response (Kolte et al., 2011). At physiological concentrations HMWK displayed no effect on endothelial permeability but demonstrated cardioprotective properties due to the ability to sustain normal endothelial function and cardiovascular circulation (Kolte et al., 2011). These novel findings give a clear understanding of the KKS; however, further investigation is required in order to identify the molecular events that contribute to dysregulation of the system and pathologic disorders.

KKS: the implications in carcinogenesis and cancer progression

The KKS regulates many physiological processes and dysregulation and imbalance within this metabolic cascade contributes to the development of various pathological conditions (Barbasz et al., 2008). The role of the KKS, as a complex multifunctional cascade, is still poorly defined in cancer. The first studies which demonstrated the possible relevance of the KKS in cancer suggested that enhanced vascular permeability, nitric oxide synthase production and prostaglandin production in cancer tissue could be promoted by the activated KKS (Czokalo et al., 1996; Maeda et al., 1996). This leads to the suggestion that KLKs and possibly other members of the KKS could be novel cancer biomarkers (Dlamini & Bhoola, 2005; Greco et al., 2004; Rittenhouse et al., 1998; Yousef & Diamandis, 2002). Recent evidence has demonstrated a convincing role of the KKS in the pathogenesis of cancer. Several proteins generated within the KKS have been confirmed to exhibit proinflammatory properties and to be involved in aetiology and genesis of inflammation. Chronic inflammation is known to be a major cause of cancer due to the liberation of powerful oxidants which serve as mutagens and also stimulate pathogenic cell multiplication. The KKS has become a target of intense investigation by scientific researchers and the pharmaceutical industry in order to discover novel cancer biomarkers and anti-cancer therapy targets. Third generation kinin receptor inhibitors have been demonstrated to be cytotoxic to malignant cells (Dlamini & Bhoola, 2005).

KLKs: the role and association with carcinogenesis

KLKs are a group of serine proteases. tKLKs play an important role in a wide spectrum of normal physiological events such as kinin formation, blood pressure control, skin desquamation, electrolyte kinetics, tissue remodelling and prohormone processing (Borgono et al., 2004). The dysregulated expression of several KLK-related peptidases has been associated with various malignancies. The serine protease properties of tKLKs may contribute to the malignant phenotype by promoting angiogenesis, invasiveness and metastasis of malignant cells due to dysregulation of ECM components and activation of signal transduction cascades (Avgeris et al., 2011). In addition, multiple studies have demonstrated that KLKs may contribute to cancer development by the stimulation of malignant cell proliferation, enhancement of vascular permeability and malignant cells dissemination via mitogenic and cellular activities of the kinins (Dlamini & Bhoola, 2005). tKLKs have been demonstrated to promote prostate cancer cell migration, invasion and proliferation via the activation of protease-activated receptor-1 and through cross-activation of EGFR, an effect which could be downregulated by EGFR inhibitors or ERK inhibitors (Gao et al., 2010).

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Several individual members of the KLK family have been identified as potential biomarkers for various malignancies. Human tKLK 1 (KLK1) was reported to play an important role in post-ischaemic neovascularisation (Stone et al., 2009) and was associated with progression and invasiveness in pancreatic carcinoma (Wolf et al., 2001), oesophageal carcinoma (Dlamini & Bhoola, 2005), gastrointestinal stromal tumours (Dominek et al., 2010) and lung malignancies (Chee et al., 2008). Human KLK 3 (KLK3), which is also termed prostate-specific antigen (PSA), along with human tKLK 2 (KLK2) are biomarkers for prostate carcinoma (Chan et al., 1987; Rittenhouse et al., 1998). Down regulation of tissue PSA was found to be associated with more aggressive forms of prostate cancer (Stege et al., 2000) and PSA can be utilised as a diagnostic marker in this malignancy (Partin et al., 1990). The role of KLKs as prognostic biomarkers in prostate cancer became a catalyst for further research of KLK expression in other hormone-related malignancies including breast cancer. It was reported that PSA expression was significantly enhanced in more aggressive forms of breast cancer when compared with the indolent form or healthy breast tissue (Yousef & Diamandis, 2001). However, PSA-positive tumours were reported to be associated with favourable prognosis, earlier disease stage, longer median survival and reduced risk of disease relapse (Diamandis et al., 1996). Other studies have reported KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14 and KLK15 upregulation in ovarian carcinoma tissues, serum and/or cell lines (Borgono et al., 2004).

In colorectal cancer, the overexpression of KLK4, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK15 has been reported (Avgeris et al., 2010; Kontos & Scorilas, 2012). KLK5, KLK7 and KLK14 have been demonstrated to be independent prognostic factors (Talieri et al., 2009). Recently KLK4 transcript expression has also been reported to be of clinical relevance in colorectal cancer and may predict poor disease free survival (Kontos et al., 2013).

It has been reported that the quantification of KLK5 expression in breast tissue biopsies may represent a novel biomarker for the differential diagnosis between malignant and benign tumours of the mammary gland and may also be useful in the monitoring of breast cancer patients (Avgeris et al., 2011).

However, there are a few studies which have reported that KLKs can also inhibit carcinogenesis (Goyal et al., 1998; Roman-Gomez et al., 2004). For example, KLK10 (*NES1* gene) is a candidate tumour suppressor gene on chromosome 19q13.3-4 which was demonstrated to be downregulated in acute lymphoblastic leukaemia (Roman-Gomez et al., 2004) and breast cancer (Dhar et al., 2001). Despite many reports which indicate a convincing association between dysregulation of KLK expression and carcinogenesis, several key questions still remain.

KNGs: implications in cancer

It has been discussed earlier in this review that the PK/HMWK complex, which circulates freely in plasma, yields BK and HKa upon binding to the uPAR–CK1–gC1qR complex (Figure 8). HMWK expression was reported to be

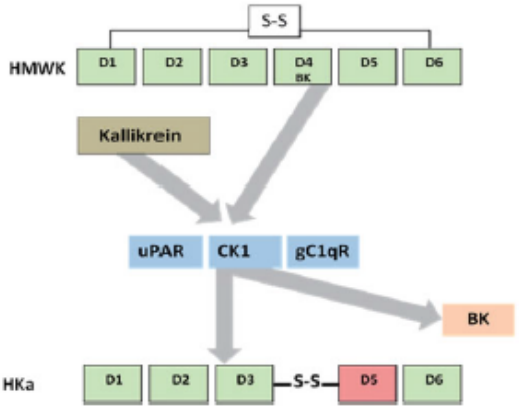

Figure 8. Mechanism of proteolytic hydrolysis of HMWK by plasma PK. Upon binding of HK–PK complex to multiprotein receptors comprising of uPAR/CK1/qC1qR on the endothelial cells membrane, PK is immediately transformed to KLK, which in turn digests KNG to release BK and HKa.

downregulated in the urine of patients with ovarian carcinoma in comparison to healthy controls (Abdullah-Soheimi et al., 2010). However, it has also been demonstrated that two-chain HMWK (D_1, D2, D3–D5 and D6) promotes endothelial cell apoptosis and inhibits angiogenesis (Colman et al., 2000; Zhang et al., 2000).

An underlying paradigm that the KKS plays an important role in promoting angiogenesis by proteolytic liberation of BK, the pro-angiogenic molecule, was contradicted by the report that HKa can serve as an angiogenic inhibitor (Browder et al., 2000). Since the interaction between urokinase and urokinase receptors was demonstrated to initiate intracellular signalling leading to stimulation of mitogenesis and HKa was reported to bind to uPAR with high affinity, the mechanism of HKa and uPAR/CK1/Gc1qR interaction has been intensively assessed *in vivo* and *in vitro*. uPAR has been shown to be involved in inhibition of cell adhesion, migration, proliferation, angiogenesis and tumour metastasis by inducing apoptosis and cell signalling failure (Bior et al., 2007). uPAR is membrane-anchored receptor which is widely distributed in monocytes and endothelial cells but is upregulated in many cancer cells (Carroll & Binder, 1999). The inhibitory activity of HKa was found to be Zn^{2+}-dependent and the interaction between HKa and uPAR was mediated via HKa D5 (the high affinity endothelial cell binding region within D5 was allocated to amino acids sequence 479–498 of HMWK) with uPAR D2 and D3 (Colman et al., 2000; Zhang et al., 2000). D5 of HKa can block vascular endothelial growth factor (VEGF) and angiogenesis in the chick chorioallantoic membrane (Zhang et al., 2000). Several studies were conducted in order to confirm D5-dependent inhibitory properties in various malignancies. HKa D5 caused dramatic inhibition of the growth of HT-1080 human fibrosarcoma cells (Colman et al., 2000) and HCT-116 colorectal carcinoma cells (Bior et al., 2007). It has been suggested that D5 possesses inhibitory properties against malignant cell migration and invasion of prostate cancer (Liu et al., 2009). It has been hypothesised that HKa disrupts

the urokinase plasminogen activator–uPAR complex, inhibits ERK activation and blocks the internalisation of uPAR, which lead to cell death and cell motility arrest (Colman et al., 2010). However, the pathway/pathways by which D5-HMWK signals and regulates the cell cycle still remains the subject for future research.

The involvement of kinin and kinin receptors in tumourigenesis

Under "normal" physiological conditions the active peptide and inflammatory mediator BK has a very short half-life before its enzymatic degradation; however, overexpression of BK and the B_1, and B_2 GPCRs have been demonstrated in various pathological conditions including carcinogenesis and cancer progression. B_1 receptors were reported to be overexpressed in prostate cancer (Taub et al., 2003; Zhang et al., 2008) whilst B_2 receptors were expressed at elevated levels in brain, gastric, lung and liver cancer (Zhang et al., 2008; Zhao et al., 2005). B_1 and B_2 receptors were overexpressed in human chondrosarcoma cells, in comparison to healthy cartilage cells (Yang et al., 2010). Immunohistochemical detection of B_2 receptor overexpression in head and neck cancer samples was recently reported (Beck et al., 2012). The expression of BK receptors in human lung adenocarcinoma, gastric adenocarcinoma, lymphoma, squamous cell lung carcinoma and head and neck cancer suggests involvement in mediation of pathologic signal transduction, nitric oxide production and increased vascular permeability (Wu et al., 2002) thus potentially leading to cancer growth, progression and tumour dissemination.

It has been reported that BK stimulates angiogenesis in endothelial cells due to B_2-dependent increase in vascular permeability in the early stages and B_2-dependent up-regulation of VEGF in the stromal fibroblasts in the later phases (Ishihara et al., 2002). Other researchers have confirmed this BK-induced increase in vascular permeability and elevated levels of VEGF in solid tumours (Maeda et al., 2003). The involvement of BK in angiogenesis via B_2-dependent stimulation of cell proliferation and cell migration has been reported (Seegers et al., 2004). BK has been demonstrated to promote B_2-dependent stimulation of growth and migration of head and neck squamous cell carcinoma cells via EGFR transactivation and also to lead to the production of cyclooxygenase-2 and PGE2 through the MAPK signalling cascade in human airway cells (Zhang et al., 2008).

GPCR-dependent activation of the actin–cytoskeleton pathway is associated with cell migration/invasion. The initial migratory cell response requires a formation of cellular protrusions, such as actin microspikes, filopodia and lamellipodia, which promotes cell movements and invasion into surrounding tissue (Erices et al., 2011). This process involves the polymerisation of actin, induced by the actin-cytoskeleton signalling cascade, and is reported to be dependent on Cdc42 and Rac1 molecules. BK promotes the formation of Cdc42-dependent membrane protrusions in fibroblasts provoked by PGE2 secretion (Erices et al., 2011). BK exhibits multiple activities which can modulate several cell signal transduction pathways, initiated via B_1 and B_2 receptors, and therefore may play a significant role in the regulation of neovascularisation, tumourogenesis and tumour progression.

Conclusion

The KKS represents a complex network of interactions produced by multiple constituents within the system itself and the action of the KKS upon other important systems within the human body. We have described the KKS network and the implication of the KKS in carcinogenesis and cancer progression.

Declaration of interest

This work was not supported by external funding. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

The authors report no declaration of interest. The authors alone are responsible for the content and writing of this article.

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