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The clinical and biological relevance of CD38 expression in chronic lymphocytic leukaemia

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Abbreviations

AGC	Named after family members protein kinases A/G/C
ANOVA	Analysis of variables
APC	Allophycocyanin
ATM	Ataxia telangiectasia mutated
B2M	β2 microglobulin
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma protein 2
BCR	B cell receptor
BH	Bcl-2 sequence homology
BSA	Bovine serum albumin
cDNA	complementary DNA
CO_2	Carbon dioxide
CLL	Chronic lymphocytic leukaemia
CLP	common lymphoid precursors
CMP	common myeloid precursors
dATP	deoxy adenine triphospahte
dCTP	deoxy cytosine triphospahte
dGTP	deoxy guanine triphospahte
DMSO	Dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxy nucleotide triphospahte
dTTP	deoxy thymine triphospahte
ds	double stranded
DTT	dithiothreitol

EDTA	ethylene diamine tetra-acetic acid
FACS	Flow assisted cell sorting
FADD	Fas-associated death domain
FCR	Fludarabine, cyclophosphamide and rituximab
FCS	Foetal calf serum
FITC	fluorescein isothiocyanate
Flk-1	fetal liver kinase 1
Flt-1	fms-like tyrosine kinase 1
Flt-4	fms-like tyrosine kinase 4
FRAP	FKBP12-rapamycin associated protein
GF	Growth factors
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSC	Haemopoiesis stem cells
IgM/IgG	Immunoglobulin M/G
IL-1β	Interleukin-1β
IRS-1	Insulin receptor substrate-1
IVT	In vitro transcription
KDR	kinase insert domain-containing receptor
LD_{50}	Lethal dose for 50% of the cells
LDT	Lymphocyte doubling time
Mcl-1	Myeloid cell leukaemia sequence 1
M-CSF	monocytes colony stimulating factor
MESF	molecules of equivalent soluble fluorochrome
$MgCL_2$	Magnesium chloride

MFI	Mean fluorescence intensity
MPP	multipotent progenitors
mTOR	Mammalian target of rapamycin
NK	Natural killer
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide dependent kinase 1
PE	Phycoerythrin
PECAM-1	Platelet endothelial cells adhesion molecule-1
PFS	Progression free survival
PIF	PDK1 interacting fragment
PI3K	Phosphoinositol-3 kinase
PIKK	Phosphoinositide kinase related kinase
PIKK	Phosphoinositide kinase related kinase
PIP2	Phosphainositol-3,4-bisphosphate
PIP3	Phosphainositol-3,4,5-trisphosphate
PKC	Protein kinase C
PMT	Photomultiplier tube
PRK1	PKC related kinase-1
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTK	Protein tyrosine kinase
PVDF	poly vinylidene difluoride
RAFT	Rapamycin and FKBP12 target
Raptor	regulatory-associated protein of mTOR

Rictor	rapamycin-insensitive companion of mTOR
RIP	Receptor-interacting protein
RNA	Ribose nucleic acid
SAM	Significance analysis of microarray
SAPE	Streptavidin phycoerythrin
sCD23	Soluble CD23
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SGK	serum and glucocorticoid-related kinase
siRNA	small interference RNA
SM	Statistical method
Ss	Single stranded
S6	S6 ribosomal protein
S6K	p70 ribosomal S6 kinase
TBS	Tris buffered saline
TCR	T cell receptor
Thr	Threonine
TK	Thymidine kinase
TTFT	Time to first treatment
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
ZAP-70	Zeta associated protein-70

Abstract

CD38 is a type II transmembrane glycoprotein that is associated with poor prognosis in chronic lymphocytic leukaemia (CLL), but to-date there is no biological rationale for this association. This study was designed to investigate the role of CD38 within CLL and answer some of the controversial questions relating to CD38 in this leukaemia.

Since the identification of CD38 as a prognostic marker in CLL there has been great debate about whether CD38 is a proxy marker for V_H gene mutation status. In addition, there has also been controversy over the most appropriate percentage threshold to use in order to categorise a patient as $CD38^+$. In this study I found a strong correlation between CD38 and V_H gene mutation status but the degree of discordance between these parameters precludes CD38 from being used a reliable surrogate marker for V_H gene mutation status. Previous studies have listed three different percentage thresholds for the classification of patients with CD38 expression; 7%, 20% and 30%. In this study I evaluated all three threshold levels; 20% was the most prognostically significant threshold. Another question relating to CD38 expression is whether it remains stable over time. Longitudinal analyses showed that CD38 expression remains constant over time and after treatment. Although CD38 expression was independently prognostic, the combination of CD38 expression and V_H gene mutation status for each patient provided a more robust measure of the patient's clinical course.

In the next part of this study I employed microarray analysis to compare $CD38^+$ and $CD38^-$ cells from the same patient purified by high speed cell sorting. $CD38^+$ CLL cells demonstrated a unique gene expression profile when compared to their $CD38^-$ counter-parts with 61 genes found to be consistently statistically different. Six genes were investigated further, VEGF, IL-1β, CXCL2, CCR3, ZAP-70 and Akt. Using flow cytometry and Western blotting techniques it was confirmed that these proteins were expressed at significantly higher levels in $CD38^+$ sub-populations when compared to the $CD38^-$ sub-

populations. $CD38^+$ and $CD38^-$ cells from the same patient showed similar drug sensitivity to fludarabine in the majority of samples tested. However, samples from five patients who had received the most prior treatment showed greater resistance to drug therapy in their $CD38^+$ CLL cells. The apoptotic proteins Bcl-2, Bax and Mcl-1 were up-regulated in the $CD38^+$ cells.

The combination of CD38 expression data with other prognostic factors such as V_H gene mutational status and ZAP-70 expression will be beneficial in determining the prognosis of the patients and the course of treatment. Also determining the role of CD38 within CLL cells could lead to new therapeutic targets.

Chapter 1: Introduction

1.1 Haemopoiesis

Haemopoiesis is the process by which blood cells are produced and it mainly occurs in the bone marrow of the central skeleton and proximal ends of long bones. A stem cell found in the bone marrow can self replicate, proliferate and differentiate (Mayani, Alvarado-Moreno, & Flores-Guzman 2003). After many divisions in the bone marrow, mature cells are formed which are then released into the peripheral blood.

The haemopoietic (pluripotent) stem cell gives rise to at least 10 different functional cell types; neutrophils, monocytes/macrophages, basophils, eosinophils, erythrocytes, platelets, mast cells, dendritic cells, B- and T-lymphocytes (Mehts & Hoffbrand 2000). Around $2x10^{11}$ erythrocytes and $1x10^{10}$ white blood cells must be replaced everyday to maintain haemopoiesis (Bellantuono 2004) (see Figure 1.1). Haemopoiesis is regulated by many growth factors, cytokines and interleukins. These are usually produced by stromal cells, T-lymphocytes, the liver and the kidney. They affect primitive cells, cells that have committed to a particular lineage and later on the function of mature cells. The growth factor/ cytokine/ interleukin binds to the receptor on the cell surface and initiates a signalling cascade that activates transcription factors which in turn activate or inhibit gene transcription. This process can lead to activation, replication, proliferation, differentiation or cell survival.

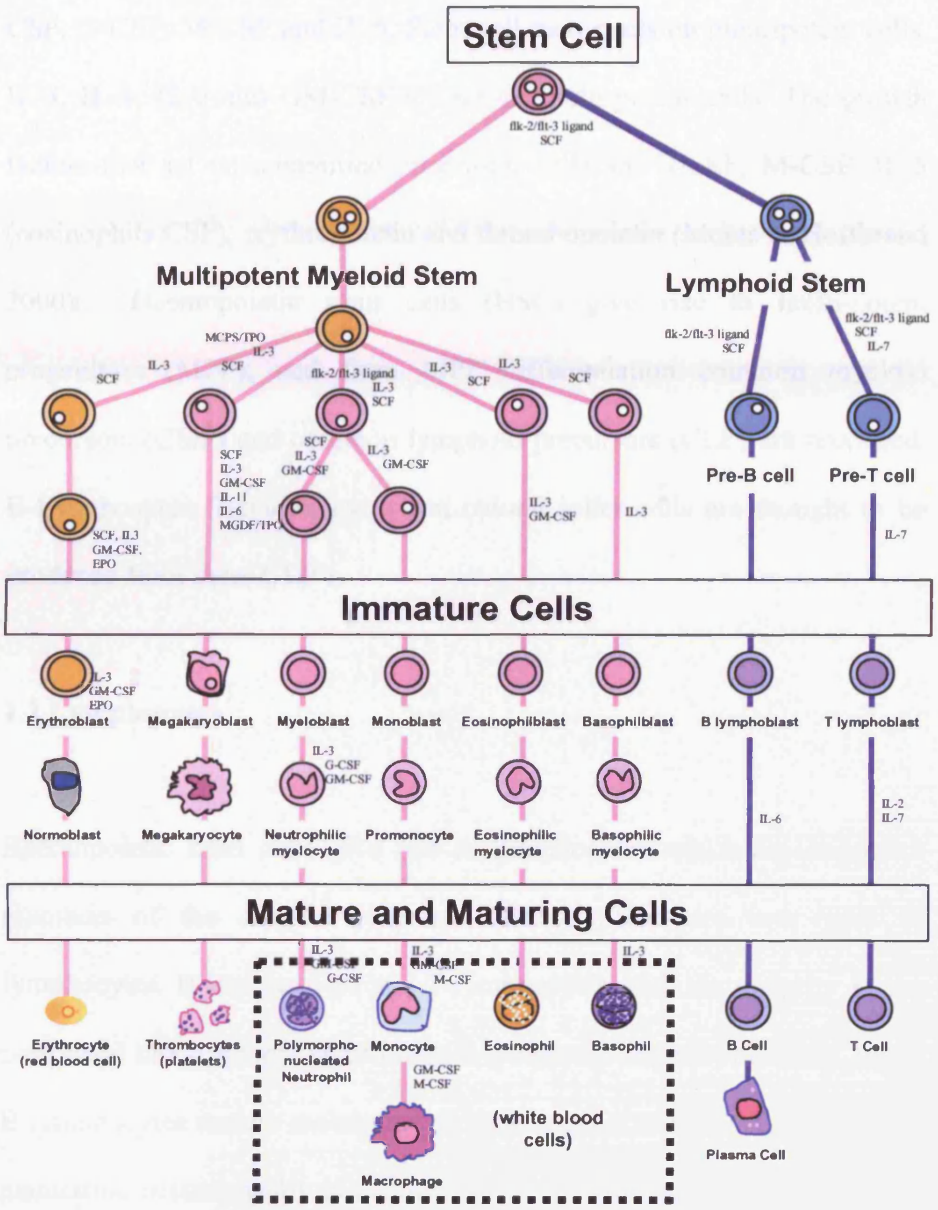


Figure 1.1: Normal haemopoiesis. The production of different cell lineages from a pleuripotent stem cell.**(Walsby 2004).**

Many growth factors are involved during haemopoiesis including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte colony stimulating factor (M-CSF), Interleukin (IL)-1, IL-3, IL-4, IL-6 and IL-8 (Ribatti et al. 2001). IL-1 and TNF act on stromal cells and stimulate the production of GM-

CSF, G-CSF, M-CSF and IL-6. Stem cell factor acts on pleuripotent cells. IL-3, IL-4, IL-6 and GM-CSF all act on multi-potent cells. The growth factors that act on committed progenitor cells are G-CSF, M-CSF, IL-5 (eosinophils CSF), erythropoietin and thrombopoietin (Mehts & Hoffbrand 2000). Haemopoietic stem cells (HSC) give rise to multi-potent progenitors (MPP), and from MPP differentiation common myeloid precursors (CMP) and common lymphoid precursors (CLP) are produced. B-lymphocytes, T-lymphocytes and natural killer cells are thought to be produced from these CLP's.

1.2 Lymphocytes

Haemopoietic stem cells give rise to lymphocytes which are important members of the immune response system. There are two types of lymphocytes, B-lymphocytes and T-lymphocytes with the majority of the peripheral blood lymphocytes being T-lymphocytes (approximately 70%). B-lymphocytes mature mainly in the bone marrow whereas T-lymphocyte maturation occurs mainly in the thymus, but lymph nodes, liver and spleen also play an important part. B-cells mediate humoral (antibody-mediated) immunity and T-cells are involved in cell mediated immunity.

1.3 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the Western world (Dighiero 2005), predominantly affecting individuals

over the age of forty (median age of diagnosis is 68 years) with a higher incidence in men than women in approximately a 2:1 ratio (Montserrat & Rozman 1994). It is characterised by the over expression of mature-looking $CD19^+/CD5^+$ B-lymphocytes, which also express the pan B-cell markers CD20 and CD23 (Dighiero 2005). These cells have weak expression of surface immunoglobulins such as IgM and IgD and have weak expression of CD79β (part of the B-cell receptor (BCR)) (Dighiero 2005). Low expression of the BCR leads to reduced tyrosine kinase activity, which in turn leads to reduced calcium mobilisation and therefore reduced responsiveness of the cells when signalling through this receptor. Peripheral blood CLL cells are mainly found in G_0/G_1 of the cell cycle, but the presence of cyclins D and E within these cells suggest that the cells are not truly quiescent but are more likely in early G_1 (Decker et al. 2003;Decker et al. 2004). The clonal expansion of B-lymphocytes in CLL is due, in part, to dysregulation of survival pathways which results in failed apoptosis rather than a high proliferation rate (Shanafelt, Geyer, & Kay 2004). Messmer *et al* (2005) found that B-CLL cells have quite a high birth rate and so suggests that the build up of CLL cells is due to increased proliferation as well as failed apoptosis. The clinical course of CLL is variable, ranging from a mild disease, with very few symptoms, to an aggressive disease with poor clinical outcome. Approximately a third of CLL patients have an indolent disease and never receive treatment (median survival 12 years), another one third initially have an indolent disease that after a period develops into progressive disease (median survival 5 years), and the final third have an aggressive and progressive disease from

diagnosis (median survival 2 years) (Montserrat & Rozman 1994;Shanafelt, Geyer, & Kay 2004).

1.4 Staging

There are two main staging systems within CLL which are summarised in Table 1. These staging systems were devised as CLL has a variable clinical course and before the introduction of these systems there was no way of determining which patients would have a favourable clinical course and which patients would have poor clinical outcome. Both the Binet and Rai system provided a staging system separating the patients into a good, intermediate and poor clinical outcome.

Binet	***Rai***
Stage A <3 enlarged areas*, haemoglobin ≥10g/dL and platelets ≥100,000/mm^3	**Stage 0** Peripheral and bone marrow lymphocytosis without lymph node enlargement or splenomegaly
	Stage I Stage 0 with lymph node enlargement but without splenomegaly
Stage B ≥3 enlarged areas, haemoglobin ≥10g/dL and platelets ≥100,000/mm^3	**Stage II** Stage 0 without lymph node enlargement but with palpable splenomegaly
	Stage III Stage 0 with lymph node enlargement and palpable splenomegaly
Stage C any number of enlarged areas, haemoglobin <10g/dL and/or platelets <100,000/mm^3	**Stage IV** Stage 0 with either fairly severe anaemia (haemoglobin < 10g) or thrombocytopenia (platelets < 100,000/mm^3)

Table 1: Binet and Rai staging systems for CLL (Binet et al. 1981;Rai et al. 1975)

* each of cervical, axillary, inguinal areas (whether unilateral or bilateral), spleen and liver count as one area: therefore the number of enlarged areas can take any value between 0 and 5.

Throughout this investigation, the Binet system was used. When comparing the two systems, it is considered that Binet stage A is equivalent to Rai stage 0, Binet stage B is equivalent to Rai stage I/II and Binet stage C is equivalent to Rai stage III/IV.

1.5 Clinical symptoms and diagnosis

Some patients display fatigue, fever, night sweats, weight loss (>10% body weight), repeated infections, autoimmune haemolytic anaemia, splenomegaly, hepatomegaly, lymphadenopathy or extra nodal infiltrates, but a high percentage of the patients are asymptomatic (Rai 1996). The disease is usually found within the asymptomatic patients after routine blood tests for unrelated matters (Montserrat & Rozman 1994). Diagnosis of CLL usually occurs when a patients displays a persistent absolute lymphocyte count (ALC) >5.0 x 10^9/L (Montserrat & Rozman 1994). This is followed by immunophenotyping using flow cytometry and sometimes cytogenetic evaluation, usually by fluorescence *in situ* hybridization (FISH), which help to distinguish CLL from other similar B-cell malignancies (Kalil & Cheson 1999).

1.6 Markers of Disease

The Binet and Rai staging systems were the first way of segregating patients into different prognostic groups. Today there are many different markers used which are prognostic. The most commonly used are

lymphocyte doubling time (LDT), β2 microglobulin, serum thymidine kinase, soluble CD23, cytogenetics, V_H gene mutation status, CD38 expression and ZAP-70 expression.

1.6.1 Lymphocyte Doubling Time

Lymphocyte doubling time (LDT) is a clinical measurement of the time in months it takes for the patients' lymphocyte count to double. It is split into two groups; patients with a LDT >12 months and patients with a LDT <12 months. The patients with a LDT <12 months have a more aggressive and progressive disease and shorter survival than the patients with a LDT >12 months (Shanafelt & Call 2004). It has been reported that CLL patients with a LDT >12 had a median survival of 118 months whereas the patients with a LDT <12 months had a median survival of 61 months (Shanafelt, Geyer, & Kay 2004).

1.6.2 β2 microglobulin

One marker that correlates with Binet stage is β2 microglobulin (B2M). B2M is a protein that is associated with class I major histocompatibility complex (Montillo et al. 2005). It is thought that high B2M, which is detected in the serum, is prognostic in CLL as it has been associated with shorter survival (Shanafelt, Geyer, & Kay 2004). Wierda *et al* (2007) included B2M in a nomogram for prognosis. Out of the six variables that were used in the nomogram, age and B2M gave the greatest contribution to

the test. This nomogram helps to identify the patients who are high risk and should receive treatment (Wierda et al. 2007).

1.6.3 Thymidine kinase

Thymidine kinase (TK) is an enzyme involved in the complementary alternative salvage pathway of DNA synthesis (Shanafelt, Geyer, & Kay 2004). It converts deoxythymidine to deoxythymidine monophosphate. TK levels have been associated with poor prognosis in CLL patients, and give valuable prognostic information in regard to response to therapy. High TK levels have been shown by Matthews *et al* (2006) to correlate with other prognostic markers such as V_H gene mutation status and CD38 expression. The dispute over the most appropriate cut-off value and the fact that until recently the assay required the use of radio-isotopes has limited the use of TK as a prognostic marker in the clinic.

1.6.4 Soluble CD23

CD23 is surface molecule that is found on CLL B-cells (Montillo et al 2005). Soluble version of CD23 (sCD23) has been shown to be a marker of poor prognosis in CLL with an association with shorter progression-free survival and overall survival (Molica 1997).

1.6.5 Cytogenetics

There are many cytogenetic abnormalities that are associated with CLL. The most common is a deletion at 13q14 which is usually associated with patients that have good prognosis (Thornton et al. 2004). These patients have the same prognosis as those patients who have no cytogenetic abnormalities. In contrast, patients that express a 17p deletion (17p-) or an 11q deletion (11q-) have a poor clinical outcome (Dighiero 2003). A 17p deletion often involves deletion or mutation of the p53 gene and an 11q deletion usually disrupts the ATM gene (Mehes 2005). Mutations of the p53 gene occurs in 10% - 15% of CLL patients but Pettitt *et al* (2001) stated that dysfunction of the p53 protein can occur in CLL through alternative mechanisms. One example of this is some patients have a extra copy of chromosome 12 which codes for the p53 inhibitory protein, MDM2. Also inactivation of ATM gene is another cause of p53 dysfunction in CLL patients. Patients that express trisomy 12 or 6q deletion (6q-) have an intermediate prognosis as they have a better clinical outcome than patients with 17p or 11q deletion, but they have shorter overall survival than those with 13q14 deletion or no cytogenetic abnormalities (Cuneo et al. 2004;Dohner et al. 1995). Patients with p53 mutations have poor clinical outcome and drug resistance (Carter et al. 2004).

1.6.6 V_H genes

There is a three step process for the maturation of a B-cell: the process begins with a naïve B-cell; one that has not yet encountered antigen. The second step occurs mainly in the germinal centre. After encountering antigen, the B-cell travels to the germinal centre where rearrangement or somatic hypermutation occurs in the heavy region of their immunoglobulin genes. The B-lymphocytes are then termed "mutated". During somatic hypermutation, individual nucleotides within the variable (V) region, diversity (D) region or joining (J) region are replaced with alternatives nucleotides in order to increase antibody diversity (Goldsby, Kindt, & Osbourne 2000). The third step is termed the post-mutational stage. A memory B-cell leaves the germinal centre and can either join the circulating B-cells, go to the antigen draining site (e.g. marginal zone of the spleen), or can differentiate into a plasma cell (Stevenson et al. 1998).

On closer inspection of CLL, it was found that the disease could be sub-divided into two prognostic groups, dependent on the mutational status of their cells' immunoglobulin heavy chain variable genes (V_H genes). If there is a difference of 2% or less from the closest germ line sequence, then the cells are said to be "unmutated". The cells are classed as "mutated" when there is a difference of >2% from the closest germ line sequence (Oscier et al. 2002). There is controversy in the literature over this 98% threshold level, as some studies have shown that a 97%, 96% or 95% (Krober et al. 2002;Wiestner et al. 2003) cut-off level are better representatives of the

patients' clinical outcome but usually the 98% threshold level is used clinically. It was generally believed that the mutated cells are more mature cells that have transited the germinal centre, whereas the unmutated lymphocytes, although they have experience antigen and therefore are not naïve, it is thought that they have not transited the germinal centre, so they are not as mature as mutated CLL cells (Dighiero 2003). It is now believed that the unmutated cells do actually transit the germinal centre but for an unknown reason, do not undergo somatic hypermutation and hence fail to rearrange their V_H genes (Stevenson & Caligaris-Cappio 2004). Patients with mutated V_H genes have a better prognosis and longer time to first treatment. In contrast, patients with the unmutated V_H genes have a more progressive disease and poorer clinical outcome (Hamblin et al. 1999;Lin et al. 2002;Vilpo et al. 2003).

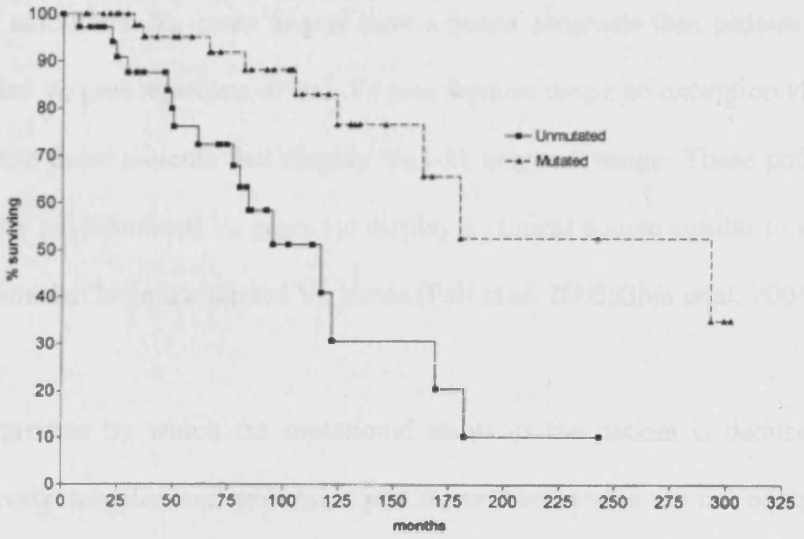


Figure 1.2 Kaplan-Meier curve showing the overall survival for patients with either unmutated V_H gene mutation status or mutated V_H gene mutation status (Hamblin, Davis, Gardiner, Oscier, & Stevenson 1999)
Patients with unmutated V_H genes had a shorter overall survival than the patients with mutated V_H genes.

During B-cell maturation, rearrangement of the variable region occurs, starting with the heavy chain variable region, then the light chain variable region. With the heavy chain region, a D_H gene segment joins to a J_H segment. This resulting D_HJ_H segment then joins with a V_H segment to generate a $V_HD_HJ_H$ segment that encodes the entire variable region (Goldsby, Kindt, & Osbourne 2000). CLL patients appear to use a limited repertoire of V_H gene segments. The most common ones are V_H4-34, V_H1-69 and V_H3-21. The patients can be sub-divided depending on what V_H segment usage they display (how the gene segments are rearranged). The most commonly displayed V_H gene usage is V_H4-34 and these patients usually have a mutated V_H genes and a good prognosis. In contrast, patients that use V_H1-69 usually have unmutated V_H genes and have aggressive disease with short overall survival (Kienle et al. 2006). Although patients with unmutated V_H genes usually have a poorer prognosis than patients with mutated V_H gene regardless of their V_H gene segment usage, an exception to this rule are those patients that display V_H3-21 segment usage. These patients usually have mutated V_H genes but display a clinical course similar to those patients that have unmutated V_H genes (Falt et al. 2005;Ghia et al. 2005).

The process by which the mutational status of the patient is deduced is relatively complex and expensive and many laboratories are not equipped to carry out the technique. As a consequence, there has been considerable interest in identifying a surrogate marker for V_H gene status. A few suggestions have been made including CD38 expression and ZAP-70 expression.

1.6.7 CD38

CD38 (also known as T10) (Ibrahim et al. 2001) is a 45kDa type II transmembrane marker that is involved in cell signalling (Morabito et al. 2001), and has also been linked to proliferation within normal lymphocytes (Zupo et al. 1994). The CD38 gene is found on chromosome 4p15 (Malavasi et al. 1994) and can be expressed in B- and T-lymphocytes, plasma cells, macrophages and erythrocytes (Sun et al. 2006). It is a multifunctional cyclase that is thought to allow extracellular adenosine diphosphate ribosyl cyclase (cADPr) to traverse the cell membrane and activate intracellular ryanodine receptors that release Ca^{2+} into the cytoplasm from endoplasmic reticular stores (Sun et al 2006). cADPR is involved in signalling and has the ability to control calcium levels (Deaglio et al. 2006). The ligand for CD38 is CD31 (also known as platelet endothelial cells adhesion molecule-1 (PECAM-1)) (Ibrahim et al 2001;Morabito et al 2001) a 130kDa molecule that is a member of the immunoglobulin super family and is involved in the modulation of leukocyte adhesion to the vessels (Deaglio et al 2006). It has been shown that CD31/CD38 interactions control an active signalling pathway in lymphocytes (Deaglio et al. 2003). It is thought that CD38 is located in the plasma membrane in close proximity to the BCR and TCR (Deaglio et al 2006).

As well as proliferation, CD38 has been associated with apoptosis, differentiation, T-lymphocyte signalling, neutrophil migration, insulin

secretion and neurotransmission depending on the cell type (Lande et al. 2002;Zupo et al 1994). It has also been shown that CD38 is involved in disease pathophysiology (Sun et al 2006). Up-regulation of CD38 on different T-lymphocyte sub-sets plays a role in the ability of HIV therapies to reconstitute $CD4^+$ cells (Sun et al 2006). Another function of CD38 is that it can induce phosphorylation of CD19 (there is thought to be a physical association between CD38 and CD19) and this prevents apoptosis through up-regulation of the Bcl-2 family proteins (Deaglio et al 2006;Pittner et al. 2005). It has also been shown that CD38 signalling involves c-Cbl which then associates with the p85 sub-unit of PI3K which activates the PI3K pathway (Shubinsky & Schlesinger 1997). The tyrosine phosphorylation of CD19 by CD38 is also associated with Lyn and it is thought that Ca^{2+} flux due to CD38 signalling can only occur when CD19 is activated (Deaglio et al 2006).

CD38 has been shown to be prognostic in CLL (Ghia et al. 2003;Hamblin et al. 2002). There are essentially three types of CD38 expression in CLL cells; entirely positive, entirely negative, or bimodal expression, where the patient has distinct populations of both $CD38^+$ and $CD38^-$ cells (Ghia, Guida, Stella, Gottardi, Geuna, Strola, Scielzo, & Caligaris-Cappio 2003). There is much controversy over the level at which patients that express CD38 are classed as positive (Damle et al. 1999;Ibrahim et al 2001;Krober et al 2002). Suggested "cut-off" levels from previous studies are 7%, 20% and 30% (Damle et al 1999;Ibrahim et al 2001;Thornton et al 2004). However, Ghia *et al* (Ghia et al 2003) found that any presence of $CD38^+$

cells, no matter how small in number, is indicative of poor overall survival. There is also great debate whether the expression levels of CD38 remain constant throughout the course of the disease, or whether the levels fluctuate with disease progression and/or after treatment (Hamblin et al 2002;Ibrahim et al 2001). In this regard, patients who express CD38 appear to respond poorly to treatment (Pittner et al 2005).

1.6.8 ZAP-70

Zeta associated protein-70 (ZAP-70) is a member of the protein tyrosine kinase family (PTK) (Hus et al. 2006). It is found in T-lymphocytes and natural killer (NK) cells and is a member of the signalling pathway that is activated by the T-cell receptor (TCR) (Crespo et al. 2003;Schroers et al. 2005). ZAP-70 is generally considered to be the T-cell equivalent of the BCR-associated protein Syk, which also belongs to the PTK family and is involved in B-lymphocyte signalling (Nolz et al. 2005). ZAP-70 has also been found in normal B-lymphocytes but only those showing a more activated phenotype and it has also been shown to be expressed in B-cell malignancies such as CLL and non-Hodgkin's lymphoma (Nolz et al 2005).

ZAP-70 was first identified in CLL samples following microarray experiments. Importantly, it was shown to be differentially expressed in patients with unmutated V_H genes. These patients showed five times

greater transcription of the gene than the mutated cases (Orchard et al. 2004;Wiestner et al 2003). More recently, ZAP-70 has been shown to enhance the signalling capacity of CLL cells following stimulation of the BCR (Chen et al. 2006;Schroers et al 2005). Previous research groups have suggested that the absence of ZAP-70 rather than its presence might be considered abnormal in CLL B-lymphocytes (Scielzo et al. 2006). In addition, Rassenti *et al* (2004) stated that the presence of ZAP-70 (>20%) was a stronger predictor of the patient needing treatment than V_H gene mutation status.

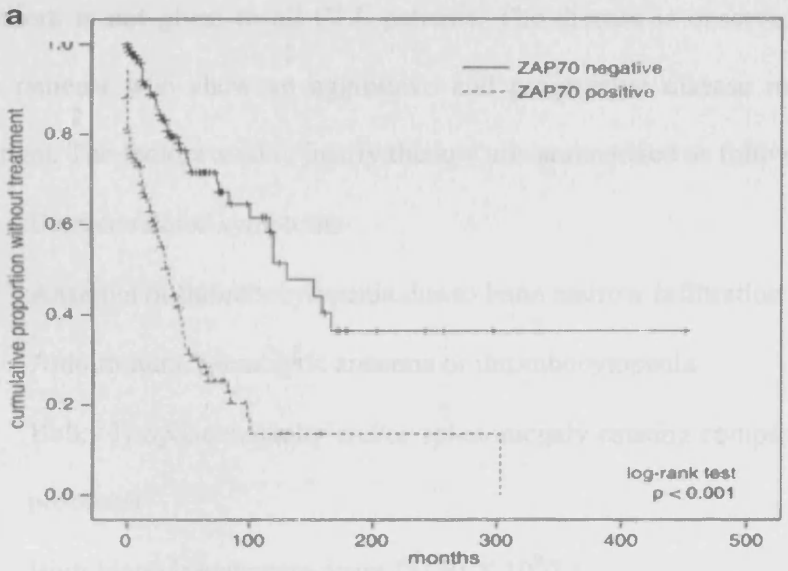


Figure 1.3 Kaplan-Meier curves showing time to first treatment for patients who expressed ZAP-70 and those who were ZAP-70 negative (Schroers, Griesinger, Trumper, Haase, Kulle, Klein-Hitpass, Sellmann, Duhrsen, & Durig 2005).
The patients who had ≥ 20% ZAP-70 expression had a shorter time to first treatment than those patients who had < 20% ZAP-70 expression.

It has been shown that ZAP-70 is a marker for poor prognosis as it is associated with rapid progression, short time to first treatment and poor survival (Durig et al. 2002;Wiestner 2005). It has been suggested that ZAP-

70 expression is a superior marker of prognosis than CD38 expression because it is thought the ZAP-70 expression levels do not change over time and has been shown to have strong predictive power of those CLL patients who will need treatment (Rassenti et al. 2004). However, there is still much controversy about the reliability of the methods used for measuring ZAP-70.

1.7 Treatment

Treatment is not given to all CLL patients. The disease is observed and those patients who show an aggressive and progressive disease receive treatment. The factors used to justify therapy are summarised as follows:

- Disease related symptoms
- Anaemia or thrombocytopenia due to bone marrow infiltration
- Autoimmune haemolytic anaemia or thrombocytopenia
- Bulky lymphadenopathy and/or splenomegaly causing compressive problems
- High blood lymphocyte count (>150 X 10^9/L)
- Rapidly increasing blood lymphocytosis (i.e. LDT <12 months)
- Increased susceptibility to bacterial infections
- Massive lymphocytic infiltration of bone marrow (i.e. diffuse histopathological pattern or >80% lymphocytes in bone marrow aspirate)
- Complex cytogenetic abnormalities
- Advanced clinical stage (Montserrat & Rozman 1994)

If these factors are not displayed then the patient is usually monitored for disease progression but does not receive immediate treatment (Rai 1996). It has been shown that patients with early stage CLL do not display a survival advantage if they receive chemotherapy before they show disease progression (Brugiatelli et al. 2006).

Chlorambucil was the first line treatment for patients with CLL but it was suggested that long term toxicity, for example secondary cancers, was slightly increased in patients that had been treated with chlorambucil. Purine analogues were then produced, such as fludarabine, and these were found to produce higher complete remission rates and quality of life for the patient. Treatment with fludarabine increased the risk of opportunistic infection (Brugiatelli et al 2006).

Monoclonal antibodies were then developed such as Alemtuzimab (anti-CD52) and Rituximab (anti-CD20) (Wierda 2006). Rituximab is used in combination therapies such as FCR (fludarabine, cyclophosphamide and rituximab). This combination therapy showed a higher complete remission and overall response.

1.8 Fludarabine

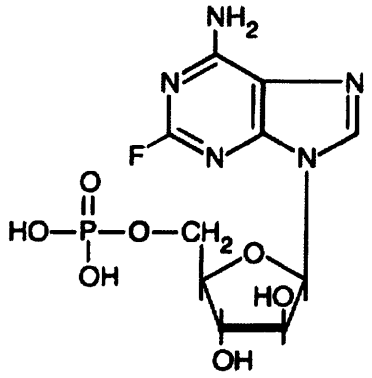


Figure 1.4: Structure of fludarabine (http://www.neuro.wustl.edu/neuromuscular/pics/diagrams/drugs/fludarabine2.jpg)
Fludarabine has been shown to be an effective single agent in the treatment of CLL when considering complete remissions as an end-point (Brugiatelli et al 2006).

Fludarabine (9-β-D-arabinofuranosyl-2-fluro-adenine 5' monophosphate) (Keating et al. 1994) is a purine nucleoside analogue used in the treatment of CLL. Within 5 minutes of intravenous infusion fludarabine monophosphate is dephosphorylated into 9-β-arabinofuranosyl-2-fluoroadenine (F-Ara-A). Once transported into the cell, F-Ara-A is converted into its active state F-Ara-A triphosphate (F-Ara-ATP) (Ross, McTavish, & Faulds 1993). Purine analogues promote apoptosis by inhibiting DNA polymerase and ribonucleotide reductase. Fludarabine can also inhibit transcription by its ability to be incorporated into RNA as well as DNA. The major side effects of fludarabine are myelosuppression, haematological and immunological toxicities, autoimmune haemolytic anaemia and at very high doses, there is significant neurotoxicity. When used in combination chemotherapy such as FC (Fludarabine and cyclophosphamide), the side effects are alopecia, vomiting, diarrhoea and cardiac toxicity (Dighiero & Binet 2000).

Fludarabine was originally administered in an intravenous form. However, an oral form of fludarabine has been recently introduced (a 10mg tablet) that has been found to be a more convenient way to administer the therapy. Previous studies have shown that fludarabine induced complete or partial remission in most CLL patients (Ross, McTavish, & Faulds 1993). Montserrat and Rozman found that 83% of previously untreated patients responded to treatment with fludarabine and 75% achieved complete remission, whereas in previously treated patients there was a response rate of 57% with only 29% achieving complete remission (Montserrat & Rozman 1994).

Recently fludarabine has been used in combination therapy such as FC (fludarabine and cyclophosphamide) and most recently FCR (fludarabine, cyclophosphamide and rituximab). FCR therapy has been shown to have an improved complete response rate with the German CLL study group finding that this combination therapy produced a response rate of 95% and a complete response rate of 71% (Hallek 2005). Similarly Keating *et al* also found that treatment with FCR had a complete response rate of 70% (Brugiatelli et al 2006;Keating et al. 2005).

1.9 Apoptosis

There are two main pathways by which apoptosis is induced; the intrinsic pathway and the extrinsic pathway. These two pathways are in no way mutually exclusive as they interact at various points throughout both

pathways. They initiate apoptosis via the activation of specific proteases known as caspases (cysteine proteases with aspartate specificity), which exist as zymogens in the cytosol and become activated upon cleavage.

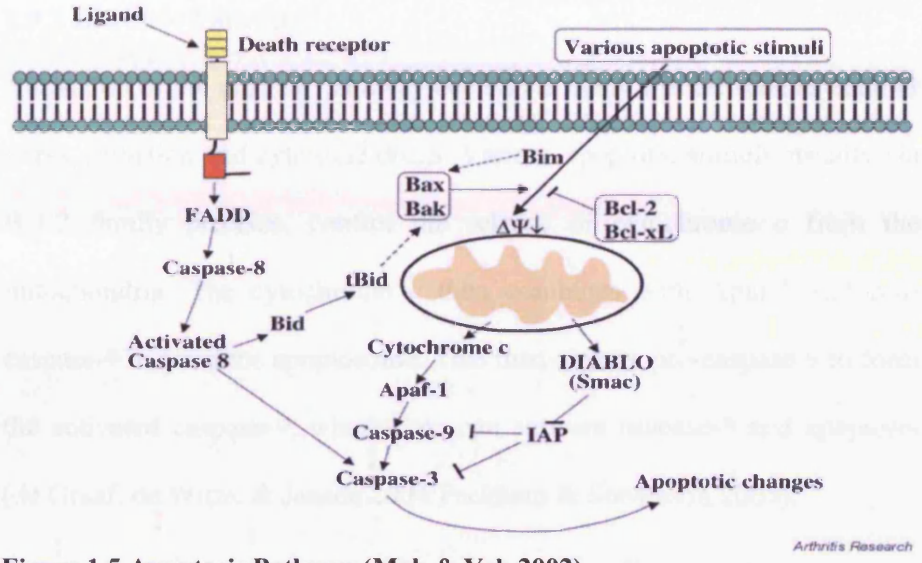


Figure 1.5 Apoptosis Pathway (Mak & Yeh 2002)
The extrinsic pathway and intrinsic pathway are the pathways that lead to apoptosis. They are not independent pathways as they interact via the apoptotic protein Bid.

1.9.1 Extrinsic Pathway

The extrinsic pathway is activated by cell-surface death receptors, such as the Fas receptor (CD95). CD95 contains a death domain in the receptor tail which allows protein-protein interactions. The death receptor recruits intracellular adaptor proteins that also contain a death domain i.e. Fas contains a death domain which can bind to both Fas-associated death domain (FADD) protein and receptor-interacting protein (RIP) (Mak & Yeh 2002). FADD then interacts with caspase-8, and through aggregation and close proximity caspase-8 is cleaved thereby activating it. Once activated, caspase-8 can either activate caspase-3 and hence apoptosis, or it

can activate Bid and initiate apoptosis via the intrinsic pathway (Packham & Stevenson 2005).

1.9.2 Intrinsic Pathway

Activation of the intrinsic pathway occurs via many stimuli such as cellular stress, radiation and cytotoxic drugs. Various apoptotic stimuli, usually via Bcl-2 family proteins, control the release of cytochrome *c* from the mitochondria. The cytochrome *c* then combines with Apaf-1 and pro-caspase-9 to form the apoptosome. This then cleaves pro-caspase-9 to form the activated caspase-9, which then can activate caspase-3 and apoptosis (de Graaf, de Witte, & Jansen 2004;Packham & Stevenson 2005).

1.10 Bcl-2 family

Bcl-2 family proteins are key molecules in the control of apoptosis. There are at least 20 family members that have been identified and they can be divided into 3 groups dependent on their structure and function (Schimmer et al. 2003). Group I are the anti-apoptotic proteins which include Bcl-2 and Bcl-X_L and they have four Bcl-2 sequence homology (BH) domains, BH1, BH2, BH3 and BH4 (Packham & Stevenson 2005). Mcl-1 (myeloid cell leukaemia-1) is also a Group I protein but does not contain the BH4 domain (Packham & Stevenson 2005). Bax, Bok and Bak are the pro-apoptotic members of Group II and have sequence homology at BH1, BH2 and BH3 domains and hence are termed multidomain pro-apoptotic proteins (Packham & Stevenson 2005). Group III only share the BH3

domain and include the pro-apoptotic proteins Bid, Bik, Noxa and Bim (Bellosillo et al. 2002;Cory, Huang, & Adams 2003).

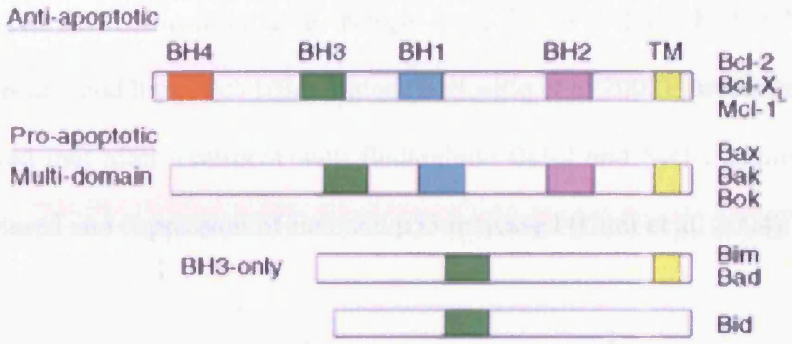


Figure 1.6: Structure of the Bcl-2 family members (Packham & Stevenson 2005)
Group I (anti-apoptotic), Group II (pro-apoptotic) and Group III (BH3 only) members of the Bcl-2 family. The Bcl-2 homology (BH) and transmembrane (TM) domains are indicated for each group. These BH domains are short sequence motifs less than 20 amino acids in length. The TM domain is used in the targeting of the proteins to the mitochondrial membrane.

Bax, Bid and Bad are usually found in the cytoplasm whereas Bcl-2, Bcl-X_L and Bak are usually associated with the mitochondrial membrane (Bellosillo et al 2002). Bax is translocated to the mitochondrial membrane on the induction of cell death (Dewson et al. 2003). When this translocation occurs Bax undergoes a conformational change that exposes the NH_2-terminus and the COOH-terminus that target the mitochondria. Bak is also an important protein which can also release cytochrome *c* from the mitochondria, and conformational changes or up-regulation are thought to be necessary for the induction of apoptosis (Bellosillo et al 2002).

Within CLL cells there are high levels of Bcl-2 (Bairey et al. 2001;Hanada et al. 1993;Packham & Stevenson 2005;Pepper et al. 1999) and increased Bcl-2/Bax ratios have been associated with clinical outcome and patients

with low Bcl-2/Bax ratios respond better to chemotherapy than patients with high Bcl-2/Bax ratios (Bannerji et al. 2003;Moshynska et al. 2004;Pepper, Hoy, & Bentley 1997). Patients who failed to achieve complete remission after drug therapy were found to have higher Mcl-1 expression and high Mcl-1/Bax ratios (Bellosillo et al 2002). Investigations showed that after treatment with fludarabine Bcl-2 and Mcl-1 expression decreased and expression of Bax and p53 increased (Cuni et al. 2004).

1.11 Survival pathways

Many survival pathways are constitutively activated in CLL cells. Cuni *et al* (Jiang et al. 2000) showed that NF-κB is constitutively active in CLL lymphocytes and this may lead to activation of either the MAP kinase pathway or the phosphatidylinositol-3 kinase (PI3K) pathway. It is thought that dysregulation of these survival pathways leads to failed apoptosis and therefore contributes to the accumulation of CLL lymphocytes.

1.11.1 PI3 Kinase pathway

Insulin and many other growth factors can activate the PI3 kinase pathway. PI3 kinase catalyzes the phosphorylation of inositol phospholipids at the 3 position to generate phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate (Vara et al. 2004). The PI3Ks are heterodimers consisting of a catalytic subunit, p110, and a regulatory subunit, p85 (Jiang, Zheng, Aoki, & Vogt 2000). This pathway is involved

in cell adhesion, vesicular trafficking, protein synthesis and cell survival (Waite & Eng 2002).

1.11.2 PTEN

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a 54kDa protein and a member of the protein tyrosine phosphatase family. PTEN plays a major role in the control of the PI3K pathway by de-phosphorylating PIP_3 at the 3' position, which is essential for translocation of Akt to the plasma membrane for activation (Leslie & Downes 2004). With PIP_3 deactivated, Akt activation is reduced and therefore downstream signalling is also reduced. PTEN has a relatively high constitutive activity as it has been shown to lack obvious regulatory domains (Leupin et al. 2002). In CLL there has been shown complete loss of heterozygosity at 10q23.3 and allelic imbalances in PTEN expression which lead to loss in PTEN protein expression (Ballif et al. 2001).

1.11.3 PDK1

PDK1 (3-phosphoinositide-dependent kinase 1) is a serine/threonine kinase which is involved in the phosphorylation and activation of the AGC kinase super family (named after the family members protein kinase A, protein kinase G and protein kinase C) (Gao & Harris 2006) by phosphorylation of threonine (Thr) in the conserved activation loop region (Ballif et al 2001;Feldman et al. 2005). These include Akt/PKB, protein kinase C (PKC), PKC-related kinases (PRK1 and PRK2), p70 ribosomal S6-kinase (S6K) and serum and glucocorticoid-related kinase (SGK) (Balendran et al.

1999). It has also been suggested that when PDK1 interacts with PIF (PDK1 interacting fragment) it can also phosphorylate Akt at Serine 473 (Carver, Aman, & Ravichandran 2000).

The phosphorylation activity of PDK1 is often dependent on the presence of PIP3. PIP3 has a pleckstrin homology (PH) domain, which PDK1 also possesses(Piccolo et al. 2004). PDK1 binds to PIP3 and is translocated to the plasma membrane where it can phosphorylate Akt on threonine 308 (Thr308) (Balendran et al 1999). PDK1 can phosphorylate certain molecules independently of PIP3. One such molecule is S6K which is downstream of mTOR and is involved in protein synthesis (Balendran et al 1999). PDK1 can phosphorylate S6K at Thr 252 and it has also been suggested that it can phosphorylate Thr 412 as well (Hay 2005).

1.11.4 Akt

Akt is an important member of the PI3K pathway and its activation is associated with resistence to apoptosis, increased cell growth and cell proliferation (Kawakami et al. 2004). It has a catalytic domain at its C-terminal and a pleckstrin homology domain at its N-terminal (Cuni et al 2004). Akt is activated by phosphorylation at two sites, threonine 308 (Thr308) and serine 473 (Ser473) (Kawakami et al 2004). Is it firstly phosphorylated on Thr308 in the activation loop of the kinase domain by PDK1 and then phosphorylated on Ser473 in the C-terminal hydrophobic motif by an unknown molecule that has been termed PDK2 (Bayascas & Alessi 2005;Feng et al. 2004). Phosphorylation at both sites is needed for

activation of the Akt molecule. Suggested molecules for the elusive PDK2 have been mTOR-rictor, PDK1 and Akt itself (Falt et al 2005;Hay 2005). Once activated, Akt can phosphorylate over 9000 proteins (Barragan et al. 2002). One of these molecules is the apoptotic protein Bad (Datta et al. 1997;Downward 2004). Akt phosphorylates Bad at Ser 136 preventing it from binding to the anti-apoptotic protein Bcl-2 and inactivating it, therefore the cell is protected from apoptosis (Franke et al. 2003).

Yano *et al* (1998) described a PI3K independent activation of Akt and its survival responses. They explain that Ca^{2+}/calmodulin-dependent kinase kinase (CaM-KK) can phosphorylate Akt on Thr 308 residue independently of the PI3K pathway (Cuni et al 2004). Akt was found to be constitutively active within CLL lymphocytes providing an explanation to why CLL cells have an abnormally long life-span (Petroulakis et al. 2006).

1.11.5 mTOR

Mammalian target of rapamycin (mTOR) (also known as RAFT (rapamycin and FKBP12 target) or FRAP (FKBP12-rapamycin associated protein)) (Hay & Sonenberg 2004;Petroulakis et al 2006) is a very large protein which belongs to the family of Phosphoinositide kinase related kinases (PIKKs). It is involved in the control of translation by phosphorylating and activating S6K and 4E, an eukaryotic initiation factor (Guertin & Sabatini 2005). mTOR phosphorylates and activates S6K which in turn can activate S6 ribosomal protein. Phosphorylation of 4E-BP by mTOR allows 4E to become free to bind to the cap structure of mRNA as a

part of the translation initiation complex (Hay 2005). There are at least 2 distinct complexes in which mTOR can exist in: mTOR-Raptor (regulatory-associated protein of mTOR) which is rapamycin sensitive, and mTOR-rictor (rapamycin-insensitive companion of mTOR) which is rapamycin insensitive (Hay 2005). It is mTOR-rictor that has been put forward as the elusive "PDK2" which phosphorylates Akt on Ser-473 residue and it is the mTOR-raptor that is involved in the phosphorylation of S6K and 4E-BP, and has also been indicated in a negative feedback loop with Akt (Petroulakis et al 2006).

1.11.6 S6K and S6

S6K is involved in the control of different steps of protein synthesis, cell growth and storage of amino acids. It has also been associated with cell cycle regulation. S6K directly phosphorylates insulin receptor substrate 1 (IRS-1) which inhibits PI3 kinase and Akt activation. Through this negative feedback loop, S6K can regulate mTOR (Petroulakis et al 2006). S6 activation has been linked to enhanced translation of TOP mRNAs (mRNA that contains a terminal 5' oliopyrimidine tract) which encode ribosomal proteins, elongation factors and many other factors that are involved in translation in response to growth factors.

1.12 Project aims and objectives

CD38 has been shown to be an important prognostic factor within CLL, but to date there is no biological rationale for why CD38 is associated with poor prognosis. Therefore, the aims of this present study were as follows:

- Determine whether CD38 is prognostic in our cohort of CLL patients
- Establish whether CD38 expression changes over time in CLL patients
- Isolate purified $CD38^+$ and $CD38^-$ cells from the same CLL patient and establish whether they have a distinct transcriptional signature?
- Investigate whether $CD38^+$ cells regulate the PI3K pathway differently to $CD38^-$ cells?
- Determine whether $CD38^+$ cells regulate their response to apoptotic signals differently when compared to $CD38^-$ cells?

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Primary antibodies

Antibody	Clone	Volume (µl)	Company	Catalogue No.
Akt		2.0	Cell Signaling Technologies via New England Biolabs, Ipswich, MA, USA	9272
Akt-1	B-1	5.0	Santa Cruz Biotechnology, CA, USA	sc-5298
Bax-FITC	B-9	7.0	Santa Cruz Biotechnology	sc-7480
Bcl-2-FITC	124	7.5	Dako Cytomation, Ely, UK	F7053
β-actin		2.0	Cell Signaling Technologies	4967
CD19-APC	SJ25-C1	4.0	Caltag Laboratories, Botolph Claydon, UK	MHCD1905
CD38-RPE	HIT2	4.0	Caltag Laboratories	MHCD3804
IL-1β	H-153	4.0	Santa Cruz Biotechnology	sc-7884
Ki67	MIB-1	8.0	Dako Cytomation	F7268
Mcl-1	S-19	7.5	Santa Cruz Biotechnology	sc-819
PDK1		2.0	Cell Signaling Technologies	3062
Phospho-Akt1/2/3 (Ser 473)		5.0	Santa Cruz Biotechnology	sc-7985-R
Phospho-Akt 1/2/3 (Thr 308)		5.0	Santa Cruz Biotechnology	sc-16646-R
Phospho-Akt (Ser 473)		2.0	Cell Signaling Technologies	4051S
Phospho-		2.0	Cell Signaling Technologies	9275L

Akt (Thr 308)				
Phospho-S6 ribosomal protein (Ser235/236)		2.0	Cell Signaling Technologies	2211L
p-Tyr	PY20	8.0	Santa Cruz Biotechnology	sc-508
S6 Ribosomal Protein		2.0	Cell Signaling Technologies	2212
VEGF	C-1	4.0	Santa Cruz Biotechnology	sc-7269
VEGFR2 (Flk-1)	A-3	4.0	Santa Cruz Biotechnology	sc-6251
ZAP-70-Alexa Fluor 488 conjugate		4.0	Caltag laboratories	MHZAP7020

Table 2.1: Primary Antibodies. Details of supplier, catalogue number, clone and experimentally-determined titration of each antibody.

2.1.2 Secondary antibodies

Antibody	Volume (µl)	Company	Catalogue No.
ECL anti-mouse IgG_1, peroxidase linked species-specific whole antibody (from sheep)	1.0	Amersham Biosciences	NA931
ECL anti-rabbit IgG_1, peroxidase linked species-specific whole antibody (from donkey)	1.0	Amersham Biosciences	NA934
Polyclonal rabbit anti-mouse immunoglobulins/FITC	7.5	Dako Cytomation	F0313
Polyclonal swine anti-rabbit immunoglobulins/FITC	7.5	Dako Cytomation	F0054

Table 2.2: Secondary antibodies. Details of supplier, catalogue number and experimentally-determined titration of each antibody.

2.1.3 Amersham Biosciences, Buckinghamshire, UK

- ECL advance™ enhanced chemiluminescence western blotting detection kit. Catalogue number: RPN2135
- Hyperfilm™ ECL high performance chemiluminescence film 18 x 24cm 75
- sheets. Catalogue number: RPN3103K

2.1.4 BioRad, Hemel, Hempstead, UK

- Quick Start™ Bradford dye reagent (1X). Catalogue number: 500-0205
- Quick Start™ Bovine serum albumin (BSA) 2mg/ml. Catalogue number: 500-0206

2.1.5 Calbiochem via Merck Biosciences Ltd., Nottingham, UK

- SU1498 VEGFR2 inhibitor 5mg. Catalogue number: 572888

2.1.6 Cell Signaling Technologies via New England Biolabs, Ipswich, MA, USA

- Jurkat extracts Calyculin A treated. Catalogue number: 9273
- LY294002 treated Jurkat Extracts. Catalogue number: 9273

2.1.7 Dako Cytomation, Ely, UK

- Polyclonal rabbit anti-human IgM 1.0mg/ml Catalogue number: A0426

- Intrastain: Fixation and permeabilisation kit for flow cytometry. Catalogue number: K2311

2.1.8 Invitrogen, Paisley, UK

- Heat inactivated foetal calf serum (FCS). Catalogue number: 10108-157
- Invitrolon™ PVDF filter paper sandwich 0.45µm pore size. Catalogue number: LC2005
- MagicMark™ Western protein standards. Catalogue number: LC5600
- MultiMark™ Multi-coloured standards (1X), 500µl. Catalogue number: LC5725
- NuPAGE™ 4-12% Bis-Tris gels 1.0mm x 10 wells. Catalogue number: NP0321BOX
- NuPAGE™ Antioxidant 15ml. Catalogue number: NP0005
- NuPAGE™ LDS sample buffer (4X) 10ml. Catalogue number: NP0007
- NuPAGE™ MOPS SDS running buffer (20X) 500ml Catalogue number: NP0001
- NuPAGE™ sample reducing agent (10X) 10ml. Catalogue number: NP0009
- NuPAGE™ transfer buffer (20X) 1L. Catalogue number: NP0006-1

2.1.9 Qiagen, Crawley, West Sussex, UK

- QIAamp DNA blood midi kit. Catalogue number: 51183

2.1.10 Sigma Aldrich, Poole, Dorset, UK

- Ammonium chloride. Catalogue number: A-4514
- Histopaque-1077. Catalogue number: 10771-500ml
- Mammalian Cell Lysis Kit. Catalogue number: MCL-1
- Monoclonal anti-vascular endothelial growth factor, antibody produced in mouse. Catalogue number: V4758-5MG
- Paraformaldehyde. Catalogue number: P-6148
- Phosphate buffered saline tablets, 100 tablets. Catalogue number: P-4417
- Polyoxyethylenesorbitan Monolaurate (Tween 20). Catalogue number: P-1379
- RPMI-1640 Medium Sterile filtered with L-glutamine and $NaHCO_3$. Catalogue number: R8758
- Sodium chloride. Catalogue number: S-5886
- Trizma hydrochloride, SigmaUltra, >99% titration. Catalogue number: T6666-250G
- Vascular endothelial growth factor 121, human recombinant. Expressed in E-coli. Catalogue number: V3388-5UG

2.1.11 Thermo Electron Corporation, Cambridge, United Kingdom

- Finntip5ml sterilized 5 x 54 pcs/rack. Catalogue number: 9402073
- Finntip 10 10 x 96 pcs/rack. Catalogue number: 9400300
- Finntip 250 universal sterilized 10 x 96 pcs/rack. Catalogue number:9400263

- Finntip 1000 sterilized 10 x 96 pcs/rack. Catalogue number: 9401113

2.1.12 Fludarabine was kindly donated by Dr Christopher Fegan, Department of Haematology, Llandough Hospital, Penlan Road, Penarth, Cardiff, UK.

2.2 Equipment

Agilent bioanalyzer (Agilent Technologies UK Limited)

Beckman Coulter Z2 Cellular Counter (Beckman Coulter)

DNA Thermo Cycler (ABI)

FACScan (Becton Dickinson)

FACSCalibur (Becton Dickinson)

MoFlo high speed cell sorter (Dako Cytomation)

2.3 Methods

In a number of the kits that were used in these experiments the exact composition of the different solutions were not provided by the manufacturers.

2.3.1 Patients and samples

Ethical approval for this project was obtained from Bro Taf Local Research Ethics Committee panel B (# 02/4806). Peripheral blood was obtained from unselected patients attending outpatient clinics at Llandough Hospital and Birmingham Heatlands Hospital with their informed consent. None of the

patients who had previously undergone chemotherapy had received treatment within 3 months of their study sample being taken.

2.3.2 Isolation of lymphocytes

Lymphocytes were separated by density centrifugation on Histopaque-1077 (300 x g for 30 minutes). This is a polysucrose (5.7g/dl) and sodium diatrizoate (9.0g/dl) solution which is adjusted to a density gradient 1.077g/cm^3. This allows the erythrocytes and granulocytes to sediment whilst trapping mononuclear cells (including lymphocytes) at the plasma-histopaque interface. The lymphocytes were aspirated into a sterile centrifuge tube, to which phosphate buffered saline (PBS) was added, and the cells were spun at 300 x g for 10 minutes. Subsequently, 0.87% ammonium chloride was added to lyse any contaminating erythrocytes and spun for 10 minutes at 300 x g. The cells were washed again in PBS and centrifuged again at 300 x g for 10 minutes. This washing step was then repeated and the numbers of lymphocytes were counted using a Beckman Coulter Z2 Cellular Counter. The cells were subsequently aliquoted (1 x 10^6 cells) into 5ml Falcon tubes.

2.3.3 Determination of V_H gene mutation status

The BIOMED-2 procedure was carried out as described previously. (Matthews *et al.* 2004) This procedure was carried out by the Molecular Diagnostics Department of the Department of Haematology. The key elements of this procedure are outlined in the subsequent sections.

2.3.3.1 DNA extraction

DNA was extracted using QIAamp DNA blood midi kit. In a 15ml centrifuge tube, 2ml of white blood cells (2 X 10^6 cells) was added to 200µl of QIAGEN protease and was mixed briefly. To this 2.4ml of Buffer AL was added and the solution was mixed thoroughly by vortexing. The solution was incubated at 70°C for 10 minutes. Ethanol (2ml) was added to the sample and mixed by vortexing. Half the solution was added to the QIAamp midi column and this was centrifuged at 1850 x g for 3 minutes. The flow-through was discarded, and the rest of the solution was added to the column which was spun at 1850 x g for 3 minutes. The filtrate was discarded. 2ml of Buffer AW1 was added to the QIAamp midi column and this was centrifuged at 4500 x g for 1 minute. 2ml of Buffer AW2 was then added to the QIAamp midi column and this was centrifuged at 4500 x g for 15 minutes. The flow-through and collection tube was discarded and the QIAamp midi column was placed in a fresh 15ml centrifuge tube. 300µl of Buffer AE was pipetted directly onto the membrane of the QIAamp midi column, and with the cap closed, it was incubated at room temperature for 5 minutes and then centrifuged for 5 minutes at 4500 x g. To obtain maximum DNA concentration, 300µl of eluate containing the DNA was reloaded onto the QIAamp midi column and was incubated at room temperature for 5 minutes. It was then centrifuged at 4500 x g for 5 minutes. The DNA was kept for further down stream assays such as polymerase chain reaction.

2.3.3.2 Polymerase Chain Reaction (PCR)

The DNA previously extracted was then used in a multiplexed PCR reaction with the BIOMED-2 primers.

V_H1-FRI	5' GGCCTCAGTGAAGGTCTCCTGCAAG 3'
V_H2-FRI	5' GTCTGGTCCTACGCTGGTGAAACCC 3'
V_H3-FRI	5' CTGGGGGGTCCCTGAGACTCTCCTG 3'
V_H4-FRI	5' CTTCGGAGACCCTGTCCCTCACCTG 3'
V_H5-FRI	5' CGGGGAGTCTCTGAAGATCTCCTGT 3'
V_H6-FRI	5' TCGCAGACCCTCTCACTCACCTGTG 3'
J_H consensus	5' CCAGTGGCAGAGGAGTCCATTC 3'

Table 2.3: Sequences of BIOMED-2 primers

A 50µl solution was made up of 0.5µg of DNA sample, 10 pmol of each primer, 2 nM of dNTPs (deoxy nucleotide triphosphates), 1 U AmpliTaq Gold and 10X PCR buffer II. The DNA Thermo Cycler (ABI) was used as follows: denaturation at 94°C for 15 minutes; 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds; and a final cycle of 10 minutes at 72°C.

2.3.3.3 Analysis

The PCR products were analysed on the Agilent bioanalyzer. The products were then sequenced directly using 3' J_H consensus primer in an automated ABI Prism 3100 genetic analyser using Big-Dye terminators. Comparison of the derived sample sequence was made with germline sequences stored

on the Ig Blast database (http://www.ncbi.nlm.nih.gov/igblast/) and the percentage sequence homology to the closest germline sequence was determined. Both the percentage sequence homology and the V_H gene segment usage for each patient sample were noted.

2.3.4 Cell culture

Cells were cultured in RPMI-1640 Medium supplemented with penicillin (10,000 units/ml), streptomycin (10mg/ml) and 10% foetal calf serum (FCS). These cells were then incubated at 37°C, in 5% CO_2 for a fixed period of time.

2.3.4.1 Drug sensitivity

The cells were set up in culture as described in 2.3.4 and the purine nucleoside analogue drug, fludarabine, was added at final concentrations of 2μM – 8μM. In addition, control cultures were set up without the addition of fludarabine. The samples were incubated at 37°C for 48 hours, after which the cells were spun at 300 x g for 5 minutes and used in a number of downstream assays.

2.3.4.2 Inhibitors

Lymphocytes were set up in culture as described in 2.3.4 and inhibitors were added as in Table 2.1. Control cultures contained no inhibitor.

Inhibitor	*Concentration Added*
SU1498	10μM
Monoclonal anti-VEGF	1μg/ml

Table 2.4: Type of inhibitors added to the cultures
The name and type of inhibitor is given in this table along with the concentrations used.

The cells were incubated for 24 hours at 37°C. They were then harvested by centrifugation and analysed by flow cytometry.

2.3.4.3 Recombinant VEGF

The cells were set up as described in 2.3.4 and cultured for 24h with or without 100ng/ml recombinant VEGF. The cells were then used in subsequent phenotyping experiments (2.3.5) and apoptosis assays (2.3.8)

2.3.5 Flow cytometry

Flow cytometry enables the discrimination of distinct cellular populations based on differential light scatter and fluorescence characteristics. The process of flow cytometry involves focusing cells into a unicellular stream of fluid and passing the stream through a laser beam. With early flow cytometry systems there were great problems with large cells or clumps of cells blocking the capillary fluidics system. In modern day flow cytometry systems this is overcome by the principles of laminar flow (The Bernoulli effect) and hydrodynamic focusing. The Bernoulli effect results in the cells remaining in the centre of the fluid as this is where there is least pressure and the velocity is greater. Velocity is slower at the edges as there is viscous drag along the walls of the tubing. Hydrodynamic focusing

involves introducing cells in suspension through a large tube with a larger tube around it containing sheath fluid. By reducing the size of the tube, and maintaining laminar flow, a unicellular stream of cells can be produced (Figure 2.1A).

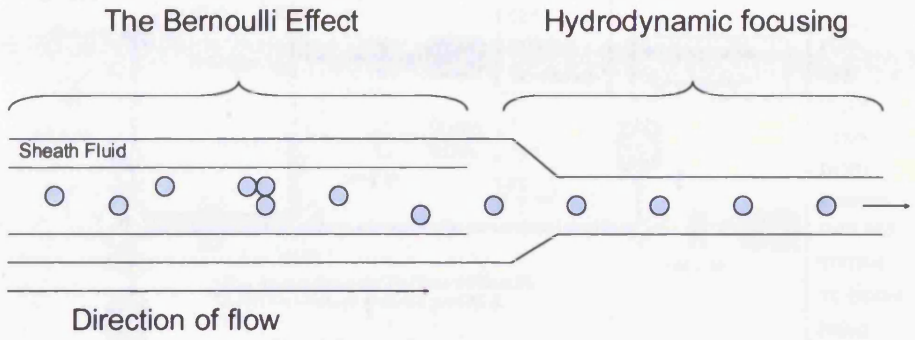


Figure 2.1A: Principles of flow cytometry (http://www.cardiff.ac.uk/medicine/haemotology/cytonetuk/introduction_to_fcm/fluidics.htm)
By combining the principles of laminar flow and the Bernoulli Effect a stream of cells can be produced with reduced risk of blocking by large cells and clumps.

The cells are then passed through a laser beam. As the cell passes through the beam there is absorption, diffraction, refraction and reflection of light as well as fluorescence which is the emission of longer wavelengths following excitation and relaxation of electron states. The characteristics of the cell can be determined by the amount of light scatter and fluorescence identified by a range of detectors (Figure 2.1B). Differential forward light scatter informs on the size of the cell and the amount of side scatter indicates the granularity of the cell. A photomultiplier tube (PMT) detects the fluorescence emitted from the cell and converts it into electrical pulses. Different filters are used to discriminate between the different wavelength fluorescent signals e.g. FL1 – fluorescein isothiocyanate (FITC), FL2 – R-

phycoerythrin (PE) and FL4 – allophycocyanin (APC). Computer software is used to analyse the electrical pulses.

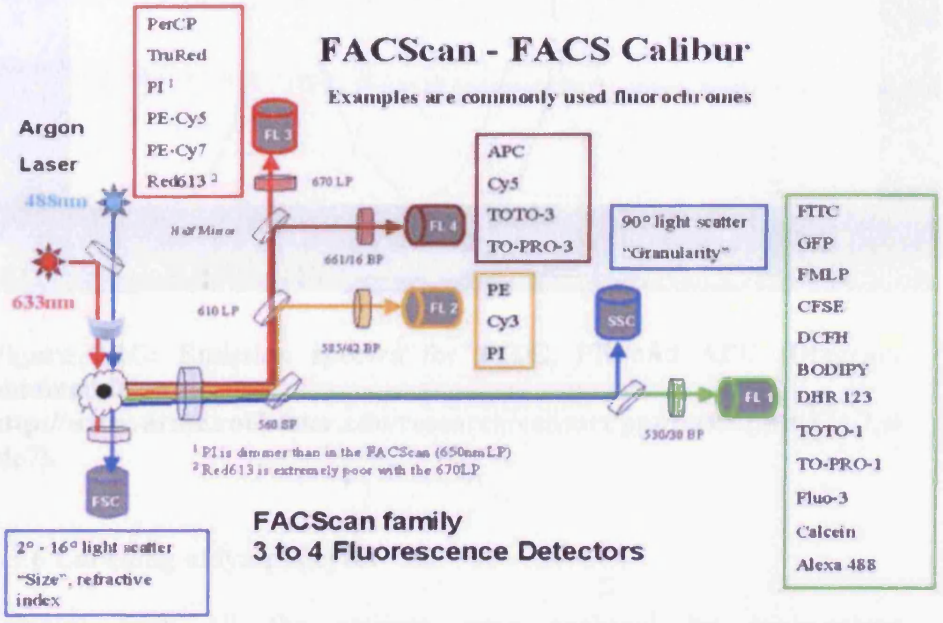


Figure 2.1B: Basic optical layout of a FACS Calibur flow cytometer
Diagram of the layout of the lasers and the flourochromes that can be quantified by each detector. This diagram was produced by The Scripps Research Institute and was found at www.facs.scripps.edu/facslab.html.

The emission maxima for the fluorochromes that were routinely used in this study are; FITC 530nm, PE 570nm and APC 650nm (Figure 2.1C). The emission spectra for the 3 fluorochromes have very little overlap, so little compensation needs to be carried out. If there was a great amount of overlap then compensation need to be carried out in which the computer applies an algorithm to define the overlap between the 2 fluorescent signals.

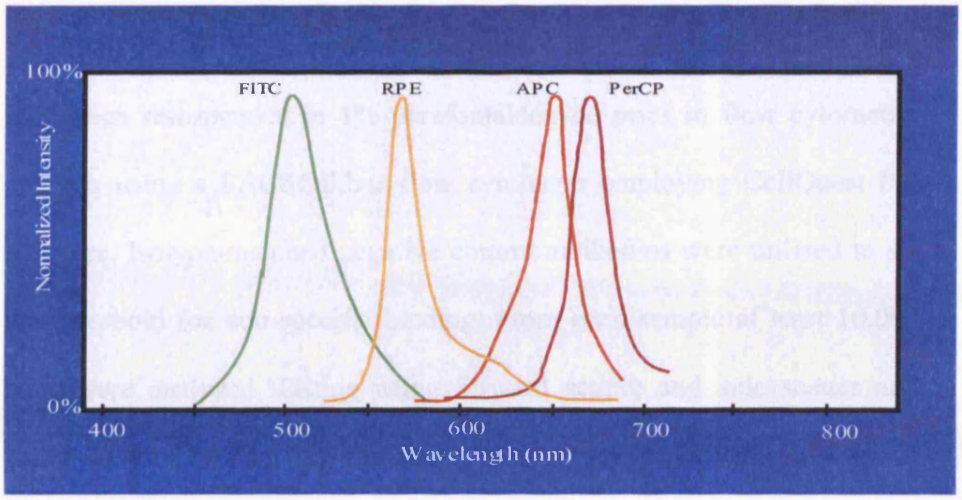


Figure 2.1C: Emission spectra for FITC, PE and APC (Diagram obtained from http://www.urmc.rochester.edu/research/cellsort/ppt/basic.ppt#274,7,slide7).

2.3.6 Labelling of lymphocytes

Samples from all the patients were analyzed by triple-colour immunofluorescent staining using a combination of the protein under investigation in conjunction with CD38 and the pan B-cell marker CD19. Isotype-matched controls were also set up for each antibody. 1 x 10^6 cells were incubated with 4µl of CD38 R-phycoerythrin (PE) conjugated antibody and 4 µl of CD19 Allophycocyanin (APC) conjugated antibody. The cells were then fixed using a commercially available kit (caltag) and resuspended in permeabilisation solution together with titration-determined volumes of each antibody. Briefly, 50µl of Solution A was added to the samples and they were incubated in the dark for 15 minutes. The cells were washed in PBS and spun at 300 x g. Solution B (50ml) was added to the cells along with titration-determined volumes of the desired antibody, incubated in the dark for 15 minutes and then washed in PBS and spun at 300 x g. A fluorescein isothiocyanate (FITC) labelled secondary antibody

was added to all the samples that were not pre-conjugated and the cells were then resuspended in 1% parafomaldehyde prior to flow cytometric analysis using a FACSCalibur flow cytometer employing CellQuest Pro software. Isotype-matched negative control antibodies were utilized to set the threshold for non-specific binding. From each sample at least 10,000 cells were analyzed. Gating using forward scatter and side scatter and gating of the $CD19^+$ cells ensured that protein expression was quantified in the viable B-lymphocyte population. The mean fluorescent intensity (MFI) was calculated for each protein using WinMDI software (J. Trotter, Scripps Research Institute, La Jolla, CA) (Figure 2.2)

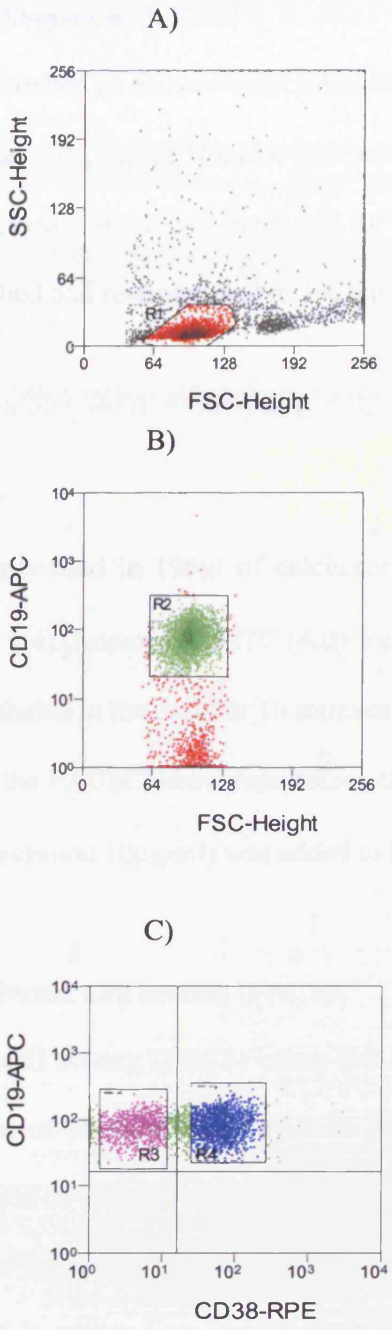


Figure 2.2: Analysis of FACSCalibur data.
These three dot plots show the gating strategy employed throughout the investigation. A) Gating of the total viable lymphocyte population (R1-red). B) Gating of the $CD19^{+}$ B-lymphocyte population only (R2-green). C) Gating of $CD38^{+}$ (R4-blue) and $CD38^{-}$ (R3-purple) cells from the viable B-lymphocyte population.

2.3.7 Labelling of lymphocytes with Ki-67

The lymphocytes were labelled as above except when the permeabilisation solution was added to the cells, 1µl of Nonidet P40 was also added, along with the Ki-67-FITC antibody, and it was incubated for 15 minutes at 4°C. The cells were then washed and re-suspended in 1% paraformaldehyde and analysed as above.

2.3.8 Annexin V Assay

1 x 10^6 cells were resuspended in 196µl of calcium-rich binding buffer (which had been diluted 1:4). Annexin V-FITC (4µl) was then added to the cells, and they were incubated in the dark for 10 minutes. Within 1 hour the cells were analysed on the FACSCalibur. Just before the analysis, 8µl of propidium iodide (concentration 10µg/ml) was added to the cells.

2.3.9 Fluorescence Activated Cell sorting (FACS)

Fluorescence activated cell sorting (FACS) using the MoFlo high speed cell sorter is the process of physically sorting cells into defined groups. The MoFlo uses the stream in air method where the nozzle is vibrated with a transducer which helps the stream to break up into droplets. The flow of sample and sheath fluid is adjusted so that the cells can be placed into droplets separated by droplets not containing any cells. The sheath fluid is usually PBS, and the stream is electrically charged just before the droplet separates from the stream which allows three populations to be selected after passing through the electronic field (positive, negative or neutral). Three droplets are sorted per cell allowing the cell to be in adjacent drops

to that expected, and the sort is aborted electronically if this set of droplets is followed directly by another cell as this helps to avoid contamination with the unwanted cells. By increasing the number of droplets per cell the purity can be improved and by over-riding the abort mechanism, the recovery is increased (Figure 2.3).

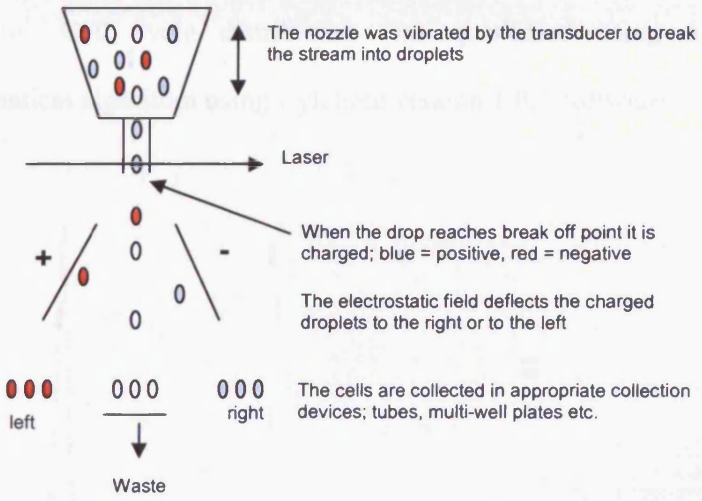


Figure 2.3: Principles of MoFlo high speed cell sorting.
The MoFlo high speed cell sorter can sort up to 60,000 events/second.

Cells were labelled with CD5-FITC, CD38-PE and CD19-APC. These cells were physically sorted into $CD5^+/CD38^+/CD19^+$ and $CD5^+/CD38^-/CD19^+$ sub-populations by high speed cell sorting (up to 35,000 events/second) using a MoFlo High Speed Cell Sorter. This was carried out by Dr. Chris Pepper, Department of Haematology. The resultant cell populations were aliquoted into appropriate volumes for downstream assays such as cell cycle analysis (1×10^6 cells) and RNA extraction (1×10^7 cells) for micro-array analysis.

2.3.10 Cell cycle analysis

Aliquots (1 x 10^6 cells) of the cell sorted $CD38^+$ and $CD38^-$ sub-groups were fixed in ice cold 70% ethanol and were stored at –20°C for at least 30 minutes. They were then washed in PBS, treated with RNase A (1mg/ml) and stained with propidium iodide (400µg/ml) for 30 minutes at 37°C. The cells were analysed using a FACScan flow cytometer using 488nm excitation. Cell cycle distribution was quantified using a complex mathematical algorithm using Cylchred version 1.0.2 software.

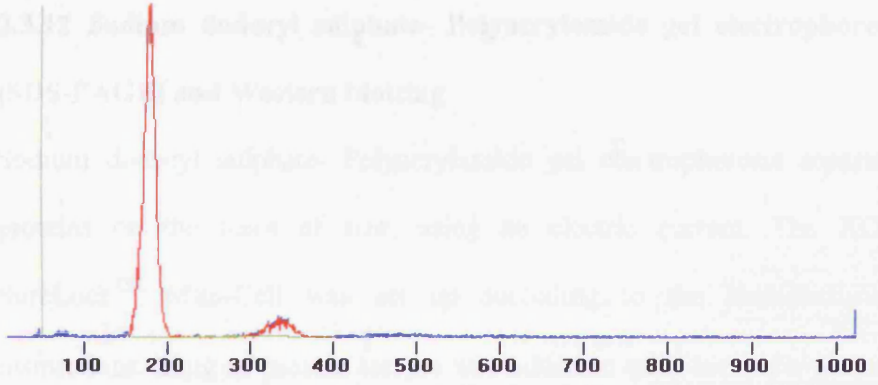


Figure 2.4 Cell Cycle distribution
The first red peak at 200 represents the cells that are in G_0/G_1. The green represents the cells that are in S phase. The second red peak at 400 represents the cells that are in G_2.

2.3.11 Protein extraction

3 x 10^7 cells were lysed in 100µl of Mammalian Cell Lysis kit lysis buffer (Sigma Aldrich, Poole, Dorset, UK), and incubated on ice for 15 minutes. The cells were then sonicated (3 x 5 second bursts followed by 5 second intervals), and the cell debris was collected by density centrifugation (300 x g for 5 minutes). The supernatant was transferred into a new sterile tube.

For each sample a 1:20 dilution was carried out and 5µl of this was added to a 96 well plate in duplicate. Dilutions of bovine serum albumin (BSA) (0-1.0µg/ml) (BioRad, Hemel, Hempstead, UK) were also added in duplicate to the 96 well plate. The protein concentration was determined using the Bradford Assay (BioRad) and this was read at 595nm. Using the absorption values, a standard curve was produced for the dilutions of BSA. From the curve the quantity of protein for each sample was deduced.

2.3.12 Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis separates proteins on the basis of size, using an electric current. The XCell SureLock™ Mini-Cell was set up according to the manufacturer's instructions. 20µg of protein sample was added to each lane of a pre-cast Novex 4% - 12% Bis-Tris (Bis (2 - hydrpxyethyl) iminotris (hydroxymethyl) methane) gel, along with 1µl of MagicMark™ and 5µl of MultiMark™. Appropriate positive and negative controls were also used to ensure correct band identification. The MOPS (3-(N-morpholino)propanesulfonic acid) SDS running buffer was diluted to a 1:20 solution and was added to the XCell SureLock™ Mini-Cell. The gel was run for 50 minutes at a constant voltage of 200V. While the gel was running, a poly vinylidene difluoride (PVDF) membrane was pre-soaked in methanol. It was then soaked, along with the blotting pads and filter paper, in transfer buffer (consisting of transfer buffer (50ml), NuPage™

Antioxidant (1ml), methanol (100ml per gel) and deionised water to a final volume of 1L). Once the SDS-PAGE was complete, the gel was removed from its plastic casing, and the XCell SureLock™ Mini-Cell was rinsed of all running buffer. The gel/membrane sandwich was set up as follows (Figure 2.5):

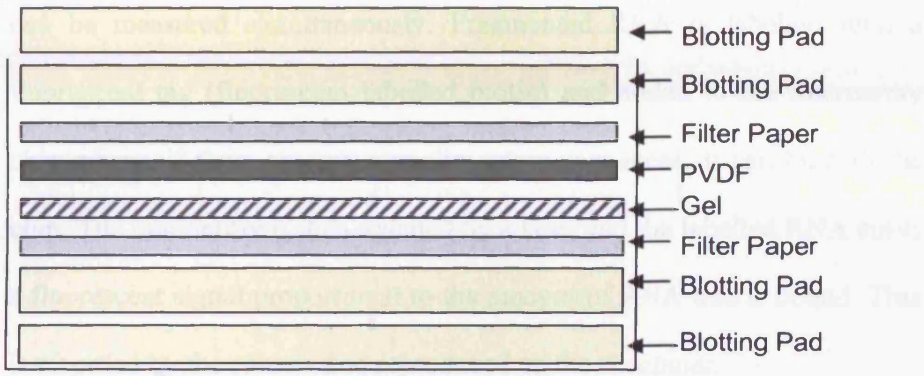


Figure 2.5: Layout of Western blotting apparatus
Two blotting pads next to the cathode core followed by filter paper, the gel, PVDF membrane, another filter paper, and 2 more blotting pads next to the anode lid.

Deionised water was added to the XCell SureLock™ Mini-Cell's "outer core" to dissipate the heat. The blotting conditions were 30V for 1 hour. Once transfer was complete, the membrane was washed in deionised water and then placed in 5% non-fat milk blocking solution (made in TBS (Tris buffered saline) with 0.1% Tween-20) for an hour. The primary antibody was diluted in a 5% non-fat milk solution according to the manufacturer's recommendations. This was added to the membrane, which was placed at 4°C on a gel rocking platform overnight.

Membranes were then rinsed in TBS-Tween (0.1% Tween 20), and a secondary antibody (diluted in a 5% non-fat milk solution) was added to the membrane for an hour. This was rinsed off using TBS-Tween (4x 5 minute washes). The ECL™ (Amersham) advance kit was used to detect

the bands on the gel, which was developed onto Hyperfilm™ ECL high performance chemiluminescence film (Amersham).

2.3.13 Microarray

Microarray is the process in which the expression of thousands of genes can be measured simultaneously. Fragmented RNA is labelled with a fluorescent tag (fluorescent labelled biotin) and added to the microarray chip where, if there are any complimentary sequences, it can bind to the chip. The microarray is then scanned by a laser and the labelled RNA emits a fluorescent signal proportional to the amount of RNA that is bound. This is recorded by the camera and reproduced on the computer.

The microarray chips contain 11 to 20 probes for every transcript which match different parts of the 3' end of the mRNA sequence. The probes are made up of a pair of 25 residue oligonucleotides, one that is a perfect match to the transcript, and the other has had the middle residue changed and so is a "mismatch". This helps to reduce the background signal and any non-specific binding. There are many different Microarray chips available and for a variety of different organisms. In this study, the human U133A array was used which contains 22,283 gene transcripts.

The process of Microarray (from RNA extraction to Scanning the chip) was carried out by Megan Musson in the Central Biotechnology Services. The key component steps involved in this procedure are described.

2.3.13.1 RNA extraction

Trizol reagent was added the cell-sorted populations (1 x 10^7 cells), and this was placed in tubes containing Phase Lock Gel-Heavy and were incubated for 5 minutes at 37°C. 0.2ml of chloroform was added and the tubes were shaken. The samples were spun at 12,000 x g for 10 minutes at 2-8°C, to separate the phases (the clear aqueous phase was on top and cloudy chloroform phase was on the bottom). The aqueous phase was transferred to a clean tube, and to which 0.6ml of isopropyl alcohol was added. The tubes were shaken, and spun as before. The pellet of RNA was re-suspended in 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 2-8°C. The supernatant was removed, and the pellet allowed to air dry. The pellet was re-suspended in RNase-free water, and incubated at 65°C for at least 30 minutes. The RNA was quantified using the Nanodrop 1000 (Nanodrop Technologies), and the purity was checked by running an Agilent chip on the Agilent bioanalyzer (Agilent Technologies).

2.3.13.2 One-cycle cDNA synthesis

A 1:20 dilution of Poly-A (poly-adenine) control stock was made in Poly-A Control Dilution Buffer from the commercially available kit (Affymetrix). From this solution, 2µl was added to Poly-A control dilution buffer to make a 1:50 dilution. Again, 2µl was taken from this solution and added to Poly-A control dilution buffer to make a 1:10 dilution. 2µl of this final dilution was added to the sample of RNA.

T7-Oligo(dT) Primer was added to the RNA and the volume was made up to 12µl with RNase-free water. The reaction was incubated at 70°C for 10 minutes and then cooled for at least 2 minutes. The First-Strand master mix was made up as follows: 4µl of 5X 1st strand master mix, 2µl 0.1M Dithiothreitol (DTT) and 1µl 10mM dNTP. This was added to the RNA/T7-oligo(dT) primer mix and incubated at 42°C for 2 minutes. The appropriate amount of SuperScript II was added to each RNA sample and incubated for 1 hour at 42°C. The sample was then cooled at 4°C for at least 2 minutes. The Second-Strand master mix was made up as follows: 91µl of RNase-free water, 30µl 5X 2nd strand reaction mix, 3µl 10mM dNTP, 1µl *E. coli* DNA ligase, 4µl of *E.coli* DNA polymerase I and 1µl RNase H. 130µl of this master mix was added to each RNA sample and it was incubated for 2 hours at 16°C. T4 DNA Polymerase (2µl) was added to the sample and was incubated at 16°C for 2 minutes. 10µl of 0.5M ethylene diamine tetra-acetic acid (EDTA) was added to the sample. cDNA (compliment DNA) Binding Buffer (600µl) was added to the sample making the mixture yellow. The sample was applied to the cDNA Cleanup Spin Column and spun at ≥ 8,000 x g for 1 minute. The spin column was transferred into a new collection tube and cDNA Wash Buffer was added to the column. This was then spun at ≥ 8,000 x g for 1 minute and the flow through was discarded. The caps of the spin columns were opened, centrifuged for 5 minutes at maximum speed and the collection tube and flow-through were discarded. The column membranes were dried by centrifugation and the spin columns were placed in clean collection tubes. cDNA elution buffer was added directly onto the column membrane and

incubated for 1 minute at room temperature. This was then spun at maximum speed for 1 minute to elute.

2.3.13.3 Synthesis of biotin-labelled cRNA

The following solution was made up; 4µl 10X IVT labelling buffer, 12µl IVT (in vitro transcription) labelling NTP (nucleotide triphosphate) mix, 4µl IVT labelling enzyme mix, required amount of template cDNA and the volume made up to 40µl with RNase-free water. The mixture was incubated at 37°C overnight. The following day 60µl of RNase-free water, 350µl cRNA binding buffer and 250µl ethanol was added to the sample, which was applied to the IVT cRNA Cleanup Spin Column. This was spun at >8,000 x g for 15 seconds, and the flow through and collection tube was discarded. The spin column was placed in a new collection tube. 0.5ml IVT cRNA wash buffer was added to the column and was spun as before with the flow through being discarded. 80% ethanol (0.5ml) was added to the sample and was spun as before. The spin column's cap was opened and it was spun at maximum speed for 5 minutes. The flow through and collection tube was discarded. After a new collection tube was added, 16µl of RNase-free water was added directly onto the membrane and the column was spun at maximum speed for 5 minutes. This step was then repeated. To quantify the cRNA the absorbance was read at 260nm and 280nm. The yield was determined using the following equation;

Adjusted cRNA yield = RNA_m – (total RNAi)(y)

Where: RNA_m = amount of cRNA measured after IVT (µg)

Total RNAi = starting amount of total RNA (µg)

y = fraction of cDNA reaction used in IVT

2.3.13.4 Fragmenting the cRNA for target preparation

The fragmentation buffer was made up as follows; 20µg cRNA, 6µl 5X fragmentation buffer, and the volume was made up to 40µl with RNase-free water. This solution was incubated for 35 minutes at 94°C, and then placed on ice. An aliquot was run on the Agilent Bioanalyzer to check the process had occurred.

2.3.13.5 Eukaryotic target hybridization

The probe array (Affymetrix U133A gene chip) was equilibrated to room temperature immediately before it was used. Hybridization cocktail was made up of fragmented RNA (10µg), control oligonucleotide B2 (3nM) (3.3µl), 20X eukaryotic hybridization controls (bioB, bioC, bioD, cre) (10µl), Herring Sperm DNA (10mg/ml) (2µl), bovine serum albumin (BSA) (50mg/ml) (2µl) 2X hybridization buffer (100µl), dimethyl sulfoxide (DMSO) (20µl) and water to make the final volume 200µl. This was heated to 99°C for 5 minutes in a heat block.

While the hybridization cocktail was heating, the 130µl of 1X Hybridization buffer was added to the array and it was incubated at 45°C for 10 minutes with rotation. The hybridization cocktail was transferred to a 45°C heat block for 5 minutes, and then was spun to remove any insoluble material. The buffer was removed from the array, and the hybridization cocktail (200µl) was added, and the array was placed in the Hybridization oven set to 45°C for 16 hours.

2.3.13.6 Washing, staining and scanning

The fluidics station was primed with Non-stringent wash buffer and Stringent wash buffer before use. After hybridization, the cocktail was removed from the probe array, and the array was filled with non-stringent wash buffer. Three staining solutions were made up and place onto the fluidics station. Staining solutions 1 and 3 were 600µl Streptavidin Phycoerythrin (SAPE) solution made up of 600µl 2X stain buffer, 48µl of 50mg/ml BSA, 12µl 1mg/ml SAPE, 540µl water. The 2nd stain solution is Antibody solution made up of 2X stain buffer (300µl), 50mg/ml BSA (24µl), 10mg/ml goat Immunoglobulin G (IgG) stock (6µl), 0.5mg/ml biotinylated antibody (3.6µl) and water (266.4µl). The fluidic protocol was as follows:

Post hybridization wash #1	10 cycles of 2 mixes/cycle with wash buffer A at 30°C
Post hybridization wash #2	6 cycles of 15 mixes/cycle with wash buffer B at 50°C
Stain	Stain the probe array for 5 minutes in SAPE solution at 35°C
Post stain wash	10 cycles at 4 mixes/cycle with wash buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes in antibody solution at 35°C
3rd Stain	Stain the probe array for 5 minutes in SAPE solution at 35°C
Final wash	15 cycles at 4 mixes/cycle with wash buffer A at 35°C. The holding temperature is 25°C

Table 2.5: Fluid protocol for the fluidics station
The protocol states which wash buffer or stain is used, the amount of cycles and at what temperature this occurs.

2.3.13.7 Analysis

Analysis of the microarray data was carried out on GeneSpring software (Agilent Technology). Firstly, any genes that were not present in at least one of the samples were removed from the gene list. Then any genes that had less than a 2 fold change were also removed. Statistical analysis was carried out (ANOVA test and Student's *t* test) and a gene list produced after comparing the gene lists from the two tests and finding the genes present in both. The gene list was exported into Pathway Assist (Stratagene), where pathways were produced with any genes that were directly connected. Pathway assist connects to Pubmed (http://www.ncbi.nlm.nih.gov) and searches for any associations between the genes input. It then uses this information to build the pathway.

2.3.14 Statistical analysis

Statistical analyses were performed using both parametric and non-parametric methods depending on whether the data obeyed a Gaussian distribution. Statistical analyses were carried out using GraphPad Prism 3.0 software.

If the data obeyed a Gaussian distribution the main parametric test that was carried out was an unpaired Student's t test. If the data was derived from the same patient (such as when $CD38^+$ and $CD38^-$ cells were analysed from a bimodal patient) then a paired Student's t test was carried out. Correlation tests were also carried out on most of the data. If the data did not obey a Gaussian distribution then, depending on the type of data, different non-parametric tests were carried out such as a Mann-Whitney test, a Fisher's

Exact test or a Chi Squared test. Also, when analysing the CD38 longitudinal data, an ANOVA was carried out. For each statistical test the value of significance used was $P \leq 0.05$. When carrying out the correlations, the r^2 value was also investigated to determine how well the data clustered around the regression line.

Chapter 3: CD38 expression as a prognostic marker in CLL

CD38 is now widely regarded as an important prognostic marker in CLL (Damle et al. 1999;Ghia et al. 2003;Hamblin et al. 2002;Schroers et al. 2005;Thornton et al. 2004). However, areas of controversy remain that hamper its confident use namely: (1) the debate about whether CD38 is a surrogate marker of V_H gene mutation status (Damle et al 1999;Hamblin et al 2002); (2) the discordance in the literature about which cut-off value for CD38 expression best discriminates between $CD38^+$ and $CD38^-$ cases in defining prognosis (Damle et al 1999;Ghia et al 2003;Ibrahim et al. 2001;Krober et al. 2002); (3) the possibility that CD38 expression changes over the course of the disease and hence may be an unstable marker (Ghia et al 2003;Krober et al 2002); (4) the relationship between CD38 and the other well known prognostic and clinical markers used in CLL.

Aims: To investigate whether CD38 expression is a surrogate marker for V_H gene mutation status and whether CD38 expression is individually a prognostic marker.

Hypothesis: CD38 may correlate with V_H gene mutational status but could not be used as a surrogate marker. However CD38 could be used as an individual marker of prognosis.

	Number of patients				
Sex (Male/Female)	102		66		
Treated/Untreated	119		49		
Stage (Binet A/B/C)	111	25	32		
V_H Gene Satus (Mutated/Unmutated)	106		37		
LDT (>12/<12)	138		28		
Cytogenetics (13q14/Trisomy 12/6q-/11q-/17p-)	5	6	5	10	3

Table 3.1: Characteristics of my patient cohort (n = 168)
The table show the number of patients who were male/female, whether they had been treated or not, what they V_H gene mutation status was (Unmutated ≥98%/Mutated <98%), Binet stage, lymphocyte doubling time (LDT) (>12 months/<12months) and any cytogenetics present.

3.1 CD38 expression as a surrogate marker of V_H gene mutation status

Damle et al (1999) not only identified V_H gene mutational status as a prognostic marker in CLL but also suggested that the 30% threshold for CD38 expression could be used as a surrogate marker for identifying patients with an unmutated V_H gene profile. Since then this assertion has been questioned (Ghia et al 2003;Hamblin et al 2002;Ibrahim et al 2001). Therefore, in this study, we investigated the relationship between V_H gene status and CD38 expression using the previously described cut-off levels (7%, 20% and 30%) in a cohort of 125 patients with at least 7% CD38 expression and for whom V_H gene mutation status was available. The V_H gene status was determined using the method described previously

(Matthews et al. 2004) and V_H gene sequences were compared to the nearest germ line sequence on the Igblast website (http://www.ncbi.nlm.nih.gov/igblast/). CD38 expression was determined by three-colour flow cytometry (CD5-FITC/CD38-PE/CD19-APC).

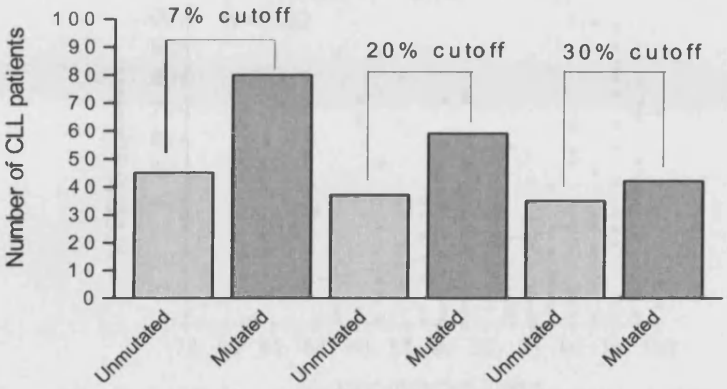
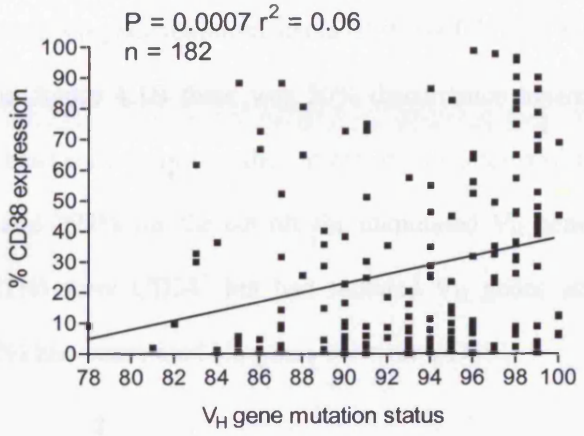


Figure 3.1: Comparison of the V_H status of the patients at different CD38 threshold levels.
The V_H gene mutation status of 125 $CD38^+$ patients (with at least 7% CD38 expression) was analysed at each threshold level. All of the CD38 thresholds examined showed marked discordance with V_H gene mutation status indicating that in this cohort at least, CD38 cannot be used as a reliable surrogate marker of V_H gene mutation status.

In contrast to the findings of Damle et al (1999), at each defined threshold for CD38 expression there were a higher number of patients with mutated V_H genes. At the 7% threshold level, 125 patients were investigated and 79 had mutated V_H genes and 46 had unmutated V_H genes. At the 20% threshold level 37 out of 90 patients (41%) had an unmutated profile and 53 out of the 90 patients (59%) had a mutated profile. At the 30% threshold level out of the 77 patients investigated 35 had unmutated V_H genes (45%) and 42 had mutated V_H genes (55%). Therefore, in this cohort of CLL patients CD38 was not a faithful surrogate marker for V_H gene mutation status, as at each threshold level a significant number of patients with mutated V_H genes were positive for CD38 expression. 63% of the patients

at the 7% threshold level had mutated V_H genes, 58% were mutated at the 20% level and 55% of the patients had mutated V_H genes at the 30% threshold level.

A



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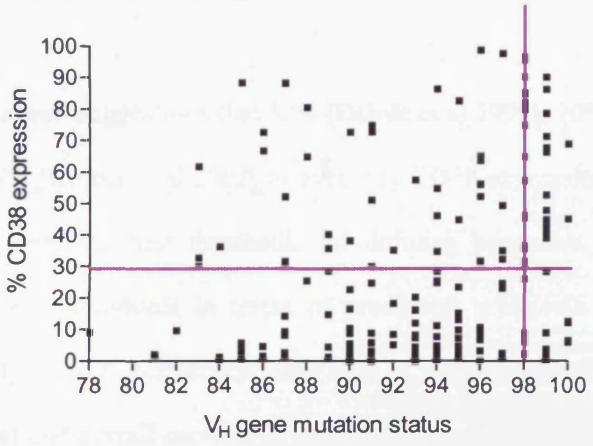

Figure 3.2 (A) Correlation between CD38 and V_H gene status (B) Discordance between CD38 and V_H gene mutation status

V_H gene mutation status was determined using the method previously described and compared to the nearest germline sequence and CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC). There was a positive correlation between CD38 expression and V_H gene mutation status although there was 30% discordance when looking at the arbitrary threshold levels (≥30% expression of CD38 and ≥ 98% V_H gene sequence homology)

Although none of the CD38 cut-off levels tested in this study were shown to be predictive of V_H gene mutation status, when the entire cohort (n = 283) was analysed regardless of the CD38 status of the patients (Figure 4.1A) there was a positive correlation between the percentage of CD38 expression and V_H gene mutation status (P = 0.0007; r^2 = 0.06). However, as shown in figure 4.1B there was 30% discordance between these two prognostic markers when using the cut-off of ≥30% for positive expression for CD38 and ≥98% for the cut-off for unmutated V_H genes. 39 of 182 patients (21%) were $CD38^+$ but had mutated V_H genes and 16 of 182 patients (9%) had unmutated V_H genes but were $CD38^-$.

3.2 CD38 expression and prognosis

There have been suggestions that 30% (Damle et al 1999), 20% (Ibrahim et al 2001), 7% (Krober et al 2002) or even any CD38 expressing cells (Ghia et al 2003) are the best thresholds for defining prognosis in CLL. To examine these thresholds in terms of predicting prognosis in our CLL cohort analyses were performed using time to first treatment, progression-free survival and overall survival as end-points.

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3.2.1 The threshold level for CD38 and Time to first treatment

The time to first treatment (TTFT) is a useful measure of advancing disease in CLL as therapeutic intervention is only indicated following evidence of disease progression. Therefore, if treatment is required early, then by

definition, the disease is progressive and the patient is likely to have a poorer clinical outcome. In this study, the patient cohort (those with both CD38 expression data and TTFT data available, n = 267) was divided according to the three published cut-off levels for CD38 and the TTFT was plotted for each group using Kaplan Meier analysis. Figure 3.3 shows the Kaplan Meier curves for TTFT using (A) 7%, (B) 20% and (C) 30% CD38 expression cut-off levels

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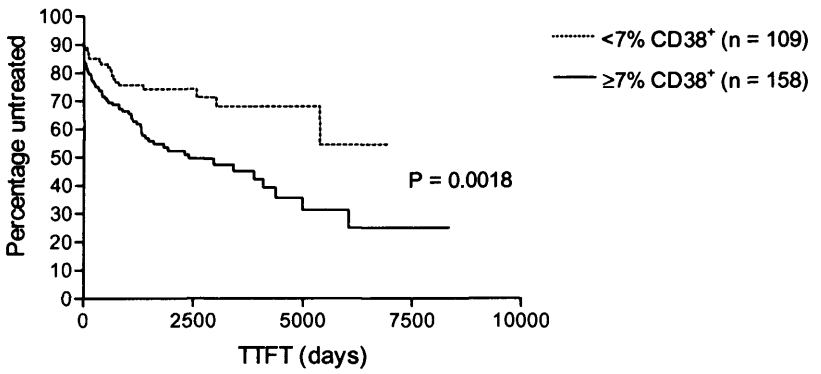

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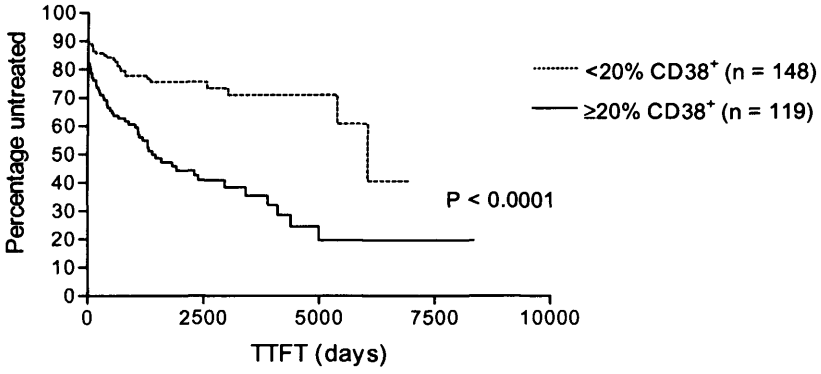

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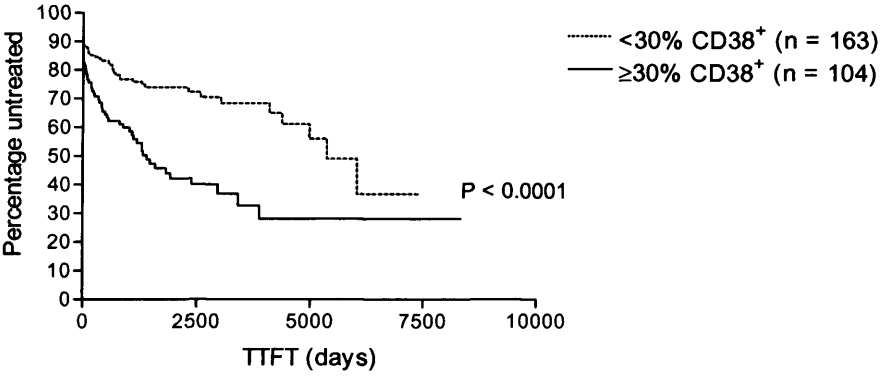

Figure 3.3: Time to first treatment for $CD38^+$ and $CD38^-$ patients at the (A) 7% (B) 20% and (C) 30% threshold levels

CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and the TTFT was derived from a database of clinical information. The TTFT for each threshold level was plotted using Kaplan Meier analysis. The 7% threshold level showed the least statistical difference in TTFT between $CD38^+$ and $CD38^-$ patients ($P = 0.0018$) and the 20% and 30% threshold levels showed the same statistical difference in TTFT between $CD38^+$ and $CD38^-$ patients ($P < 0.0001$).

In agreement with previous studies, all three threshold levels used (7%, 20% and 30%) showed the $CD38^+$ patients had a significantly shorter TTFT than the patients who did not express CD38 above the defined thresholds (Rosenwald et al. 2001). The 7% threshold showed the smallest statistical difference in TTFT between $CD38^+$ and $CD38^-$ CLL patients ($P = 0.0018$). Statistically the 20% and 30% threshold levels showed the same difference between the TTFT for $CD38^+$ and $CD38^-$ patients ($P < 0.0001$ and $P < 0.0001$ respectively). However there was a difference between the median TTFT for the $CD38^+$ and $CD38^-$ patients between these two cut-off levels. At the 30% cut-off level the $CD38^-$ patients had a median TTFT of 5375 days (~14.7 yrs) and the $CD38^+$ patients had a median TTFT of 1397 days (~3.8 yrs) whereas at the 20% cut-off level the $CD38^-$ patients had a median TTFT of 6034 days (~16.5 yrs) and the $CD38^+$ patients had a median TTFT of 1397 days (~3.8 yrs). As the 20% threshold showed the greatest difference in mean TTFT, this suggests that this threshold was superior to the 30% threshold even though their P values were the same.

3.2.2 The threshold level for CD38 and progression-free survival

Progression-free survival (PFS) is an alternative marker of activity of the disease. It can be used to define whether a patient has indolent or active disease. If a patient has progressive disease then usually they require early therapeutic intervention and have an inferior clinical outcome. Figure 3.3 shows the Kaplan Meier curves for PFS using (A) 7%, (B) 20% and (C) 30% CD38 expression cut-off levels.

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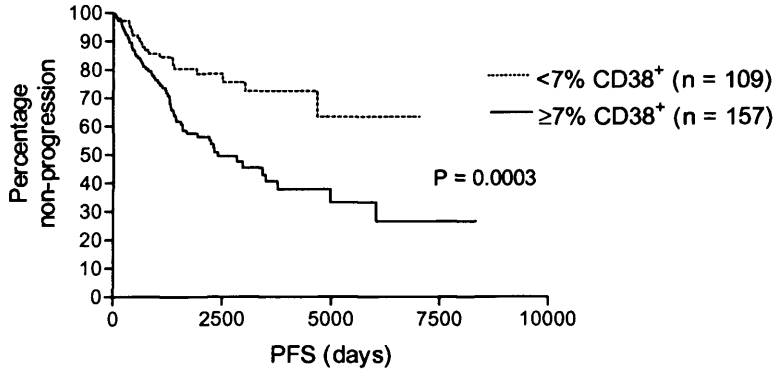

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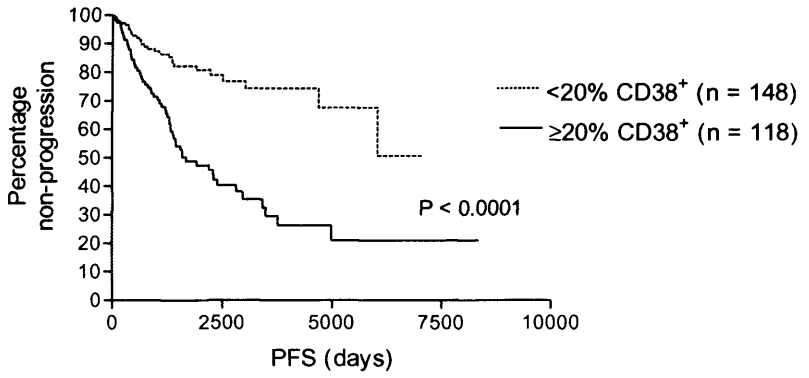

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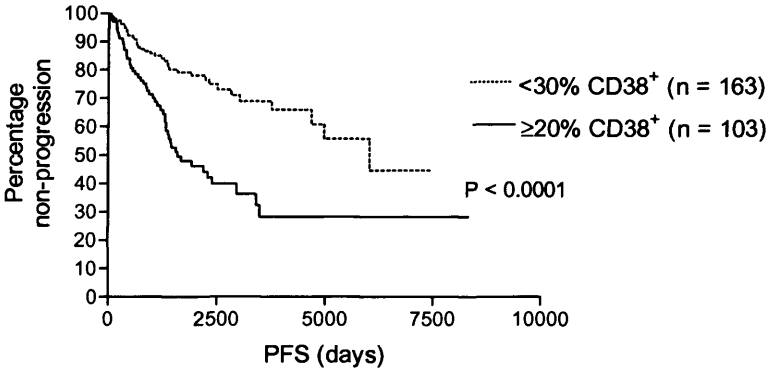

Figure 3.4: Progression-free survival for $CD38^+$ and $CD38^-$ patients at (A) 7% (B) 20% and (C) 30% threshold levels.
CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and the PFS was derived from a database of clinical information. The PFS for each threshold level was plotted using Kaplan Meier analysis. The 7% threshold level showed the least statistical difference in PFS between $CD38^+$ and $CD38^-$ patients ($P = 0.0003$) and the 20% and 30% threshold levels showed the same statistical difference in PFS between $CD38^+$ and $CD38^-$ patients ($P < 0.0001$).

$CD38^+$ patients had a shorter PFS than patients who were $CD38^-$ at all three threshold levels. The difference in PFS for $CD38^+$ and $CD38^-$ patients was statistically significant (P = 0.0003) at the 7% CD38 threshold level and was highly significantly different at the 20% and 30% CD38 threshold levels (P < 0.0001 and P < 0.0001 respectively). Table 3.2 shows the difference in median PFS between the three threshold levels.

	CD38 threshold level		
	7%	**20%**	**30%**
$CD38^-$	Undefined	Undefined	6034 days (~ 16.5 yrs)
$CD38^+$	2386 days (~ 6.5 yrs)	1682 days (~ 4.6 yrs)	1589 days (~ 4.4 yrs)

Table 3.2: The median PFS for $CD38^+$ and $CD38^-$ patients at all three threshold levels (7%, 20% and 30%).
CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and the median PFS was determined using Kaplan Meier analysis. Undefined means the median time to disease progression had not been reached and therefore a figure could not be given.

In two of the cut-off levels (7% and 20%) after 8265 days (22.6 years) the $CD38^-$ patients had not reached the median time to disease progression. The median PFS for the $CD38^+$ patients at the 7% and 20% cut-off levels were 2386 days (~6.5 years) and 1682 days (~4.6 years) respectively. At the 30% threshold level the median time to disease progression was 6034 days (16.5 years) for $CD38^-$ patients and 1589 days (4.4 years) for $CD38^+$ patients.

Both the 20% and 30% threhold level have the same statistical significance when investigating PFS but which threshold level is superior? The 30% threshold level showed the $CD38^+$ patients had a worse prognosis with a mean PFS of 4.4 years in comparison to the 20% threshold level where their PFS was 4.6 years. However, the $CD38^-$ patients had a better prognosis at the 20% threshold level as they had not reached the median PFS whereas at the 30% threshold level it was 16.5 years. This data indicates that the 20% threshold level was superior as it showed the greatest difference between the median PFS in $CD38^+$ and $CD38^-$ patients and it appears that the patients with CD38 expression between 20% and 30% caused this difference.

3.2.3 The threshold level for CD38 and Overall survival

Overall survival (OS) is the ultimate statistic in medicine! However, particularly in the context of clinical trials in CLL this parameter is rarely measured because of the relatively long natural history of CLL in many patients. In this study CD38 expression was measured in archived material from patients that had died from CLL-related causes was used to increase the number of events and allow meaningful OS analysis.

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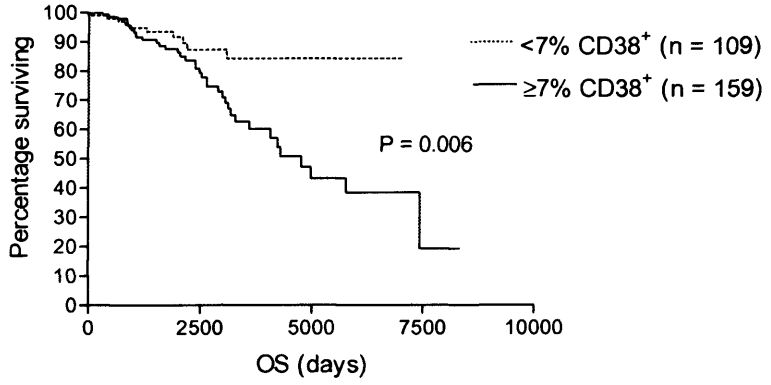

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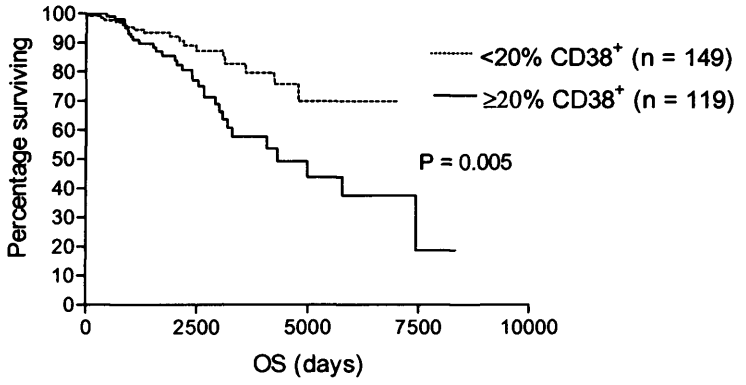

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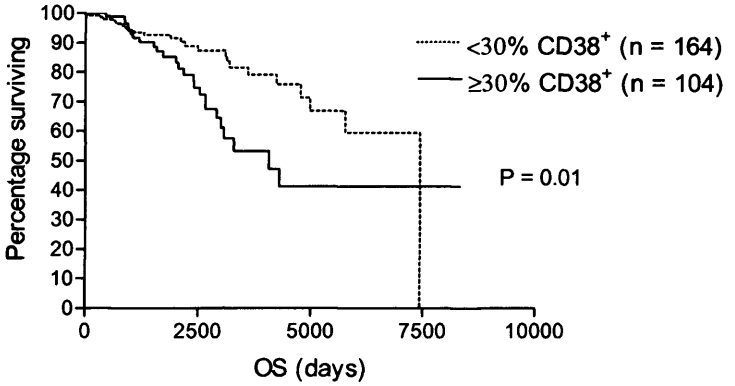

Figure 3.5: Overall survival curves for $CD38^+$ and $CD38^-$ patients at (A) 7% (B) 20% and (C) 30% threshold levels.

CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and the OS was derived from our database of clinical information. The OS for each threshold level was plotted using Kaplan Meier analysis. The 7% threshold level showed an intermediate statistical difference in OS between $CD38^+$ and $CD38^-$ patients (P = 0.006) and the 20% threshold level showed the greatest statistical difference between the OS of $CD38^+$ and $CD38^-$ patients (P = 0.005) and 30% threshold levels showed the least statistical difference in OS between $CD38^+$ and $CD38^-$ patients (P = 0.01).

At the 7% and 20% threshold levels for CD38 expression the median survival time for the patients who were classified as $CD38^-$ was not reached after 8265 days (22.6 years). At the 30% threshold level the median survival time for the $CD38^-$ patients was 7437 days (~20.4 years). In contrast, the patients who were $CD38^+$ had a median OS of 4770 days (13.1 years) at the 7% threshold level, 4304 days (11.8 years) at the 20% threshold level and 4065 days (11.1 years) at 30% threshold level.

At all three threshold levels the $CD38^-$ patients survived for a significantly longer time than patients who were $CD38^+$. The 20% threshold level was the most prognostic out of the three cut-off levels with the greatest significance with regard to overall survival between the two groups (P = 0.005).

3.2.4 Prognostic sub-groups defined by CD38 expression and V_H gene mutation status

Hamblin *et al* (1999) and Damle *et al* (1999) showed that V_H gene mutation status was prognostic; those patients with unmutated V_H genes had a shorter overall survival than those patients with mutated V_H genes. Subsequently, Rosenwald *et al* (2001) showed that patients with unmutated V_H genes also had a shorter time to first treatment than those patients with a mutated V_H genes. A number of studies have suggested that additional prognostic information may be gained by analysing CD38 expression and V_H gene mutation status in combination (Bilous et al. 2005;Jelinek et al.

2001). Therefore, the patient cohort was divided into four groups; $CD38^-$ / mutated V_H genes, $CD38^+$ / mutated V_H genes, $CD38^-$ /unmutated V_H genes and $CD38^+$ / unmutated V_H genes. Figure 3.6 shows that when CD38 expression is analysed in conjunction with V_H gene status the sub-group of patients with $CD38^+$ / unmutated V_H genes have the shortest mean TTFT (250 days) and the $CD38^-$ / mutated patients have the longest mean TTFT (6034 days).

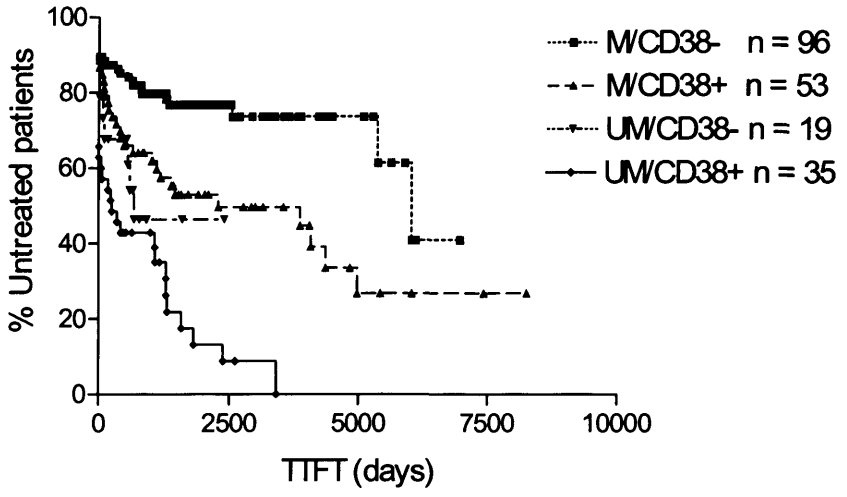

Figure 3.6 Time to first treatment for the different mutational status and CD38 expression
V_H gene mutation status was determined using the method previously described and compared to the nearest germline sequence and CD38 expression was determined using triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC). Patients were classed as unmutated if their V_H genes were ≥ 98% homologous to the closest germline sequence and were classed as $CD38^+$ if they had ≥ 20% expression of the CD38 antigen. The mutated / $CD38^-$ sub-group had the longest time treatment-free and the unmutated / $CD38^+$ sub-group had the shortest TTFT.

Importantly, CD38 separated the mutated group into 2 sub-groups and the unmutated group into 2 sub-groups. Despite the Kaplan Meier curves showing clear separation, there was no statistical difference between

unmutated patients who expressed or did not express CD38 ($P = 0.19$). However, the numbers in both groups were relatively small and this may contribute to the lack of statistical significance. In contrast, patients who had a mutated profile and were $CD38^+$ had a significantly shorter TTFT than the mutated patients who were $CD38^-$ ($P < 0.0001$).

V_H gene mutation status is a known prognostic marker in CLL but there are a group of patients who have mutated V_H genes but still have aggressive disease. The classic sub-group that fit this description are the V_H 3-21 segment usage group which invariably have poor prognosis but usually have mutated V_H genes (Falt et al. 2005;Ghia et al. 2005). As V_H 3-21 has been shown to be associated with poor prognosis in the mutated group (Falt et al 2005) this was investigated in this patient cohort. Seventeen patients were found to have V_H 3-21, 8 were mutated and $CD38^+$ (47%), 6 were mutated and $CD38^-$ (35%), 1 was unmutated and $CD38^-$ (6%) and 2 were unmutated and $CD38^+$ (12%). The high number of mutated and $CD38^+$ patients with this V_H gene usage may provide another explanation for why this group has a shorter TTFT than the mutated and $CD38^-$ patients, although there was still a high number of patients with V_H 3-21 who were mutated and $CD38^-$.

This data suggests that analysis of CD38 along with V_H gene mutational status can help to predict those patients with mutated V_H genes at risk of disease progression. Given that the patients with mutated V_H genes still had a shorter TTFT than the patients with unmutated V_H genes irrespective of their CD38 expression status (positive or negative), this suggests that

individually, V_H gene mutation status is a superior prognostic marker than CD38 expression.

	CD38 expression (≥ / <20%)	
	$CD38^-$	**$CD38^+$**
Mutated	6034 days (16.5 years)	2304 days (6.3 years)
Unmutated	680 days (1.9 years)	250 days (0.7 years)

Table 3.3 Median TTFT for each group.
V_H gene mutation status was determined by the method described previously and then compared to the nearest germline sequence and patients with ≥98% homology were classed as unmutated. CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and patients with ≥ 20% CD38 expression were classed as positive. Patients who were mutated / $CD38^-$ had the longest median TTFT (16.5 years) and patients who were unmutated / $CD38^+$ had the shortest median TTFT (0.7 years).

The median TTFT also separates the mutated and unmutated into 2 groups giving four groups in total. The patients who had mutated V_H genes and were $CD38^-$ had a median TTFT of 6034 days (16.5 years) whereas the patients who had mutated V_H genes and were $CD38^+$ had a median TTFT of 2304 days (6.3 years). The TTFT for patients with unmutated V_H genes and were $CD38^-$ was 680 days (1.9 years) and the TTFT patients with unmutated V_H genes and $CD38^+$ was 250 days (0.7 years). Taken together, this data suggests that the V_H gene mutation status of the patient is a more important determinant of TTFT than CD38 expression. However, the integration of these two parameters creates a hierarchy of risk that separates patients into four distinct categories. This approach could be used in the future to more accurately inform clinicians and patients about the likely course of an individual's disease.

3.3 Does CD38 expression change over time?

There has been great controversy over whether CD38 expression changes over the course of the disease and/or after treatment; Hamblin *et al* (2002) suggested that CD38 expression does change over time whereas Ibrahim *et al* (2001) argued that expression of CD38 remains fairly constant throughout the course of the disease. To investigate these contradictory claims, samples from individual patients were taken at different time points over the course of this study. A total of 21 patients were serially investigated; at least three measurements of CD38 expression were made for each patient at various time intervals (1 month – 24 months). Statistical analysis was carried out using analysis of variance (ANOVA) to establish whether the intra-patient variation in CD38 expression was significant.

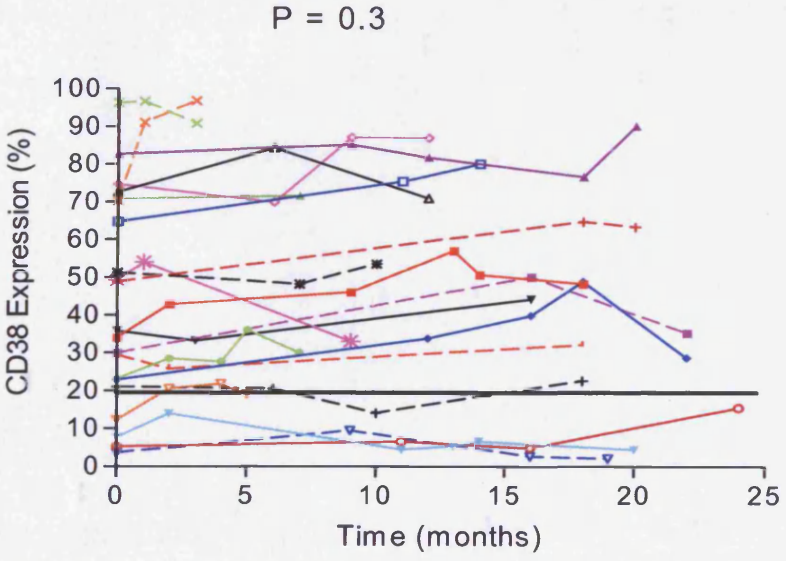

Figure 3.7 Longitudinal analysis of CD38 expression in 21 patients.

CD38 expression was analysed in 21 patients at different time points throughout the investigation. Only patients with 3 or more time points were included in the study so that statistical analysis could be carried out. An ANOVA test showed no significant change in CD38 expression over time (P = 0.3).

The intra-patient CD38 expression did vary to some extent over time (1% - 27%). However the ANOVA test showed no significant change in CD38 expression (P = 0.3). One out of 21 patients investigated (9.5%) crossed the 20% CD38 threshold thereby changing their CD38 status from negative to positive. This may be due to the fact that this patient had CD38 expression near the 20% threshold and so slight fluctuations in their CD38 expression pushed them over the threshold. This might be due to a number of factors including experimental error in the measurement of CD38 expression and intrinsic variation in numbers of circulating $CD38^+$ CLL cells. This indicates a problem in the arbitrary cut-off system currently employed as although both patients changed from being classed as $CD38^-$ to being $CD38^+$ their disease did not change and neither patient showed disease progression over the period of the study.

3.4 ZAP-70 expression and CD38 expression

Zeta associated protein-70 (ZAP-70) is a protein tyrosine kinase that has been shown to be an important prognostic marker in CLL (Orchard et al. 2004) and Rassenti *et al* also suggested that it was more prognostic than V_H gene mutational status (Rassenti et al. 2004). ZAP-70 was first identified as being important in CLL following micro-array studies comparing V_H gene mutated and unmutated CLL samples (Rosenwald et al 2001). Consequently, it has also been suggested as a surrogate marker for V_H gene mutation status. Furthermore, Wiestner *et al* (Wiestner 2005) suggested that when investigating TTFT, ZAP-70 is a superior prognostic marker

when compared to V_H gene mutation status and CD38 expression. To determine whether our data agreed with this suggestion, ZAP-70 was investigated in relation to CD38 and V_H gene status. Cells were triple labelled with ZAP-70-Alexa fluor 488, CD38-PE and CD19-APC. The percentage of cells expressing ZAP-70 and CD38 were derived from gated histogram plots using the $CD19^-$ population to define the lower limit of positivity for ZAP-70 expression and using a conventional isotype control method for determining the percentage of CLL cells that were CD38 positive.

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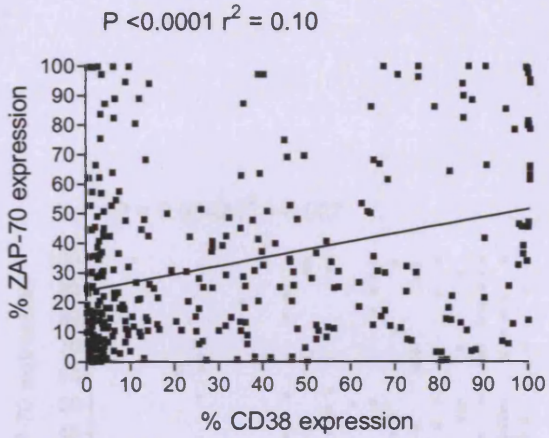

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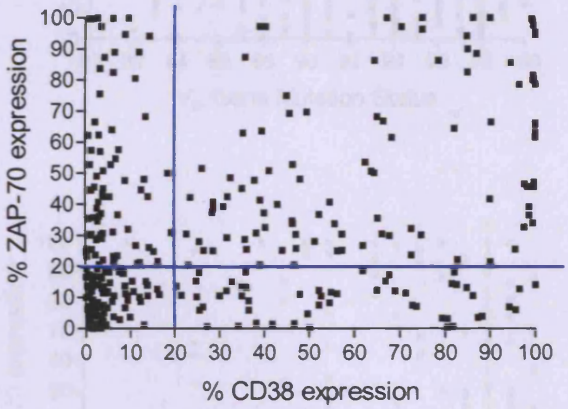

Figure 3.8 (A) Correlation between CD38 and ZAP-70 (B) Discordance between CD38 and ZAP-70 using a 20% threshold for both parameters.
CD38 expression and ZAP-70 expression was determined by triple colour flow cytometry (ZAP-70-Alexa fluor 488, CD38-PE and CD19-APC) on fixed and permeabilised cells. There was a positive correlation between CD38 and ZAP-70 expression although there was 40% discordance when investigating the arbitrary threshold levels (≥20% expression of both CD38 and ZAP-70)

CD38 and ZAP-70 were positively correlated ($P < 0.0001$; $r^2 = 0.1$) but again there was discordance between the two parameters (40% discordance). Using ≥20% cut-off for positive expression of CD38 and ≥20% for positive expression of ZAP-70, 16% of patients (52 out of 327)

were $CD38^+$ but $ZAP\text{-}70^-$, and 24% of patients (77 out of 327) were $CD38^-$ but $ZAP\text{-}70^+$.

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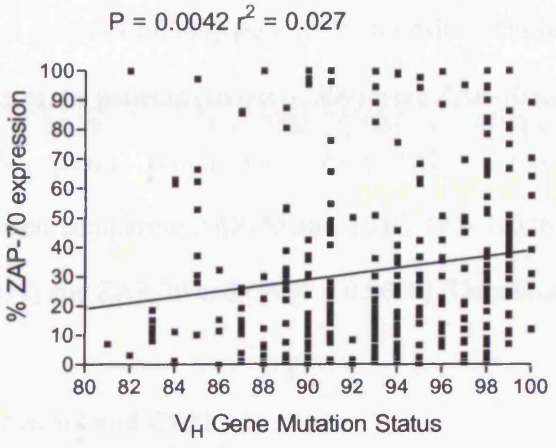

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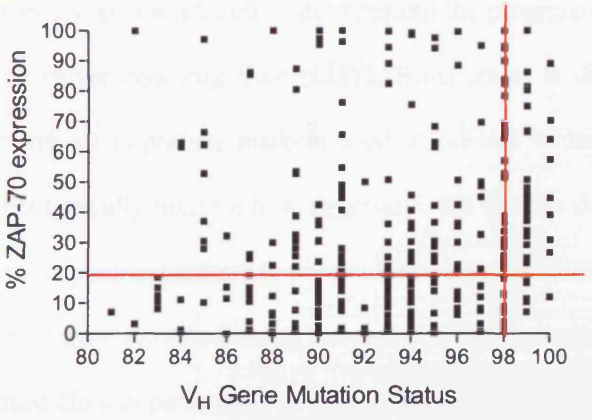


Figure 3.9 (A) Correlation between ZAP-70 and V_H gene status (B) Discordance between ZAP-70 and V_H gene status using a 20% threshold for ZAP-70 and a 2% threshold for V_H gene mutation status.
V_H gene mutation status was determined using the method previously described and compared to the nearest germline sequence and ZAP-70 expression was determined by two colour flow cytometry (ZAP-70-Alexa fluor 488 and CD19-APC). There was a positive correlation between V_H gene mutation status and ZAP-70 expression although there was 43% discordance when investigating the arbitrary threshold levels (≥98% V_H gene sequence and ≥20% ZAP-70 expression)

ZAP-70 expression was positively correlated with V_H gene mutation status ($P = 0.0042$; $r^2 = 0.027$). There was however 43% discordance between the two prognostic markers. Using ≥98% cut-off for unmutated V_H gene mutation status and ≥20% cut-off for positive expression of ZAP-70, 38% of the patients (118 out of 309) were ZAP-70 positive but had mutated V_H genes and 5% of the patients (16 out of 309) were ZAP-70 negative but had unmutated V_H genes. Within the cohort ZAP-70 was found to be prognostic when comparing ZAP-70 and TTFT ($P = 0.026$), ZAP-70 and PFS ($P = 0.017$) and ZAP-70 and OS ($P = 0.0008$) (Unpublished data).

3.5 Clinical factors and CD38

Clinical factors are also important in determining the prognosis of the CLL patient. Lymphocyte doubling time (LDT), Binet stage at diagnosis and cytogenetics are all important markers used at present within the clinic. These markers typically indicate how aggressive the disease is but they are not useful as prospective markers to predict disease progression. This study set out to investigate the relationship between CD38 expression and other commonly used clinical parameters. Lymphocyte doubling time (LDT) was split into 2 groups for the analysis; the patients who had a LDT <12 and the patients who had a LDT >12 as these are the parameters used in the clinical practice (Shanafelt, Geyer, & Kay 2004).

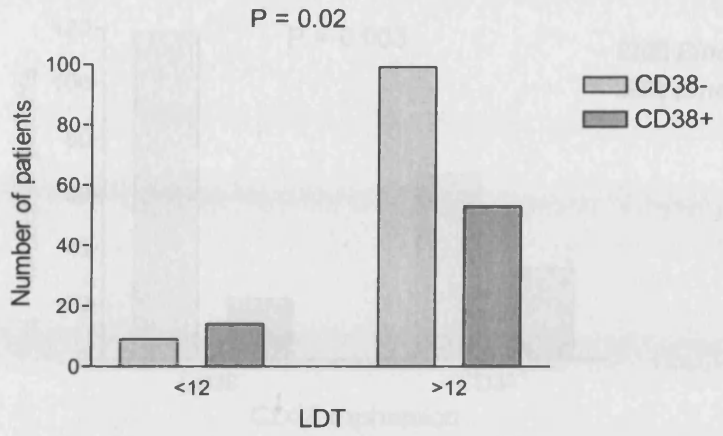


Figure 3.10. The number of CD38$^+$ and CD38$^-$ patients based on their LDT. 175 patients were analysed for CD38 expression using triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and lymphocyte doubling time (LDT) was determined using a clinical database. A Fisher's exact test was carried out and CD38 expression was associated with <12 months LDT.

Out of the 23 patients with a LDT <12, 9 patients (39%) were CD38$^-$ and 14 (61%) were CD38$^+$. 99 patients (65%) out of 152 had a LDT >12 and were CD38$^-$ whereas there were 53 patients (35%) out of 152 who were CD38$^+$ and had a LDT >12. There was a significantly higher proportion of CD38$^+$ patients with a LDT of less than 12 months and a higher proportion of CD38$^-$ patients with a LDT greater than 12 months (P = 0.02).

The Binet staging system was split into 2 groups for this analysis; Binet stage A (good prognosis) and Binet stage B/C (poor prognosis) (Binet et al. 1981)

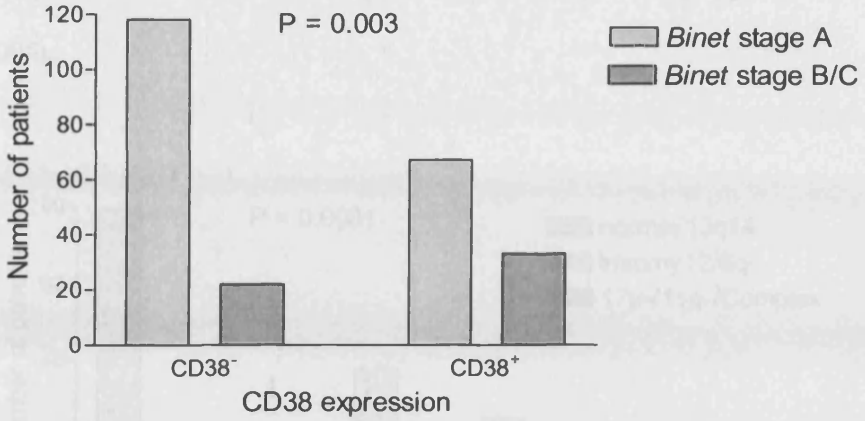


Figure 3.11 The number of $CD38^+$ and $CD38^-$ patients in *Binet* Stage A and *Binet* Stages B/C
240 patients were analysed for CD38 expression using triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and Binet stage was determined from a clinical database. The Binet staging system was split into 2 groups for this analysis; Binet stage A (good prognosis) and Binet stage B/C (poor prognosis). A Fisher's exact test was carried out and CD38 expression was found to be associated with Binet stages B/C.

140 patients were $CD38^-$ and 118 of these patients (84%) were in Binet Stage A and 22 patients (16%) were in Binet stages B/C. Out of the 100 patients that were $CD38^+$ 67 patients (67%) were Binet stage A and 33 patients (33%) were Binet stages B/C. There was a significantly higher proportion of $CD38^+$ patients in Binet Stage B/C and a significantly higher proportion of $CD38^-$ patients in Binet Stage A ($P = 0.0005$).

The cytogenetic abnormalities described within the patient cohort were categorised into 3 groups; the good prognostic cytogenetic group contained the normal karyotype (i.e. no cytogenetic lesion detected by fluorescence *in situ* hybridisation) and 13q14 deletion (Mehes 2005), the intermediate prognostic cytogenetic risk group contained trisomy 12 and 6q deletion (Cuneo et al. 2004;Mehes 2005) and the poor prognostic cytogenetic risk

group contained 17 deletion, 11q deletion and complex karyotype (Mehes 2005).

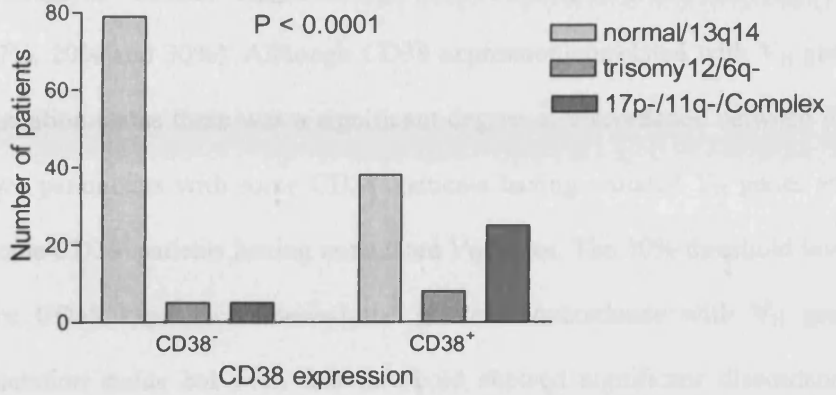


Figure 3.12 Cytogenetics of CD38$^+$ and CD38$^-$ patients
168 patients were analysed for CD38 expression and cytogenetic abnormalities. CD38 expression was determined by triple colour flow cytometry (CD5-FITC, CD38-PE and CD19-APC) and the cytogenetic abnormalities were determined from a clinical database. The abnormalities were split into 3 groups; good risk (normal/13q14), intermediate risk (trisomy 12/6q$^-$) and poor risk (17p$^-$/11q$^-$ /complex) prognostic groups. A Chi Squared test was carried out to analyse CD38 expression and the three prognostic cytogenetic risk groups. CD38 expression was associated with the intermediate and poor prognostic cytogenetic groups.

89 patients were classified as CD38$^-$ and 79 of these patients (88%) were in the good prognosis cytogenetics risk group, 5 patients (6%) had the intermediate prognosis cytogenetics and 5 patients (6%) had the poor prognosis cytogenetics. 71 patients were CD38$^+$ and 38 of these patients (54%) had the good prognosis cytogenetics, 8 patients (11%) had the intermediate prognosis cytogenetics and 25 patients (35%) had the poor prognosis cytogenetics. There were a greater proportion of CD38$^-$ cells with a normal karyotype or the good prognosis cytogenetic marker 13q14 whereas a greater proportion of CD38$^+$ patients had the poor cytogenetic markers 17p deletion and 11q deletion ($P < 0.0001$).

3.6 Conclusion

In this investigation CD38 expression was not found to be a reliable surrogate marker for V_H gene mutation status at any of the thresholds tested (7%, 20% and 30%). Although CD38 expression correlated with V_H gene mutation status there was a significant degree of discordance between the two parameters with some $CD38^+$ patients having mutated V_H genes and some $CD38^-$ patients having unmutated V_H genes. The 30% threshold level for CD38 expression showed the greatest concordance with V_H gene mutation status but even this threshold showed significant discordance between the two parameters. These findings were in agreement with Krober *et al* (2002) who showed that 30% of the patients within this study were discordant for CD38 expression and V_H gene mutation status.

The threshold for defining CD38 status with the greatest prognostic value was 20% expression and this was demonstrated by significant differences in TTFT ($P < 0.0001$), PFS ($P < 0.0001$), and OS ($P = 0.005$) between $CD38^+$ and $CD38^-$ patients. The $CD38^+$ patients had a significantly shorter TTFT, a significantly shorter PFS and a significantly shorter OS. This was in agreement with an earlier study by Ibrahim *et al* (2001) who investigated 218 CLL patients and also found the 20% threshold for CD38 expression was the most prognostic. This data indicates that patients who express CD38 have a more aggressive and progressive disease (as shown by PFS data), a higher probability of requiring early treatment intervention (as shown by the TTFT data) and a shorter survival (as shown by the OS data).

V_H gene mutation status is a known prognostic marker in CLL but there are a group of patients who have mutated V_H genes but still have aggressive disease. The classic sub-group that fit this description are the V_H 3-21 segment usage group which invariably have poor prognosis but usually have mutated V_H genes (Falt et al 2005;Ghia et al 2005). As V_H 3-21 has been shown to be associated with poor prognosis in the mutated group (Falt et al 2005) this was investigated in this patient cohort. The high number of mutated and $CD38^+$ patients with this V_H gene usage (8 out of the 17 patients; 47%) may provide another explanation to why this group has a shorter time to first treatment than the mutated and $CD38^-$ patients, although there was still a high number of patients with V_H 3-21 who were mutated and $CD38^-$. This data implies that the combination of CD38 and V_H gene status is required for a more accurate clinical assessment, as this would help to identify the patients with mutated V_H gene who will have progressive disease and may require treatment. The data in this study shows when looking at V_H gene status and the CD38 expression is included the cohort can be split into four sub-groups. The patients with mutated V_H genes that also expressed CD38 (≥20%) had a shorter TTFT than patients with mutated V_H genes that did not express CD38 (<20%). Interestingly, VH gene mutation status appeared to be more important than CD38 expression in determining TTFT as patients with unmutated V_H genes had a shorter TTFT than patients with mutated V_H genes. However, the unmutated patients could also be split into 2 groups based on CD38 expression; patients with unmutated V_H genes and $CD38^+$ patients having the shortest TTFT.

The longitudinal data generated in this study indicated the CD38 expression does not change significantly over time which was in agreement with Ibrahim *et al* (2001) but in disagreement with Hamblin *et al* (2002) and Oscier *et al* (2002). Although CD38 expression did not change significantly over time, 1 out of the 21 patients did cross the 20% threshold barrier changing their CD38 status from negative to positive. This may be due to the fact that this patient had CD38 expression near the 20% threshold and so slight fluctuations in their CD38 expression pushed them over the threshold. This might be due to a number of factors including experimental error in the measurement of CD38 expression and intrinsic variation in numbers of circulating $CD38^+$ CLL cells. This indicates a problem in the arbitrary cut-off system currently employed as although both patients changed from being classed as $CD38^-$ to being $CD38^+$ their disease did not change and neither patient showed disease progression over the period of the study.

None of the patients investigated in this longitudinal study had received treatment within 3 months of the study. Krober *et al* (2002) found that treatment induced variations in CD38 expression. This is an area that would have to be investigated within this CLL cohort in the future.

There was a correlation between CD38 expression and the other well known prognostic markers in CLL, V_H gene mutation status and ZAP-70 expression. In agreement with Wiestner et al (2003) ZAP-70 also correlated with V_H gene mutation status. This suggests that analysis of all 3

prognostic markers, V_H gene status, CD38 and ZAP-70, would be more informative to the clinician of the patient's prognosis than any one marker in isolation. The discordance between the three prognostic markers highlights the problems when using arbitrary thresholds for defining positive or negative status. The data in this study suggests that each prognostic marker should be regarded as a continuous variable to provide more information to the clinician.

CD38 expression was associated with Binet Stage B/C, a lymphocyte doubling time of <12 and the cytogenetic abnormalities 17p deletion (p53 mutation) and 11q- (mutation of the ATM gene). All of these factors are indicative of aggressive disease with poor clinical outcome (Mehes 2005;Shanafelt, Geyer, & Kay 2004) which strengthens the idea that CD38 expression is a poor prognostic marker in CLL.

Chapter 4: Microarray analysis of $CD38^+$ and $CD38^-$ cells derived from the same patient.

CD38 is an important prognostic marker in CLL (Ghia et al. 2003;Hamblin et al. 2002), but to-date there is no biological rationale for the association between CD38 and poor prognosis. In order to address this issue directly, an investigation of the transcriptional signatures of purified $CD38^+$ and $CD38^-$ cells derived from the same patient was carried out.

Aims: To investigate whether $CD38^+$ cells have a different transcriptional signature to their $CD38^-$ counterparts.

Hypothesis: $CD38^+$ cells will have a differential transcriptional signature to their $CD38^-$ counterparts.

$CD38^+$ and $CD38^-$ cells were physically cell sorted from samples derived from individual patients using a MoFlo high speed cell sorter. This approach ensured that downstream microarray experiments were performed on highly purified (>98%) sub-populations of CLL cells. In this way, the intrinsic inter-patient variation in global gene expression would be negated and the risk of contamination of the CLL cell gene signature by other peripheral blood mononuclear cells was effectively eliminated.

To determine the characteristics of the cell sorted sub-populations, their light chain expression and V_H gene sequences were investigated. Light

chain restriction was measured by flow cytometry. Percentage V_H gene sequence homology to the closest germline sequence and V_H gene segment usage was determined by DNA analysis using the previously described BIOMED-2 Standardized Primers and Protocols (Matthews et al. 2004) followed by direct sequence analysis using Igblast (http://www.ncbi.nlm.nih.gov/igblast/) of amplified DNA derived from the same patient's sorted $CD38^+$ and $CD38^-$ sub-populations. The paired $CD38^+$ and $CD38^-$ cells from each patient showed the same light chain restriction i.e. they expressed either κ or λ light chains and therefore the cells were deemed monotypic. In addition, the paired $CD38^+$ and $CD38^-$ cells from each patient had identical V_H gene sequences and were therefore monoclonal (Table 4.1). This indicated that, at least in this limited cohort, the $CD38^+$ and $CD38^-$ cells derived from the same patient were derived from a single clone that had undergone a malignant transformation and the only detectable difference between them was the absence or presence of the expression of the CD38 antigen.

Patient #	Light chain restriction		% sequence homology and V_H gene usage	
	$CD38^+$	$CD38^-$	$CD38^+$	$CD38^-$
1	λ	λ	98% (1-69)	98% (1-69)
2	κ	κ	94% (4-34)	94% (4-34)
3	λ	λ	95% (3-21)	95% (3-21)
4	λ	λ	91% (3-23)	91% (3-23)
5	κ	κ	89% (4-59)	89% (4-59)
6	λ	λ	99% (3-72)	99% (3-72)
7	κ	κ	100% (1-18)	100% (1-18)
8	λ	λ	99% (2-70)	99% (2-70)

Table 4.1: Light chain restriction and sequence homology for 8 patients with bimodal expression of CD38
For each patient the light chain restriction was investigated by flow cytometry and was found to be the same for both $CD38^+$ and $CD38^-$ cells. In addition, the paired $CD38^+$ and $CD38^-$ cells had identical V_H gene sequences thereby revealing their monoclonal pedigree.

Microarray analyses were carried out using U133A gene chipsets which contain 22,283 human gene sets (Agilent technologies 2006). Highly purified (>98%) $CD38^+$ and $CD38^-$ cells were analysed on separate gene chips. Prior to individual gene analysis, a global normalization technique was employed which involved the determination of the mean gene expression on each chip and this value was used as the denominator to produce a normalized value for each gene. This was followed by normalization for each gene in all the samples. The median value was determined for each gene and then for each sample the gene expression was divided by the median value. There are many techniques for analysing microarray results, for example, Statistical tests such as ANOVA (analysis of variance) and *t*-tests and SAM (significance analysis of microarray) analysis (Gale et al. 2005;Tusher, Tibshirani, & Chu 2001). In this study,

the results were analysed using Genespring software (Agilent Technologies) and two different methods of analysis were employed. The first method used two statistical tests; ANOVA and the student's *t*-test. The second method used a technique specifically designed for microarray analysis called SAM.

4.1 Statistical Method 1 (SM1)

Any genes that were not present or deemed marginal (Affymetrix 2006) in at least one of the samples (15122 genes remained) as employed by Gale *et al* (2005), or genes that did not have a ≥ 2 fold difference in transcription between $CD38^+$ and $CD38^-$ subsets derived from the same patient were removed from the gene list (1383 genes remained) as per the method previously used by Schmelz *et al* (2005) and Hüttmann *et al* (2006).

An ANOVA was carried out followed by a Student's *t*-test on the remaining 131 genes. For both tests $P \leq 0.05$ was considered to be statistically significant, differences in gene transcription with P values greater than 0.05 were removed from the subsequent gene list. The 62 genes that remained following this process are shown in the gene cluster heat map in figure 4.2. A red colour denotes genes that were relatively highly expressed in $CD38^+$ CLL cells and a green colour represent genes that were relatively under expressed in $CD38^+$ CLL cells when compared with their $CD38^-$ counter-parts. The $CD38^+$ samples clustered together and the $CD38^-$ samples clustered together suggesting that the gene transcription

signature associated with the expression of the CD38 antigen was consistent between patients. The raw data from these analyses can be found via the internet (http://www.ncbi.nlm.nih.gov/geo/) with the following accession numbers: GSM146300-GSM146335.

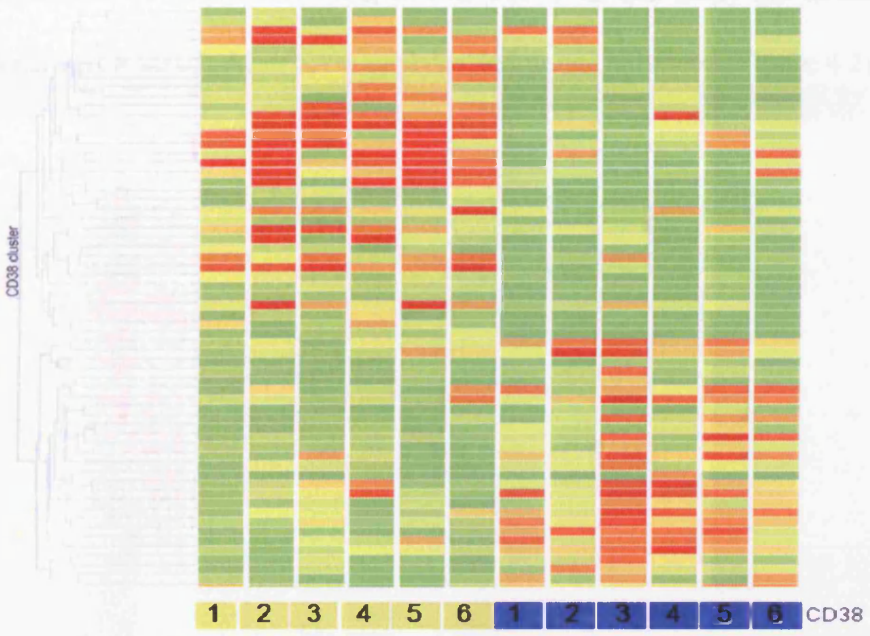


Figure 4.1 Gene cluster heat map showing the final 62 genes that are differentially expressed between $CD38^+$ and $CD38^-$ cells from the same patient.
The heat map showed that the $CD38^+$ and $CD38^-$ samples clustered together and expressed a relatively consistent differential gene expression profile. The red represents genes that were relatively highly expressed and the green represents genes that were relatively under expressed in $CD38^+$ CLL cells when compared with $CD38^-$ cells derived from the same patient.

4.2 Statistical Method 2 (SM2)

All the genes that were not present or deemed marginal in at least one of the samples were removed from the gene list (15122 genes remained). The remaining genes were analysed using SAM (significance analysis of

microarray) which was employed by Hüttmann *et al* (2006). This method was specifically designed for microarray analysis (Tusher, Tibshirani, & Chu 2001). In contrast to SM1, SAM analyses all the genes and designates a threshold level at which the differences in gene transcription are deemed significant (Tusher, Tibshirani, & Chu 2001). In this study the threshold level was P ≤0.05. After SAM analysis 467 genes remained (Figure 4.2)

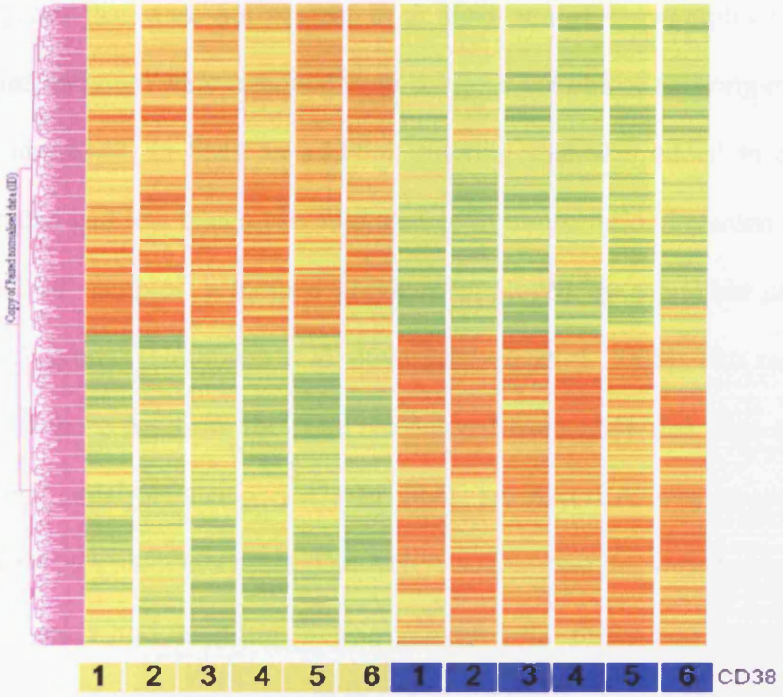


Figure 4.2 Gene expression profiling of $CD38^+$ and $CD38^-$ cells derived from the same patient
467 genes were differentially expressed between the $CD38^+$ and $CD38^-$ sub-clones derived from the same patient. The red represents genes that were relatively highly expressed and the green represents genes that were relatively under-expressed in $CD38^+$ CLL cells when compared with paired $CD38^-$ CLL cells.

In accordance with SM1, the samples revealed a clear gene expression pattern with the $CD38^+$ samples clustering together and the $CD38^-$ samples clustering together. However, following SAM analysis 467 genes were

considered to be differentially expressed between the $CD38^+$ and $CD38^-$ sub-clones derived from the same patient.

4.3 Comparison of Statistical Methods 1 and 2

The genes that were not present in at least one of the samples or were deemed marginal were removed from the gene list before carrying out both SM1 and SM2. In SM1 an addition filtering step was added to remove genes that had less than a two fold change in transcription between $CD38^+$ and $CD38^-$ samples; a method previous employed by a number of other research groups (Huttmann et al. 2006;Schmelz et al. 2005). This removed some of the genes from the gene list derived from SM1 that were present following analysis using SM2. In SM1 an ANOVA was carried out followed by a student's *t*-test. The ANOVA analysed the variance between all the genes and the *t*-test then investigated the difference between the $CD38^+$ and $CD38^-$ genes. In SM2 SAM analysis was carried out which looked at the genes and determines a threshold at which the genes are deemed significant. After SM1 62 genes were differentially expressed between the $CD38^+$ and $CD38^-$ sub-clones whereas 467 genes were differentially expressed in $CD38^+$ and $CD38^-$ sub-clones after SM2. The first method appears more stringent as the genes had to pass two $P \leq 0.05$ filters and consequently fewer genes were present after the analyses. Out of the 62 genes that were present after SM1, 61 genes were also found in the

list of 467 genes after SM2. The one gene missing from SM2 (but present in SM1) had very significant individual P-values for patients #2 and #3 which allowed it to pass the ANOVA and Student's *t*-test filters but since SAM uses different algorithms, the highly non-significant individual P-values for patients #1, #4, #5 and #6 did not allow this gene (thromboxane A2 receptor isoform 2) to pass its significance threshold. The question remains as to whether the genes missing from SM1 (but present in SM2), or the gene in SM1 (but missing from SM2) are biologically significant? However, this question was not addressed in this present study due to time constraints.

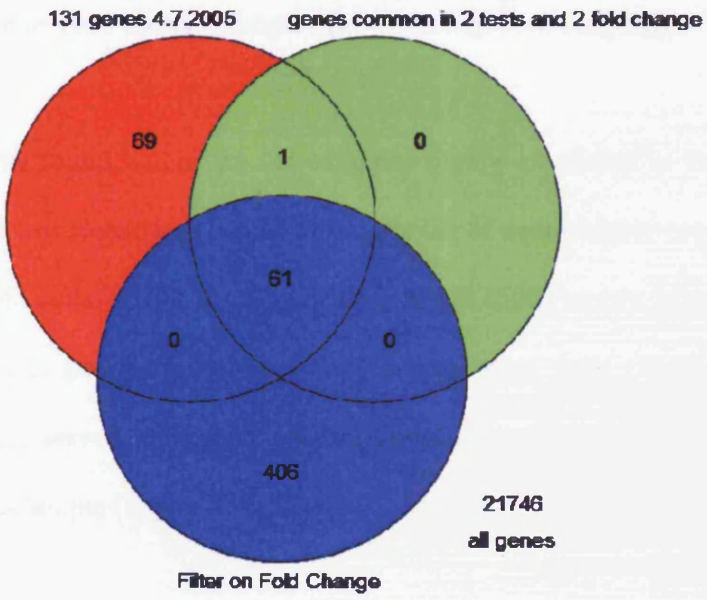


Figure 4.3 Venn diagram showing the comparison of the genes that were present after SM1 and SM2.
The red circle shows the 131 genes that were present after the ANOVA in SM1. The green circle shows the 62 genes that were present after the ANOVA and the student's *t* test (SM1) and the blue circle shows the 467 genes that were present after SAM analysis (SM2). The intersection of the three circles reveals that 61 genes were common to both the SM1 and SM2 analyses.

4.4 Pathway Architect

The gene list containing the 61 genes present after both statistical methods was exported to the software programme called Pathway Architect 1.1.0 (Stratagene). Two pathways were drawn; a pathway using all the genes relatively over-expressed in the $CD38^+$ cells (Figure 4.5A) and a pathway using all the genes that were relatively over-expressed in the $CD38^-$ cells (Figure 4.5B). Pathway Architect performs a literature review on the imported gene list via Pubmed (http://www.ncbi.nlm.nih.gov/Pubmed) and using this information draws an appropriate pathway. The arrows represent genes that are directly associated with one another either by activation, de-activation or via a protein complex (given in Figure 4.4 legend).

CD38 was found within the list of genes highly expressed in the $CD38^+$ cells and was reassuringly absent from the list of genes highly expressed in the $CD38^-$ cells. CD38 gene transcription was significantly higher in the $CD38^+$ cells (mean fold change; 9.1) compared to their $CD38^-$ counter-parts. This served as a good internal control for the efficacy of the cell sorting technique (Figure 4.4).

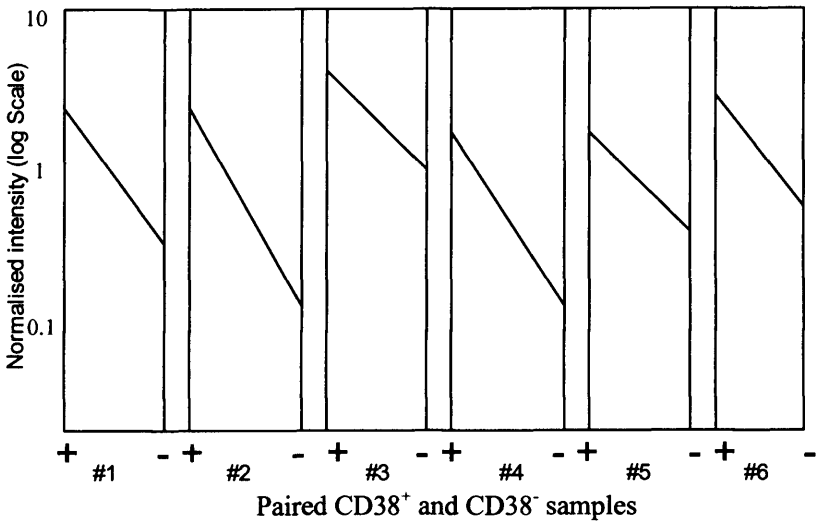


Figure 4.4 Diagram showing the expression levels of CD38 in the $CD38^+$ and $CD38^-$ samples from each of the patients.
In each case CD38 gene transcription was significantly higher in the $CD38^+$ sample (on the left) when compared to the paired $CD38^-$ sample (on the right) for each patient. This finding confirms the success of the cell sorting procedure and shows the relative consistency of the gene expression differences between $CD38^+$ and $CD38^-$ CLL cells derived from different patients.

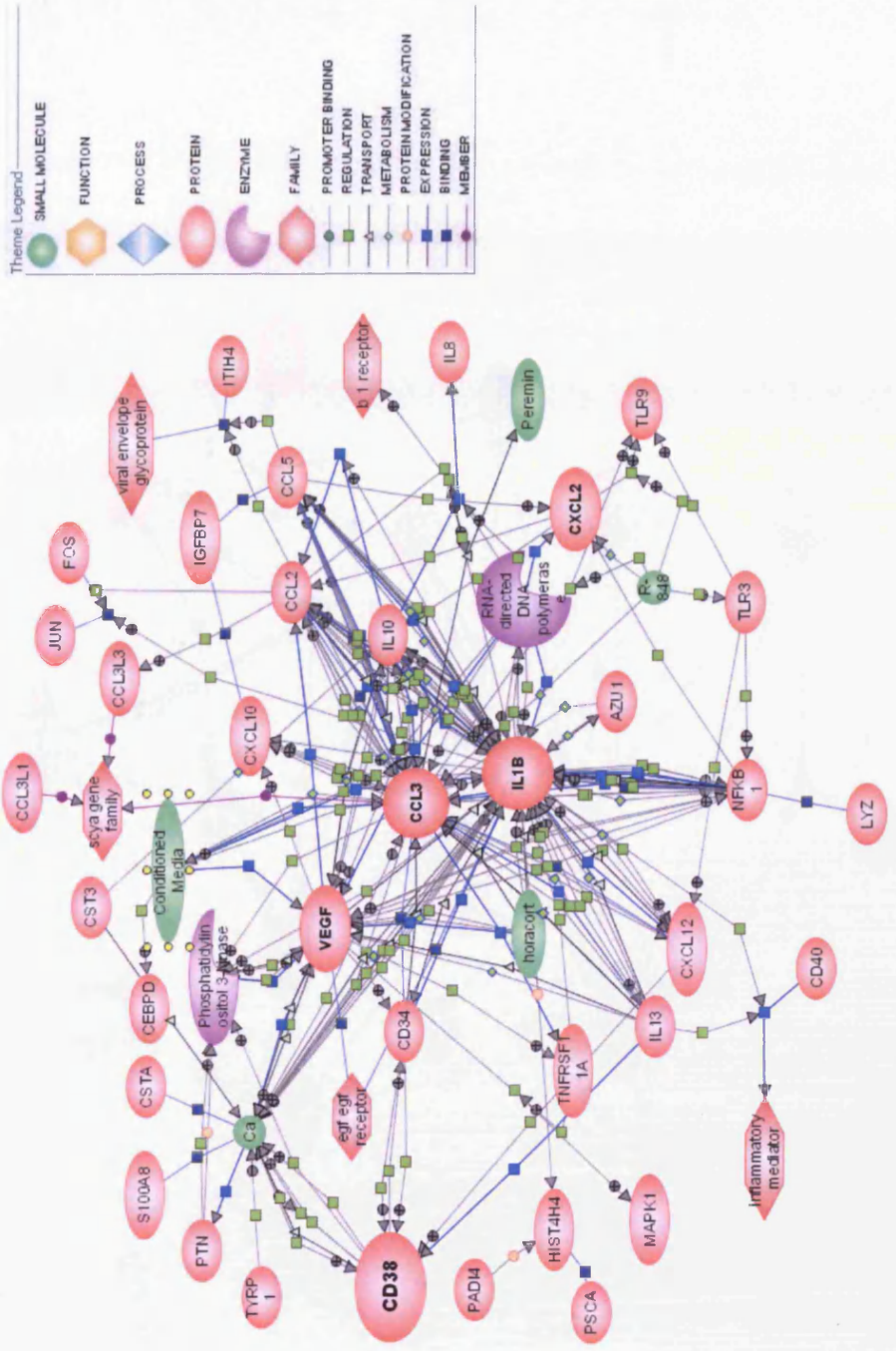


Figure 4.5A Pathway produced from the genes highly expressed in the CD38$^+$ cells when compared to their CD38$^-$ counter-parts

CD38$^+$ and CD38$^-$ cells from the same patient were analysed by microarray. The gene list produced after SM1 and SM2 was imported into Pathway Architect and the genes that were relatively over-expressed in the CD38$^+$ cells were analysed for direct associations. This was achieved by interrogating PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed). Subsequently, a pathway with the genes that were directly associated with one another was produced.The significance of the colours and shapes are described in the theme legend.

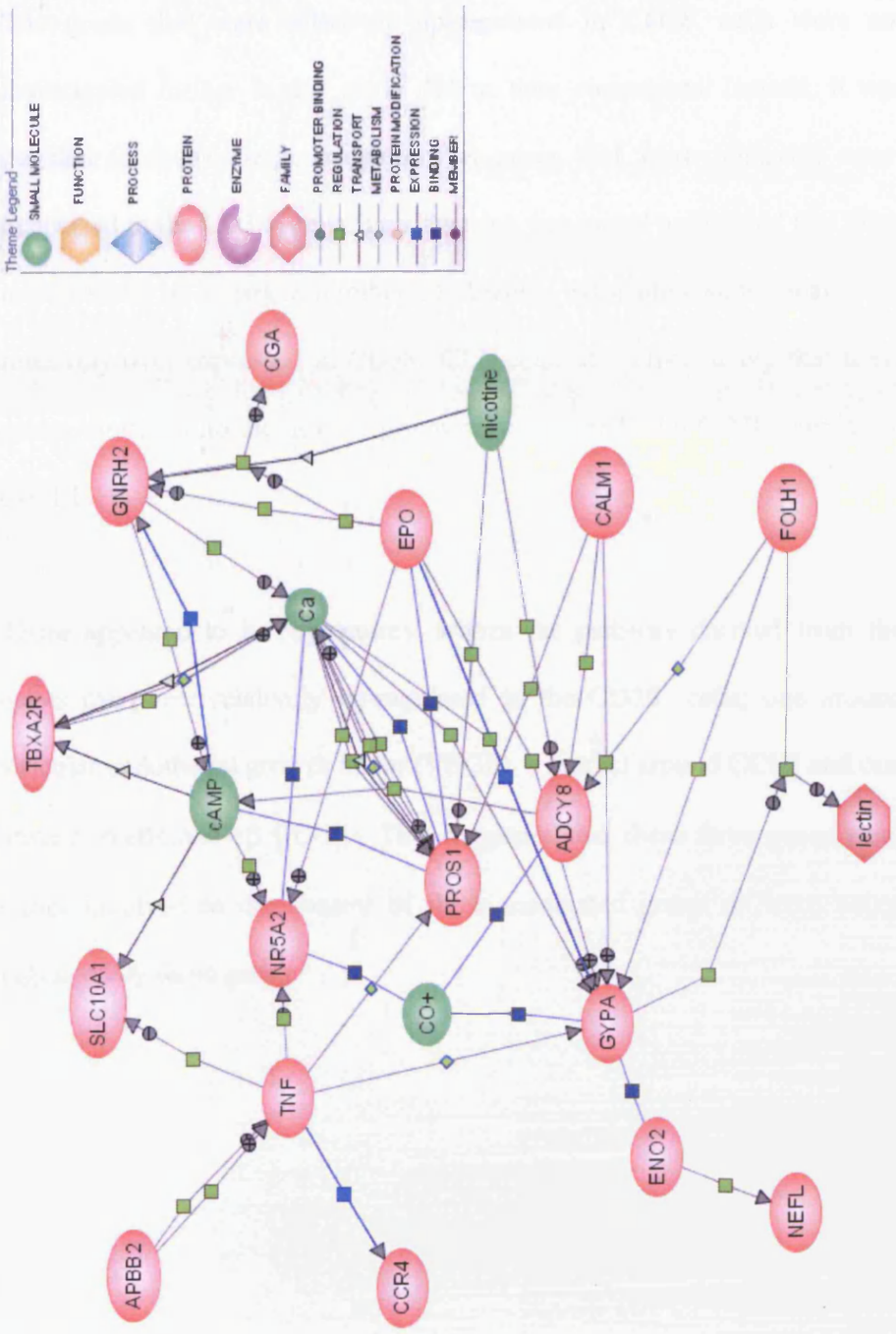


Figure 4.5B Pathway produced from the genes highly expressed in the CD38$^-$ cells when compared to their CD38$^+$ counter-parts

CD38$^+$ and CD38$^-$ cells from the same patient were analysed by microarray. The gene list produced after SM1 and SM2 was imported into Pathway Architect and the genes that were relatively over-expressed in the CD38$^-$ cells were analysed for direct associations. This was achieved by interrogating PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed). Subsequently, a pathway with the genes that were directly associated with one another was produced.The significance of the colours and shapes are described in the theme legend.

The genes that were relatively up-regulated in $CD38^-$ cells were not investigated further in this study due to time constraints. Instead, it was decided to focus the investigation on genes that were relatively over-expressed in the $CD38^+$ cells. This decision was based on the fact that there appeared to be a larger number of directly associated genes that were relatively over-expressed in $CD38^+$ CLL cells. It seemed likely that these genes contribute to the inferior prognosis associated with CD38 expression in CLL.

There appeared to be 'epicentres' within the pathway derived from the genes that were relatively up-regulated in the $CD38^+$ cells; one around vascular endothelial growth factor (VEGF), a second around CCL3 and one around interleukin-1β (IL-1β). This suggested that these three genes were either involved in the control of these associated genes or were being regulated by these genes.

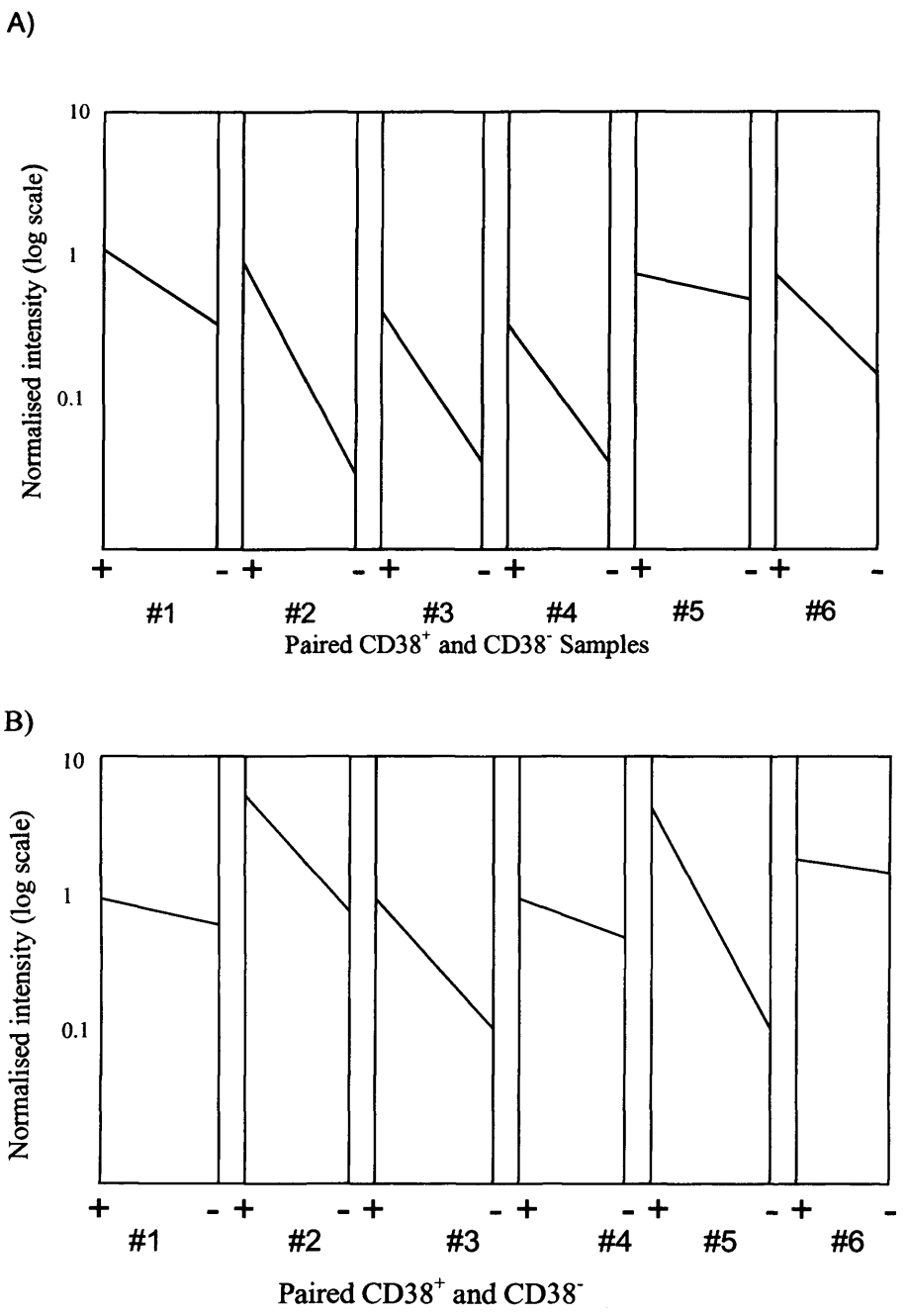


Figure 4.6: Diagram showing the expression levels of (A) VEGF and (B) IL-1β in the $CD38^+$ and $CD38^-$ paired samples from each of the patients.
In every case VEGF and IL-1β were relatively over-expressed in the $CD38^+$ samples (on the left) when compared to the paired $CD38^-$ samples (on the right).

VEGF has been associated with tumour vascularisation in the bone marrow of CLL patients and has been shown to be expressed at high concentrations in some CLL cells. (Ferrajoli et al. 2001). In this regard, McCabe et al (

2004) also suggested a link between CD38 expression and VEGF expression levels. IL-1β is well known to be associated with VEGF along with the cytokines CXCL2 and CCL3 (Saijo et al. 2002). These four genes were of particular interest due to the connections already made between these genes and CLL, and they were found to be highly expressed in the $CD38^+$ cells; VEGF (mean fold change; 5.8. 14/62 genes from SM1 gene list and 15/467 genes from SM2 gene list), IL-1β (mean fold change; 4.1. 21/62 from SM1 gene list and 36/467 genes from SM2 gene list), CXCL2 (mean fold change; 9.4. 3/62 from SM1 gene list, 3/467 genes from SM2 gene list), and CCL3/MIP-1α (mean fold change; 5.4. 16/62 from SM1 gene list and 17/467 genes from SM2 gene list). These four genes were found to be directly associated as shown in Figure 4.7.

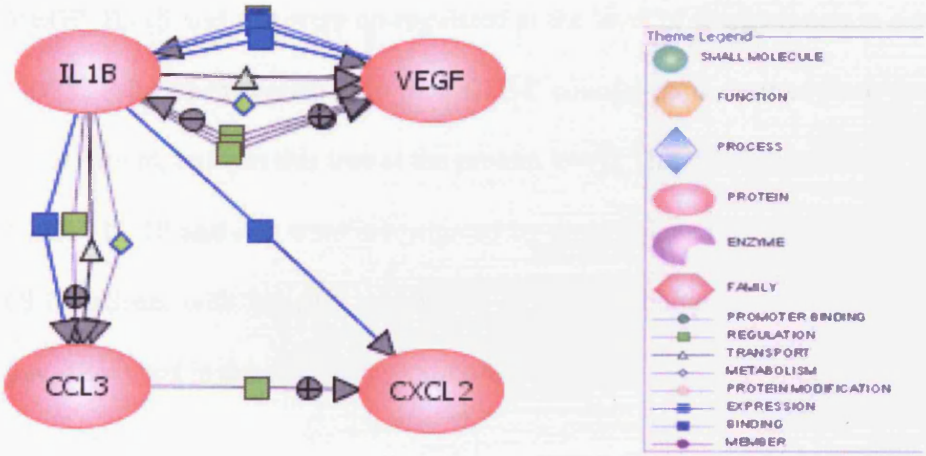


Figure 4.7: Pathway showing the direct interaction between these 4 genes. This pathway was derived from Pathway Architect showing the direct interactions between VEGF, IL-1β, CXCL2 and CCL3. These four genes were highly expressed in the $CD38^+$ sub-clones when compared to their $CD38^-$ counter-parts. The significance of the colour of the shapes and lines is described in the theme legend.

Akt and ZAP-70 were also found to be highly expressed in $CD38^+$ cells when compared to $CD38^-$ cells; Akt (mean fold change; 2.5. 139/467 from

SM2 gene list) and ZAP-70 (mean fold change; 2.2. 214/467 from SM2 gene list). Akt is up-regulated in CLL cells and is associated with resistance to apoptosis (Hay 2005) and its reported constitutive activation in CLL cells (Cuni et al. 2004) encouraged a more thorough investigation of its expression in $CD38^+$ CLL cells. ZAP-70, an important prognostic marker in CLL (Rassenti et al. 2004), was also shown to be relatively over-expressed in $CD38^+$ CLL cells. This again encouraged further investigation.

4.5 Protein Validation

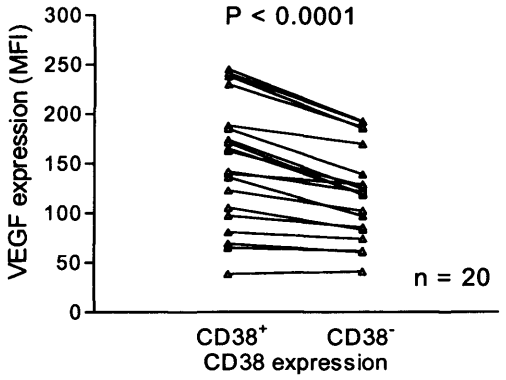
4.5.1 Analysis of VEGF, IL-1β, Akt in paired $CD38^+$ and $CD38^-$ samples

VEGF, IL-1β and Akt were up-regulated at the level of transcription in the $CD38^+$ cells when compared to their $CD38^-$ counter-parts derived from the same patient, but was this true at the protein level? The expression levels of VEGF, IL-1β and Akt were investigated by three colour flow cytometry in CLL patients with bimodal expression of CD38 (including all 6 patients that were used in the microarray experiments).

There was a wide range of expression of VEGF, IL-1β and Akt in $CD38^+$ and $CD38^-$ samples derived from the same patient. The level of expression of VEGF ranged from 38.5 – 244.8 mean fluorescence intensity (MFI) units, IL-1β ranged from 6.1 – 41.6 MFI units and Akt ranged from 61.3 – 276.2 MFI units. Using paired Student's *t*-test analyses, the expression of

VEGF, IL-1β and Akt were all found to be significantly higher in $CD38^+$ cells when compared to $CD38^-$ cells derived from the same patient ($P < 0.0001$, $P = 0.0002$ and $P < 0.0001$ respectively).

A



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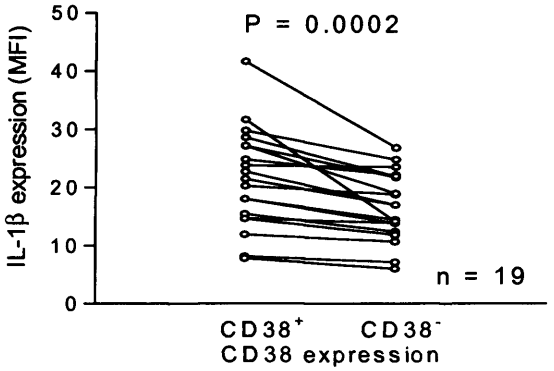

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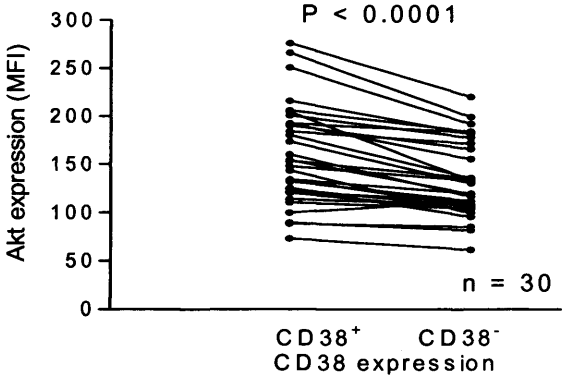


Figure 4.8 Protein expression in $CD38^+$ and $CD38^-$ cells derived from the same patient

$CD38^+$ and $CD38^-$ cells from patients with bimodal expression of CD38 were investigated for (A) VEGF expression (B) IL-1β expression and (C) Akt expression by flow cytometry. In each case the $CD38^+$ cells had significantly higher protein expression than their $CD38^-$ counter-parts as determined by paired Student's *t*-test. The horizontal lines in each plot denote the median value for each cell population.

4.5.2 ZAP-70 analysis in $CD38^+$ and $CD38^-$ sub-clones derived from the same patient

ZAP-70 gene transcription was found to be relatively over-expressed in the $CD38^+$ cells when compared to their $CD38^-$ counter-parts. ZAP-70 is an important prognostic marker within CLL and its expression is associated with poor prognosis and shorter overall survival than patients with no ZAP-70 expression (Schroers et al. 2005;Wiestner et al. 2003). It has also been shown that ZAP-70 is associated with activated B-cells and cells containing ZAP-70 have the ability to signal more effectively via the BCR (Chen et al. 2006). ZAP-70 expression was investigated in $CD38^+$ and $CD38^-$ cells derived from 36 individual CLL patients by triple colour flow cytometry (ZAP-70-Alexa fluor 488, CD38-PE and CD19-APC). Regions were drawn around the $CD38^+$ and $CD38^-$ CLL cells and these regions were forward gated into CD19/ZAP-70 dot plots.

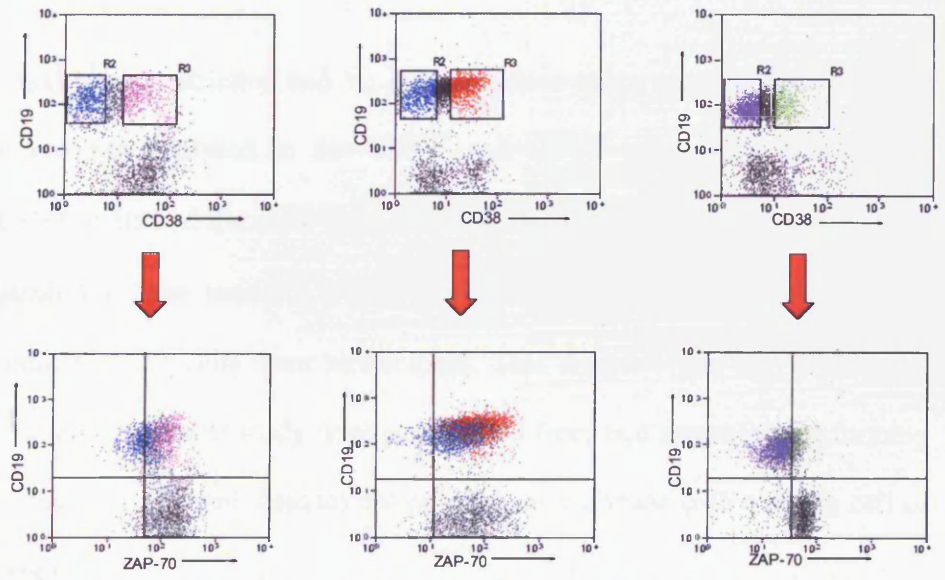


Figure 4.9: ZAP-70 expression in $CD38^+$ and $CD38^-$ cells from 3 bimodal CLL patients (Pepper et al. 2006)
CLL patients with bimodal expression of CD38 showed differential expression of ZAP-70 in $CD38^+$ (R3 gated cells in pink/red/green) and $CD38^-$ (R2 gated cells in blue and mauve) cells derived from the same patient irrespective of their percentage CD38 expression or their ZAP-70 status.

$CD38^+$ cells showed significantly higher expression of ZAP-70 when compared to their $CD38^-$ counter-parts. This was not dependent on the size of the CD38-expressing clone or the ZAP-70 status of the patient (whether they were positive (≥ 20% expression) or negative (< 20% expression)).

4.6 Conclusion

CD38 expression has been associated with poor prognosis in CLL (Ghia, Guida, Stella, Gottardi, Geuna, Strola, Scielzo, & Caligaris-Cappio 2003;Hamblin, Orchard, Ibbotson, Davis, Thomas, Stevenson, & Oscier 2002;Ibrahim et al. 2001) but to-date there is no biological rationale to explain this phenomenon. $CD38^+$ and $CD38^-$ cells derived from the same patient were analysed by microarray and were found to have characteristic differential gene expression profiles.

Light chain restriction and V_H gene mutation status and V_H gene segment usage was analysed in the $CD38^+$ and $CD38^-$ sub-groups. $CD38^+$ and $CD38^-$ cells had the same light chain restriction (either κ or λ) and had the same V_H gene mutation status and the same V_H gene segment usage indicating the cells were monoclonal. This suggests that the $CD38^+$ and $CD38^-$ cells in this study were not derived from two separate transforming events (i.e. biclonal disease) but rather from a disease with a single cell of origin.

In agreement with McCabe *et al* (2004) VEGF expression was associated with CD38 expression in this study. It has been shown that VEGF signalling through the VEGFR2 receptor results in activation of the PI3K/Akt pathway which is a cell survival pathway (Bairey et al. 2004). Since VEGF, VEGFR2 and Akt were all up-regulated in the $CD38^+$ cells, it seems likely that this contributes to the poor clinical outcome associated with CD38 expression. This concept is investigated further in Chapters 5 and 6 of this thesis.

There is no universal 'gold standard' method for the analysis of gene expression profiles derived from microarray results so in this study two techniques were used and subsequently compared. The first method applied filtering techniques (removing absent/marginal genes and removing genes that had <2 fold change) and then applied two statistical tests to the genes; ANOVA and student's *t*-test. The second method applied one filter (removing absent/marginal genes) and employed a statistical technique designed for microarray analysis called SAM. Out of the 6 genes of interest four were present using both methods. ZAP-70 and Akt were present after SM2 but not SM1 but this does not mean that they are not biologically significant. Indeed, there is a body of evident that points to a clear role for both ZAP-70 and Akt in determining the clinical course of CLL. (Rassenti et al 2004).

In concordance with the gene expression data, VEGF, IL-1β, Akt and ZAP-70 were all found to be relatively over-expressed in $CD38^+$ cells

when compared to their $CD38^-$ counter-parts. This indicated that these sub-populations have activated survival pathways such as the PI3K/Akt pathway. Furthermore, this study is in broad agreement with that of Hüttmann *et al* (2006), who showed that $CD38^+$ CLL cells relatively over express genes that are involved in angiogenesis.

$CD38^+$ cells showed consistently higher expression of ZAP-70 irrespective of the size of the CD38-expressing population or ZAP-70 positivity (whether ≥ 20% of the cells express ZAP-70). Hüttmann *et al* (2006) found that separation of patients into $ZAP\text{-}70^+/CD38^+$ and $ZAP\text{-}70^-/CD38^-$ groups could more reliably define good prognosis and poor prognosis groups than when either parameter was considered separately. This data and the data in this chapter support the conclusions from Chapter 3 of this thesis. Namely, that in the clinical setting a composite picture made up of CD38 expression, ZAP-70 expression and V_H gene mutation status provides more reliable prognostic information.

The relative over-expression of VEGF, IL-1β, CXCL2, CCL3, ZAP-70 and Akt may provide a biological rationale to why CD38 expression is associated with poor prognosis in CLL. Taken together, the data derived from these studies suggests that CD38 might be a rational therapeutic target in CLL patients as it seems likely that inhibiting/reducing the expression of this molecule may also result in the down-regulation of the genes that are relatively over-expressed in $CD38^+$ CLL cells. Ideally this notion would be

explored using RNA interference methodologies but the challenge of transfecting primary CLL cells is not trivial.

Chapter 5: Vascular endothelial growth factor

In chapter 4 vascular endothelial growth factor (VEGF) was shown to be transcriptionally activated in $CD38^+$ CLL cells when compared to $CD38^-$ CLL cells derived from the same patient. VEGF is a glycoprotein made up of two 23 kDa subunits and has been shown to play a role in cell migration, proliferation, vascular permeability, intracellular Ca^{2+} influx, and chemotaxis (Wang et al. 1999). In the context of CLL, it was recently found to be required for chemokine-dependent motility of CLL cells on and through endothelium; processes important for the invasion of lymphoreticular tissues (Till et al. 2005). Furthermore, McCabe et al (2004) showed a correlation between the expression of CD38 and VEGF indicating that VEGF may be regulated by CD38 or *vice versa*. However, their study was purely correlative and hence did not directly address this question.

Aims: To investigate the protein expression of VEGF to see whether it differed between $CD38^+$ and $CD38^-$ cells and whether there was a difference in the biological role of VEGF between the two sub-populations.

Hypothesis: VEGF will be up-regulated at the protein level in the $CD38^+$ cells when compared to their $CD38^-$ counterparts and this higher expression will help explain why $CD38^+$ cells are associated with poor clinical outcome.

There are three VEGF receptors (VEGFR): VEGFR1 (Flt-1), VEGFR2 (KDR/Flk) and VEGFR3 (Flt-4). CLL cells have been shown to express all three VEGF receptors and all are able to signal (Bairey et al. 2004). However, it appears that VEGFR2 is the key VEGFR in CLL as its ligation causes a much stronger phosphorylation response in CLL cells (Bairey, Boycov, Kaganovsky, Zimra, Shaklai, & Rabizadeh 2004;Lee et al. 2005) and its expression has been correlated with cell proliferation and survival (Lee, Shanafelt, Bone, Strege, Jelinek, & Kay 2005). In concordance with this, VEGFR2 was shown to be highly expressed in a subset of CLL patients with aggressive disease; more marked lymphocytosis, severe anaemia and shorter survival (Ferrajoli et al. 2001). Interestingly, Bairey *et al* (2004) suggested that this survival effect mediated through VEGFR2 is mediated via the PI3K/Akt pathway. It is now clear that CLL cells are capable of producing VEGF and possess functional VEGF receptors suggesting that both autocrine and paracrine loops might be important in the pathogenesis of CLL. Indeed, Farahani *et al* (2005) showed that VEGF signalling can occur through both paracrine (VEGF secreted from another cell) and autocrine (endogenous VEGF) signalling pathways in CLL cells, and this autocrine signalling produces a protective/survival effect.

Given the link between VEGF and VEGFR2 with poor prognosis in CLL, the primary aim of this investigation was to quantify the protein expression of VEGF and VEGFR2 in $CD38^+$ and $CD38^-$ CLL cells derived from the same patient. In addition, the role of both exogenous and endogenous

VEGF in the *in vitro* survival of these subsets of CLL lymphocytes was examined.

5.1 VEGF expression in $CD38^+$ and $CD38^-$ cells from patients with bimodal expression of the CD38 antigen

In chapter 4 VEGF transcription was found to be significantly higher in $CD38^+$ cells when compared to their $CD38^-$ counterparts. Since VEGF signalling through VEGFR2 is associated with shorter survival (Ferrajoli et al 2001), and McCabe *et al* (2004) found a correlation between VEGF expression and CD38 expression, it was decided to investigate whether there was a biological link between VEGF and CD38.

The level of VEGF expression was investigated in $CD38^+$ and $CD38^-$ cells derived from the same patient by flow cytometry (n = 16). The $CD38^+$ and $CD38^-$ cells were gated and the level of VEGF expression (MFI) was investigated in each sub-population (Figure 5.1A). In addition, $CD38^+$ and $CD38^-$ cells from bimodal patients were physically cell sorted (n = 4) and investigated for VEGF expression by Western blotting (4 – 12 % Bis-Tris gels and transferred for 1 hour), using the same VEGF antibody that was employed in the flow cytometry assay (Figure 5.1B). There was great inter-patient variability with the level of VEGF expression ranging from 38.5 – 244.8 MFI units (>6 fold) as measured by flow cytometry. However, $CD38^+$ cells were found to have consistently higher expression levels of

VEGF then their $CD38^-$ counterparts ($P < 0.0001$) and this was confirmed qualitatively by Western blot analysis.

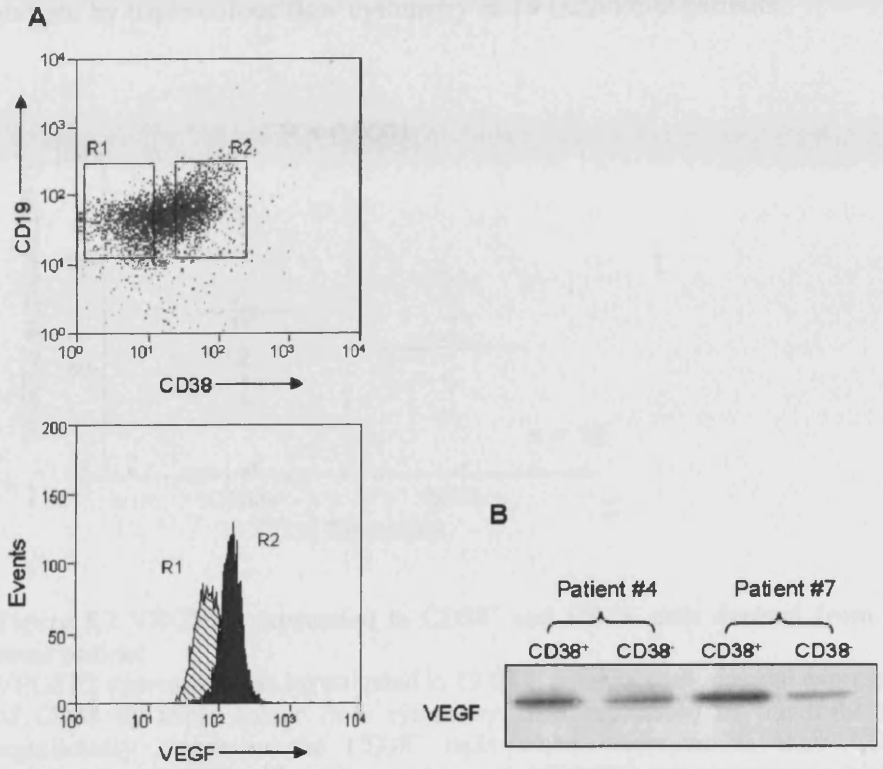


Figure 5.1: Differential expression of vascular endothelial growth factor in $CD38^+$ and $CD38^-$ sub-populations derived from the same patient (n = 16)
(A) VEGF protein expression was found to be significantly higher in $CD38^+$ CLL cells when compared with $CD38^-$ CLL cells derived from the same patient sample using flow cytometry. (B) $CD38^+$ and $CD38^-$ sub-populations were physically sorted by high speed cell sorting and were subsequently Western blotted using the same anti-VEGF antibody employed in the flow cytometry assays. $CD38^+$ CLL cells showed higher expression of VEGF than their paired $CD38^-$ counter-parts.

A number of studies have shown the importance of VEGFR2 expression in predicting aggressive disease and shorter survival in CLL (Ferrajoli et al 2001). In addition, Farahani *et al* (2005)showed that VEGF signalling can occur via both paracrine and autocrine pathways. Therefore, this study investigated the expression of VEGFR2 in $CD38^+$ and $CD38^-$ sub-populations and tried to elucidate whether these distinct sub-populations of

CLL cells responded differently to autocrine and paracrine VEGF signalling. VEGFR2 expression was quantified in $CD38^+$ and $CD38^-$ sub-populations from CLL patients with bimodal expression of the CD38 antigen by triple colour flow cytometry in 19 individual patients.

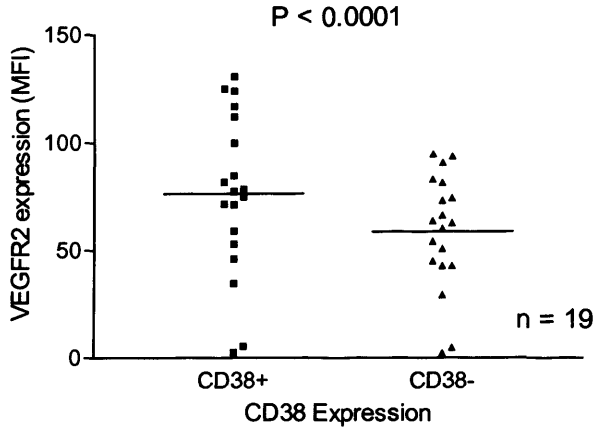


Figure 5.2 VEGFR2 expression in $CD38^+$ and $CD38^-$ cells derived from the same patient
VEGFR2 expression was investigated in 19 CLL patients with bimodal expression of CD38 by triple colour flow cytometry. The expression of VEGFR2 was significantly higher in the $CD38^+$ cells when compared to their $CD38^-$ counterparts. Horizontal bars denote the mean VEGFR2 expression in each CLL sub-population.

There was a wide range of VEGFR2 expression between patients ranging from almost no VEGFR2 expression (2.3 MFI units) to high expression of VEGFR2 (130.7 MFI units). The mean level of expression of VEGFR2 was 76.3 MFI units for the $CD38^+$ cells and 58.7 MFI units for the $CD38^-$ cells. VEGFR2 expression was significantly higher in $CD38^+$ cells when compared to $CD38^-$ cells from bimodal patients ($P < 0.0001$). Therefore, $CD38^+$ CLL cells relatively over expressed both endogenous VEGF and VEGFR2. However, it was unclear whether this expression pattern was

biologically important so a series of experiments were undertaken to elucidate this.

5.2: *In vitro* survival analysis of $CD38^+$ and $CD38^-$ cells

$CD38^+$ and $CD38^-$ cells were physically cell sorted using the MoFlo high speed cell sorter (10,000-30,000 cells/second), and then set up in culture to analyse whether the over-expression of VEGF was biologically important to $CD38^+$ CLL cells. Control cultures were also analysed in which the $CD38^+$ and $CD38^-$ cell sorting gates were combined to yield a mixed 'unsorted' population that had been subjected to the same cell sorting stresses. The levels of apoptosis was analysed in cell sorted samples derived from 6 individual CLL patients by flow cytometry using changes in forward scatter (giving an index of size of the cells) and side scatter (which measures granularity). Typically apoptotic cells are smaller with consequently higher granularity than viable cells. Employing this method for gating apoptotic cells allowed the simultaneous analysis of VEGF expression in the viable cultured CLL cells. Quantification of apoptosis was confirmed in parallel experiments using the Annexin V / propidium iodide assay. In all the cultures tested, purified $CD38^+$ cells showed the lowest percentage apoptosis at each time point. The $CD38^-$ cells showed the lowest viability and the 'unsorted' populations were less viable than the $CD38^+$ cells but more viable than the $CD38^-$ cells (Figure 5.3A).

In addition, the expression of VEGF was measured in the viable cells from each of the cell cultures by dual colour flow cytometry (VEGF-FITC / CD19-APC). The viable cells were gated using forward scatter and side scatter which was then forward gated into each $CD19^+$ dot plot so that only the viable $CD19^+$ cells were investigated. VEGF expression (MFI) was then quantified using gated histograms. Figure 5.3B shows the VEGF expression in the $CD38^+$ CLL cells (183.6 MFI units) the $CD38^-$ CLL cells (69.2 MFI units) and the 'unsorted' population (97.4 MFI units) following 3 days in culture. At all the time points measured VEGF was consistently higher in $CD38^+$ CLL cells when compared to $CD38^-$ CLL cells ($P = 0.001$) or the 'unsorted' cultures ($P = 0.003$).

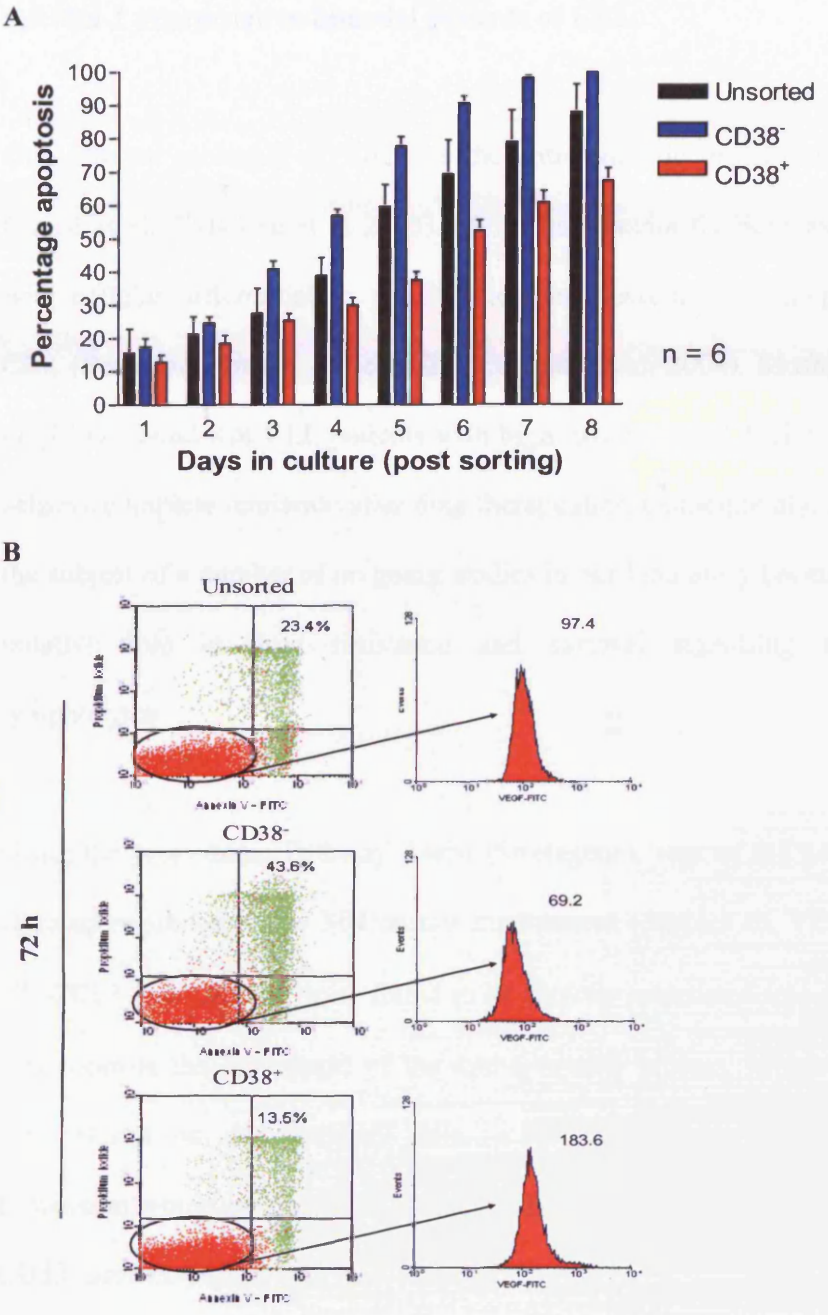


Figure 5.3: The association of VEGF with survival in CD38$^+$ and CD38$^-$ cells
(A) Cells were physically cells sorted using the MoFlo cell sorter and set up in culture. The percentage apoptosis was measured per day in each group over an eight day period by flow cytometry using forward scatter and side scatter. Apoptosis was also measured using the Annexin V / propidium iodide assay. (B) VEGF expression (MFI) was investigated by flow cytometry in the viable cells from the purified cultures at each time point. Cells were labelled with VEGF-FITC and CD19-APC and the viable cells were gated in the forward scatter/ side scatter plot. This region was then forward-gated into a CD19 dot plot. Subsequently, the level of expression of VEGF was analysed in each group. The figure shows the relative expression of VEGF in all three cultures following 3 days in culture.

5.3 Mcl-1 expression in bimodal patients of CLL

One effector molecule of VEGF is the anti-apoptotic protein Mcl-1 (Le Gouill et al. 2004;Lee et al 2005). Mcl-1 has previously been associated with cellular differentiation, proliferation and resistance to apoptosis in CLL (Pederson, Kitada, & Leoni 2002;Saxena et al. 2004). Moshynska *et al* (2004) found that CLL patients with high expression of Mcl-1 failed to achieve complete remission after drug therapeutics. Consequently, Mcl-1 is the subject of a number of on-going studies in our laboratory because of its putative role in drug resistance and survival signalling in CLL lymphocytes.

Using the programme Pathway Assist (Stratagene), four of the genes that were up-regulated in the Microarray experiments (chapter 4); VEGF, IL-1β, CCL3 and CXCL2, were found to be directly associated in a pathway that controls the expression of the anti-apoptotic protein, Mcl-1 (Figure 5.4). Based on this pathway data, it was hypothesised that Mcl-1 expression would be higher in $CD38^+$ CLL cells when compared to their $CD38^-$ counter-parts.

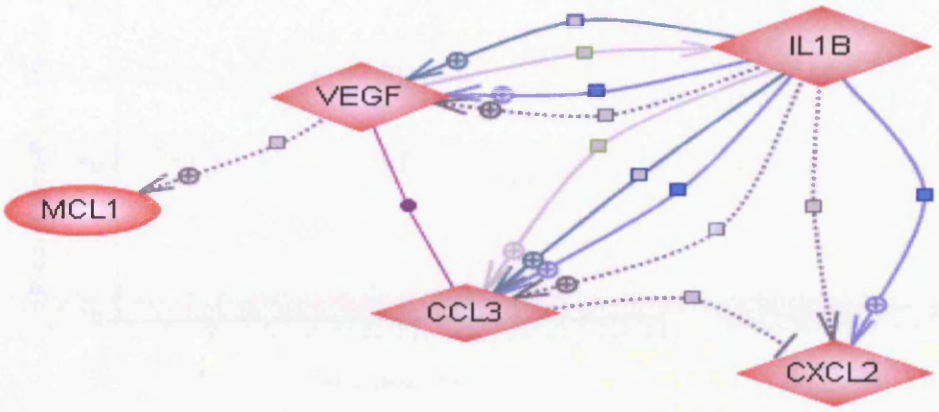


Figure 5.4: The control of Mcl-1 by the pathway involving VEGF, IL-1β, CCL3 and CXCL2
VEGF, IL-1β, CCL3 and CXCL2 were directly associated in a pathway that controls the expression of the anti-apoptotic protein Mcl-1.

In a separate study in our laboratory, Mcl-1 expression was determined by flow cytometry in 142 CLL patients. The patient cohort was then divided into 2 groups; those with Mcl-1 expression above the mean Mcl-1 expression (>1514 molecules of equivalent soluble fluorochrome (MESF)) and those patient with Mcl-1 expression below the mean Mcl-1 expression (<1514 MESF). The PFS of these two groups was then determined. Patients with high Mcl-1 expression had a significantly shorter PFS than those patients with low Mcl-1 expression ($P = 0.002$) indicating that Mcl-1 expression is prognostic within this CLL patient cohort (Figure 5.5).

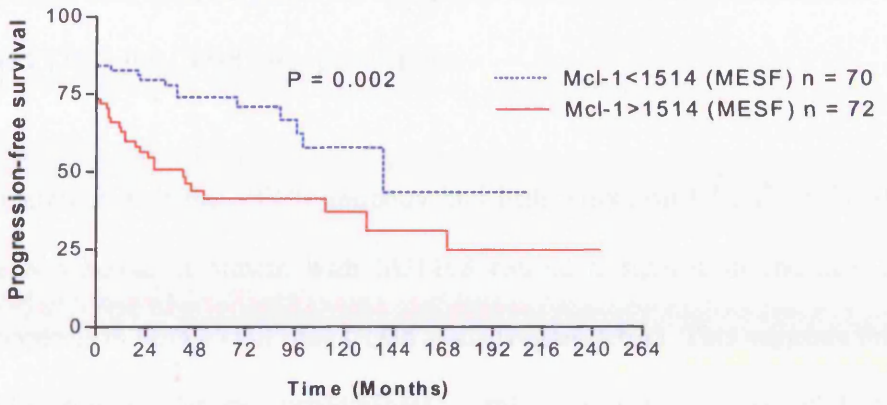


Figure 5.5 Mcl-1 expression in CLL patients (Unpublished data)
Progression-free survival for patients with high expression of Mcl-1 (>1514 MESF) and low expression of Mcl-1 (<1514 MESF). The patients with high expression of Mcl-1 had significantly shorter progression-free survival than those patients with Mcl-1 expression below the mean for the entire cohort (P = 0.002).

5.4 Inhibition of VEGF function

McCabe *et al* (2004) showed that CD38 expression correlates with VEGF expression. In addition, CLL cells have been shown to constitutively produce VEGF in culture and VEGF signalling provides some cytoprotection to CLL cells (Chen et al. 2000). In this study, the effect of inhibition of VEGF on cell survival was investigated in $CD38^+$ and $CD38^-$ sub-populations derived from 3 different patients with bimodal expression of the CD38 antigen. Cells were cultured for 24h with or without 1μg/ml of an anti-VEGF neutralising antibody and/or 10μM of the VEGF signalling inhibitor SU1498, which is a potent and selective inhibitor of vascular endothelial growth factor receptor 2 kinase activity (Strawn et al. 1996). As VEGF has been shown to signal via both paracrine and autocrine signalling mechanisms (Farahani et al. 2005), both the anti-VEGF neutralising antibody and the VEGF inhibitor SU1498 were employed to establish

whether autocrine or paracrine signalling was the predominant effector in the $CD38^+$ and $CD38^-$ sub- populations.

Treatment with the VEGF antibody had little effect on $CD38^+$ or $CD38^-$ cells, whereas treatment with SU1498 caused a significant increase in apoptosis in both $CD38^+$ and $CD38^-$ cells (Figure 5.6A). This suggests that both sub-populations predominantly rely on signals generated by endogenous VEGF as SU1498 had a larger effect on survival. However, the combination of inhibitors produced the largest increase in apoptosis in both the $CD38^+$ and the $CD38^-$ cells suggesting that both sub-populations utilise endogenous and exogenous VEGF.

5.5 Cytoprotection effect of recombinant VEGF in $CD38^+$ and $CD38^-$ sub-clones

The data presented here suggest that VEGF expression plays a role in maintaining CLL cell survival *in vitro*. To investigate this further recombinant VEGF was added to cultures of $CD38^+$ and $CD38^-$ cells to see whether there was any effect on spontaneous apoptosis. $CD38^+$ and $CD38^-$ CLL cells derived from 4 different patients were cultured for 24h with or without 100ng/ml recombinant VEGF. This concentration of VEGF has previously been shown to induce cytoprotection in unsorted CLL cells by Farahani *et al* (2005). Both sub-populations showed a decrease in spontaneous apoptosis after 24h following the addition of recombinant VEGF. However, this was only statistically significant in the $CD38^-$ sub-

populations (P = 0.02) (Figure 5.6B). In order to try to rationalise this result, Mcl-1 expression was measured by flow cytometry in the $CD38^+$ and $CD38^-$ cells derived from the same patient cultured with and without the addition of recombinant VEGF for 24h. In keeping with the previous findings, Mcl-1 expression was consistently higher in the $CD38^+$ CLL cells (Chapter 7, Figure 7.3C). However, the addition of recombinant VEGF resulted in a marked increase in Mcl-1 expression in the $CD38^-$ cells and a more modest increase in the $CD38^+$ cells (Figure 5.6C).

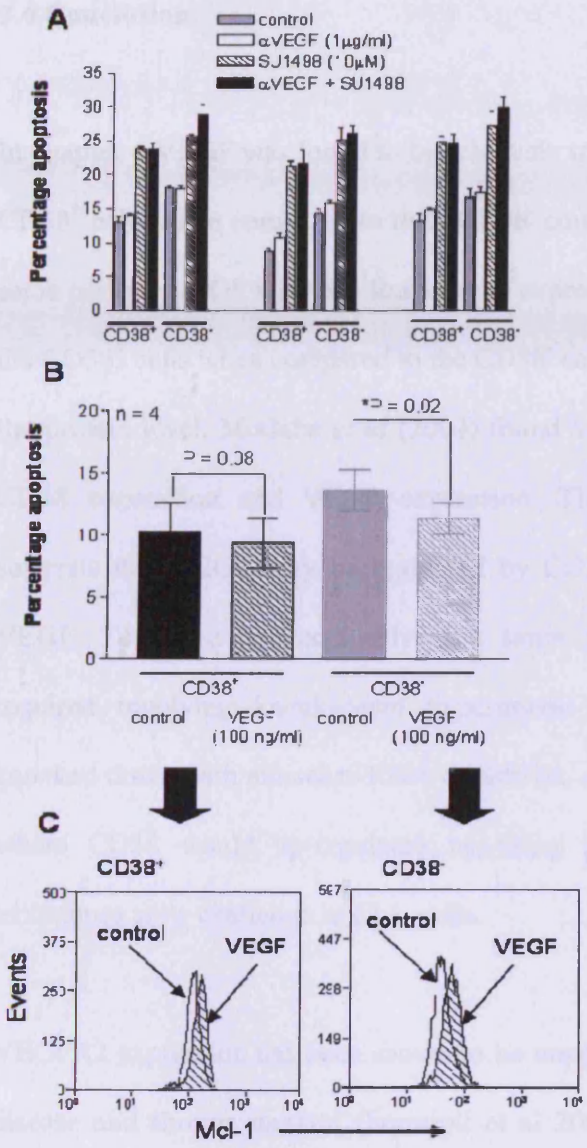


Figure 5.6: Effect of VEGF on *in vitro* CLL cell survival.
(A) $CD38^+$ and $CD38^-$ sub-populations derived from bimodal patients were cultured for 24h with or without 1μg/ml of an anti-VEGF neutralising antibody and/or 10μM of the VEGF receptor inhibitor SU1498. Treatment with the neutralising antibody had no significant effect on survival in either the $CD38^+$ or $CD38^-$ sub-populations whereas the VEGF receptor inhibitor SU1498 caused a significant decrease in cell viability in both sub-populations. (B) $CD38^+$ and $CD38^-$ sub-clones derived from 4 different patients were cultured for 24h with or without 100ng/ml recombinant VEGF. Both the $CD38^+$ and $CD38^-$ CLL cells showed a decrease in apoptosis in the VEGF-treated cultures but this only reached significance in the $CD38^-$ sub-populations (P = 0.02). (C) Mcl-1 expression was measured by flow cytometry in the sub-populations cultured with and without the addition of recombinant VEGF for 24h. Both the $CD38^+$ and $CD38^-$ sub-populations showed an increase in Mcl-1 expression following exposure to VEGF and this was most marked in the $CD38^-$ CLL cells (P = 0.01).

5.6 Conclusion

In chapter 4 VEGF was found to be relatively transcriptionally activated in $CD38^+$ cells when compared to their $CD38^-$ counter-parts derived from the same patient. VEGF was also found to be expressed significantly higher in the $CD38^+$ cells when compared to the $CD38^-$ cells from the same patient at the protein level. McCabe *et al* (2004) found a direct correlation between CD38 expression and VEGF expression. This, along with our data, suggests that VEGF may be regulated by CD38 or CD38 may regulate VEGF. To prove this conclusively a series of experiments would be required involving knock-down experiments where VEGF would be knocked down with antisense RNA or SiRNA. Also knock-in experiments where CD38 would up-regulated by using gene transfer. These two techniques are a challenge in CLL cells.

VEGFR2 expression has been shown to be important in CLL in predicting disease and shorter survival (Ferrajoli et al 2001). Farahani *et al* (2005) also showed that VEGF can signal via autocrine and paracrine pathway. In this study, VEGFR2 was found to be relatively over expressed at the protein level in the $CD38^+$ cells, but it was also found to be expressed to a lesser degree in the $CD38^-$ cells. This suggested that both sub-populations were capable of signalling via exogenous VEGF as evidenced by the experiments using recombinant VEGF in cell sorted populations of CLL cells.

Even though VEGF was shown to be relatively over expressed (both transcriptionally and at the protein level) in the $CD38^+$ cells when compared to their $CD38^-$ counter-parts, this did not indicate whether this over expression was biologically important to the $CD38^+$ CLL cells. An investigation into spontaneous apoptosis showed that $CD38^+$ cells had the lowest spontaneous apoptosis and $CD38^-$ cells had the highest levels of spontaneous apoptosis. Concomitantly, VEGF expression was found to be higher in the $CD38^+$ sub-populations, and lower in the $CD38^-$ sub-populations, indicating a correlation between VEGF expression and survival. This cytoprotection of the $CD38^+$ cells by VEGF was abrogated by the use of the inhibitor SU1498, but the anti-VEGF blocking antibody had no significant effect on the survival of either sub-populations. Addition of the recombinant VEGF caused a reduction in spontaneous apoptosis in the $CD38^-$ cells despite the fact that these sub-populations expressed less VEGFR2 than their $CD38^+$ counter-parts. This indicates that $CD38^+$ CLL cells preferentially utilise an internal autocrine survival loop whereas $CD38^-$ sub-clones are possibly more dependent on exogenous VEGF to maintain optimal cell viability as they do not produce enough endogenous VEGF. In bimodal patients, it is feasible that $CD38^+$ cells can provide exogenous VEGF for the $CD38^-$ cells. This notion is supported by the apoptosis experiments in which the unsorted population showed less spontaneous apoptosis than the purified $CD38^-$ CLL cells.

Four genes found to be highly expressed in the $CD38^+$ cells in the microarray experiments, VEGF, IL-1β, CXCL2 and CCL3, were found to

be directly associated with the control of the expression of Mcl-1. Importantly, the anti-apoptotic protein Mcl-1 has previously been shown to be a down-stream effector of VEGF signalling (Le Gouill et al 2004). Furthurmore, Mcl-1 has been shown to be associated with poor response to chemotherapy (Moshynska et al. 2004) and in an independent study within our laboratory Mcl-1 was shown to be prognostically relevant as patients with high Mcl-1 expression had shorter PFS than patients with low Mcl-1 expression. This suggests that the Mcl-1 expression may contribute to the enhanced cytoprotective phenotype exhibited by the $CD38^+$ cells, and this may be influenced by the high VEGF expression.

When recombinant VEGF was added to the $CD38^-$ cells, there was a marked elevation in their Mcl-1 expression but even this failed to achieve comparable expression to their $CD38^+$ counter-parts. This indicates that the autocrine VEGF signalling utilised the $CD38^+$ sub-populations may be a more effective inducer of Mcl-1 expression than exogenous VEGF. Alternatively, $CD38^+$ CLL cells may be subject to other Mcl-1-inducing pathways such as those triggered by BCR ligation. In this regard, some groups have shown that BCR ligation can induce Mcl-1 expression (Pepper et al. 2006;Petlickovski et al. 2005).

It has been shown that CD38 expression is higher in CLL cells in the bone marrow and lymphoid tissues compared to the peripheral blood (Ghia et al. 2003). This indicates that once the cells leave the bone marrow, the expression of CD38 may decrease and so the higher the CD38 expression

in the peripheral blood, the higher the turnover/renewal from the bone marrow and lymph nodes.

This data provides a biological rationale for the poor prognosis associated with CD38 expression in CLL. It also provides evidence that both Mcl-1 and VEGF may prove to be useful therapeutic targets in the treatment of patients with poor prognosis CLL. The control of VEGF and Mcl-1 is likely to be complex and will require further investigation.

Chapter 6: The role of the PI3K pathway.

In Chapter 4 $CD38^+$ sub-populations were shown to possess a different transcriptional signature when compared to their $CD38^-$ counter-parts derived from the same patient. Chapter 5 was in part a validation of these findings by confirming that the increased transcription of one of these genes, VEGF, resulted in elevated VEGF protein expression in $CD38^+$ CLL cells. Further validation of the microarray results is continued in this chapter by investigating the protein expression of Akt. Akt is a member of the phosphoinositol-3 kinase (PI3K) pathway, was found to be differentially expressed between $CD38^+$ and $CD38^-$ cells derived from the same patient. Interestingly, Bairey *et al* (2004) suggested that VEGF survival signalling occurs via the PI3K pathway indicating that the activation of Akt and VEGF may be biologically linked.

Aims: To determine whether the PI3K pathway is differentially regulated in $CD38^+$ CLL cells compared to $CD38^-$ CLL cells.

Hypothesis: The PI3K pathway is up-regulated in $CD38^+$ cells when compared to their $CD38^-$ counterparts, which gives the $CD38^+$ cells a survival advantage.

6.1 Validation of Microarray

Akt, a member of the PI3K pathway has previously been shown to be constitutively active within CLL cells (Cuni et al. 2004). In Chapter 4 transcription of Akt was found to be significantly higher in the $CD38^+$ cells when compared to their $CD38^-$ counter-parts by microarray analysis. To confirm this at the protein level Akt was measured by triple colour flow cytometry (Akt-FITC, CD38-PE and CD19-APC) in $CD38^+$ and $CD38^-$ cells from 31 patients with bimodal expression of CD38.

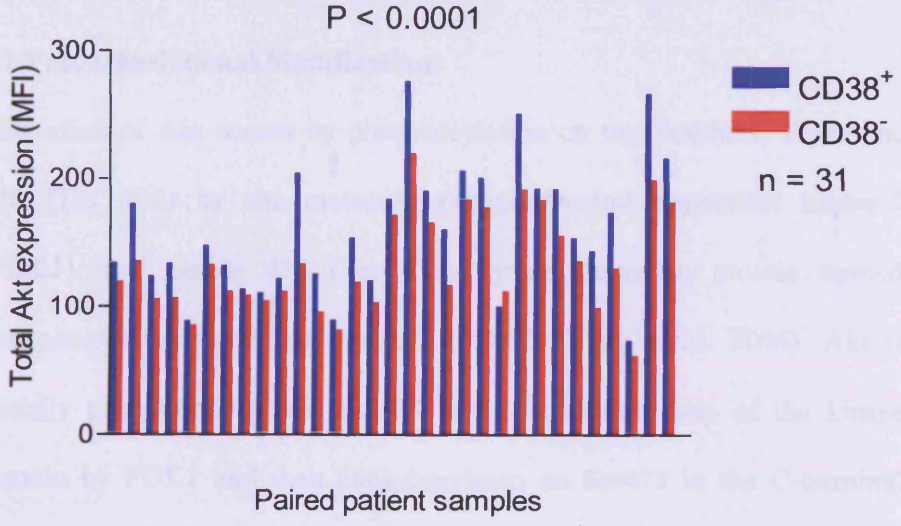


Figure 6.1 Expression of Akt in $CD38^+$ and $CD38^-$ sub-populations derived from the same patient.
Akt expression was determined in $CD38^+$ and $CD38^-$ sub-populations derived from 31 patients with bimodal expression of the CD38 antigen by triple colour flow cytometry (Akt-FITC, CD38-PE and CD19-APC) on fixed and permeabilised cells. The data was analysed using a paired Student's *t*-test and Akt was found to be expressed at significantly higher levels in $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts ($P < 0.0001$).

Most patients demonstrated higher Akt expression in their $CD38^+$ CLL cells when compared to their paired $CD38^-$ CLL cells. However, 1/31 samples showed higher Akt expression in the $CD38^-$ sub-populations when compared to the $CD38^+$ CLL cells suggesting that there may be other mechanisms that impact upon Akt expression in CLL cells that are not associated with CD38 expression. The level of expression of Akt varied greatly between patients ranging from 61.3 – 276.2 MFI units. Using a paired Student *t*-test Akt protein expression was found to be significantly higher in the $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts ($P < 0.0001$) (Figure 6.1).

6.2 Post-translational Modifications

Activation of Akt occurs by phosphorylation on two residues, Threonine 308 (Thr 308) by the molecule Phosphoinositol dependent kinase-1 (PDK1), and Serine 473 (Ser 473) by an unknown protein termed Phosphoinositol dependent kinase-2 (PDK2) (Feng et al. 2004). Akt is initially phosphorylated on Thr308 in the activation loop of the kinase domain by PDK1 and then phosphorylated on Ser473 in the C-terminal hydrophobic motif by an unknown molecule that has been termed PDK2 (Bayascas & Alessi 2005;Feng et al 2004). There have been suggestions that CD38 expression is associated with a more activated phenotype (Morabito et al. 2006;Pittner et al. 2005) and so an investigation was undertaken to determine whether $CD38^+$ cells had higher expression of p-Akt (Ser 473) and p-Akt (Thr 308) than their $CD38^-$ counter-parts.

$CD38^+$ and $CD38^-$ cells were investigated from CLL patients with bimodal expression of CD38 by triple colour flow cytometry (p-Akt (Ser 473 or Thr 308)-FITC, CD38-PE and CD19-APC).

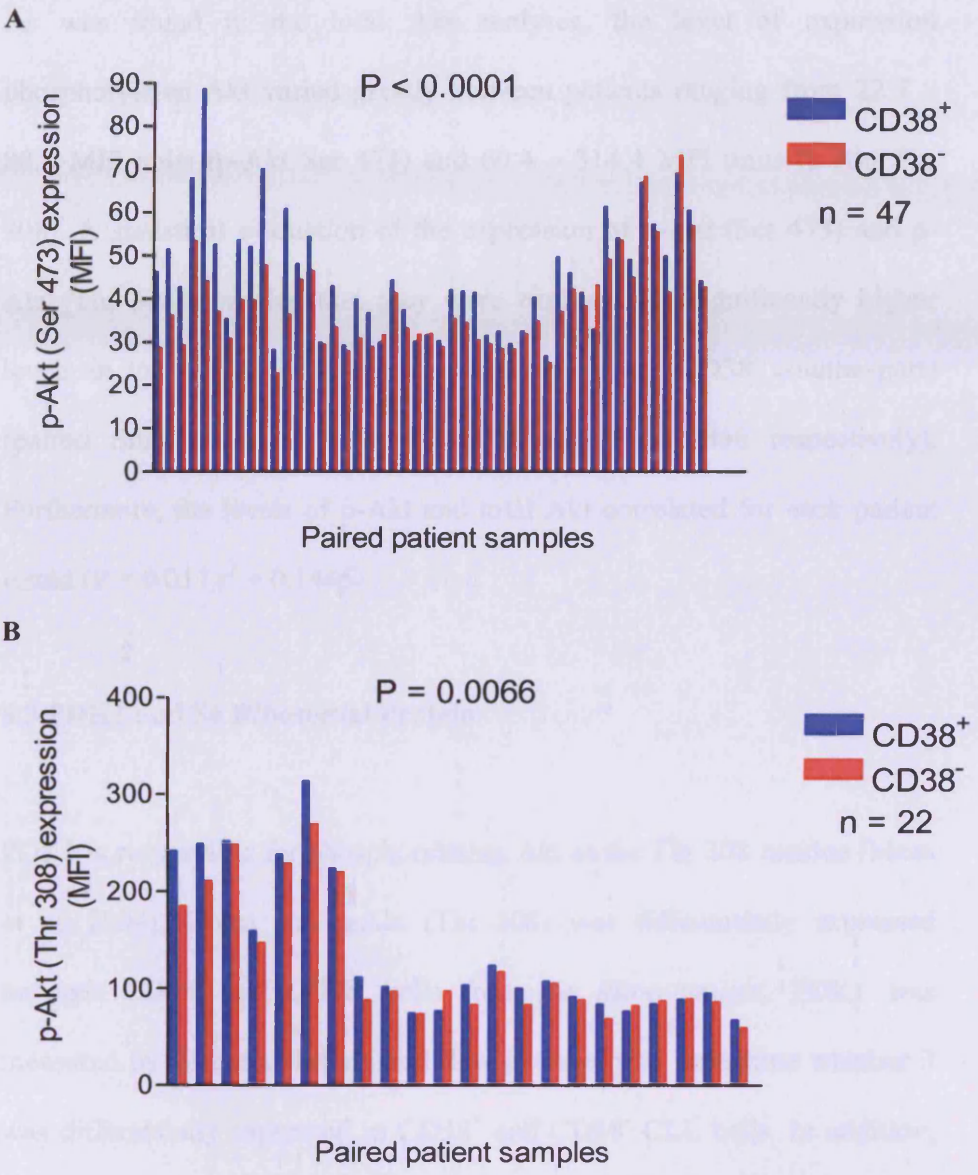


Figure 6.2 Protein expression of (A) p-Akt (Ser 473) and (B) p-Akt (Thr 308) in $CD38^+$ and $CD38^-$ cells from the same patient.
Expression of p-Akt (Ser 473) and p-Akt (Thr 308) was determined in $CD38^+$ and $CD38^-$ CLL cells derived from patients with bimodal expression of the CD38 antigen by triple colour flow cytometry (p-Akt-FITC, CD38-PE and CD19-APC). The data was analysed using a paired Student's *t*-test and both p-Akt (Ser 473) and p-Akt (Thr 308) was found to be expressed significantly higher in $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts ($P < 0.0001$ and $P = 0.0066$ respectively).

For both phosphorylation states of Akt (p-Akt (Ser 473) and p-Akt (Thr 308) there was great inter-patient variability but the majority of patients showed higher phosphorylation levels of both residues in their $CD38^+$ cells.

As was found in the total Akt analyses, the level of expression phosphorylated Akt varied greatly between patients ranging from 22.7 – 88.5 MFI units (p-Akt Ser 473) and 60.4 – 314.4 MFI units (p-Akt Thr 308). A statistical evaluation of the expression of p-Akt (Ser 473) and p-Akt (Thr 308) revealed that they were expressed at significantly higher levels in the $CD38^+$ cells when compared to their $CD38^-$ counter-parts (paired Student's *t*-test ; $P < 0.0001$ and $P = 0066$ respectively). Furthermore, the levels of p-Akt and total Akt correlated for each patient tested ($P = 0.017$ $r^2 = 0.1445$)

6.3 PDK1 and S6 Ribosomal Protein

PDK1 is responsible for phosphorylating Akt at the Thr 308 residue (Mora et al. 2004). Given that p-Akt (Thr 308) was differentially expressed between $CD38^+$ and $CD38^-$ cells from the same patient, PDK1 was measured by Western blotting and flow cytometry to determine whether it was differentially expressed in $CD38^+$ and $CD38^-$ CLL cells. In addition, an important downstream effector molecule of the PI3K pathway, S6 ribosomal protein (Petroulakis et al. 2006), was also investigated to see whether it was expressed (and phosphorylated) differentially in $CD38^+$ and $CD38^-$ cells from the same patient. Cells from 28 patients with bimodal expression of CD38 were investigated for p-S6, S6 and PDK1 expression by triple colour flow cytometry. The data were then analysed using the paired Student's *t*-test.

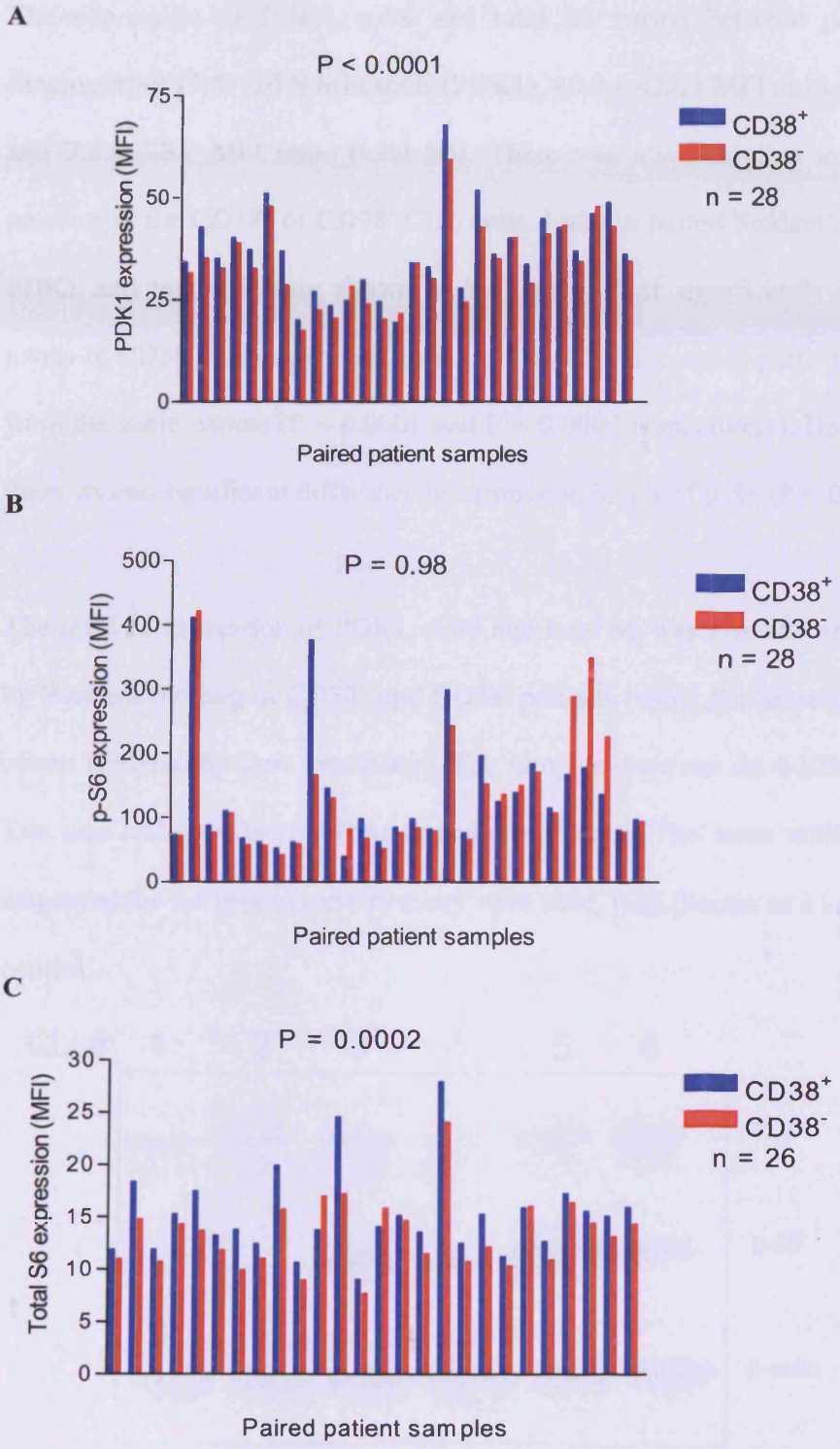


Figure 6.3: Protein expression of (A) PDK1, (B) p-S6 and (C) total S6 in $CD38^+$ and $CD38^-$ cells from the same patient.
$CD38^+$ and $CD38^-$ sub-populations were analysed using triple colour flow cytometry (PDK1 or p-S6 or S6 – FITC, CD38-PE and CD19-APC). The data was analysed using a paired student's *t*-test. The expression of PDK1 and total S6 was found to be significantly higher in the $CD38^+$ CLL cells when compared to their $CD38^-$ counter-parts. However, there was no significant difference in p-S6 expression between the two sub-populations.

The expression of PDK1, p-S6 and total S6 varied between patients ranging from 17.5 – 67.9 MFI units (PDK1), 40.0 – 422.1 MFI units (p-S6) and 7.8 – 28.1 MFI units (total S6). There was also variation in these proteins in the $CD38^+$ or $CD38^-$ CLL cells. Using a paired Student's *t*-test PDK1 and total S6 were shown to be expressed at significantly higher levels in $CD38^+$ cells when compared to their $CD38^-$ counter-parts derived from the same patient ($P < 0.0001$ and $P = 0.0002$ respectively). However there was no significant difference in expression levels of p-S6 ($P = 0.98$).

The level of expression of PDK1, p-S6 and total S6 was also investigated by Western blotting in $CD38^+$ and $CD38^-$ patients (using the same patient cohort analysed by flow cytometry). The samples were run on 4-12% Bis-Tris gels and then blotted (transferred) for 1 hour. The same antibodies employed for the flow cytometry assay were used, with β-actin as a loading control.

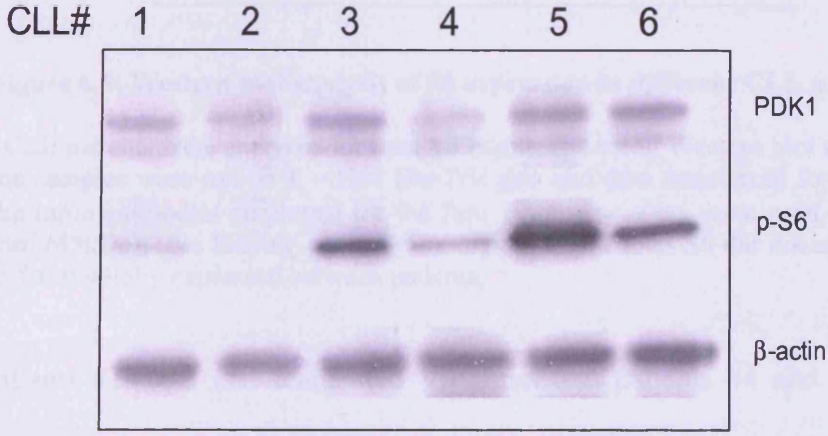


Figure 6.4: Western blot analysis of PDK1 and p-S6 expression in different CLL patients

6 CLL patients were analysed for PDK1 and p-S6 expression using Western blot analysis. The samples were run on 4 – 12% Bis-Tris gels and then transferred for 1 hour. The same antibodies employed for the flow cytometry assay were used, with β-actin (45kDa), as a loading control. The differential expression of PDK1 and p-S6 did not appear to be associated with V_H gene mutation status or CD38 expression.

Patients #1 – #5 had unmutated V_H genes and patients #1 – #3 were $CD38^-$ whereas patients #4 and #5 were $CD38^+$. Patient #6 was also $CD38^+$ but had mutated V_H genes. Both PDK1 (58 – 68 kDa) and p-S6 (32kDa) were differentially expressed between CLL patients. However, the expression profile of PDK1 and p-S6 did not appear to be associated with either V_H gene mutation status or CD38 expression when comparing the different CLL patient samples. This was also true when looking at the expression of PDK1 and p-S6 by flow cytometry. Neither PDK1 nor p-S6 were associated with V_H gene mutation status (P = 0.94 and 0.56 respectively) or CD38 expression (P = 0.34 and P = 0.87 respectively).

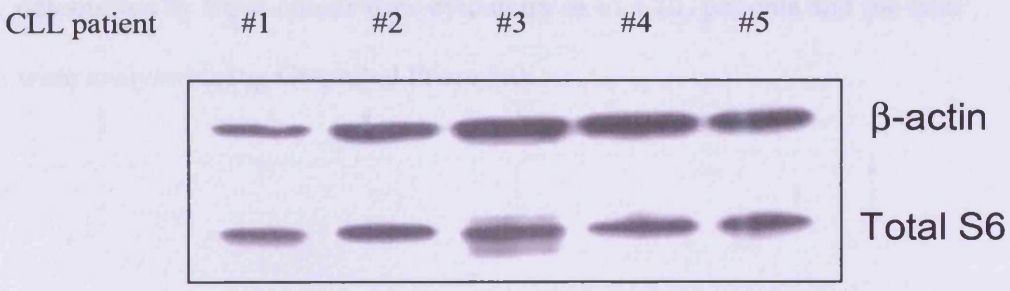


Figure 6.5: Western blot analysis of S6 expression in different CLL patients

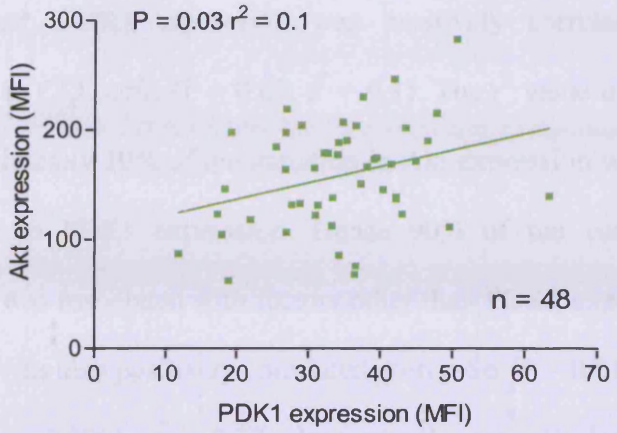
5 CLL patients were analysed for total S6 expression using Western blot analysis. The samples were run on 4 – 12% Bis-Tris gels and then transferred for 1 hour. The same antibodies employed for the flow cytometry assay were used, with β-actin (45kDa), as a loading control. The expression of total S6 did not appear to be differentially expressed between patients.

Patients #1 – #3 had unmutated V_H genes and patients #4 and #5 had mutated V_H genes. Patients #1 and #4 were $CD38^+$ and patients #2, #3 and #5 were $CD38^-$. There did not appear to be any significant difference in S6 expression (32kDa) between any of the CLL patient samples tested. Importantly, the rank order of normalised S6 expression in the patient

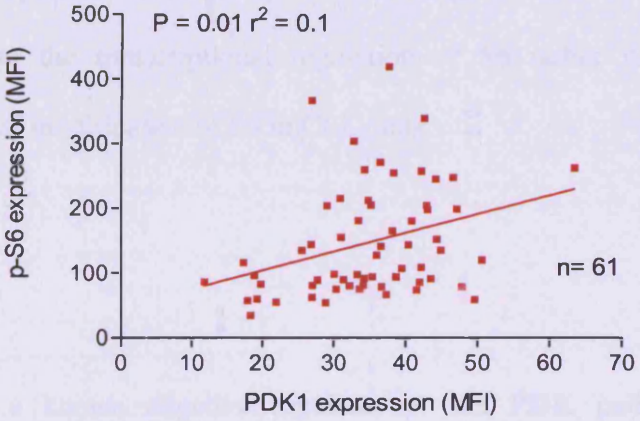
samples as determined by Western blotting was the same as the rank order of expression of S6 in these samples as measured by flow cytometry.

Previously it has been shown that PDK1 phosphorylates Akt on Thr 308 (Feng, Park, Cron, Hess, & Hemmings 2004) and there have been suggestions that PDK1 can also phosphorylate Akt on Ser 473 (Balendran et al. 1999). Therefore an investigation of the relative expression of these proteins in CLL samples was carried out. Since p-S6 is at the distal end of the PI3K pathway (Petroulakis et al 2006) and is an essential effector molecule, an investigation into whether PDK1 and S6 correlated was also carried out. PDK1, Akt, p-S6 and S6 expression levels (MFI) were determined by triple colour flow cytometry in 61 CLL patients and the data were analysed using GraphPad Prism 3.0.

A



B



C

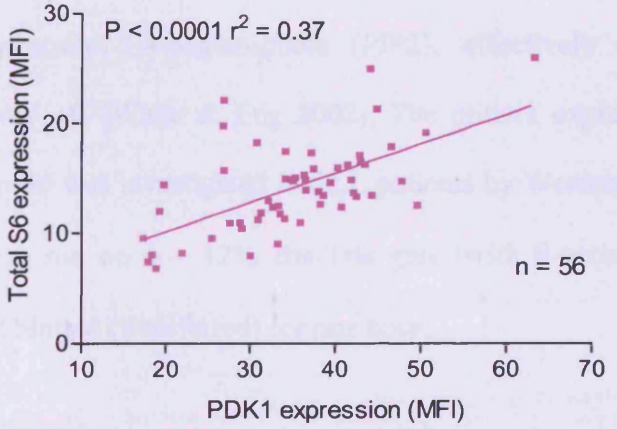


Figure 6.6 Correlation between (A) Akt and PDK1, (B) p-S6 and PDK1 and (C) Total S6 and PDK1

The level of expression of the proteins Akt, PDK1, p-S6 and total S6 was determined by dual colour flow cytometry (Akt, PDK1, p-S6 and S6-FITC and CD19-APC). Correlation analysis was carried out in GraphPad Prism 3.0 software. Akt, p-S6 and S6 all correlated with PDK1 expression. Total Akt expression was analysed due to lack of confidence in the p-Akt antibodies.

As expected, PDK1 expression was positively correlated with Akt expression in CLL cells ($P = 0.03$, $r^2 = 0.1$). The r^2 value of 0.1 indicated that approximately 10% of the variation in Akt expression was attributable to changes in PDK1 expression. Hence 90% of the variation in Akt expression was associated with factors other than PDK1 expression. PDK1 expression was also positively correlated with p-S6 ($P = 0.01$, $r^2 = 0.1$) and total S6 ($P < 0.0001$, $r^2 = 0.37$). Interestingly, total S6 levels correlated more closely with PDK1 than p-S6 levels suggesting that PDK1 may be involved in the transcriptional regulation of S6 rather than the post-translational modification of S6 in CLL cells.

6.4 PTEN

PTEN is a known negative regulator of the PI3K pathway. It de-phosphorylates phosphatidylinositol-3,4,5-trisphospate (PIP3) into phosphatidylinositol-3,4-bisphosphate (PIP2), effectively switching the PI3K pathway off (Waite & Eng 2002). The protein expression level of PTEN and p-S6 was investigated in CLL patients by Western blotting. The samples were run on 4 – 12% Bis-Tris gels (with β-actin as a loading control) and blotted (transferred) for one hour.

Figure 6.7 Western blot analysis for PTEN and p-S6 expression
6 CLL patients were analysed for PTEN and p-S6 expression using Western blot analysis. The samples were run on 4 – 12% Bis-Tris gels and then transferred for 1 hour. The same antibodies employed for the flow cytometry assay were used, with β-actin (45kDa), as a loading control. The expression of PTEN did not appear to be differentially expressed between patients, whereas p-S6 expression was differentially expressed between these patients.

Patients #2, #3 and #4 were $CD38^+$ (≥30% CD38 expression) whereas Patients #1, #5 and #6 were $CD38^-$ (<30% CD38 expression). Also patients #1, #3, #4 and #6 had mutated V_H genes whereas patient #2 had unmutated V_H genes. There appeared to be no significant difference in expression of PTEN (54kDa) between the CLL patients analysed. However the same patients showed differential expression of p-S6 (32kDa) but again there did not seem to be an association between p-S6 expression and V_H gene mutational status or CD38 expression.

6.5 Conclusion

In the microarray analysis in Chapter 4, Akt transcription was shown to be relatively increased in $CD38^+$ CLL cells when compared to $CD38^-$ CLL cells derived from the same patient. In this investigation, this was also found to be true at the protein level. Akt is activated by phosphorylation on

two residues, serine 473 and threonine 308. Both phosphorylation sites of Akt were were more frequently phosphorylated in $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts. This indicates that $CD38^+$ cells have higher levels of activated Akt, and therefore have a more activated phenotype which is in agreement with the findings of Cuni *et al* (2004). Since these CLL cells appear to be more activated, this provides a rationale for the poor prognosis of CLL patients that express the CD38 antigen.

PDK1 is the molecule that phosphorylates Akt on Thr 308 residue (Feng et al 2004;Mora et al 2004) and was also found to be expressed significantly higher in cells that express CD38 compared to those that do not. Total S6, which is a molecule at the distall end of the PI3K pathway (Petroulakis et al 2006) was also more highly expressed in $CD38^+$ cells compared to their $CD38^-$ counter-parts. Intriguingly, the phosphorylated form of S6 was not found to be significantly different between $CD38^+$ and $CD38^-$ cells from the same patient. This suggests that the PI3K pathway signalling via S6 is probably not biologically important in distinguishing between $CD38^+$ and $CD38^-$ cells.

When comparing $CD38^+$ CLL patients (≥30% CD38 expression) to $CD38^-$ CLL patients (<30% CD38 expression), the expression levels of p-S6, S6 and PDK1 were not significantly different. An explanation for this may be that the lower protein expression found in $CD38^-$ sub-populations in bimodal patients causes a reduction in the mean protein expression for the

whole sample resulting in no statistically significant difference between these PI3K proteins in $CD38^+$ and $CD38^-$ patient samples.

There was a positive correlation between Akt expression and PDK1 expression which was expected as PDK1 phosphorylates Akt on Thr 308 residue (Cuni et al 2004). It has also been suggested that PDK1 attached to PIF (PDK1 interacting fragment) may be the elusive PDK2 which phosphorylates Akt on Ser 473 residue (Balendran et al 1999). PDK1 expression was also positively correlated with p-S6 expression and total S6 expression when comparing $CD38^+$ and $CD38^-$ cells from the same patients. This was also expected as PDK1 can phosphorylate p70 S6 kinase (S6K) directly (and indirectly through Akt) which then phosphorylates S6 (Balendran, Currie, Armstrong, Avruch, & Alessi 1999). In addition, PTEN, a known negative regulator of the PI3K pathway (Waite & Eng 2002) was not differentially expressed within the CLL patient cohort. This suggests that PTEN is not likely to play a major role in the control of the PI3K pathway in CLL cells or in determining the clinical course of the disease.

Taken together, these data suggests that although the PI3K pathway/Akt is important in CLL cells (Cuni et al 2004), signalling via S6 (i.e. activation of the distall end of the canonical PI3K pathway) does not appear to significantly contribute to the altered biology of CD38-expressing CLL cells. However, the relative over-expression of Akt in the $CD38^+$ CLL cells does suggest that the PI3K pathway may result in the activation of other

molecular targets not considered in this study (e.g. NF-κB) that possibly contribute to the association of CD38 expression with poor prognosis. This notion is currently under investigation in our laboratory.

Chapter 7: Drug Sensitivity of $CD38^+$ and $CD38^-$ cells

CLL is a disease where lymphocyte accumulation is caused, at least in part, by failed apoptosis (Pepper et al. 1999). This failure of the apoptotic machinery is thought to be due to dysregulation of apoptotic proteins such as the Bcl-2 family proteins, for example, Bcl-2 (B cell lymphoma protein 2), Bax (Bcl-2 associated X protein) and Mcl-1 (Myeloid cell leukaemia sequence 1) (Pepper et al 1999;Saxena et al. 2004). Many studies have shown that high expression of Bcl-2 and Mcl-1 in CLL patients is associated with poor response to therapeutic drugs (Kitada et al. 1998;Pepper et al. 2001). In addition, the ratio of pro- and anti-apoptotic proteins has been shown to be important in disease progression and response to chemotherapy (Kitada et al 1998;Saxena et al 2004).

Aims: To investigate whether $CD38^+$ sub-clones were more resistant to drug chemotherapy than their $CD38^-$ counter-parts, and whether the apoptotic proteins Bcl-2, Bax and Mcl-1 were differentially expressed within these two sub-clonal populations.

Hypothesis: $CD38^+$ cells are more resistant to drug chemotherapy due to high expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 when compared to $CD38^-$ cells.

7.1 The sensitivity of $CD38^+$ and $CD38^-$ cells from the same patient to fludarabine.

$CD38^+$ and $CD38^-$ cells were physically cell sorted from 25 patients with bimodal expression of CD38 using the MoFlo high speed cell sorter and set up in culture. Different concentrations of fludarabine were added to the cultures for 48 hours and the amount of apoptosis was measured by flow cytometry using forward scatter and side scatter plots. Forward scatter measures the size of the cells and side scatter measure the granularity of the cells. This helps to distinguish apoptotic cells from viable cells as cells undergoing apoptosis become smaller and more granular. The data from these experiments were confirmed by using the Annexin V/ Propidium iodide assay. Dose-response curves were produced by plotting the amount of apoptosis produced by each concentration of fludarabine and the LD_{50} values (the concentration at which 50% of the cells were killed) were determined for both the $CD38^+$ and $CD38^-$ sub-clonal populations. For 20 of the 25 patient samples analysed the LD_{50} for both the $CD38^+$ and $CD38^-$ sub-clones were not statistically significantly different ($P = 0.44$) indicating that both $CD38^+$ and $CD38^-$ cells had similar sensitivity to fludarabine. Figure 7.1A shows an example of the comparative sensitivity of $CD38^+$ and $CD38^-$ cells derived from a single CLL patient. Figure 7.1B shows the relative sensitivity to fludarabine of $CD38^+$ and $CD38^-$ cells derived from 20 CLL patients.

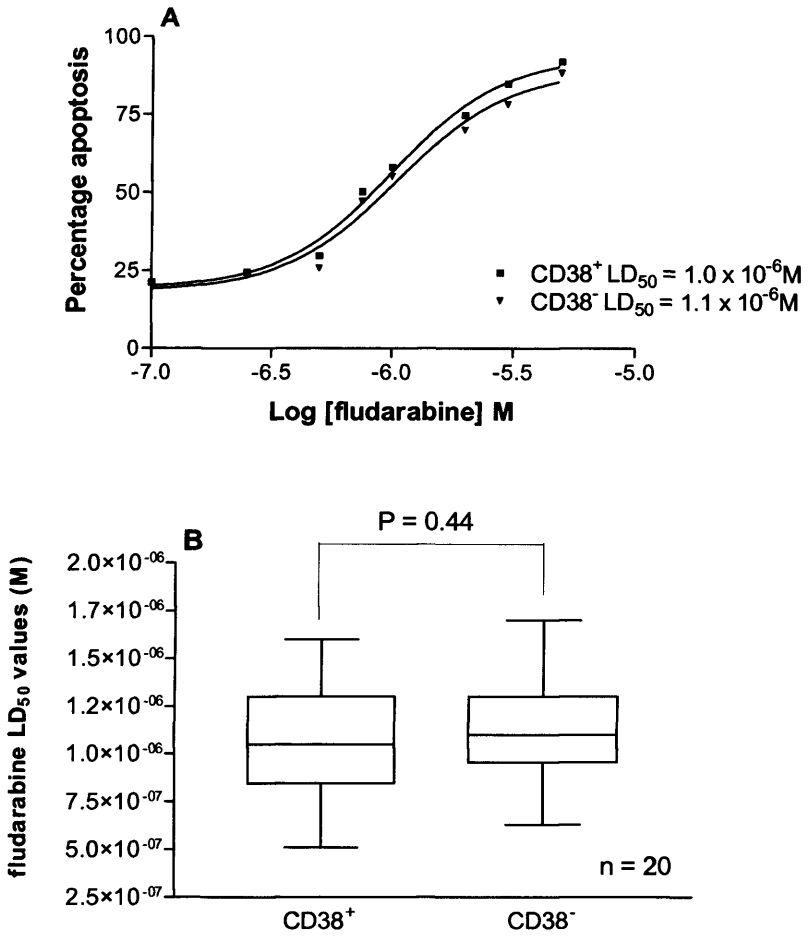


Figure 7.1 Comparative *in vitro* sensitivity to fludarabine in $CD38^+$ and $CD38^-$ CLL cells.
$CD38^+$ and $CD38^-$ cells were high speed cell sorted and set up in culture where they were incubated with or without fludarabine for 48 hours. The amount of apoptosis was determined by flow cytometry using forward scatter and side scatter plots. The concentration of fludarabine that killed 50% of the CLL cells (LD_{50}) was similar for both $CD38^+$ and $CD38^-$ cells. (A) shows the overlaid dose-response curves for $CD38^+$ and $CD38^-$ CLL cells derived from a single patient. (B) shows the comparative fludarabine sensitivity of $CD38^+$ and $CD38^-$ cells derived from 20 CLL patients.

In contrast, five patients who had previously received at least four different treatment regimens displayed differential sensitivity to fludarabine with the $CD38^-$ cells having a similar LD_{50} to those patients who had not previously received treatment whereas the $CD38^+$ cells were markedly more resistant to fludarabine. Figure 7.2A shows the overlaid dose-response curves for

$CD38^+$ and $CD38^-$ cells derived from a single CLL patient. Figure 7.2B shows the comparative sensitivity of $CD38^+$ and $CD38^-$ cells derived from the five heavily treated patients. It should be noted that the sensitivity of the $CD38^-$ cells derived from these five patients was similar to that seen in the other 20 patients in the cohort. Only the $CD38^+$ CLL cells showed a marked increase in drug resistance to fludarabine.

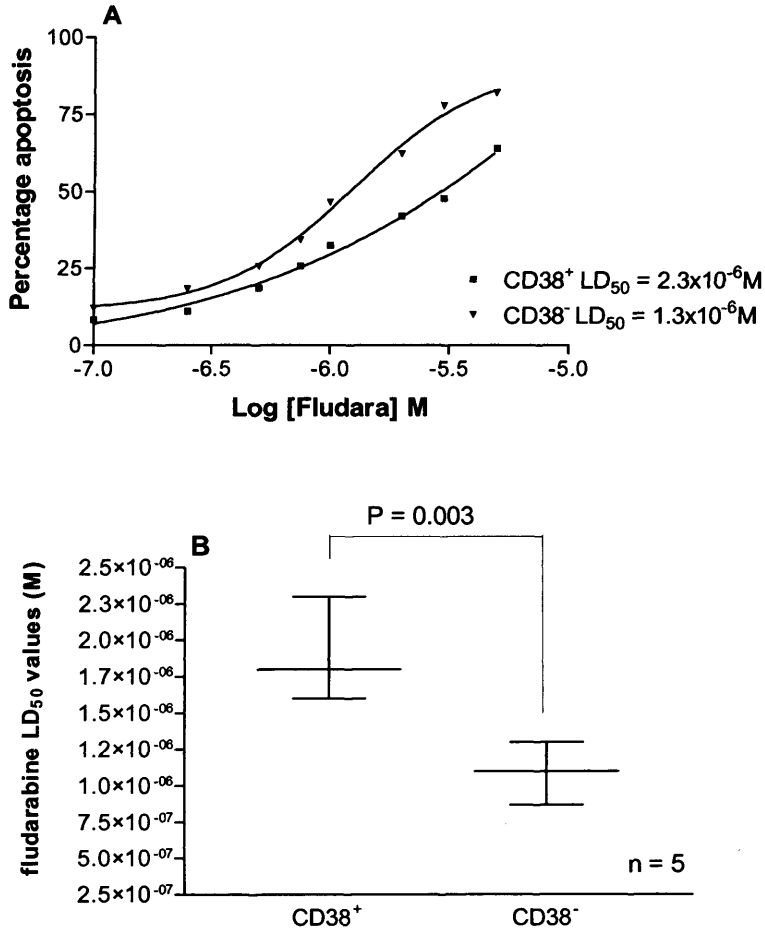


Figure 7.2 Comparative *in vitro* sensitivity to fludarabine in $CD38^+$ and $CD38^-$ CLL cells derived from heavily treated CLL patients, Apoptosis was determined in cell sorted $CD38^+$ and $CD38^-$ cells derived from the same patient by flow cytometry using forward scatter and side scatter plots. $CD38^+$ cells were more resistant to fludarabine than their $CD38^-$ counter-parts shown by their LD_{50} values. (A) shows the overlaid dose-response curves for $CD38^+$ and $CD38^-$ CLL cells derived from a single patient. (B) shows the comparative fludarabine sensitivity of $CD38^+$ and $CD38^-$ cells derived from five heavily treated CLL patients.

7.2 Expression of the apoptotic proteins Bcl-2, Bax and Mcl-1 in $CD38^+$ and $CD38^-$ cells.

Dysregulation of apoptotic proteins has been suggested as an explanation for why CLL lymphocytes fail to undergo apoptosis (Pepper, Thomas, Hoy, Cotter, & Bentley 1999;Saxena, Viswanathan, Moshynska, Tandon, Sankaran, & Sheridan 2004). Bcl-2 has been shown to be differentially expressed in CLL and plays an important role within the disease (Pepper, Thomas, Hoy, Cotter, & Bentley 1999). Bcl-2/Bax ratios have also been shown to be prognostic (Oltvai, Milliman, & Korsmeyer 1993;Pepper, Thomas, Hoy, Cotter, & Bentley 1999) along with Mc-1/Bax ratios (Bellosillo et al. 2002). Mutations in the pro-apoptotic *bax* gene also appear to influence how patients respond to drug therapies (Starczynski et al. 2005).

Recently Mcl-1 has been put forward as an important prognostic factor within CLL lymphocytes (Kitada, Andersen, Akar, Zapata, Takayama, Krajewski, Wang, Zhang, Bullrich, Croce, Rai, Hines, & Reed 1998;Saxena, Viswanathan, Moshynska, Tandon, Sankaran, & Sheridan 2004). Faderl *et al* (2002) looked at many prognostic proteins and found that Bcl-2 predicted the patients with the worst clinical outcome. We have shown greater prognostic significance than Bcl-2 or any other apoptotic protein (Unpublished data). Mcl-1 was shown to be differentially expressed between CLL patients and in Chapter 5 Mcl-1 was shown to be prognostic

as patients with high Mcl-1 expression had a shorter PFS than patients with low Mcl-1 expression.

In this study, the expression level of Bcl-2, Bax and Mcl-1 was measured in 45 patients with bimodal expression of CD38 by triple colour flow cytometry (protein of choice-FITC, CD38-PE and CD19-APC). The level of expression of each protein was investigated in the $CD38^{+}$ and $CD38^{-}$ sub-populations from the same patient with bimodal expression of CD38 using a paired student's *t*-test.

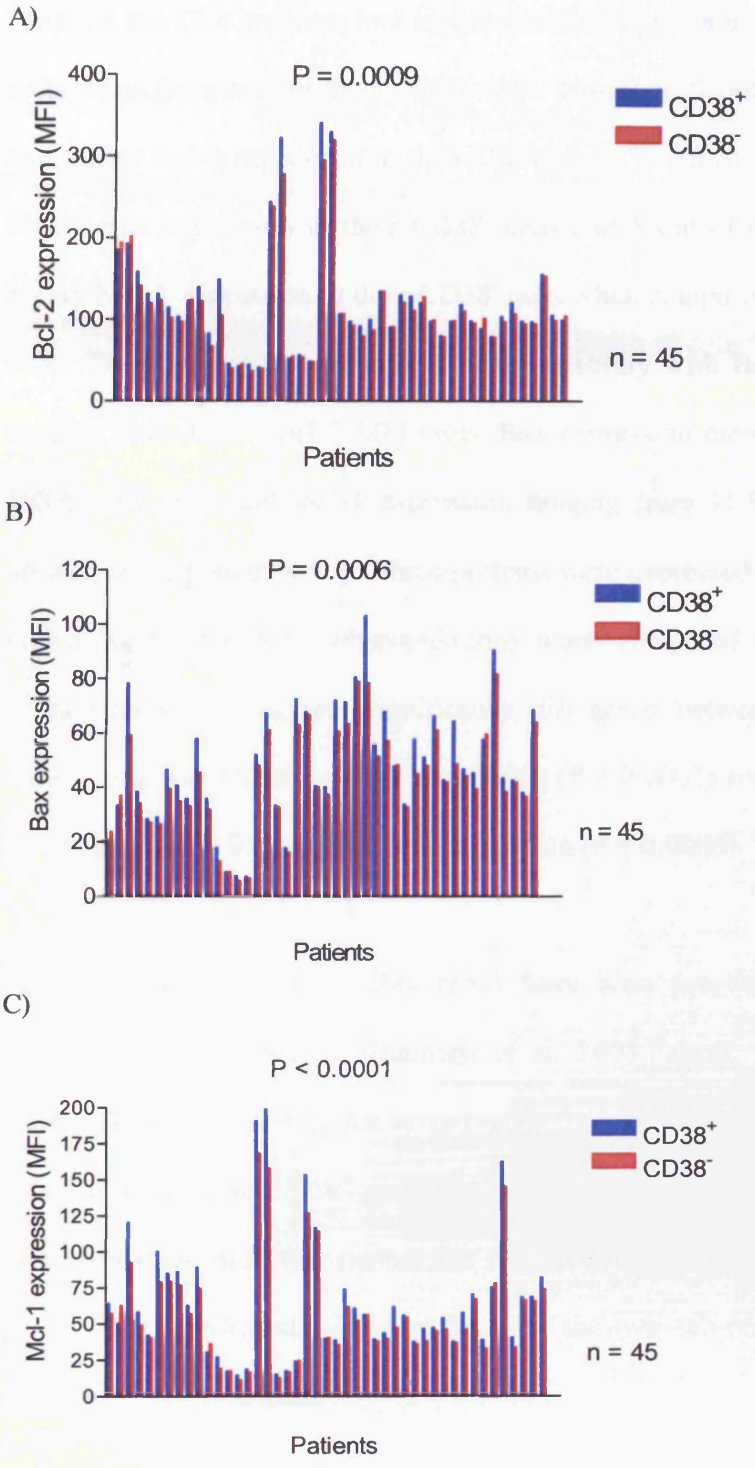


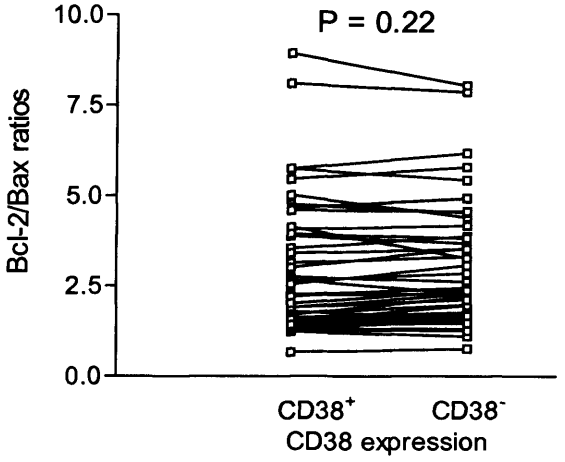
Figure 7.3 (A) Bcl-2, (B) Bax and (C) Mcl-1 expression in paired $CD38^+$ and $CD38^-$ sub-populations derived from the same patient

$CD38^+$ and $CD38^-$ cells from 45 patients with bimodal expression of CD38 were analysed by triple colour flow cytometry on fixed and permeabilised cells for expression levels of the apoptosis-regulating proteins Bcl-2, Bax and Mcl-1. The data were analysed using a paired *t* test. In all three cases the protein expression levels were found to be statistically higher in the $CD38^+$ sub-populations when compared to the $CD38^-$ sub-populations.

Most of the CLL patients had higher protein expression in their $CD38^+$ cells when compared to their $CD38^-$ cells. However, 9 out of 45 patients had higher Bcl-2 expression in their $CD38^-$ cells, 5 out of 45 patients had higher Bax expression in their $CD38^-$ cells and 6 out of 45 patients had higher Mcl-1 expression in their $CD38^-$ cells when compared to the $CD38^+$ cells. There was also great inter-patient variability with Bcl-2 expression ranging from 37.1 – 341.9 MFI units, Bax expression ranging from 6.0 – 103.6 MFI units and Mcl-1 expression ranging from 11.9 – 202.3 MFI units. Using a paired *t*-test all three proteins were expressed at significantly higher levels in $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts. The highest significance difference between $CD38^+$ and $CD38^-$ cells was found in Mcl-1 expression ($P < 0.0001$) compared to Bcl-2 expression ($P = 0.0009$) and Bax expression ($P = 0.0006$).

High Bcl-2/Bax and Mcl-1/Bax ratios have been associated with poor response to chemotherapy (Bannerji et al. 2003;Pepper, Thomas, Hoy, Cotter, & Bentley 1999). An investigation into the Bcl-2/Bax ratios and Mcl-1/Bax ratios in $CD38^+$ and $CD38^-$ sub-populations derived from the same patient showed that neither the Bcl-2/Bax ratios nor the Mcl-1/Bax ratios were significantly different between the two sub-populations ($P = 0.22$ and $P = 0.26$ respectively) (Figure 7.4).

A



B

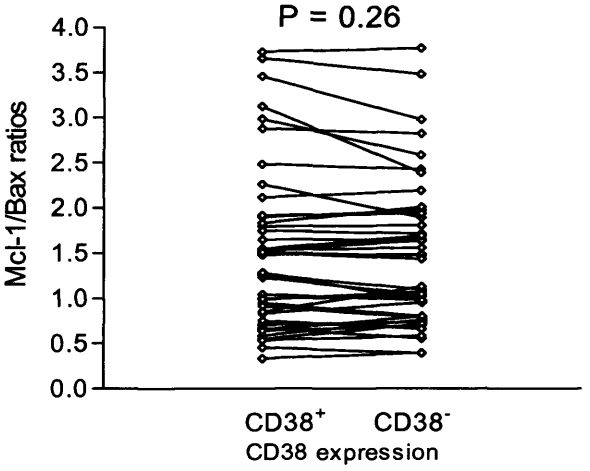


Figure 7.4 (A) Bcl-2/Bax ratios and (B) Mcl-1/Bax ratios in $CD38^+$ and $CD38^-$ sub-populations derived from the same patient.
Bcl-2, Bax and Mcl-1 expression was determined by triple colour flow cytometry in 44 patients with bimodal expression of the CD38 antigen. The Bcl-2/Bax ratios and Mcl-1/Bax ratios were compared in the $CD38^+$ and $CD38^-$ sub-populations derived from the same patient and analysed using a Student's *t* test. Neither the Bcl-2/Bax ratios nor Mcl-1/Bax ratios were significantly different when comparing the $CD38^+$ and $CD38^-$ populations.

7.3 Conclusion

Lymphocyte accumulation in CLL is due in part to dysregulation of the apoptotic proteins which leads to failed apoptosis (Pepper et al 1999). This dysregulation of the apoptotic proteins, such as Bcl-2, Bax and Mcl-1 has shown to be associated with poor responsiveness to chemotherapy (Kitada et al 1998). CD38 expression has also been associated with poor response to chemotherapy (Deaglio et al. 2006).

As investigation into the response of $CD38^+$ and $CD38^-$ sub-populations (derived from the same patient) to the therapeutic drug fludarabine revealed that in most cases (20/25) the LD_{50} for both $CD38^+$ and $CD38^-$ sub-clones were similar, indicating that neither sub-population was more resistant to fludarabine. However, in a subset of 5 patients, who had previously received at least four separate treatment regimens, the $CD38^+$ sub-clones showed a marked increase in resistance to fludarabine whereas the $CD38^-$ sub-clones retained the same sensitivity as that found in samples derived from untreated patients. These 5 patients had not been treated in a uniform fashion but had been subjected to a number of different drug treatments and so the preferential resistance found in the $CD38^+$ sub-populations did not appear to be mediated by exposure to a specific agent.

The apoptotic proteins Bcl-2, Bax and Mcl-1 were all expressed at significantly higher levels in the $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts. The high expression of Bcl-2 and Mcl-1 in the $CD38^+$ sub-clones was as expected since both Bcl-2 and Mcl-1 expression

has been associated with poor clinical response (Kitada et al 1998;Pepper et al 1999) and this may be an explanation to why CD38 expression is associated with poor prognosis. However, the high expression of Bax was unexpected, as Bax is a pro-apoptotic protein (Bannerji et al 2003). The elevated Bax expression contributed to the lack of significant difference in the Bcl-2/Bax and Mcl-1/Bax ratios between $CD38^+$ and $CD38^-$ CLL cells and this may provide an explanation for why most of the paired $CD38^+$ and $CD38^-$ sub-clones showed similar sensitivity to *in vitro* fludarabine.

Further experiments need to be carried out in which the levels of Bcl-2, Bax and Mcl-1 expression are measured in the $CD38^+$ and $CD38^-$ sub-clones pre- and post treatment in a longitudinal fashion. These experiments will address the issue of preferential clonal evolution resulting in elevated drug resistance in the $CD38^+$ sub-clones.

Chapter 8: Discussion

CD38 is a 45 kDa type II transmembrane glycoprotein that has been shown to be prognostic in CLL (Damle et al. 1999;Ghia et al. 2003;Hamblin et al. 2002;Huttmann et al. 2006). There has been controversy over the use of CD38 as a prognostic marker as there is no universally-agreed threshold to classify a patient as CD38 positive, and there is also some debate about whether CD38 expression changes over the course of the disease (or after treatment) or remains constant. The aim of this study was to address these issues and to elucidate a biological rationale for the association between CD38 expression and poor prognosis in CLL.

As an initial step, we investigated the relative prognostic capacity of the three thresholds previously published by other groups (7%, 20% and 30%) (Damle et al 1999;Ibrahim et al. 2001;Krober et al. 2002). In agreement with Ibrahim *et al* (2001) and other groups (Durig et al. 2002) the 20% threshold was found to be the most prognostic as evidenced by the most significant difference in overall survival (OS) between the $CD38^+$ and $CD38^-$ patients. There was also significant difference in PFS, TTFT and OS between the $CD38^+$ and $CD38^-$ patients at both the 7% and 30% level, but the 20% showed the greatest significant difference. An explanation for the improved prognostic power of the 20% threshold level may lie in the fact that a significant number of the patients in our cohort had CD38 expression between 20 and 30% and these patients predominantly had active/progressive disease and poor clinical outcomes. Therefore, the 30% threshold appears to exclude a significant number of patients with active/progressive disease. In contrast, the 7% threshold appears to include a significant number of patients that have stable disease with relatively long

TTFT and OS. Therefore, the intermediate threshold of 20% represents a good compromise resulting in the inclusion of most of the patients with poor outcome disease whilst excluding more of the patients with good outcome disease.

Although </≥20% CD38 expression proved to be the best threshold for defining prognosis in the patient cohort, in agreement with Ghia *et al* (Ghia et al 2003) the expression of the CD38 antigen *per se* was associated with poor prognosis. This was demonstrated by $CD38^+$ patients having significantly shorter PFS, TTFT and OS in $CD38^+$ patients. In addition, CD38 expression was associated with other poor clinical and prognostic markers such as Binet stage B/C, a LDT >12 months, 17p-/11q-/complex cytogenetics, unmutated V_H gene mutation status and ZAP-70 expression.

In agreement with Ghia *et al* (2003) (and disagreement with Hamblin *et al* (2002) CD38 expression was found not to change significantly over time which would support the use of CD38 as a prospective prognostic marker. When used in conjunction with V_H gene mutation status it was found to split the mutated and unmutated groups into two groups (giving four in total). Importantly, the combination of CD38 expression and V_H gene mutational status identified a subset of patients with a poorer prognosis i.e. $CD38^+$/mutated V_H genes. V_H gene mutation status retained its superior prognostic power as patients with mutated V_H genes still had a significantly better prognosis than patients with unmutated V_H genes irrespective of their CD38 expression. However, our data suggest that the combination of V_H gene mutation status and CD38 expression, and possibly other prognostic markers like ZAP-70, might provide added value in terms of more accurately defining the prognosis of

individual CLL patients. Indeed, a number of groups have already published work to this effect (Bilous et al. 2005;Jelinek et al. 2001;Pepper et al. 2007).

Once CD38 expression was shown to be prognostic the next step was to investigate the biological rationale behind the association between CD38 expression and poor prognosis. In order to explore this, we took advantage of the fact that a proportion of CLL patients have bimodal expression of CD38 i.e. they possess distinct subsets of CLL cells that are $CD38^+$ and $CD38^-$. By using high speed cell sorting we were able to purify these sub-populations derived from the same patient and examine their biological characteristics. As a first step, we demonstrated that these $CD38^+$ and $CD38^-$ lymphocytes had the same light chain restriction (were monotypic) and had essentially the same V_H gene sequence (were monoclonal). Therefore, we could conclude that these cellular sub-populations both originated from a single malignant transforming event. This data agreed with the findings of Ghia *et al* (2003).

Subsequently, we went on to examine the gene expression signatures of these sub-populations in order to determine whether we could derive any clues about the poor prognosis of CD38-expressing CLL patients. $CD38^+$ sub-populations were shown to have significantly different gene expression profile to $CD38^-$ sub-populations derived from the same patient with 61 genes being differentially expressed between these two sub- populations when using a two-fold cut-off to define gene expression changes.

Analysis of the gene list revealed two distinct nodes of genes that appeared to be associated with one another. One of these nodes was centred around vascular endothelial growth factor (VEGF). VEGF was consistently over-expressed in $CD38^+$

CLL cells when compared to their $CD38^-$ counter-parts. Given that a number of papers had previously suggested that VEGF might be important in CLL (Ferrajoli et al. 2001; McCabe et al 2004) and that there appears to be an association between VEGF and CD38 expression, we investigated this further in our purified sub–clonal populations. VEGF protein was shown to be relatively over expressed in $CD38^+$ CLL cells thereby confirming the transcription data. Furthermore, VEGF expression was associated with *in vitro* survival and the addition of exogenous VEGF caused an increase in the anti-apoptotic protein Mcl-1, a downstream effector of VEGF signalling. VEGF signalling was significantly inhibited by SU1498 (a VEGF kinase inhibitor) but was essentially unaffected by blocking VEGF receptor binding by using a VEGFR2 antibody. This data along with the exogenous VEGF data suggests that $CD38^+$ sub-populations signal predominantly via an autocrine signalling pathway and $CD38^-$ sub-populations signal via a paracrine survival loop. In the case of bimodal patients the $CD38^+$ sub-populations provide exogenous VEGF which the $CD38^-$ sub-populations can use to signal (Pepper et al 2007).

VEGF signalling through VEGFR2 has been shown to utilise the PI3K/Akt survival pathway (Bairey et al. 2004). In this regard, Akt was found to be relatively over expressed, in this study both transcriptionally and at the protein level, in the $CD38^+$ sub-populations when compared to their $CD38^-$ counter-parts. Although it is tempting to consider this to be an explanation for why $CD38^+$ sub-populations survive longer than the $CD38^-$ sub-populations in *in vitro* culture, the relative increase in PI3K activation in $CD38^+$ CLL cells appears not to be mediated via VEGFR2 signalling. Interestingly, expression of the down stream effector molecule of the Akt signalling pathway, p-S6, was not significantly different between the two sub-populations

suggesting that survival via this pathway was not significant between $CD38^+$ and $CD38^-$ sub-populations. Ringshausen *et al* (2002) found that protein kinase C (PKC) was important in the survival of CLL suggesting that PKC activation is possibly a more important downstream effector of the Akt pathway in CLL cells. Alternatively, investigations in our laboratory have found that the transcription factor NF-κB is differentially expressed between $CD38^+$ and $CD38^-$ patients. Previous work by Cuni *et al* (2004) found that Akt was an upstream signalling molecule of NF-κB and so this molecule may be the important effector of Akt signalling in the $CD38^+$ and $CD38^-$ sub-populations. Further investigations are currently on-going to try to elucidate the relative importance of these molecules in the maintenance of CLL cells.

As $CD38^+$ sub-populations are less susceptible to spontaneous apoptosis than their $CD38^-$ counter-parts, the next step was to investigate whether $CD38^+$ sub-populations were more resistant to chemotherapeutic agents than $CD38^-$ sub-populations. Somewhat surprisingly, in the majority of patient samples tested (20/25) both sub-clonal populations had similar sensitivity to the chemotherapeutic drug fludarabine. This indicates that $CD38^+$ CLL cells are not intrinsically more drug resistant when compared to their $CD38^-$ counter-parts. However, 5/25 patient samples derived from patients who had previously received a number of treatment regimens showed marked and differential drug resistance in their $CD38^+$ sub-populations. This suggests that these cells are preferentially undergoing clonal evolution and a longitudinal analysis of the cytogenetics of these sub-populations is currently being conducted in our laboratory.

Although there was differential expression of the apoptotic proteins Bcl-2, Bax and Mcl-1 between $CD38^+$ sub-populations and $CD38^-$ sub-populations there was no significant difference between the Bcl-2/Bax and Mcl-1/Bax ratios in these two sub-populations. This may help to explain why both the $CD38^+$ and $CD38^-$ sub-populations had similar sensitivity to drug therapeutics.

The flow cytometry methods used throughout this investigation are not without their limitations. For instance, the background staining of the isotype-matched control antibodies in cells that have been fixed and permeabilised is almost always higher than that found for surface antigen isotype controls. This suggests that the fixation and permeabilisation process promotes none-specific binding of the antibodies. However, it should be noted that the majority of the analyses performed in this study were of paired data i.e. $CD38^+$ and $CD38^-$ CLL cells derived from the same patient. As such, it is reasonable to consider that the level of background, none-specific, binding will be the same for each paired set of samples thereby diminishing the chance of measured differences in fluorescence intensity being due to artefact. Also as CD38 expression is associated with cellular activation, differences in antigen expression between $CD38^+$ and $CD38^-$ CLL cells measured by flow cytometry may, at least in part, be a reflection of the change in cell size. In this context, it has previously been reported that CD38-expressing CLL cells are larger than the cells that do not express CD38 (Manocha et al. 2003). Despite these caveats, given that the data obtained by flow cytometry was also confirmed by Western Blotting techniques it seems highly likely that the conclusions reached in this study are justified.

Taken together, this work for the first time provides a biological rationale for why CD38 expression is associated with poor prognosis in CLL. The gene expression signatures derived from $CD38^+$ and $CD38^-$ sub-populations not only shed new light on the biology of these sub-populations of CLL cells but also highlight VEGF and Akt as potential therapeutic targets. In addition, the combination of V_H gene mutation status and CD38 expression was shown to provide beneficial information about the clinical outcome of individual CLL patients.

Possible future work

- Investigate the other genes that were found to be differentially expressed between $CD38^+$ and $CD38^-$ sub-populations derived from the same patient
- Perform knock-down experiments in which CD38 expression is inhibited using antisense or SiRNA in order to establish whether a $CD38^-$ gene signature can be induced in cells that formerly expressed CD38. This would address the question of whether CD38 is merely a marker of CLL cell activation.
- knock-in experiments where CD38 would be induced using gene transfer techniques
- Perform knock-down experiments in which VEGF expression is inhibited using antisense or SiRNA in order to assertain the importance of VEGF to CLL cell survival
- Investigate the effect of treatment on the expression levels of Bcl-2, Bax and Mcl-1 in $CD38^+$ and $CD38^-$ sub-populations.

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