Preliminary Characterisation of FAM129C, a Novel Protein Identified from Proteomic Screening of CLL Samples

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Preliminary Characterisation of FAM129C, a Novel Protein Identified From Proteomic Screening of CLL Samples

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The principal aim of this thesis was to begin to determine the possible functions of a novel and B-cell specific protein, FAM129C, identified from proteomic screening of purified CLL plasma membrane fractions. Bioinformatic analysis showed that FAM129C contained a pleckstrin homology domain that probably causes the protein to be associated with the plasma membrane but lacked any other obvious domains. Using quantitative RT-PCR, I showed that FAM129C was expressed from early stages of B-cell differentiation. It was expressed at high levels in chronic lymphocytic leukaemia (CLL) and in the activated subtype of diffuse large B-cell lymphoma, where it may be a useful diagnostic marker. FAM129C was also expressed at high levels in normal B-cell populations including both naïve pre-germinal centre and memory cell populations but interestingly was rapidly down-regulated following stimulation to proliferation. Similar down-regulation was also observed in CLL cells stimulated to proliferation *in vitro*. Similar down-regulation was also observed in CLL cells stimulated to proliferate in vitro. Subcellular fraction studies of FAM129C showed wide expression in many different cell fractions, but mainly in the cytoplasm. The pattern of FAM129C expression was similar to that of CXCR4 and, therefore I have speculated that there is a potential association between these two proteins in B cell development and in B cell maturation during germinal centre reaction.

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Abbreviations

A (nucleotide)	Adenine
aa	aminoacids
ABC DLBCL	Activated B cell DLBCL
AID	Activating-induced cytidine deaminase
AIHA	Autoimmune haemolytic anaemia
ALC	Absolute lymphocyte count
ALL	Acute lymphocytic leukaemia
ALPS	Autoimmune lymphoproliferative syndrome
ANOVA	Analysis of variance
APAF	Apoptotic protease activating factor 1
APC	Antigen-presenting cell
APS	Ammonium persulphate
ATM	Ataxia–telangiectasia mutated gene
B2	Mature follicular B cells
BAFF	B cell activating factor belonging to the TNF family
BAFFR	B cell activating factor belonging to the TNF family receptor
BAK	BCL2 homologous antagonist killer
Bam32	B-cell adaptor molecule of 32kDa
BANK	B cell scaffold protein with ankrin repeats
BAX	BCL2 associated X protein
BCC	Basal cell carcinoma
BCL	B cell lymphoma
BCLXL	B cell lymphoma extra large
BCMA	B cell maturation antigen
BCNP1	B cell novel protein 1
BCR	B cell receptor
BH	BCL2 homology domain
BID	BH3 interacting domain death agonist
Bim	BCL2 –interacting mediator of cell death
BLAST	Basic local alignment search tool
BLASTP	Protein-protein BLAST
BLNK	B-cell linker
BLOSUM	Blocks of amino acids substitution matrix
B-lymphocyte/cell	Bursa dependant lymphocyte/cell
BLys	B lymphocyte stimulator
BM	Bone marrow
BSA	Bovine srum albumin
BTK	Bruton’s tyrosine kinase
C	central
C	Constant
C (nucleotide)	Cytosine
c.f.	confer
CAML	Calcium-modulator and cyclophilin ligand
CARD	Caspase recruitment domain

CB	centroblasts
CC	centrocytes
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor type
CD	Cluster of differentiation
CDA	Cytidine deaminase
cDNA	Complementary DNA
CDR	Complementarity determining region
CDS	Coding sequence
c.f.	confer
CFAR	Cyclophosphamide, Fludarabine, Alemtuzumab and Rituximab
c-FLIP	Cellular FLICE(FADD-like interleukin1β converting enzyme) inhibitory protein
CGH	Comparative genomic hybridisation
CI	Confidence interval
cIAP	Cellular inhibitor of apoptosis
CLB	chlorambucil
CLL	Chronic lymphocytic leukaemia
CLLU1	CLL up regulated
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
CMV	cytomegalovirus
CO2	Carbon dioxide
COP	Cyclophosphamide oncovine (vincristine) prednisolone
CREB	cAMP responsive element binding protein
CSK	C-termin SRC kinase
CSR	Class switch recombination
CT	Cycle threshold
C-terminal	Carboxyl-terminal
CTL	Cytolytic T lymphocytes
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CYLD	cylindromatosis
D	dimension; Diversity
DC	Dendritic cell
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
Del	Deleted
dH2O	Distilled water
DISC	Death inducing signalling complex
dL	Decilitre
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco’s modified Eagle’s medium

DMP	Dimethylpirelimidate diHCL
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DR	Death receptor
dsDNA	Double-stranded DNA
EBF	Early B cell factor
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic Acid
e.g.	Exampli gratia (*Lat.*), for example
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplastic reticulum
ERK	Extracellular signal-regulated kinase
F	Fragment; Fludarabine
FADD	FAS-associated via death domain
FAM129	Family member 129
FAS	FS-7-associated surface antigen (CD95)
FASL	FS-7-associated surface antigen ligand (CD95L)
FC	Fludarabine and Cyclophosphamide
FCR	Fludarabine, Cyclophosphamide and Rituximab
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FDC	Follicular dendritic cell
FISH	Fluorescence in-situ hybridisation
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FLIP	FLICE (caspase-8)-inhibitory protein
FLT3	Fms-like tyrosine kinase receptor-3
FOXP1	Forkhead box P1
FR	Framework region
G	Grams
G (nucleotide)	Guanine
G0	Gap 0, quiescent phase of cell cycle
GC	Germinal centre
GCET1	centrin
gDNA	Genomic DNA
GEF	Guanine-nucleotide exchange factor
Gld	Generalised disease
GM-CSF	Granulocyte macrophage colony-stimulating factor
Grb2	Growth factor receptor bound protein 2
h	hour
H_2O	Dihydrogen monoxide, water

HCDR3	Heavy chain complimentarity determining region 3
HD	Hodgkin disease
HEV	High endothelial venules
HGNC	HUGO Gene nomenclature Committee
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HR	Hazard ratio
HSC	Haemopoietic stem cells
HUGO	Human Genome Organisation
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IgD	Immunoglobulin delta heavy chain
IgG	Immunoglobulin gamma heavy chain
IGH	Immunoglobulin heavy chain
IGHV	Immunoglobulin heavy chain variable gene
IGLV	Immunoglobulin Lambda variable genes
IgM	Immunoglobulin mu heavy chain
Ig-β	Immunoglobulin beta chain
Igκ	Immunoglobulin Kappa locus
Igλ	Immunoglobulin Lambda locus
IHC	Immunohistochemistry
IKBβ	Inhibitor of kappa B beta isoform
IKK	Iκβ kinase
IL7	Interleukin 7
IL7R	Interleukin 7 receptor
IMDM	Iscove's modified Dulbecco's medium
IMS	Industrial methylated spirit
IP	immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRF	Interferon regulatory factor
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine activation motives
ITP	Idiopathic/immune thrombocytopenic purpura
J	Junctional
JNK	c-Jun N-terminal kinase
kB	Kilo base
kDa	Kilo Dalton
L	Litre
LAT	Linker for activation of T cells
LB	Luria-Bertani broth, lysogeny broth
LCDR3	Light chain complimentarity determining region 3
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
LFA	Leukocyte function antigen

LIME	Lck-interacting molecules
LN	lymphadenopathy
LNR	Leicestershire Northampton Rutland
LPL	Lipoprotein lipase
Lpr	Lymphoprolifertive
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β receptor
LZD	Leucine zipper domain
m	Membrane bound
M	mutated
MALT	Mucosa associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MBL	Monoclonal B cell lymphocytosis
MCL	Mantle cell lymphoma
MDA	Monroe Dunway Anderson
µg	Microgram
mg	Milligram
MgCl2	Magnesium Chloride
MHC	Major histocompatibility complex
mHC	Haevy chains of m isotypes
Mili-Q	Purified and deionised water by Milipore system
min	Minutes
miR	microRNA
µL	Microlitre
ml	Millilitre
ML	Maximum likelihood method
mm	milimeter
mM	Millimolar
MPP	Multipotent progenitor cell
MRC	Medical Research Council
MRD	Minimal Residual Disease
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS4A1	Membrane-spanning 4-domains
MUSCLE	Multiple sequencecomparison by log-expectation
MW	Molecular weight
NaCl	Sodium Chloride
NaF	Sodium fluoride
NB	Nota bene
NCBI	National centre for biotechnology information
NCK	Non-catalytic region of tyrosine kinase
NEMO	NF-κB essential modulator

NF-κB	Nuclear factor kappa B cell
ng	nanogram
NHEJ	Non-homologous-end-joining
NHL	Non-Hodgkin lymphoma
NICE	National Institute for Health and Clinical Excellence
NIK	NF-κB inducing kinase
NJ	Neighbour joining
NK	Natural killer cell
nm	nanometer
NMSC	Non-melanoma skin cancer
NP-40	Nonyl phenoxyl polyethoxylethanol
NTAL	Non-T-cell activation linker
N-terminal	Amino-terminal
NT-L	Non-transfected mouse fibroblasts Ltk-
^{o}C	Degrees centigrade
OD	Optical density
Oligo dT	oligodeoxythymidylic acid
ORF	Open reading frames
OS	Overall survival
p	peripheral
P	promotor
PAG	Phosphoproteins associated with glycosphingolipid-microdomains
PAGE	Polyacrylamide gel electrophoresis
PAL	Periarteriolar lymphoid sheath
PARP	Polu (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with tween
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pfam	Protein families
PFS	Progression free survival
PGE2	Prostaglandin E2
pH	Potential of hydrogen
PHD	Pleckstrin homology domain
PHOSIDA	Phosphorylation site database
pI	Isolectric point
PI	Prognostic index, phosphoinositides
PI3K	Phosphoinositide 3-kinase
PIRB	Paired immunoglobin-like receptor
PKC	Protein kinase C
PKK	Protein kinase C associated kinase
PLCγ2	Phospholipase-Cγ2
pleckstrin	Platelet and leukocyte C kinase substrate

PLL	Prolymphocytic leukaemia
PMBL	Primary mediastinal B cell lymphoma
POP-4	Performance optimised polymer-4
Pre-B	Precursor B cell
Pre-pro-B	Pre-progenitor B cell
pro-B	Progenitor B cell
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
RA	Rheumatoid arthritis
Rac2	Ras-related C3 botulinum toxin substrate 2
RAG	Recombination activating gene
RANK	Receptor activator of NF-κB
R-CHOP	Rituximab Cyclophosphomide Hydroxydaunorubicine Oncovine Prednisolone
Rf	Relative mobility
RNA	Ribonucleic acid
rpm	Revolutions per minute
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RR	Relative risk
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Reverse transcriptase quantitative PCR
S	Synthesis (cell cyle phase)
s	secreted
S/N	supernatant
S1P	Sphingosine-1-phosphate
SCC	Squamous cell carcinoma
SCF	Stem cell factor
scFv	Single-chain variable fragment
SDF1	Stromal cell-derived factor-1
SDS	Sodium Dodecyl Sulfate
sec	Seconds
SH	SRC-homology
SHM	Somatic hypermutation
sIg	Surface immunoglobulin
SIT	SH2-interacting transmembrane adaptor protein
SLE	Systemic lupus erythematosus
SLL	Small lymphocytic lymphoma
SMAC/DIABLO	Supermolecular activation clusters/ direct IAP binding protein with low PI
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
SPSS	Statistical package for social sciences
SRC	sarcoma
SYK	Spleen tyrosine kinase

t	Translocation, trisomy
T (nucleotide)	Thymine
T1	Transitional 1
T2	Transitional 2
TACI	Transmembrane activator and CAML
TAE	triethanolamine
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris base, boric acid, EDTA
TBP	TATA box binding protein
TC	Cytotoxic T-cell
TCL1	T-cell leukaemia/lymphoma1
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
TetO2	Tetracycline operator 2
TetR	Tetracycline repressor protein
TLR	Toll like receptor
T-lymphocyte/cell	Thymus dependant lymphocyte/cell
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRAF	TNF receptor-associated factor
TRAIL	Tumour necrosis factor Related Apoptosis Inducing Ligand
T-Rex	Tetracycline regulated expression system
TRIM	T cell receptor interacting molecule
Tris	Tris(Hydroxymethyl)aminomethane
TTFT	Time to first treatment
UK	United Kingdom
UM	unmutated
US	United States
UTR	Untranslated region
UV	Ultra violet
v.	Version, versus
V	Variable; volt
v/v	Volume/volume
VCAM	Vascular cell adhesion mediator
VEGF	Vascular endothelial growth factor
VH	Variable region of heavy chain
VL	Variable region of light chain
VLA	Very late antigen
w/v	Weight/volume
WCC	White cell count
WHO	World Health Organisation
WT1	Wilms Tumour

XIAP	X-linked inhibitor of apoptosis protein
ZAP-70	Zeta associated protein-70
β2M	β microglobulin
β-ME	β-mercaptoethanol
ΔCT	Delta CT (difference between two CT values)
ΔΔCT	Delta delta CT (difference between the two delta CT values)

Chapter 1: Introduction

B cell malignancies are common disorders presenting as 90% of all mature lymphoid neoplasm worldwide. Overall they represent 4% of all new cancers per year. Amongst mature B cell malignancies chronic lymphocytic leukaemia (CLL) is the most common leukaemia in the western world, with an incidence of 2-6 cases per 100,000 per year (World Health Organization, 2008).

CLL can have variable clinical presentation ranging from a chronic, indolent condition not initially requiring any therapy for many years or as an aggressive disease needing early treatment. Identifying the patients falling into the latter group at diagnosis is potentially important to improve outcomes. Despite recent developments in prognostic markers, it is not possible to predict with certainty the course of the disease and there is still a need for further development. There are a number of approaches to identify key molecule/s that might improve prediction of a patient's course and therefore hopefully improve the eventual outcome.

This thesis describes the preliminary characterisation of a novel B-cell specific protein, initially termed BCNP1 (B-cell novel protein 1, now renamed FAM129C) and its possible roles in CLL. Secondly, I describe some observations of progrnostic significance in CLL derived from a novel population-based, CLL database from patients attending the clinic in Leicester.

FAM129C was identified from proteomic screening of chronic lymphocytic leukaemia (CLL) plasma membrane fractions (Boyd *et al.*, 2003). Although FAM129C was identified in CLL patient samples and is expressed at high protein level in all cases, it became apparent that it is also expressed specifically in an aggressive form of diffuse large B cell lymphoma (DLBCL): Activated B cell DLBCL (ABC DLBCL). To understand what role FAM129C might play in B cells, I will first discuss B cell development and the germinal centre reaction. Since the activation of NF-κB is central to several aspects of B cell function: development, proliferation, activation and cell death, hence I present a brief introduction to its role.

Understanding normal B-cell development is a crucial for developing new prognostic markers and specific, targeted treatments and therefore understanding role of FAM129C in this process. The subsequent sections in this introduction review the current state of knowledge on the life and death of a B cell.

1.1 B cell development

B cell development (Figure 1.1) in humans can be detected from the 20th week of gestation and continues throughout life. Liver, spleen and foetal omentum are the major sites of foetal haematopoiesis, but in adult life the sole site of the production is the bone marrow (Paul, 2008). Pluripotent haemopoietic stem cells (HSC) (Figure 1.1) generate all blood cell types through progenitor cells which differentiate into lineage specific progenitors. Multipotent progenitor cells (MPP) commit to either common myeloid progenitors (CMP) or common lymphoid progenitor (CLP) through earliest lymphocyte progenitor (ELP).

Early B cell development consists of at least three phases:

1) Progenitor B cells rearrange their immunoglobulin (Ig) genes: this process is dependent on stromal bone marrow interaction and independent of antigen. This phase produces immature B cells with surface IgM expressed initially in conjunction with surrogate light chains.
2) Auto-reactive IgM+ cells are therefore removed through tolerance mechanisms and receptor editing. The latter process spares autoreactive B cells by replacing their receptors (Nemazee, 2000; Amin & Schlissel, 2008). Apoptotic regulators compromising the pro-apoptotic Bim and anti-apoptotic BCL2 and BCLXL are involved in deletion of immune autoreactive cells. Bim is a critical inducer of death triggered by BCR ligation in immature B cells, independent of FAS (member of death receptor family inducing extrinsic apoptotic pathway, and can be inhibited by BCL2 and BCLXL (Marsden & Strasser, 2003).
3) Finally B cells leave the bone marrow, express IgM and IgD on the cell surface and are ready to encounter antigen to become activated, positively selected cells which proliferate and differentiate into antibody producing plasma cells or into memory cells.

Pre-pro-B cells (early B) are characterised by the immunophenotype CD34+ CD10+ CD19- TDT- (terminal deoxynucleotidyl transferase). These cells are the earliest recognised B cell precursors in the bone marrow (Siebenlist *et al.*, 2005). As soon as the immunoglobulin (Ig) heavy chain (Igμ) is re-arranged in these cells, they become pro-B cells. Somatic rearrangement of the *IGH* locus is a very early event in B-cell differentiation.

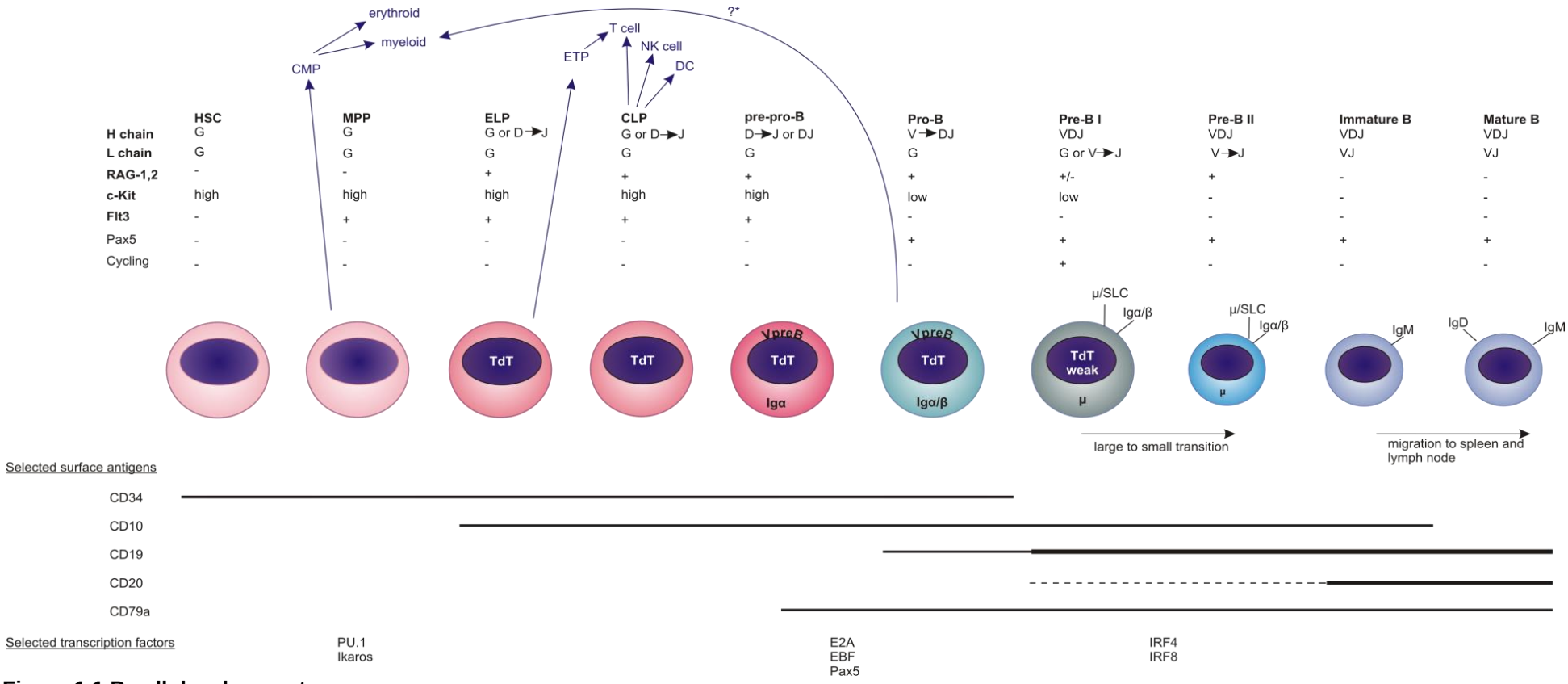

Figure 1.1 B cell development.

Stages of developing B lineage cells in human bone marrow delineated on expression of combination of cell surface proteins. RAG (recombination activating genes, expression fluctuates and is expressed when either Ig heavy chain and light chain are rearranged). Myeloid progenitors originate from MPP, T cell progenitors from ELP.

HSC- haemopoietic stem cell, MPP-multipotent progenitor, ELP- early lymphoid progenitor, CLP-common lymphoid progenitor; G-germline, μ/SLC and Igαβ form pre-BCR

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Pro-B have the immunophenotype CD34+ CD10+ CD19+ TdT+. This stage is characterised by production of the pre-B cell receptor (BCR). The pre-BCR is composed of two membrane anchored heavy chains of the μ isotype (μHC), which binds to Ig-α/Ig-β (CD79α/β) dimer and λ-light chain like surrogate chains (Figure 1.1). RAG-1 and RAG-2 (recombination activating genes) are early lymphocytes specific enzymes initiating DNA strand breakage within the *IGH* locus: first in D-J segments, than in V-DJ.

Formation of the pre-BCR heralds transition from the late pro-B cell to the large pre-B cell and is also characterised by loss of CD34 and TdT (Lebien *et al.*, 1990).

Pre-BCR ligation in immature cells can have the following outcomes: apoptosis, anergy (unresponsiveness) or receptor editing. Expression of the pre-BCR is an important check point for the transition from the pro-B to the pre-B cell stage.

Pre-B cells (immunophenotype: CD34- CD10+ CD19+ TdT-), this fraction is both IgM- and IgM+; they also express heavy chains (μHC) in the cytoplasm and have downregulated TdT. At this stage pre-BCR signalling promotes:

- Allelic exclusion at the *IGH* locus Induction of proliferation.
- Induction of further differentiation, transition from large to small pre-B cells, which start recombining light chains (Martensson *et al.*, 2007).

The pre-BCR has an important effect on cell growth and survival (Lam *et al.*, 1997) and the stromal cell interaction (Hess *et al.*, 2001). These cells begin to proliferate in the bone marrow as large pre-B cells and then arrest in G1 and become non-cycling cells. Non-cycling pre-B cells are small, induce transcriptional silencing of surrogate light chains (Kurosaki *et al.*, 2010) and no longer express pre-BCR. However, the next step is re-arrangement of Igκ and Igλ light chains which bind Igμ heavy chains to form the BCR (surface IgM), characteristic of immature B cells.

Immature B cells acquire surface IgD, CD21 and CD22 and migrate from bone marrow to the periphery and enter the spleen as **transitional 1 and 2 cells** (T1 and T2). ŧThese are short lived and only 10-30% contribute to the mature peripheral B cell compartment (Kurosaki *et al.*, 2010). During the transitional period some cells become splenic marginal zone B cells, which respond quickly to a limited number of antigens; finally they enter the peripheral circulation as **mature follicular B** (B2) cells. The final stage of B-cell maturation is the development of a functional BCR and acquiring additional survival signal from BAFF (B cell activating factor belonging to the TNF family). BAFF (also known as B Lymphocyte Stimulator (BLyS)) is critical for maintenance of normal B-cell development and homeostasis. BAFF promotes B cell differentiation and survival by upregulating BCL2 (Batten *et al.*, 2000).

Tonic BCR signalling (i.e. without being activated by ligand) is important for transition from transitional B cells to mature B cells. Further development occurs after antigen exposure in the germinal centre (GC), see below.

1.2 B-cell signalling

1.2.1 Antigen encounter and immunological synapse

The BCR consists of an antigen binding membrane immunoglobulin (IgM) in complex with a CD79α/β sheath these protein contain~~ing~~ immunoreceptor tyrosine activation motifs (ITAMs), which recruit signalling kinases similarly to the pre-BCR (Figure 1.2) (Reth, 1989).

On resting, antigen-naïve B cells, the BCR is in the monomeric form (Tolar *et al.*, 2005). After antigen encounter BCRs are concentrated into one area of the outer membrane and generate so-called immunological synapses.

Antigen binding leads to the phosphorylation of ITAM motifs by LYN (Src family kinase) which creates a signalosome. A signalosome is an assembly of the SYK kinase phospholipase-Cγ2 (PLCγ2), phosphoinositide 3-kinase (PI3K), Bruton's tyrosine kinase (BTK), VAV and adaptor molecules e.g. B cell linker (BLNK).

Signalosomes coordinate downstream events through calcium flux, induction of gene expression and the internalisation of antigen. Once internalised antigen is processed in the endosomes, loaded onto the major histocompatibility complex (MHC class II) and presented on the B cell surface to CD4+ T cells, which in turn are activated and proliferate. T cells help to complete activation of B cells and induce B cell proliferation (Harwood & Batista, 2010).

In the follicular area of the lymph node the average speed of naïve B cells is 6µm/min (Okada & Cyster, 2006). This motility is probably dependent on CXCL13/CXCR5 interaction (Han *et al.*, 2005) and the follicular dendritic cells (FDC) network (expressing CXCL13) which serves as a net for the B cell movement (Bajenoff *et al.*, 2006). Both macrophages, located in the subcapsular sinus (SCS) (equivalent to the marginal zone in spleen), and FDC play an important role in the transport of the antigen from the SCS to the follicle and therefore present antigen to the naïve cells (Junt *et al.*, 2007).

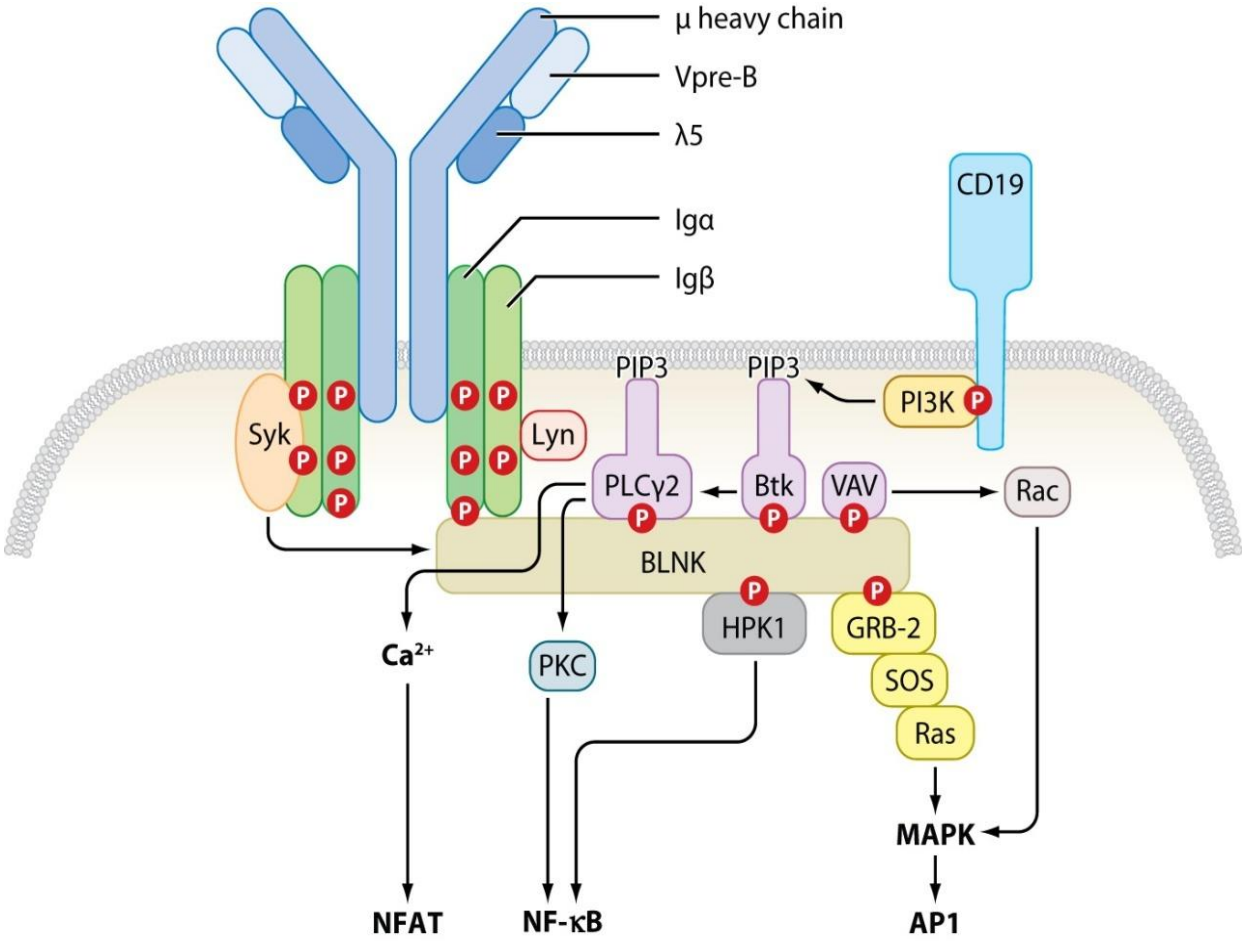

Figure 1.2 Signal transduction through the pre-B cell receptor.

With activation, there is clustering of the signal transduction molecules CD79α and CD79β. Their ITAM motifs are phosphorylated by a SRC family member, shown here as LYN. SYK is then activated by binding to the phosphorylated ITAM motifs. Activated SYK phosphorylates multiple tyrosine residues in the scaffold protein BLNK. LYN also phosphorylates Btk and CD19. Phosphorylated CD19 serves as a docking site for phosphatidylinositol 3-kinase (PI3K), which produces PIP3. PIP3 acts as a docking site for the PH domains of Btk and PLCγ2. The SH2 domains of Btk and PLCγ2 bind to phosphorylated tyrosines in BLNK, which allows Btk to phosphorylate PLCγ2. Phosphorylated tyrosine residues that act as docking sites for SH2 domains are shown as red circles. NFAT, NF-κB, and AP1 are transcription factors.

However smaller antigens may diffuse through the small gaps of the subscapular sinus floor (Carrasco, 2010) (Figure 1.3). Antigen on the membrane of the antigen presenting cell (APC) creates a stable, interaction between the B cell and the APC, also referred as a immunological synapse (IS)(Batista *et al.*, 2001; Carrasco *et al.*, 2004). The main feature of the IS is the central rearrangement of antigen-engaged BCRs, called supramolecular activation clusters (SMACs) at the contact zone.

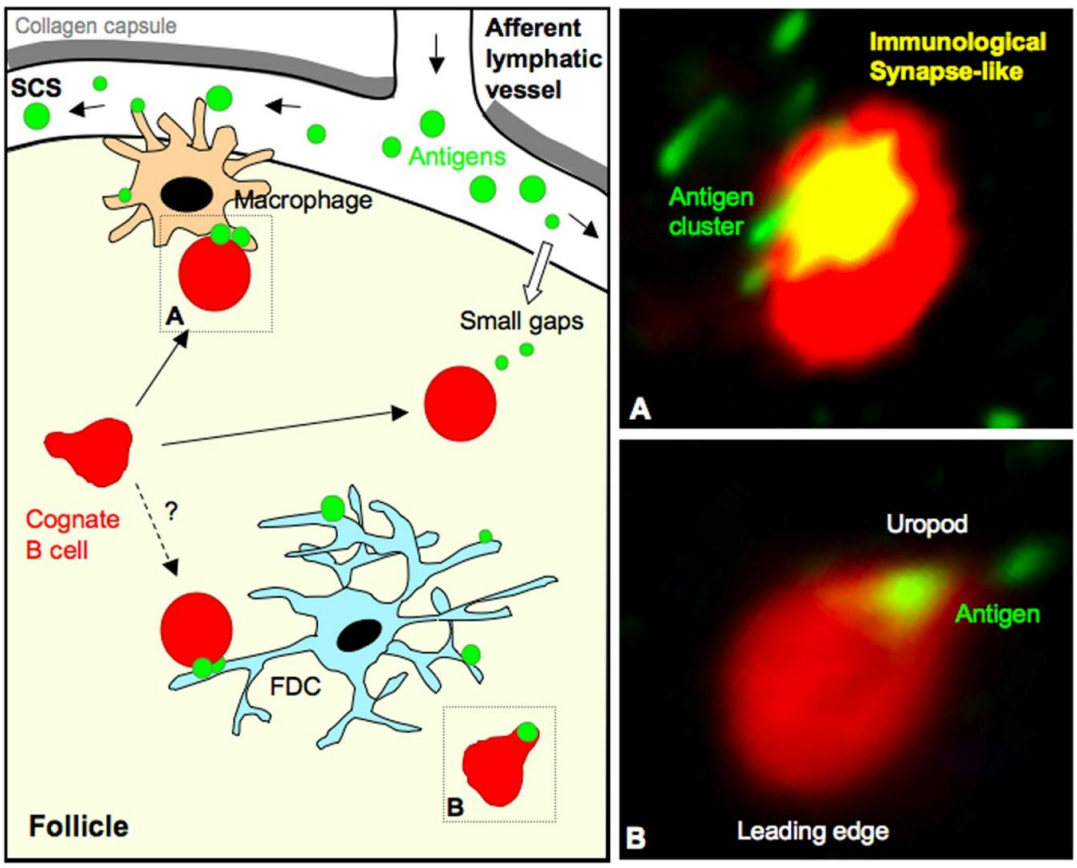

Figure 1.3 Priming of naïve B cells with antigen in lymph node follicules.

Cartoon on the left: naïve B cells can encounter antigen from subscapular sinus (SCS) macrophages and probably follicular dendritic cells, in addition small antigen can diffuse through the small gaps of the SCS floor. Dashed line boxes A and B in the cartoon correspond to multiphoton microscopy images on the right. (red: naïve B cells, green: antigen, yellow: immunological synapse) A. Naïve B cells of circular shape are attached to the antigen binding site. B. Polarised naïve B cell (migratory cell shape) actively migrating and carrying antigen in the uropod..

Central clusters (cSMAC) of antigen receptors (BCR/antigen) are surrounded by a peripheral ring of the integrins LFA-1 (leukocyte function-associated antigen-1, ligand to ICAM-1, intracellular adhesion molecule-1) and VLA-4 (very late antigen-4, ligand to VCAM-1, vascular cell adhesion mediator-1) called peripheral SMAC (pSMAC) (Carrasco & Batista, 2006). The adhesion molecules ICAM-1 and VCAM-1 are highly expressed on the surface of the APC, macrophages, FDC and dendritic cells and promote interaction with B cells (Koopman *et al.*, 1991; Kushnir *et al.*, 1998). The role of the IS is currently controversial it was initially thought to be a platform for BCR signalling:, however now the IS is considered to be important in the efficient BCR-mediated antigen internalisation, which in turn leads to optimal activation through the recruitment of T cell help, even when antigen is limited (Carrasco, 2010).
Membrane bound antigen triggers ~~a~~ two distinct responses in B cells (Figure 1.4)., in phase 1, B cells spread rapidly over the surface of the antigen-presenting membrane and re-distribute antigen into small clusters (Fleire *et al.*, 2006). The BCR microclusters contain IgM and IgD (Depoil *et al.*, 2008) and contain approximately 100 BCRs. Phase 2, the contraction phase, is where the microcluster of BCR/antigen is moved to the centre of the contact site. This phase ends in the formation of IS. BCR microclusters are hot spots for the recruitment of signalling molecules, containing proteins phosphorylated by tyrosine and lacking the inhibitory phosphatase CD45 (Depoil *et al.*, 2008).

Formation of the IS requires re-organisation of the B-cell cytoskeleton and the key roles in the spreading phase have the tyrosine kinases LYN, SYK and guanine-nucleotide exchange factor (GEF) for Rho GTPases VAV1 and VAV2. The GTP-ase Rac2 plays an important part in the assembly of the F-actin platform and of calcium signalling initiation through PLCγ2 (Arana *et al.*, 2008; Lin *et al.*, 2008). BTK and BLNK are positioned further downstream of the intracellular signalling (Weber *et al.*, 2008). CD19 is a regulatory co-receptor, a membrane adaptor protein which enhance BCR signalling. The transient recruitment of CD19 to microclusters induces signal amplification by moving signalling molecules to the surface (Depoil *et al.*, 2008). The events following microcluster formation are further discussed in the next paragraph, where it is reviewed together with the role of B-cell signalling adaptors.

1.2.2 Adaptor molecules in B-cell signalling

Adaptor proteins play an important role in the creation of the immunological synapse and in B-cell signalling. Adaptors interact with multiple signalling proteins and serve as

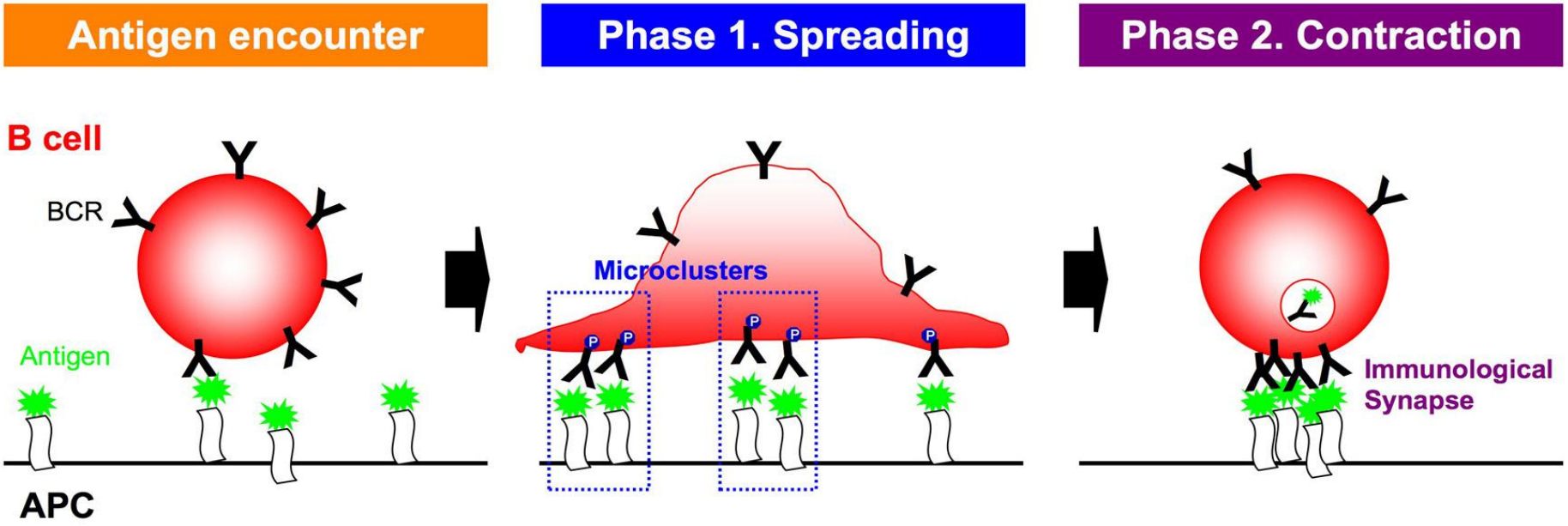

Figure 1.4 Spreading-contraction response drives Immunological Synapse (IS) formation.

The encounter of the membrane-immobillised antigen on the surface of antigen presenting cell (APC) activates BCR-signalling and initiates Phase 1: spreading where B cell maximises antigen gathering by increasing the contact surface with the APC; BCR/antigen microclusters are formed, which are the core of the microsignalosomes assembly.

Phase 2: B cell contracts, localises the antigen at the centre of the B cell-APC contact, internalises it, and establishes the IS.

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molecular “scaffolds” to localise proteins to specific subcellular sites, to control enzyme activities and direct the formation of multiprotein complexes and can have both positive and negative regulatory effect on B cell signalling (Kurosaki, 2002).

Adaptors modulate signalling by protein-protein or protein-lipid interaction through specific domains (SRC-homology2 (SH2), SH3; phosphotyrosine-binding (PTB) or pleckstrin homology domain (PHD: FAM129C contains such a domain and, a detailed analysis of PHD is presented in Chapter 5)). Usually adaptor proteins lack intrinsic enzymatic activity but some kinases e.g. LYN, BTK and CSK are SRC-family protein-tyrosine kinases, containing the SH2 and SH3 domain; other examples are lipid-metabolising enzymes (e.g. PI3K, PLCγ2) and VAV. There are also co-receptors which behave like adaptor proteins, namely CD19 and PIRB (paired immunoglobulin-like receptor).

A large number of BCR signalling adaptors protein have been identified (Table1.1) which can be divided into cytoplasmic and transmembrane. They are either positive or negative regulators of BCR signalling. Some including Grb2 can have both positive and negative regulatory functions (Table 1.1). I mention these in some detail since bioinformatic analysis of FAM129C suggested that it might also function as a B-cell specific adaptor protein.

BLNK is a docking site/scaffold for NCK, BTK and PLCγ2. BLNK organises microsignalosomes in the spreading phase through the recruitment of VAV and PLCγ2 (Weber *et al.*, 2008). VAV plays an important role in the B cell spreading phase by its GEF activity on actin modulators. The B lymphocyte adaptor molecule of 32 kDa (Bam32), after phosphorylation by a SRC family kinase and recruitment to the plasma membrane, becomes another potential mediator of cytoskeleton reorganisation. The co-receptor CD19 has an essential role in mediating B cell spreading. CD19 forms a complex with VAV, PI3K, LYN, PLCγ2 (Li X. JI, 1997) and is transiently associated with microclusters (Depoil *et al.*, 2008) .

Abnormalities in adaptor proteins have been associated with immunodefiencies in man (Table 1.1), e.g. BLNK mutation causes neutropenia and recurrent infections and CD19 leads to antibody deficiency syndromes (Conley *et al.*, 2009).

Tedoldi (Tedoldi *et al.*, 2006) examined six transmembrane adaptor proteins (LAT, NTAL, PAG, LIME, TRIM, SIT, see Table 1.1) in an immunohistological study of B cell lymphomas. PAG was selectively expressed in germinal centre subsets of DLBCL and absent in ABC DLBCL. Follicular cell lymphomas were mainly negative for SIT and positive for PAG and NTAL, whereas CLL and MCL tended to be positive for SIT and negative for PAG and NTAL. LAT, LIME, TRIM are restricted to T cells. Tedoldi *et al.*

indicated that transmembrane adaptor proteins may represent a new type of markers for DLBCL. Presence of PHD in FAM129C and its B cell specificity implies that FAM129C may have adaptor function.

Table 1.1 Adaptor molecules in B cell signalling

Cytoplasmic (alternative names)	Domain	Function	Disease or Knock-outs
BLNK	SH2	Activated Btk , PLCγ2 and Vav bind to BLNK via SH2 domain and hence allow Btk to phophorylate PLCγ2, which in turn leads to calcium influx and activation of JNK and ERK (MAP kinases).	Human mutation: Neutropenia, recurrent infection, lymphocyte phenotype similar to Btk (variable and weaker CD19 and high IgM) (Conley *et al.*, 2009) BLNK-/- spontaneous pre-B cell lymphoma (Flemming *et al.*, 2003)
Grb2)	PHD Sh2,	Negative regulator of BCR-induced calcium signalling (Stork B, 2004), promotes CD22 phosphorylation and positive modulation of BCR-induced Vav activation. Grb2 forms a complex with BLNK and Vav and moves Vav to the plasma membrane on BCR activation (Jang *et al.*, 2009)	Grb2-/- mice:Reduced number of mature B cells, increased production of autoimmune antibodies (Jang *et al.*, 2009).
Bam32	SH2, PHD	GC associated (Zhang *et al.*, 2010), PI3K effector molecule, , recruited to the plasma membrane. Implicated in activation of the GTP-ase Rac1, MAPKs ERK and JNK regulating signalling pathways of cell survival, proliferation, cytoskeletal rearrangement, receptor internalisation (Zhang *et al.*, 2009)	Bam32-/- show normal development of B and T cell subsets, but impairment of BCR-induced proliferation(reduced activation of MAPK, JNK and ERK pathways; failure to mature to follicular cells (Allam & Marshall, 2005). Bam32 plays an important role in production of IgG3 in response to *Streptoccocus pneumonia,* therefore maybe a cause of immunodeficiencies predisposing to infections by polysaccharide-encapsulated organisms (Fournier *et al.*, 2003)
BCAP	Ankrin repeat, proline rich, coiled coil	Binds PI3K and connects BCR to the PI3K pathway Probably important for the survival of peripheral mature B cells by acting on c-Rel (Yamazaki & Kurosaki, 2003)	BCAP-/- mice: decreased follicular B cells, (Simeoni *et al.*, 2004), loss of B1 cells, reduced mature B cells, reduced c-Rel, poor responses to T cell independent type II antigens (Yamazaki & Kurosaki, 2003)
BANK	Ankrin repeat, coiled coil	Participates in BCR-mediated calcium homeostasis (Yokoyama *et al.*, 2002), attenuates CD40-mediated Akt activation, therefore preventing hyperactive B cell responses.	BANK-/- increased GC formation, cd40 mediated proliferation and survival increased, enhanced Akt activation (Aiba *et al.*, 2006).
[VAV1 and VAV2]	DH, PHD, SH2, 2xSH3	Acts as guanine nucleotide exchange factors for many small GTPases: Rac1/2, RhoA/G, can promote site specific actin polymerisation (Turner & Billadeau, 2002)	Overexpression was seen in 13q deleted CLL (Prieto-Sanchez *et al.*, 2006) Vav1-/-Vav2-/-: B cell development severly impaired (Tedford *et al.*, 2001)
3BP2	PH, SH2	Complexes with Vav1, vav2, PCLγ and Syk in B cells; binds to cytoplasmic tail of CD19 (Chen *et al.*, 2007)	Cherubism: AD disorder, excessive osteoclast activity causing erosions in maxillar and mandibular bone and giant cell granuloma formation 3BP2-/- defects in Syk phosphorylation and calcium flux (Chen *et al.*, 2007)
DOK1 and DOK3	PHD, PTB	Dok1: negative regulator of Res-ERK pathway, Dok3: negative regulator of the activation of JNK and mobilisation of calcium in BCR signalling, interacts with SHIP-1 and Grb2 (Mashima *et al.*, 2009)	Dok-1 -/-and dok-2-/- develop CML-like myeloproliferative disease (Niki *et al.*, 2004) unable to find data on Dok-1 and Dok-3 and B cells

Act1	Helix-loop-helix, coiled coil	Regulator of signalling pathways mediated by CD40-CD154 interaction and BAFF (Qian *et al.*, 2002)	Act1-/- mice: increase of peripheral B cells, lymphadenopathy, splenomegaly, hypergammaglobulinaemia, autoantibodies (Qian *et al.*, 2002), other group: features of Sjogren's syndrome (Qian *et al.*, 2008).
Transmemebrane			
NTAL	proline reach, palmitylation site	Localised in rafts, after phosphorylation by Syk-family protein kinases, recruits Grb2 and enhances ERK phosphorylation (Brdicka *et al.*, 2002) it was proposed to have a role in internalization of the BCR (Mutch *et al.*, 2007)	
PAG	proline reach	PAG is phosphorylated, recruits CSK (carboxy-terminal SRC kinase), suppresses activity of LYN and other B-cell SRC kinases; mediate cross-talk between lipid rafts and the actin cytoskeleton (Horejsi *et al.*, 2004)	
[CD19]		Reduces threshold of BCR activation by 10^4 fold (Fearon & Carroll, 2000); engages and activates PI3K, Vav, Lyn and Fyn; is required for maximum induction of calcium influx	Human: antibody deficiencies syndromes (Conley *et al.*, 2009) CD19-/- compromised immune responses to antigen (Rickert *et al.*, 1995)

Green font, positive regulators; red font, negative regulators; []-coreceptors and signalling molecules assimilating adaptor function, CD19 and Vav. BCAP, B-cell adaptor for phosphoinositide 3-kinase; BLNK, B-cell linker; DH, DBL-homology; Dok, downstream of tyrosine kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase); MAPK, mitogen-activated protein kinase; NTAL/LAB (non-t-cell activation limker/linker for activation of B cells); PAG, Phosphoprotein Associted with Glycosphingolipid enriched microdomains; PI3K, phosphatidylinositol 3-kinase; PTB, phosphotyrosine binding domain; Sos, son of sevenless

1.2.3 The germinal centre reaction and CD40 signalling

The first description of germinal centre (GC) was published in 1885 by Walther Flemming (cited in (Brink, 2007)), the light and dark zones of the germinal centre were described much later in 1930 by Röhlich (Allen *et al.*, 2007a); the model of a GC reaction was proposed by MacLenan in 1994 (Maclennan, 1994) and its basis is still valid today.

The GC consists of a dark and light zone and is surrounded by the mantle and marginal zones (Figure 1.5). The mantle zone is the site of most of the naïve B cells, representing pre-GC cells, whereas marginal zone is the location of memory cells, post-GC.

Antigen-induced B cell activation and differentiation is a dynamic process involving changes in gene expression that give rise to germinal centres (GC). The GC reaction is characterised by clonal expansion, class switch recombination (CSR) at the *IGH* locus, somatic hypermutation (SHM) of *IGHV* genes and selection according to the affinity of the BCR. This is reminiscent of the theory of Darwinian selection (LeBien & Tedder, 2008). Initiation of the GC reaction occurs by follicular dendritic cells acquiring antigen and migrating to the T zone (periarteriolar lymphoid sheath, PAL). These FDC mature

and stimulate naïve CD4 T cells. These antigen specific T cells migrate to the B cell follicle and activate antigen specific follicular cells.
Activated B cells can follow two paths, either enter the GC and become centroblasts (follicular pathway) or move to the extrafollicular areas (extrafollicular pathway), proliferate, and undergo terminal differentiation into short-lived plasma cells secreting antibodies. Short-lived plasma cells express IgM only and lack somatic mutation.

The dark zone of GC consists mainly of centroblasts, a small number of follicular dendritic cells and occasional T cells (c.f. light zone: there is a large number of T cells and follicular dendritic cells). There is a high degree of proliferation in the dark zone of the GC. The centroblasts (CB) have very little of surface Ig of any isotype. The majority of centroblasts do not receive any CD40-CD154 signalling (Basso *et al.*, 2004). This observation was supported by the staining of lymphoid tissue which showed lack of expression of proteins required for NF-κB activation like c-Rel, p65, TRAF1, JUNB.

CD40-CD154 signalling is important in the centrocyte stage: it affects B cell selection according to its antigen affinity (Rathmell *et al.*, 1996) and in the immunoglobulin class switching (Liu *et al.*, 1996). Furthermore, CD40-CD154 signalling is crucial for B cells exiting from the GC to become memory cells. Memory B cell development was impaired by in vivo blocking of the CD40-CD154 interaction and the GC was normally formed (Gray *et al.*, 1994).
Therefore in summary the authors concluded that GC expansion occurs in the absence of CD40 signalling and may act only in the initial and final stages of GC reaction. There are number of systems available to mimic the germinal centres and microenvironment (discussed in the Materials and Methods chapter). In my laboratory the set up system was mouse fibroblasts stably transfected with human CD40L (CD154) cultured in presence of IL-4 as described by Banchereau (Banchereau *et al.*, 1991).

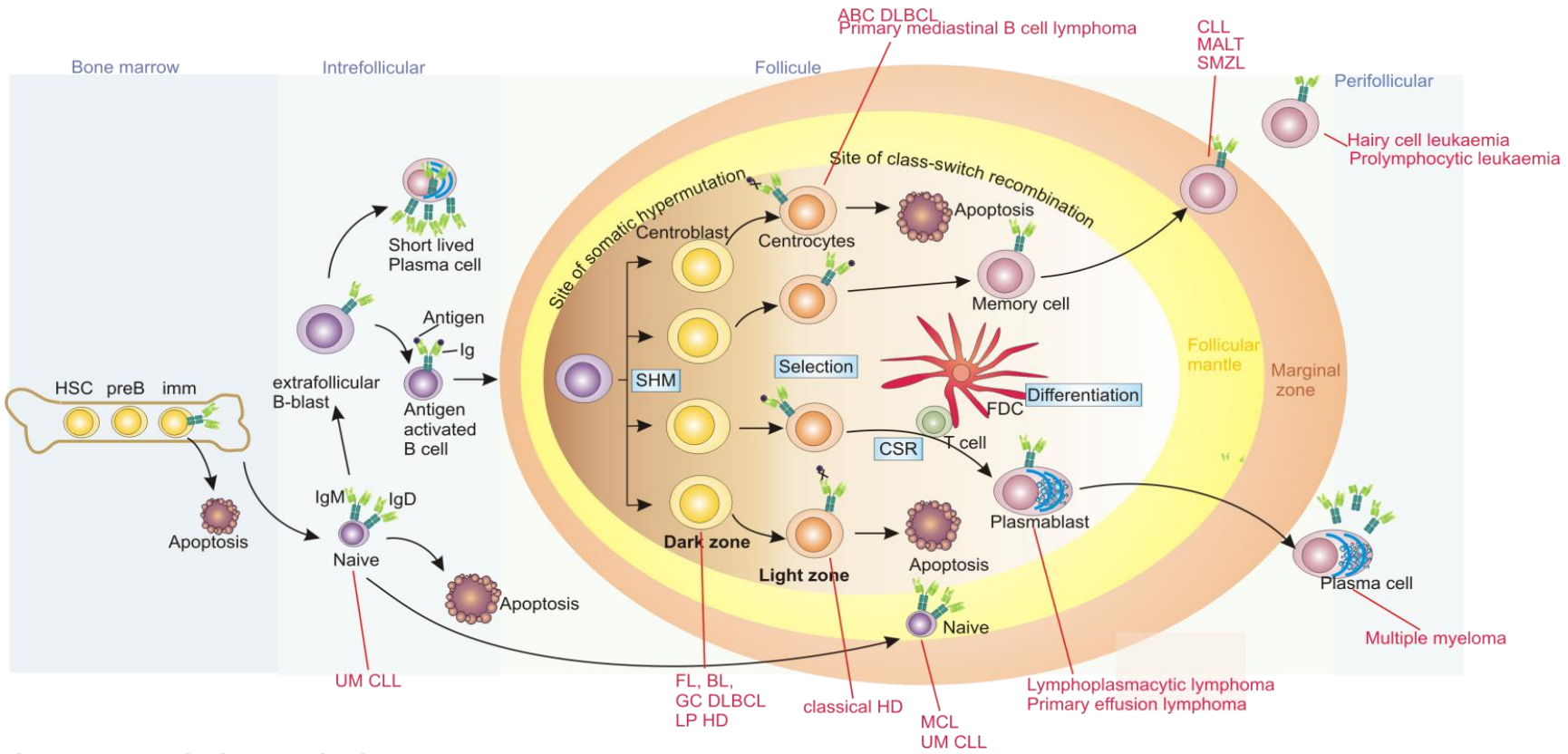

Figure 1.5 Germinal Centre (GC).

B cells develop from haemopoietic stem cells (HSC) in bone marrow and remain there until the immature B cell with formed B cell receptor (BCR, IgM), immature B cell enter the spleen (not shown and progress through transitional T1 and T2 stage and enter the peripheral circulation as mature (naïve) follicular B cells. Naïve B cells activated by antigen, travel to primary follicules and establish GC. IgM+IgD+ naïve B cells surround GC and form mantle zone; in GC dark zone centroblasts (CB) start proliferating, here clonal expansion and somatic mutation takes place. The majority of CB which acquire disadvantageous mutations apoptose and only a few which have achieved high affinity to antigen by mutations are positively selected

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The main process occurring in the dark zone is the somatic hypermutation of V genes regulated by the enzyme activation induced deaminase (AID (Muramatsu *et al.*, 2000). It is currently thought that *IGHV* mutations occur at the DNA level (Reynaud *et al.*, 2003). This is a highly risk-prone process: AID was shown to have a role in tumourigenesis by generating chromosomal translocations between *c-myc* and the IgH locus in some murine B cell lymphomas (Okazaki *et al.*, 2007). There was a suggestion that there are different types of CD40 signalling in different parts of GC (Siepmann *et al.*, 2001).

The migration to and through the GC is dependent on the expression of cytokines. Naïve B2 B cells upregulate CCR7 after binding antigens, and are attracted by a gradient of CCL21 to the outer T cell zone of secondary lymphoid tissues where they obtain T cell help (Reif *et al.*, 2002). In the dark zone CB express high levels of the chemokine receptor, CXCR4, which is crucial to position the CB in the dark zone (Allen *et al.*, 2004).

Only GC B cells form a stable interaction with the GC T cells. They present the highest number of peptide-antigen-MHC class II complexes compared with the other B cells. Only B cells that bind, internalise and present antigen can induce CD154 expression by T cells (Casamayorpalleja *et al.*, 1995). There is a fail-safe mechanism, where cells which receive a CD40 signal without any signal via the surface Ig seem to be susceptible to FAS-ligand mediated lysis (Rothstein *et al.*, 1995). BCL2 and BCLXL may be important in GC rescue. The whole process is rapid and strictly controlled and GC B cells exit the GC within hours (Allen *et al.*, 2007b) (c.f. chapter 1.3.3).

The CB exits the cell-cycle and becomes a non-proliferating centrocyte (CC) which re-expresses Ig and moves to the light zone where it undergoes class switch recombination. B cell selection is a two stage process: CCs compete for binding to the antigen presented on FDC (presented as peptides on MHC class II molecules), the majority of the B cells die in this selection process (there is loss of BCL2 in these cells); these selected CC present antigen to the T cells, which in turn enhance survival or promote differentiation into antibody secreting plasma cell or memory B cells.

1.2.4 FAS (also termed CD95 or APO-1)

FAS killing was simultaneously discovered by the Peter Krammer group and a Japanese group by Nagata and Yonehara in 1989. Krammer's group generated a monoclonal antibody (APO1 specific) which induced apoptosis in lymphoid cells (Trauth *et al.*, 1989). At the same time the Japanese group immunised mice with the human fibroblast cell line FS-7 to produce monoclonal antibodies against the interferon

receptor, during the screening phase they found a cytotoxic antibody to the FS-7 cells, the antigen was called FAS (FS-7-associated surface antigen (Yonehara *et al.*, 1989). The Nagata group cloned and sequenced the *FAS* gene (Itoh *et al.*, 1991) and discovered it as the underlying mutation in *lpr* (lymphoproliferative) mice (Adachi *et al.*, 1993).

FAS belongs to the tumour necrosis factor receptor (TNFR) type family (e.g. TNF-R1, TRAIL-R1, -R2, DR3 and DR6). A common feature of death receptors is a conserved 80 amino acid sequence, the death domain, in the cytoplasmic tail. It has one extracellular domain rich in cysteines that bind FASL, and a cytoplasmic domain involved in death signals (Henkler *et al.*, 2005).

FAS signals the apoptotic pathway but also a non-apoptotic pathway (the NF-κB and MAP kinase pathway), which induces tumourigenic or pro-survival genes (Wajant *et al.*, 2003).

Effector T cells and NK cells express FASL (CD178) and target cells express FAS (CD95 or Apo-1) (Chen *et al.*, 1998; Zhang *et al.*, 2000). FASL is a molecule of 40 kDa, expressed on T cells but also constitutively on cells of immune privileged organs (brain, anterior chamber of the eye, testes), therefore, these sites are protected from the action of immune system (Ramaswamy & Siegel, 2007).

This process is tightly regulated by activators and inhibitors:

- c-FLIP (cellular FLICE-inhibitory protein: interferes with the recruitment of pro-caspase 8 to the DISC (death inducing signalling complex) (Scaffidi *et al.*, 1999);
- endothelial cells (Wang *et al.*, 2002) and hepatocytes (Huh *et al.*, 2004) withstand FAS stimulation by expression of tyrosine kinase receptor c-Met. It prevents self-association of FAS receptor and FAS/FASL interaction;
- decoy receptor 3 (DcR3), a soluble receptor for FASL, a member of the TNFR superfamily and plays important role in immune suppression and tumour progression by escaping immune surveillance by neutralising FASL (Wu *et al.*, 2003);
- downstream regulation by XIAP (X-chromosome linked inhibitor of apoptosis protein); lymphocytes from patients with XIAP deficiency have enhanced apoptosis after stimulation of FAS (Rigaud *et al.*, 2006).

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Figure 1.6 Pro-survival members of BCL-2 family preserve mitochondrial integrity by stopping activation of Bax and /or Bak.

Anti-apoptotic protein are inactivated by BH3-only proteins, this leads to changes in mitochondrial membrane potential and outer membrane permabilisation, release of cytochrome c, the latter is required to form the apoptosome and induces the caspase cascade. Death-receptor pathway, activates caspase 8, which in turn cleaves BID to tBID, hence connecting both pathways.

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http://www.biooncology.com/bioonc/research/apoptosis/index.m

Mutations in CD95 (FAS) lead to autoimmune lymphoproliferative syndrome (ALPS) (Fisher *et al.*, 1995; Rieuxlaucat *et al.*, 1995). Observations in human ALPS patients and respective abnormalities in mouse models show the *lpr*-mutation of *FAS* which causes defective expression of CD95, the lpr^{CG}-point mutation in the DD (death domain) of CD95 stops recruitment of FADD and a *gld*- mutant form of *FASL*. These mice present with lymphoproliferation, the accumulation of abnormal lymphocytes and autoantibody production eventually leads to systemic autoimmunity (Watanabefukunaga *et al.*, 1992; Takahashi *et al.*, 1994).

These observations, both in mice and human models, suggest that FAS plays an important role in lymphocyte homeostasis i.e. it is critical for abrogating the chronic immune response (Hughes *et al.*, 2008) and prevention of autoimmunity (Peter *et al.*, 2007).

A recent paper from the A. Strasser group (O' Reilly *et al.*, 2009) appears to resolve the functional divide between the membrane bound (mFASL) and the secreted FASL (sFASL) by generating respective knock-out mice. They showed that mFASL is essential for cytotoxic activity and prevents lymphadenopathy, autoimmunity and cancer; the sFASL appears to promote autoimmunity and tumourigenesis through non-apoptotic pathways.

FAS appears to play a key role in earlier described germinal centre (GC) clonal selection and entry of GC B cells into the memory compartment. The exact mode of this is not fully understood. Follicular dendritic cells contain FASL (Hur *et al.*, 2000). Hennino (Hennino *et al.*, 2001) showed that GC B cells contain high levels of FLIP, these cells have reassembled FAS into complexes with FADD and caspase 8, without interaction with FASL. FLIP levels fall *in vitro* culture and leads to apoptosis. FLIP levels are maintained by CD40 engagement and contact with FDC in germinal centres, both protect against cell death (van Eijk *et al.*, 2001).

Autoreactive B cells are FAS-sensitive, hence eliminated during the GC reaction. FAS expressing B cells are in danger of being eliminated by activated, FASL presenting T cells, therefore inducible FAS-resistance probably protects B cells during their T cell interaction, until B cell immune response maturity is achieved. Therefore, FAS-resistant B cells are more effective in presenting antigen than FAS-sensitive B cells (Ozdemirli *et al.*, 1996). FAS-resistant B cells block also FAS-induced cell death of T cells (Wang *et al.*, 2000).

Primary B cells express low levels of FAS and must be stimulated to upregulate FAS expression and become sensitive to apoptosis (Daniel & Krammer, 1994).

The upregulation can be achieved by lipopolysaccharide (LPS) treatment or by stimulation with CD40L (CD154)(Garrone *et al.*, 1995; Schattner *et al.*, 1995; Lagresle *et al.*, 1996; Rathmell *et al.*, 1996). Interestingly, BCR stimulation by crosslinking with F(ab)2 fragments induces moderate FAS increase but cells are resistant to FAS induced apoptosis (Rothstein *et al.*, 1995). Treatment with IL4 can also induce FAS-resistance (Foote *et al.*, 1996). This is relevant as the CD154 cellular system used in this thesis included IL4 in the culture.

Ig stimulation of B cells induces resistance to FAS killing which is mediated by NF-κB induction of anti-apoptotic genes (Schram & Rothstein, 2003; Sen, 2006).

Dalla-Favera's group showed the relationship between CD40, NF-κB, BCL-6 and IRF4. NF-κB binds to the *IRF4* promoter and induces it, in turn IRF4 binds to the *BCL-6* promoter and suppresses BCL-6 expression. Primary GC B cells, after CD40-CD154 stimulation and induction of NF-κB, show upregulation of *IRF4* transcripts and protein, downregulation of *BCL-6* transcripts and BCL-6 protein (Basso *et al.*, 2004; Klein *et al.*, 2006). GC centrocytes that express nuclear NF-κB lack BCL-6, and the ones which lack NF-κB, express BCL-6 (Saito *et al.*, 2007).

FAS and FASL expression has been associated with good prognosis in DLBCL (Kojima *et al.*, 2006). Another Japanese group showed that soluble FAS level determines clinical outcome of patients with diffuse large B-cell lymphoma (Hara *et al.*, 2009). Mollinedo (Mollinedo *et al.*, 2010) used compound Edelfosine which induced coclustering of FAS/CD95 death receptor and rafts in CLL cells and caused selective edelfosine-induced apoptosis.

1.2.5 BCL2 in B cells and CLL

The *BCL2* (B-cell lymphoma 2) gene was described in 1985 by Krosmeyer's group (Bakhshi *et al.*, 1985) after cloning a chromosome breakpoint at the t(14;18)(q32;q21) present in follicular lymphomas (FL). As a consequence, BCL2 transcription becomes driven by the immunoglobin VH gene promoter and enhancer on chromosome 14. Follicles in FL are strongly positive for BCL2 although within the normal GC BCL2 is usually extinguished. Inappropriate overexpression of BCL2 promotes survival by inhibiting apoptotic cell death (Vaux *et al.*, 1988). BCL2 is at the centre of the intrinsic (mitochondrial) apoptosis pathway.

BCL2 controls apoptosis in many conditions. How this is mediated is not entirely clear. It regulates the release of apoptotic proteins from mitochondria, by altering the permeability of the outer mitochondrial membrane. BCL2 can prevent release of

cytochrome c (Kluck *et al.*, 1997; Hengartner, 2000), and therefore it is believed to control mitochondrial membrane integrity. The anti-apoptotic BCL2 is localised predominantly within the outer mitochondrial membrane by a hydrophobic C-terminal membrane spanning domain (Lithgow *et al.*, 1994). The role of BCL2 in the ER and nuclear envelope are not fully understood. ER-localised BCL2 is regulated by JNK phosphorylation, whereas a mitochondrial pool of ceramide can cause dephosphorylation of BCL2 by the protein phosphatase 2A and activate apoptosis (Ruvolo *et al.*, 1999).

BCL2 deficient mice were observed to exhibit abnormal death of renal epithelial progenitors, melanocyte progenitors, as well as mature B and T lymphocytes. BCL2 deficiency has been stipulated to cause fatal polycystic kidney disease (100% mortality by 6 weeks), premature greying and lymphopenia. Indicating that normally all these cell types are critically dependent on BCL2 All of these effects can be rescued by concominant loss of BIM (BH3 only, pro-apoptotic protein) (Veis *et al.*, 1993). On the other hand, Eμ-BCL2 transgenic mice, which should recapitulate the BCL2/IGH chromosomal translocation, have only a low incidence of lymphomas and only after a long period, suggesting therefore that BCL2 itself is only weakly tumourigenic (McDonnell & Korsmeyer, 1991). Another transgenic mice strain, where the BCL2 levels are much higher (under the *vav* gene promoter) (Egle *et al.*, 2004) causes GC hyperplasia and progresses to a condition similar to follicular lymphoma. However, interestingly, most of the mice died prematurely, before development of lymphoma, from immunoglobulin nephritis which leads to kidney failure.

There are 12 core members of BCL2 family proteins. The common factor is the presence of a BH3 motif (BH3 has the amino acid sequence of LXXXGD, where X is any other amino acid) and they are structurally similar to BCL2. They can be broadly divided into antiapoptotic and pro-apoptotic categories. BH3 only proteins are sensitisers that binds to anti-apoptotic proteins and inducing release of proapoptotic proteins (Chonghaile & Letai, 2008).

BCL2 also has several other possible functions including suppression of the cell cycle. The connection between cell cycle and apoptosis is generally accepted: cycling cells are more susceptible to cell death and quiescent cells more resistant to killing. The first observation of the cell cycle inhibitory effect of BCL2 was made by Korsmeyer's group (Linette *et al.*, 1996) by observing *bcl2*-deficient, *bcl2*-heterozygous, wild type and transgenic *BCL2* T cells; they observed that the onset of S phase was the quickest in cells *bcl2*-/- and there was a difference in size between these cells. These genotype

comparisons presented evidence that BCL2 plays a role in regulating cell cycle entry. Mutation of the conserved residue tyrosine 28 (Y28) in the N-terminal BH4 antiapoptotic domain of BCL2 enhances G1/S transition without any impact on the antiapoptotic function of BCL2 (Janumyan *et al.*, 2003). The antiapoptotic function of BCL2 makes it an oncogene whereas its effect on the cell cycle makes it a tumour suppressor (Zinkel *et al.*, 2006); this is usually dependent on type of tissue and genetic context. In lymphoid cells an antiapoptotic effect is thought to be dominant over an antiproliferative function and therefore in this situation BCL2 is an oncogene.

Although BCL2 expression in CLL is high the significance and prognostic value in CLL is unclear. It is not driven by the t(14;18) as only small minority of patients: 2% of cases have this translocation (Dyer *et al.*, 1994). BCL2 expression is much higher than other BCL2 family members, e.g. BAX (Pepper *et al.*, 1997) and an increased BCL2/BAX ratio (favouring cell survival) was shown to be associated with treatment response, but not associated with Rai staging (Saxena *et al.*, 2004). There was no proven correlation between the worse prognosis in BCL2 positive patients and resistance to chemotherapy (Kitada *et al.*, 1998).

The regulation of BCL2 expression is still unclear. It can be modulated at both transcriptional and post-transcriptional levels. Seto (Seto *et al.*, 1988) described two promoters regulating gene transcription. The major transcriptional site of P1 is located 715bp upstream from the BCL2 translational site; promoter P2 is located 1.3 kb downstream of the P1 promotor. P2 has TATA and CAAT boxes. A new promoter with p53 dependent activity was found between P1 and P2 (Bredow *et al.*, 2007). In the majority of CLL patients the promoter region of BCL2 is hypomethylated, which contributes to the increased transcription and protein expression (Hanada *et al.*, 1993).

Several transcription factors are known to be involved in the positive regulation of BCL2 transcription: cAMP responsive element binding protein (CREB) (Wilson *et al.*, 1996) and NF-κB (Heckman *et al.*, 2002). The negative regulatory sites are: π1 binding sites in pre-B cells (Chen & Boxer, 1995), WT1 binding sites (Heckman *et al.*, 1997), and p53 binding sites (Miyashita *et al.*, 1994; Wu *et al.*, 2001).
Singh *et al.* (Singh & Bhat, 2004) suggested that transcription factor NF-κB plays a role in downregulation of BCL2 when cells are treated with the drugs 5-fluorouracil and carboplatin. The microRNAs 15 and 16 were thought to be initially involved in regulation of BCL2 expression, however mir-15 and mir-16 knock-out mice do not have increased levels of BCL2. BCL2 is post transcriptionally modified by phosphorylation at

the putative MAPK (mitogen-activated protein kinase) kinase sites which leads to ubiquitination, proteosome-dependant degradation, caspase-dependant cleavage, hence resulting in the loss of anti-apoptotic activity (Breitschopf *et al.*, 2000).

There is a number of BCL2 mutations published in the literature. Tanaka identified 6 mutations in five untreated patients, Pappa (Pappa *et al.*, 1997) described 43 somatic mutations within the first 582 bp of the opening reading frame of *BCL2* in $DOHH_2$ follicular lymphoma cell lines, eight cases of non-Hodgkin lymphoma with t(14;18) and one case of benign follicular hypeplasia. There was a high frequency of mutations in the BH1 domain (12/43, 28%) and there were only 2 mutations found in the BH2 domain. More recently Park identified six polymorphisms; one in P2 promoter, three in exon 1 and two in exon 2. Nuckel *et al.* (Nuckel *et al.*, 2007) proposed that the -938 polymorphism in promoter P2 has a negative regulatory effect and screened 123 CLL patients and 120 healthy volunteers. In this study polymorphism -938AA was associated with more aggressive disease with the worse overall survival and reduced time to first treatment. Discussion of this work is presented in detail in the BCL2 Chapter (Appendix).

1.3 CLL biology

1.3.1 What is a normal B cell counterpart of CLL?

The cellular origin of CLL cells has not been clearly defined. However there is growing evidence that CLL with both unmutated and mutated *IGHV* originate from cells that encountered antigen.

CLL cells are characterised by ~~co~~expression of CD5 on the cell surface and therefore it was initially thought that CLL cells originated from CD5+ normal B cells (Zenz *et al.*, 2010). However this appears to be unlikely as normal CD5+ B cells rarely have any mutations in *IGHV* (Fischer *et al.*, 1997), whereas the majority of CLL carry these mutations. Also, gene expression profiling showed no similarities between CLL and CD5+ cord B cells but the closest correlation is to memory B cells (Klein *et al.*, 2001).

The origin of CLL with mutations in *IGHV* seems to be post germinal centre memory cells, more specifically marginal zone-like B cells that underwent SHM during T independent response (Chiorazzi & Ferrarini, 2003). These cells are similar to B1a cells in mice: they are capable of self-renewal and produce polyreactive IgM antibodies with low avidity. The mechanism of mutation in these cells is unknown, as it was observed that these cells lack AID (activation induced deaminase) (Willenbrock *et al.*, 2005).

Further evidence that CLL cells originate from the post-GC cells is the presence of mutations in BCL6 in CLL cases with mutated *IGHV* (Pasqualucci *et al.*, 2000). There is good evidence that CLL with unmutated *IGHV* also originates from cells which encountered antigen. Firstly, as mentioned above, unmutated *IGHV* CLL has a gene expression pattern similar to the post-GC memory cells (Klein *et al.*, 2001). The lack of *IGHV* mutations does not necessarily imply lack of transit through the GC since some memory B cells lack somatic mutations(Seifert & Kuppers, 2009). UM *IGHV* CLL cells express polyreative and autoreactive antibodies (Chu *et al.*, 2008; Lanemo Myhrinder *et al.*, 2008), which makes them closely related to B1 cells in mice. In addition they have an activated, post antigen encountered cell phenotype (Damle *et al.*, 2002). Many UM CLL have stereotypic BCR, i.e. suggesting exposure to the similar antigen and presenting almost identical epitopes and are more commonly found in (Stamatopoulos *et al.*, 2007). Autoantigen specificities in these B cells suggest both T dependent and T independent reactions (Zenz *et al.*, 2010). The T cell dependent response involves entering B-cell follicules in GC, whilst T cell independent response refers to the marginal zone, where B cells spontaneously produce low avidity IgM antibody (Tumang *et al.*, 2005), without cooperation with T cells.

Whether there are one or two cells of origin for M- and UM-CLL presently remains unclear.

As mentioned above UM CLL probably represents the equivalents of B1 equivalent cells from mice, on the basis of producing autoreactive antibodies; whereas M CLL seems to be derived from mouse B2 equivalent cells and undergoes T cell dependent GC reaction (O'Brien & Gribben, 2008).

Although M and UM CLLs differ not only in *IGHV* mutations, expression of activation markers and marked clinical outcome differences, gene expression profile data suggest that they probably originate from a common precursor, as there are very few genes differentially expressed (Klein *et al.*, 2001; Rosenwald *et al.*, 2001) between the two forms.

1.3.2 BCR signalling in CLL

BCR stimulation appears to be a central event in CLL, as suggested by stereotyped HCDR3 region and the preferential usage of *IGHV* segments with specific *IGLV* (Chiorazzi & Ferrarini, 2003).

The sustained activation of the BCR is likely to causes proliferation of CLL cells, similar to physiological responses in normal B cells (Petlickovski *et al.*, 2005). BCR signalling in CLL was associated with extended activation of the MEK/ERK and PI3K/AKT

pathways with AKT-induced elevated expression of the antiapoptotic MCL-1 protein, (Longo *et al.*, 2008).

However not all CLL cases maintain responsesd to BCR stimulation. CLL cells can be divided into 'responders' and 'non-responders' after BCR stimulation. 'Non-responders', i.e. B cells, mostly (63%) comprised patients with indolent, disease with M *IGHV* whilst BCR sensitive 'responders' were mainly (66%) patients with UM *IGHV* (Stevenson & Caligaris-Cappio, 2004). Responders retained the capacity for activation and had overall poor prognosis (Lanham *et al.*, 2003).

The molecular basis for differential BCR signalling in CLL remains unclear. Importantly, UM-IGHV CLL mostly express ZAP70 where ZAP70 plays unknown functions in BCR-CLL signalling. Also surface IgM is generally expressed at higher levels in UM-CLL. Downstream adaptors were described in the earlier section.

It was observed that 'responders' have significantly higher SYK levels than anergic CLL (Lankester *et al.*, 1995).

One of the markers of anergy in 'non BCR responding CLLers' is constitutive activation of MEK and ERK and phosphorylated ERK was associated with a favourable prognosis (Muzio *et al.*, 2008). The anergy of M *IGHV* cells may be caused by chronic exposure to autoantigens with a lack of concurrent co-stimulatory signals (Efremov *et al.*, 2007) leading to BCR desensitisation. There may also be a difference in how BCR associates with lipid rafts in M and UM *IGHV:* BCR in M *IGHV* CLL was excluded from lipid rafts and this in turn probably interferes with cytoskeletal rearrangements required in activation (Allsup *et al.*, 2005).

For example LYN was reported to be aberrantly expressed and activated and present in cytosol and is a major contributor to antigen-independent (tonic) BCR signalling (Contri *et al.*, 2005). UM *IGHV* cells have an increased tendency to phosphorylate SYK and to recruit and phosphorylate ZAP-70 (Mockridge *et al.*, 2007). ZAP 70 enhances and prolongs BCR signalling independent of tyrosine kinase function, probably acting as an adaptor protein (Gobessi *et al.*, 2007).

CD38 plays an important role in BCR signalling and was demonstrated to act as a co-receptor and a modifier of BCR signalling by inducing calcium influx, lipid raft formation, proliferation, cocapping with CD79a/b, IgM and IgD (Deaglio *et al.*, 2003). Also CD38 ligation triggers ZAP70 phosphorylation (Deaglio *et al.*, 2007).

1.3.3 CLL as a disease of proliferation rather than suppressed apoptosis subpopulations

Galton (Galton, 1966) and Dameshek (Dameshek, 1967) proposed that CLL represents is an accumulation of functionally inert lymphocytes which fail to die. This hypothesis was based on the observation of natural history of CLL patients. However recent data have indicated that CLL is also a disease of altered proliferation.

Although the idea of cell proliferation kinetic studies was first published in 1968 (Zimmerma.Ts *et al.*, 1968) using ^{3}H Thymidine incorporation, the Galton-Dameshek hypothesis was only questioned recently by the Chorrazzi group (Messmer *et al.*, 2005) when they demonstrated that proliferation kinetics in B cells (using heavy water to track proliferating cells) was dynamic rather than simply an accumulation of lymphocytes failing to die.

The method is based on giving ^{2}H (deuterium) to the patients as heavy water ($^{2}H_2O$) which was incorporated into replicating DNA and the deuterium content is quantified by mass spectrometry (Hellerstein, 1999).

Messmer showed that the lymphocyte pool in CLL patients shows a dynamic change between cellular birth and death. The CLL birth rates, i.e. the direct measurement of deuterium enrichment, were a small percentage of the clone per day, 0.1-1.75% (10^{9}-10^{12} cells born each day). The clone expansion was measured by the WBC count; the death rate was the difference of the growth rate and the birth rate. The death rate ranged from 0-2% of clone per day, which suggests that most of the patients do not have an apoptotic defect. Patients differed in kinetics of the proliferating subsets.

There was a suggestion from this study, that UM CLL have higher birth rates, but this observation was not statistically significant. The same group (Damle *et al.*, 2007; Calissano *et al.*, 2009) analysed the phenotypes in the groups of different deuterium content and found that there are three distinct populations of cells (see Figure 1.7):

The highest level of ^{2}H labelling was representative of the proliferative compartment i.e. 'young cells' (in solid tissue), as defined by the phenotype CXCR4 ^{dim} CD5 ^{bright} CD38 ^{hi} and consists of a very small fraction of all cells.

$CD38^{hi}$ cells were also positive for Ki-67, confirming that these cells are the cycling fraction.

In the newly produced cells, increased levels of CXCR4 caused longer retention in solid tissues and therefore prolonged exposure to survival factors and stimulatory ligands. CLL proliferation centres are found in the lymphoid tissues where there is CD38 upregulation and Ki-67 expression (Patten *et al.*, 2008), , as well as cyclin D1,

and antiapoptotic molecule survivin expression (Lampert *et al.*, 1999; Granziero *et al.*, 2001).

Callisano proposed a model where after CXCL12/SDF1(stromal cell-derived factor-1) ligation cells downregulate CXCR4 and increase expression of CD5 and CD38.The interaction of CXCR4 and SDF1 not only protects CLL from apoptosis, but also facilitates infiltration of the bone marrow by spontaneous migration beneath the bone marrow stromal cells (Burger *et al.*, 2000)

'Mature cells' ('Bulk') are the majority of the CLL clone in the peripheral blood (phenotype: $CXCR4^{int}CD5^{int}$ $CD38^{int}$), these cells have downregulated CD38 and CD5 (Chiorazzi, 2007).

The lowest level of ^{2}H labelling was seen in 'old cells' from the resting (re-entry) compartment, with CXCR4 ^{bright} $CD5^{dim}$ $CD38^{low}$. This group of cells either re-enters lymphoid tissue where the cycle of proliferation begins again (see proliferative compartment above) or die. CD38 low expressing cells are more prone to induced apoptosis than the cells which express high levels of CD38.

1.3.4 CLL prognostic markers

1.3.4.1 Prognostic factors

There are a number of prognostic factors currently used in clinical practice (summarised in Table 1.2). The ideal prognostic factor is one which is easy to assess reproducible in different centres, with good sensitivity and specificity, is disease dependent and stable.

Prognostic markers can be divided into the following categories:

- Clinical factors (Thurmes *et al.*, 2008);
- Serum markers
- Immunophenotypic markers – utilising proteins expressed both within the cytoplasm and at the cell surface.
- Gene expression markers (ie RNA markers)

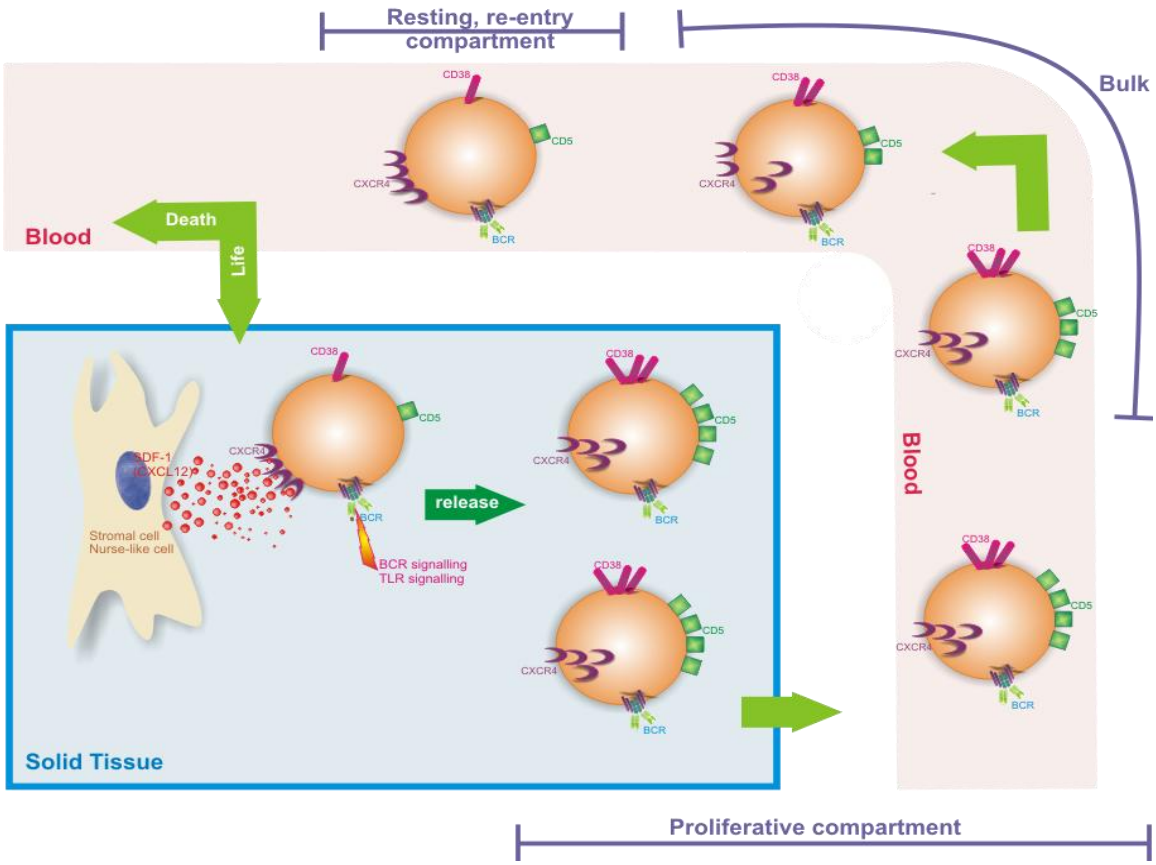

Figure 1.7 Dynamics of the CLL clone based on deuterium studies (Messmer, 2005)

In solid tissue CLL cells receive proliferating signals through the BCR and are stimulated by SDF-1/CXCL12, which downregulates CXCR4 and increases CD38. High CD38 markes recently divided cellswhich are resistant to several apoptotic stimuli (NB.these may be paradoxically BCL2 negative). These proliferative cells represent a very small stem-cell like pool fraction, and are released to the periphery. Cells in the periphery 'Bulk' are lose their CD38 and CD5 expression. "Resting" cells express high levels of CXCR4 and low CD5 and CD38, they can re-enter the lymphoid tissue and be re-stimulated by stromal cells or die.
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- Serum markers

There are number of new serum markers under investigatigation. Oscier *et al.* compared a number of serum markers (thymidine kinase, free light chain ratios, soluble CD23) with well-established β_2M in cohort of patients participating in the UK CLL4 trial (Oscier *et al.*, 2009). The cut off for β_2M was chosen arbitrarily as 4mg/L. Patients with values above 4mg/L had significantly poorer overall survival (OS) and progression-free survival (PFS). Serum BAFF inversely correlates with lymphocyte counts and may have independent prognostic significance (Ferrer *et al.*, 2009; Molica *et al.*, 2009). Serum APRIL (a proliferation inducing ligand) correlates with CD38 expression. Serum free light chains, a well-established marker in multiple myeloma (MM), were shown to be a prognostic marker in CLL (Pratt *et al.*, 2009). The cut off of 50mg/L identified patients with significantly poorer outcome and it was shown to be independent of both ZAP70 and *IGHV* mutational status as an indicator of time to first treatment. Serum levels of vascular endothelial growth factor (VEGF) were associated with advanced clinical stage and disease progression (VEGF receptor, see below immunophenotype paragraph)(Ho *et al.*, 2006).

- Immunophenotype (CD38, CD49d)

Damle (Damle *et al.*, 1999), in his seminal paper, first described CD38 as a surrogate marker for unmutated *IGHV* and as a prognostic marker. The use of CD38 as a surrogate marker was extensively discussed in the literature and some groups found discrepant results ((Hamblin *et al.*, 2000; Thunberg *et al.*, 2001)). CD38 as an independent prognostic marker is widely accepted.

A new marker CD49d is an α4-integrin shown in a cohort of 432 patients to be an independent prognostic marker for TTFT and OS.

Mulligan's group (Huang *et al.*, 2009) extensively analysed surface immunophenotype and showed that progressive CLL patients have increased CD38 and CD123 and decreased CD57, CD71, CD185. The Vintilescu group (Bumbea *et al.*, 2009) suggested stratifying patients into 2 main groups: poor prognosis (cyclin $D1^{+}$, $CD38^{+}$, $CD20^{high}$, $FMC7^{+}$, $CD23^{weak}$) and good prognosis (cyclin $D1^{-}$, $CD38^{-}$,$CD20^{low}$, $FMC7^{-}$, $CD23^{high}$). These patterns have a strong association with ZAP70.

Another important surface marker is the surface immunoglobulin isotype: CLL cases that express "unswitched" surface immunoglobulin isotype (IgM and IgD) are associated with unmutated heavy chain, increased expression of CD38 and ZAP70 and short lymphocyte doubling times (Lanasa *et al.*, 2009). However, TTFT and OS were not significantly statistically different.

VEGFR (Vascular endothelial growth factor receptor) was shown to be a prognostic predictor in CLL even in the early Binet stages patients and was comparable to *IGVH* mutation stages (Barreto *et al.*, 2009).

- Genetics
    - Conventional cytogenetics and FISH (fluorescence in situ hybridisation),

The chromosomal aberrations in CLL were described as early as the 1970s (Autio *et al.*, 1979). Chromosomal abnormalities can be detected by conventional chromosome banding analysis after stimulating cells with mitogens to achieve metaphases. This is a very laborious and expensive technique. Currently FISH, a technique developed in the late 1980's and 1990's, is the standard. A more detailed and sensitive analysis (Bentz *et al.*, 1995), currently used only as a research tool, is comparative genomic hybridisation (CGH). The most common chromosomal abnormalities are the 13q deletion, trisomy 12, 11q deletion and the 17p deletion, these abnormalities were stratified according to prognostic outcomes as good 13q and normal cytogenetics, intermediate: trisomy 12 and 11q deletion and poor: 17p deletion (Table 1.2) (Dohner *et al.*, 2000).

Abnormalities on chromosome 14 have been shown in a small cohort of patients (49) to be associated with shorter treatment-free survival (24 months v. 93 months) (Mura *et al.*, 2009; Travella *et al.*, 2009); ATM mutations confer poorer overall and treatment-free survival and were associated with 11q deletion and *IGHV*1-69 (Stankovic *et al.*, 2009).

- *IGHV*

The Bournemouth group first showed (Oscier *et al.*, 1997) an association between the level of somatic mutation and different cytogenetic abnormalities. This was followed by a paper from the same group (Hamblin *et al.*, 1999) and (Damle *et al.*, 1999) showed there were two distinct groups, mutated and unmutated (Table 1.2). Since then, *IGHV* mutation status became one of the most important

prognostic factors currently used in clinics. Oscier's (Davis *et al.*, 2009) group identified a correlation between *IGHV* gene usage and cytogenetic abnormalities: 13q was associated with *IGHV*3-7, t12 with 4-39, 11q del with *IGHV*1-69, lack of 17p del was associated with *IGHV*3-11, 3-21 and 3-48. *IGHV*3-21 was not associated with poor prognostic outcome in a Czech population (Schwarz *et al.*, 2009; Turcsanyi *et al.*, 2009), unlike previously reported in a Scandinavian population (Tobin *et al.*, 2002a). *IGHV* is discussed in detail in Chapter 1.4.

- *p53* mutations

Mutations in *p53* are detected in 4-15% of CLL patients. The presence of mutations in *p53* was associated with poor prognosis in retrospective trials (not confirmed in

prospective trials). The presence of mutations is particularly important in patients with 17p deletions (Zenz *et al.*, 2007).

- Molecular (ZAP70, Lipoprotein lipase, CLLU1, miR-34a)

The Rosenquist group compared the validity of RNA-based markers (Sevov *et al.*, 2009) and found that lipoprotein lipase (LPL) has the strongest correlation for the prediction of clinical outcome in CLL amongst other markers (CLLU1, TCL1, MCL1, ZAP70) tested.

Table 1.2 Prognostic markers

Prognostic factors		% Total CLL cases	Median Survival	Reference
Clinical Stage	A	55	Same as age matched controls	(Binet *et al.*, 1981)
	B	30	84 months	(Binet *et al.*, 1981)
	C	15	24 months	(Binet *et al.*, 1981)
Markers of tumour burden	Lymphocyte count and LDT			(Montserrat *et al.*, 1986)
	Serum LDH Serum β2M Bone marrow infiltration pattern			(Han *et al.*, 1984; Rozman *et al.*, 1984)
***IGHV* mutation status**	Mutated	60	310 months	(Damle *et al.*, 1999; Hamblin *et al.*, 1999; Lanham *et al.*, 2003)
	Unmutated	40	119 months	(Lanham *et al.*, 2003)
"Surrogate" markers for *IGHV* status	CD38 ZAP-70	 20%		(Damle *et al.*, 1999; Crespo *et al.*, 2003)
Cytogenetic Abnormalities (in 82% of all CLL)	13q del	55	133 months (92 months of treatment free interval)	(Dohner *et al.*, 2000)
	Normal karyotype		111 months	(Dohner *et al.*, 2000)
	17p del	7	32 months (9 months of treatment free interval)	(Dohner *et al.*, 2000)
	11q del	18	79 months	(Dohner *et al.*, 2000)
	Trisomy 12	16	114 months (median treatment free interval)	(Dohner *et al.*, 2000)
p53 abnormalities	Presence	15	47 months	(Lanham *et al.*, 2003)

17p deletion, 11q deletion, and unmutated *IGHV* have the strongest adverse prognostic value. Patients diagnosed with Binet stage A frequently had unmutated *IGHV*, high risk genomic aberration 17p del, 11q del occurred more frequently in the unmutated subgroup, whereas a good prognostic marker(13q deletion) was overexpressed in the mutated *IGHV* subgroup (Krober *et al.*, 2002).

1.4 Activated B cell diffuse large B cell lymphoma (ABC DLBCL).

Germinal centre (GC DLBCL), activated B cell (ABC DLBCL) and primary mediastinal B cell lymphoma (PMBL) are very diverse genetically, with different clinical presentation, response to therapy and outcome. These distinctive types of DLBCL were first identified from global gene expression microarray studies (Alizadeh *et al.*, 2000; Rosenwald *et al.*, 2002). The GC B cell-like DLBCL subgroup expresses genes present in normal GC B cells, whereas the ABC group expresses genes observed when B cells are activated through the BCR. Most importantly these subtypes of DLBCL are also separated by differences in 5 years overall survival (OS): for GC DLBCL the OS is 60% and for ABC DLBCL the OS is significantly reduced to 35%.
The precursor cell of ABC DLBCL is unclear. These subtypes probably originate from post GC memory cells (Seifert & Kuppers, 2009) or extrafollicular, pre-GC B cells with high level of AID (Cattoretti *et al.*, 2006). The ABC cells are blocked during plasmacytic differentiation due to genetic lesions affecting *Blimp-1*, which is a 'master regulator' of the terminal differentiation of B cells into plasma cells (Pasqualucci *et al.*, 2006). ABC DLBCL was shown to be a more aggressive form of DLBCL than GC DLBCL (Rosenwald *et al.*, 2002). In every-day practice it is difficult to diagnose ABC DLBCL.

Proposed events in lymphomagenesis implicated in ABC DLBCL are constitutive NF-κB activation, (as outlined below) BCL2 overexpression by amplification of *BCL2* locus (Lenz *et al.*, 2008c), overexpression of AID, heavy mutational load of *IGHV* genes and lack of class switch recombination, *INK4A-ARF* deletion, which leads to loss of tumour suppressor *p16* and $p14^{ARF}$, trisomy 3 (causing overexpression of FOXP1), 19q gain or amplification (and consequent upregulation of SPIB) (Lenz & Staudt, 2010).

The constitutive upregulation of NF-κB in ABC DLBCL is thought to be induced by at least two distinct mechanisms. Firstly, mutations in coiled-coil domain of CARD11 in 10% of ABC-DLBCL result in NF-kB activation (Lenz *et al.*, 2008a).-CARD11 (Caspase recruitment domain-containing protein 11) is a signalling scaffold protein that coordinates the activation of IkB kinase b, a positive regulator of the NF-kB pathway. Mutated CARD11 stimulates the NF-κB signalling pathway in ABC DLBCL in the absence of activation by antigens. The mechanisms are not well understood, however Lenz identified eight missense mutations in exons encoding the coiled-coil domain in 9.6% of ABC DLBCL biopsies. The coiled-coil domain enables CARD11 oligomerisation and therefore activation of NF-κB pathway. The cell lines with

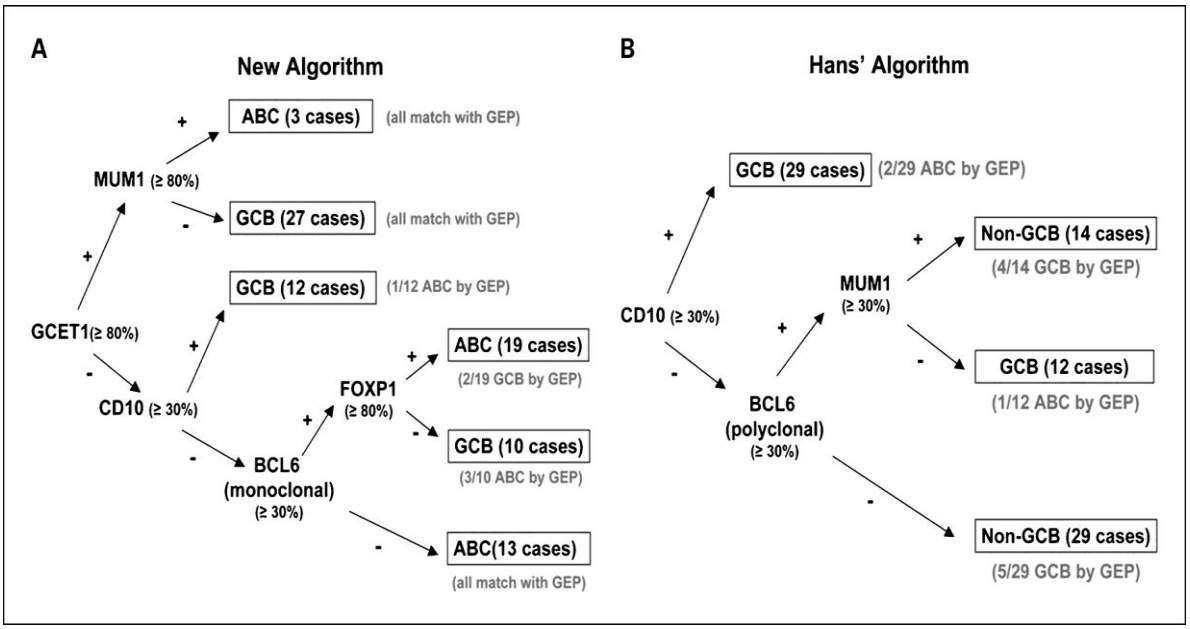
mutations introduced in this domain of CARD11 caused constitutive NF-κB activation and augmented NF-κB activity on antigen receptor stimulation. Lenz et al. hypothesised that the coiled-coil domain normally keeps CARD11 in the latent state in resting B cells, however after antigen encounter coiled-coil causes CARD11 protein to aggregate and stimulate the NF-κB pathway. The mutations in CARD11 cause the pathological aggregation without the presence of foreign antigen. In contrast, in cases with wild-type of CARD11, activation of NF-κB may occurs through chronic active B cell receptor signalling (Davis *et al.*, 2010).

The evidence for the chronic active B cell receptor signalling hypothesis was provided by identification of mutation in ITAMs of CD79a and CD79b (Davis *et al.*, 2010). This was further proven by induction of apoptosis in cell lines with chronic active BCR signalling (but not in BCR-independent cell lines) using inhibitors of BTK and SRC-family kinases. It was proposed that CARD11 mutants would respond to inhibition of IKK (IkappaB kinase) (Lam *et al.*, 2005). In this thesis I show that FAM129C is preferentially expressed in ABC-DLBCL and might be a clinically useful new marker for diagnosis of this aggressive subtype of disease.

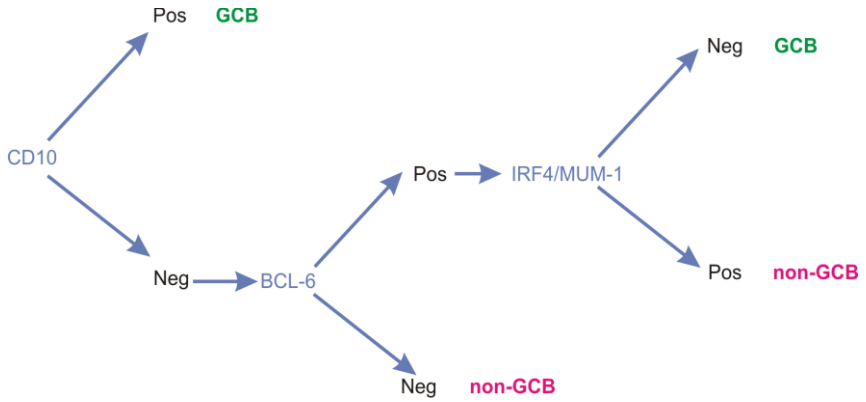
Is a highly restricted marker for a subset of GC lymphoma and the FOXP1 antibody (that differentiates a BCL6 positive group) (Montes-Moreno *et al.*, 2008).

Genetic profiling is not widely available, it is expensive, labour-intensive, impractical and requires fresh tissue. Therefore, efforts have been made to translate the results from genetic array data to immunohistochemistry. The most successful attempt to date was Hans classification using CD10, BCL-6 and MUM1 (multiple myeloma oncogene-1) staining (Hans *et al.*, 2004). MUM1 is a lymphoid member of the interferon regulatory factor (IRF) family of transcription factors. The positive predictive value of this classification is 87% for GC DLBCL and 73% for ABC DLBCL, 20% of cases were misclassified. The alternative algorithm was proposed by Muris (Muris *et al.*, 2006) where BCL-2, CD10 and MUM1 were used. BCL-2 was found to have significant adverse effect on survival within ABC DLBCL (Iqbal *et al.*, 2006).

More recently, the Chan group developed a new immunohistochemical algorithm which had 93% concordance with gene expression profiling (Choi *et al.*, 2009). The new algorithm uses five markers: MUM1, CD10, BCL6, FOXP1 and a new antibody to GC marker: germinal centre B cell expressed transcript (GCET) (Montes-Moreno *et al.*, 2008) (Figure 1.8).



C.



	GCB DLBCL	ABC DLBCL
CD10	+	-
BCL-6	+	+/-
IRF4/MUM1	-	+

Figure 1.8 Hans and new algorithm

A. The new algorithm uses five markers with 78 out of 84 cases were concordant (93%) with gene expression data, **B.**Hans' algorithm 72/84 cases were concordant (86%) in the same patient set.
Reprinted with permission Choi W. CCR, 2009; 15(17), Copyright (2009), with permission from American Association for Cancer Research.
C. Simplified Hans' Algorithm IHC profile of germinal center (GC) and activated B cell (ABC) types of DLBCL, the Hans' algorithm.

GC are CD10 and/or BCL-6 positive, ABC are CD10 negative, IRF4 positive, can be BCL-6 positive. Table summarises the flow-chart above.

There are only two published examples of ABC DLBCL cell lines: OCI Ly3, OCI Ly10 (Lenz *et al.*, 2008a). The identity of the majority of DLBCL cell lines is not widely available. Therefore DLBCL cell lines were screened using Hans' algorithm (Hans *et al.*, 2004) using immunobloting (Figure 3.11). The Hans' algorithm was developed for immunohistochemistry (IHC), but using it on immunobloting, made analysis easier than IHC, as cell lines were single clonal population. Muris *et al.* modified the original Hans' algorithm by adding BCL2 to the panel (Muris *et al.*, 2006). Expression of BCL2 identifies the aggressive subtype of ABC DLBCL.

1.5 Identification of FAM129C (BCNP1)

FAM129C (previously known as BCNP1 (B cell novel protein 1)) was originally identified by mass spectrometry in CLL plasma membrane preparations (Boyd *et al.*, 2003). Plasma membrane preparation was separated on a 1D gel (Figure 1.9), sequential bands excised in 0.5mm intervals and proteins in each slice digested with trypsin. The resulting peptide fragments were analysed by mass spectrometry. The expression of novel molecules in different cell types was studied using quantitative RT-PCR analysis *BCNP1* was highly expressed in CLL and lymphoma samples and restricted to B cell containing tissues**.** Subsequently, BCNP1 has been renamed FAM129C by the HUGO Gene Nomenclature Committee (HGNC). as it has now been shown to be a member of a family of three closely related genes .

FAM129C remains protein of unknown function. FAM129C levels appeared to be elevated in lymphoid malignancies in comparison to levels found in normal B cells (peripheral blood, spleen, and tonsil) (Boyd *et al.*, 2003). This expression profile suggests that FAM129C might have, diagnostic and/or prognostic roles in CLL and in the other B cell malignancies.

FAM129C is a member of gene family including FAM129A (NIBAN) and FAM129B. There is a 28% sequence identity between FAM129C and FAM129A and similarly 27% identity between FAM129B and FAM129C. The common feature of this family is the presence of pleckstrin homology domain (PHD) (for the detailed discussion of PHD see Chapter 5). FAM129A was first described using Eker rat model of hereditary renal carcinoma (Majima *et al.*, 2000). FAM129A was identified in the pre-neoplastic lesions of renal carcinomas, in hepatic angiomyolipoma (Kannangai *et al.*, 2005), in various types of thyroid tumours (Matsumoto *et al.*, 2006) and in head and neck squamous carcinoma (Ito *et al.*, 2010).

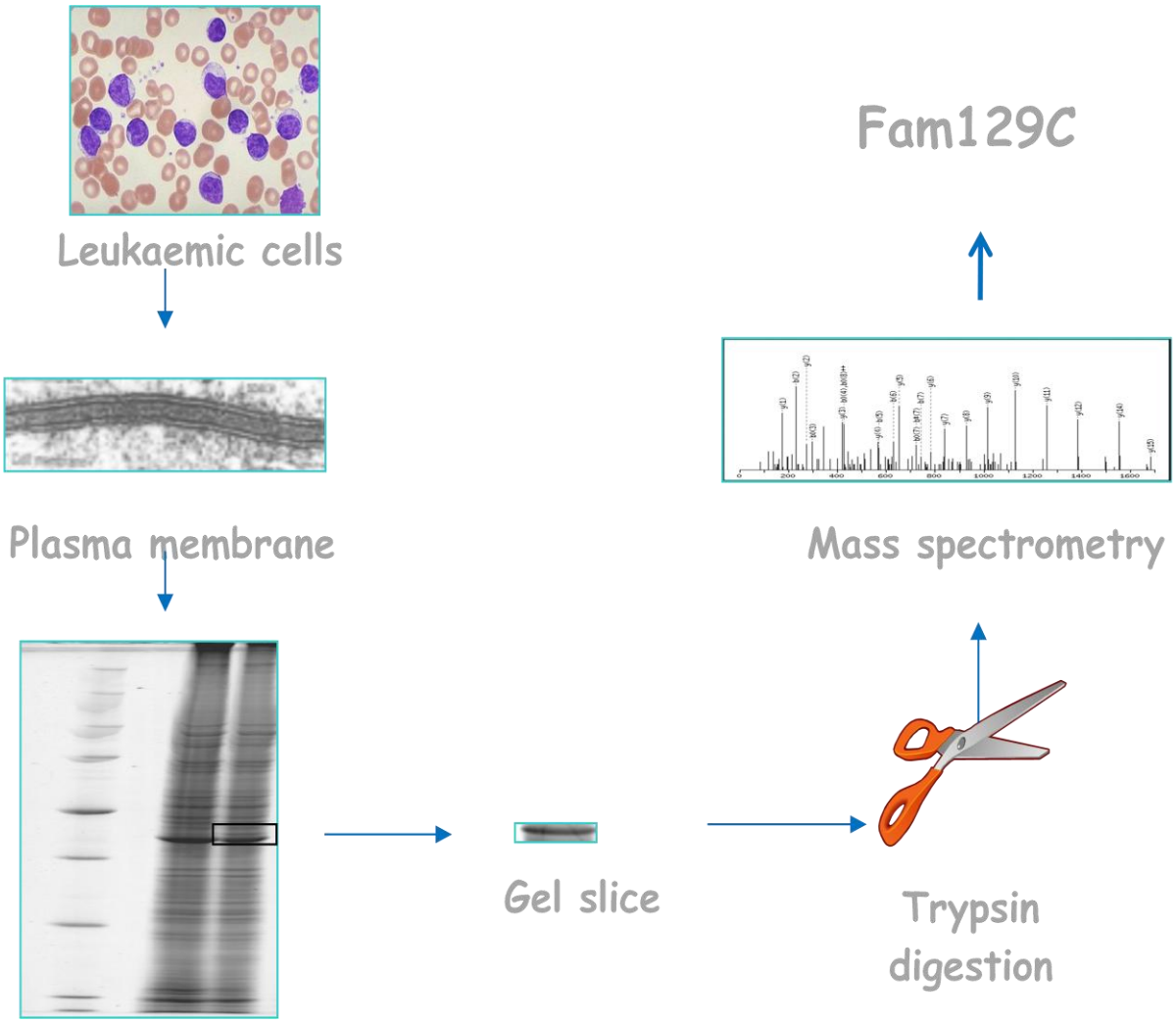


Figure 1.9 Identification of FAM129C.

The plasma membrane preparation from CLL cells were separated on 1D gel, sequential bands were excised in 0.5mm intervals and each slice was further digested with trypsin. The resulting peptide fragments
Modified with kind permission of Springer Science+Business Media. Methods Mol Biol. 2007; 370:135-46

The functions of FAM129A are not yet known.It was postulated to be involved in the ER stress response, probably protecting tumour cells from death-inducing stress by modulating protein translation (Sun *et al.*, 2007). However, FAM129A knock-out mice did not show any obvious abnormal phenotype, perhaps due to functional overlap with other family members.

FAM129B was recently identified as a B-Raf phosphorylation target in melanoma using functional proteomics (Old *et al.*, 2009). FAM129B had six out of 90 identified phosphorylation events using phosphoprotein profiling. Old et al. showed that FAM129B controlled cell invasion into three-dimentional extracellular matrix in a phosphorylation-dependent manner. However the phophorylation sites are not preserved in FAM129C.

1.6 Aims of the thesis

My project was firstly to characterise the functions of and attempt to understand its role in B-cell biology; and its relevance in the context of CLL and ABC DLBCL

Secondly, I was involved in creating the Leicester CLL database where I collected clinical information together with biological parameters, to identify new markers in large sets of patients.

Chapter 2: Materials and Methods

2.1 Cell lines and related techniques

2.1.1 Materials

Unless otherwise stated all chemicals were from Fisher Scientific (Loughborough, UK). Propidium Iodide (PI), imidazole, ethylenediaminetetraacetic acid (EDTA), Histopaque, kanamycin-sulphate, ethidium bromide, Luria-Bertani broth (LB), LB agar, isopropyl β-D-1-thiogalactopyranoside (IPTG), agarose, bromophenol blue, ammonium persulphate, saponin, glycerol, Hepes, paraformaldehyde and formaldehyde were purchased from Sigma (Poole, UK). PE-conjugated CD19 were from Becton Dickenson (Oxford, UK). SOC buffer, Hoechst-33342, Goat-anti-Mouse Alexa-488 antibody, RPMI, DMEM, Foetal Calf Serum (FCS), Glutamax and primers were purchased from Invitrogen (Paisley, UK). FITC-conjugated Annexin V and CD19-FITC combined with CD5-PE were from Caltag Medsystems (Buckingham, UK). Bradford protein assay reagent and ECL were from Pierce (Cramlington, UK). Acrylamide from ProtoGel: National Diagnostics (Atlanta, GA Distributor Geneflow (EC-890)) and Triton X-100 from Fisher (BP151-100). Nickel-labelled Agarose beads were from Amersham Biosciences (Little Chalfort, UK). Complete Mini Protease inhibitor tablets and EDTA-Free mini-protease inhibitor tablets were from Roche (Burgess Hill, UK). Hyperladder I was from Bioline (London, UK) and the Protein Assay Reagent was from BioRad (Hemel Hempstead, UK).

2.1.2 Cell Culture

Cell culture procedures were carried out aseptically in a class II laminar flow cabinet. Cells were maintained in a Heraeus CO_2 Auto-Zero incubator at 37°C with 5% CO_2. The majority of the cell lines were routinely cultured in Roswell Park Memorial Institute-1640 media (RPMI) supplemented with 10% (v/v) heat inactivated FCS, 2mM glutamax (L-glutathione) (Sigma) (unless specified in the Table 2.1-2.3. The human cell lines and their origins used in these studies are compiled in the Tables 2.1-2.3 together with any special growing conditions for their culture (specified in the tables' footnotes).
Cells were passaged before they reached confluence to maintain a logarithmic growth.

Table 2.1 Human acute lymphocytic leukaemia B-cell lines

Cell line	Pathology	Reference
380	human B cell precursor leukemia	Pegoraro L. et al., Proc Natl Acad Sci USA 81: 7166-7170 (1984).
697	human B cell precursor leukemia	Findley HW et al., Blood 60: 1305-1309 (1982).
CALL2	human B cell precursor leukemia	Tomeczkowski et al., Br J Haematol, 89: 1995, 771,
CALL4	human B cell precursor leukemia	Tomeczkowski et al., Br J Haematol, 89: 1995, 771,
Cemo1	human B cell precursor leukemia	Silva MLM et al., Leukemia 10: 575-578 (1996).
HAL-01	human B cell precursor leukemia	Ohyashiki K et al., Leukemia 5: 322-331 (1991).
HB	human B cell precursor leukemia	From Prof L. Boxer
Karpas231	human B cell precursor leukemia	Nacheva E et al., Blood 82: 231-240 (1993).
Kasumi-2	human B cell precursor leukemia	From Prof MJS Dyer cell line bank
KOPN8	human B cell precursor leukemia	Matsuo Y & Drexler HG, Leukemia Res 22: 567-579 (1998).
LILA	human B cell precursor leukemia	Salvaris E et al., Leukemia Res 16: 655-663 (1992)
LK63	human B cell precursor leukemia	Salvaris E et al., Leukemia Res 16: 655-663 (1992).
MUTZ5	human B cell precursor leukemia	Meyer C et al., Leukemia 15: 1471-1474 (2001)
Nalm-6	human B cell precursor leukemia	Hurwitz R et al., Int J Cancer 23: 174-180 (1979).
Nalm27	human B cell precursor leukemia	Ariyasu T et al., Human Cell 11: 43-50 (1998).
RCH-ACV	human B cell precursor leukemia	Jack I et al., Cancer Genet Cytogenet 19: 261-269 (1986).
REH	human B cell precursor leukemia	Rosenfeld C et al., Nature 267: 841-843 (1977).
RS4;11	human B cell precursor leukemia	Stong RC et al., Blood 65: 21-31 (1985).
SD1PD3	human B cell precursor leukemia	From Prof MJS Dyer cell line bank
SEM	human B cell precursor leukemia	Greil J et al., Brit J Haematol 86: 275-283 (1994).

All cells were grown in RPMI and 10% FCS

Table 2.2 Human mature B-cell lines

Cell line	Pathology	Reference
BJAB	Burkitt's lymphoma	Menezes J et al., Biomedicine 22: 276-284 (1975).
BL100	Burkitt's lymphoma	From Prof MJS Dyer cell line bank
Boar88	Folicular lymphoma	Yonetani, N., *Oncogene*, **17**, 971–979(1998).
Daudi	Burkitt's lymphoma	Nadkarni JS et al., Cancer 23: 64-79 (1969).
DB	Mature EBV negative	Beckwith M et al., J Natl Cancer Inst 82: 501-509 (1990).
DG75	Burkitt's lymphoma	Ben-Bassat H et al., Int J Cancer 19: 27-33 (1977).
Elijah	Burkitt's lymphoma	From Prof MJS Dyer cell line bank
GRANTA519	Mantle cell lymphoma	Jadayel DM et al., Leukemia 11: 64-72 (1997).
HBL1	Diffuse large B cell Lymphoma	Abe M et al., Cancer 61: 483-490 (1988).
HBL2	Diffuse large B cell Lymphoma	Abe M et al., Cancer 61: 483-490 (1988).
HDLM-2	Hodgkin	Drexler HG et al., Leukemia Res 10: 487-500 (1986).
HRC57	EBV immortilised normal B cells	Karin E. Summers, JCO Jan 15 2001: 420-424.
JVM2	Mantle cell lymphoma	Melo JV et al., Int J Cancer 38: 531-538 (1986).
Karpas422	Mature EBV negative	Dyer MJS et al., Blood 75: 709-714 (1990)
KMH2	Hodgkin disease	Kamesaki, H. et al. (1986) Blood 68, 285-292.
L591	Hodgkin disease	Diehl V et al., Cancer Treat Rep 66: 615-632 (1982).
MD901	Diffuse large B cell Lymphoma without t(14; 18)	From Prof MJS Dyer cell line bank
MD903	Diffuse large B cell Lymphoma without t(14; 18)	From Prof MJS Dyer cell line bank
Mek1	Prolymphocytic leukaemia	Stacchini A et al., Leukemia Res 23: 127-136 (1999)
Mek2	Prolymphocytic leukaemia	Stacchini A et al., Leukemia Res 23: 127-136 (1999)
Molp8	Multiple myeloma	Matsuo Y et al., Leukemia Res 28: 869-877 (2004).
MUTU111	EBV positive Burkitt lymphoma	**Gregory et al. 71 (7): 1481. (1990)**
Namalwa	Human Burkitt lymphoma	Nadkarni et al., Cancer 23: 64-79 (1969), PubMed ID 4178827
Nappi 88	Follicular transformed to Burkitt lymphoma	From Prof MJS Dyer cell line bank
NUDUL-1	DLBCL	Epstein AL et al., Int J Cancer 35: 619-627 (1985).
OCI-Ly3	ABC Diffuse large B cell Lymphoma	Tweeddale ME et al., Blood 69: 1307-1314 (1987).
OCI-Ly10	ABC Diffuse large B cell Lymphoma	From Prof MJS Dyer cell line bank
OCI-Ly19	ABC Diffuse large B cell Lymphoma	Chang H et al., Leukemia Lymphoma 19: 165-171 (1995).
Oz	Burkitt's lymphoma	Nagai M et al., Hematol Oncol 15: 109-119 (1998).
P32	Burkitt's lymphoma	From Prof MJS Dyer cell line bank
Per 365	Mature from paediatric ALL	Kees UR et al., Genes Chromosomes Cancer 12: 201-208 (1995).
Per 377	Mature from paediatric ALL (sister cell line to Per 377, second	Kees UR et al., Genes Chromosomes Cancer 12: 201-208 (1995).

	relapse)	
PR1	Diffuse large B cell lymphoma with t(14; 18)	From Prof MJS Dyer cell line bank
Raji	Burkitt's lymphoma	Pulvertaft JV, Lancet i: 238-240 (1964).
Ramos	Caucasian Burkitt's lymphoma	Intervirology 1975;5:319; Int. J. Cancer 1977;19:337; J. Immunol 1982;129:1336
RCK8	Mediastinal DLBCL	Kubonishi I et al., Jpn J Cancer Res 76: 12-15 (1985)
Rec1	Mantle cell lymphoma	From Prof MJS Dyer cell line bank
RIVA	Mature EBV negative	Th'ng KH et al., Int J Cancer 39: 89-93 (1987).
RPMI8226	Multiple myeloma	Matsuoka Y et al., Proc Soc Exp Biol Med 125: 1246-1250 (1967).
Sc1	Follicular cell lymphoma	Th'ng KH et al., Int J Cancer 39: 89-93 (1987)
SSK41	Follicular lymphoma	Sideras P, 1989, Int Immunol, 1, 631-
Sp53	Mantle cell lymphoma	Daibata M et al., Cancer 64: 1248-1253 (1989)
SUDHL4	Mature EBV negative	Epstein AL et al., Cancer 42: 2379-2391 (1978)
SUDHL9	Mature EBV negative	Epstein AL et al., Cancer 42: 2379-2391 (1978)
Tanoue	Burkitt's lymphoma cell line	El-Sonbaty SS et al., Leukemia Res 19: 249-256 (1995).
U266	Multiple myeloma	Nilsson K et al., Clin Exp Immunol 7: 477-489 (1970)
Z138	Mantle cell lymphoma	Estrov Z et al., Leukemia Res 22: 341-353 (1998).

All cells are grown in RPMI with 10%FCS,
except for OCI-LY3 in Iscove's modified Dulbecco's medium (IMDM) with 10%FCS,
OCI-Ly10 in culture in IMDM and 20% human plasma with β-mercaptoethanol (55mmol/L) and 20% heparinised normal human plasma.
Granta in Dulbecco's Modified Eagle's Medium (DMEM) with 4500mg/ml

Table 2.3 Non-B-cell lines

Cell line	Pathology	Reference
293	Human embryonal kidney	Graham et al., J Gen Virol 36: 59-72 (1977), PubMed ID 886304
COS7	African green monkey kidney	Gluzman, Cell 23: 175-182 (1981)
DT40	Chicken B lymphocyte	Baba et al., Virology 144: 139-151 (1985),
HL60	Human acute myeloid leukaemia	Collins SJ et al., Nature 270: 347-349 (1977).
Hct-116	Human colon carcinoma	Baba et al., Virology 144: 139-151 (1985),
HeLa	Human cervix carcinoma	Scherer et al., J Exp Med 97: 695-710 (1953)
Hep-3B	Human hepatocellular carcinoma	Aden et al., Nature 282: 615-616 (1979),
J82	Human bladder carcinoma TCC	O'Toole C, Price ZH, Ohnuki Y and Unsgaard B. (1978). Br. J. Cancer, 38, 64–76.
Jurkat	Human T cell leukaemia	Schneider U et al., Int J Cancer 19: 621-626 (1977).
MCF7	Human breast carcinoma	Soule et al., J Natl Cancer Inst 51: 1409-1413 (1973)
NIH-3T3	Mouse embryo, mouse fibroblasts	Jainchill, J Virol 4: 549-553 (1969)
SAOS-2	Human osteosarcoma	Fogh et al., J Natl Cancer Inst 58: 209-214 (1977),
THP1	Human acute monocytic leukaemia	Tsuchiya S et al., Int J Cancer 26: 171-176 (1980).
U2OS	Human osteosarcoma	Nature 1986 319:511-514 Pub Med 86118705

Cells were grown in DMEM with 4500mg/ml, except for HL60, Jurkat and THP1 in RPMI with 10% FCS; DT40: RPMI with 10% FCS, 1% chicken serum and 50μM β 2-mercaptoethanol.

Cells were routinely frozen in liquid nitrogen during culture to ensure stocks of all passages. To detach and subculture cells they were washed with warm sterile phosphate buffer saline (PBS, Sigma) and detached with a solution of 1X trypsin/EDTA (Sigma). The trypsin activity was stopped by adding double the volume of medium. After centrifugation at 200*g* for 5 minutes, cells were resuspended in appropriate medium, counted with a haemocytometer and seeded at the desired density.

2.1.2.1 Long term storage of cells

To generate and maintain batches of cells, exponentially growing cells were washed twice with PBS and centrifuged at 200*g* for 5 minutes at ambient temperature. The cell pellet was resuspended in freezing medium (50% (v/v) FBS, 10% (v/v) DMSO and 40%

(v/v) DMEM) to a desired density of cells/ml. Aliquots of 1ml were placed in cryogenic boxes at -80°C overnight and then immersed in liquid nitrogen. Cells were recovered from the cell bank by rapid thawing to 37°C in a water bath, and then instantly resuspended in the appropriate culture medium and transferred to a tissue culture flask.

2.1.2.2 Purification of CLL cells from whole blood

Peripheral blood was collected into Lithium-Heparin tubes from patients diagnosed with CLL staged according to the Binet's system. Blood was diluted 1:2 in RPMI and loaded on a Ficoll density gradient (Histopaque, Sigma-Aldrich). The gradients were centrifuged at 1600 rpm for 30 minutes at ambient temperature and the lymphocyte fraction was collected (Figure 2.1). After collection, cells were washed in RPMI and centrifuged at 1200 rpm for 10 minutes and resuspended in RPMI containing 10% FCS and 2mM glutamax. Cells were counted on a Sharfe Coulter cell counter and resuspended to $5x10^6$ per ml.

2.1.2.3 Transfection of cells using Amaxa (now Lonza) nucleofection

Cells were split 24 hours prior to transfection. On the day of transfection the cells were washed in PBS (at ambient temperature) twice to remove any medium containing FCS. It was important to avoid temperature fluctuations, therefore all solutions used in transfection were at ambient temperature. $2x10^6$ cells were used per transfection and 2µg linearised plasmid (ScaI (New England Biolabs) was used to linearise the pcDNA4 expression vector and FspI (NEB) for pcDNA6/TR). Before resuspending the pellet in the buffer provided by the manufacturer, the remaining PBS was completely removed. All handling of the cells was performed using a 1ml Gilson pipette. Once cells were resuspended in suitable transfection buffer (all transfections were done in buffer V), the cells were electroporated within a maximum of 10 minutes in the cuvettes provided by the manufacturers and with the electroporation program suggested on the Amaxa website http://www.lonzabio.com/ (Table 2.4).

Table 2.4 Transfection programmes and selection

Cell line	Transfections used	1st selection: Blasticidine (InvivoGen)	2nd selection: Zeocin (InvivoGen)
Jeko1	X1	10µg/ml	50µg/ml
Mec1	C5	10µg/ml	100µg/ml
Ramos	O6, C5	10µg/ml	250µg/ml
Nalm6	T20, C5	10µg/ml	100µg/ml
RCH-ACV	X1, C5	10µg/ml	50µg/ml

Blasticidine was used to select cells containing pcDNA6/TR, Zeocin to select cells containing pcDNA4

Figure 2.1 Isolation of mononuclear cells using a Ficoll density gradient.

A. Prior to centrifugation blood was diluted 1:2 in RPMI and loaded on a Ficoll density gradient (Histopaque) in a 1:2 ratio. **B.** Post centrifugation at 1600 rpm for 30 minutes at room temperature and the lymphocyte fraction (from buffy coat) was separated between Ficoll and plasma and floating on top of the Ficoll.
From http://www.axis-shield-density-gradient-media.com with kind permission.

Immediately after electroporation the “V” buffer was diluted with 20% FCS in phenol red-free RPMI. Cells were spun and resuspended in medium containing 20% FCS in phenol red-free RPMI. Between 24-48h after transfection a suitable selection antibiotic was started and the transfected cells were cloned using the minimal dilution method.

2.1.2.4 Single cell cloning using minimal dilution method

The transfected cells were plated in 96-well plates (flat-bottomed) at concentrations 0.3-, 1-, 3-, 10- and 100-cells per well. Each well contained 200µL of cell suspension, the medium contained selection antibiotic, e.g. for plates 0.3 cell per well the cell suspension was prepared at 1.5 cell/well; for plates 1-cell per well, at 5 cells/ml, for 3 cell per well, at 15 cells/ml, for 10 cells per well, at 50 cell/ml and for 100 cell per well, at 500 cells/ml. Once a colony was visible and the medium turned a yellowish colour, the clone was expanded into a 24-well plate and subsequently a 12-well plate. At this stage the clones were frozen.

2.1.2.5 Tetracycline regulated expression system (T-REx)

Tetracycline-regulated Expression System for Mammalian cells (T-REx™, Invitrogen, Paisley, UK) is summarised in Figure 2.2. The T-REx™ system is based on regulation of expression by tetracycline, using elements from the *E.coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994, Hillen, 1983). Tetracycline binds the Tet repressor and the promoter of the gene of interest becomes active. The studied cells were transfected with two plasmids: pcDNA6/TR and pcDNA4. Plasmid pcDNA6/TR is a regulatory vector expressing high levels of the *Tet*R gene controlled by the CMV promoter. The *Tet*R gene encodes the Tet repressor protein (tetR) in cultured cells which forms homodimers. TetR homodimers bind to the Tet operator 2 ($TetO_2$) sequences in the inducible expression vector with very high affinity and therefore repress transcription of the gene of interest. However, if tetracycline (tet) is added to medium it binds to tetR homodimers and changes their conformation. Homodimers lose the high affinity to the Tet operator and are released from $TetO_2$ binding which, in turn, induces transcription of the gene of interest.

2.1.2.6 Synchronisation of mammalian cells

To understand if FAM129C affects the cell cycle and how these changes occur it was important to synchronise the cells in the same phase of cell cycle (Liu, 2005). There are number of methods available to synchronise the cell culture, broadly divided into

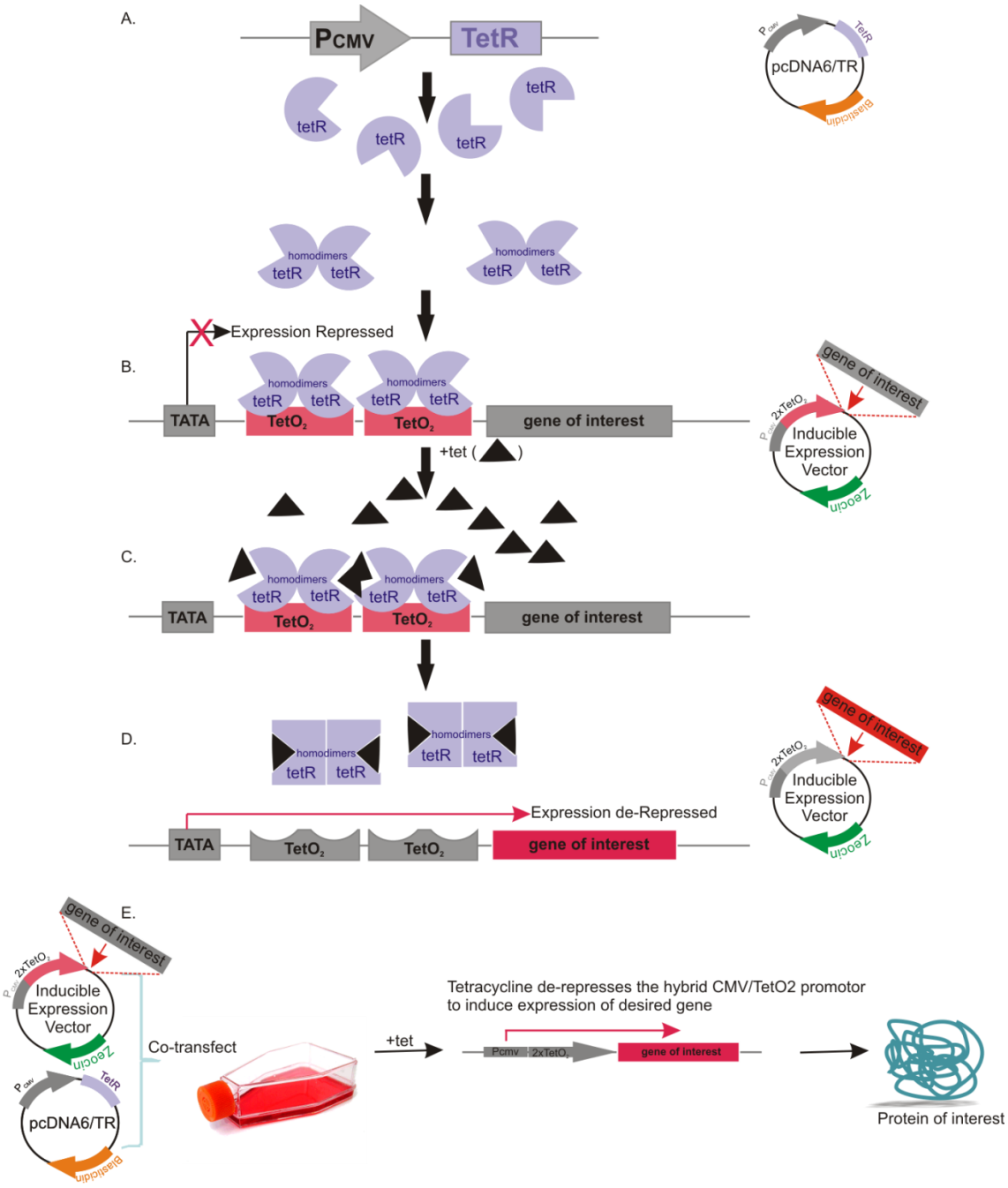


Figure 2.2 Diagram of the principle of tetracycline-regulated expression system (T-REx).

A. Tet repressor protein (tetR) is expressed from pcDNA6/TR in cultured cells and form homodimers. B. TetR homodimers bind to Tet operator 2 ($TetO_2$) sequences in the inducible expression vector with very high affinity, repressing transcription of the gene of interest. C. Added tetracycline (tet) binds to tetR homodimers. D. Bound tetracycline to tetR homodimers changes their conformation, hence loses the high affinity to Tet operator and is released from $TetO_2$ binding, induces transcription from the gene of interest. E. Gene of interest is cloned into inducible expression vector and the inducible and regulatory vector (pcDNA6/TR) are co-transfected to mammalian cells. Tetracycline de-represses CMV/$TetO_2$ promoter in the inducible vector and induces transcription of the gene of interest.

(Modified from Invitrogen User manual, versionF, 25-0271, with permission)

biological and pharmacological. Biological methods include serum deprivation, contact inhibition (specific to fibroblasts) and centrifugal elutriation (Davis *et al.*, 2001a). Serum deprivation was unsuccessful in the Ramos cell line: cells died before synchronisation was achieved after various (0.1-1%) concentrations of FCS were used, therefore an alternative method was optimised, Ramos cells were treated with mimosine at 50mM for 24h (Mosca *et al.*, 1992) which synchronised Ramos cells in G1. The G1 arrest was assessed using DNA analysis using flow cytometry (see below Chapter 2.1.2.4). The medium containing mimosine was removed after 24h, to allow cells to progress through the cell cycle.

2.1.2.7 Stimulation systems

CLL cells appear to be dependent on the microenviroment both cellular and soluble (Ghia & Caligaris-Cappio, 2000), whilst CLL cells cultured alone tend to die within few days and will be discussed below. To mimic the process described above many *in vitro* systems have been introduced.

1) Stromal systems were shown to support viability of CLL cells in culture derived from bone marrow (Panayiotidis *et al.*, 1996; Lagneaux *et al.*, 1998) and from blood (Burger *et al.*, 2000) these could be from primary patients (Granziero *et al.*, 2003) or from immortalised cell lines HS5 and HS27A) (Ghia *et al.*, 2005a).
2) T cell support: interactions with activated T cells via CD40L (Buske *et al.*, 1997; Kitada *et al.*, 1999).
    a. Autologous T cells co-cultured with CLL cells (Ranheim & Kipps, 1993).
    b. To simulate T cell help a CD40L system was developed by transfecting CD40L into mouse fibroblasts in the presence of IL-4 (T helper cytokine) (Buske *et al.*, 1997).
3) CLL cells themselves secrete chemokines to create their own microenviroment (Ghia *et al.*, 2005a).

CD40L system

A CD40L system was established in our laboratory (Banchereau *et al.*, 1991). CLL cells were isolated from heparinised venous whole blood using density gradient centrifugation as described above. CLL cells ($3x10^6$/ml) were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with FCS (Invitrogen) and Penicillin/Streptomycin (Invitrogen) in a 37°C and 5% CO_2 incubator. CLL cells were cultured alone on tissue culture plastic or co-cultured with 80-90% confluent and 35Gy irradiated non-transfected mouse fibroblasts Ltk- (NT-L) cells or transfected Ltk- cells with human CD40-Ligand (CD40-L) and rh-IL4 (10ng/ml) (R&D Systems, Minneapolis,

MN, USA)(Banchereau *et al.*, 1991). CLL cells were removed from the L cells by gentle washing with RPMI before treatment.

This system was used to upregulate the Fas receptor in B cells in the experiment.

NFkB inhibition (blocks constitutive IkB) with BAY-117082 (Merck)

CLL cells were incubated with BAY-117082 for 1h before being placed onto CD40L layer.

BCR stimulation

Cells were prepared at a concentration of $20x10^6$/ml, $F(ab')_2$fragment goat anti-human IgM (Jackson Immunoresearch Laboratories, West Grove, USA) was added to the medium at a working concentration of 20µg/ml, kept on ice for 30 minutes (negative control), then the cells were removed and incubated at 37^0C for 20 minutes. The cells were put on ice and washed with ice-cold PBS twice. Post stimulation, cells were mechanically fractionated (see below, Chapter 2.44) to check the localisation of FAM129C.

BAFF stimulation

BAFF was a kind gift from Dr A. Craxton (MRC Toxicology, Leicester). BAFF supernatant was obtained from HEK293T cells stably transfected with Strep Tag II-tagged BAFF (amino acids 133-285), containing an isoleucine zipper, which has been shown to induce NF-κB2 p100 processing to its p52 subunit in isolated CLL cells.

CLL samples were treated with Strep Tag purified BAFF and with media containing BAFF reagent ('p' and 'S/N' respectively in Figure 5.25) for 18h. Post stimulation, cells were mechanically fractionated to check the localisation of FAM129C. The positive control of treatment was achieved by monitoring the fate of p52 and p100, specifically by seeing an increase of p52 and reduction in p100.

2.1.2.8 Inducing apoptosis

Reagents to induce apoptosis are presented in Table 2.5. The cells were incubated for 6-8 hours and the level of apoptosis was measured using AnnexinV/PI flow cytometry.

Table 2.5 Reagents used to induce apoptosis

Reagent and Supplier	Stock	Working concentration
MG132, proteasome inhibitor (Calbiochem)	20mM	5µM
Etoposide (Sigma)	100mM	200µM
TRAIL (kind gift from Dr N. Harper)	1.4mg/ml	100ng/ml
Staurosporin (Sigma)	1mM	1µM
Anti- Fas activating antibody (CH11 clone, Millipore, Temecula)	500mg/µl	100ng/µl

2.1.2.9 Flow cytometry methods

Measurement of apoptosis induction by AnnexinV/PI binding

In non-apoptotic cells, phosphatidylserine (PS) is localised to the inner side of the plasma membrane. When apoptosis is induced, PS is externalised to the outer surface of the plasma membrane and provides an engulfment signal to macrophages and NK cells (Fadok *et al.*, 1998). In 1995, Annexin V was discovered to preferentially bind to PS and therefore can mark apoptotic cells (Vermes *et al.*, 1995). Fluorescent-tagged Annexin V (conjugated with FITC) can measure the extent of apoptosis in a given population (Martin *et al.*, 1995). In conjunction with Propidium iodide (PI), to establish membrane integrity, apoptotic cells can be identified by flow cytometry.

For each measurement, $1x10^6$ cells were collected and washed. Cells were resuspended in Annexin Buffer (10mM Hepes/NaOH (pH7.4), 140mM NaCl, 2.5mM $CaCl_2$) and 1.5µl of FITC conjugated Annexin V was added to each sample. Cells were incubated for 10 minutes at ambient temperature. After incubation, cells were placed on ice and 250ng of PI was added to each sample. Samples were read on a Becton Dicknson FacScan flow cytometer immediately.

Measurement of surface Fas expression

The amount of Fas present on the surface of a cell can be measured using receptor specific primary and fluorescent secondary antibodies. To achieve this measurement, $1x10^6$ cells were resuspended in 10% v/v goat serum in PBS and incubated with 5ng/µl (1:100 of the stock antibody) of mouse monoclonal anti-Fas human activating antibody (Clone CH-11, Upstate) for 1h on ice. Cells were washed and resuspended in fresh 10% goat serum and PBS. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin ($F(ab')_2$ fraction (Dako (F0479)) was added to a final concentration of 50ng/µl. Following incubation on ice for 1h cells were washed and the level of receptor expression analysed using the FACSCalibur™ machine with excitation/emission wavelengths of 488/525nm and the CellQuest Pro® software. The acquisition settings were set out by cells stained with secondary antibody only in the first quadrant of the log scale. Receptor expression could be determined by a rightward shift in the curve and an increase in the geometric mean fluorescence intensity.

Cell cycle analysis

When cell division is triggered the amount of RNA increases and new DNA is synthesised (in the S phase). When the DNA is doubled the cell reaches G2 and then the cell enters mitosis (M phase), after division the DNA content returns to its original

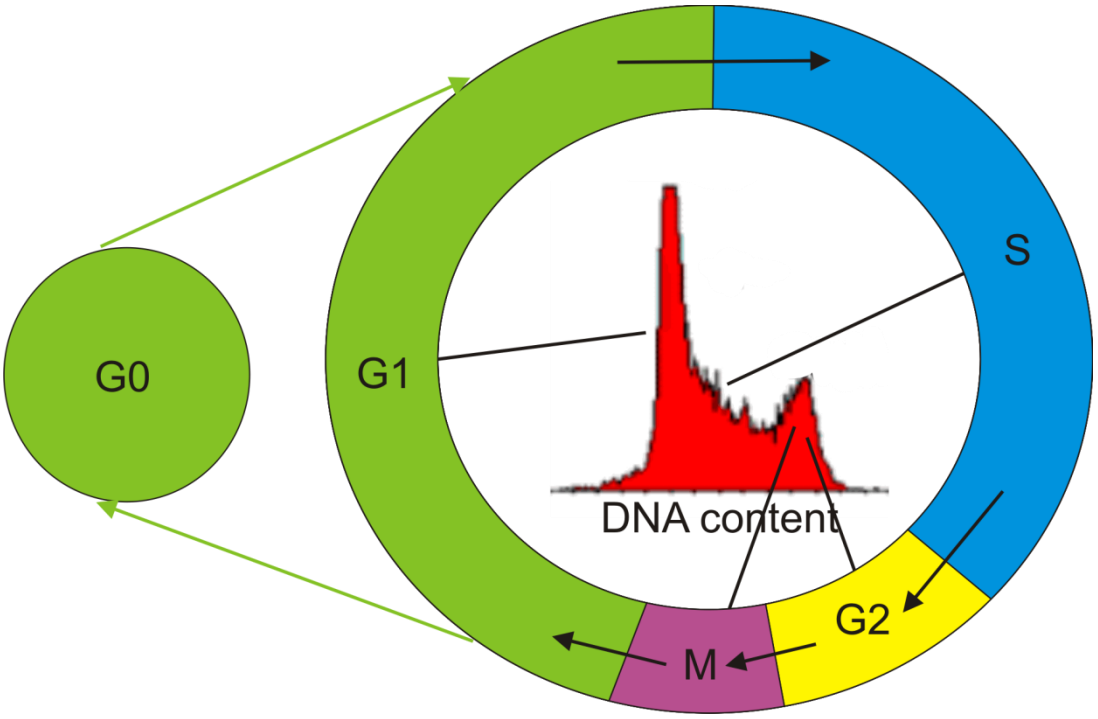


Figure 2.3 Measurement of DNA content and stages of cell cycle.

G0 (not shown, 'out of cycle' has the same amount of DNA as G1. When the division is trigged RNA increases, the new DNA is synthesized in S phase, when DNA is doubled when the cell reaches G2 and enters the mitosis, the DNA content returned to the level of G0/G1. *Modifiedwith permission. from* http://*flowbook.denovosoftware.com/Flow_Book/Chapter_6%3a_DNA_Analysis*

level of G0 or the cell repeats the division process. Figure 2.3 shows the relationship between DNA content and cell cycle.

To measure the DNA content, the cells have to be stained with PI that binds to DNA and reflects accurately the amount of DNA present. PI has red fluorescence and can be excited at 488nm. PI stains all double stranded nucleic acids therefore the cells have to be incubated with RNase to remove any double stranded RNA and the cells have to be fixed or permeabilised to allow PI to enter the cells.

Method

A suspension was prepared of single cells in 200µl of PBS; 2ml of ice-cold 70% ethanol with 30% PBS were added whilst vortexing vigorously. The suspension was left for a minimum 30 minutes on ice. After the incubation the cells were spun and resuspended in 700µl of PBS. If clumps were visible under microscope, the suspension was passed through a 25-gauge syringe needle. Finally 100µl of RNase (Ribonuclease

A, Sigma, 1mg/ml) and 200µl of PI (400µg/ml) was added and incubate at 37^0C for 30 minutes. The sample was analysed using an argon-ion laser tuned to 488nm and measuring forward and orthogonal light scatter and red fluorescence.

2.1.3 Immunocytochemistry

Immunocytochemistry can be described as the staining of fixed cells allowing the detection of specific antigenic epitopes. This method permits the visualisation of the localisation of specific antigens using antibodies, which have been raised against the protein of interest.

2.1.3.1 Buffers and Reagents

Table 2.6 Buffers used in immuno histochemistry

10% Goat Serum
Goat Serum (Sigma) 1ml
Made to 10ml with 1X PBS

4% Paraformaldehyde (PFA)
1X PBS 100ml
Paraformaldehyde 4g (Sigma)

2.1.3.2 Cell Preparation

After several washes with ice-cold PBS, the cells were fixed using 4% PFA, for 10 minutes, and washed with PBS then permeabilised with ice-cold 0.1% Triton X-100 for 3 minutes. Once fixed and permeabilised, cells were blocked with 10% goat serum in PBS and incubated with a primary antibody (anti-MYC at dilution 1:1000) for 1h and subsequently with a secondary antibody for 1 h at ambient temperature. All antibodies were diluted in 10% goat serum–PBS. Alexa Fluor 488 and 568 (Molecular Probes) conjugated secondary antibodies were used at 1:1000. Cells were then washed, counterstained with 49, 69-diamidino-2-phenylindole (DAPI), and mounted by using the ProLong Anti-fade kit (Molecular Probes). Slides were analysed by confocal microscopy.

2.2 Protein Electrophoresis and Western Blotting

2.2.1 Principle of Protein Electrophoresis

Electrophoresis is the name given to the movement of charged particles in solution by applying an electrical field across the mixture. The speed at which the molecules move is dependent on their size, shape and charge, making this a useful tool for the separation of large molecules such as proteins. Most proteins are separated using

polyacrylamide gels. These cross-linked, sponge-like structures act as size selective sieves such that molecules smaller than the gel pore size move more freely through the gel than those that are larger. Gel pore size is regulated by the concentration of polyacrylamide used where the higher the concentration the smaller the pore size. SDS polyacrylamide gel electrophoresis (SDS-PAGE) is the technique we chose to use as this allowed us to separate proteins on the basis of their molecular weight.

Sodium dodecylsulfate (SDS) is an anionic detergent that is used to denature proteins to individual polypeptides. It does this by wrapping around the polypeptide backbone and conferring a net negative charge in proportion to the length. It binds at the constant weight-ratio of 1.4g/g of polypeptide and makes proteins of similar molecular weights migrate at similar rate by unifying their isoelectric point.

2.2.2 Principle of Immunoblotting

The transfer of proteins separated by SDS-PAGE from a gel to a membrane by electrophoretic elution is known as immunoblotting. Nitrocellulose membranes are the most commonly used material. For a wet transfer, the membrane was soaked in water and 2 pieces of 3mm paper were soaked in transfer buffer and a sandwich of membrane/paper and sponge was then assembled. The separated proteins were transferred onto nitrocellulose (at 65V for 2h) using BioRad Mini transfer blot equipment in 1x transfer buffer. Following transfer the membrane blots were placed in a box with 5% non-fat dried milk dissolved in PBS-T 0.1% and shaken for 30 minutes at ambient temperature to block non-specific binding. The blot was then probed with a protein-specific primary antibody (for either 1h at ambient temperature or overnight at 4°C to the desired antibodies diluted to the optimal working solution) followed by a secondary antibody specific for the general class of primary antibodies. After each incubation the blots were washed three times for 5 minutes in PBS-T 0.1%. Secondary antibodies are generally tagged with either a peroxidase or alkaline phosphatase thus allowing immunoreactive bands to be detected by colour development of enhanced chemoluminescence (ECL, Amersham-Life Science) upon application of the appropriate enzyme substrates.

2.2.3 Principle of Enhanced Chemiluminescence (ECL) Detection

Chemiluminescence is the emission of light without heat as a result of a chemical reaction. One of most well characterised systems is the horseradish peroxidase (HRP)/hydrogen peroxide catalysed oxidation of luminol under alkaline conditions. The

emission of light is due to the excitation decay of the luminol. To enhance the light emission the oxidation reaction can be done in the presence of chemical enhancers such as phenols. This process is known as enhanced chemiluminescence (ECL). The blots were exposed to film for a different time ranging from 15 seconds to 20 minutes depending on the primary antibody and developed.

2.2.4 Buffers and Reagents

Table 2.7 Buffers used in western blotting

10X Buffer stock solution
30g TriZma Base (Sigma)
140g Glycine (Sigma)
Made up to 1L with distilled water.

1X Running buffer
100ml of Buffer stock solution
5ml 20% SDS
Made up to 1L with distilled water

1X Transfer buffer
100ml of Buffer stock solution
200ml 100% methanol (Sigma)
Made up to 1L with distilled water

20% SDS
40g powdered SDS (Sigma)
200ml distilled water

Phosphate Buffered Saline
1 tablet/100ml distilled water (Gibco)
PBS-Tween (0.1%) (PBS-T)
1L PBS
5ml of a 20% Tween-20 solution (Sigma)

Membrane Blocking solution
PBS
0.1% Tween-20
5% dry-powdered milk (Marvel)

Primary/Secondary antibody solution
PBS
0.1% Tween-20
3 % dry-powdered milk (Marvel)
Primary/ secondary antibody

2.2.5 Protein Extraction

Total proteins were extracted from cells growing in culture by a lysis method. Cell pellets were washed once in PBS and excess liquid was aspirated and pellets frozen in dry ice and stored at -80°C until needed. Thirty microlitres of lysis buffer were directly added to the cell pellets. Frozen pellets were left to thaw on ice in lysis buffer for 10 minutes before mixing with a pipette. Non-frozen samples were mixed as soon as buffer was added and then left on ice for 10 minutes before centrifuging at 13,000 rpm for 5 minutes at +4°C. Cellular debris was pelleted and the total protein present in the supernatant was recovered and placed in a fresh Eppendorf tube (1.5ml). An aliquot (1µl) was used for determination of protein concentration.

Table 2.8 Lysis buffer

Lysis Buffer	*Final Concentration*
Triton X-100	*0.5%*
Tris 1M, pH7.6	*50mM*
NaCl	*150mM*
Potease inhibitor cocktail 100X (Sigma)	*1X*
Sodium Fluoride	*2.5mM*
Sodium Ortovenadate	*10mM*
Make to 200ml with distilled water.	

2.2.6 Protein Concentration Measurement

Protein concentration in the cellular extract was determined according to the Bradford protocol. Firstly, a stock solution of bovine serum albumin (BSA) of 2mg/ml, and dilutions ranging from 1 to 20µg/µl, were prepared by adding the appropriate amount of stock solution directly to 1ml aliquots of Bradford reagent (BioRad). The blank sample was Bradford reagent alone. The absorbance at 595nm was measured in the spectrophotometer and a calibration curve generated. The concentration of proteins in the samples was determined by adding 1µl of protein extract to 1ml of Bradford reagent and the absorbance read on a spectrophotometer. Samples were processed as for the calibration curve and the amount of protein calculated.

2.2.7 SDS-PAGE

Stacking and separating gels were prepared shortly before pouring. Ammonium persulphate (APS) catalysed polymerisation and TEMED accelerated the reaction and therefore these reagents were added last.

Table 2.9 Sample buffer

5X Sample buffer	*Final concentration*
SDS	*10%*
Tris pH6.8	*312.5mM*
Glycerol (Sigma)	*50%*
β-mercaptoethanol (Sigma)	*25%*
Bromophenol-blue (Sigma)	*0.025%*

The samples were prepared with one fifth of the volume of sample buffer (Table 2.9) were added to the protein lysates and were boiled at 95°C for 5 minutes.

After a brief spin, 60µg of prepared lysates were loaded into the wells of a 5% stacking gel on a 10% polyacrylamide gel (Table 2.10). A prestained commercially available protein marker (ladder) (BenchMark^{TM}, BioRad) was also loaded.

Table 2.10 Stacking and running gel

Stacking gel	*Final concentration*
Acrylamide mix (Protogel, Gene Flow National Diagnostic)	*5%*
Tris pH6.8	*0.13M*
SDS	*0.01%*
APS	*0.01%*
TEMED	*1000x*
dH_2O	

10%Running Gel	*Final Concentration*
Acrylamide mix (Protogel, Gene Flow National Diagnostic)	*8%*
Tris pH8.8	*0.4M*
SDS	*0.01%*
APS	*0.01%*
TEMED	*1600x*
Distilled Water	

The individual samples were loaded into wells and one lane on each gel contained the marker. Proteins were resolved on a minigel apparatus (BioRad) and run at 30mA per 1.5mm plates thickness.

2.2.8 Antibodies

2.2.8.1 Primary Antibodies

Table 2.11 Primary antibodies

Antibody	Host	Source (catalogue number)	Concentration
Covalab1	Rabbit	Covalab (custom order)	**1:1000**
Antibody 15	Rabbit	Covalab (custom order)	**1:1000**
Anti-IRF4	Rabbit	Cell Signaling Technology (4948)	**1:1000***
Anti-BCL6	Rabbit	Santa Cruz (sc-858)	**1:1000**
Anti-BCL2	Mouse	Dako (MO887)	**1:1000**
Anti-CD10	Mouse	Vector laboratories (VP-C328)	**1:500**
Anti-MYC (clone 9B11)	Mouse	Cell Signaling Technology (2276)	**1:1000**
Calnexin	Rabbit	Santa Cruz (sc-11397)	**1:1000**
COX IV	Rabbit	Cell Signaling Technology (4844)	**1:1000**
EEA1	Mouse	BD Transduction Labs, (610457)	**1:5000**
Na K ATP	Mouse	Abcam, (ab7671)	**1:2000**
LDH	Goat	Abcam, (ab2101)	**1:1000**
PCNA	Mouse	BD Transduction Labs, 610665	**1:10000**
CD79b	Rat	Serotec (MCA2209)	**1:1000**
pAKT	Mouse	Cell Signaling Technology (4060)	**1:2000**
tAKT	Rabbit	Santa Cruz (sc-8312	**1:1000**
pERK	Mouse	Cell Signaling Technology (9106)	**1:2000**
tERK	Mouse	Cell Signaling Technology (4696)	**1:2000**
Anti-Lyn	Mouse	Santa Cruz (sc-7274)	**1:1000**
Flotilin	Mouse	Abcam (ab41927)	**1:1000**
P100/p52	Rabbit	UpstateBiotechnology (06-413)	**1:10000**
Anti-Fas (clone CH11)	Mouse	Millipore (05-201)	**1:10**
F(ab')2 fragment anti-human IgM	Goat	Jackson Laboratories (109-006-129)	**10µg/ml**
Actin	Mouse	Sigma (A3853)	**1:5000**

***** overnight at 4^0C in 5%BSA

2.2.8.2 Secondary Antibodies conjugated to HRP

Table 2.12 Secondary antibodies

Antibody	Host	Source	Concentration
Anti-mouse	Sheep	Amersham Bioscience	1:10,000
Anti-rabbit	Donkey	Amersham Bioscience	1:10,000
Anti-rat	Goat	Cell Signaling Technology	1:3,000

2.2.8.3 Raising FAM129C antibodies

FAM129C proved to be a difficult protein to raise antibodies against; this may be due to a lack of strong antigenic epitopes in the sequence. Bioinformatic expertise was utilized (Dr. R. Schmid) to predict peptides which were situated on the outer structure of the pleckstrin homology domain or extracellular region. For instance, one of the peptides used by Eurogentec and the LRF Oxford Antibody Facility (residue 168-185) was designed to be an exposed peptide of the pleckstrin homology-like domain. This attempt did not produce any antibody. The initial aim was to generate an antibody that would recognize all isoforms listed within the database, with the exception of isoform e, f and g (where no information on translation was available).

Only one specific antibody was raised: a rabbit polyclonal raised against peptides (Antibody 15, Covalab) which proved to be particularly suitable for western blotting.

However it was not suitable for both immunochemistry and flow cytometry techniques. Figure 2.4 summarises various approaches, the peptide sequences and protein fragments used.

The first polyclonal antibody generated was produced in rabbits by the inoculation of a mixture of two peptides (Covalab, Villurbanne, France). This antibody (Covalab 1) recognised FAM129C protein, however there were a number of non-specific bands detected by western blotting (please see Chapter 5, Figure 5.8). In order to resolve the origin of the non-specific bands, a blocking experiment was conducted (Figure 2.5). A peptide situated towards the C terminus of the protein blocked both specific and non-specific bands. The other peptide slightly reduced the specific signal. A second attempt, employing a similar strategy, was devised based upon results obtained with Covalab 1 antibody in the design of the peptides used for the inoculation, this method generated Antibody 15. Antibody 15 was purified by affinity chromatography using both peptides from rabbit 15, as rabbit 16 had non-specific response at the same molecular weight as FAM129C (Figure 2.6). The two antibodies were compared using a panel of ALL and DLBCL cell lines (please see Figure 2.7 in the results chapter) and Antibody 15 proved to be more sensitive and specific, giving fewer non-specific bands than Covalab 1, although neither of these antibodies proved useful with either immunochemical or flow cytometric techniques (intracellular or surface staining).
A further attempt was made to raise a rabbit polyclonal antibody by Eurogenetec (Liege, Belgium), this antibody recognised neither exogenous (FAM129C transfected cells) or endogenous FAM129C (OCI-Ly10, OCI-Ly3, HBL1, NuDUL-1).

Four attempts were made to generate a monoclonal antibody. I was unable to generate the full size protein as the recombinant protein proved insoluble. At the time the bioinformatic prediction suggested three transmembrane domains, therefore I produced a GST-protein fragment with the sequence between predicted transmembrane domains (residue 190-343, MW=17.5kDa) the protein was generated in E.coli; both attempts at mice immunisation failed. A further two trials were attempted to generate a mouse monoclonal antibody from peptides, these were also unsuccessful.

```
mgpdrkevpl srgtqavvvg kgrgapgdds smggrpsspl dkqqrqhlrg qvdtllrnfl
 61 pcyrgqlaas vlrqisrelg pqeptgsqll rskklprvre hrgpltqlrg hpprwqpifc
121 vlrgdgrlew fshkeeyeng ghclgstalt gytlltsqre ylrlldalcp eslgdhtqee
181 pdsllevpvs fplflqhpfr rhlcfsaatr eaqhawrlal qqgirlqgtv lqrsqapaar
241 afldavrlyr qhqghfgddd vtlgsdaevl tavlmreqlp alraqtlpgl rgagrarawa
301 wtelldavha avlagasagl cafqpekdel laslektirp dvdqllrqra rvagrlrtdi
361 rgplesclrr evdpqlprvv qtllrtveas leavrtllaq gmdrlshrlr qspsgtrlrr
421 evysfgempw dlalmqtcyr eaersrgrlg qlaapfgflg mqslvfgaqd laqqlmadav
481 atflqladqc lttalncdqa aqrlervrgr vlkkfksdsg laqrrfirgw glciflpfvl
541 sqlepgckke lpefegdvla vgsqaltteg iyedvirgcl lqridqelkk tlgandvsct
601 ldgclevpwe qegadeetea ereggacprq pdsgaqiqpl cpppspgtfr s
```

	Peptides sequences (unless specified)	Company	Type	Source	Suitability
1.	ldavrlyrqhqghf cllqridqelkktlgandv	Covalab (referred as Covalab1)	polyclonal	rabbit	ELISA, WB
2.	rpsspldkqqrqhl vrgrvlkkfksdsg	Covalab (referred as Antibody 15)	polyclonal	rabbit	ELISA, WB, poor IP
3.	elgpqeptgsqll	MRC Toxicology, Dr J Embleton	scFv	N/A	ELISA
4.	mgpdrkevpl srgtqa cpeslgdhtqeepdsll	Eurogenetec	polyclonal	rabbit	ELISA
5.	lcpeslgdhtqeep andvsctldgclevp	LRF Antibody facility (Oxford)	monoclonal	mouse	weak ELISA
6.	sfplflqhpfrrhlcfsa at… (protein fragment)	LRF Antibody facility (Oxford)	monoclonal	mouse	-

Figure 2.4 Strategies used to raise antibodies against FAM129C.

scFV -single chain variable fragments; ELISA- Enzyme-linked immunosorbent assay; WB-western blotting, IP-immunoprecipitation

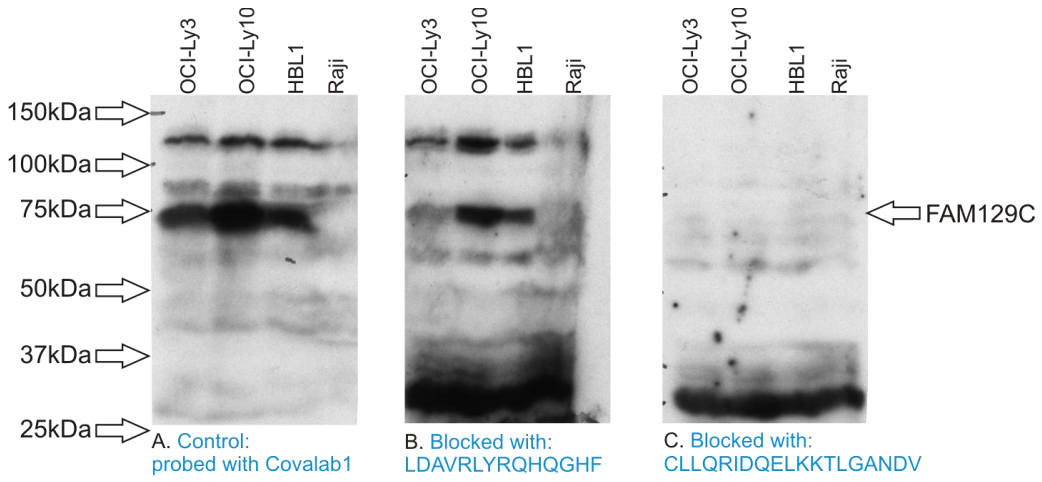


Figure 2.5 Covalab1 antibody and peptide blocking.

Blots B and C were first blocked with peptides before probing with Covalab antibody. The peptide placed at the C terminal part of the protein (C) was the most efficient in blocking both specific and non-specific bands. Peptide LDAVRLYRQHQGHF partially blocked FAM129C signal (B). Band at 130kDa is nonspecific.

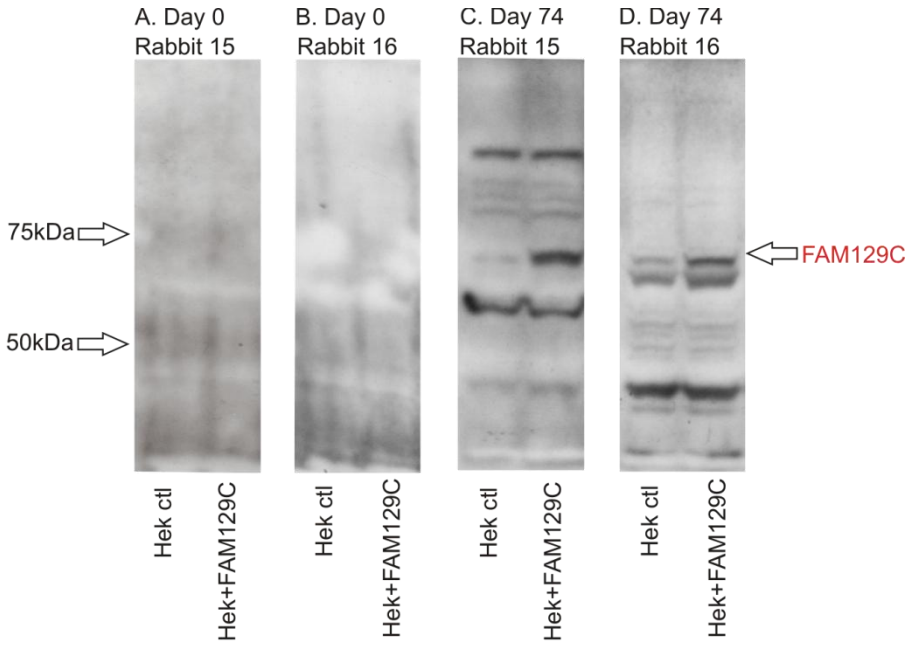


Figure 2.6 Rabbit sera test, day 0 (A and B) and day 74 bleed (C and D).

Hek ctl-does not express FAM129C, Hek+FAM129C is Hek cell line transfected with FAM129C containing mammalian expression construct. Rabbit 15 developed a specific response to FAM129C, there is the appearance of immunoreactive bands of the correct molecular weight (as seen on C, but not on A). Rabbit 16 showed a non-specific response at the same molecular weight as FAM129C , the band was present in Hek ctl as well as Hek transfected with FAM129C(D), therefore only sera from rabbit 15 were purified (C).

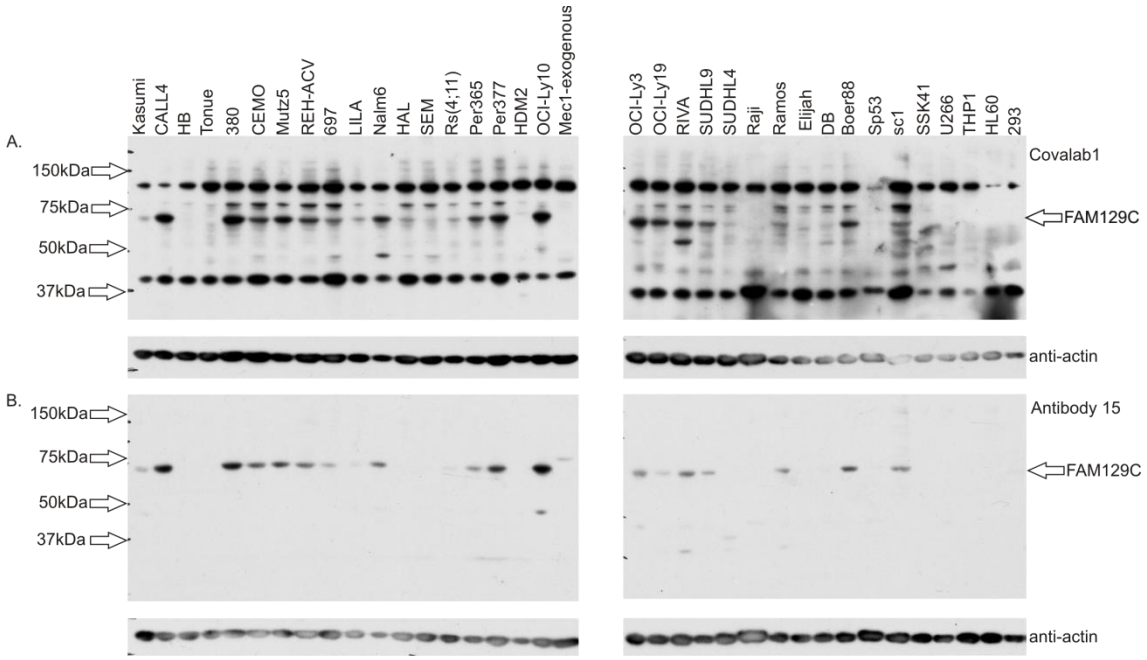


Figure 2.7-Comparison of Covalab1 (A) antibody and Antibody 15 (B) on the panel of ALL and DLBCL cell lines.

Mec1-exogenous- Mec1 stably transfected with FAM129C tetracycline inducible construct. Please see Chapter 2 for the list of cell lines and the differences between Covalab1 and Antibody 15.

In addition, an attempt was made to generate a scFv antibody in-house from phage display libraries (Tomlinson, I&J, Nissim, Griffin) (Nissim *et al.*, 1994; Griffin & Ouwehand, 1995). This work was performed under the supervision of Dr J. Embleton. The peptide used to screen the library was taken from the original publication by R. Boyd (Boyd *et al.*, 2003). Although positive bacterial clones were found by ELISA, they did not work in western blotting (WB), flow cytometry or immunohistochemistry.

The antibody of choice was Covalab Antibody 15 for its specificity. The majority of the work presented in this thesis was performed using this antibody. Cell lines expressing *FAM129C,* as determined by RT-qPCR, were used to test the specificity of the antibody.

2.2.8.4 Calculating molecular weight

The relative mobility of proteins in an SDS-PAGE gel is related to their molecular weight. A standard curve was created from protein ladder (selection of proteins of known MW) by plotting their molecular weight expressed in $\log_{10}$ against the relative mobility (Rf) of the proteins. In order to calculate Rf it is important to know the top of the running gel and the position of the tracking dye bromophenol blue at the end of the run (in Figure 2.8 this distance is 60mm). Relative mobility is calculated as a ratio of a distance of a given protein to 60mm. The relative mobility of FAM129C was fitted to the curve to identify the molecular weight. The point where the extrapolated curve crosses the y axis determines the limit of the given gel. In the example shown in Figure 2.8 the gel limit, i.e. zero motility is 199.5kDa (y intercept is 2.3, therefore $10^{2.3}$=199.5), therefore proteins large than 199.5kDa will be excluded.

The specific band to FAM129C was seen in primary cells, patient samples and cell lines and appeared to be approximately 70kDa. This would correspond to Isoform b (Aceview) and Isoform 2 (Boyd *et al.*, 2003), see Table 2.13. The template which was used to clone FAM129C was purchased from Origine and it was isoform a, which therefore should run at 77.4. The expressed protein was migrating at the level of 75kDa (the tags account approximately 5kDa). The calculated value based on the relative mobility on the western blotting was 70.79kDa (Figure 2.8).

Table 2.13-Predicted molecular weight of FAM129C isoforms as suggested by NCBI database and described by Boyd (2003).

Isoform	a=3	b	c	d	e	f	g	1	2
aa	679	620	596	579	180	111	83	666	651
pI	8.99	8.28	6.97	9.82	?	?	?	8.71	8.41
kDa	77.4	69.11	66.06	64.72	?	?	?	73.8	72.19

Isoforms a-g as listed on Aceview, isoforms1-3 were originally described by Boyd, 2003. Molecular weight (kDa) and theoretical isoelectric point (pI) were predicted using http://au.expasy.org/tools/protparam.html. There are no data on translation sequence of isoform e-g.

2.3 Immunoprecipitation

2.3.1 Immunoprecipitation with CD79b

Cells were lysed for a minimum of 30 minutes on ice in a solution of 1% digitonin, 20mM HEPES, pH7.6, 137mM NaCl, and a protease inhibitor 'cocktail' (Sigma-Aldrich). After pelleting of nuclei and cell debris, supernatants were used.

Preparation of the beads: Protein G beads were washed three times with PBS-T (with 0.05% Tween) , 6.25µl of antibody was added to 100µl of beads and incubated for 1h at 4^0C. The amount of beads for each immunoprecipitation was optimised, an example of optimisation is shown in Figure 2.9

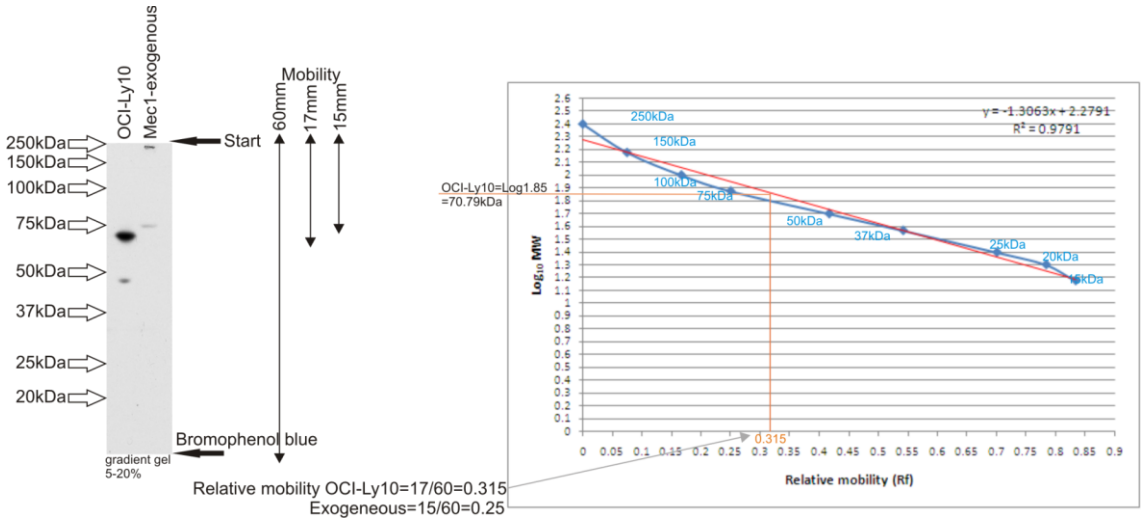


Figure 2.8 Calculating molecular weight based on relative mobility (Rf).

Rf is calculated as the ratio of distance travelled to distance marked by bromophenol blue, e.g. for OCI-Ly10 it is 17mm/60mm=0.315. Rf for bromophenol blue=1. A standard curve is drawn on the basis of the relative mobility of the markers and their Log_{10} value. The molecular weight for the endogenous FAM129C read from the standard curve is 70.8kDa.

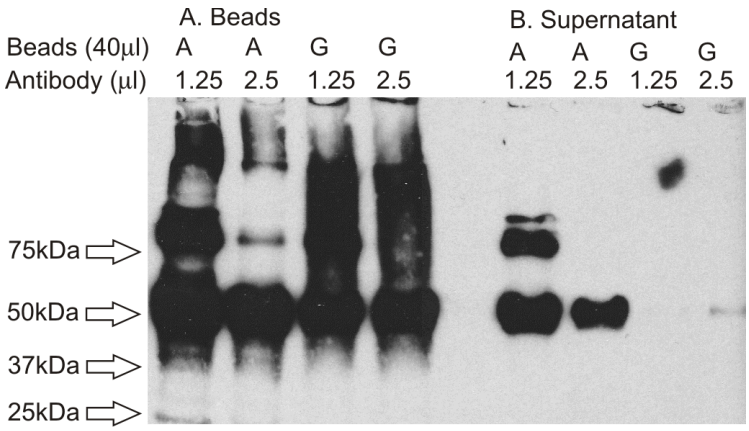


Figure 2.9 Optimisation of amount of FAM129C antibody and type of beads

A. Material eluted from beads **B.** Antibody present in supernatant. The best combination of beads and antibody were in 40µL protein G-beads and 1.25µL , as shown in B. lane 3, there is no detectable antibody in the supernatant.

Crosslinking: Beads bound to antibody were washed twice in 1ml 0.2M fresh triethanolamine pH8.2 (TEA, T9534)) the beads were resuspended in 1ml of 20mM dimethylpirelimidate diHCL (DMP, Fluka, 80490) in 0.2M TEA pH8.2 (prepared immediately before use). The suspension was incubated at ambient temperature on a roller for 30min. The beads were centrifuged, washed with 50mM Tris pH7.5, left for 15 minutes on a roller, and washed a further three times with PBS/0.05% Tween. The beads were transferred into lysate. Controls were beads conjugated to rat IgG (Santa Cruz Biotechnology).

The lysates were captured by incubation with crosslinked beads overnight and subsequently washed three times with PBS + 0.05% Tween. The proteins of interest were eluted from the bead-antibody complex with 100mM Glycine buffer pH2.5 for 30 minutes on a daisy wheel (4^{o}C). The eluate was prepared with 2x sample buffer and analysed by immunoblotting.

2.3.2 Immunoprecipitation with anti-MYC

Table 2.14 Lysis buffer

Lysis Buffer Constitutes	Stock	For 200ml of lysis buffer
50mM TrisHCL pH8	1M	10ml
150mM NaCl	5M	6ml
1mM $MgCl_2$	1M	0.2ml
1mM EGTA	0.5M	0.4ml
100mM NaF	0.5M	40ml
10% Glycerol	100%	20ml
0.05% Tween		0.1ml
1mM Na Vanadate	200mM	
Protease Inhibitors (Roche)	1 tablet per 10ml	
H_2O		115.4

The cells were lysed for 1h with 0.5% Triton TX-100 with the lysis buffer (Table 2.14) and the sample was gently mixed. Spermine was added (1: 50 dilution, Sigma, S3256) for 20 minutes, the lysate was then spun in a microcentrifuge for 40 minutes at 30,000x*g*. The lysate was concentrated on a vivaspin column and fractionated over a sucrose density gradient (centrifuged for 18h). Fractions 12-16 and 17-22 were used for the immunoprecipitation (IP) which were dialysed prior to use.

Beads were washed three times with PBS, pH8. The washed beads were resuspended in 75µl of PBS pH8 and 5µl anti-MYC antibody was added and incubated for 1h. The proportion of antibody to beads was optimised for each IP. For anti-MYC IP: 15µl beads with protein A for 1µl anti-MYC antibody; and for FAM129C IP: 30µl protein G Dynabeads for 1µl of anti-FAM129C antibody.

After incubation of beads and antibody, beads were washed with lysis buffer, then the earlier prepared lysate was added to the washed bead-antibody complex and incubated for 2h at 4^0C. After incubation the beads were washed with Net gel (Table 2.15) for a minimum three times, and finally beads were resuspended in 2xSDS sample buffer if protein A was used or elution buffer (200mM Glycine, 200mM NaCl, pH2, for 15min 800rpm shaker at 20^0C) if protein G were used.

Table 2.15 NetGel buffer for washing beads

NetGel buffer	Stock	For 200ml
50mM TrisHCL pH7.5	1M	10
150mM NaCl	5M	6
1mM EDTA	0.5M	0.4
0.25%gelatin	2%	25
0.1% NP-40 (IGEPAL)	100%	0.2
H_2O		158.4

2.4 Fractionation methods

2.4.1 Sucrose density gradient centrifugation of cell homogenates

This technique separates proteins according to their molecular weight. The first step was to break cells using a ball-bearing homogeniser in detergent-free buffer followed by a 10 minutes 1000x*g* spin (Figure 2.10). The pellet consisted of intact cells and nuclei. The supernatant was then centrifuged further on a 60% sucrose cushion to concentrate the membranes and separate the cytosol and plasma membranes. The latter was placed on a sucrose gradient. This was spun at 100,000x*g* for 18h. Fractions (0.5ml) were collected and analysed by SDS and WB (Figure 2.11.A). The percentage sucrose was measured by refractometry

2.4.2 Sucrose fractionation with mild detergent

This technique is very similar to above described sucrose fractionation with the exception that the cells are lysed in 1%Triton X-100 (Figure 2.11). This mild lysis allows the separation of protein complexes according to the molecular weight. Molecular weight markers are used to identify the weight of complexes in certain fractions: Aldolase 128kDa, Catalase 250kDa, Thyroglobulin 770kDa (all from Amersham HMW markers for chromatography). The complexes were destroyed by treating the sample with the strong detergent, SDS, as shown in Figure 2.12.

2.4.3 Generation of lipid rafts

Lipid rafts were prepared from 100 × 10^6 cells that were solubilised in 1ml of 1%Triton X-100, 1mM EDTA, 50mM Tris-HCl, pH7.4 (with protease inhibitors), vortexed for 3-5 seconds and incubated on ice for 30 minutes, mixing every 15 minutes and purified by sucrose density gradient purification (Saeki *et al.*, 2003). The whole cell lysate was diluted with 80% sucrose (in homogenization buffer) to a final concentration of 40% sucrose (total of 4ml) and was overlaid with 30% (4ml) and 5% (4ml) sucrose buffer (without Triton X-100) (Figure 2.13). The discontinuous sucrose gradient was

centrifuged for 18h at 100,000x*g* at 4°C, and lipid rafts were harvested from the interphase of the 5 and 30% sucrose bands and centrifuged at 100,000x*g* for 1h at 4°C. The lipid raft pellet was then solubilised in SDS sample buffer and analysed as described below.

2.4.4 Mechanical fractionation

Cells were washed with PBS and sonicated (Soniprep 150, MSE) at the amplitude of 10 microns, on ice, twice for 5 seconds; the cell destruction was checked by light microscopy (to check if >90% of cells were destroyed and large parts of the membrane were still present). The sonicated cells were spun briefly in a benchtop centrifuge for 1 minute at 13,000 rpm to remove unbroken cells, the supernatant was further spun in the Personal Benchtop Ultracentrifuge Beckman Optima TLX for 30 minutes at 55,000 rpm (100,000x*g*). The supernatant contains cytoplasm, pellet plasma membranes and intact cells. The pellet was washed with PBS to remove cytoplasm. The pellet is composed of plasma membranes and some unbroken cells.

2.4.5 Nuclear fractionation

Nuclear fractionation was performed using a commercial kit NE-PER® (Thermo Scientific, Rockford, USA). This method is based on the sequential lysis and centrifugation steps.

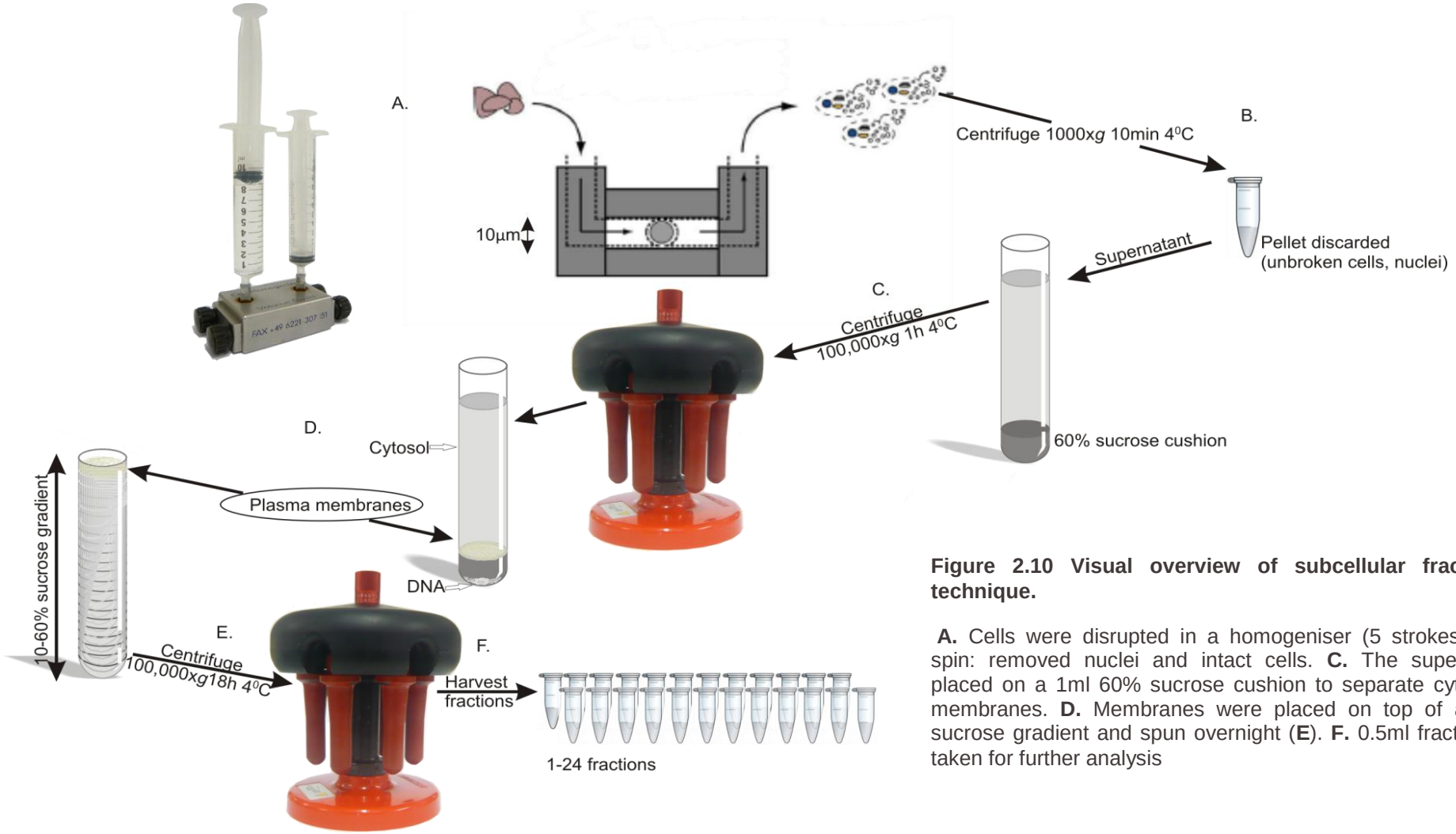


Figure 2.10 Visual overview of subcellular fractionation technique.

A. Cells were disrupted in a homogeniser (5 strokes) **B.** First spin: removed nuclei and intact cells. **C.** The supernatant is placed on a 1ml 60% sucrose cushion to separate cytosol from membranes. **D.** Membranes were placed on top of a 10-60% sucrose gradient and spun overnight (**E**). **F.** 0.5ml fractions were taken for further analysis

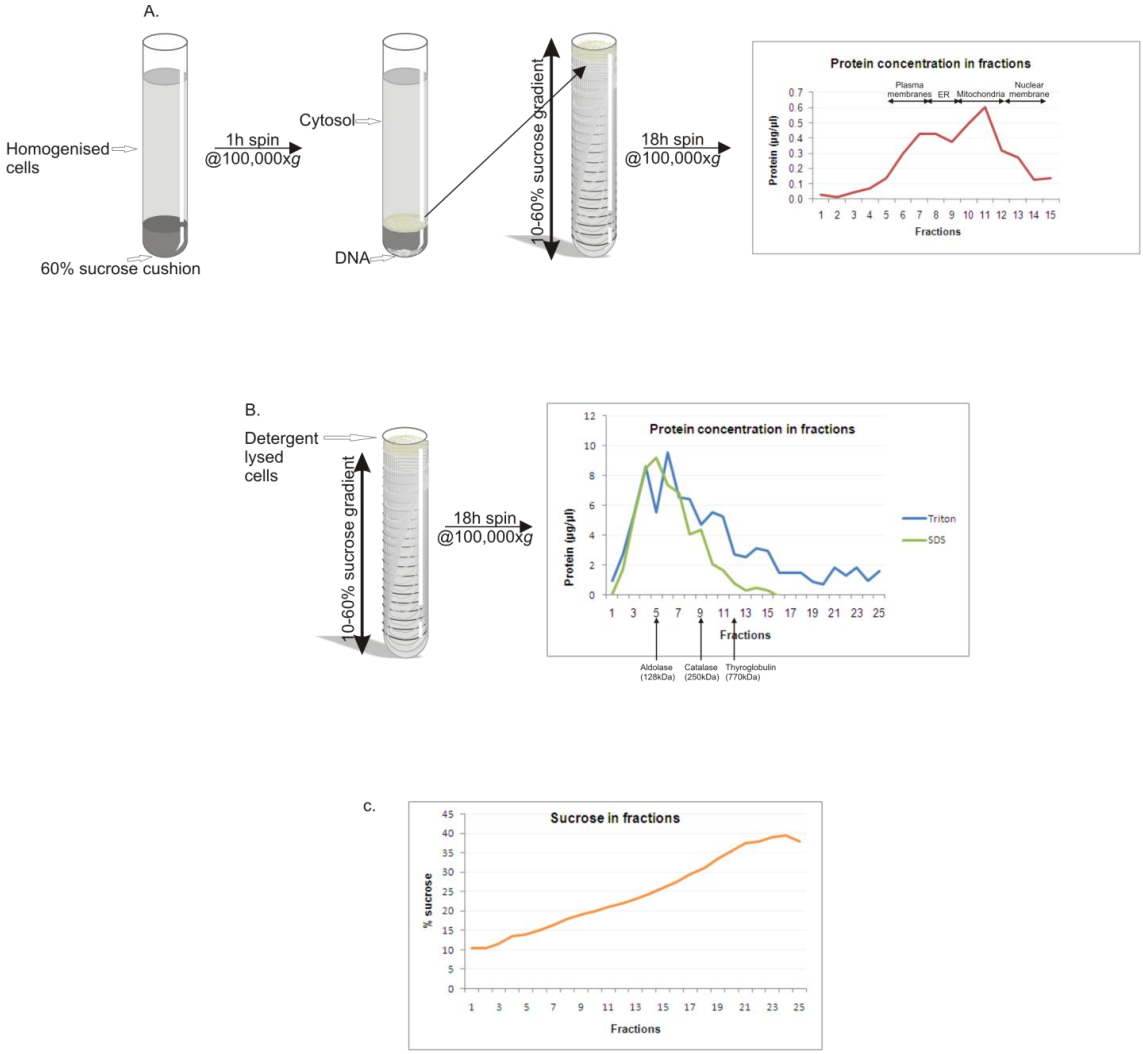


Figure 2.11 Comparison between sucrose fractionation without detergent (A) and with detergent (B).

A. Sucrose density gradient centrifugation of cell homogenates, cytosol and heavy membranes are separated by spin on a 1ml 60% sucrose cushion, the heavy membranes were then spun on a sucrose gradient. Graph shows protein distribution across fractions, organelle position was based on the immunoblotting as shown in the chapter 3, Figure 3.13 **B.** Sucrose fractionation with detergent to separate large molecular complexes, graph shows difference in protein distribution across fractions of SDS v. Triton X100 lysates. Protein's of molecular weight of 128kDa were positioned in fraction 5 (of Triton lysis, by position of Aldolase) and of 250kDa in fraction 9 (Catalase) , 770kDa in fraction 12 (Thyroglobulin); N.B. Fractionation in A. and B. were done in different size tubes. **C.** Sucrose concentration across fractions after 18h spin at 100,000xg.

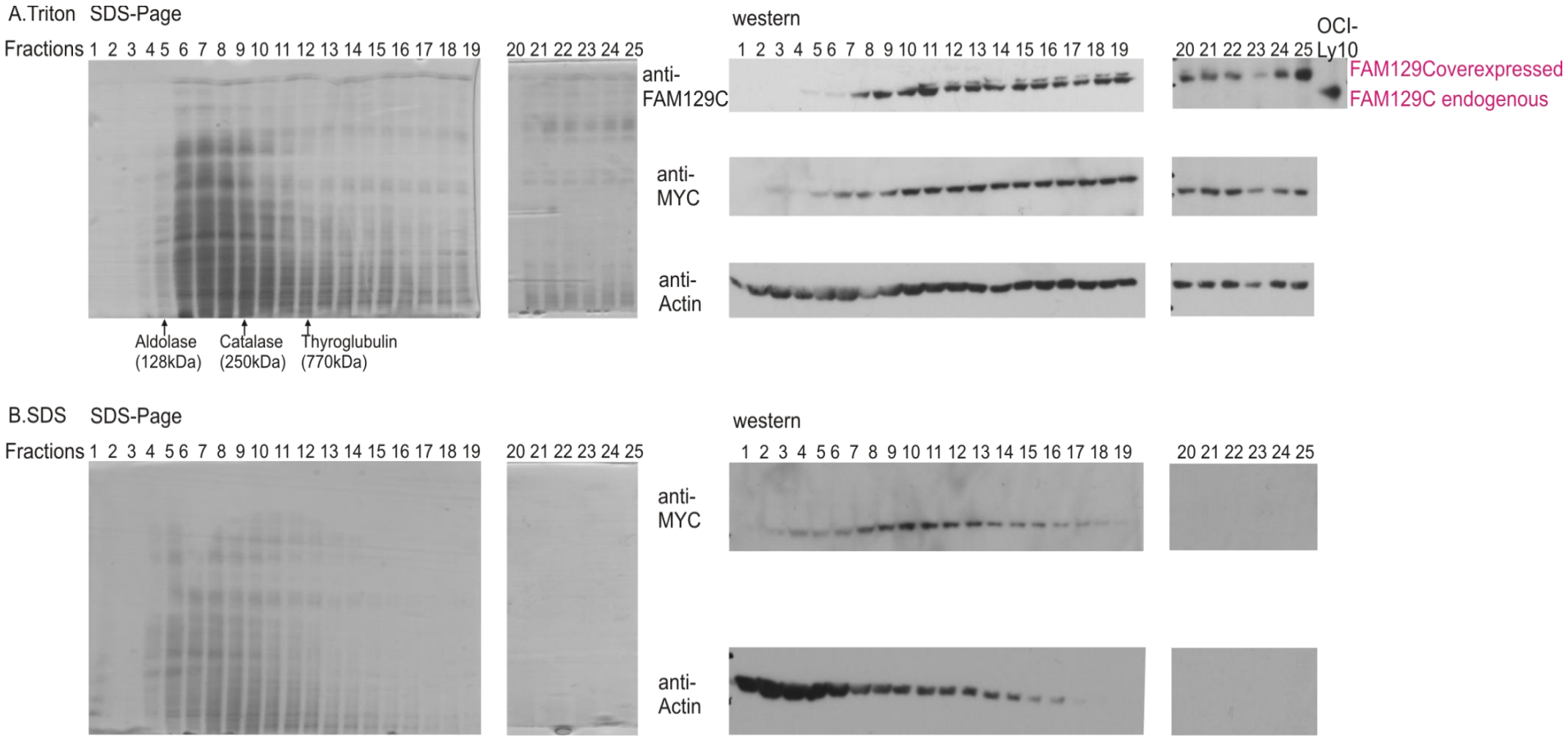


Figure 2.12 Comparison of protein distribution in sucrose fractionation of Triton v. SDS lysed cells.

Marker proteins Aldolase (128kDa), Catalase (250kDa) and Thyroglobulin (770kDa) identify MW in given fractions . A. In Triton treated cells the actin is distributed uniformly throughout all the fractions, whereas in B. protein in SDS lysed cells are localised in early fractions as shown in SDS-Page and in actin blots

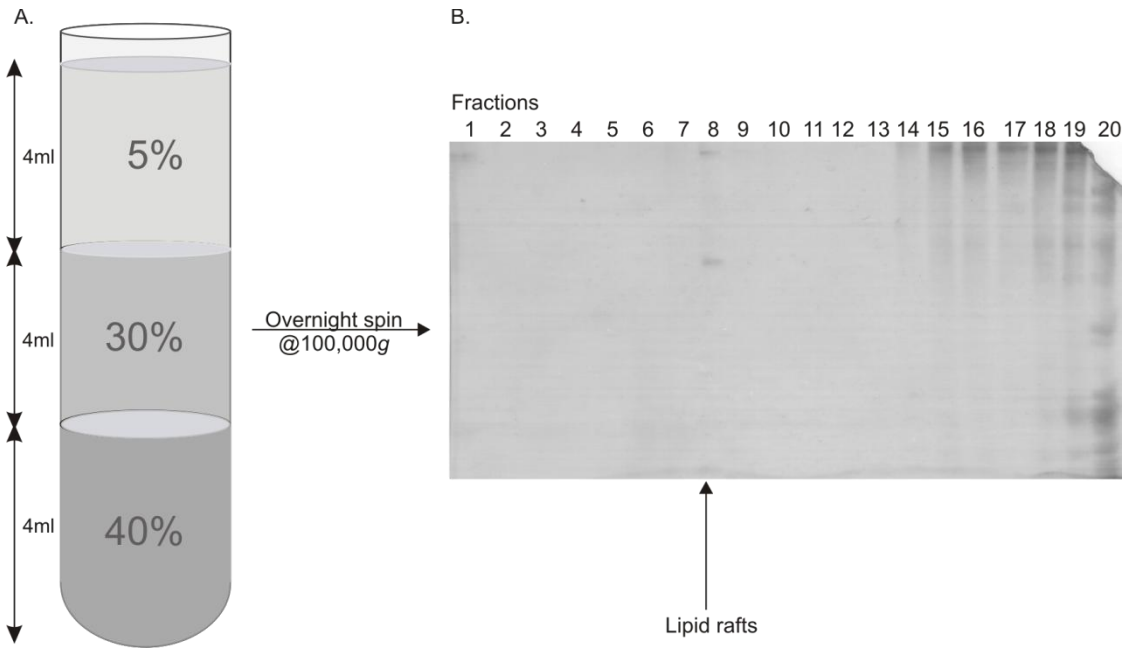


Figure 2.13 Lipid rafts (detergent resistant membrane , DRM) isolation.

A. Triton lysed cells were mixed with sucrose to make a 40% sucrose-lysate solution. 30% and 5% sucrose solutions were carefully overlaid as shown in the diagram. **B.** SDS Page gel, after 18h spin at 100, 000x*g* majority of the sample remained in fraction 15-20, lipid rafts were identified in fraction 8, as shown in the immunoblots in chapter 3, Figure 3.17.

2.5 Molecular Biology Protocols

2.5.1 Plasmids

pcDNA4 was obtained from Invitrogen (Paisley, UK). pGEX 4T.1 was obtained from Amersham Pharmacia Biotech, pCMV6NEO from Origine (Rockville, USA).

2.5.2 Bacterial Strains and Culture Conditions.

Escherichia Coli strains DH5-α and BL-21 (DE3) were obtained from Life Technologies Ltd. and were routinely grown and maintained on Luria agars containing the required antibiotic. For liquid cultures Luria-Bertani (LB) medium (10g bacto-tryptone, 5g bacto yeast extract, 10g NaCl in 1L sterile water) Anachem (Beds, UK) was used and bacteria were grown at 37 °C with shaking.

2.5.3 Transformation of *E.Coli*

DH5-α (subcloning efficiency cells 1×10^8 transformants/μg DNA) were used for routine transformations. For transformation of ligation mixes (2.4.11), library efficiency DH5α cells (1×10^8 transformants/μg DNA) (Life Technologies Ltd.) were used. All procedures

were carried out under aseptic conditions. DNA (200-500ng) was added to cells which were then incubated on ice for 30 minutes prior to heat shock (42 °C, 45 secs). After being allowed to recover on ice for 2 minutes, 1ml of SOC medium (Life Technologies Ltd.) was added and cells grown for 1h at 37 °C. Cells were then plated onto the appropriate selective medium and grown overnight at 37 °C.

2.5.4 Preparation of Plasmid DNA

Plasmid DNA was prepared using kits supplied by Qiagen Ltd. (Surrey, U.K). Kits used depended on the quantity or quality of DNA required. Generally, mini-preps kits provided sufficient quantities of DNA for everyday work (10-15 μg), and for general stocks, however for transfection experiments larger quantities were required and hence maxi-preps were carried out. All kits are based on a standard protocol (Sambrook J *et al.*, 2002). Bacteria are first subjected to alkaline lysis which denatures plasmid DNA and chromosomal DNA as well as cellular protein. Chromosomal DNA and proteins are then "salted out" and removed by centrifugation. Plasmid DNA is then collected from the lysate on a membrane and eluted using Milli-Q water.

2.5.5 Quantification of DNA

DNA was quantified by first diluting in Milli-Q water then measuring A_{260nm} using a DNA/RNA calculator (Amersham/Pharmacia Biotech). Double-stranded DNA (dsDNA) concentration was then calculated using the formula:

$$\text{dsDNA concentration (μg/ml)} = (A_{260} \times 100 \text{ (dilution)} \times 50)/1000$$

The purity of isolated DNA could be assessed using the A_{260nm}/A_{280nm} ratio. Plasmid DNA has an A_{260nm}/A_{280nm} ratio of 1.8.

2.5.6 DNA Electrophoresis on Agarose Gels

Purified and digested plasmid DNA was analysed using agarose gel electrophoresis essentially as described (Sambrook J *et al.*, 2002). Briefly, agarose (0.5-2% w/v) was dissolved by heating in TAE buffer (40mM Tris:Acetate (pH 8.5), 2mM EDTA) and after cooling to ~50 °C ethidium bromide 0.5μg/ml was added and the gel poured. DNA samples were prepared with 10× Orange G loading buffer (0.5% (w/v) Orange G, 25% (w/v) Ficoll-400, 20mM EDTA) in Milli-Q water and applied directly to wells in the gel. Gels were electrophoresed in TAE buffer at 100 V for 30-60 minutes.

2.5.7 Construct Generation

pcDNA4 FAM129C plasmids were constructed using standard molecular biology protocols (Sambrook J *et al.*, 2002).For the generation of Fam129C a construct containing the full sequence of FAM129C (NM_173544.2) with 5' UTR in CMV6Neo from Origine was purchased. The coding sequence was amplified by Polymerase Chain Reaction (PCR) as described in 2.4.9. using the following primers: upstream (5' CGC GGA TCC ACC ATG GGG CCT GAC CGG AAG GAG G 3') and downstream (5' CCG GAA TTC TTG TCT TGG GGT AGG CCG AAA 3') obtained from the Invitrogen (Paisley, UK). To facilitate cloning the upstream primer contained a BamH1 restriction site and the downstream an ECOR1 site. PCR product and pcDNA4 vector were cut with ECOR1 and BamH1 (NEB), following digestion the PCR and vector were ligated.

2.5.7.1 Polymerase Chain Reaction (PCR)

Amplification of DNA fragments by PCR was performed using *Pfx* Platinum® (Invitrogen) according to the manufacturer's instructions with the appropriate primers in a Perkin Elmer GeneAmp 9700 thermal cycler (Perkin Elmer, CA USA). All PCR reactions were gel purified before further manipulation.

2.5.7.2 Restriction Digestion of Plasmid DNA/PCR Products

Restriction enzymes and buffers were obtained from New England Biosciensce and used according to manufacturer's instructions. Digestions were generally set up and left for up to 16 h (37°C), depending on the amount of DNA and enzyme efficiency. Digested DNA was purified by gel electrophoresis followed by gel extraction using a Qiaquick™ gel extraction kit (Qiagen).

2.5.7.3 Ligation of Digested DNA Fragments

Gel-purified restriction-digested DNA with cohesive ends was ligated using T7 DNA ligase (Life Technologies Ltd.) according to the manufacturer's instructions. A number of ligation reactions were performed with differing vector: insert ratios. Ligation reactions were then incubated at 16°C overnight in a Perkin Elmer 480 DNA Thermal cycler. Control ligations containing vector only and vector with ligase were also set up to ensure that the vector had been effectively linearised by both restriction digests. Ligation reactions were then used to transform DH5α cells (*E.Coli* library efficiency) as described above. Plasmid DNA was purified from bacterial suspension using the miniprep system (Qiagen) as described in 2.5.4. The sequences of all constructs were verified before use (PNACL).

2.5.7.4 Large scale preparation of plasmid DNA by maxiprep

To obtain sufficient DNA to transfect cells, a maxiprep (Qiagen kit, as per manufacturer's instructions) purification was performed. Using a sterile tip, 100µl of bacterial suspension obtained from the miniprep (Qiagen kit, as per manufacture's instructions) were added to 250ml of liquid LB containing 50µg/ml ampicillin (contained in a 500ml conical tube), and allowed to grow at 37°C in a shaking incubator at 220rpm overnight. Bacterial cells were then pelleted by a 30min centrifugation at 9,000 rpm at 4°C. After removal of LB medium, plasmid DNA was purified with the Qiagen Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The DNA recovered from the maxi preparation was quantified using a spectrophotometer, by reading the absorbance at 260nm and 280nm. The quality of the DNA prepared was determined by the ratio between 260nm and 280nm absorbances, a ratio of 1:8 was judged to be acceptable.

2.5.7.5 Generation of FAM129C constructs

Production of FAM129C-overexpressing cell lines was challenging as there was:

1. No available FAM129C template, I searched for it in the primary cells.
2. Difficulties in transfecting B cells.

A summary of the cloning efforts are shown in Table 3.3. At the beginning of this project, there was no commercially available full length cDNA. The available IMAGE clones were for partial cDNA only. Initially, the cloning work was performed on the template received from Oxford Glycosience (OGS), where FAM129C was originally identified. This template was missing 27aa at the N terminus, as initially there were problems in identifying the first exon (Dr R. Boyd, personal communication).

Table 2.16 Generated constructs containing FAM129C

	Vector	Template	Problems
1.	CMV10 (C-terminal 3xFLAG) and CMV14 (N-terminal 3xFLAG	Oxford GlycoScience	Missing 27aa at the N terminus
2.	pcDNA3.1	CLL cDNA	Multiple artifacts: mutations and insertions
3.	pcDNA4 N-terminal epitope tags (TR system)	Origine cDNA clone	In frame, expressing insert but not expressing tags
4.	pcDNA4 C- terminal epitope tags (TR system)	Origine cDNA clone	None, used in this chapter

To clone the full length protein, cDNA from a CLL patient was used. It was the highest FAM129C expressing material available at the time. Five different patient samples were used. There were multiple artefacts in the cloning sequence and insertions, despite using proof reading *Taq* polymerase. In 2007 a full length cDNA (including non-coding region) became available from Origine. This was purchased and used as a template for further cloning attempts.

Different human B-cell lines produced variable results when transfected. The maximal transfection efficiency was 10% using nucleofection (Lonza AG, Cologne) and the best transfection efficiency was achieved in the BJAB cell line (DLBCL cell line) 50% efficiency. However, FAM129C expression was rapidly downregulated to undetectable mRNA levels and protein within 24h. (Figure 3.25) BJAB did not express endogenous FAM129C. I attempted to transfect Jurkat cells (T cells) and I failed to detect any expression of FAM129C. This suggests that FAM129C is degraded or expression is not sustained in FAM129C negative cells.

These pilot experiments suggested that stable expression in B cells may be only achievable in the tetracyline inducible systems, alternatively there could have been mRNA stability issue. To simulate the situation in B cells *in vivo* I chose cell lines with a small, detectable level of FAM129C expression: Mec1, Jeko, Ramos, Rec1, Tonue, Nalm6 and RCH-ACV. These cells have previously been stably transfected with the tetracycline repressor (TetR) gene (kind gift of Dr T. Akasaka).These cell lines were transfected by nucleofection, cultured with Zeocin antibiotic to select positive clones and single-cell clones established by limiting dilution. There were a number of clones generated. However, over extended periods of weeks the expression of FAM129C was lost. Even soon after transfection the FAM129C expression was weaker than observed in endogenous controls, despite strong CMV promoter regulating transcription.
The construct which was used in the following results section is a tetracycline repressor system in pcDNA4 with tags at the C terminus of the FAM129C.

2.5.8 Real time PCR

2.5.8.1 RNA Extraction

RNA was extracted from cells using the Trizol Reagent method. All benches were swabbed with 1%SDS/70% IMS to reduce the chances of RNase contamination. Trizol reagent (1ml) was added to $10x10^6$ cell pellets which were fully homogenised. The lysate was incubated for 5 minutes at ambient temperature before 200µl of chloroform

was added. The lysate was then incubated for a further 10 minutes at ambient temperature before being spun for 10 minutes at 13,000x*g* at 4^{o}C.

After centrifugation, the aqueous phase was transferred to a new 1.5ml microcentrifuge tube and 1ml of isopropanol added. The sample was mixed by gentle vortexing and then left to incubate for 10 minutes at ambient temperature. The samples were then spun at 13,200rpm for 20 minutes to pellet the RNA. After centrifugation the supernatant was removed and the pellet washed twice with 70% ethanol (2x1ml, centrifuged at 13,000rpm for 10 minutes). The remaining ethanol was completely removed by air drying the pellet in a laminar flow hood and the pellet resuspended in 40µl of RNase-free water. The extracted RNA was then quantitated as described in section 2.3.3.

Quantitation and Quality Control

Quantitation was carried out by the OD260 absorbance method, 1 OD unit was taken as 40µg of RNA. Two different UV-Vis spectrophotometers were used for the quantitation, a BioPhotometer (Eppendorf, Germany) and the NanoDrop ND-1000 (NanoDrop Technologies, USA).

Quality control was carried out by agarose gel electrophoresis and by calculation of the OD260/280 ratio, taking between 1.8 and 2.0 to be good quality for DNA and RNA.

2.5.8.2 Real time: Overview of the Process

Real time PCR is an accurate method for the quantification of gene expression. Although Microarray technology provides insight into the intricacies of gene expression changes it cannot provide the sensitivity of real time PCR. Real time PCR monitors the progress of a PCR reaction as it occurs using the detection of a fluorescent dye which increases in emission the more PCR product is present. This allows characterisation of samples by the point of time at which the reaction can be detected (the cycle threshold, CT) as opposed to the amount of target accumulated after a fixed number of cycles as in a Northern blot. The higher the copy number of a species of RNA the sooner an increase in fluorescence emission will be detected. Real time PCR was carried out using the ABI 7000 Sequence Detector and SYBR Green Dye (Applied Biosystems Inc.) which fluoresces when bound to double stranded DNA.

Primer Design

Primers were designed (Table 2.16) using Primer Express 2.0 Software (Applied Biosystems Inc.) so that they span an exon/exon boundary (to circumvent potential signal arising from gDNA contamination) and a melting temperature just below 60^{o}C.

Table 2.17 Real time PCR primers

	mer sequence
FAM129C forward	5'-CCTGAATCCTTGGGAGACCAT
FAM129C reverse	5'-GAACAGCGGGAAGCTCACA
TBP forward*	5'-CACGAACCACGGCACTGATT
TBP reverse	5'-TTTTCTTGCTGCCAGTCTGGAC

* Bieche I et al 1999. Clinical chemistry 45 (8) 1148- 1156

2.5.8.3 cDNA First Strand Synthesis

Prior to real time PCR the whole RNA sample was reverse transcribed to cDNA. It is the cDNA that is used as the template in the real time detector. To reverse transcribe the sample, 1µl whole RNA (100ng/µl) was added to 8.25µl First Strand Master Mix. The samples were denatured at 95^{o}C for 5mins and then cooled on ice to allow annealing of the random hexamer primers in the master mix.

After the sample had cooled, 0.25µl RNasin (Promega 1unit/µl) and 0.5µl Superscript III Reverse Transcriptase (Invitrogen) were added. The samples were then placed in an MJ Research DNA engine to be incubated at 23^{0}C for 10mins, 50^{0}C for 45mins, 99^{0}C for 10mins and then held at 4^{0}C.

2.5.8.4 SYBR Green Real Time PCR Reaction and Analysis

SYBR Green Real Time PCR reactions were carried out in triplicate for each sample and a non-template control was used to check for contamination. Reactions were set up in 96-well optically clear microtitre plates (Applied Biosystems Inc.) as per Table 2.17 to a final volume of 25µl.

Table 2.18 Components of the SYBR Green Real Time PCR Reaction.

	Reagent volume (µL)
SYBR Green PCR Master Mix (2x) (ABI)	12.50
Sense Primer (50µM)	0.15
Anti-Sense Primer (50µM)	0.15
cDNA Template from 2.5.8.3	1.00
PCR Grade Water	11.20
Total	**25.00**

The plates were then mixed by vortexing and spun down in a centrifuge before being loaded into the real time PCR machine. The real time PCR was run on the default thermal program for the detector (Table 2.18) with a dissociation protocol at 60^{0}C to check for the presence of non-specific amplification. PCR product was measured in real time by the increase in SYBR green fluorescence and the data were analysed using the Sequence Detector program (PE Biosystems, USA). To confirm amplification specificity, PCR products were subjected to melting curve analysis.

Analysis of the results was carried out using the ΔΔCT relative quantitation method (Livak & Schmittgen, 2001). It is a comparative method, where the CT (cycle threshold) values of the samples of interest are compared with CT values of a calibrator (control sample). Both CT values of the samples of interest and the calibrator are normalised to a housekeeping gene. The mean CT was calculated for each gene and normalised against the endogenous control gene, the TATA box binding protein (TBP). To ensure a correct choice of endogenous reference, TBP and the gene of interest FAM129C rates of amplification were assessed using sequentially diluted template. The amounts of cDNA from 0.0781ng to 10ng per reaction were tested and CT values were analysed. The results showed a parallel rate of amplification for both PCR products. Therefore the housekeeping gene encoding TBP and the target genes had compatible amplification efficiencies that allowed quantification of the gene expression by the ΔΔCT method.

I tested several cell lines in order to show that *FAM129C* expression is characteristic of B cells. Since the highest expression was expected to be found in B cells it was necessary to introduce a cell type which could serve as a calibrator (reference) to enable cross lineage comparison. The HRC57 cell line was selected for this purpose.

The HRC57 is a “normal” B-cell line immortalised with EBV (a kind gift from Dr Jude Fitzgibbon (CRUK, London) (Summers *et al.*, 2001)). *FAM129C* expression in HRC57 was detected reproducibly and used as value of 1. HRC57 cells provided a constant, uniform and simple evaluation standard for the normalisation of *FAM129C* expression. The relative expression of *FAM129C* in the investigated cells and in the calibrator (HRC57) was first normalised against the housekeeping *TBP* gene and then the ratio of *FAM129C* expression in the cells of interest and in the HRC57 cells was calculated. For consistency and internal quality control, I chose only tests which gave similar CT (cycle threshold) for cDNA values for the house keeping gene (arbitrarily I chosed cut off of CT=26.15 or less), i.e. similar expression of the TBP gene, as reflected by CT values. Therefore these samples had comparative amounts and quality of RNA. Primary haematopoietic cells were obtained from healthy volunteers and from haematological patients attending Leicester Royal Infirmary. The B cells were purified using $CD19^+$ magnetic beads or negative selection beads (both from Miltenyi Biotec), the purities of the pools from positive selection were 95-97%, negative purification 90-95%.

Table 2.19 ABI 7000 Sequence Detector thermal profile for SYBR Green Real Time PCR.

Time	Temperature	Role
2mins	50 ^{O}C	Initial Denature
10mins	95 ^{O}C	Annealing
For one cycle		
15s	95^{O}C	Denature
1min	60^{O}C	Anneal, Extend and Read
For 40 cycles		
15s	95^{O}C	Denature
20s	60^{O}C	Dissociation Protocol Read
15s	95^{O}C	For one cycle
For one cycle		

2.6 Statistical analysis

Statistical analysis was done with the help and advice of Dr C. Weston (Medical statistician) and were done using GraphPad Prism 5.0 and SPSS17 (SPSS Inc. Chicago, Illinois, USA). Significance between proportions was tested using the Fisher exact (for small samples) test or χ^2 test (for large samples). For data with Gaussian distribution, the unpaired t-test (for two groups of variables) and one way ANOVA (for more than two groups of variables) were used. For the non-parametric data the Mann Whitney (for two groups of variables) or Kruskal Wallis test (for more than two groups of variables) were employed. The survival analysis and Kaplan Meier graphs were generated using the log-rank method in the form of the Mantel-Cox test. The Cox regression analysis was performed using SPSS software. All tests were two-tailed and the significance of $p<0.05$ was assessed as significant.

Time to first treatment (TTFT) was defined as the time from diagnosis to the first treatment or death. TTFT can be used synonymously to progression free survival (PFS). Almost all patients who progressed required therapy and only a small number of patients (<5) would not be included by TTFT but counted if PFS was used, these are the patients who progressed, but were not fit for any therapy and were offered palliative care. Overall survival (OS) is the time from diagnosis to death. In treated patients 'time to the first treatment' (TTFT) is the time from diagnosis to the first treatment and PFS in these patients was the time form the first treatment to the subsequent treatment or death. OS, TTFT and lymphocyte doubling time (LDT) was analysed using the Kaplan-Meier method. Patients were censored (i.e. assigned 0) at the last update of the database (May 2009), for OS when patients were still alive, for TTFT if treatment or

death had not occurred. (LDT) was the time defined in months within which lymphocyte count doubled.

CD38 staining was performed on frozen samples by collaborators in Bournemouth. The gating was set up by CD2-PE, CD5-FITC, CD19-PE-Cy5, the test is run on CD38-PE, CD5-FITC, CD19-PE-Cy5. CD38 positive cells were defined as >30% of mean fluorescence intensity by flow, negative as<30%.

The significant difficulties in analysis of the data were missing data, especially when doing multivariate analysis. Table 2.19 shows available numbers in each category.

Table 2.20 Clinical information and number of patients with available data.

Information collected	No of cases	% of cases
Total number of patients	496	
Male	321	65
Female	175	35
Age	457	92
WCC	466	94
Lymphadenopathy	425	86
Stage	471	95
Ethnicity	496	100
Other cancers assessment	468	94
Autoimmunity	468	94
Treatment status	469	95
LDT	346	70
Mutation status	418	84
Cytogenetics	330	67
β2M	212	43
CD38	375	76
TTFT	468	94
OS	468	94

Chapter 3: Characterisation of Novel B Cell Specific Protein FAM129C

3.1 Introduction

A key aim in leukaemia research is to identify and characterise new targets with therapeutic, diagnostic and prognostic value. Proteomics screening conducted by our group on leukaemic cells offers the opportunity of identifying new molecules and this approach has been used to study the plasma membrane proteome isolated from B cells obtained from MCL and CLL patients (Boyd *et al.*, 2003; Boyd *et al.*, 2008; Boyd *et al.*, 2010).

Using this shotgun proteomic approach many proteins of either unknown functions and/or aberrant subcellular localisation in B cells have been identified from CLL and/or MCL membrane preperations including FAM129C/BCNP1, MIG2b, EVI2b (Boyd *et al.*, 2003) as well as Oxytocinase, from MCL: HVCN1, TRPV2, Raftilin and FAM3C. So far fully characterised are HCVN1, Raftilin and MIG2b (Boyd *et al.*, 2009; Meves *et al.*, 2009; Capasso *et al.*, 2010). The functions of HCVN!, the voltage proton channelin B cell have been partially elucidated (Capasso *et al.*, 2010)

In this chapter I describe some preliminary screening, functional and biochemical studies on FAM129C/BCNP1, a protein of unknown functions, identified by proteomic screening of purified CLL plasma membranes (Boyd *et al.*, 2003). Initially termed *BCNP1* (Bcell novel protein 1), this gene has subsequently been renamed *FAM129C* by the Human Genome Nomenclature committee as there are three related genes, which presumably share some common functions.Initial quantitative mRNA expression studies on FAM129C showed it to be upregulated in CLL patients and other lymphoid malignancies in comparison to normal B cells (peripheral blood, spleen, and tonsil). This expression profile and the fact that the protein was initially thought to harbour three transmembrane domains, suggested that FAM129C might have a, diagnostic/prognostic and possible therapeutic role in CLL and other B-cell malignancies.

To begin to understand the potential role of FAM129C in B cells it was necessary to establish the expression of FAM129C throughout the B-cell life cycle, starting from the common lymphoid progenitor, through antigen-independent development in the bone marrow to antigen-dependent selection in lymphoid tissue, and finally undergoing selection in the germinal centre (GC) to produce a memory B cell or plasma cell (Figure 3.1).

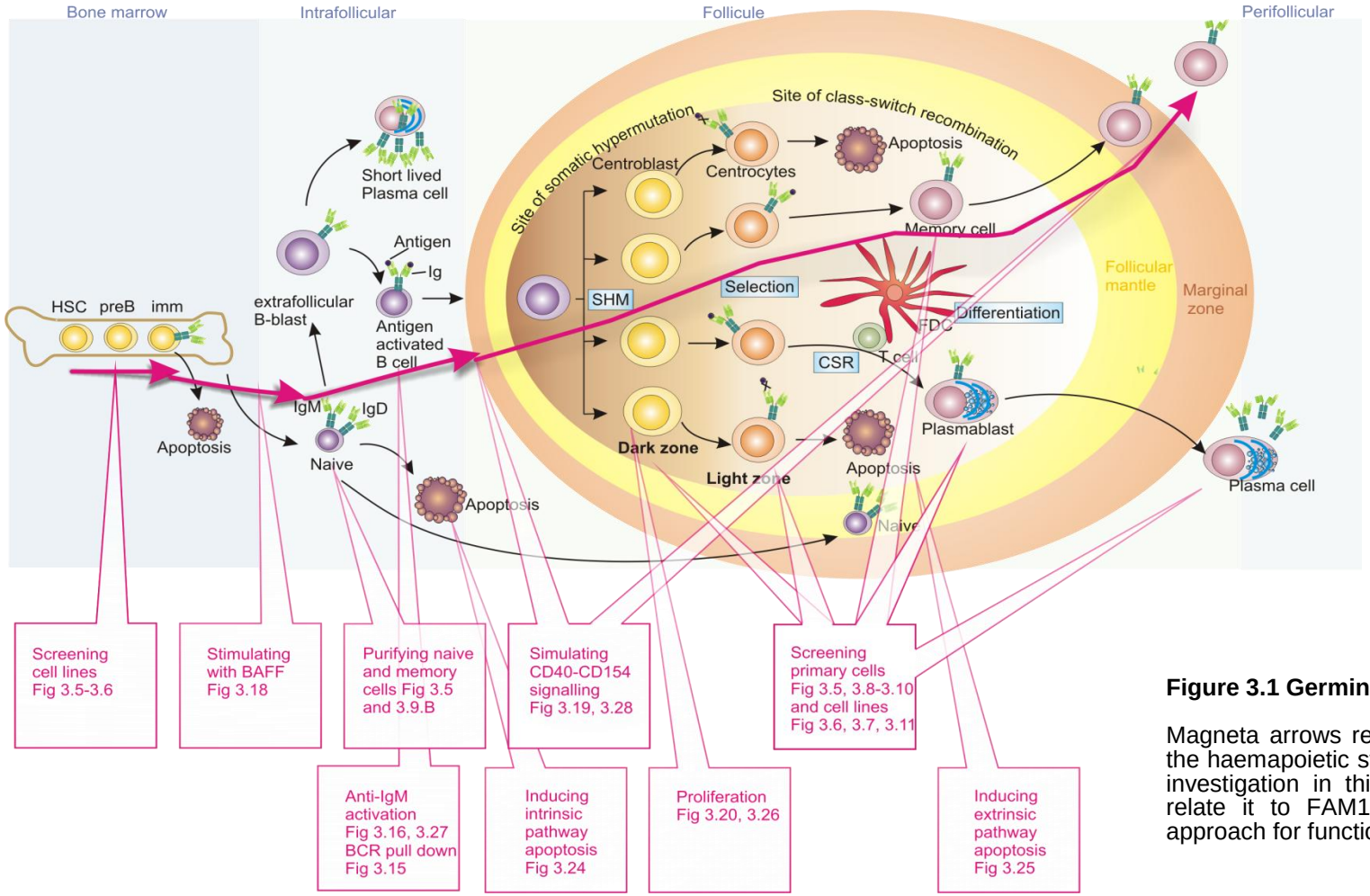
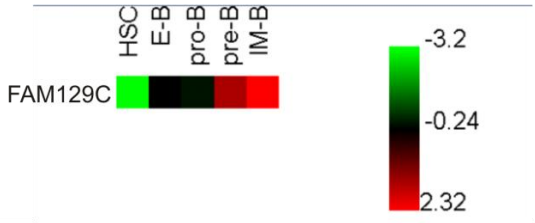


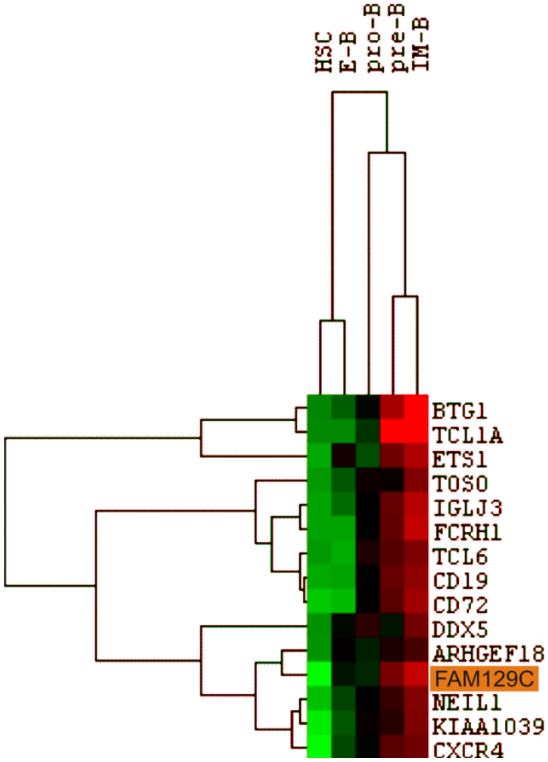
Figure 3.1 Germinal centre and FAM129C.

Magneta arrows represent the “journey” of a B cell from the haemapoietic stem cell to the memory cell. My plan of investigation in this thesis is to track this journey and relate it to FAM129C. Boxes track the investigative approach for functional characterisation.



GeneSymbol	Gene expression (median)				
	HSC	E-B	pro-B	pre-B	IM-B
FAM129C	-3.20	-0.24	-0.46	1.43	2.32
Fold Change	0.108517528	0.844547224	0.72663361	2.696335463	4.982949662
Abs Fold Change	9.215101139	1.184066411	1.3762094	2.696335463	4.982949662

Figure 3.2 FAM129C and B cell development.
Pictorial analysis of the early stages of human B cell gene expression profiling (data analysed from Hystad, 2007), FAM129C appeared in pre-B cell stage.



Gene	Function
BTG1, ETS1	1-positive regulation of cell differentiation 2-negative regulation of cell proliferation 3-transcription factor binding
BTG1, DDX5	cell growth
CD72, ETS1	immune response

Figure 3.3 FAM129C and B cell development.
Pictorial analysis of the early stages of human B cell gene expression profiling (data Hystad, 2007), FAM129C and other genes which appeared in pre-B cell stage.

Recently, a number of microarray studies were published and gave an insight into FAM129C during B-cell development and differentiation. The Smeland group demonstrated that FAM129C is observed in pre-B cells (Hystad *et al.*, 2007) (Figure 3.2 and 3.3) Secondly analysis of the Rosenwald *et al.* microarrays showed it is highly correlated in ABC DLBCL (Rosenwald *et al.*, 2002). The nature of ABC DLBCL precursors' is not well understood and and may represent memory B cells (Seifert & Kuppers, 2009). In contrast, FAM129C is downregulated in lymphocyte predominant Hodgkin lymphoma (Brune *et al.*, 2008) a malignancy thought to originate from late germinal centre B cells differentiating towards memory B cells.

In order to characterise FAM129C and to begin to understand its role in B-cell development and in differentiation, I have used a number of approaches. Firstly, bioinformatic analysis was used to investigate the predicted protein structure and relationship between the other family members, FAM129A and FAM129B. Secondly, I studied FAM129C expression during B-cell development in primary malignant cells and in derived malignant cell lines. I also sought to characterise the biochemical and molecular functions of FAM129C in order to identify a possible signalling roles in B cells. Specifically, the following experiments were performed:

1. Levels of FAM129C mRNA and protein expression were determined in primary normal B cell populations and in malignant cells and leukaemic cell lines, representing distinct stages of B-cell development.

2. The other stages of B-cell differentiation were replicated by stimulating normal and malignant B-cells as shown in Figure 3.4, namely,

    a) reproducing antigen-dependant B-cell selection by activation with anti-IgM, i.e. simulating activation of B cell with antigen before entering GC;
    b) mimicking T cell-B cell interaction in GC by using CD154 transfected feeder-layer;
    c) treating cells with BAFF, which facilitates production and maintenance of follicular and marginal zone B cells.
    d) Apoptosis induced by FasL is important in GC selection and therefore another important pathway involved in the B-cell life cycle.

Levels of FAM129C were determined following each stimulus.

In the second part of this chapter I present a preliminary functional characterisation of FAM129C and attempts to identify potential FAM129C binding partners.

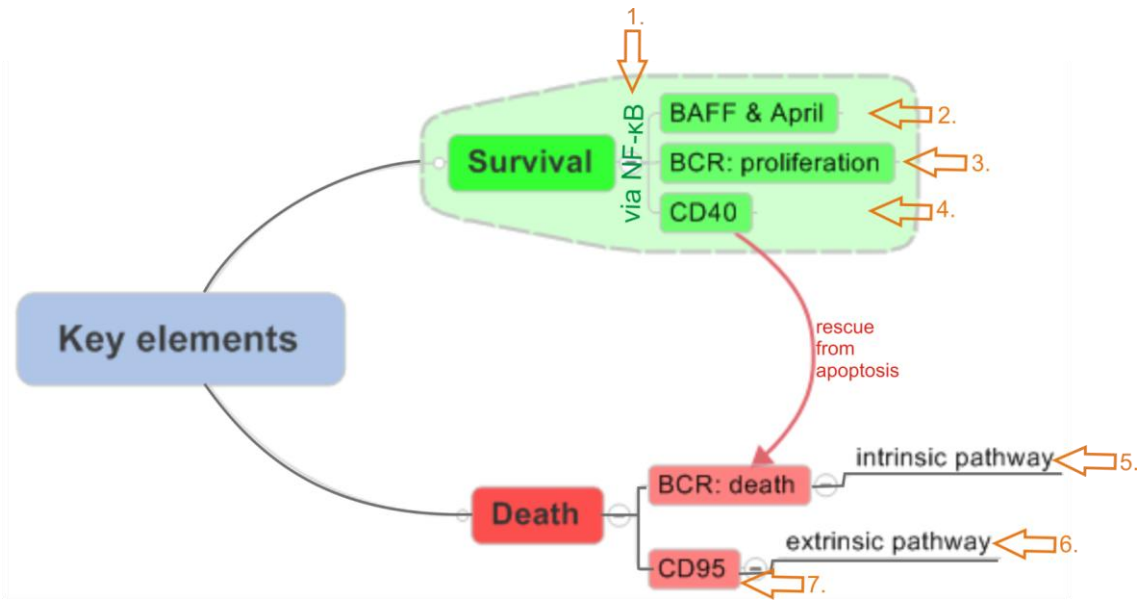


Figure 3.4 Investigating 'Key elements' in B cell homeostasis.

The key signalling pathways in survival are: BCR, BAFF and CD40 signalling and they all utilise the NF-κB dependent pathway (1). The key death signals are BCR (only in early B cell stages up to T1 stage of B-cell development) and Fas (CD95). CD40 signalling is able to rescue cells from apoptosis (red arrow). Orange arrows mark corresponding sections in this chapter (2-chapter3.3.2.4; 3- 3.3.2.2; 4-3.3.2.5; 5-3.3.4.1 (Figure 3.24); 6-Figure 3.25).

.

3.2 Results: expression of FAM129C

3.2.1 Transcription of *FAM129C* gene

To assess transcription of *FAM129C* relative quantitative RT-PCR (RT-qPCR) was used (using SYBR® Green). Primers for *FAM129C* were designed across exon-exon boundaries to amplify the common (5'UTR) sequence of all known isoforms. TATA box binding protein (*TBP*) gene expression was used as the internal reference as TBP expression is consistently high in B cells (Lossos *et al.*, 2003).

The mRNA expression data is presented in Figures 3.3-3.7. Figure 3.5 shows levels of *FAM129C* RNA expression in peripheral B cells derived from five different healthy volunteers. The range of expression is 100-132 with the mean of 114.7. in comparison with the EBV transformed normal B-cell line HRC57 (standardised value of 1.0).

B cell populations in peripheral blood consist of 60% naïve B cells and 40% memory B cells (Klein *et al.*, 1998), I therefore purified peripheral B cells further from a normal donor to $CD27^+$ (memory) and $CD27^-$ (naïve) fractions. *FAM129C* expression in $CD27^-$ was 3.5-fold higher than in $CD27^+$ cells (Figure 3.5).

Other primary haemopoietic cells express low levels of FAM129C in comparison to B cells. Figure 3.5 shows expression in other compartments of haemopoietic lineage: dendritic cells ($CD304^+$), myeloid ($CD14^+$), T cells ($CD3^+$) and fraction of cells $CD19^-$, $CD3^-$ and $CD14^-$ (this pool of cells consists mainly of NK cells). The expression levels bserved in purified plasmacytoid dendritic cells (purified using $CD304^+$ magnetic beads), was low. In addition I obtained immature and mature myeloid dendritic cells; immature myeloid dendritic cells were cultured from $CD14^+$ purified cells incubated with IL4, GM-CSF and mature dendritic cells by maturation of immature myeloid dendritic cells with GM-CSF, PGE2 and TNF-α. Low levels of expression in both myeloid dendritic cells were similar to the plasmacytoid dendritic cells. Similarly, the levels in $CD14^+$ and $CD3^+$ were low. Interestingly, the levels in the $CD19^-$ $CD3^-$ $CD14^-$ pool were higher indicating that NK cells express FAM129C albeit at lower levels than in B-cells (rightmost column, this experiment was only performed on the one occasion).

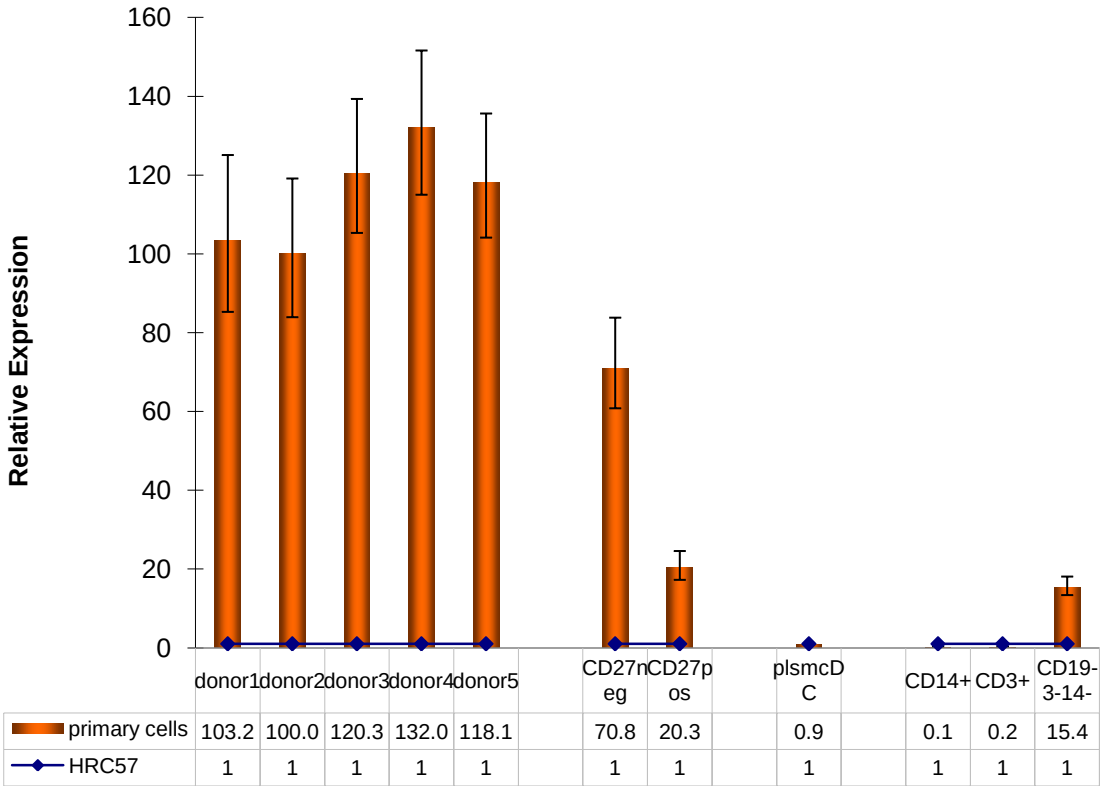
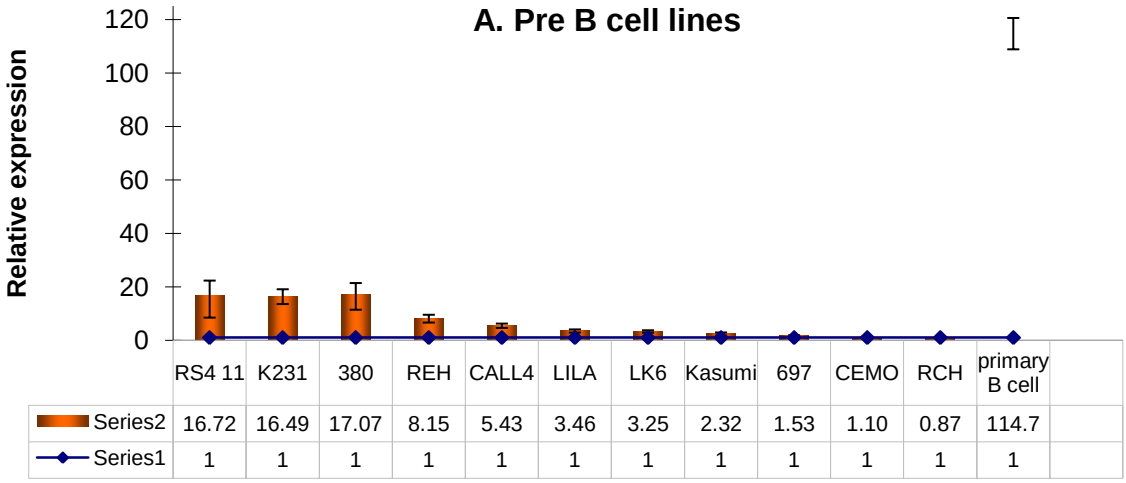
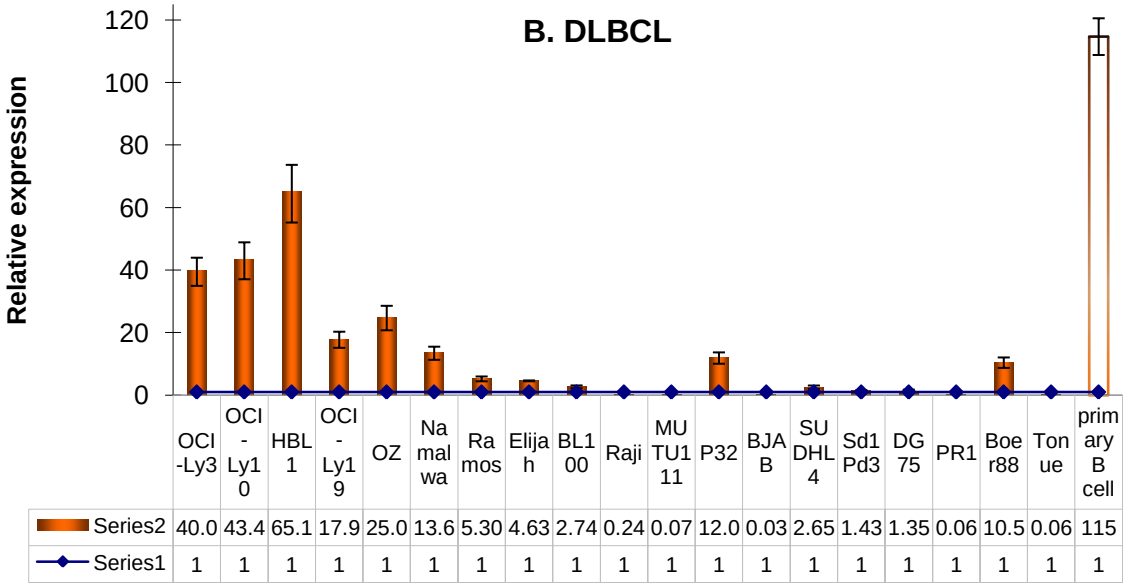


Figure 3.5 Relative expression in primary haematopoietic cells.

The columns represent mean values, error bars: standard error of the mean(SEM). Donor1-4 is mRNA extracted from CD19 positive purified cells from healthy volunteers (donor 1: male 50 years old, 2: male 60, 3: male 30, 4: male 40, 5: male 29), CD27: B cells further purified into CD27 positive and negative fraction; plDC: plasmacytic dendritic cells (CD304 positive). The last column represents CD19- CD3- CD14- fraction, which is mainly composed by NK cells All presented fractions were purified using Miltenyi Biotec beads from blood obtained from healthy volunteers.

FAM129C RNA expression levels were determined in a panel of haemopoietic cell lines, representing different lineages at different stages of differentiation. *FAM129C* was expressed only in a subset of derived malignant B-cell lines (Figure 3.6) (see Chapter 2.1.2 for the list of cell lines used in these studies) and was not detected in multiple myeloma, T cell, myeloid and non-haematopoietic cell lines. (data not shown). The levels of *FAM129C* in B-cell lines varied markedly and were much lower than those observed in either normal B-cell levels or CLL cells (see below). The B cell lines expressing *FAM129C* were a subset of B-cell precursor ALL cell lines (Figure 3.6..A) and activated diffuse large B-cell lines (ABC DLBCL) (Figure 3.6.C: OCI-Ly3, OCI-Ly10, HBL1). The former expressed relatively low levels of *FAM129C*, whereas ABC DLBCL showed a higher level (c.f. hollow bar Figure 3.6). Other groups showed similar data with the high levels of FAM129C in ABC DLBCL (Figure 3.7).





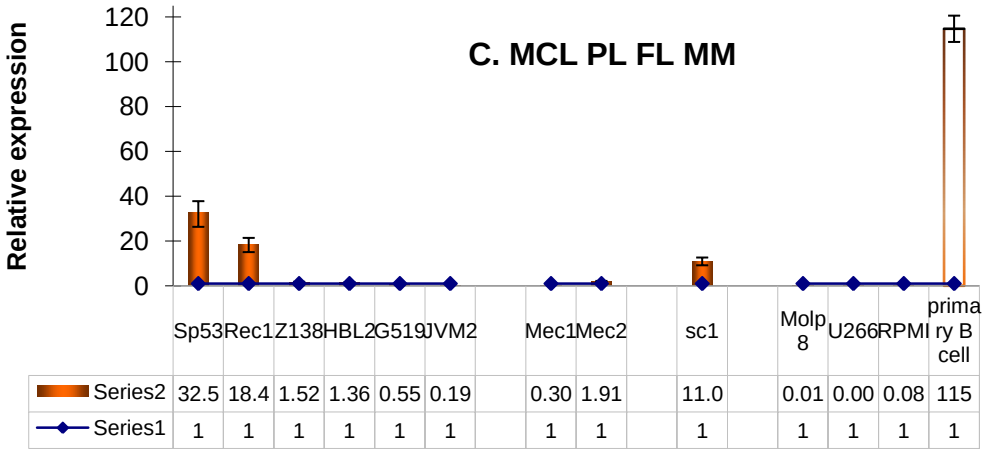


Figure 3.6 FAM129C mRNA expression in B cell line
Panel A presents examples of tested pre-B cell lines, B: DLBCL and C: examples cell lines derived from mature B cells (MCL, PL, FL, MM)
DLBCL-diffuse large B cell line, MCL-mantle cell lines, PL-prolymphocytic cell line, FL-Follicular lymphoma cell line, MM-multiple myeloma cell line. Ly3: OCI-Ly3; Ly10: OCI-Ly10; Ly19: OCI-Ly19, M111: MUTU111; G519: Granta519. Data tables show relative values. Hollow bar represents expression in primary B cells.

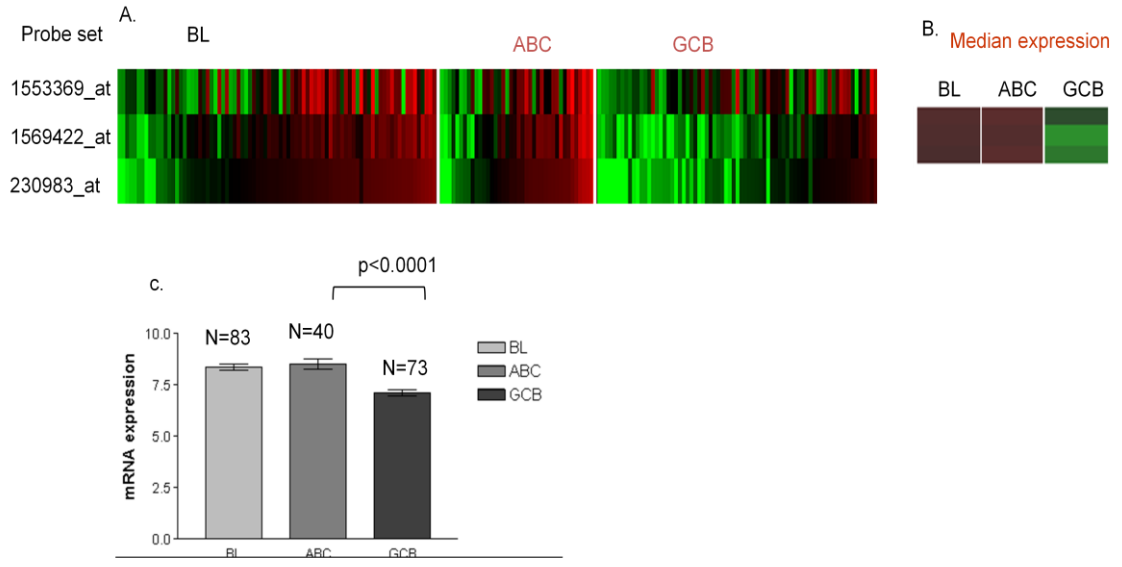


Figure 3.7 Expression in aggressive B-cell lymphoma.

GC shows significantly lower expression of the FAM129C compared to ABC-DLBCL or BL; BL: Burkitt Lymphoma; ABC: activated B-cell like DLBCL; GC: germinal center B-cell lymphoma; A. difference in the microarray expression using three probe sets (1553369_at1569422_at; 230983_at); B. Median mRNA expression; C. Comparison of mRNA expression presented by histograms The figure and data curtesy of Dr J. Iqbal. (from Lenz paper N Engl J Med. 2008 Nov 27;359(22):2313-23)

Next, I investigated the expression levels in the primary haematological malignancies, mainly in CLL , (see Figure 3.8). Figure 3.8 shows varied expression levels in the CLL patients ranging from 0.93 to 102.3 with the mean of 32.9. in comparison with the HRC57 cell line. There was therefore considerable heterogeneity in the levels of expression of FAM129C in CLL (Figure 3.8).

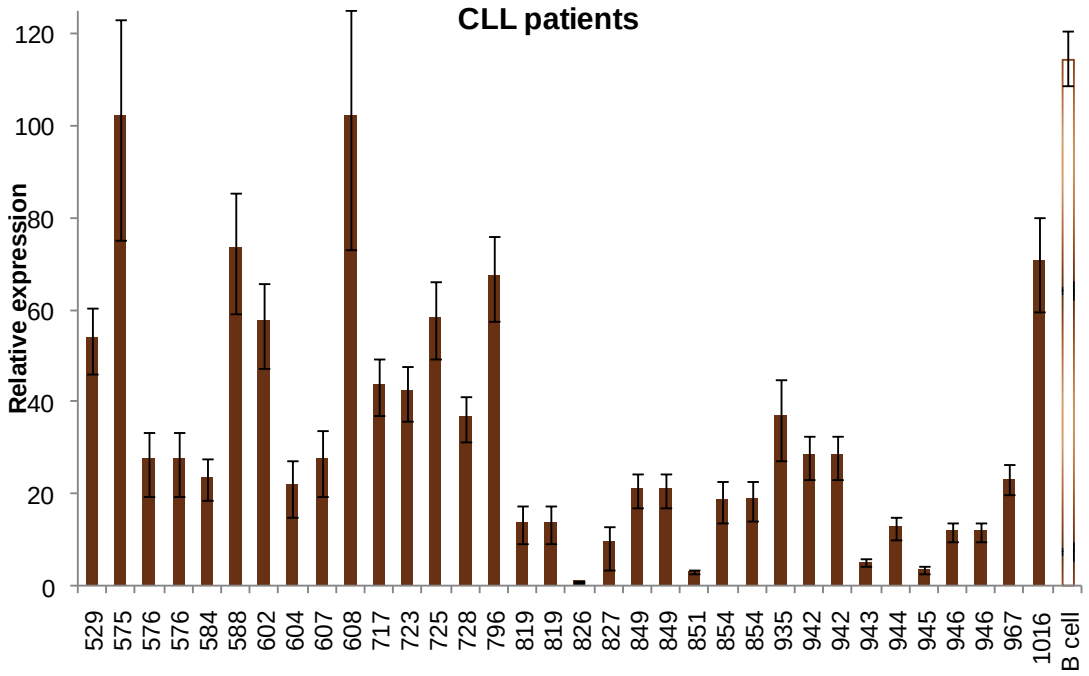


Figure 3.8 The range of mRNA expression levels in CLL patients.
Example of mRNA expression of CLL patients compared to normal primary B cells (hollow bar represents expression in primary B cells).

I attempted to identify correlations between the expression levels of *FAM129C* and clinical or molecular parameters. A statistical analysis using Prism v.5 (Graphpad) was undertaken. There were no significant associations with gender of the patient, *IGHV* status or *IGHV* family, stage of disease, treatment or progression of the disease and *FAM129C* levels (data not shown).

In conclusion, these mRNA studies taken in conjunction with publicly available gene expression data show that *FAM129C* expression is B cell specific and expressed at high levels in normal B cells. Further, naïve B cells have higher levels than memory B cells. None of the cell lines tested showed comparable levels to normal B cell levels and the highest expressing cell lines originated from ABC DLBCL. Levels in CLL cases were heterogenous but did not correlate with any of the commonly utilised prognostic

markers. Most importantly there was no correlation with *IGHV* mutation status, therefore it indicates lack of involvement of FAM129C in BCR signalling.

3.2.2 Protein expression

To confirm the RNA experiments I sought to determine FAM129C protein expression levels using multiple different antisera, which I attempted to generate. As outlined in Materials and Methods chapter, genuinely FAM129C-specific antisera and antibodies proved impossible to obtain and there remains an urgent need for such reagents. For the experiments described in this section, I used the most specific reagent, an affinity-purified antiserum 15, polyclonal rabit raised against peptides (Figure 2.4).

Protein expression broadly confirmed the results seen in the mRNA expression profiles (see 3.2.1). FAM129C protein expression was B cell specific (Figure 3.9), highest expression was detected in normal primary B cells (personal observation). FAM129C expression was absent in myeloid, T cell lineage, Hodgkin disease (HDLM2, L591) and in non-haematopoietic cell lines (data not shown). A panel of CLL patients was tested (Figure 3.10) and the levels of FAM129C protein appear to be uniform in this condition.

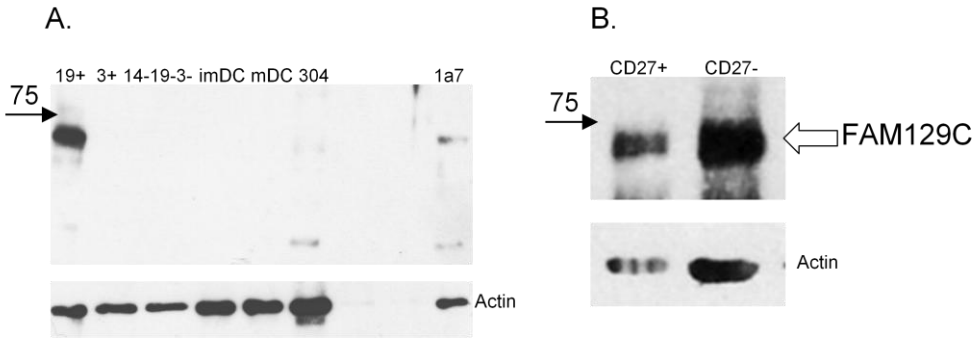


Figure 3.9 FAM129C expression in primary cells.
A. There is strong expression in primary B cells, there is very weak band in plasmacytoid DC (CD304),19+: primary B cells purified with CD19 beads; 3+: CD3 purified T cells; 14-19-3-, cells which are negative for CD14, CD3, CD19: NK cells; dendritic cells; 304: CD304 purified plasmacytoid dendritic cells, 1a7:positive control; Raji transfected with FAM129C, **B.** Naïve versus Memory cells: band <50kDa is equally represented in both types of the cells, CD27negative cells (naïve) have higher expression to that seen in CD27 positive (positive). These data confirm earlier presented mRNA expression data in Figure 3.5. Both blots were probed with Covalab1

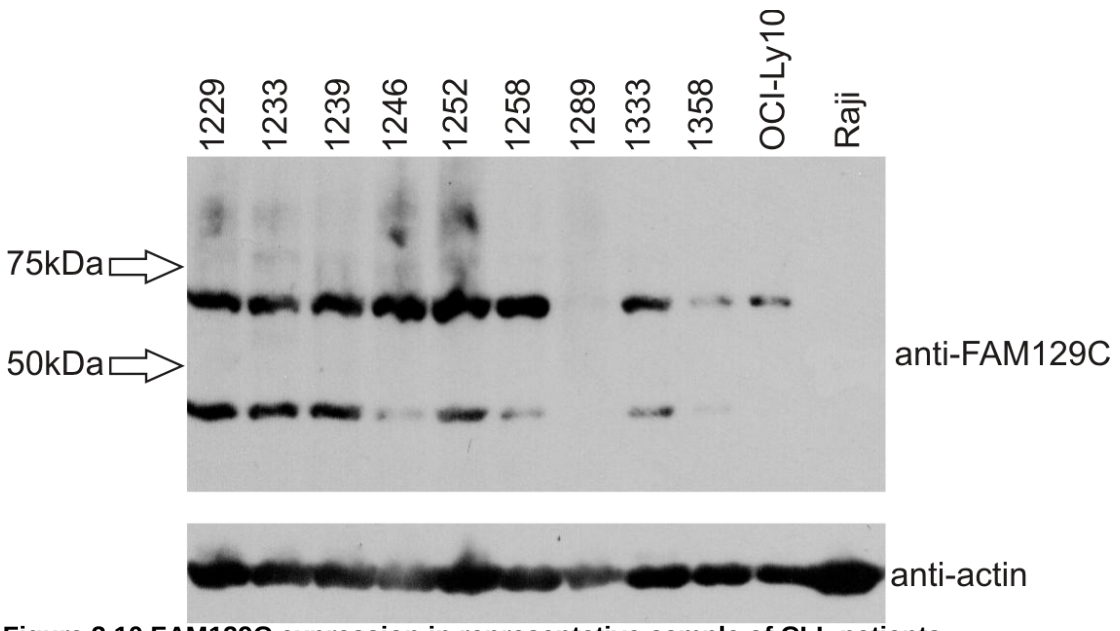


Figure 3.10 FAM129C expression in representative sample of CLL patients.
Numbers represent patient samples; LY10: OCI Ly10 cell line, positive control; Raji: negative control. FAM129C band runs<75kDa. NB patient 1289 does not express either of the bands. This patient had stable, low level CLL and had developed acute lymphocytic leukaemia (ALL) the lack of expression in this patient is explained by the vast majority of cells in this samples are ALL . Loading was normalised to protein concentration, probed with Antibody 15.

FAM129C protein was detected in only a few B-cell lines and at lower levels than in primary B cells. It was observed that the highest protein levels in cell lines were present in ABC DLBCL cell lines (OCI-Ly3, OCI-Ly10, HBL1, RIVA, NUDUL, MD903, SUDHL9) and in some ALL cell lines (CALL4, 380, CEMO, Kasumi, Mutz5, REH-ACV, 697, Nalm6, Per366, Per377). This pattern corresponded to the results found during the investigation of mRNA expression (Chapter 3.2.1).

A large number of cell lines were screened, the summary of these results are shown Table 3.1 and the representative WB shown in Figure 3.11. FAM129C appeared to correlate with IRF4/MUM1 expression: seven ABC DLBCL cell lines were positive for FAM129C (marked in red in Figure 3.11). A further three cell lines, MD901, Nappi and SD1PD3, were positive for IRF4 and FAM129C and therefore are likely to be ABC DLBCL derived. It is therefore likely that FAM129C could be used as a potential marker for ABC DLBCL.

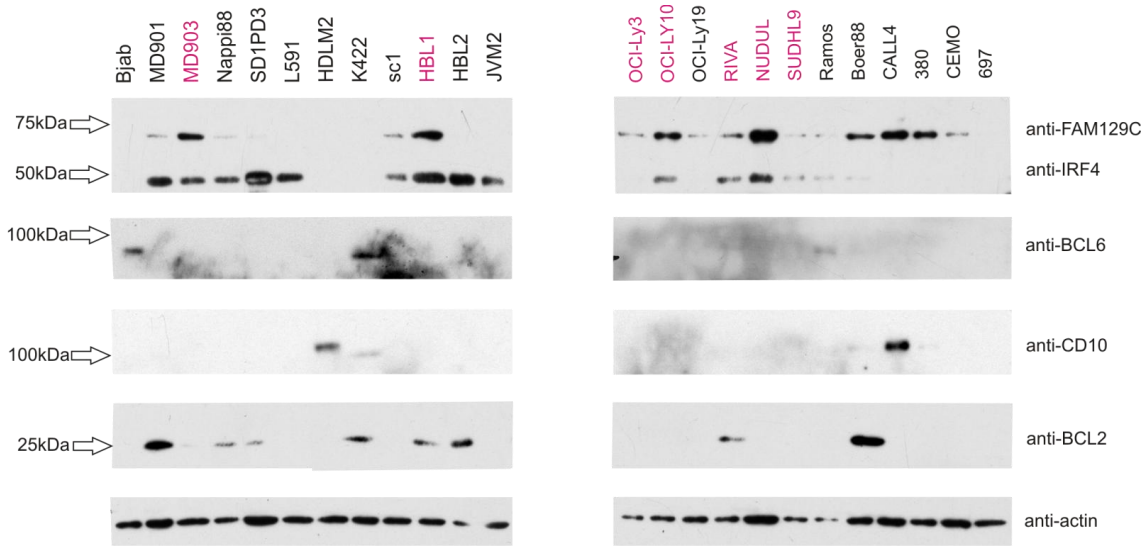


Figure 3.11 Screen of cell lines to identify ABC DLBCL according to Hans' algorithm.

BCL-2 identifies aggressive forms of ABC DLBCL and was introduced in modified Hans' protocol described by Muris (c.f. Introduction). Cell lines in red are ABC DLBCL origin. The results of cell line screening were summarised in Table 5.2

Table 3.1 Cell lines screened according to the Hans' algorithm

cell lines	origin	FAM129C	IRF4	BCL6	CD10	BCL2	RNA
377	ALL	+	-	-	-	nd	nd
380	ALL	++	w	-	+++	-	17
697	ALL	w	w	-	+	-	2
CALL4	ALL	++	w	-	++++	-	5
CEMO	ALL	w	w	-	+	-	1
Kasumi	ALL	-	-	-	+++	nd	2
LILA	ALL	-	-	-	-	nd	3
Mutz5	ALL	+	-	-	+++	nd	nd
Mutz111	ALL	-	-	-	-	nd	nd
REH	ALL	+	-	-	++++	nd	8
RS(4;11)	ALL	w	w	-	-	nd	17
SEM	ALL	-	-	-	-	nd	nd
BJAB	BL	-	-	+	-	-	0.03
Daudi	BL	-	-	-	+	nd	nd
DG75	BL	-	-	-	++	nd	1
Elijah	BL	-	-	-	w	nd	5
Raji	BL	-	-	-	-	nd	0.2
Ramos	BL	+	+	+	-	-	5
sc1	FL	w	+	-	++	-	11
G518	MCL	-	-	-	-	nd	nd
HBL2	MCL	w	+	w	-	+	1
JVM2	MCL	-	+	w	-	-	0.2
Sp53	MCL	-	-	-	-	nd	33
Z138	MCL	-	-	-	w	+	2
HDML2	HD	-	-	-	+	-	nd
KMH2	HD	-	+	-	-	nd	nd
L591	HD	-	+	-	-	-	nd

HBL1	ABC DLBCL	+	+	-	-	+	65
NUDUL	ABC DLBCL	++++	++	-	-	-	nd
OCI-Ly3	ABC DLBCL	+	+	-	-	-	40
OCI-Ly10	ABC DLBCL	++	++	-	-	-	43
RIVA	ABC DLBCL	+	+	-	-	+	nd
SUDHL9	ABC DLBCL	+	+	-	-	-	nd
Boer88	GC DLBCL	+	+	+	w	++	11
OCI-Ly19	GC DLBCL	w	-	-	-	-	18
PR1	GC DLBCL	-	-	w	w	nd	0.06
SUDHL4	GC DLBCL	-	-	-	-	nd	3
RCK8	Mediastinal NHL	-	+	-	-	-	0.03
DB	DLBCL	-	-	-	-	nd	nd
K422	DLBCL	-	-	+	+	+	nd
MD901	DLBCL	w	+	-	-	++	nd
MD903	DLBCL	+	w	-	-	-	nd
Nappi	DLBCL	w	++	-	-	+	nd
SD1PD3	DLBCL-EBV trans	w	++	-	-	+	1.43
Tonue	DLBCL	-	-	-	-	nd	0.06

++++levels of protein expression; w-weak expression; nd-not done. Orange highlighted cell lines are the candidates for ABC DLBCL. ALL: acute lymphocytic leukaemia; DLBCL: diffuse large B cell lymphoma (unspecified); MCL: mantle cell lymphoma; HD: Hodgkin disease, GC germinal centre. Table represent summary of the cell line screening by immunoblotting, example of which is shown in Figure 3.11. RNA levels presented as relative values, as in Figure 3.3.

3.2.3 Subcellular localisation of FAM129C.

3.2.3.1 Immunofluorescence

None of the available antibodies were suitable for immunohistochemistry (IHC). In sections of normal tonsils, Antibody 15 strongly stained non-specifically a small number of plasma cells (data not shown). This is a well recognised non-specific binding staining pattern found in IHC (personal communication, Dr K. West).

The generation of FAM129C-overexpressing cell lines enabled the study of cellular localisation using antibodies to the tag (MYC) and confocal microscopy (Figure 3.10). The staining appeared to be predominantly cytoplasmic, but there was also weaker plasma membrane staining present, this was further confirmed by biochemical fractionation (not shown).

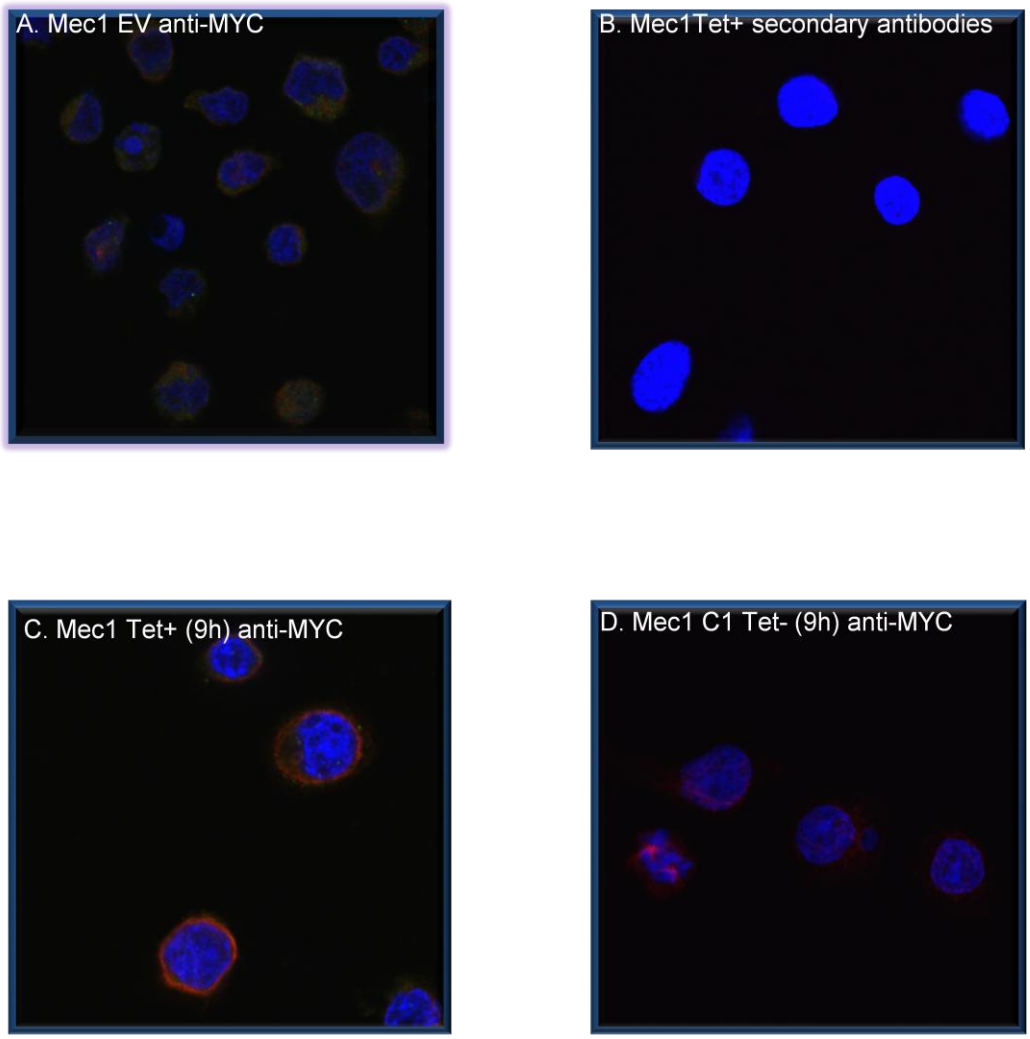


Figure 3.12 Immunoflourescence showing induced and un-induced clone Mec1 using anti-MYC antibody.
EV: empty vector, Tet+: induced with tetracycline; Tet-: uninduced; MYC tag; magnification x63. A &B control slides; C: Myc staining induced cells for 9h, expressing FAM129C and the MYC tag; D: uninduced transfected clone, showing very little staining cf.panel C. Nuclei were counterstained with Hoechst (blue).

3.2.3.2 Subcellular Fractionation

Various methods of fractionation were employed to establish the subcellular localisation FAM129C. FAM129C enriched plasma membranes were examined using sucrose density gradient centrifugation of cell homogenates (Figure 3.13). The results obtained by the subcellular fractionation of CLL samples were similar to normal primary B cells (not shown). FAM129C appears to distributed as two separate pools.

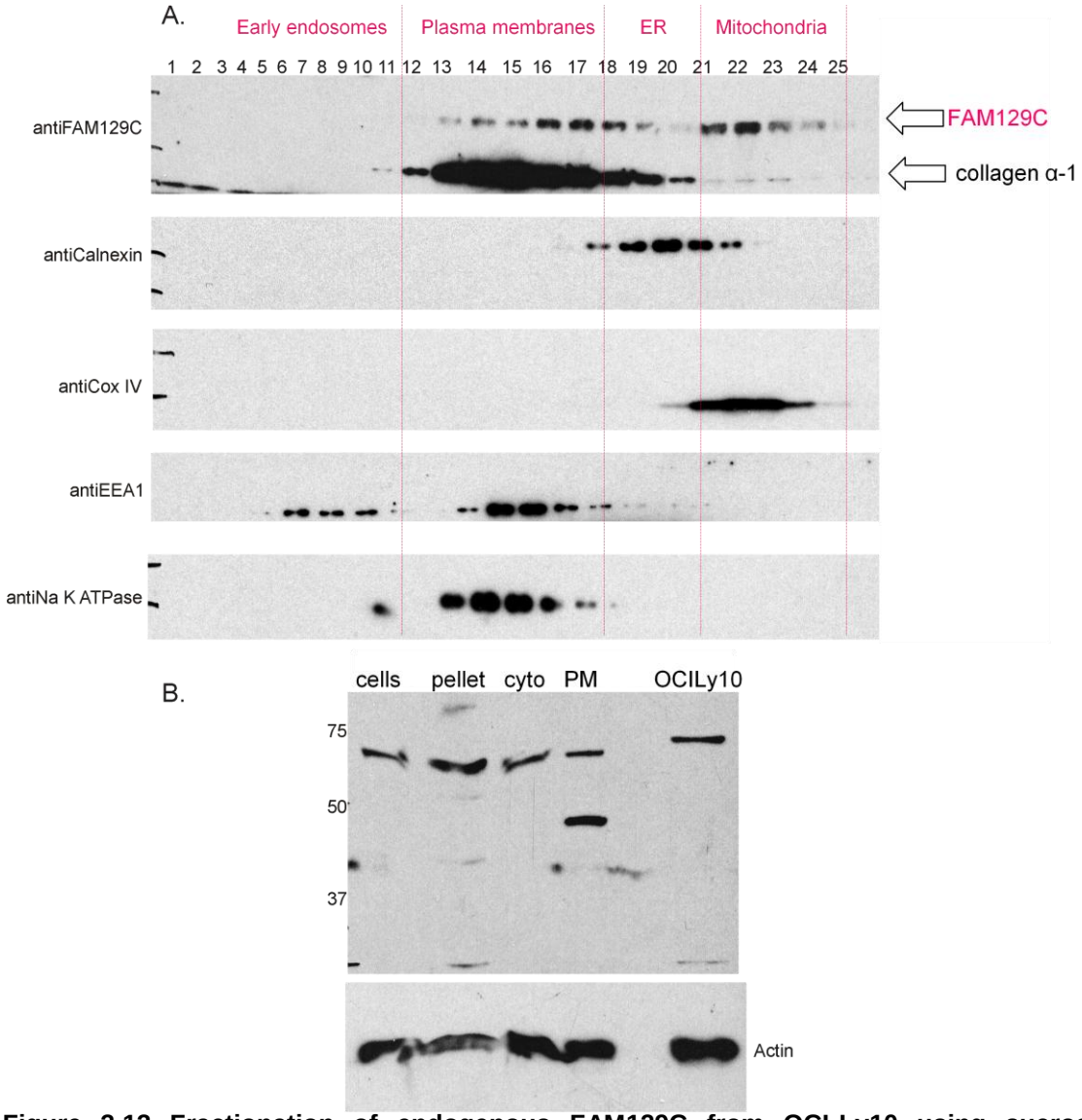


Figure 3.13 Fractionation of endogenous FAM129C from OCI-Ly10 using sucrose gradient.

This figure shows two steps of sucrose gradient fractionation: the fractionation of FAM129C with distribution immunoblots of other memebrane protein (A) and pre-fractionation step (B) where plasma membranes were isolated from cytoplasm and nucleus to be further separated by sucrose gradient (see Figure 2.10 and 2.11a and section 2.5.1)

A. Calnexin: probed to visualise endoplasmic reticulum (ER); Calnexin: Endoplastic reticulum specific; CoxIV: to show mitochondrial fraction: EEA1: early endosomes, second pool of EEA1 running with plasma membrane marker, represents probably late endosomes; $Na^{+}K^{+}$ ATPase: to show plasma membrane, Collagen α-1 is an additional and internal control in the sample for plasma membrane.

B. shows pre-fractionation step (see text). Cyto: cytoplasm, PM: plasma membranes; OCILy10: the positive control.

The first, lighter fraction did not appear to co-localise with the endoplasmic reticulum ER, mitochondria, early endosomes but most likely with plasma membranes, the picks of FAM129C and plasmamembrane did not overlay. It might co-localise with the Golgi apparatus whereas the second pool clearly co-localises with mitochondria. I was not able to confirm these results by other means, i.e. confocal microscopy because of lack of FAM129C antibody suitable for this technique.

To investigate the presence of FAM129C in the nuclear fraction, detergent fractionation was performed to actively enrich the nuclear fraction with detergents and centrifugation (NE-PER nuclear and cytoplasmic reaction reagent, PIERCE) (Figure 3.14). The effectiveness of the fractionation was assessed by lactate dehydrogenase (LDH) expression in the cytosolic fraction and Proliferating Cell Nuclear Antigen (PCNA) in the nuclear fraction. FAM129C was found in largest quantities in the

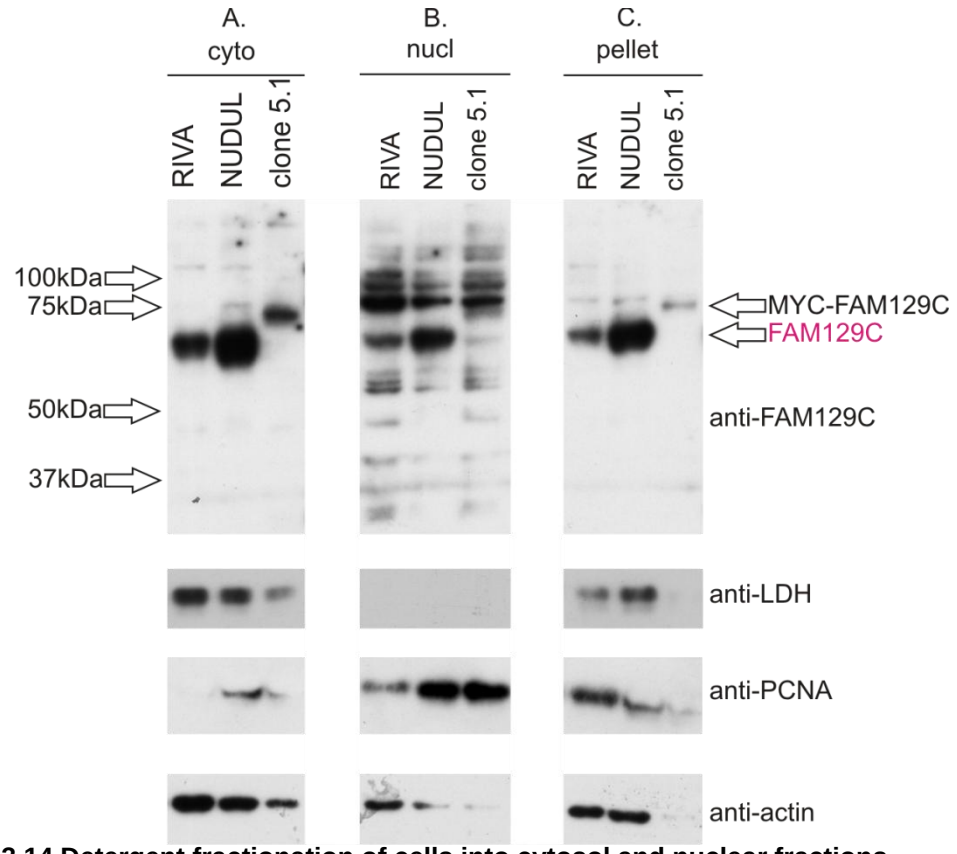


Figure 3.14 Detergent fractionation of cells into cytosol and nuclear fractions.
The effectiveness of the fractionation was assessed by LDH expression in the cytosolic fraction and PCNA in the nuclear fraction. FAM129C was present mainly in cytosol (A) and in the lesser degree in the nucleus (B). RIVA, NUDUL: ABC DLBCL cell lines, clone 5.1: Tetracycline induced FAM129C protein in Ramos cell line. Pellet: whole cell lysate pre-fractionation.

cytosol and, by blotting, to a lesser degree in the nucleus. In the nuclear fraction there were a large number of bands other than 70kDa FAM129C. The fractionation may have exposed new epitopes present in the nuclear fraction for reaction with Antibody 15, or, alternatively, Antibody 15 identified different isoforms of FAM129C, that are not present in cytosol or plasma membranes. There are bands at 64, 66 and 77kDa which may possibly represent isoform d, c and a, but this remains to be confirmed.

To summarise FAM129C appears to be mainly localised in the cytoplasm, probably associated with the mitochondria and may also possibly be present in the Golgi apparatus. It is also found in the nucleus, although at lower levels than in the cytoplasm. It is not an integral part of the plasma membrane, as its fractionation pick did not overlay with the pick of the plasma membrane control.

3.3 Functional studies on FAM129C

To perform functional studies I first set out to create an *in vitro* model. The ideal system would have been primary CLL cells, where FAM129C was first identified. However this presented several problems. Firstly there were no *bona fide* CLL cell lines. An added difficulty with this approach is that FAM129C mRNA expression in CLL is very diverse, although this was not confirmed in the protein data.

Secondly, CLL cells are difficult to genetically modify (Pearce *et al.*, 2010), as they do not proliferate and tend to die in culture within few days. Therefore I selected cell lines which express low levels of FAM129C and transfected them with an inducible gene expression system. Characterisation of this model is described in 3.3.4. I also used ABC DLBCL cell lines expressing the highest levels of FAM129C cell lines to assess endogenous expression of FAM129C. The experiments characterising FAM129C using endogenous models (ABC DLBCL cell lines and CLL patients' samples) are described in section 3.3.1 and 3.3.2.

3.3.1 Putative role of FAM129C in the B-cell development

Gene expression profile analysis indicated that FAM129C is expressed first in pre-B-cells and then maintained until the centroblast stage, when gene expression is down-regulated, only to be re-expressed in memory B-cells (also see Figures 3.5 and 3.32). Given the broad expression of FAM129C in B-cells I sought to understand the possible role of the protein following various pro- and anti- apoptotic signals.

3.3.2 FAM129C in B-cell signalling, survival and death

To investigate a possible role of FAM129C in BCR signalling I investigated whether there was an intracellular translocation for FAM129C between compartments after BCR activation by anti-IgM. I also investigated if FAM129 co-immunoprecipitated with the BCR complex using an anti-CD79b antibody.

Finally, I studied whether there was role for FAM129C in BAFF signalling.

3.3.2.1 FAM129C does not co-immunoprecipitate with BCR complex

For the BCR pull-down, the HBL1 cell line was chosen (Figure 3.15). It is an ABC DLBCL cell line, does respond to activation with anti-IgM and expresses high endogenous levels (for cell line) of FAM129C.

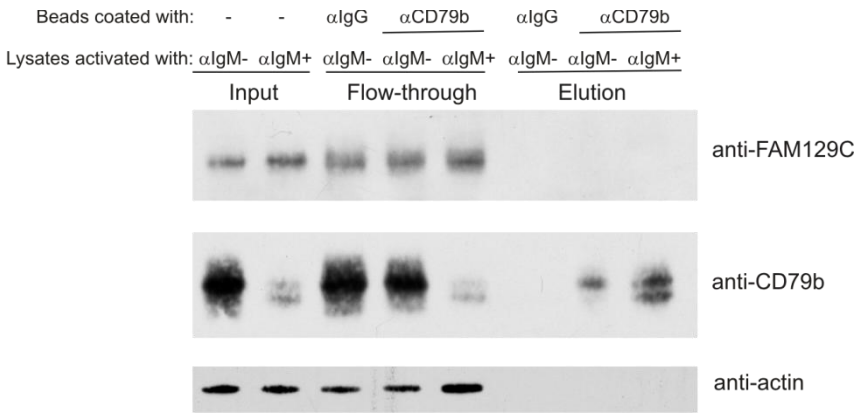


Figure 3.15 BCR pull-down.
HBL1 was activated with anti-IgM (αIgM+), controls were: non-activated cells lysate (αIgM-) on anti-CD79b coated beads and HBL1 cell lysate with rat anti-IgG beads. BCR pull down was successful as shown by CD79b bands (last two lanes in anti-CD79b blotin the elution of IgM- and IgM+ lane) and depletion of CD79b in lane 5 in comparison to the starting material in lane 2. There is dramatic loss of total CD79b following BCR crosslinking with anti-IgM in input samples(cf. lane 1 and 2) and also in the unbound fraction (cf. lane 4 and 5). The doublet seen in the lane 2, 5 and 8 may represent phosphorylated and unphosphorylated form of CD79b. The samples were loaded as equal proportion of each step.
FAM129C did not co-immunoprecipitate with BCR complex.
'αIgM-': lysis from not activated cells; 'αIgM+':lysis from activated cells

The cell line was activated by anti-IgM for 20 min at 37^{0}C and subsequently gently lysed with 1% digitonin (mild detergent to maintain cellular interactions). Anti-CD79b antibody was covalently crosslinked to beads by dimethylpirelimidate diHCl (DMP). The BCR pull down was effective as shown by CD79b bands in the elution of IgM- and IgM+ lanes. However, FAM129C did not immunoprecipitate with the BCR complex, in this cell line either before or after anti-IgM stimulation, demonstrating there was no direct association of FAM129C and the BCR complex.

3.3.2.2 Anti-IgM activation did not displace FAM129C from cytoplasm

I asessed the effect of IgM activation and cellular displacement of FAM129C in CLL patients (Figure 3.16). Firstly, patient CLL cells were screened to assess their ability to activate, by AKT following cross-linking of surface IgM. Cells were treated with an anti-F(ab')$_2$ anti-IgM antibody at 20µg/ml for 20 minutes at 37^{0}C. Cells were separated into cytosol and membranes fractions by brief sonication followed by a high velocity (100,000x*g*) spin. The efficiency of the fractionation was checked for the presence of

LDH (lactate dehydrogenase, cytosolic marker). BCR activation by anti-IgM did not cause changes in FAM129C expression nor lead to any displacement from cytoplasm to membranes, on the basis of mechanical fractionation.

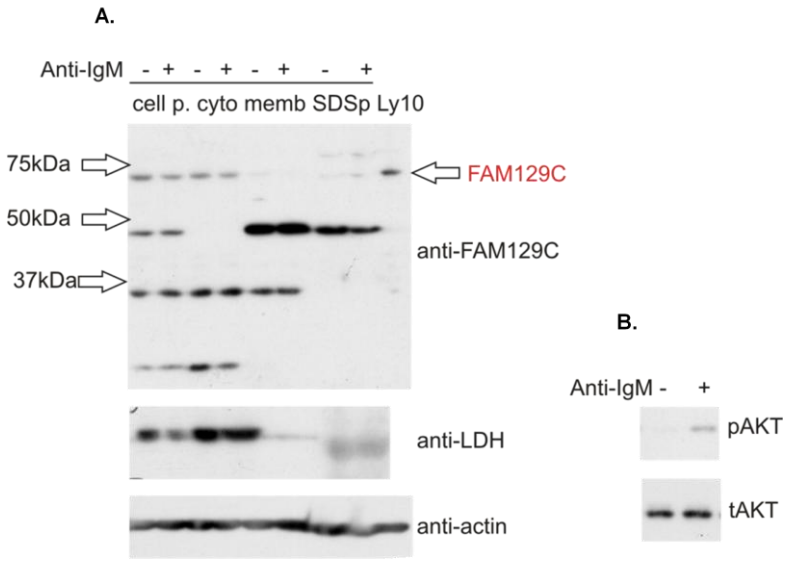


Figure 3.16 IgM activation of CLL sample and subcellular fractionation.
A. There were no changes in level of expression or localisation with anti-IgM stimulation. Cell p.: cell pellet, control samples; cyto: cytosol; memb: membrane; SDSp: pellet lysed with SDS, insoluble fraction; Ly10: OCI Ly10, endogenous positive control. **B.** Control for activation, pAKT: phospho AKT, tAKT: total AKT.

Taken together, FAM129C is not likely to be directly involved in BCR signalling, as BCR stimulation does not cause downregulation or displacement in the cell, and the BCR pull-down did not contain FAM129C. These data fit with earlier presented *IGHV* status and mRNA level in CLL patients.

3.3.2.3 FAM129C is not displaced to lipid rafts after B cell activation.

Lipid rafts are specialised cholesterol- and glycosphingolipid-rich microdomains in the plasma membrane, which segregate important signalling molecules during the different stages of B cell activation. Unstimulated BCR is present in the non raft fraction of the plasma membrane, when BCR is clustered after engagement of antigen, they associate with the membrane raft fraction (Dykstra *et al.*, 2003). The activated fraction of tyrosine kinase-LYN, a key protein in early stages of activation is localised in lipid rafts (Young *et al.*, 2003). Lipid rafts were generated from HBL1 cell line pre- and post anti-IgM stimulation (Figure 3.17). FAM129C was not present in the lipid rafts of either control cell lines HBL1 (ABC DLBCL cell line) nor in anti-IgM-stimulated HBL1.

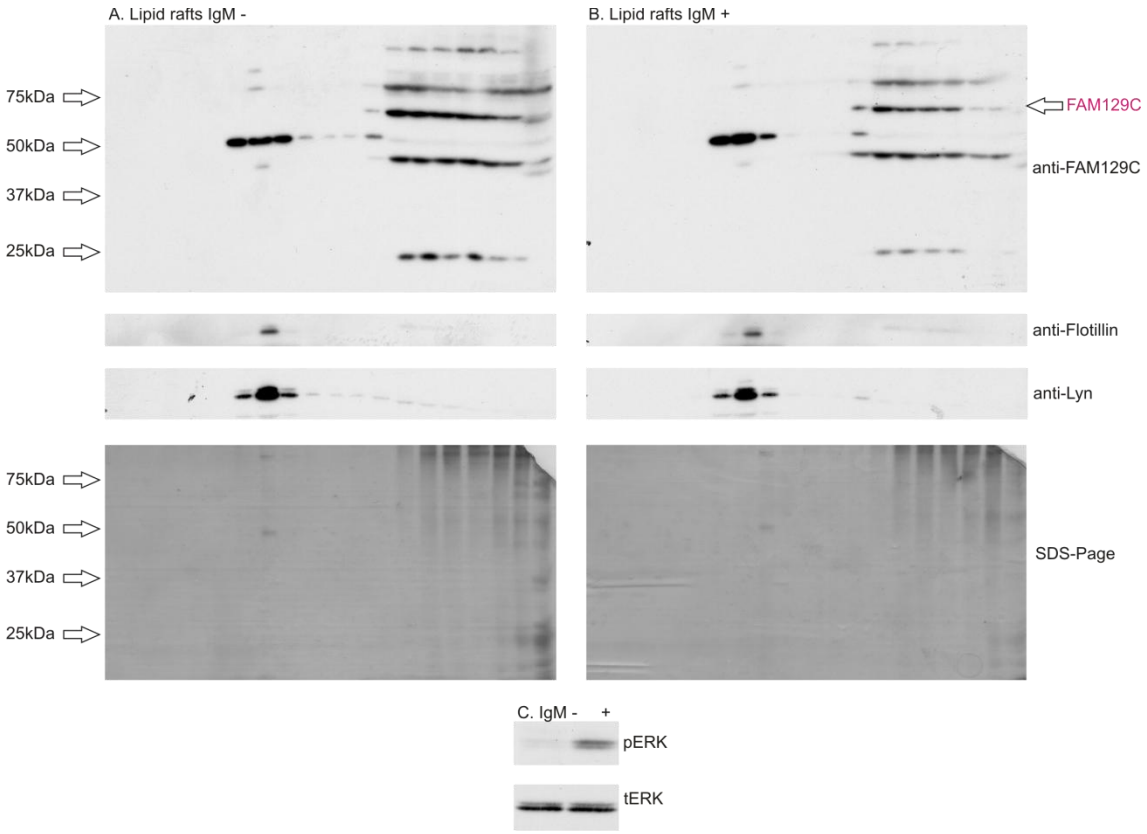


Figure 3.17 Isolation of lipid rafts in HBL1 cell line.
FAM129C was not present in the lipid rafts fractions in the control cells nor in the cells activated with anti-IgM. Blots with Flotillin and Lyn identified position of lipid rafts in fractions. Panel C showed successful IgM activation through phosphorylation of ERK.NB. Strong 50kDa band (visible on the SDS page) was enriched in rafts and identified as probably human collagen α-1 (by mass spectrometry).

The likely identity of the 50kDa band was revealed from analysis of lipid raft preparations. A 50kDa protein detected by Antibody 15 was mainly in the patient samples (Figure 3.10); it appeared to be further enriched in preparation of plasma membranes (Figure 3.13.B). There was a suggestion that this band might be an isoform of FAM129C or its breakdown product. The 50kDa band was localised in the lipid rafts fractions and even visible on the SDS-Page, this band was cut and analysed using mass spectrometry. The most abundant protein identified with good confidence was human collagen α-1 (mascot score 152).

3.3.2.4 Does the intracellular localisation FAM129C changes following BAFF stimulation?

BAFF plays a key role in the peripheral B-cell selection and survival for both pre-antigen and antigen-induced B cells and is especially effective in transitional B cells. BCR induced signals together with BAFF receptor (BR3) allows production and maintenance of immunocompetent pools of mature follicular B cells and marginal zone

B cells, at the same time remaining self-tolerant (Stadanlick & Cancro, 2008). BAFF also plays a role in immunoglobulin switching and GC maintenance (Kalled, 2006).

BAFF was a kind gift from Dr A. Craxton (MRC Toxicology, Leicester). BAFF supernatant was obtained from HEK293T cells stably transfected with Strep Tag II-tagged BAFF (amino acids 133-285), containing an isoleucine zipper, which has been shown to induce NF-κB2 p100 processing to its p52 subunit in isolated CLL cells.

CLL samples were treated with two forms of BAFF reagent: Strep Tag purified BAFF (marked in Figure 3.20 as 'p') and with media containing BAFF reagent (marked in Figure 3.18 as S/N) for 18h. Post stimulation, cells were mechanically fractionated to check localisation of FAM129C. On treatment with BAFF reagent, there was an increase of p52 and reduction in p100. This observation was particularly evident in the cytosolic fraction on stimulation with the purified form of BAFF.

Similar to the previously discussed activation with anti-IgM, there was no displacement of FAM129C between cytosol and membranes as shown by mechanical fractionation.

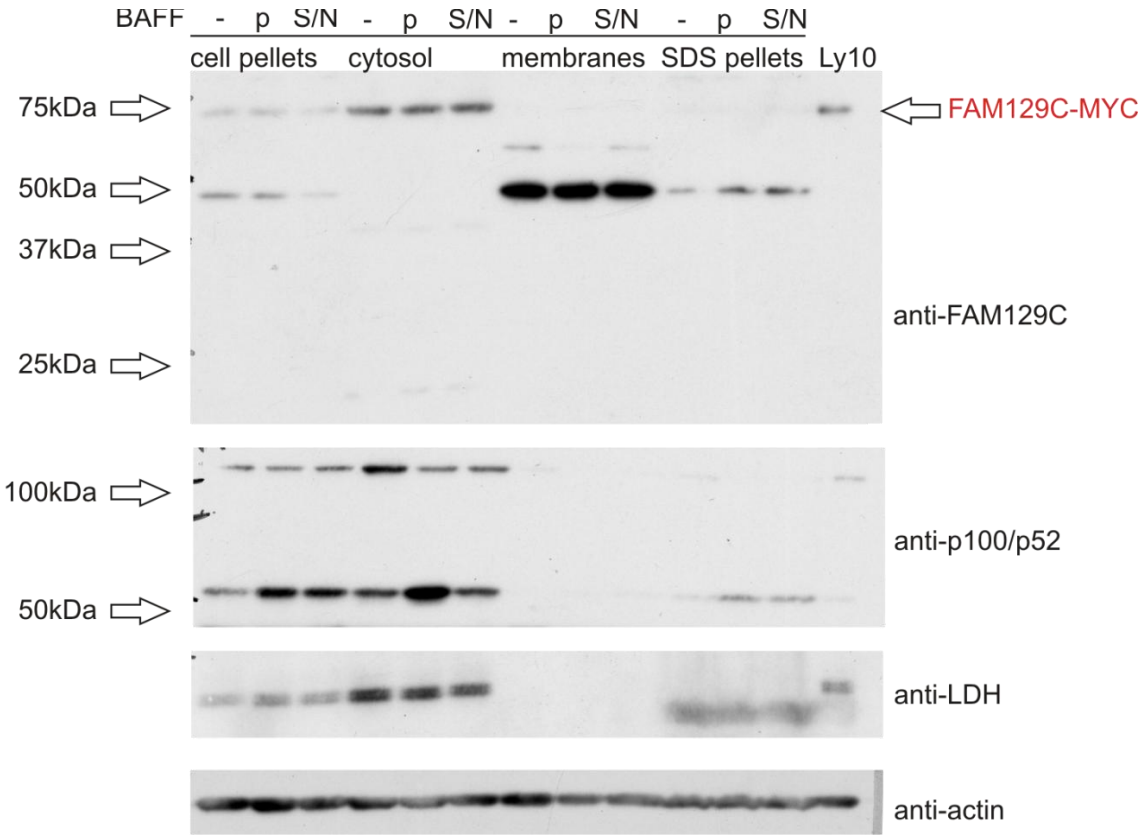


Figure 3.18 CLL samples treated with BAFF and subcellular fractionation.
There were no changes in the level of expression or localisation with IgM stimulation. p: purified BAFF; S/N: cell supernatant, media containing BAFF. SDS pellets: cell pellets lysed in SDS, insoluble fraction. Ly10: OCI Ly10, endogenous positive control sample. Blotting with p100/52 shows NF-κB processing.

3.3.2.5 FAM129C was down-regulated upon CD40-CD154 signalling.

To mimimic the germinal centre microenviroment, the CD154/IL4 system was utilised. This method induced consistent downregulation of endogenous FAM129C in cell lines (see below). The protein downregulation was observed as early as 24h. One of major pathways induced by CD40 signalling is non-canonical NF-κB activation, *NF-κB2*. Figure 3.19 shows CLL cells on CD40L system with and without inhibition with BAY-11-7082 (10μM for 24h) (Merck, Darmstadt, Germany). BAY-11-7082 selectively and irreversibly inhibits the phosphorylation of IκB-α (a primary regulator of p65/p50 complexes of classical pathway).

In order to check the effectiveness of the CD154-IL4 system I tested for the expression of the precursor p100 and its processing to p52; p100/p52 is one of the five subunits of NF-κB signalling pathway and is partially degraded by the proteosome and cleaved, following activation of NIK (NF-κB inducing kinase) and subsequent phosphorylation of IKKα to generate active DNA binding product, p52 in the alternative pathway. Upon stimulation of CD40L there is accumulation of p100, which is processed to p52 by NIK (Coope *et al.*, 2002), (Senftleben *et al.*, 2001). Castro (JI, 2009) showed recently that c-Rel upregulates p100, therefore blocking classical pathway with BAY-11-7082 ((E)-3-(4-methylphenylsulfonyl)-2-propenenitrile) effects alternative pathway. Figure 3.19 showed effective CD40L signalling by increased p100 and p52 in the CD40L lane. The inhibition was effective as represented by complete abrogation of p100 and reduction of p52. FAM129C expression was downregulated on CD40L system in comparison to control cells ('T0' and 'Plastic') and on cells incubated with the non-transfected fibroblasts ('NTL'). BAY-11-7082 appears to completely block p100 (α p52) expression suggesting p100 expression requires canonical NF-κB activation.

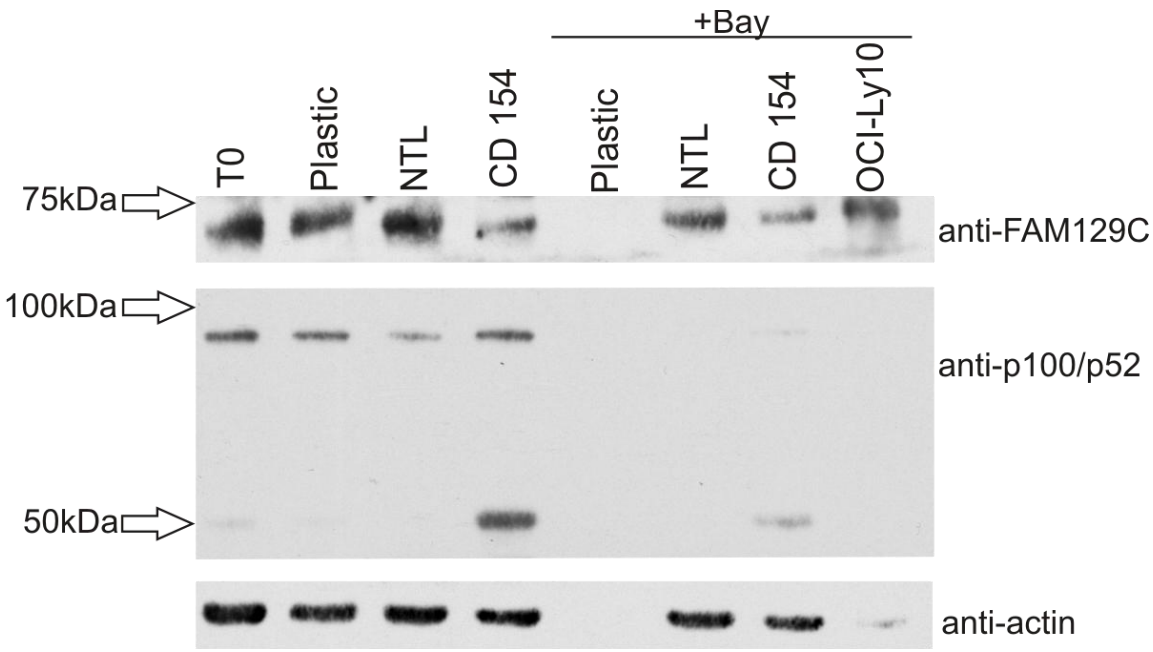


Figure 3.19 CLL samples stimulated with CD40L and IL4, with, and without, NF-κB inhibitor Bay11-7082.
Time-course: 24h. Blot with p100/52 showed processing of p100 and appearance of p52, whereas with BAY11-7082 blocking there was no p100 and reduced p52. BAY11-7082 blocks p100 expression but not CD40L induced FAM129C downregulation.

3.3.2.6 FAM129C and its role in the quiescent cells.

The downregulation of FAM129C protein levels within the germinal centre and in proliferating CLL cells suggested that FAM129C might be involved in maintaining B-cells in G1/G0 phase of the cell cycle. I investigated this hypothesis further using other stimuli to promote growth of normal and malignant B-cells. Figure 3.20 shows primary cells (both normal B cells and CLL cells) stimulated to proliferate with phorbol 12-myristate 13-acetate (PMA). Down-regulation of FAM129C was seen both in normal and CLL B-cells stimulated with PMA. Similarly, an MCL cell line with endogenous expression of FAM129C (Rec) showing down regulation of this protein after exposure to PMA (Figure 3.20.C). PMA affects a variety of cell processes, including proliferation and differentiation. In most cell types, PMA enhances proliferation by a protein kinase C (PKC)- dependent mechanism. However when the Rec cell line was treated with PMA and the PKC inhibitor Bisindolylmaleimide I, there was no inhibition of the FAM129C downregulation. This suggests that the down-regulation of the FAM129C is regulated by other mechanisms.

I have attempted to downregulate endogenous FAM129C in ABC DLBCL and GC cell lines. The knock down of FAM129C in ABC DLBCL cell lines OCI-Ly10, OCI-Ly3 and HBL1 was unsuccessful. The knock down was successful in a GC DLBCL cell line, OCI-Ly19 (Figure 3.22). The cells with downregulated FAM129C stopped proliferating in culture and did not apoptose, however this experiment was not repeated.

The CD38 positive population in CLL is associated with proliferation (Dealaglio MolMed 2010), therefore the separated populations of CLL CD38 positive and negative populations. In the example were the separation was better, there were similar amounts of FAM129C in CD38 positive fraction. Less than 10 patients were separated into CD38 positive and negative fractions and no obvious differences were noted. These two fractions were further treated on CD154/IL4 system with no observable difference in FAM129C downregulation in CD38 positive and CD38 negative CLL fractions (data not shown), i.e. both fractions down-regulated FAM129C with similar kinetics.

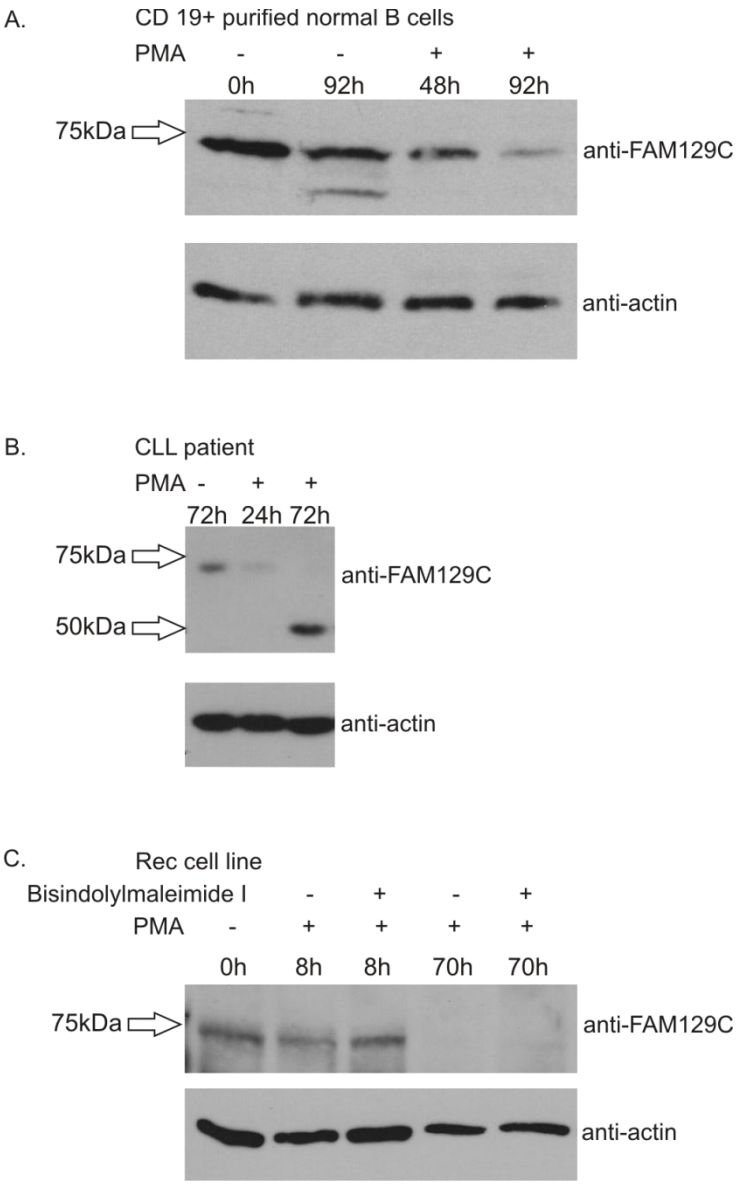


Figure 3.20 FAM129C downregultion after stimulating cells to proliferation with PMA.

A. CD19+ purified normal B cells and PMA treatment; B. CLL cells post PMA treatment; C. Rec cell line expressing endogenous FAM129C is downregulated by PMA and the inhibition of PK C does not reverse the process.

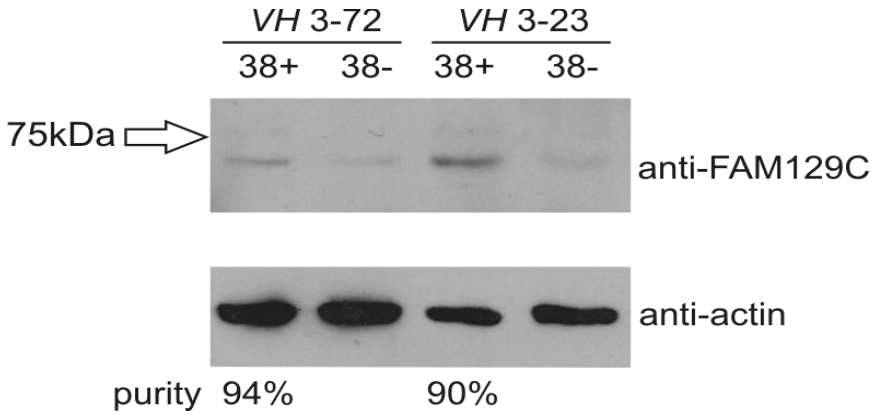


Figure 3.21 FAM129C expression in CD38 positive and negative fractions in two different patients.
The densitometry (CD38/actin for VH 3-72 was 0.68 (CD38+) and 0.64 (CD38-) and for VH3-23 case was was 1.2 (CD38+) and 0.7 (CD38-)

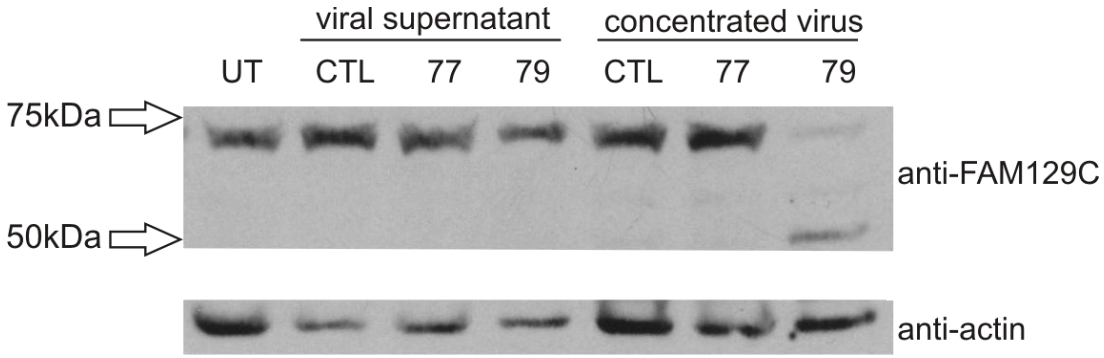


Figure 3.22 Downregulation in OCI-Ly19 cell line with SHU-RNA (Origine) using retrovirus

CTL: scrumbled vector; the most efficient downregulation was observed in vector 79.

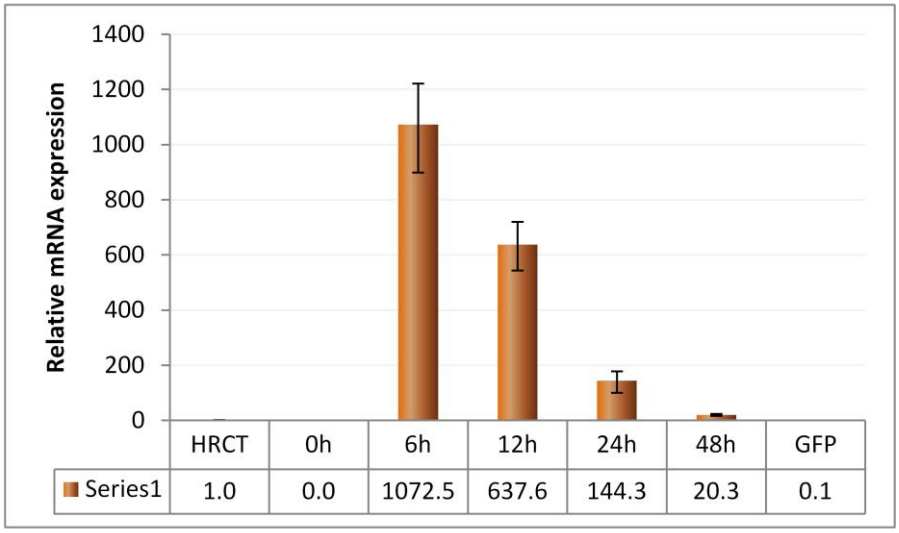
3.3.3 Characterisation of the overexpressing clones

The work below was produced using Ramos cells stably expressing FAM129C in a tetracycline-regulated expression system. Ramos cells represent germinal centre centroblasts. They had a small, detectable level of FAM129C expression, however the endogenous FAM129C was not seen in the lysis of the overexpressing cell lines.

I attempted to characterise the generated clones according to the key B-cell elements described in the introduction of this chapter. I subjected FAM129C expressing cells lines to apoptosis-inducing extrinsic (by Fas and TRAIL), and intrinsic, pathways (using MG132, etoposide and staurosporine). I studied the rate of proliferation in FAM129C expressing clones and I investigated the response to BCR via anti-IgM and CD40L

signalling. Finally, I looked at the association of FAM129C with large complexes and attempted to immunoprecipitate FAM129C to identify protein partners.

A.



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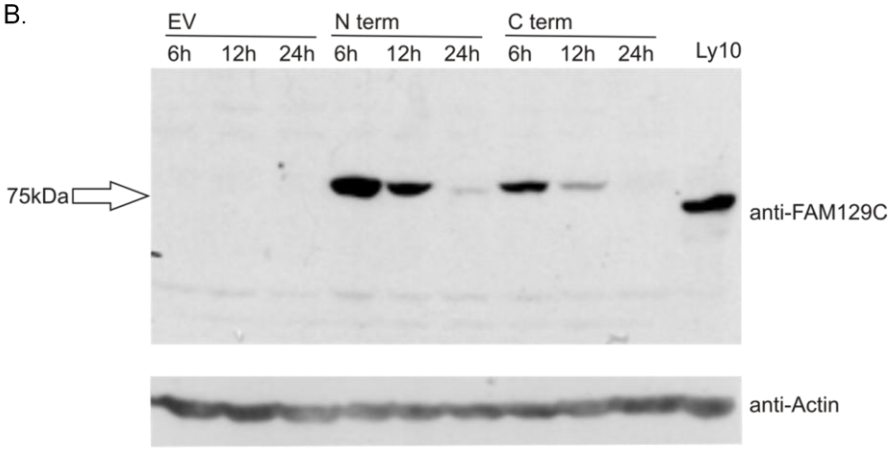


Figure 3.23 Time course of FAM129C expression in the BJAB cell line post transfection.

A. mRNA expression pre and post nucleofection NB. Scale was increased 10 fold in comparison to endogenous data**. B.** WB of FAM129C in BJAB showing downregulation of expression protein (paralleled by mRNA) using both constructs: N-terminus and C-terminus; EV: empty vector, N-term and C-term.

3.3.3.1 Apoptosis in overexpressing clones

To investigate the role of FAM129C in apoptosis I subjected FAM129C clones to apoptosis by the intrinsic pathway using etoposide (200µM), staurosporine (1µM) and MG132 (5µM). The control cell line was the same clone but not induced with tetracycline and therefore not expressing FAM129C (Figure 3.24.A). The treatment was repeated on different clones and apoptosis was measured at 6h (Figure 3.24.B).

The extrinsic pathway was investigated by stimulation with TRAIL ligand (100ng/ml, courtesy of Dr N. Harper, MRC, Leicester) and anti-Fas agonistic antibody (100ng/ml) (clone CH11, Upstate). As shown in Figure 3.24 expression of FAM129C did not sensitise, or protect, the clones to apoptotic stimuli.

Ramos clones were resistant to Fas-induced killing; this is a well-described phenomenon as non-activated B cells express small amount of Fas on the surface.

Spontaneous apoptosis in CLL patients was investigated (Figure 3.24) The loss of expression of FAM129C was correlated to the amount of apoptosed cells. There appears to be a degradation product on the day 2 and 4, but not on day 7. There were no differences in CLL cells harvested from the bone marrow *vs* peripheral blood

I was particularly interested in Fas-mediated killing and FAM129C's role in this process. The cells downregulate FAM129C as they enter GC, cf. naïve v. memory cells. To test this hypothesis I cultured Ramos clones in a CD40L system in the presence of IL4 (Figure 3.25.A) to induce surface Fas expression. Fas was upregulated in both tetracycline-induced and uninduced cells (Figure 3.25.B) in the cells cultured on the CD40L transfected cells by comparison to cells cultured on mouse fibroblasts.

The Ramos cells were then removed from the mouse fibroblast layer and repeatedly washed (IL4 inhibits Fas-mediated apoptosis (Foote *et al.*, 1996). Fas agonistic antibody (CH11) was utilised to induce apoptosis using three different concentrations (3, 10 and 30ng/ml) and apoptosis was assessed by flow cytometery using Annexin V and Propidium Iodine (PI) staining at 9h and 24h. There was no significant difference in apoptosis between clones expressing FAM129C and control clones (EV: empty vector).

One of the main problems of this system was that the amount of apoptosis induced in Ramos differs between the transfected and untransfected cells; after 24 hours incubation with CD40L some 40-45% of transfected fibroblasts had died whilst the value for the untransfected cells was significantly less at 25%. This problem was previously observed in my laboratory (Dr. M. Butterworth, personal communication).

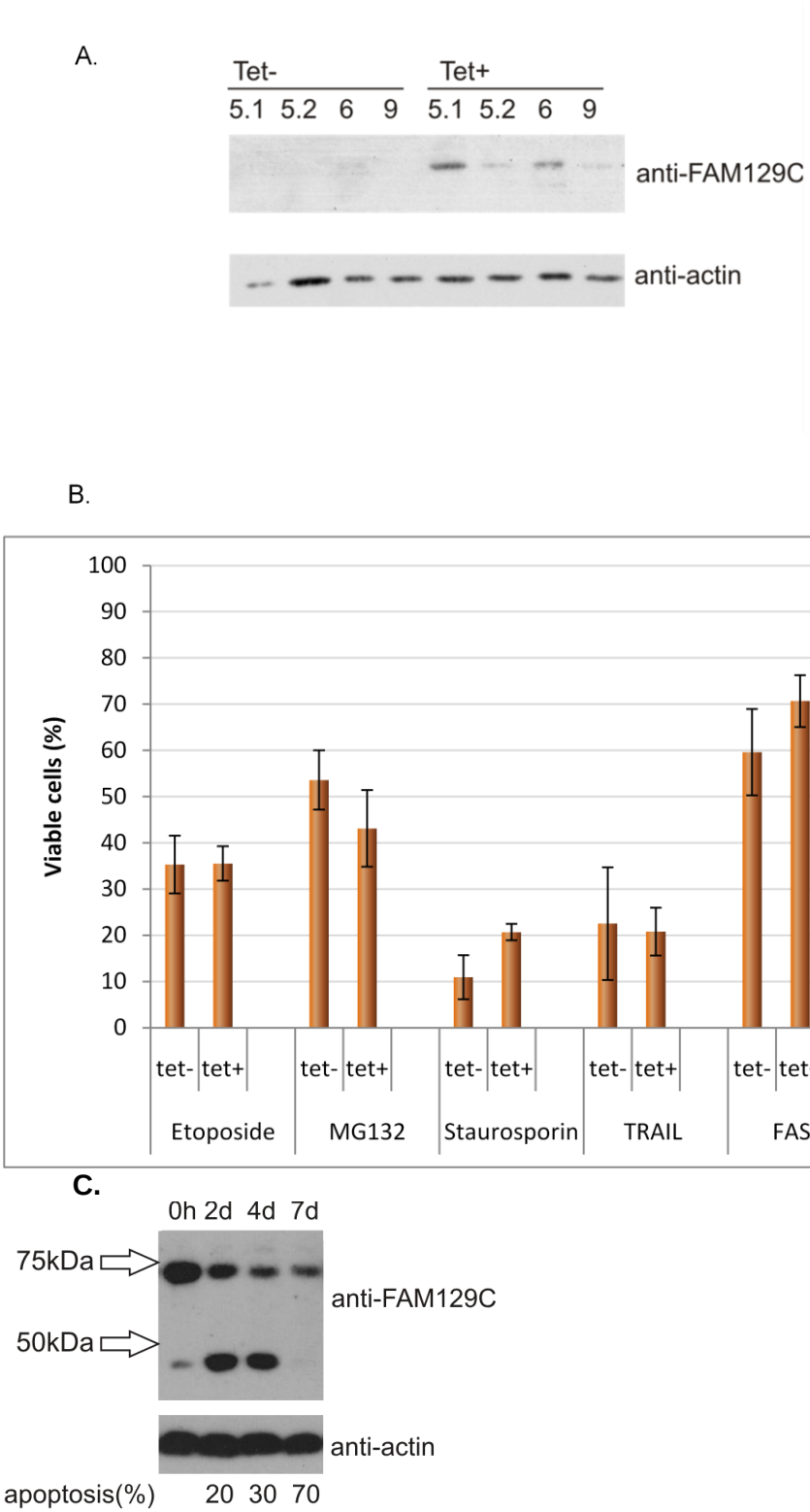


Figure 3.24 Apoptosis in Ramos cell clones overexpressing FAM129C and spontaneous apoptosis in CLL patient.

A. Clones used in testing for apoptosis, tet- uinduced clones, tet+ induced clones with tetracycline. Clones 9 and 5.2 were the weakest, there were no 'leaking'clones, i.e. there was no expression seen in uninduced clones. **B.** Apoptosis was measured by flow cytometry (Annexin V and Propidium Iodine (PI)) at 8h.There was no difference in apoptosis between induced and uninduced clones. C. Changes in FAM129C expression in CLL patient undergoing spontanous apoptosis whilst in culture over

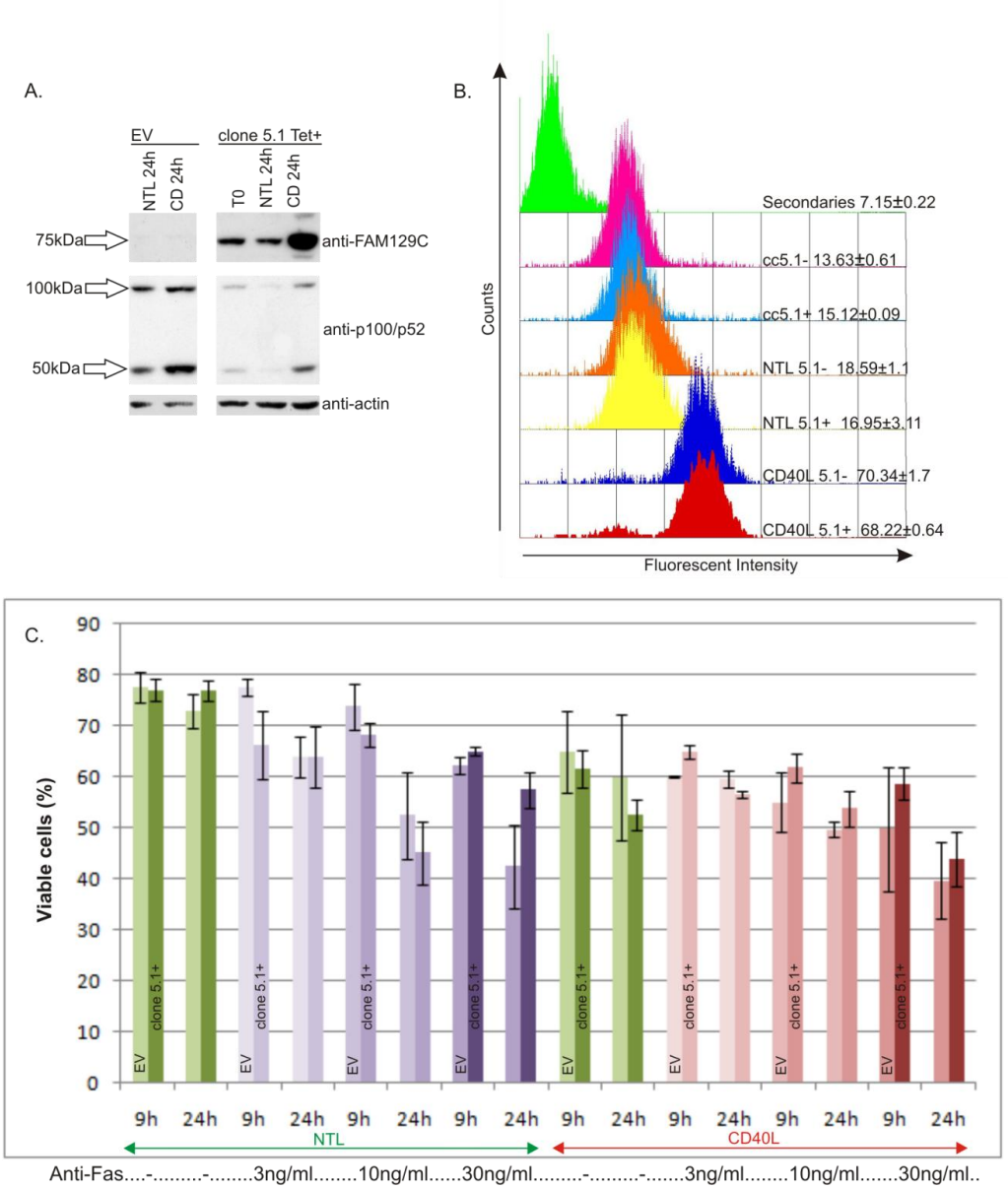


Figure 3.25 FasL induced apoptosis.

A. WB of EV (Empty Vector) and induced clone 5.1. cultured on non-transfected mouse fibroblasts (NTL) and fibroblasts overexpressing CD40L in presence of IL4 (CD); CD40 signaling through NF-κB, as shown by increase in p52; there is some degree of processing (p100 to p52) on untransfected L cells. **B.** Flow cytometry of upregulation Fas expression, cc: cells grown alone, NTL: clone 5.1 grown on non-transfected mouse fibroblasts (NTL) and mouse fibroblasts transfected with CD40L and in the presence of IL4, values represent geomentric mean of fluorescence intensity with standard errors. C. measurement of living cells using Annexin V and propidium iodine (PI), paired columns: on the left: representing EV, on the right: clone 5.1+ (induced). The cells were treated with different concentrations of Fas agonistic antibody (CH11): 3ng/ml, 10ng/ml and 30ng/ml. There were no differences in Fas upregulation in clone 5.1 (induced and uninduced with tetracycline). In this system there were no differences between NTL and CD40L and EV and clone 5.1 in Fas mediated killing.

3.3.3.2 Proliferation

I have tested the rate of proliferation of clones expressing FAM129C, against clones not induced with tetracycline (Figure 3.26). I attempted to synchronise cells first, i.e. to have all the cells in the tested pool in the same phase of the cell cycle (G_0), then

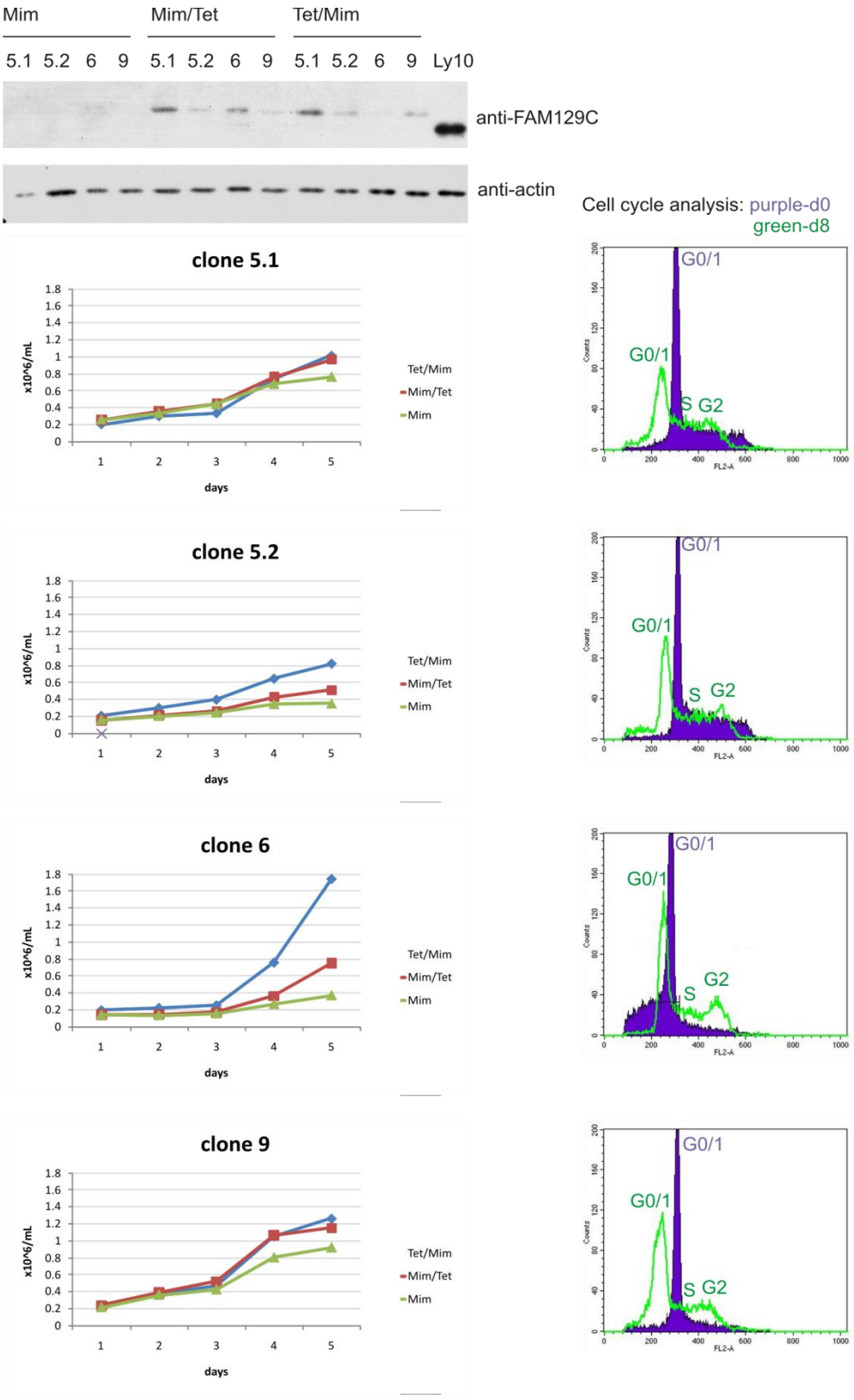


Figure 3.26 Proliferation experiment.

Clones were treated with mimosine to synchronise cells in the pool to the same cell cycle phase. **A.** FAM129C protein expression in uninduced clones , but treated with Mimosine(Mim), induced when cells were synchronised (Mim/Tet), induced prior to cell synchronisation, i.e. prior to mimosine treatment (Tet/Mim). **B.** Proliferation rates from day 1 to day 5. C. Cell cycle assessment using DNA/propidium iodine flow cytometry method.

analyse proliferation rate of the cells. Serum starvation was used initially, but the Ramos cell line does not tolerate this method and significant cell death was observed before the majority of the cells reached G_0. I then used the reversible cell cycle inhibitor, mimosine. Cells were incubated with 150µM mimosine for 24h and cell cycle was assessed by DNA and flow cytometry using the propidium iodide method. As shown in Figure 3.26, the best synchronisation was achieved in clone 6 and clone 9. The strongest expressing clone, 5.1 was not as well synchronised as clone 6 and clone 9. I have experimented on inducing FAM129C at the different time points during synchronisation: 'Mim/Tet' FAM129C was induced after synchronisation, i.e. tetracycline was added after mimosine was removed. 'Tet/Mim': FAM129C was induced prior to the mimosine treatment; tetracycline was added 24h before mimosine. There was no difference on proliferation depending on when FAM129C was induced (before or after synchronisation). There appeared to be small increase in proliferation in cells expressing FAM129C only in cells weakly expressing FAM129C. Despite synchronisation of the cells in tested culture I did not observe significant differences between FAM129C expressing cells and the control cells.

3.3.3.3 Signalling in FAM129C-Ramos clones

I have repeated BCR signalling experiments which were already performed on the cell lines with the endogenous expression of FAM129C. I tested B-cell signalling in Ramos clones using anti-IgM stimulation to assess the translocation of FAM129C after stimulation (Figure 3.27) and CD40L signalling (Figure 3.28). The anti-IgM stimulated cells were fractionated into cytosol and membrane to assess protein trafficking. FAM129C was not downregulated on IgM stimulation and I did not observe cytosol to membrane trafficking. Similar to previous experiments (3.3.2.2) on the endogenously expressing cells, I did not observe changes in FAM129C induced by BCR stimulation with IgM antibody.

Next, I cultured Ramos clones on CD40L transfected mouse fibroblasts in the presence of IL4 (Figure 3.28) for total of 6 days. The end point was the alternative pathway processing as tested by transition of p100 to p52 protein. There was a marked increase of p52 protein as well as p100 on CD40L transfected fibroblasts cells (CD), this was not seen on non-transfected mouse fibroblasts (NTL). This suggested NF-κB activation in cells grown on CD40L cells. In this experiment I observed 'leakiness' of clone 5.1, i.e. FAM129 expression was seen in non-tetracycline induced cells. Endogenous FAM129C is not detected in the overexpressed clones (Figure 3.28.B).

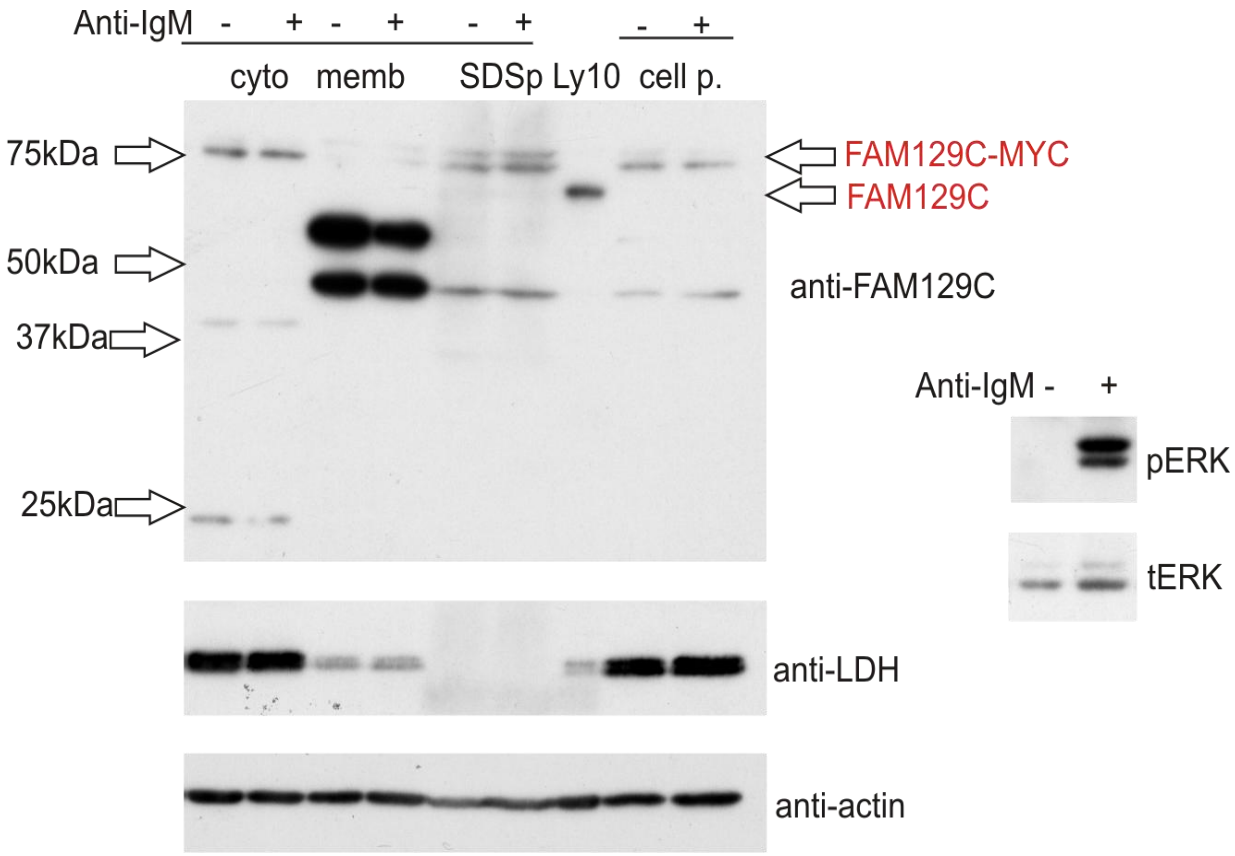


Figure 3.27 IgM and stimulation on Ramos clone 5.1

A. Cells were stimulated with IgM (IgM+) and fractionated to cytosol (cyto), membrane (memb) fraction and insoluble fraction (SDSp: SDS lysed pellet); OCI-Ly10(Ly10) provides endogenous positive control for the antibody; cell p.: cells pre-fractionation. **B.** control for B cell stimulation with phosphoERK against total ERK.

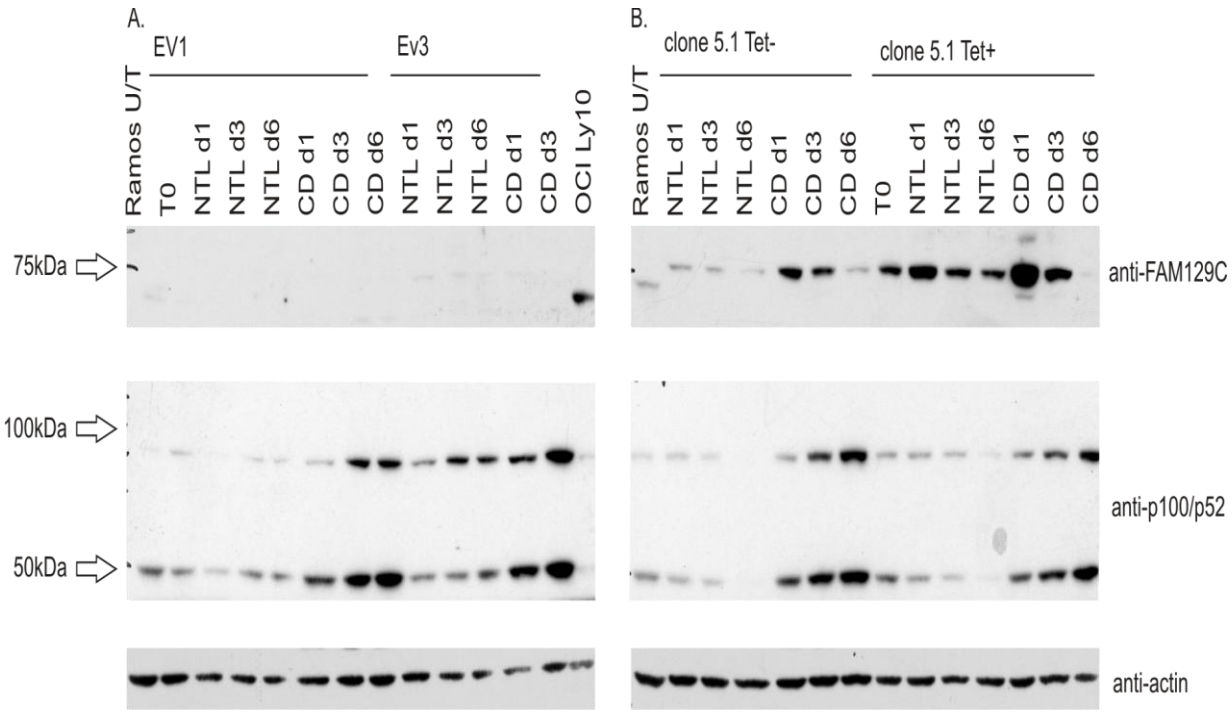


Figure 3.28 Clones expressing FAM129C and CD40L signaling.

A. Two control clones (with empty vectors) on untransfected mouse fibroblasts (NTL: non-transfected L cells) and CD40L transfected cells (CD) on day 1, 3, 6. The effectivenes of the system was checked by non-canonical NF-κB processing of p100 to p52. **B.** FAM129C Ramos expressing clones uninduced (Tet-) and induced (Tet+) on CD40L system. Clone 5.1 expressed FAM129C without tetracycline induction. There was marked upregulation of FAM129C expression in uninduced and induced clones with complete down regulation of protein expression by day 6, as seen in CLL patients' samples. There was no difference in NF-κB responses between control cells and FAM129C expressing clones.

Interestingly, FAM129C protein expression was initially upregulated by CD40 signalling, it was particularly striking in uninduced cells. However, it was downregulated to undetectable levels by day 6, similar to cells with the endogenous expression.

I did not observe differences in NF-κB pathway processing between cells expressing FAM129C and control cells.

3.3.3.4 Association with high molecular complexes

In the attempt to identify FAM129C functional partners first I investigated if FAM129C is associated with high molecular protein complexes (Figure 3.29). I used the method of sedimentation of protein complexes in a sucrose density gradient centrifugation as described by Hartman and Dudkina (Dudkina *et al.*, 2005; Hartman *et al.*, 2007). NU-DHL1 of cells was lysed in 0.5% Triton to preserve complexes and, as a control, two other fractions were also treated with SDS and SDS together with βME.

SDS disrupts protein complexes, whereas βME breaks disulphide covalent bonds. Each of three lysate was loaded on the 10-40% sucrose gradient and spun overnight at 100 000x*g*. I used commercially available markers to assess the molecular weight of each fraction of Triton lysed cells: aldolase (126kDa) was found in the fraction 5, catalase (232kDa) travelled to fraction 9, and thyroglobulin (669kDa) was found in fraction 16. FAM129C was found to be spread across all of the fractions: from fraction 3 to 24 (only 18 fractions are shown for the purpose of clarity, Figure 3.31) with the peak in fraction 4.

This confirmed the validity of the result and experiment shown in Figure 3.29 is that of the distribution of endogenous distribution of FAM129C. FAM129C appeared to forms a dimer: as the commonest form of the protein: the peak is between fraction 3 and 6, which corresponds to the position of aldolase (MW=128kDa).

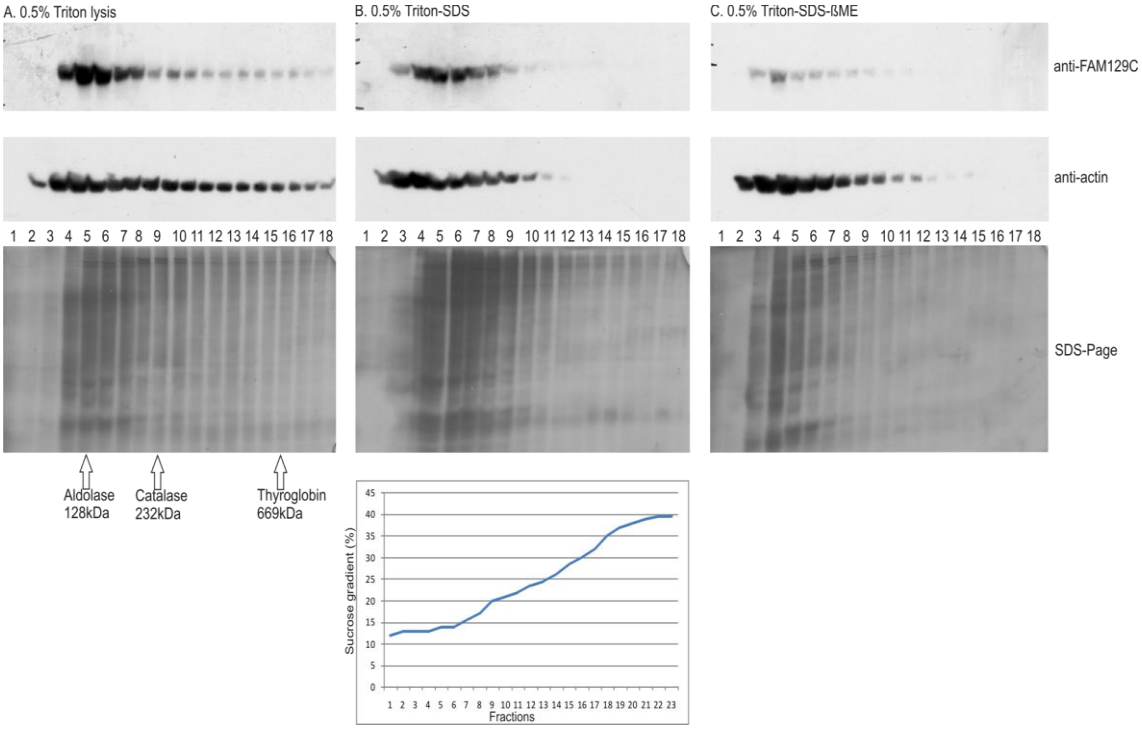
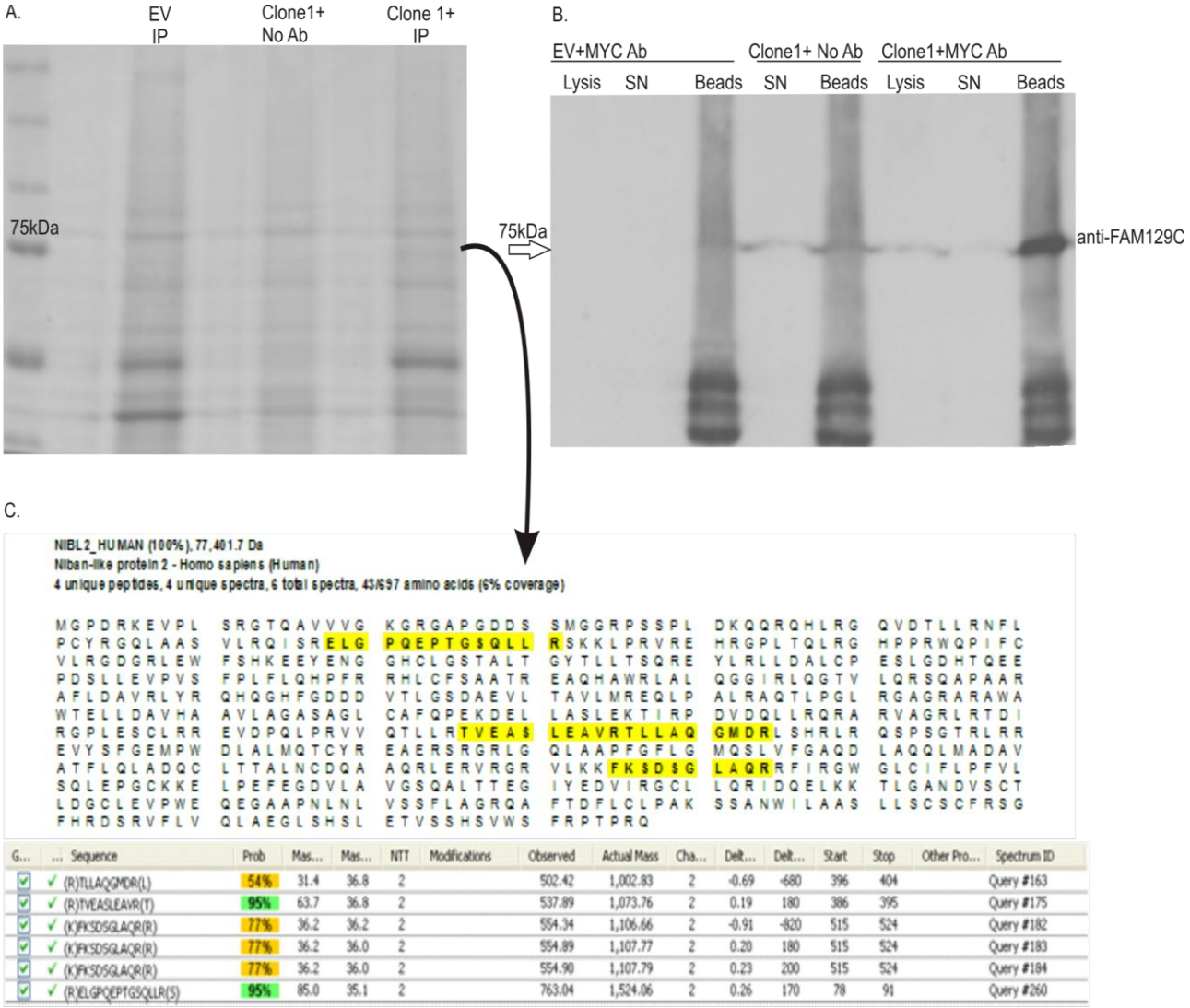


Figure 3.29 Sedimentation of protein complexes in a sucrose density gradient centrifugation in NuDUL-1 cell line.

Proteins in their native state are separated on a sucrose gradient (10-40%), fractions were collected from the top of the tube, proteins were denatured and run on WB and on SDS-Page. To show the disruption of the complexes, the sample was treated with SDS and SDS with βME. Catalase (232kDa) and Thyroglobin (669kDa) marked molecular weights of the complexes in respective fractions. Endogenous FAM129C behaves similarly to actin upon fractionation. This figured confirmed the observation in the endogenous system, the same results were initially obtained in clone 5.1, results not shown.

3.3.3.5 Co-immunoprecipitate protein partners

An immunoprecipitation (IP) of FAM129C was performed. The available polyclonal rabbit antibody did not immunoprecipitate FAM129C. I attempted to immunoprecipitate MYC-tagged FAM129C induced Ramos clone using weak detergent of 1%Triton (Figure 3.30). In an effort to optimise the IP, I 'pre-cleared' lysate with sucrose fractionation, as described in previous paragraph (3.3.4.4). The initial fractions were discarded, as these fractions contained most of cellular protein. The IP was performed on pooled fractions 9-12 and 12-24, after the sucrose was dialysed. I was able to IP FAM129C (based on both western blotting and mass spectrometry, Figure 3.30), however despite introduced optimisations, I was not able to identify clearly co-precipitated protein partners as there were no differential bands on SDS-Page.



G...	...	Sequence	Prob	Mas...	Mas...	NTT	Modifications	Observed	Actual Mass	Cha...	Delt...	Delt...	Start	Stop	Other Pro...	Spectrum ID
☑	✓	(R)TLLAQGMDR(L)	54%	31.4	36.8	2		502.42	1,002.83	2	-0.69	-680	396	404		Query #163
☑	✓	(R)TVEASLEAVR(T)	95%	63.7	36.8	2		537.89	1,073.76	2	0.19	180	386	395		Query #175
☑	✓	(K)FKSDSGLAQR(R)	77%	36.2	36.2	2		554.34	1,106.66	2	-0.91	-820	515	524		Query #182
☑	✓	(K)FKSDSGLAQR(R)	77%	36.2	36.0	2		554.89	1,107.77	2	0.20	180	515	524		Query #183
☑	✓	(K)FKSDSGLAQR(R)	77%	36.2	36.0	2		554.90	1,107.79	2	0.23	200	515	524		Query #184
☑	✓	(R)ELGPQEPTGSQLLR(S)	95%	85.0	35.1	2		763.04	1,524.06	2	0.26	170	78	91		Query #260

Figure 3.30 Immunoprecipitation using MYC tag in overexpressed FAM129C.
A. SDS gel, EV: empty vector control, Clone 1+: induced clone, incubated with beads without the antibody; Clone 1+ IP: capture: induced cells lysate incubated with beads and anti-MYC antibody. **B.** protein blot of IP; SN: supernatant, **C.** mass spectromery of the immunoprecipitated band; FAM129C (shown as Niban-like protein 2, NIBL2_HUMAN; 4 unique peptides, 6% protein coverage. mass spectromery of the identified bands, showing 3 peptide coverage.

3.4 Discussion

3.4.1 Expression of FAM129C

3.4.1.1 FAM129C in haematological malignancies

FAM129C was found to be B cell specific on mRNA and protein level. FAM129C is expressed from the earliest stages of B-cell differentiation up to the germinal centre, where the gene is down-regulated, only to be re-expressed at high levels in memory B cells once again (see Figure 3.32). Among mature B-cell malignancies, high level FAM129C expression was observed in both ABC DLBCL and CLL, both of which may represent transformation of activated memory B cell.

FAM129C was isolated from theplasma membrane from CLL patients, therefore I investigated the level of FAM129C expression and relation to prognostic parameters.

There was no correlation between the relative levels of *FAM129C* mRNA and stage at diagnosis, gender, time to progression and most importantly with *IGHV* mutation status, therefore indicating that FAM129C does not play a role in BCR signalling. deleted The levels of protein were comparable to each other whereas mRNA levelwas varied, however this observation should be further investigated in a large number of patients and protein/mRNA extracted in parallel.

An important observation was that FAM129C was consistently expressed in all tested ABC DLBCL cell lines, suggesting a potential role as a marker for this type of lymphoma. Together, these data suggest that FAM129C should be a useful marker for ABC DLBCL subtype. However, at the moment the lack of suitable antibodies for IHC precludes its routine adoption in screening panels.

3.4.1.2 FAM129C Subcellular Localisation

FAM129C is not localised in a specific cell compartment and is localised mainly in the cytosol (possibly mitochondria) and, to a lesser degree, in the nucleus. It was also found associated with the membrane probably by the pleckstrin homology domain and this is probably why it was identified in the plasma membrane preparations by Boyd *et al.* The move of FAM129C between the cellular compartments post stimulation was investigated using the mechanical fractionation and it did not identify any displacement. This method in comparison to the confocal microscopy is very crude and may have missed subtle changes between cellular compartments.

3.4.2 Function of FAM129C

The investigation of the function of FAM129C was difficult, there were no indications in either bioinformatics or in the literature as to the possible pathway in which it might be involved. From bioinformatic analysis (Chapter 4) I knew that:

- FAM129C probably was not a transmembrane protein as it was previously thought (Boyd *et al.*, 2003);
- It contained a pleckstrin homology domain, which probably docks the protein to the membrane;
- The phylogenetic analysis suggested that FAM129C was present only in mammals and underwent significant evolution from its family members: FAM129A and FAM129B, therefore most likely evolved its function as well.
-

3.4.2.1 FAM129C and quiescence

Our original hypothesis for the possible function of FAM129C was in maintaining cellular quiescence and preventing cellular proliferation. For example, FAM129C was isolated from circulating CLL cells from the peripheral blood, which are predominantly in G0/G1 state and rapidly down-regulated when CLL cells are stimulated to undergo proliferation on the CD154/IL4 *in vitro* system or by PMA. However, set against this, is the expression of FAM129C in both B-cell precursors and ABC DLBCL both of which proliferate rapidly. The expression of FAM129C in ABC DLBCL is unanticipated and likely to be pathological given the constitutive activation of NF-κB seen in these malignancies (Davis *et al.*, 2001b; Lenz *et al.*, 2008b). In both normal B cells and in CLL, FAM129C is rapidly down-regulated once NF-κB is activated. How ABC DLBCL avoid this down-regulation is not clear. Preliminary whole genome sequencing data indicate that FAM129C may be mutated in this subgroup of disease (Prof R Gascoyne BCCA Vancouver, Canada, personal communication).

I reanalysed the gene expression microarray data (Davis *et al.*, 2001b; Lenz *et al.*, 2008b) to look for correlations between signature ABC DLBCL genes (NF-κB target genes) and FAM129C and I did not find any correlation with *TRAF2, BCL2, CFLAR* (encoding c-FLIP), with *CARD11* the correlation with *IRF4* is weak: R^2=0.3 (Rosenwald *et al.*, 2002)

The Chan group (Shen *et al.*, 2004) reported gene expression profiles in different types of mature B cells: in GC, mantle zone and marginal zone. The authors first microdissected the three compartments from normal snap frozen tonsil and spleen. The cells from each of the three compartments were purified by both magnetic beads

purification and FACS sorting before total RNA was extracted and further amplified with T7 RNA. Analysis of gene expression was performed using Lymphochip cDNA microarray containing 7399 genes. I analysed their gene expression data for *FAM129C* (Figure 3.32).

This analysis confirms my QRT-PCR data showing that the highest level of expression is in the naïve B cell, there was a significant difference when cells from GC were compared to mantle zone (p=0.0163, unpaired student t-test) or when GC, mantle and marginal zone were compared together (p=0.0057, one way ANOVA).

There are a number of proteins which were upregulated at the pre-B cell stage, for instance, CXCR4 (C-X-C motif chemokine receptor 4) (Figure 3.3).

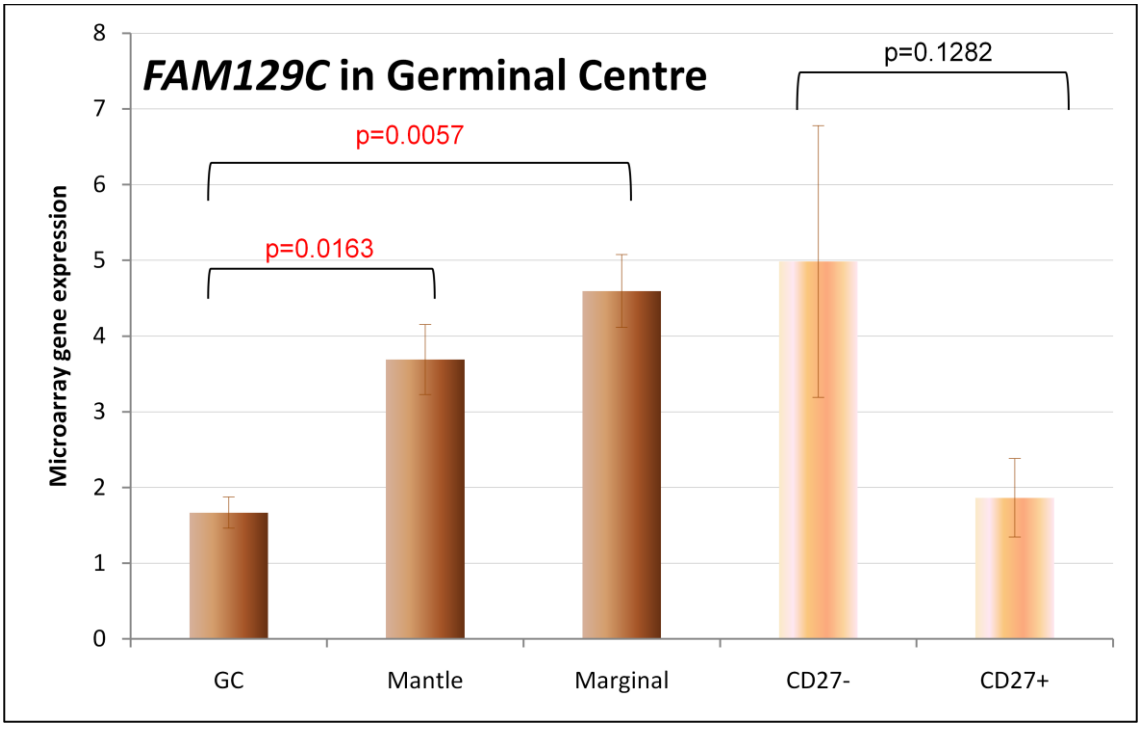


Figure 3.31 *FAM129C* expression in normal dissected tonsil (dataset from Shen *et al.*)

GC, germinal centre; Mantle, mantle zone cells; Marginal, marginal zone cells. CD27-, purified naïve cells; CD27+, purified memory cells. Student unpaired t-test performed on analysis between GC, Mantle and CD27- ,CD27+, One way ANOVA to compare GC, Mantle and Marginal zones. Significant p values are shown in red.

CXCR4, similarly to FAM129C, appears at the pre-B stage. CXCR4 is a chemokine receptor (ligand: SDF-1) and is required for GC B cell positioning in the dark zone. SDF-1 is highly expressed in the dark zone and low in the light zone and therefore appears to be an important factor controlling cell positioning in the dark zone (Allen *et al.*, 2004).–Similar expression of CXCR4 and FAM129C support the suggestion that FAM129C might be involved in GC reaction.

3.5 Summary

1. FAM129C was highly B cell specific both on the RNA and protein levels
2. The highest levels were seen in normal B cells
3. It may be a potential diagnostic marker for ABC DLBCL.
4. FAM 129C is mainly residing in the cytosol, and probably associated with the cytoskeleton as a part of large protein complexes
5. FAM129C first appears at the pre-B stage, reaches its peak in naïve cells, levels fall at the entry to GC and particularly at the centroblasts stage and is re-expressed again in the memory cells.(Figure 3.33)
6. It contains PHD which suggests it might play a role as signalling adaptor
7. FAM129C is downregulated by NF-κB and upregulated in cells with constitutive NF-κB activation, therefore FAM129C might play a role as a NF-κB signalling adaptor protein, modulating NF-κB signalling.

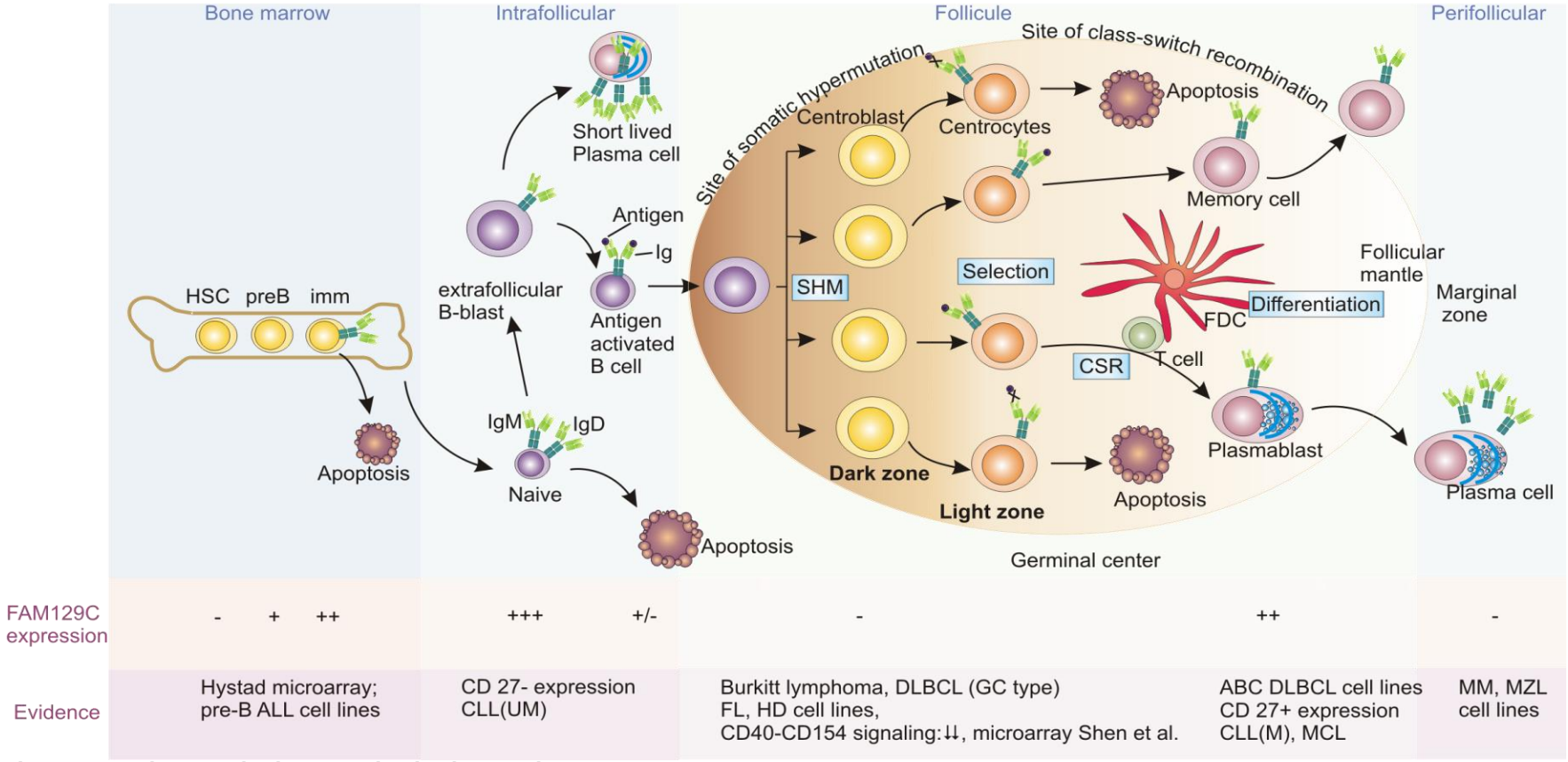


Figure 3.32 The germinal center (GC) microenviroment.
Antigen activated B cells differentiate into centroblasts in the dark zone of the GC, during the proliferation somatic hypermutation introduces mutations into V(D)J regions; centroblasts differentiate into centrocytes and move into the light zone. In the light zone the selection takes place: only GC B cells with high affinity to the antigen and the ones which successfully competed for the T cell help survive. The GC B cells with the low affinity, autoreactive or intermidiate affinity which failed to acquire T cells help die through Fas mediated apoptosis. FAM129C appeared at the pre-B stage and persisted throughout B cell development with the exception of dark zone at the GC.HSC: haemopoietic stem cells, imm: Immature B cell; ALL: acute lymphocytic leukaemia; CLL (UM): unmutated chronic lymphocytic leukaemia, CLL (M): mutated CLL, DLBCL: diffuse large B cell lymphoma; HD: Hodgkin disease; FL: follicular lymphoma; MM: multiple myeloma; MZL: marginal zone lymphoma. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer 5(4):251-262 copyright (2005) http://www.nature.com and World Health Organization (2008). WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon, International Agency for Research on Cancer

Chapter 4: Bioinformatic analysis of FAM129C

4.1 Introduction

FAM129C was identified during proteomic screening of purified CLL plasma membranes for novel cell surface markers (Boyd *et al.*, 2003). From the initial characterisation, FAM129C was thought to have three transmembrane domains and quantitative mRNA expression studies showed it to be upregulated in CLL patients. It also appeared to be elevated in lymphoid malignancies in comparison to normal B cells (peripheral blood, spleen, and tonsil). As FAM129C presented a potential for a prognostic factor I carried out a bioinformatic analysis of FAM129C with its aim to understand the structure and possible predictive characteristics and functions for this new protein which could explain experimental work presented in Chapter 3 and further help to understand function of this protein.

The bioinformatic methods are described in this chapter rather than in the Materials and Methods chapter for clarity and because they are an integral part of the analysis.

4.2 Gene and Isoforms

As FAM129C/BCNP1 (B cell novel protein 1) was recently identified by the analysis of plasma membrane fractions purified from CLL samples (Boyd *et al.*, 2003) and in 2006 the protein was renamed FAM129C by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) (Table 4.1). FAM129C is a member of a family of proteins which also include FAM129B and FAM129A. FAM129C is homologous to FAM129A which is also called NIBAN and has been described in mouse (Majima *et al.*, 2000). The sequence identity between FAM129C and A is 25.75%, and FAM129C and B is 25.23% (Figure 4.1).

Table 4.1 Nomenclature of the FAM129C family.

New Gene Symbol	Gene Name	Aliases	Gene ID
FAM129A	family with sequence similarity 129, member A	C1orf24, NIBAN	116496
FAM129B	family with sequence similarity 129, member B	C9orf88, DKFZP434H0820, FLJ13518, FLJ22151, FLJ22298, bA356B19.6	64855
FAM129C	family with sequence similarity 129, member C	BCNP1, FLJ39802	199786

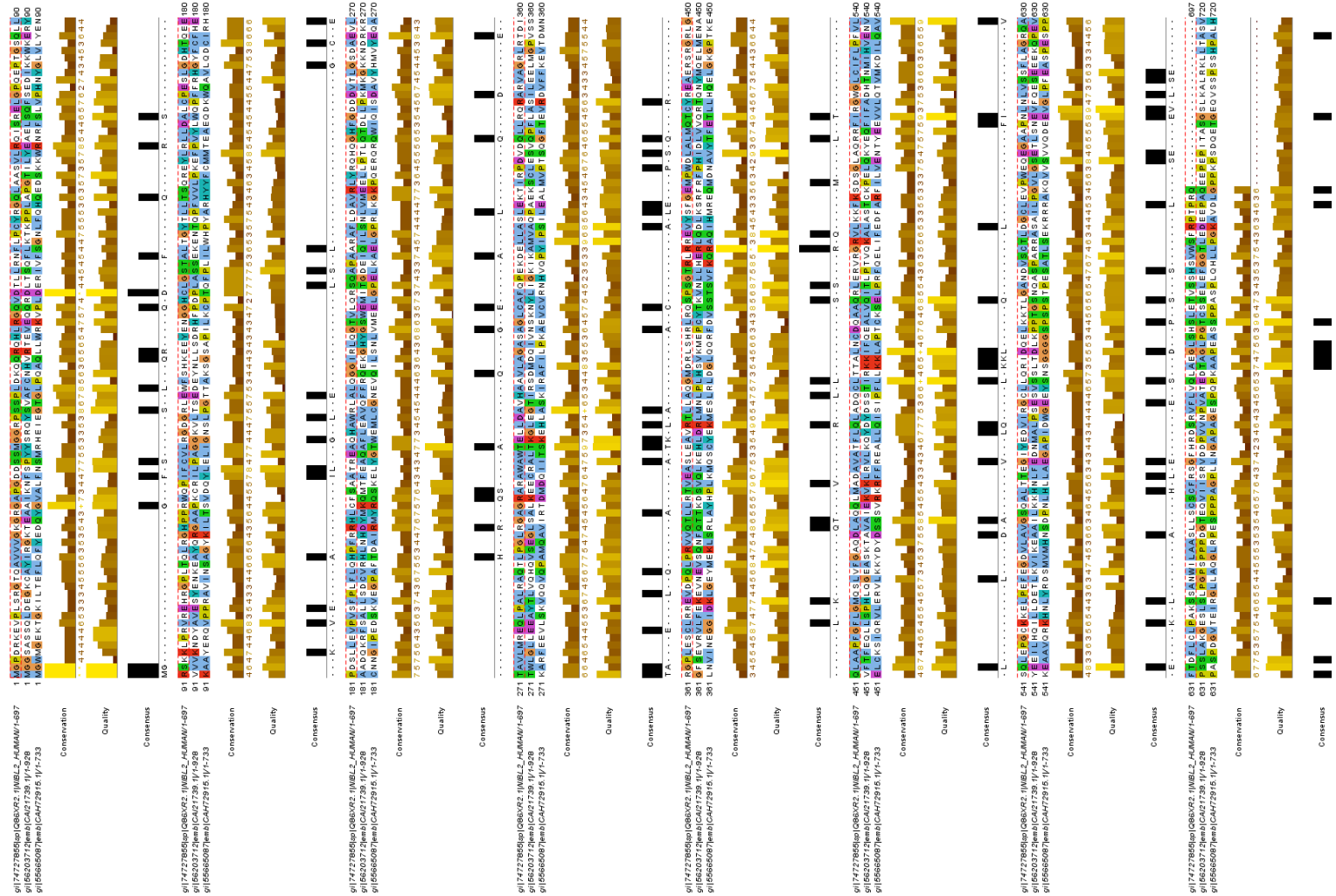


Figure 4.1 The alignment of FAM129 family members using Jalview.

The first sequence is FAM129C (74727855), second FAM129A (56203712) and third FAM129B (55665087). Conservation, Quality and Consensus histograms below the alignments are part of Jalview output. Conservation is calculated after sequences were clustered. Each column in the alignment is given a score from 0 to 10 (fully conserved) based on the common physico-chemical properties of the residues. Quality of the alignments is the BLOSUM62 score based on observed substitutions. Consensus shows which residues are most abundant in the alignment at each position and gives percentage for each column of the alignment.

The *FAM129C* gene is located on chromosome19p13.11 and consists of 16 exons (isoform a, Table 4.2), this gives rise to a cDNA of 2,073 bp long and encodes a protein of 620 amino acid residues. There is some discrepancy over the number of isoforms. Boyd *et al.* originally described three main isoforms, which share the same sequence up to position 1981, but differ at the 3' UTR. Isoform 1 (AY254197) has 666 residues, isoform 2 (AY254198): 651 and isoform 3 (AY254199): 697 residues. Aceview (NCBI) lists seven possible transcripts (Table 4.2 and 4.3, Figure 4.2 (Thierry-Mieg & Thierry-Mieg, 2006)). The existence of isoform c is questionable because there is only one partial clone identified and contrary to the data on expression (Boyd *et al.*, 2003) and in this thesis, (see results chapter) isoform c was only identified from testis. Alternatively it may suggest it is either cell specific or with a limited tissue distribution. Isoform d is the most credible, as it was identified from 17 clones. The Japanese database H-InvDB: http://www.jbirc.aist.go.jp/hinv/spsoup/locus_view?hix_id=HIX0020023&status=full lists seven isoforms, similar to Aceview: these include the same protein isoforms: a, b, c, d, e protein, but there are no isoforms f and g. The isoforms originally submitted by R. Boyd (AY254198: isoform 2 and AY254197: isoform1) are included. Ensembl (Table 4.4) lists 4 isoforms: 3 are corresponding to AceView isoforms a, b, c and the fourth is originally described isoform 2.

Table 4.2 mRNA structure and expression (ACEview accessed April 2007) with permission

mRNA variant	# of exons	# of clones	Tissue	5' completeness evidence	3' completeness evidence	coordinates on gene
a	16	8	germinal center B cell , serous papillary carcinoma, high grade, pooled tumors, uterus, dorsal root ganglia	aggregated clones	validated polyA	1 to 30547
b	15	3	spleen	capped	3' stop	3964 to 28730
c	14	1	embryonal carcinoma, cell line, testis	no evidence	3' stop	4004 to 28732
d	15	17	germinal center B cell, b-cell, chronic lymphocytic leukemia, liver and spleen, spleen	capped	validated polyA	3969 to 30345
e	3	3	lymph , lymphoma, cell line, lymphoma	5' stop	3' stop	16852 to 20691
f	4	2	Burkitt lymphoma,	5' stop	validated polyA	4001 to 13438
g **(unspliced)**	1	2	B-cells, primary B-cells from tonsils (cell line), spleen	5' stop	3' stop	10197 to 11071

Table 4.3 Predicted proteins
(Aceview accessed April 2007) with permission.

Protein	Protein quality	Exons in CDS	Predicted localisation	Completeness	Extends from	coordinates on mRNA	Min set of supporting clones
a	697 aa	16	cytoplasmic	complete	Met (ATG) to Stop	139 to 2232	AY254199
b	620 aa	15	cytoplasmic	complete	Met (ATG) to Stop	78 to 1940	AK097121
c	596 aa	14		COOH complete	1st codon to Stop	2 to 1792	BC053606
d	562 aa	13	cytoplasmic	complete	Met (ATG) to Stop	73 to 1761	AK074069
e	180 aa	3		complete	Met (ATG) to Stop	372 to 914	BC033225
f	111 aa	3		complete	Met (a..CTG.) to Stop	99 to 434	BE513656
g	83 aa	1	mitochondrial	complete	Met (ATG) to Stop	182 to 433	BQ707062

Protein c has a partial cDNA according to Unigene (highlighted in red). LZD-leucine zipper domain), d is the predicted target of nonsense mediated mRNA decay (NMD) candidate.

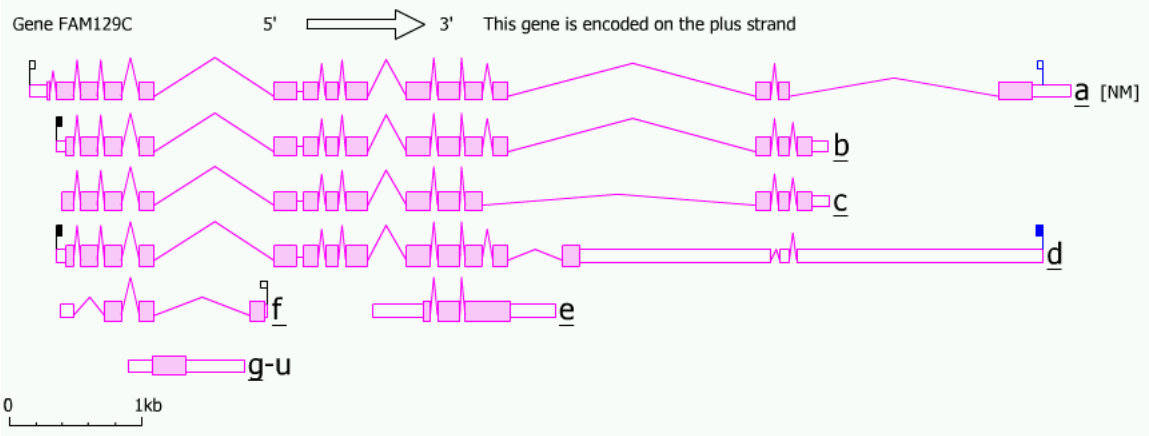


Figure 4.2 Diagram of alternative mRNA of FAM129C aligned from 5' end to 3' end (from AceView with permission) Isoform names represent variants listed in the Table 1.2. Pink rectangles are exons, size of the rectangle is proportional to their length; joining lines are introns, the height of the joining line reflects the number of cDNA clones supporting the introns. Black flag at the 5' end on variant b and d represent capped 5'end. Flags at the 3' shows validated 3' end. Isoform a, is a representative transcript, it was originally described by Boyd as isoform 3. Isoform c is only supported by one clone, isoform f and g by two clones.

Table 4.4 Isoforms listed by Ensembl compared to AceView isoforms and original isoforms described by R. Boyd.

Clone	Protein	Corresponding AceView Isoform (AceView)	Corresponding Original Isoform (R. Boyd)
AK074069	Q8TEQ3	d	n/a
NM_001098524	NP_001091994.1 (isoform b)	n/a	Isoform 2 (AY254198)
NM_173544	NP_775815 (isoform a)	a	Isoform 3 (AY254199)
BC053606,	AAH53606.1	c	n/a

Isoform 1, described originally in the Boyd paper, has not been mentioned in the AceView

As described above, there is contradictory information in the databases about existing isoforms and splice variants. The work described in the Results section is based on Isoform a (Aceview) or Isoform 3 (Boyd *et al.*, 2003) which is the longest form (Table 4.4).

4.3 Protein alignment

There are a variety of bioinformatic tools for investigating the putative structure/function relationship in a new protein.

BLAST (Basic Local Alignment Search Tool) is one of the most popular algorithms for searching sequence databases (Altschul *et al.*, 1990). BLASTP searches for protein sequences using BLOSUM matrices to score the alignments. The aligned sequences are scored by the amount of credit for each aligned pair of residues. Hydrophobicity, charge, electronegativity and size are implicitly taken into account, e.g. similar aromatic amino acids receive a significant positive score. BLOSUM-62 is appropriate for comparing sequences of approximately 62% sequence similarity.

In order to search a large database efficiently, BLASTP breaks down the sequence into 4 letter words. Each word will overlap with the subsequent one with 3 characters. Words composed of the most common aminoacids will be discarded. The database sequences are searched for the identified words. Each time a word is matched, the match is extended in both directions from the matching word until the alignment score falls below given thresholds. Gaps are inserted to optimize the alignment (Krane & Raymer, 2002).

To improve the specificity of database search, the Position Specific Iterative BLAST (PSI-BLAST) can be used http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. PSI-BLAST analysis is useful both for identifying the distant members of a protein family, whose relationship is not recognisable by straight sequence comparison, and also for

deducing the function of hypothetical proteins that are not annotated in the database. The program first performs a gapped BLAST search. Then another BLAST search is performed, using hits from the profile sequence to produce a position-specific scoring matrix based on which positions evolution has conserved *v.* which positions evolution has allowed to vary over time. The sequences found after the first round are added to the profile The PSI-BLAST program uses the information from any significant alignments returned to construct a position-specific score matrix, which replaces the query sequence for the next round of database searching. PSI-BLAST may be iterated until no new significant hits are found.

I applied the above bioinformatics tools to analyse FAM129C. The NCBI database was searched using FAM129C protein sequence (annotation NP_775815). E values of 10^{-3} and below are considered as statistically significant results and therefore to increase the likelihood of evolutionary relationship between the query sequence and the search results the PSI-BLAST threshold was set at 0.000001. Two hundred and thirty two sequences were collected, saved in FASTA format and aligned using Muscle v.3.6 (Multiple sequence comparison by log-expectation, (Edgar, 2004). The sequences were then viewed using http://www.jalview.org/ (Clamp *et al.*, 2004)**.**

The generated alignment was visualized in Jalview. The number of aligned sequences was reduced to 40 by removing duplicate and partial sequences. FAM129C differs from other aligned sequences by 30 residues at the N terminus. There is a second Methionine in FAM129C (residue 32) which appears to be a start codon in other aligned sequences. There was close alignment between residues 32 and 600 within these 40 selected sequences. The C terminus beyond residue 608 was less conserved. FAM129C group has the shortest C terminus in the alignment, whereas the FAM129B had the longest.

4.4 Phylogenetic tree

Phylogenetic trees allow understanding of the relationship of the protein of interest with other proteins within their family and show possible ancestral relationship among sequences. It may also help to understand biological functions, if the functions of related proteins are known.

The alignment generated by the Muscle programme (see above) was further modified either manually or using G blocks http://molevol.ibmb.csic.es.

Gblocks is a computer program that eliminates poorly aligned positions and divergent regions of an alignment of DNA or protein sequences. Gblocks selects conserved blocks from a multiple alignment according to a set of features of the alignment positions. The use of Gblocks reduces the necessity of manually editing multiple alignments and makes the automation of phylogenetic analysis of large data sets feasible. The final alignment was used to generate a phylogenetic tree using 2 different methods.

1- distance method Neighbour Joining (NJ) (ClustalW);

2- maximum likelihood method (ML) (http://www.tree-puzzle.de v.5.2) (Schmidt *et al.*, 2002). The phylogenetic trees were visualized in TreeView v.1.6.6 program http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

Neighbor Joining (NJ) algorithm sets neighbors that minimise the total length of the tree. NJ starts with a star-like tree with all species coming off a single central node. Neighbors are than sequentially found that minimise the total length of the branches. Maximum likelihood (ML) is a purely statistically based method of phylogenetic reconstruction. ML method searches for the optimal choice by assigning probabilities to possible evolutionary change, therefore maximising probability of the tree. The tree is shown in Figure 4.3.

Using the two different methods resulted in essentially the same tree. As both methods gave the same prediction, confidence in the phylogenetic prediction is high. The tree presented in the Figure 4.3 is an unrooted phylogenetic tree. Rooted trees start from the single common ancestor (root), whereas unrooted trees only specify relationship between nodes and they do not specify the direction of evolution in which evolution occurred. Proteins shown in the Figure 4.3 have a common ancestor. The set of aligned sequences does not provide sufficient information to determine where the root is but this tree helps in understanding the relationship between similar sequences.

There are four clear groups visible in the tree: FAM129C (BCNP1 group, red), NIBAN group (FAM129A, green), FAM129B (blue) and ‘the fish group’ (black). Three main groups; FAM129A, FAM129B and FAM129C have similar representation in mammals. The ‘fish group’ is represented by three proteins in jawed fish: Danio rerio and Tetraodon nigroviridis. All the three proteins present in fish appear to be evolutionary distant from each other. Unlike FAM129B and the FAM129C group, FAM129A (NIBAN family) has a bird representative (Gallus gallus). The only example of reptiles (frog: Xenopus laevis) is seen in the FAM129B group. The frog protein has a long branch in

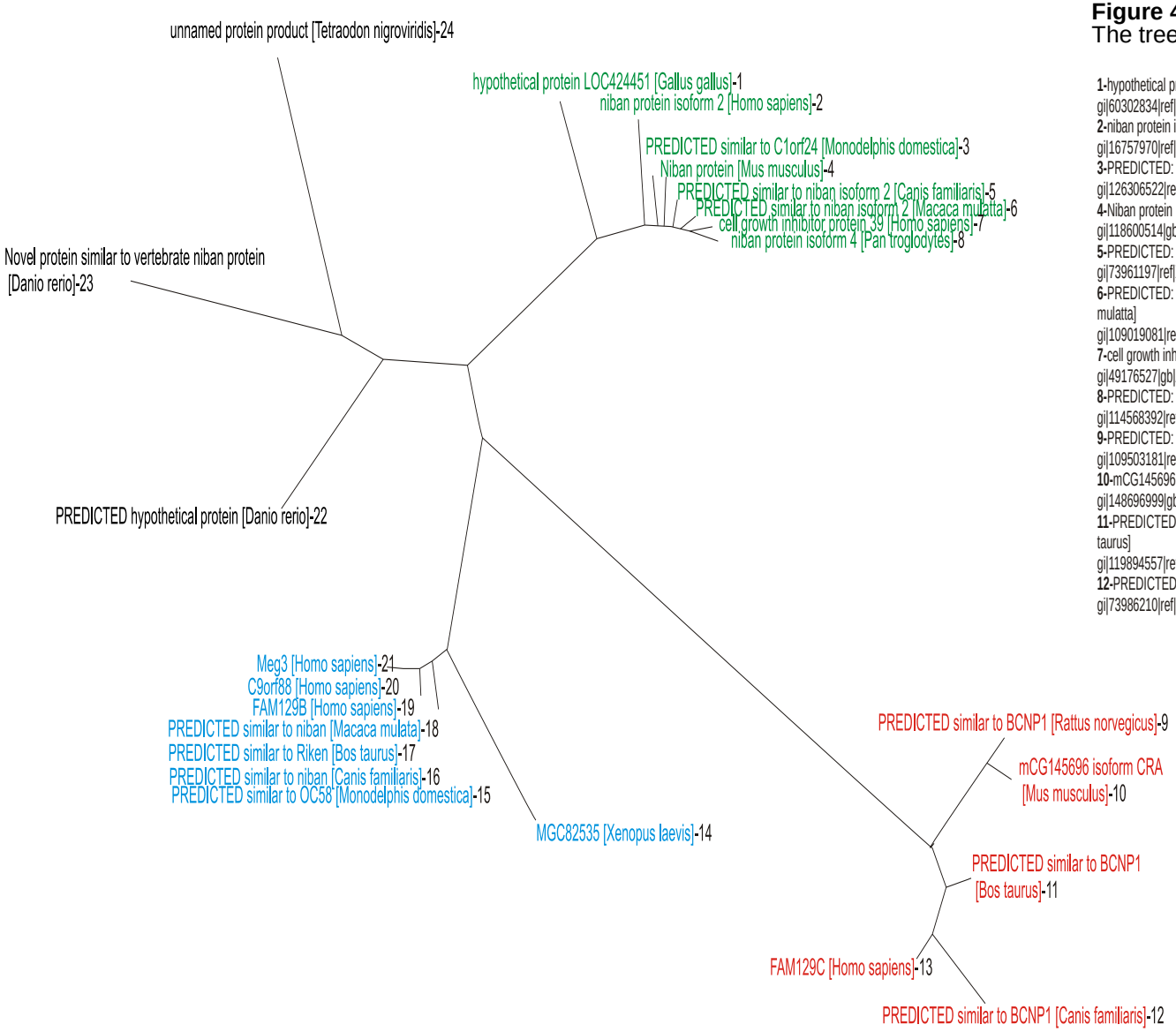


Figure 4.3 FAM129C phylogenetic tree.
The tree was viewed by Treeview

1-hypothetical protein LOC424451 [Gallus gallus]
gi|60302834|ref|NP_001012614.1
2-niban protein isoform 2 [Homo sapiens]
gi|16757970|ref|NP_443198.1
3-PREDICTED: similar to C1orf24 [Monodelphis domestica]
gi|126306522|ref|XP_001375714.1
4-Niban protein [Mus musculus]
gi|118600514|gb|AAH21332.1
5-PREDICTED: similar to niban protein isoform 2 [Canis familiaris]
gi|73961197|ref|XP_537163.2
6-PREDICTED: similar to niban protein isoform 2 isoform 2 [Macaca mulatta]
gi|109019081|ref|XP_001113897.1
7-cell growth inhibiting protein 39 [Homo sapiens]
gi|49176527|gb|AAT52218.1
8-PREDICTED: niban protein isoform 4 [Pan troglodytes]
gi|114568392|ref|XP_001164202.1
9-PREDICTED: similar to B-cell novel protein 1 [Rattus norvegicus]
gi|109503181|ref|XP_573881.2
10-mCG145696, isoform CRA_a [Mus musculus]
gi|148696999|gb|EDL28946.1
11-PREDICTED: similar to B-cell novel protein isoform 1; BCNP1 [Bos taurus]
gi|119894557|ref|XP_593682.3
12-PREDICTED: similar to B-cell novel protein 1 [Canis familiaris]
gi|73986210|ref|XP_852572.1
13-FAM129C protein [Homo sapiens]
gi|31657200|gb|AAH53606.1
14-MGC82535 protein [Xenopus laevis]
gi|148236293|ref|NP_001085586.1
15-PREDICTED: similar to OC58 [Monodelphis domestica]
gi|126297627|ref|XP_001365096.1
16-PREDICTED: similar to niban protein [Canis familiaris]
gi|73968197|ref|XP_851563.1
17-PREDICTED: similar to RIKEN cDNA 9130404D14 gene isoform 1 [Bos taurus]
gi|119904139|ref|XP_869626.2
18-PREDICTED: similar to niban protein [Macaca mulatta]
gi|109112214|ref|XP_001095814.1
19-FAM129B protein [Homo sapiens]
gi|45501018|gb|AAH67366.1
20-C9orf88 variant protein [Homo sapiens]
gi|68533087|dbj|BAE06098.1
21-MEG3 [Homo sapiens]
gi|14248495|gb|AAK57556.1
22-PREDICTED: hypothetical protein [Danio rerio]
gi|68355884|ref|XP_689912.1
23-novel protein similar to vertebrate niban protein [Danio rerio]
gi|56207511|emb|CAI21333.1
24-unnamed protein product [Tetraodon nigroviridis]
gi|47212454|emb|CAF94106.1

comparison to other FAM129B members, which suggest that the reptile form of FAM129B may have slower evolution from its mammal counterparts. The NIBAN family members in mammals appear to be very closely related. The human NIBAN is similarly different from the whole group as its bird counterpart. FAM129B and FAM129C evolved from the same ancestor, most likely by gene duplication. The FAM129C family has the longest branch out of all four groups. The length of the branch signifies either a lapsed time, measured in years or number of molecular changes (e.g. mutations) that have taken place between the two nodes. FAM129C appears to be more distinct from the other 3 groups. Unlike in other groups, members of FAM129C are represented only in mammals. The longest branch and present only in mammals suggest that FAM129C may have changed function during evolution (paralog) or could result from a mammalian duplication of the NIBAN gene. In the phylogenetic tree based on FAM129C alignment the lowest animals are cartilaginous fish. Searches of Drosophila and nematode protein databases did not identify homologous proteins.

FAM129C may be involved in adaptive immunity. It is known from previous work (Boyd *et al.*, 2003) and data presented in this thesis (see Results) that FAM129C is B cell specific. Information from the phylogenetic tree and the experimental data together support the notion that FAM129C has a role in immunoglobulin rearrangement and/or on further fate of immunoglobulins in B cells. Adoptive immunity appears suddenly in cartilaginous fish. Innate immunity appears very early in evolution: it is present in early eukaryotes like amoeba and it is well developed in *Drosophila melanogaster* and in many invertebrates including *Caenorhabditis elegans* (Janeway, 2001; Murphy *et al.*, 2008). The presence of RAG (recombination-activating-genes) enables jawed vertebrates and other higher vertebrates to encode enzymes essential for the rearrangement of the immunoglobulin and T cell receptor genes. Other important features appearing in the cartilaginous fish are: somatic hypermutation, polymorphic MHC class I and class II molecules, presence of thymus and secondary lymphoid tissues (Flajnik, 2002). Further in the evolution, amphibians acquire class switch recombination but birds are the first to have germinal centre formation.

A phylogenetic tree may help in hypothesising a possible function of the unknown protein if the functions of related proteins are known and the evolutionary process is short. The physiological function of FAM129A (NIBAN) is unknown. FAM129A has been associated with renal cell carcinoma in both animals and human (Adachi *et al.*, 2004). The NIBAN knockout mice do not show an obvious phenotype(Sun *et al.*, 2007). In the same study NIBAN was shown to be involved in endoplasmic reticulum (ER)

stress response and has an effect on apoptosis via regulating translation (via phosphorylation of eukaryotic translational initiation factor-eIF). FAM129B was only recently identified as a B-Raf phosphorylation target in melanoma using functional proteomics and it was proposed it controlled cell invasion into three-dimensional extracellular matrix in a phosphorylation-dependent manner (Old *et al.*, 2009).

4.5 Secondary and tertiary structure predictions

The secondary structure was predicted using neural networks computational techniques (PROF) provided by PredictProtein. Protein domains were identified by searching the Interpro, Superfamily (Gough *et al.*, 2001) and Pfam databases (Finn *et al.*, 2006). Identified domains were the pleckstrin homology domain. Creating an alignment of the PH domain to FAM129C was done by collection of PH sequences has been uploaded from Pfam. These sequences were copied to HHpred http://toolkit.tuebingen.mpg.de/hhpred (Biegert *et al.*, 2006). The HHpred software identifies conserved residues. With this information the FAM129C PH sequence has been manually aligned to the collection of other PH containing proteins.

4.5.1 Does FAM129C contain the Pleckstrin homology domain?

The FAM129C protein is predicted to contain a Pleckstrin homology domain (PHD). The PHD was originally identified in 1993 (Haslam *et al.*, 1993; Mayer *et al.*, 1993) and its name is derived from platelets and **l**eukocytes C kinase substrate protein. PH is a domain of approximately 100-120 residues and is found in a variety of organisms from yeast to humans (there are 27 yeast protein and 252 human proteins containing PH domain) and is the 11th most abundant domain in the human genome (Lemmon *et al.*, 2002). Its function appears to be very diverse (Lemmon, 2004) and it is found in many molecules involved in cellular signalling, cytoskeleton organisation, membrane trafficking and phospholipid modification (Lemmon & Ferguson, 2000). PH domains were classified according to phosphoinositide binding affinity and specificity (Table 4.5) (Maffucci & Falasca, 2001).

Table 4.5 Classification of PH domain

Group characteristics	PHD examples	Phosphoinositides
Group 1, **high affinity to specific phosphoinositide, interaction is sufficient to target the host protein to plasma membrane.**	BTK	PtdIns-3,4,5-P_3
	PLC-δ1	PtdIns-4,5-P_2
	Grp1	PtdIns-3,4,5-P_3
	GAP1^{IP4BP}	PtdIns-3,4,5-P_3
	PKB	PtdIns-3,4,5-P_3, PtdIns-3,4-P_2
	Bam32/DAPP1	PtdIns-3,4,5-P_3, PtdIns-3,4-P_2
Group 2, **low specificity and/or affinity, unlikely to be sufficient to drive a translocaion of the protein to the plasma memebrane**	PLC-β1	PtdIns-3-P>PtdIns-3,4,5-P_3, PtdIns-4,5-P_2
	Dbl	PtdIns-4,5-P_2, PtdIns-3,4,5-P_3
	Gab1	PtdIns-3,4,5-P_3>PtdIns-4,5-P_2, PtdIns-3,4-P_2
	IRS-1	PtdIns-3,4,5-P_3>PtdIns-4,5-P_2, PtdIns-3,4-P_2
	PLC-γ1	PtdIns-3,4,5-P_3>PtdIns-3-P, PtdIns-4,5-P_2
Group 3, **non-specific binding**	Pleckstrin	non-specific
	Dynamin	non-specific
	DAG K- δ	non-specific

BTK, Bruton's tyrosine kinase; PLC-δ1, phospholipase C; Grp1, general receptor for 3-phosphoinositides; GAP1: GTPase activating protein 1; PKB, protein kinase B; DAPP1, dual adaptor for phosphotyrosine and 3-phosphoinositides1 (Bam32, B cell adaptor molecule of 32kDA); PLC-β1, phospholipase C- β1; Dbl, dbl oncoprotein; Gab1, Grb-2-associated protein 1; IRS-1,insulin receptor substrate 1; PLC-γ1, phospholipase C- γ1; DAG K- δ, diacylglycerol kinase-δ
Modified from FEBS Letters, 506(3): 173-9, Maffucci T. Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide–protein co-operative mechanism, Copyright (2001), with permission from Elsevier.

Figure 4.4 compares eight different PH domains. They have essentially the same highly conserved tertiary structure but the sequence alignments would show only 7%-23% primary sequence identity (Lemmon & Ferguson, 2000). The characteristic structure is the β sandwich structure composed of two nearly perpendicular β-sheets, one β sheet consists of four strands (β1 to β4) and the other from three (β5 to β7), they are connected with six 'variable' loops and at the C-terminal amphipathic α-helix.

'Variable loops' display hypervariable sequences in PH alignments (Cozier *et al.*, 2004). The loops join the membrane surface and PH domain binds the phosphoinositides located inside the lipid bilayer.

Several PH domains recognize particular phosphoinositides (Table 4.5) with high affinity and specificity (Lemmon & Ferguson, 2001), this recognition is important in phosphoinositidine 3-kinase signalling (Vanhaesebroeck *et al.*, 2001). Phosphoinositides are a family of phosphorylated derivatives of the membrane lipid phosphatidylinositol, they are present in cell's plasma membranes, endosomes and nucleus. Only a small fraction of the PH domains, approximately 10%, binds specifically phosphoinositides (see Table 4.5) (Rebecchi & Scarlata, 1998; Yu *et al.*, 2004) and probably the PH domain needs assistance from other domains to achieve

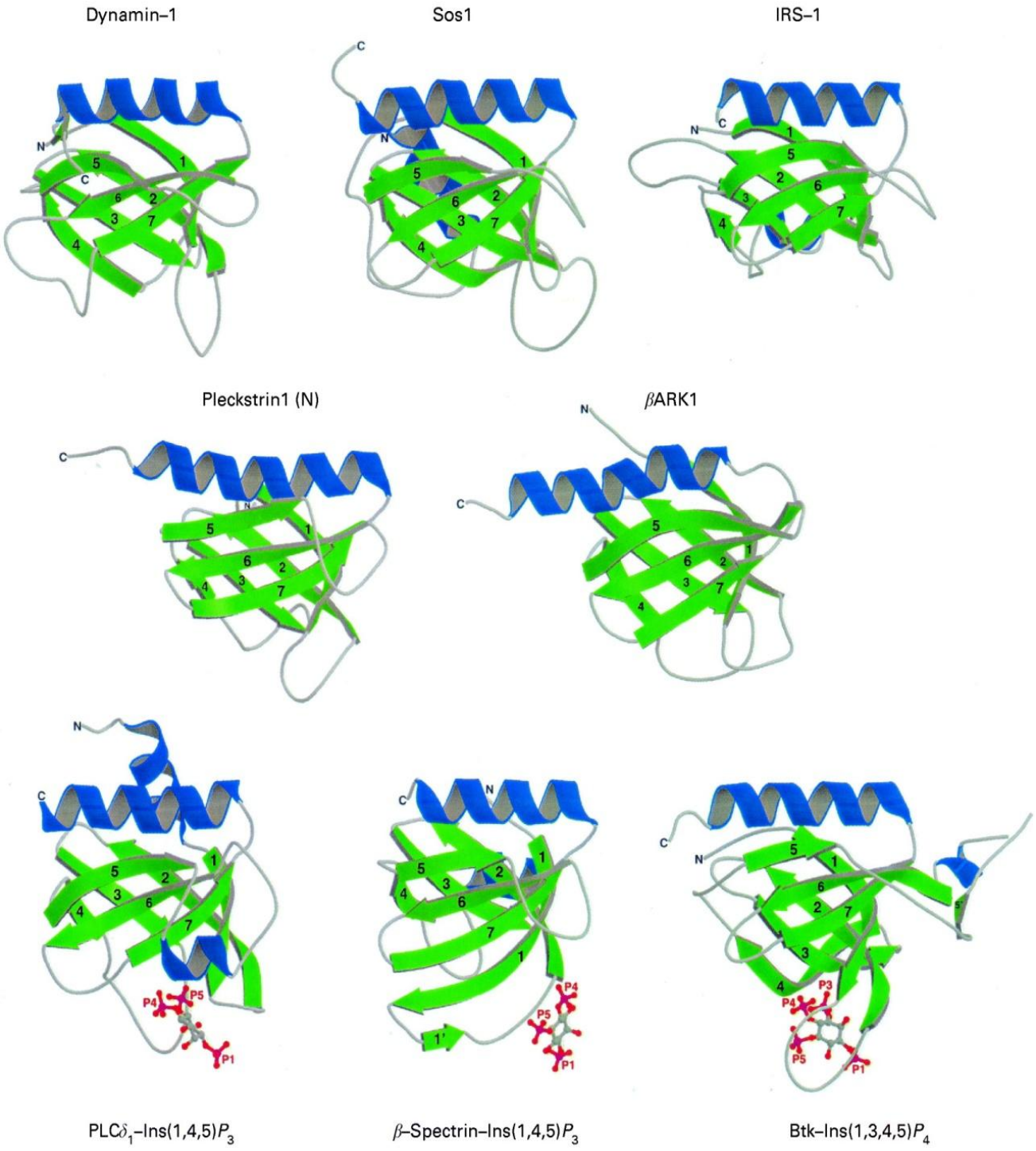


Figure 4.4 Comparison of PH domains with known structures.

Blue are α-helices and green are β strands. In red and grey is the marked position of phosphoinositide Ins(1,4,5)P_3, phosphate groups in red, inositol moiety in grey. Pleckstrin 1 and βARK1 are NMR derived structures, others: X-ray crystallography. With a kind permission from Lemmon and Ferguson, 2000.

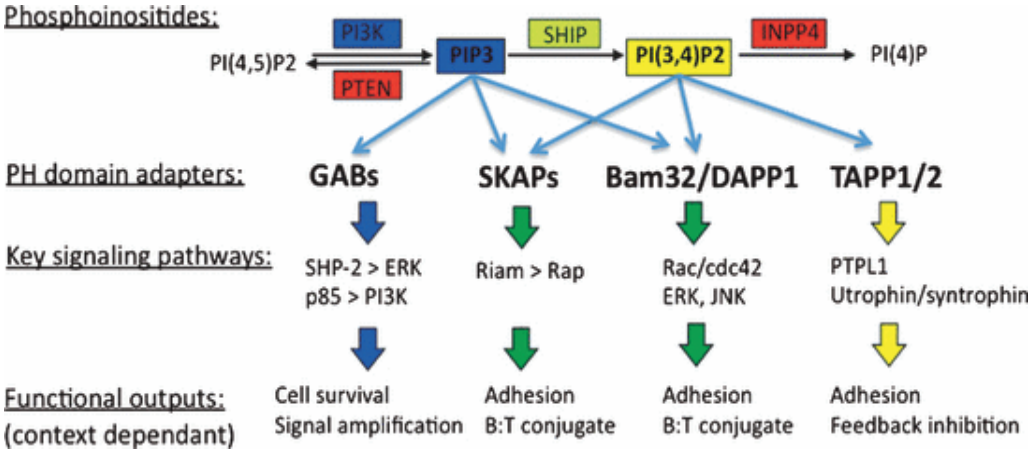


Figure 4.5 Schematic illustrating the regulation and functions of 3-phosphoinsitides and PH domain adapters in immune cells.

In the top portion, a simplified model of the PI3K signaling pathway is presented, indicating the roles of lipid phosphatases in controlling levels of specific phosphoinositides. In the bottom portion, PH domain adapters are indicated (note many other PH domain proteins are present in immune cells). Blue arrows indicate direct binding of PH domain adapters to specific 3-phosphoinositides present in the plasma membrane. Block arrows indicate causal signaling linkages, which in some cases involve multiple intermediary steps. PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase.

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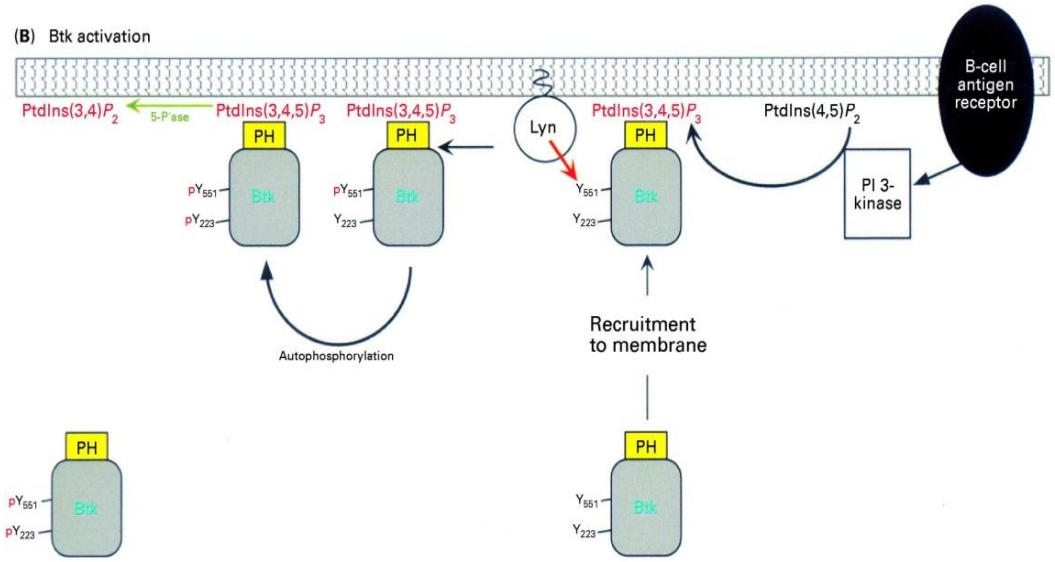

Figure 4.6 Role of PH domain in Btk: Btk activation by BCR-induced PI3-kinase.

SRC-family kinase LYN is constitutively membrane associated. PH domain recruits BTK to the membrane by recognising PI3-kinase products, brings close to LYN, which in turn phosphorylates BTK. BTK autophosphorylates itself and becomes active.

From Lemmon and Ferguson, 2000

their function. The majorities of PH domains bind phosphoinositides with low affinities and with low specificity (Takeuchi *et al.*, 1997; Kavran *et al.*, 1998) and are unable to bind membranes independently *in vivo* (Yu *et al.*, 2004). Table 4.6 gives examples of proteins containing PH domain and therefore a range of functions the PH domain might be involved in.

Table 4.6 Examples of proteins containing PHD
(modified with kind permission from Springer Scince +Media Business from (Cozier *et al.*, 2004)

Proteins containing PHD	Examples
Tyrosine kinases	Bruton's tyrosine kinase (BTK)
Serine/threonine kinase	Protein kinase B (PKB) Phosphoinositide-dependent protein kinase 1B (PDK-1)
Regulators of small GTP-binding proteins (GTPase activating proteins and guanine nucleotide exchange factors)	Phosphatidylinositol 3,4,5 triphosphate Rac exchanger 1 (P-Rex 1) protein ARAP1 (Centaurin) Cytohesin 2 (ARNO protein)
Signalling adaptor molecules	Growth factor receptor-bound protein 7 (Grb7), Insulin Receptor Substrate1 (IRS1) B cell adaptor molecule of 32kDA (Bam32/ DAPP1)
Proteins modifying membrane phospholipids (phospholipase C isoforms)	Phospholipase C-β1 Phospholipase C**-** δ1
Cytoskeletal proteins	Spectrin C. elegans UNC-104 protein
Proteins involved in the regulation of membrane traffic	Dynamin Oxysterol binding protein

GTPases are thought to be PH domain targets (Lodowski *et al.*, 2003). The PH domain probably binds membranes by other factors than binding phosphoinositides. 25-33% of PH domains target membranes by binding simultaneously to phosphoinositides and GTPases, or other molecules (Yu *et al.*, 2004). It is thought that there is probably another unknown factor that is similarly weak but probably more specific (Lemmon, 2004). Isoforms of protein kinase C (PKC) and associated proteins (Lemmon & Ferguson, 2000) are proposed to be a second main ligand for the PH domain. Proteins containing PH are phosphorylated by PKC. Good examples are pleckstrin in platelets and dynamin, both are phosphorylated by PKC.

As mentioned above a subset of PH domains which bind selectively PI (3,4)P_2 and/or PI (3,4, 5)P_3 are found in the following signalling proteins including (Zhang *et al.*, 2009):

- protein kinases: Bruton's tyrosine kinase (BTK), AKT, PDK1 (3-phosphoinositidine-dependent kinase 1);
- guanine nucleotide exchange factors (GEFs): VAV, Tiam1, GRP, SOS, P-Rex1
- guanosine triphosphatase (GTPase)-activating proteins (Arap3, Gap1m, centaurin-α),

- scaffold/adapter molecules: GRB-associated binder 1/2/3,
- SRC kinase-associated phosphoprotein (SKAP)/adhesion and degranulating-promoting adaptor protein (ADAP).
- PH domain adapters (Bam32/TAPP1/2) (regulation and functions of adaptors is summarised in Figure 4.5).

Subsets of PH domains which bind phosphoinositides non-specifically are pleckstrin, dynamin and DAG K-δ (Maffucci & Falasca, 2001) which suggests that the PH domain might serve more complex modulating factors with both lipids and protein recognition. Varnai speculated that PH domains might serve as molecular switches regulated by phosphoinositides (Varnai *et al.*, 2005). The importance of the PH domain is evidenced by mutations in the PH domain of the tyrosine kinase BTK leading to diminished kinase activity and presenting as immunodeficiency in both mice and humans (Rawlings *et al.*, 1993). The function of the PH domain in BTK is presented in Figure 4.6. The PH of BTK have highly selective binding to PIP3, which localizes to the membrane and to SRC kinases (LYN) which phosphorylate BTK and induces further autophosphorylation and hence activation.

Carpten described a mutation in the PH domain in AKT1, promoting aberrant membrane association by a PH domain and causing cancer (Carpten *et al.*, 2007).

The PH domain identified in the FAM129C sequence using Interproscan, Superfamily and Pfam. The PH domain is located between residues 99-227 of FAM129C (Figure 4.7) with the following probabilities: Interproscan 6.9e-4, Superfamily 5.3e-9. Probabilities more than 0.0001 were considered significant. PH was identified by Pfam with 0.0034. Figure 4.7.A shows a prediction of the secondary structure in FAM129C: the N terminus of the protein consists of β sheets (which represent PH domain) and the majority of the protein is formed by α-helices.

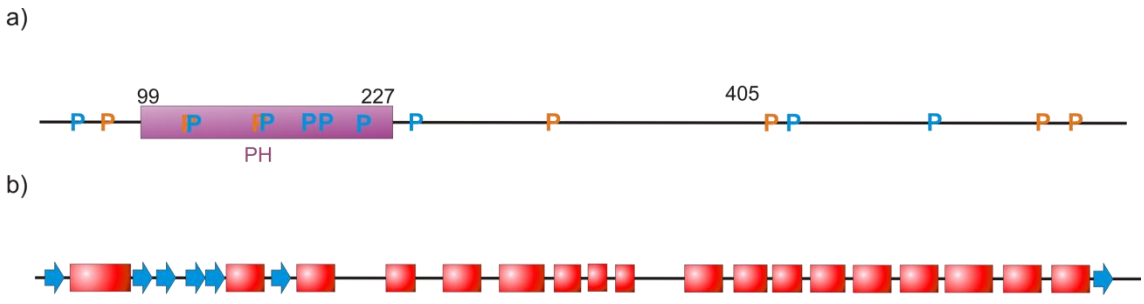

Figure 4.7 Schematic representation of domains found in FAM129C and secondary structure
A) domains: PH: Pleckstrin homology domain, P: putative phosphorylation sites, orange P: prediction for protein kinase C (residues: 92, 132, 157, 337, 406, 690, 697); blue P: prediction for casein kinase II (residues: 38, 132, 157, 172, 177, 183, 265, 424, 541); **B)** secondary structure red rectangles are α helices, blue arrows: β sheets.

Although PH domains have little similarities on the level of primary sequence, the known structure of PH shows remarkable conservation of 3 dimensional organisations as mentioned above. FAM129C's pleckstrin homology domain sequence was aligned to other examples of PH (Figure 4.8). There are certain residues strongly conserved, mainly hydrophobic (blue highlight in Figure 4.8). There are seven predicted β sheets aligned to other PH domains and an α-helix at the C terminus. It is very difficult from the sequence alone to classify FAM129C to a recognized class of PH domain and to predict its possible function. The currently available evidence for classification is based on the presence of single, highly conserved aminoacids. The Skolnik group suggested that PH domains binding with high affinity to phophoinositides have lysine at the end of strand β1 and arginine in the middle of strand β2 (Isakoff *et al.*, 1998). Levine *et al.* suggested that arginine in β2 is important for translocation to the Golgi apparatus in oxysterol-binding protein (Levine & Munro, 1998). The FAM129C PH domain does not have lysine in strand β 1 or arginine in β 2. I also compared conserved residues present in Bam32/DAPP1 and FAM129C did not show any similarities in conserved residues (not shown). From this I propose that the FAM129C PH domain probably does not bind phosphoinositides with high affinity, is not associated in Golgi and is not an adaptor protein.

The PH domain is also present in FAM129B with a probability of 5.5e-5, for the superfamily predicted with a likelihood of 1.2e-11. FAM129A (NIBAN) contains only a short fragment of the PH domain: when the N-terminus sequence of the protein is searched using Pfam there are 31 aminoacids (residue 75-106) which correspond to β1 and β2 strands of the PH domain, with the likelihood of 0.014 (a search of the whole sequence did not identify any identifiable domains). This therefore suggests that FAM129A probably contains a PH domain with an insertion. Alignment of the PH like domains in FAM129A, FAM129B and FAM129C are shown in Figure 4.9. There are certain highly conserved residues in these two sequences similar to a PH like domain. However the identity between the two sequences was 27.87%, marginally better than pairwise alignment between the full length sequences (25%, see above). This is consistent with the fact that PH domain identification is based on sequence search fold recognition and not on pairwise alignment.

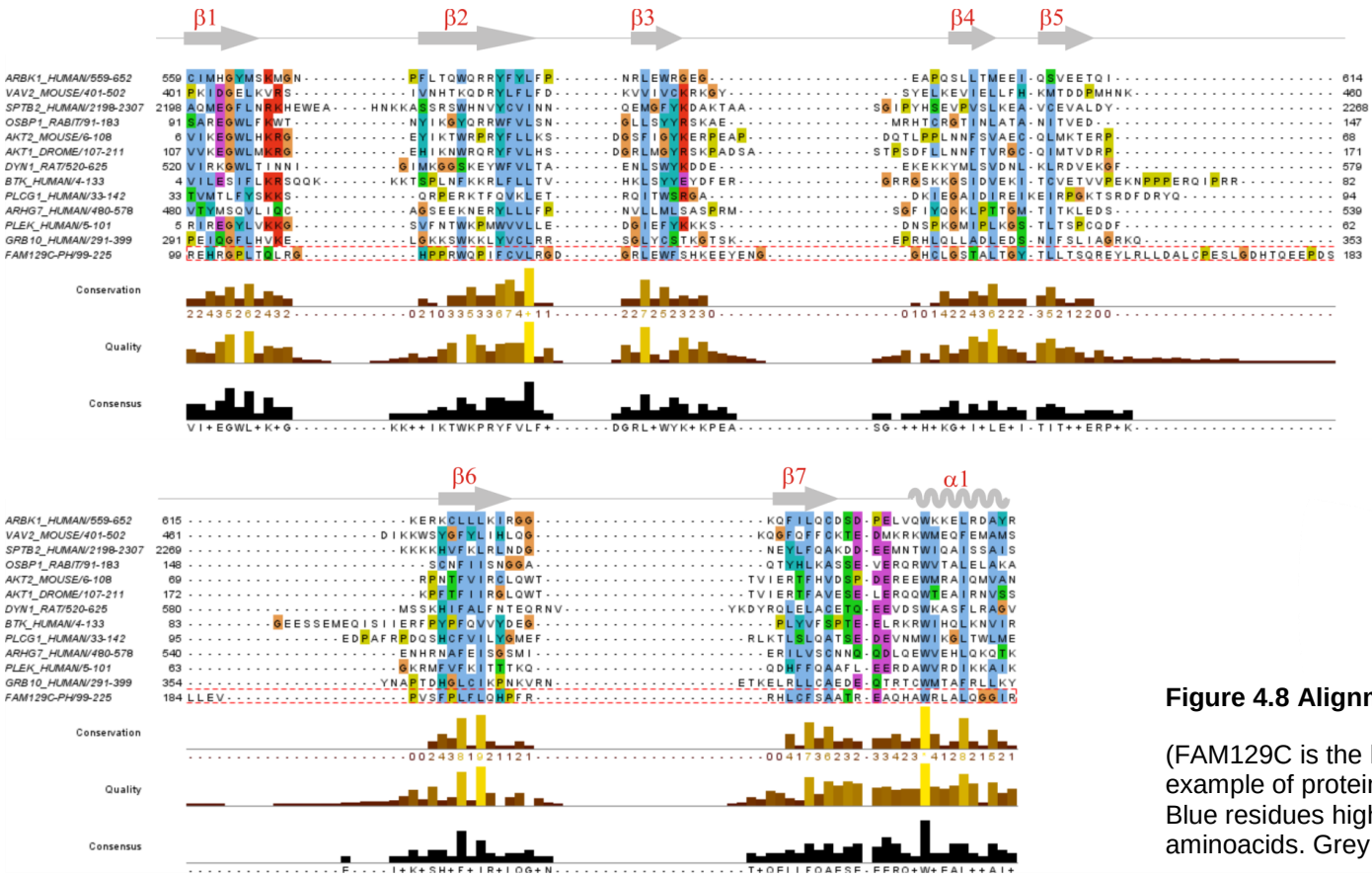

Figure 4.8 Alignment of FAM129C PH domain

(FAM129C is the last sequence in red frame) against example of proteins with PH domain from Pfam search. Blue residues highlight hydrophobic, strongly conserved aminoacids. Grey arrows depict β sheets of PH domain

To generate a model of the tertiary structure of the protein the information is used from experimental data of X-Ray crystallography of the known domains. To model the domain of FAM129C the X-Ray crystallography data of Bruton Tyrosine Kinase 1 (BTK1) was used as templates. An alignment of the protein sequences was initially generated by MUSCLE software and refined manually. Models were created using MODELLER software (Sali *et al.*, 1995) and the validity of the models chosen were tested using PROSAII (Sippl, 1993). Models were visualized using PyMOL (DeLano Scientific).

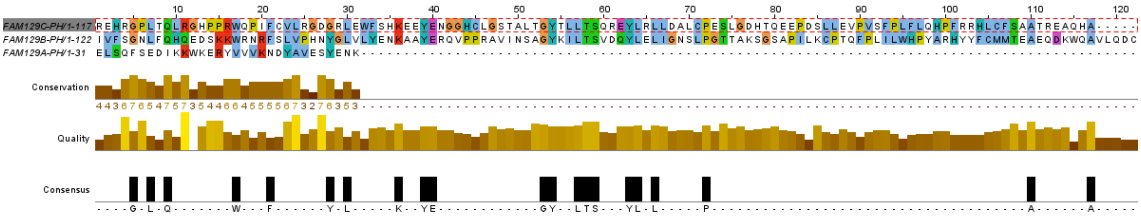

Figure 4.9 Alignment of PH like domain in FAM129B, FAM129A (NIBAN) and FAM129C
PH (residues 99-225) of FAM129C (top sequence in red frame) and PH (residues 69-192) of FAM129B (second sequence) and of FAM129A (NIBAN) bottom sequence. Sequence identity between PHD sequences is 27.87%.

Figure 4.10 shows the predicted tertiary structure of the FAM129C PH domain. This homology model is built on BTK as a template. The underlying template-target pairwise sequence alignment is based on a series of multiple sequence alignments, secondary structure prediction and fold recognition results. BTK is one of a list of plausible templates, however none of the potential templates is very close to FAM129C in terms of sequence identity (less than 7%) and hence any functional conclusions (apart from the idea that the FAM129C PH domain is structurally similar to the BTK PH-domains) are highly speculative. There are seven predicted β sheets at the N terminus which will form the tertiary structure of the pleckstrin homology domain.

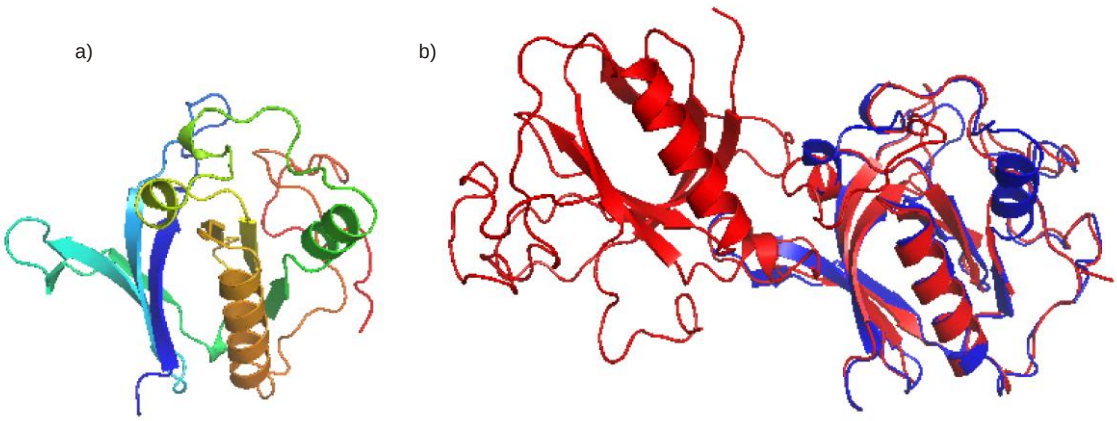

Figure 4.10 Tertiary structure
a) Cartoon representation of FAM129C PH domain, blue represent N terminus, red: C terminus, arrows are β strands, spiral represent α helix; b) FAM129C (in blue) and the template used for modeling the PH-domain, BTK1(in red). BTK1 contains two PH domains. As expected for models generated by using the homology modeling technique, the model and its template look rather similar (courtesy of Dr R Schmid).

The core structure consists of a pair of nearly perpendicular sheets of four and 3 anti-parallel strands resembling a collapsed β barrel. A long C terminal α helix (shown orange in Figure 4.10 a) packs against one edge of the β sheet structure, stabilising the entire fold. β sheets are closely packed (Rebecchi & Scarlata, 1998). The loops connecting the beta-strands differ greatly in length, making the PH domain relatively difficult to detect.

4.6 Posttranslational modification

Localisation and a signal sequence

For the identification of targeting signals and predicting protein localisation, the PSORT prediction programme has been used (http://psort.nibb.ac.jp). PSORT uses a nearest neighbor classifier; this is a statistical method that classifies according to similarity. The unknown sequences are compared to the database of known signal and non-signal peptides. Another tool to predict localisation using artificial neural networks is http://www.cbs.dtu.dk/services/TargetP/ , Artificial neural networks and hidden Markov model, Signal P http://www.cbs.dtu.dk/services/SignalP/ looks for signal peptides.

Phosphorylation, N-myristoylation sites and disulphide bonds

Phosphorylation, N-myristoylation sites and disulphide bonds were predicted using www.predictprotein.org. This website uses PROSITE database motif sequence search. For the phosphorylation sites it uses protein C kinase and casein kinase II preferences

for the phophorylation of serine or threonine. Casein kinase II requires also the presence of acidic residues. The myristoylation sites were also run using NMT-The Myr Predictor http://mendel.imp.ac.at/sat/myristate/SUPLpredictor.htm

Other websites used for phosphorylation sites were: NetPhos2 and PHOSIDA. NetPhos2 http://www.cbs.dtu.dk/services/NetPhos/ uses a neural network based approach (it postulates that it has a sensitivity greater than 70% in predicting phosphorylation targets base on sequence and structural information. PHOSIDA http://www.phosida.com/ is a phosphorylation site database, integrates thousands of high-confidence *in vivo* phosphor sites identified by mass spectrometry-based proteomics in various species. For each phosphor site, PHOSIDA lists matching kinase motifs, predicted secondary structures, conservation patterns, and its dynamic regulation upon a stimulus. Using support vector machines (a method used in the machine learning process), PHOSIDA also predicts phosphosites (Gnad *et al.*, 2007). The accuracy of predicting phosphoserines is 90% and phosphothreonines 75%.

Transmembrane regions (TM)

Transmembrane regions were predicted using the following webside:
TMHMM http://www.cbs.dtu.dk/services/TMHMM/ . TMHMM has been described as the best predicting programe for TM regions (Moller *et al.*, 2001).

Proteolytic cleavage

Proteolytic cleavage sites by proteosomes have been predicted using PAProCII http://www.paproc2.de/ (Toes *et al.*, 2001). These sites can be predicted with the high level of accuracy of >98% using neural networks.

Glycosylation

Glycosylation sites were predicted using http://www.cbs.dtu.dk/services/NetNGlyc/ (predicts with >75% accuracy), http://www.cbs.dtu.dk/services/NetOGlyc/ (predicts with >85% accuracy). This server uses neural networks to predict the sites.

Function prediction

ProtFun http://www.cbs.dtu.dk/services/ProtFun-2.1/ uses Gene Ontology (GO) and neural networks to predict function on the basis of the sequence. The GO project tries to classify genes and gene products. Each protein can be assigned a molecular function, biological process and localisation. GO assigns a number to each protein that

will represent these 3 levels of functional description. Currently there are hundreds of protein categories. ProtFun uses 347 of these categories.

4.6.1 Targeting signals and localisation

PSORT identified the presence of a leucine zipper domain (LZD) and cytoplasmic localisation. PSORT does not provide probabilities of its predictions. The LZD sequence found in FAM129C is: LRTVEASLEAVRTLLAQGMDRL between 384 and 405 aminoacids. This domain has not been identified by any other prediction programme. This prediction was not confirmed by any other searches. The LZD is a helical stretch of amino acids rich in leucine residues (typically occurring once every seven amino acid residues, i.e. once every two turns of the helix), which readily forms a dimer. In addition to forming homodimers, leucine zipper proteins can occasionally form heterodimers depending on the compatibility of the hydrophobic surfaces of the two different monomers. Such heterodimer formation provides an important combinatorial control mechanism in gene regulation (Strachan & Read, 2003) .TargetP did not identified localisation of the protein. SignalP did not identify any signal peptides.

4.6.2 Phosphorylation, Myristoylation Sites and Disulphide Bonds

There are 7 phosphorylation sites predicted based on protein C kinase predictions and 9 if casein kinase II is used (Figure 4.9 and Table 4.7). There is a wide variation in phosphorylation sites using different methods. The most accurate prediction is given for serins using a PHOSIDA search (>90%). The overlapping sites from both searches were following serine sites 31, 37, 38, 76, 412, 414, 519, 655, 687, 690.

There are also seven myristoylation sites according to Prosite prediction. Similarly to LZD, phosphorylation and myristoylation sites are putative and tend to be overpredicted. Using another server (NMT), there were no myristyolation sites identified.

N-myristyolation sites are thought to modulate the interaction of a modified protein with the intercellular membranes or with other proteins and therefore have similar functions to pleckstrin homology domains. As there are no transmembrane regions in FAM129C (see below) and there is experimental evidence that FAM129C is attached to the membrane, these sites may be important. There are eight predicted disulphide bonds distributed between residue 436 and 635 of the protein. This prediction is given with very high confidence (9/9).

Table 4.7 Phosphorylation sites.

Protein Kinase C (PROSITE)	Casein Kinase II (PROSITE)	PHOSIDA	NetPhos2
92, 132, 157, 337, 406, 690, 697	38, 132, 157, 172, 177, 183, 265, 424, 541	Serines: 31, 37, 38, 76, 412, 414, 519, 655, 659, 679, 685, 687, 690; Threonines: 177, 694	Serines: 11, 30, 31, 37, 38, 70, 76, 92, 132, 157, 172, 183, 234, 333, 390, 406, 412, 414, 424, 445, 517, 519, 665, 687, 690; Threonines: 14, 286, 337, 382, 694

List of phosphorylation sites predicted by three internet sites identification engines: PROSITE, PHOSIDA and NetPhos2. In red are marked sites with score >0.9.

4.6.3 Transmembrane regions

TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) is currently predicting transmembrane regions based on α-helices and their length and hydrophobicity. It did not identifie any transmembrane domains.

Proteolytic cleavage

PAProCII predicted 232 cleavage sites using constutive proteosome 20S and 167 if used immunoproteosome 20S. Out of 167, 43 were strongly predicted.

Glycosylation

Presence of glycosylation residues can have a significant effect on protein folding, localisation, function and protein interaction.

Function

ProtFun predicted that FAM129C is involved in energy/metabolism, is an enzyme, probably isomerase (Table 4.8), however the odds ratios in the prediction are low, which make these predictions weak. The summary of significant predictions is summarised in Table 4.8. The prediction programs did not identify transmembrane domains, peptide cleavage sites, or strong glycosylation sites. The function prediction is very weak and suggests that FAM129C might be an enzyme involved in energy producing pathway.

Table 4.8 Summary of significant predictions

Feature	Output	Probabilities and Scoring
SignalP 3.0	No signal peptide cleavage site predicted	
ProP 1.0	1 propeptide cleavage predicted at position 420	Score: 0.543
TargetP 1.1	No high confidence targeting prediction	
NetOGlyc 3.1	1 putative O-glycosylated site at position 694	Score 0.578
NetNGlyc 1.0	No N-glycosylated sites predicted	
TMHMM 2.0	No transmembrane helices predicted	
DISULFIND	8 sites predicted	Confidence 9/9
ProtFun 2.2	Energy-Metabolism	0.355
	Enzyme	0.742
	Enzyme class:isomerase	0.104

The score >0.5 indicates positive prediction. N.B. ProtFun results were with very low predicitive probability.

4.7 Summary

Bioinformatic analysis identified that FAM129C is a part of a gene family where the key three members are FAM129A (NIBAN), FAM129B and FAM129C (BCNP1); all three proteins contain a pleckstrin (PH) domain (the FAM129A PH domain has probably a large insertion). The phylogenetic tree of the family showed that FAM129C has undergone substantial evolution from the other family members and is closer related to FAM129B than FAM129A (likely to be caused by a fragmented PH domain in FAM129A). The PH domain is a ubiquitous domain with multiple functions; it interacts with phosphoinositides with different levels of specificity but also recently it has been suggested that it has important interactions with proteins. Therefore it makes this domain very difficult to characterise and I was not able to assign the specificity and function of the FAM129C PH domain on the basis of its sequence alone.

In the paper describing the discovery of FAM129C the authors used a bioinformatic program (not specified in the paper) which predicted transmembrane regions in FAM129C; my application of more recent and multiple transmembrane prediction algorithms (TMHMM server) could not find any convincing predicted transmembrane regions in FAM129C.

The important characteristics of diagnostic/prognostic/therapeutic targets in B cell malignancies are ease of detection (i.e. presence on the cell surface, therefore accessible by flow cytometry or immunohistochemistry), ability to generate antibodies to the target, specificity to the B cells, but different level expression between normal and malignant cells and finally reproducible and consistent results. FAM129C did not appear to have a transmembrane domain, but did have a pleckstrin homology domain

which helps association with the membrane and therefore suggest internal membrane and cytoplasmic localisation, rather than cell surface localisation. Bioinformatic analysis did not reveal any possible function for FAM129C it did show however it was distinct from other family members; possibly with distinct functions. The presence of a PH domain indicates that the protein may have a role in B-cell signalling, probably as an adaptor protein. Differential expression or localisation of FAM129C between normal cells and malignant cells may point to its utility as a diagnostic/prognostic marker.

Chapter 5: Leicester CLL Database and its Outcomes

5.1 Discussion

5.1.1 Introduction

One of the first attempts to identify factors influencing CLL patients' survival was work by the London haematologist Dr David Galton, which was first presented in his MD thesis in 1963 and published in 1966 (Galton, 1966). The thesis was based on 12 years of his observation of 88 CLL cases in an attempt to map out a natural history of this condition. This work was published long before the Binet and Rai staging and before any prognostic factors were used in clinical practice (Rai *et al.*, 1975; Binet *et al.*, 1981). Galton made a number of observations. The majority of the patients he observed (52/88) presented incidentally as a result of an abnormal full blood count. Patients had two trends of their lymphocytosis: progressive increase (I), reaching plateau and stabilising (IIa) and 12 patients had lymphocyte count which remained constant (IIb). Type II was associated with indolent disease. It is tempting to compare Galton's division of type I (aggressive) and type II (indolent), disease to unmutated and mutated CLL. Galton also noticed that female patients had a less aggressive course of the disease. To explain the basis of pathophysiology of CLL he proposed the hypothesis that an increase in the lymphocyte count was due to "the loss of the normal degree of responsiveness to homeostatic control".

Since 1963 CLL practice has dramatically changed. Each CLL patient is staged at the diagnosis by Binet (mainly used in the UK) or Rai (preferred in North America). There are many prognostic factors currently available in clinical practice: pathological changes in the B cell receptor (immunoglobulin heavy chain gene (*IGHV*) mutation), cytogenetic abnormalities, CD38, serum β2M etc. Despite a plethora of prognostic markers currently used in clinical practice, and although clinicians are able identify some 'high risk' patients. We remain unable to discriminate prospectively at diagnosis most patients who are destined to follow Galton's type 1 or type 2 disease. This has major implications for the rational management of the disease

In this chapter I analysed currently available prognostic factors in a large cohort of patients attending the CLL clinic in Leicester and related them to the overall survival and time to the first treatment time to identify the most significant prognostic factors. I present a preliminary analysis of the Leicester CLL database with the main focus on

prognostic factors. This database was created together with Dr A. Majid, a postdoctoral scientist, in an effort to categorise samples brought from the clinic to the laboratory. The whole database contains a total of 710 patients with various haematological disorders and the majority (n=496) were those with the diagnosis of CLL. These data were unique since they represent unselected, population-based records, allowing unique insights into the pathogenesis of CLL and the outcomes of therapy.

5.1.2 Creation of the database

I collected data both prospectively and retrospectively on 496 patients attending Leicester Royal Infirmary, which is a primary referral centre for Leicester and Leicestershire and a tertiary referral centre for the Leicestershire, Northampton and Rutland (LNR) cancer network. Data was accumulated from 2004-2009, prospectively for the new patients and retrospectively for those already under follow-up. A summary of the data of the Leicester cohort is presented in the Table 7.1.

I coordinated ethical approval for this project; informed written consent was acquired from patients and *IGHV* mutation analysis was performed by Dr A. Majid, FISH was done by the Institute of Human Genetics in Kiel, Germany, and CD38 staining was performed on defrosted cells by Dr D.G. Best and Mr I. Tracy (Bournemouth). The data presented here are used with their permission.
Clinical data were collected and analysed by the author.
The results and other data are presented as the Appendix in Chapter 7.

5.1.3 Incidence and prevalence

Interestingly CLL shows wide ethnic variations indicating that a strong genetic background. Similar variation is also seen in follicular lymphoma (Biagi & Seymour, 2002).
The incidence of CLL is highest in the White population and the lowest in Asians. It has been well documented that the incidence of CLL in Asian countries, Japan and China was lower than in a European-descended population (Asou *et al.*, 1993). In the USA, CLL accounts for 30% of all leukaemia cases (Molica *et al.*, 1999b). In India CLL was reported to be between 1.95-8.8% of all diagnosed leukaemia and the majority of cases (90%) were present in the intermediate or high risk stage ((Bhutani *et al.*, 2002).There are variations in incidence amongst the White population reported. Redaelli presented the highest incidence in Australia 9 per 100,000 and lowest in Netherlands 3.2 per

100,000. In the UK the reported incidence was 4.5 per 100, 000 (Redaelli *et al.*, 2004). I was not able to find data on incidence and prevalence of CLL in the Black or Chinese immigrant population in Europe. There was one publication describing a southern Asian population with CLL in Birmingham (Gunawardana *et al.*, 2008). The low incidence of CLL among the Asian population does not appear to change with migration to North America or among second or later generation descendants (Yonehara *et al.*, 1989; Gale *et al.*, 2000). This observation seems to confirm racial susceptibility

From the Leicester database the Incidence of CLL in Leicester in the white population was similar to the previously reported. The incidence ratio in our cohort calculated over the period of eight years was 5 per 100,000 person-year. The overall prevalence in the Leicester and Leicestershire cohort was 69 per 100,000 and recalculated excluding ethnic minorities rose to 75 per 100,000 in the white population.

The prevalence based on a Leicester and Leicestershire population (17.6 per 100,000) showed that Asians were 3.6 times less likely to have CLL than White even allowing for different population demographics. Similar data was previously published based on a North American population (Morton *et al.*, 2006) and in a UK South Asian CLL population (Gunawardana *et al.*, 2008): 28 cases per 240,000 in the Asian population in Birmingham (i.e. 12 per 100,000).

According to the 2001 Census data there were approximately 4000 Chinese people in Leicester and there was a population of 8,000 Black people (including mixed race), however there were no Black or Chinese patients in our CLL cohort. Dores (Dores *et al.*, 2007) estimated from US data that the incidence of CLL in Blacks was 75% of those in Whites and in Asians 23% of those in Whites. On this basis I should have seen 3-4 Black patients with CLL in our cohort ($p<0.0001$). It is difficult to understand why I have not recorded any Black patients with CLL. The Dores data is based on a North American population. Probably similar to a White population incidence in Blacks will vary. It has been estimated that there are 10,000 Somalian living in Leicester (from 16 June 2008: http://www.bbc.co.uk/leicester/content/articles/2006/09/29/leicester_somali_community_feature.shtml) and approximately 2,000 Ethiopians (from 2006, http://www.iomlondon.org/doc/mapping/IOM_ETHIOPIA.pdf) (NB. 2001 Census quotes total of 8,000 Blacks and does not provide information on Black origin). On the basis of the estimated Somalian population the majority of the Black population living in Leicester comes from North-East Africa and on the basis of this finding the incidence of CLL is very small.

5.1.4 CLL in the Asian population

There are scarce publications on CLL in the Asian population, particularly on the Asian migrant population in the Western world. Analysis of the Leicester CLL population gave a unique opportunity to understand characteristics of CLL in southern Asians from the Indian subcontinent.

The Birmingham group published an observational study on a South Asian population in the UK. Birmingham has similar demographics to Leicester. The Asian cohort was only marginally bigger than Leicester's cohort, (28 *v.* 19). Similarly to our cohort they found that Asian patients present at younger age than the White counterparts (Birmingham 61, Leicester: 58) and male preponderance was more marked than in Caucasian patients (79% v. 56%). Unlike the Birmingham cohort, Leicester's cohort appears to have a good prognosis: TTFT in Leicester was 207 months whereas this was remarkably short in Birmingham (Gunawardana *et al.*, 2008) 24 months This is remarkably short for any CLL population and suggests a very different aetiology.

However, a significant weakness of both studies was the small numbers.. Although there were very discrepant results, both cohorts were very small and therefore any general conclusions should be made with caution.

5.1.5 Clinical staging

The majority of CLL patients present at the early stages (BinetA0 56% and A 24%), however the staging alone does not identify patients who may progress quickly. The Binet staging has evolved, and stage A0 was introduced into clinical practice, this combines stage 0 of Rai into stage A of Binet, i.e. specifies group of stage A patients who have lymphocytosis only without any significant lymphadenopathy. The distinction between stage A0 and A is valid on the basis of Leicester cohort: I compared A0 and A: time to first treatment was significantly different $p<0.0001$ and greater need for therapy in patient with A stage disease (52% v. 15%, $p<0.0001$). Other variables differentiating stage A0 from A were higher proportion of mutated *IGHV* (77% v. 66%, $p=0.0415$), more patients with normal cytogenetics (30% v. 17%, $p=0.0401$) and less patients with t12, 11q and 'other' cytogenetics abnormalities (11% v. 24%, $p=0.0153$; 6% v. 17%, $p=0.0084$; 7% v. 13%, $p=0.0454$ respectively). However, overall survival of stage A and A0 were similar (243 and 216 months, $p=0.2349$).

5.1.6 Role of gender

Gender in CLL appears to play an important role, similarly to other haematological malignancies (Cartwright *et al.*, 2002). There is not only reduced incidence in females but also women with CLL tend to have a more indolent course of disease. The biological causes of these differences are not known.

Females presented at older age median of 67 versus 65 years in males. A high proportion of female patients were diagnosed older, 43% (67/156) of females presented at the age of ≥70 in comparison to 33% males, although this observation did not reached statistical significance (101/299, p=0.0544). Similar proportions were diagnosed at the age of >50 and <70 (females 51% and males 57%). There was only small number of patients <44 (n=10), 8 men and 2 women, giving ratio of 4:1. This confirms the previously found male preponderance in CLL aged 40-44 (5.4:1) described by Cartwright (2002). The reasons for this interesting difference are not known.

In our cohort I found that women had a better TTFT (p=0.0011), confirming Molica's findings (Molica *et al.*, 2005), but OS was not statistically significant (p=0.0964). Men were more likely to require treatment (40% v. 30%, p=0.04); women have longer LDT (65 v. 59 months although it did not reach statistical significance), and were more likely to be diagnosed as a stage A0 (p=0.038), whereas men tend to be at stage B (p=0.008).

Our group has observed male preponderance in *IGHV*1-69 (Walewska *et al.*, 2007), a gene segment associated with poor prognosis, discussed in detail below (paragraph 3.2.1.5)

5.1.7 Lymphocyte doubling time

Doubling of lymphocytes is one of the oldest prognostic factors used in practice. Galton observed significance of progression in lymphocyte count in patients with Type I disease, i.e. progressive increase in WCC had worse survival in comparison to Type II. LDT gives a crude assessment of the speed of the disease. The 12 months limit was set by Montserrat in 1986 (Montserrat *et al.*, 1986).

In the Leicester cohort as expected there were significant differences between the two analysed groups, especially in TTFT and OS. Analysis of the Leicester cohort showed that patients with short doubling time tend to be older, with higher WCC at diagnosis; they were more likely to have mutated *IGHV* genes, 11q deletion, 17p deletion, trisomy 12 and 'other' abnormalities. They were less likely to have '"good" cytogenetics. Thirty

percent of these patients were stage B and C at diagnosis. Although, interestingly, in our group, they were more likely to be stage A than the patients with more than 5 years of doubling time. Presentation with lymphadenopathy was important: patients with short doubling time tend to have large lymphadenopathy and less frequently present with no palpable lymph nodes.. The TTFT of 25 months in LDT<12 months was similar to data published by a German group (20 months) ((Bergmann *et al.*, 2007).
Leicester patients with <12 months LDT had better OS (118months) than presented by Montserrat (60 months) (Montserrat *et al.*, 1986). LDT was significant in univariate analysis for both OS and TTFT ($p<0.001$).

5.1.8 Beta$_2$-microglobulin (β2M) as a prognostic marker

Beta$_2$-microglobulin is a small, 11.6 kDa polypeptide that stabilises the tertiary structure of the major histocompatibility complex class I molecule (Bjorkman & Burmeister, 1994). Free β2M is a well established prognostic marker in multiple myeloma (Durie *et al.*, 1990) and has also been used in CLL (Hallek *et al.*, 1996; Molica *et al.*, 1999a). It was one of the first prognostic markers used in CLL. It is inexpensive, easily available in many non-specialised laboratories and despite these factors it has been partly forgotten in the recent race to find new CLL markers and was omitted from the most recent CLL guideline as a prognostic marker (Hallek, 2008).
β2M in CLL has been used in different cut-off levels: 2mg/L (Tsimberidou *et al.*, 2007) and 4mg/L (Wierda *et al.*, 2007; Oscier *et al.*, 2009). Recently, Oscier (Oscier *et al.*, 2009) found that the cut-off level of 4mg/L was a better prognostic serum marker over thymidine kinase, free light chain ratio and soluble CD23

In my analysis of the Leicester cohort I used both arbitrary levels: a cut-off of 4mg/L and 2.5 mg/L and excluded from analysis all the patients with renal impairment as β2M levels dependent on glomerular filtration rate of the patient. (Delgado *et al.*, 2009),
I found that β2M was a strong prognostic marker regardless of the chosen cut-off level.
. However in my analysis of the Leicester cohort I found that the cut-off of 2.5mg/L identified more statistically significant variables and this was probably due to very small numbers in the >4mg/L cohort (n=17).
The variables which were significant in both cut-offs were age and WCC at diagnosis, stages A0, B and C, presence and the size of lymphadenopathy. The latter three were expected as they are markers of disease volume. There was a difference in the proportion of treated patients, TTFT and OS (Figure 7.13-7.14). The variables which

were not significant in both analyses were: stage A, proportion of patients with autoimmune conditions, normal and 'other' cytogenetics, 17p deletion (the latter was represented by very small numbers). The analysis using 2.5mg/L cut off firstly identified additional statistically significant variables: gender, mutation status, 13q deletion ($p<0.0001$) and trisomy 12 ($p=0.0116$), secondly improved significance of p values of the variables which were significant in >4mg/L cut off. Interestingly I observed that in 2.5mg/L analysis 11q and LDT became statistically insignificant. This suggests that both 11q deletion and LDT are dependent on high levels of β2M.

I found that older patients had higher values of β2M in analysis with both cut-offs and in univariate analysis β2M had an adverse effect on OS and TTFT. Age at diagnosis did not have significant effect on TTFT, but did influence OS and LDT (Table 7.3). This confirms Delgado observations, which showed that age is significant in both univariate and multivariate analysis of β2M, after correcting glomerular filtration rate (this equation corrects for age).

These data show that β2M was a reliable marker. I did not have data on changes in levels of β2M during the course of disease. It is possible that regular measurements may identify progressive disease before other parameters.

5.1.9 *IGHV* gene segments usage and mutational status

The Bournemouth group first showed (Oscier *et al.*, 1997) an association between the level of somatic mutation and different cytogenetic abnormalities. This was followed by a seminal paper from the same group (Hamblin *et al.*, 1999) and Damle (Damle *et al.*, 1999).Studies of immunoglobulin variable heavy chain usage (*IGHV*) in the B cell receptor not only stratified CLL into two prognostically important groups: mutated (M) and unmutated (UM), but also helped identifying normal B cell counterparts to the chronic lymphocytic leukaemia cells and other lymphoproliferative disorders.

Mutation status is defined currently by 98% or more homology to the germline sequence. This cut off was initially derived from observation by Matsuda that polymorphisms may lead up to a 2% difference in the DNA sequence (Matsuda *et al.*, 1993). Other groups suggested cut off of 97% (Krober *et al.*, 2002) and 95% (Lin *et al.*, 2002). However more recently Tobin *et al.* showed that the 2% cut off is the best discriminator of outcome (Tobin *et al.*, 2005).

Table 5.1 summarises the number of studies and differences in *IGHV* mutation status.

Table 5.1 Prognostic value of the *IGHV* mutation status (Van Bockstaele *et al.*, 2009)

Reference	*n*	*M* (%)	*UM* (%)	Median OS (years) all patients		Median OS (years) early stage patients	
Damle et al.(Blood 1999)	47	50	50	NR	9.0	17.0	9.0
Hamblin et al (Blood 1999)	84	55	45	24.4	9.8	24.4	7.9
Jelinek et al (BJH 2001)	131	57	43	NR	5.7	ND	ND
Matrai et al (Blood 2001)	40	40	60	NR	9.0	ND	ND
Hamblin et al. (Blood 2002)	145	66	34	24.4	9.1	27.0	12.1
Kröber et al.[a](Blood 2002)	300	37	63	NR	6.6	NR	6.6
Lin et al.[b] (Blood 2002)	69	38	43	NR	7.5	ND	ND
Oscier et al. (Blood 2002)	205	60	40	25.8	9.9	27.3	9.6
Tobin et al.(Blood 2002)	112	44	56	10.3	5.9	ND	ND
Ghia et al. (Blood 2003)	82	60	40	NR	15.3	ND	ND
Magnac et al.(Leukemia 2003)	58	57	43	NR	7.0	ND	ND
Tobin et al.(Blood 2003)	244	41	59	10.0	5.8	ND	ND
Vasconcelos et al. (JCO 2003)	145	57	43	NR	7.0	NR	8.1
Aleskog et al.(EJH 2004)	46	46	54	8.0	5.3	ND	ND
Orchard et al. (Lancet 2004)	167	68	32	24.4	9.8	ND	ND
Marasca et al.(J Mol D 2005)	146	57	43	17.2	10.6	ND	ND
Tobin et al. (Hematologica 2005)	323	42	58	10.2	5.9	ND	ND
Nikitin et al. (Leuk Lym 2007)	134	43	57	18.1	6.4	ND	ND
Stamatopoulos et al.(ClinChem 2007)	105	51	49	NR	12.7	ND	ND

OS, overall survival; M, mutated *IGHV* genes; UM, unmutated *IGHV* genes.
NR, not reached; ND, not determined.
[a] Mutation status based on 97% cutoff level.
[b] Unmutated cases show more than 98% homology with the germline sequence, mutated cases show less than 95% homology with the germline sequence
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Using the 2% cut-off I observed statistically significant differences in WCC at the diagnosis and LDT, proportion of treated patients and TTFT, β2M, CD38, some cytogenetic abnormalities and OS. Our cohort's overall survival confirmed the Bournemouth data in unmutated *IGHV* patients: 94.57 moths *vs.* Leicester 111 (p=0.0891). Interestingly Leicester mutated patients had a better survival than in Bournemouth (180 months v. Leicester 216, p=0.0477, Figure 7.6.C). I was not able to explain this difference by comparing proportions of stage A, A0, treated cases, median age, frequency of 13q, t12, 11q: Bournemouth had a statistically significant higher

proportion of patients with stage A0, less treated cases, less males, but more cases with 17p deletion (n=16). The median age was similar in both groups.

In our cohort I have confirmed Hamblin's (Hamblin *et al.*, 1999) observation that poor prognosis cytogenetic abnormalities were associated with unmutated *IGHV* genes (Table 7.11), but the association with trisomy 12 (p=0.3) and 13q (p=0.1) was very weak in the Leicester cohort. (Oscier *et al.*, 1997). There was a significant difference between mutated and unmutated *IGHV* and the need for chemotherapy (p=0.004).

I have also confirmed an association between lack of lymphadenopathy at diagnosis (p=0.01) and mutation status (Catovsky *et al.*, 2007).

Nevertheless there were a significant proportion of *IGHV* mutated patients who progressed, I observed that 31% of patients with mutated *IGHV* gene segments required treatment. Therefore I analysed this group further and found significant differences between mutated progressive and non-progressive (Table 7.13 and Figure 7.7). There were highly significant differences in WCC at diagnosis, stage, particularly stage A0, B and C (c.f. there were no differences in stages between mutated and unmutated *IGHV*), absence of lymphadenopathy, LDT, 13q deletion and β2M. OS did not reach statistical significance (p=0.556). Mutated untreated patients appear to have a distinctly different disease. This is an important observation as it suggests that a certain group within mutated patients should have close follow up as they may progress quickly.

5.1.9.1 IGHV1-69

The commonest *IGHV* segment in CLL is *VH*1-69, the data from Leicester cohort confirms findings from Mediterranean population (Ghia *et al.*, 2005b) (Figure 7.4.B). In addition *IGHV1-69* has been associated with poor clinical outcome (Kipps *et al.*, 1989; Hamblin *et al.*, 1999). I showed that unmutated *IGHV*1-69 gene segment usage in the Leicester Bournemouth and CLL4 cohorts was associated with a significant male preponderance (Figure 7.8) (Walewska *et al.*, 2007) The CLL4 cohort had a higher proportion of *IGHV*1-69 than the Leicester (p=0.0057) and Bournemouth cohort (p=0.01). This could represent selection bias as all patients in CLL4 were selected on the need for treatment basis. Unmutated 1-69 has the same OS and TTFT as other UM *IGHV* families (Figure 7.10), this was confirmed by the Efermov group (n=25/106) (Panovska-Stavridis *et al.*, 2007) and an Italian group (n=46/379) (Orlandi *et al.*, 2009b).

Interestingly unmutated *IGHV*1-69 may progress after variable period of stable disease with accelerated LDT presumably due to acquisition of secondary genetic events (Figure 7.9).

Ghia et al replied to our letter (Ghia *et al.*, 2007) and investigated *IGHV*1-69 in a cohort of 1902 CLL patients (collective data from France, Greece, Italy and Sweden). Their male to female ratio was 2.75:1, with the lowest ratio in an Italian cohort (2.2) and highest in France (3.72). The biggest difference in male to female ratio was found in the *IGHV*3-48 group: it was 9 in a Swedish group, 4.2 in a French, overall it was 2.9. Interestingly, in the CLL4 group the ratio was 4.5 (male to female=4:18) (Leicester and Bournemouth: 1.25 (5:4) and 2.2 (13:6) respectively), although the numbers in this *IGHV* group were small in our cohort. Since the publication of Ghia's response however, the Irish group reported also a male prevalence in their cohort (Galligan *et al.*, 2008)

A proportion of 1-69 (8/37, 21%) is mutated and this group has an indolent course with a long lymphocyte doubling time (Figure 7.9.A). However this did not translate to any significant difference in overall survival in the Leicester cohort (Figure 7.9.B) and could be explained by the very small numbers in the mutated group.
The unmutated group was associated with the following cytogenetic abnormalities: 11q (n=9), 17p (n=3), t12 (n=2), 13q (n=9), normal (n=1), whereas in the mutated group there were no poor prognostic chromosomal abnormalities and only 3 cases with 13q and 2 with normal cytogenetics. The observation of mutated *IGHV* 1-69 having an indolent course was confirmed by an Irish cohort (n=330) (Galligan *et al.*, 2008).

5.1.9.2 IGHV3-21

IGHV*3-21* is associated with poor prognosis and there is variable prevalence amongst published cohorts. In the Scandinavian population *IGHV*3-21 frequency was reported as 11% (n=265, (Tobin *et al.*, 2003)), 9.6% in British cohort (n=83, (Lin *et al.*, 2003)). Belgian 8.8% (n=91, (Philippe *et al.*, 2003)), Mediterranean origin 2.9% (n=553, ghia, blood, 2005). In our cohort the frequency was 4.8%, different from the Italian group (Figure 7.4.B), but did not reached significance (Ghia: 16/553, Leicester 24/496, (p=0.0509). *IGHV*3-21 was the fourth most common used *IGHV* family in our cohort, but tenth in the Mediterranean group (Figure 4.4.B). Ghia argues that utilisation of *IGHV*3-21 shows geographical bias.

There were no significant differences in patients characteristics and OS, TTFT between patients with unmutated *IGHV* gene segments and 3-21 and this confirms previous data; (Thorselius *et al.*, 2006); *IGHV*3-21, regardless of the mutation state confirms poor prognosis (Ghia *et al.*, 2008). Interestingly only LDT was significantly longer in

*IGHV*3-21 patients (85 versus 52 months, p=0.0351) but it did not translate into better OS or TTFT.

5.1.10 CD38 expression

Damle (Damle *et al.*, 1999) proposed in 1999 CD38 as a surrogate marker for unmutated *IGHV* and as a prognostic marker. The cut-off for positive and negative CD38 was established arbitrarily as 30%. Damle concluded that CD38 positivity had a 82% predictive value for mutated *IGHV* genes, and a 100% predictive value for unmutated genes.

In the following two years the discussion ensued in the literature regarding suitability of CD38 marker as a surrogate for *IGHV* mutations (Thunberg *et al.*, 2001) (Hamblin *et al.*, 2000). Thunberg argued that a low level of CD38 expression could not predict mutation status in *IGHV* genes and did not find prognostic significance when the CD38 expression in his cohort was analysed. Hamblin acknowledged that CD38 may be an important prognostic marker, however found that it was independent of *IGHV* mutation status (Hamblin *et al.*, 2000).
Concerning the levels of CD38 expression denoting positivity 20% was proposed by Ibrahim (Ibrahim *et al.*, 2001). In the more recent literature Gentile et al (Gentile *et al.*, 2005) argues the use of a lower, 7% cut-off on fresh cells at the diagnosis. Ghia argues that the presence of distinct population of CD38, irrespective of its size, rather than a numerical cut-off, identifies progressive patients (Ghia *et al.*, 2003).

Our cohort had 25% CD38 positive patients, similar to the observed frequency in the literature (Chevallier *et al.*, 2002). I have observed the same representation of cytogenetic abnormalities in CD38+ population as presented by Chevallier (percentages in brackets): 11q: 12% (14%); 13q: 27% (40%); 17p: 4% (7%); proportion of patients with trisomy12 is higher in the Leicester cohort than published data: 21% (14%).

In our cohort CD38 did not have an influence on OS, this was discrepant to the published data. Hamblin found differences in OS between CD38 positive and negative patients studying 50 patients (293 months v 105 month, p=0.0124)(Hamblin *et al.*, 2000). In Damle's paper (n=64) the values for the OS were undefined for CD38- and 108 months in CD38+. There was a difference however in TTFT of the Leicester cohort between two groups with median of 65 (CD38 positive) v 91 months (p=0.045), and not

surprisingly, as CD38 is the marker for proliferation, there was also difference in LDT (CD38 positive: 49 months *v.* 62 months, p=0.0007) (Figure 7.12).

In order to check if CD38 had an effect in our cohort when selected for stage, as previously described by Chevalier (Ibrahim *et al.*, 2001; Chevallier *et al.*, 2002; Domingo-Domenech *et al.*, 2002). OS in stage A patients CD38+ and CD38- (Figure 7.12.C) did not distinguish between two groups as previously reported. The level of significance of CD38+ and CD38- patients for overall survival was 0.087(stage A and A0); I further re-analysing our patients separating stage A0 from stage A. OS significance for stage A (without A0) was only slightly improved to p=0.07, but not statistically significant.

The discrepancy of OS between the Leicester cohort and published data may be explained by the fact that Leicester's CD38 flow cytometry was assessed from thawed frozen samples. Although Damle's original data was based on both frozen and fresh samples, the freezing process and transport introduces additional problems, especially both freezing and defrosting is operator dependant.

An interesting observation of the Leicester data was the relation between CD38 and autoimmune conditions. There was higher proportion of autoimmune conditions in CD38 positive patients (18% v. 8%, p=0.03). CD38+ patients had equal proportion of AIHA and ITP (39% each), whereas in CD38- patients the most common is AIHA (60%) and ITP only 8%, the differences were not statistically significant because of small numbers. Ghia *et al.* correlated increased autoimmune disease with CD38 positivity, which may suggest that the conditions with a higher proliferative index were more prone to autoimmune presentations (Ghia *et al.*, 2003). Ghia presented that patients with even a small population of CD38 positive cells were more prone to develop autoimmune conditions. Visco reported, similar to our cohort, a frequency of ITP of 5% in the Italian cohort (n=1278) and found that these patients have poorer survival than other CLL patients and that this was independent from clinical prognostic variables (Visco *et al.*, 2008).

5.1.11 Interphase FISH abnormalities

The frequencies of the commonly observed interphase FISH abnormalities observed in CLL seen in our cohort are presented in Figure 7.15.A. These were similar to those reported in the literature. Similar to published data before, the 13q deletion was associated with good prognosis: in our cohort it was mostly associated with stage A0

and patients with long doubling time, normal cytogenetics were more common in mutated *IGHV* gene segments and 11q with 17p with unmutated.

I compared median OS of the Leicester cohort cytogenetic groups with published data from Ulm (Dohner *et al.*, 2000) (Figure 7.16). The most significant differences were seen in good prognostic abnormalities: the 13q deletion and normal cytogenetics. The 13q deletion did not reach the median survival in the Leicester cohort (follow up over 20 years) whereas in Döhner's cohort was 134 months (p=0.0002), for the normal cytogenetic the OS was136 months (Ulm) *vs*. 211 (Leicester, p=0.0004). Similarly, in patients with the 11q deletion, the OS was longer by 3.4 years (p=0.017) and in the case of 17p deletion survival was similar: 37 months (Leicester) *vs.* 34 months (p=0.0588). The OS for the trisomy 12 was 4 years longer than reported by Döhner, but did not reach a statistically significant difference (p=0.3246). The median time to first treatment for all these groups were similar to the TTFT reported in the German study. Döhner's data has the 'disadvantage' of being ten years old and being one of the first publications associating prognostic significance to specific cytogenetic abnormalities. The Leicester data has an 'advantage' of the clinician's awareness of prognostic factors and that management decisions are tailored according to the prognostic factors.

Analysis of the Leicester cohort confirms published data: good prognostic abnormalities (13q deletion and no detectable abnormality) were mainly seen in stage A0 (Table 7.2). An interesting analysis provides the division of mutated *IGHV* into treated and untreated group (*IGHV*3-21 was excluded from mutated group) (Table 7.13). The mutated untreated group is identified by a high proportion of 13q deletion (20 *v.* 53%, p<0.0001) and low trisomy 12 (29 *v.* 9%, p=0.0012). The 13q findings confirm previously published data (Oscier *et al.*, 1997; Dohner *et al.*, 2000), whereas observation on trisomy 12 contradicts published data, it was suggested to have a 'neutral' effect on prognosis (Gardiner *et al.*, 2005).

'Other' cytogenetic abnormalities in our cohort appear to have TTFT and OS similar to that of patients with the 11q deletion with TTFT and OS of 23 and 204 months respectively. This observation confirms published data that 'other' complex abnormalities are associated with poor prognosis (Dicker *et al.*, 2008).

I observed eight (2.42%) patients with the t(14;18) (q32;q21) involving BCL2 on chromosome 18q21 and they appear to have an indolent disease course with TTFT 87 months and OS undefined (follow up of over 10 years). The Belgian group (Put *et al.*,

2009) presented a cohort of 40 patients with the t(14;18), their TTFT was 48 months and OS 182 months. In this series the t(14;18) was not associated with advanced disease, most patients were stage A and responded well to therapy.

5.1.12 Analysis of 13q deletion, what matters: hemi-, homo and mosaic deletions or percentage of cells affected?

There was only scarce literature on different forms of the 13q deletion: from the Mayo clinic (Dewald *et al.*, 2003; Van Dyke *et al.*, 2008) and from a very small cohort (n=6) from Buenos Aires (Chena *et al.*, 2008). In the most recent series of 259 patients (15 years of follow up) with isolated 13q deletion from the Mayo clinic (Van Dyke *et al.*, 2008)), OS for the whole group was 9.3 years and TTFT 6.9 years. In our cohort (significantly smaller, n=127), frequencies of 13 q abnormalities were 68% hemizygous, 12% homozygous and 20% mosaic (i.e. homo- and hemizygous). Van Dyke did not find significant differences in age and gender between the three groups, there was no difference in OS (p=0.313) nor TTFT (p=0.53), whereas in the Leicester group I found a marginally significant difference in OS, were homozygous and mosaic did not reach significance. (p=0.14). I compared our data to the Mayo clinic and found a significant difference in OS in the mosaic group (Figure 7.18.C-E). The mosaic group in the Leicester cohort presented only as a stage A0 or A and had the longest doubling time. Although our cohort was smaller, there appears to be a survival advantage in homozygous and, in particular, in mosaic deletions.

The Bournemouth group investigated the influence of 13q chromosomal deletion size and its prognostic value (Strefford *et al.*, 2009). A large 13q deletion at diagnosis was associated with disease progression and appeared to influence response to treatment, whereas patients with a small deletion achieved complete rather than partial response to treatment.

I checked another hypothesis: does the size of 13q clone, i.e. percentage of affected cells influence the patients' outcome? Table 7.25 and Figure 7.19 summarise the data. The chosen cut-off of 80% affected cells showed a significant difference in TTFT between the two groups. There were also statistical differences between stage A (p=0.036), presence of lymphadenopathy (0.0149) and proportion of treated patients (p=0.002), TTFT (p=0.005) and no difference between the WCC at diagnosis (p=0.073). This suggests that size of the clone matters and although 13q carries good prognostic influence, its effect may be altered with the clone size. Patients who had >80% effected cells are more likely to present with higher WCC, have lymph nodes and require treatment sooner. This implies that large 13q clone may lose its 'good'

prognostic characteristics. This finding confirms recently reported observations from Salamanca and the Mayo clinic ((Hernandez *et al.*, 2009; Van Dyke *et al.*, 2010). Both groups identified the significance of a large clone size and short TTFT, however it did not effect OS. These observation points to the fact that the 13q clone size is more important rather than if patients are heterozygous or homozygous.

5.1.13 Multivariate analysis of the above prognostic marker

Identification of reproducible independent prognostic factors is important for rational management of disease. It is paramount to stratify patients early, before progressive disease, prior to treatment. This would have significant influence on the management of these patients and help to identify patients who would be suitable for follow up in GP surgeries and therefore would have not only an economic impact on hospital services, but also on patients' quality of life.

There are a number of studies which attempt to combine these markers into the prognostic scores on the basis of multivariate analysis. One of the most validated prognostic models is a weighted prognostic model (Wierda *et al.*, 2007) constructed from six variables: age, β2M, absolute lymphocyte count, gender, Rai stage, and number of involved lymph nodes (LN) groups. This was predictive for 5 and 10-years survival. Wierda developed a 'normogram score' to estimate the 5-year survival probability for each individual patient. This prognostic index (PI) was tested on the independent cohort in the Mayo clinic (Shanafelt *et al.*, 2009a). Shanafelt confirmed that Wierda's normogram had TTFT prediction and OS when applied to Rai 0 patients, independent factors for OS in Mayo clinic were age, β2M, stage, number of LN regions. However they recommend refining the PI by adding biological prognostic markers like: interphas FISH, *IGHV*, ZAP-70, CD38 and CD49d.Scoring systems, e.g. Morabito *et al.* (Morabito *et al.*, 2009) proposed a prognostic score where stage A patients from Genova were stratified into three distinct groups based on the combination of ZAP70, CD38 expression and *IGHV* mutational status. However Orlandi EM *et al.* (Orlandi *et al.*, 2009a) was not able to validate this system on a cohort from Pavia.

Therefore I attempted to identify independent prognostic factors specific for the Leicester cohort. Univariate analysis identified a number of significant prognostic factors in the Leicester cohort (Tables 7.19a-c). Multivariate analysis identified independent prognostic factors of shorter OS: advancing age, unmutated *IGHV*, high β2M; for shorter TTFT: advanced stage and unmutated *IGHV*; for shorter LDT:

advanced age and high WCC. The multivariate analysis was done on patients with the complete datasets and therefore dramatically reduced the number of available patients for OS and TTFT to n=182, for LDT to n=164 (Table 7.37). This may undermine the validity of the analysis as only 36% of the whole cohort were analysed for OS and TTFT and 33% for LDT.

IGHV mutation and β2M (identified as important prognostic factors in univariate analysis) were also confirmed in multivariate analysis: *IGHV* mutation was a significant independent prognostic factor for OS and TTFT, whereas is only β2M for OS. An Italian group (Laurenti *et al.*, 2008) concluded that β2M>2.5mg/L together with age >65, Rai stage>0, deletion 17p or 11q carry independent prognosis for shorter OS. The prospective German CLL1 trial identified prognostic factors in early CLL in multivariate analysis for both OS and PFS: β2M, absolute lymphocyte count, level of thymidine kinase, age and sex (Bergmann *et al.*, 2007).

Stage at diagnosis was an independent prognostic factor for TTFT and most importantly stage A and stage A0 were both independent in the Leicester cohort. Stage was significant in univariate analysis for OS, however was not confirmed in the multivariate analysis. Molica *et al.* on behalf of the Italian study group published (Molica *et al.*, 2005) a 3-stage risk system, including advanced Rai stage, short LDT, high peripheral blood lymphocytosis. Also in Weinberg's *et al.* analysis stage was important (Weinberg *et al.*, 2007). Authors found in the multivariate analysis modified Rai stage, CD38 and serum LDH to predict time to treatment and OS and these factors were the basis of the prognostic score. In this study univariate analysis identified stage, WCC, LDT, *IGHV* mutation status and CD38 as significant for TTFT and OS. In this analysis cytogenetics did not statistically correlate with TTT (time to therapy) or survival.

Although it appears an obvious finding, but advancing age was significant in univariate analysis of the Leicester cohort in OS and LDT and it was confirmed in the multivariate analysis to be an important independent prognostic marker for survival. The Mayo group analysed the influence of age further (Shanafelt *et al.*, 2010b): CLL patients <75 had indeed reduced OS than normal population, however patients ≥75 survival did not differ to an aged matched general population. In the younger group (<75) *IGHV* mutation was the best predictor of OS, similar to our cohort.

Interestingly in Leicester WCC at presentation was significant in OS and TTFT but not LDT in univariate analysis, multivariate analysis identified WCC as an independent

prognostic factor for LDT but not for OS or TTFT. B cell count was identified as independent prognostic factor for TTFT in the cohort from the Mayo clinic (Shanafelt *et al.*, 2009b), also as mentioned above Molica *et al.* identified absolute lymphocyte count in his 3-stage risk system.

Absolute lymphocyte count was also an important independent factor in the German CLL1 trial mentioned above (Bergmann *et al.*, 2007).

5.2 Summary

In this chapter I analysed the Leicester CLL cohort and compared it with available prognostic markers.

I identified a number of interesting observations:

1. The independent prognostic factors from multivariate analysis for the Leicester cohort were age, *IGHV* mutation and β2M. β2M was the most significant prognostic marker in the Leicester cohort in multivariate analysis for both OS and TTFT. Serial measurements during disease progression may provide further insights.
2. Current prognostic markers were not able to identify prospectively Fludarabine-resistant patients. Patients who respond well to Fludarabine and refractory patients had the same prognostic parameters at diagnosis including time to the first treatment (Table 7.32 and Figure 7.23).
3. Patients with mutated *IGHV* gene segments requiring treatment appeared to be a distinctly different group to mutated untreated (Table 7.13).
4. Our CD38 data did not show strong prognostic significance and therefore current methodology should be reviewed (Table 7.15 and Figure 7.12)
5. There were a number of significant differences between stage A and stage A0 (Figure 7.1 and Table 7.2). Stage A and A0 were identified as independent prognostic factors for TTFT using multivariate analysis. On the basis of the analysis of the Leicester CLL cohort there was justification for clinical use of the stage A0, stage A patients should be followed up more closely.
6. Asian CLL patients appeared to have good prognosis in our cohort, although the reports from the Indian subcontinent and Gunawardana's report claim a more aggressive disease than in the European population (Table 7.27, Figure 7.20)(Gunawardana *et al.*, 2008). There are no studies with significant numbers of patients to confirm either of these observations. There were no Black CLL cases in our cohort, although from population numbers in Leicester and Leicestershire I should have found 3-4 patients.

Chapter 6: Final Discussion

The development of high throughput sequencing and micro-array techniques has allowed rapid and comprehensive global assessement of all DNA mutations, chromosomal rearrangements and epigenetic changes as well as determination of RNA levels within a neoplastic clone (REF). In contrast, our ability to detect all proteins expressed within a given compartment remains rudimentary. Proteomic technology is not sufficiently developed to identify all the proteins (as well as their post translational modifications) in a sample, even in the enriched sample such as plasma membrane. The best available equipment can only identify approximately 10%-20% of the proteins in a whole cell sample or 30% of the proteins in purified plasma membrane preparations. In addition, it will only identify a fraction of all known protein modifications (Dr R. Boyd, personal communication).

From proteomic screening of CLL plasma membrane fractions, the following proteins were identified in an initial screen: FAM129C, MIG2b, EVI2b (Boyd *et al.*, 2003) and Oxytocinase,. Similar proteomic screening of MCL in leukaemic phase identified HVCN1 (Capasso *et al.*, 2010), TRPV2, Raftilin and FAM3C (Boyd *et al* 2009). Only some of these proteins including HCVN1, Raftilin and MIG2b have been chraracterised functionally (Boyd *et al.*, 2009; Meves *et al.*, 2009; Capasso *et al.*, 2010).

This thesis presents some preliminary data on the characterisation of FAM129C in normal and malignant B-cells. FAM129C lacked any defined domains other than a pleckstrin homology domain, suggesting that the protein may act as an adaptor protein; however, bioinformatic analysis did not shed any light on possible FAM129C-dependent pathways. My studies have failed to delineate clearly the functions of this protein in B-cell development and physiology but some interesting data have been generated.

One possible role of FAM129C is as an adaptor protein down-regulated following NF-κB signalling, maintaining the resting/quiescent state, as the highest levels are shown in naïve B cells in comparison to memory B cells (the levels of expression were confirmed in microarray data from Chen's group (Shen *et al.*, 2004) and I have also shown that it is down-regulated upon proliferation of both normal and malignant B-cells. FAM129C does not however appear to have a direct function in BCR signalling; this was suggested by the lack of association of FAM129C expression with *IGHV*

mutational statues in CLL and specific experiments simulating BCR signalling pathway in normal and malignant B-cells. These observations were made on the basis of biochemical fractionation due to the lack of adequate antibodies suitable for immunohistochemistry. To progress this project further such an antibody would be invaluable. Another potentially interesting observation was the similar changes of expression of FAM129C with CXCR4 in developing B cells and in cells going through GC reaction. Further investigation into relation between FAM129C and CXCR4 in germinal centre cells may be important in understanding FAM129C role in B cells.

Another aspect was the expression of FAM129C in activated diffuse large B cell lymphomas (ABC DLBCL) therefore it would be interesting to confirm this observation on an analysis of primary ABC DLBCL cases. Again, these studies were thwarted by the lack of suitable antibodies/antisera for immunohistochemistry.

To further the FAM129C functional analysis it would be useful to create a model of a cell line deficient in FAM129C. Moreover the development of a FAM129C knockout mouse would provide insight into the phenotype. Generating knock out mice is costly and may not give definitive answers, e.g. the Raftlin knock out mouse was reported in 2003 (Saeki *et al.*, 2003) but it took several years to identify its functional role and it was not in B lymphocytes, but probably involved in T cell signalling (Saeki *et al.*, 2009).

This thesis sought to integrate a CLL database, with discovery of prognostically and biologically important genes.

<u>Future experiments to complete this project:</u>

1. Production of an antibodies suitable for IHC.
2. Further characterisation of protein-mRNA in parallel in a large number of CLL patients and characterisation of translational regulation.
3. Investigation of CXCR4 and FAM129C crosstalk and potential regulation.
4. Screen of primary ABC DLBCL cases with FAM129C to confirm its possible diagnostic utility.
5. Raising of FAM129C mutant mice.

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Appendix

Chapter 7: Leicester CLL database: The Results

7.1.1 Incidence and prevalence of CLL in the Leicester cohort

CLL is the most common leukaemia in Caucasians and rare in populations from the Asian subcontinent. Leicester is an ethnically diverse city in the UK with a large Asian population and substantial representations from the Black and Chinese communities. This provided a unique opportunity to analyse incidence and prevalence of CLL in migrant communities and their off-spring. The patients who had lymphocyte counts of $>5x10^9$/L and confirmed clonal lymphocytosis consistent with CLL were included in this study. There were only a few patients with monoclonal B cell lymphocytosis (MBL) and they were excluded from the analysis (Shanafelt *et al.*, 2010a).

To calculate prevalence in the Leicester population I obtained population details from the Census 2001. In 2001 Leicester had a total of 279,800 inhabitants (www.leicester.gov.uk/your-council-services/council-and-democracy/city-statistics/census2001/) and Leicestershire population was 610,300 (www.leics.gov.uk/ethnicity_in_leicestershire_web.pdf) (total of 756,150). In Leicester there were 85,659 South Asians and in Leicestershire 22,506 (total of 108,165).

The Black population as per the 2001 Census was 7,889 and the Chinese population 3,622 in Leicester and Leicestershire combined. The prevalence of CLL in Leicester and Leicestershire was **69 per 100,000** (i.e. 496 patients in 756,150). Prevalence re-calculated for Caucasians only (i.e. excluding all minorities) was higher: 75 in 100,000 (478 in 636,474).

Table 7.1 Summary table of Leicester cohort

All cases	n=496
Age median (range)	67 (34; 97)
M:F	1.8:1 (321:175)
WCC median (range)	19.4 (4; 568)
A0 n(%)	263 (56%)
A n(%)	114 (24%)
B n(%)	65 (14%)
C n(%)	29 (6%)
Treated n(%)	179 (38%)*
FC	69 (27%)**
CLB	95 (37%)(since 2001:43)
F	42 (16%)
Campath containing Rx	42 (16%)
AlloPBSCT	5 (3 MUD) (2%)
AutoPBSCT	4 (2%)
Asians n(%)	19 (4%)
Other cancers n(%)	87 (19%)
Familial n(%)	37 (8%)
Autoimmune n(%)	50 (11%)**
AIHA	27 (50%)
ITP	20 (37%)
Other	7 (13%)
IGHV M n(%)	297 (71%)
IGHV UM n(%)	121 (29%)
13q n(%)	124 (38%)
normal n(%)	86 (26%)
t12 n(%)	60 (18%)
11q n(%)	42 (13%)
17p n(%)	11 (3%)
other n (%)	38 (12%)
β2M n(range)	2.1 (1.1;7.6)
CD38 median(range) n<or>30%(%)	3.05 (0;100) (<30%=283 (75%); >30%=92(25%)
LDT median (range)	62 months (0.3; 180)
TTFT median (range)	98 months (0.1; 346)
OS median (range)	184 months (0.1; 374)

Age, WCC, OS, TTFT, LDT, β2M are median, values in brackets represent range; n represent available values. CD38: top value, median, bottom number of positive cases (i.e. >30%) and negative (i.e. <30%).
M:F-male to female; WCC-white cell count; OS-overall survival; TTFT-time to first treatment; FC-fludarabine cyclophosphamide; CLB-chlorambucil; F fludarabine; AlloPBSCT-allogeneic peripheral blood stem cell transplant; AutoPBSCT-autologous PBSCT; MUD-matched unrelated donor; LDT-lymphocyte doubling time; AIHA-autoimmune haemolytic anaemia; ITP-immune thrombocytopenic purpura, M-mutated *IGHV*; UM-unmutated *IGHV;* β2M β-2-microglobulin.
*NB. Total of 179 patients received treatment and 61% of these had multiple courses of treatment.
** percentages are of all used modalities (n=257)

In the period of 8 years there were 302 new patients seen in the Leicester Royal Infirmary, an average of 37.8 per year. The calculated mean annual incidence ratio (in 2000-2008) was 5 in 100,000 person-years. The majority of the patients (80%, 377 out of 471) were diagnosed incidentally on haematological investigation, these were represented by stages A0 and A.

There were 19 South Asian (Indian) patients in our Leicester cohort (19 patients in 108,165 total number of South Asians), which makes the prevalence of **17.6 per 100,000.** In our cohort there were no Chinese or Black patients (including Black mixed race). From the prevalence data I expected to identify four Black patients in the Leicester Black population of 8,000. The difference between the expected prevalence to the observed was statistically significant, $p<0.0001$ (Chi-squared, two-tailed).
The male to female ratio was 1.8:1 (321 males and 175 females). Median age at diagnosis was 67 years (34-97). Thirty eight percent (179 out of 469) needed treatment at some point during the follow up period.

7.1.2 Clinical staging

Clinical staging is important for evaluation and planning the management of patients with CLL. There are two systems used over the last 30 years in CLL staging: Binet and Rai, although the former is the most common used in the UK. A summary of the staging groups is presented in Table 7.2 and Figure 7.1. SLL cases were excluded. Stage A0 was defined by patients who present with lymphocytosis only with normal haemoglobin and platelets levels (therefore A0 is combination of A Binet and stage I of Rai. Significant differences were observed between the stages in WCC at diagnosis, frequencies of 13q deletion, normal cytogenetics, 11q deletion, 17p deletion and with trisomy 12 showing marginal significance. Differences in OS and TTFT were significant by analysis using logrank statistics, LDT was not significant.
Patients with mutated (M) and unmutated (UM) *IGHV* and levels of β2M were also statistically significant between the groups (non parametric test, Kruskal Wallis).

Table 7.2 Characteristics of Binet staging

	A0 56% (n=263)	A 24% (n=114)	B 14% (n=65)	C 6% (n=29)	Statistics
Age median (range) n available	65 (35;91) n=257	64 (34-92) n=107	65 (42;97) n=64	70.5(44-87) n=28	p=0.2355[b]
M:F (n:n)	1.5:1 (159: 104)	2:1 (77:37)	4:1 (52:13)	1.9:1 (19:10)	p=0.6[a]
WCC median (range) n available	18 (7.8;195) n=261	19.1(4;251) n=108	37.3(4.5;568) n=64	87(4.7; 354) n=27	p<0.0001[c]
Treatment n(%) n available	40 (15%) n=263	59 (52%) n=113	52 (81%) n=64	28 (97%) n=29	P<0.0001[a]
Asians n(%)	13 (5%)	4 (4%)	1 (2%)	1 (3%)	p=0.6226[a]
autoimmune n(%)	20 (8%)	16 (14%)	10 (15%)	4 (14%)	p=0.2[a]
M	167 (77%)	64 (66%)	36 (60%)	18 (64%)	p=0.0287[a]
UM	50 (23%)	33 (36%)	24 (40%)	10 (36%)	
13q** n available for cytogenetics	82 (47%) n=176	27 (36%) n=76	9 (17%) n=53	6 (33%) n=18	p=0.052[a]
Normal	53 (30%)	13 (17%)	15 (28%)	3(17%)	p=0.044[a]
t12	20 (11%)	18 (24%)	14 (26%)	5(28%)	p=0.059[a]
11q	11 (6%)	13 (17%)	12 (23%)	4(22%)	p=0.0116[a]
17p	3 (2%)	2 (3%)	6 (9%)	0	p=0.0104[a]
others	13 (7%)	12(13%)	10 (19%)	2(11%)	p=0.133[a]
β2M median (range) n available	2 (1.1;3.7) n=126	2.15 (1.4;4.7) n=46	3.2(1.5; 7.6) n=58	3.6(1.8;5.5) n=10	p<0.0001[c]
CD38 median n (% of >30%) n available	2.76 56 (25%) n=223	7 22 (24%) n=92	1.87 7 (21%) n=34	6.18 ; 7 (41%) n=17	p=0.9 [a]
LDT (months) median n available	63 months n=253*	73 months n=97*	52 months n=35*	68 months n=6*	p=0.1605[d]
TTFT median	175 months	45 months	8 months	1 month	p<0.0001[d]
OS median	243 months	216 months	124 months	74 months	p<0.0001[d]

[a]chi-square; [b]one-way ANOVA; [c]Kruskal Wallis; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, LDT, β2M, OS and PFS are shown as median values ; CD38: top value, median, bottom number of positive cases (i.e. >30%). Lymphoadenopathy was not included as it is part of Binet staging.
*6 of stage A0, 13 of stage A, 21 of stage B and 23 of stage C patients were treated within <6 month of diagnosis
**some patients had more than one cytogenetic abnormality, % calculated from number of patients

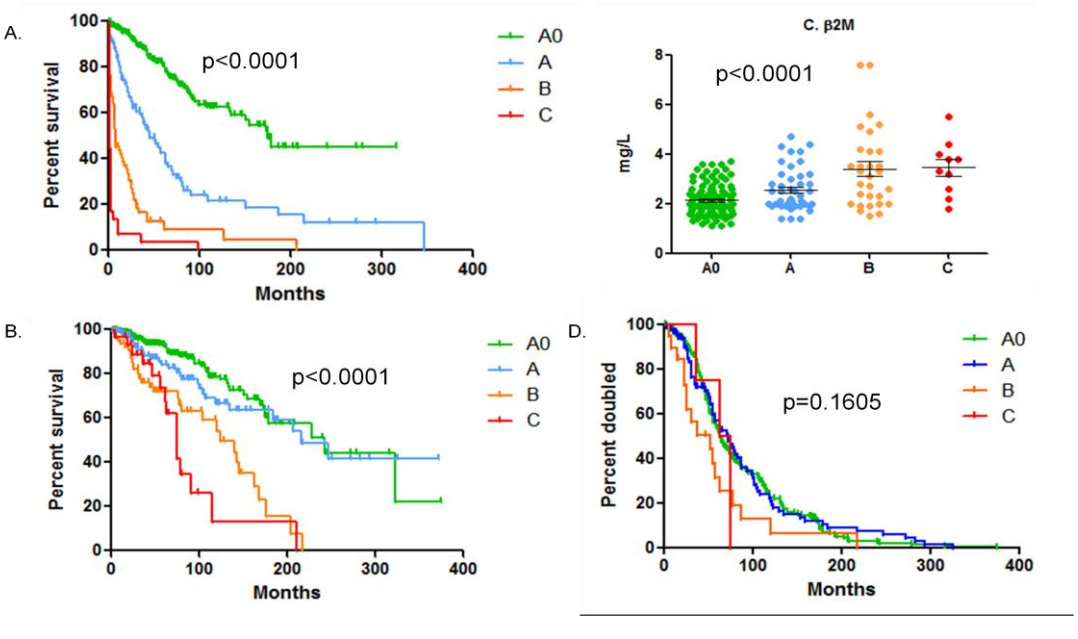


Figure 7.1 Mantel-Cox survival curve in CLL stages

A. Time to first treatment, B. Overall survival. C. β2M levels in Binet stages. D. Lymphocyte doubling time

Variables (n)	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
overall				<0.0001
A (n=114)	0.261	0.137	0.497	<0.0001
A0 (n=263)	0.191	0.105	0.350	<0.0001
B (n=65)	0.695	0.372	1.299	0.254
C (n=29)	1			

HR compares to stage C patients, for every 100 stage C patients who died: 19 stage A0 die, 26 stage A and 70 stage B(p<0.0001).

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
overall				<0.0001
A	0.144	0.09	0.232	<0.0001
A0	0.032	0.019	0.053	<0.0001
B	0.427	0.266	0.686	<0.0001
C	1			

HR compares to stage C patients, for every 100 stage C patients who require treatment 3 stage A0 needs treatment and 14 stage A and 43 stage B (p<0.0001).

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
overall				0.004
A	0.523	0.226	1.212	0.131
A0	0.552	0.244	1.249	0.154
B	1.038	0.433	2.488	0.933
C	1			

Table 7.3 Comparison of hazard ratio for OS, TTFT and LDT according to age

a. **at diagnosis**

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Age at Δ (numeric)	1.11	1.088	1.133	<0.0001

For every year increase in age at diagnosis there is a 1.11 more chance of dying: for 100 patients dying at a given age.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Age at Δ (numeric)	1.002	0.988	1.017	0.753

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Age at Δ (numeric)	1.036	1.024	1.048	<0.0001

b. **at first treatment**

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Age at Rx (numeric)	1.075	1.05	1.101	<0.0001

For every year increase in age at the first treatment there is a 1.08 more chance of dying.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Age at Rx (numeric)	1.001	0.987	1.0175	0.88

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Age at Rx (numeric)	1.022	1.002	1.042	0.031

Table 7.4 Comparison of hazard ratio for OS, TTFT and LDT for WCC at the diagnosis

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
WCC	1.005	1.003	1.007	<0.0001

For every increase in WCC by $1x10^9$/L there is a 1.005 more chance of dying.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
WCC	1.009	1.008	1.011	<0.0001

For every increase in WCC by $1x10^9$/L there is a 1.009 more chance of treatment.

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
WCC	1.005	1.000	1.009	0.039

Table 7.5 Comparison of hazard ratio for OS, TTFT and LDT for lymphoadenopathy (LN)

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
overall				0.002
LN=0	0.412	0.208	0.815	0.011
LN=1cm	0.8	0.395	1.62	0.536
LN>3cm	1			

HR compares to patients with LN>3cm. For every 100 dead patients with LN>3cm 41 patients without LN die (p=0.011), and 80 with small LN (<1cm) (p=0.536).

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
overall				<0.0001
LN=0	0.086	0.052	0.142	<0.0001
LN=1cm	0.496	0.31	0.794	0.003
LN>3cm	1			

HR compares to patients with LN>3cm. For every 100 treated patients with LN>3cm , 9 patients without LN would be treated (p<0.0001), and 50 with small LN (<1cm) would be treated(p=0.003)

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
overall				0.014
LN=0	0.453	0.266	0.769	0.003
LN=1cm	0.465	0.262	0.823	0.009
LN>3cm	1			

Table 7.6 Comparison of hazard ratio for OS and TTFT for autoimmunity

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Autoimmune	1.138	0.645	2.008	0.656

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Autoimmune	0.562	0.377	0.837	0.005

Patients with coexisting autoimmune conditions are more likely to be treated: for 100 patients with autoimmune conditions, 56 patients without autoimmune conditions will be treated..

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Autoimmune	1.149	0.801	1.646	0.451

Figure 7.1.C shows clear distinction of β2M serum levels between stage A0 (median=2.0) and A (median=2.15), there was a marginal significant difference between these two groups when compared using Student t-test, p=0.0051. Serum levels of β2M between stage B and stage C were similar (medians 3.2 and 3.55, respectively), p=0.595, however the numbers in the latter two groups were small and this may effect p-values.

Parameters which did not reach significance were: CD38, male to female ratio and the frequencies between: M and UM *IGHV* gene segments, autoimmune conditions, proportion of Asian patients.

Analysis of hazard ratios (HR) assessed using Cox regression analysis is presented in Tables 7.3-7.6. The risk of death was the highest for stage C (Table 7.3): for every 100 stage C patients who would die: 35 patients with stage A0 die and 50 with stage A ($p<0.0001$). The risk of receiving treatment was also the highest in stage C disease: for every 100 stage C patients who require treatment three stage A0 would need treatment, 14 stage A and 43 stage B ($p<0.0001$).

The age was analysed as a numeric continuous rather than categorical variable (i.e. dividing arbitrarily patients into 'young' and 'old' group). The patients who were older at diagnosis, not surprisingly had higher risk of death (Table 7.3.b; HR 1.11; 95% confidence interval (CI) 1.088-1.133) and similarly patients who were older at the first treatment (Table 7.3.c) also had higher risk of death (HR 1.075; 95%CI 1.05-1.101). Age was not statistically significant for HR when TTFT was analysed (p=0.88).

Patients with higher WCC (Table 7.4) had a higher risk of dying (HR 1.005; 95%CI 1.003-1.007) and of receiving treatment (HR 1.009; 95%CI 1.008-1.011). In this thesis I chose to define lymph nodes >3cm in diameter as 'bulky'. This diameter was chosen from Rossi *et al.* who used this diameter in the analysis of Richter's transformation in CLL (Rossi *et al.*, 2008a). Patients with bulky lymphadenopathy (Table 7.5) had higher risk of dying (LN=0, HR 0.412; 95%CI 0.208-0.815) and receiving treatment (LN=0, HR 0.086; 95%CI 0.052-0.142). Patients with coexisting autoimmune conditions are more likely to be treated (HR 0.562; 95%CI 0.377-0.0.837), but there was no statistically significant difference in overall survival.

7.1.3 Role of gender

CLL is more common in men. I investigated an impact of gender on prognostic factors and survival (Table 7.7 and Figure 7.2). There were significant differences in TTFT (p=0.001) and proportion of patients who require therapy. Females were more likely to be diagnosed in stage A0 and males in stage B. Women were older at the diagnosis (p=0.146), β2M levels were lower than in men, but did not reach statistical significance (p=0.074); OS for women was better (p=0.96), these parameters did not reach statistical significance (Figure 7.2). Females are less likely to die (p=0.085; HR 0.704; 95%CI 0.472-1.05) and less likely to receive treatment (p=0.018; HR 0.676; 95%CI 0.488-0.936).

7.1.4 Lymphocyte doubling time

LDT is a surrogate marker of B cell proliferation and LDT of <12months is a recognised adverse prognostic factor predicting patients who progress rapidly (Montserrat *et al.*, 1986). Patients whose LDT reached years have stable, non-progressive disease based on anecdotal observations. Although the convention divides lymphocyte doubling time (LDT) as >12 months and <12 months, I was interested in the extremes of disease and therefore I analysed the two spectra of the disease: aggressive CLL (LDT<12 months) and 'chronic' non-progressive CLL (LDT>5years). A period of more than 5 years was chosen in order to analyse patients with consistent non-progressive disease and secondly this group had similar numbers of patients as the <12 months group. In the Leicester cohort there were 22 patients with LDT>10years (10-15 years). The summary of the data is presented in Table 7.9 and Figure 7.3. Patients who were treated or died before they reached doubling time were excluded from analysis.

Significant differences between the 1 year- and 5 year-doubling time groups were seen in the proportion of patients with mutated and unmutated *IGHV*(p=0.013), all cytogenetic abnormalities (13q del p=0.02, 11q p=0.003, 17p p=0.017) except trisomy 12 (p=0.073), the 'normal' and 'other' cytogenetics (p=0.069); staging (A0 p=0.0013, A p=0.04, B p=0.0008, C p=0.06), level of β2M (p<0.0001), patients presenting without lymphadenopathy (p=0.002) or with lymph nodes greater than 3cm in diameter (measured clinically p=0.005),OS and TTFT (both p<0.001). No statistically significant differences were observed in the age at the diagnosis and frequency of autoimmune diseases. There were no differences in the male to female ratio, proportion of Asian patients and CD38 expression.

The longer LDT is associated with smaller risk (Table 7.10) both to die (HR 0.718; 95%CI 0.639-0.807) and for therapy (HR 0.446; 95%CI 0.371-0.537).

Table 7.7 Characteristics of male versus female patients

	Female (n=175)	Male (n=321)	Statistics
Age median (range) n available	67 (35; 91) n=157	65 (34; 97) n=299	p=0.146[b]
WCC median (range) n available	18.3 (4.3; 419) n=162	20 (4; 568) n=298	p=0.159[c]
A0 n(%)	104 (63%)	159(52%)	p=0.038[a]
A n(%)	37 (23%)	77(25%)	p=0.6[a]
B n(%)	13 (8%)	52(17%)	p=0.008[a]
C n(%)	10 (6%)	19(6%)	p=1[a]
LN=0 n(%) LN>3cm n(%)	0:106 (60%) >3cm: 9(5%)	0:178 (55%) >3cm (1%)	p=0.3[a] p=0.3
Treatment n(%)	53(30%)	128(40%)	p=0.04[a]
Asians n(%)	4 (2%)	15 (5%)	p=0.2[a]
autoimmune n(%)	18(10%)	32(10%)	p=1[a]
M n(%)	M 105(72%)	M 192(70%)	p=0.775[a]
UM n(%)	UM 41(28%)	UM 80(30%)	
13q n(%)* n available for cytogenetics	49(41%) n=119	75(36%) n=211	p=0.5[a]
normal	29(24%)	57(27%)	p=0.7[a]
t12	22(13%)	38(18%)	p=1[a]
11q	14(12%)	28(13%)	p=0.7[a]
17p	2(2%)	9(4%)	p=0.2[a]
other	13(11%)	25(12%)	p=0.86[a]
β2M median (range) n available	2.1 (1.1; 5.2) n=72	2.2 (1.1; 7.6) n=141	p=0.074[c]
CD38 median n (% of >30%) n available	2.25 33 (24%) n=140	3.83 59 (25%) n=234	p=0.59[c] p=0.9[a]
LDT median n available	65 months n=130	59 months n=245	p=0.2[d]
TTFT median	151 months	64 months	p=0.001[d]
OS median	206 months	173 months	p=0.096[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significance;
WCC, age, LDT, β2M ,OS and PFS are shown as median values
CD38: top value, median, bottom number of positive cases (i.e. >30%)
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

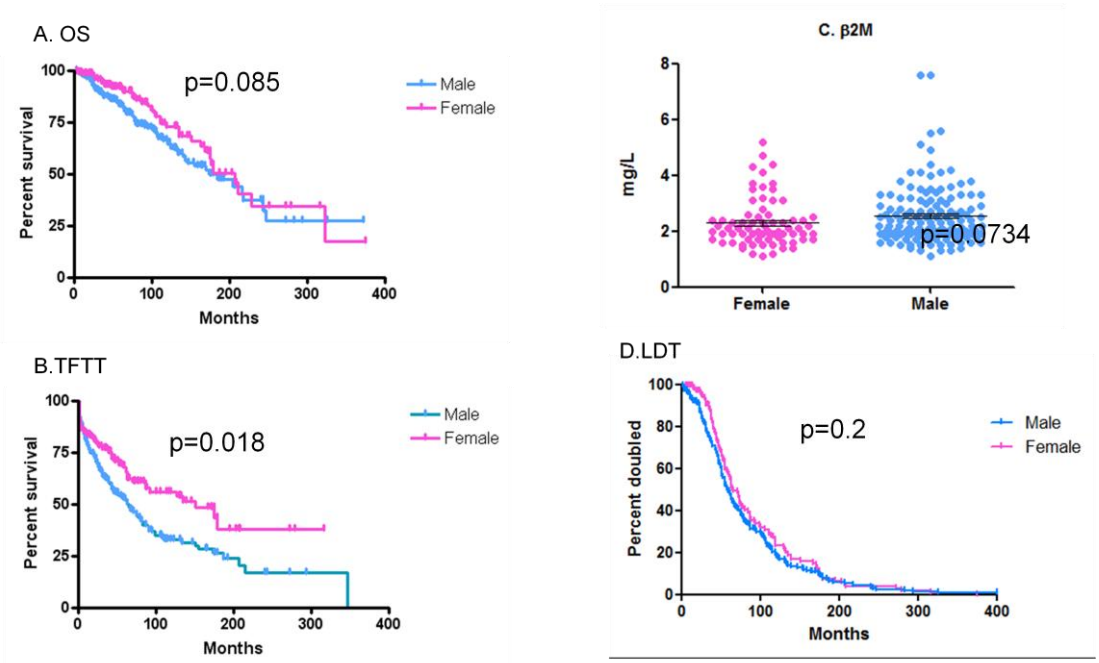


Figure 7.2 Mantel-Cox survival curve diference between gender.

A. OS and B. TFTT between male and female. C. β2M levels in male and female groups. D. LDT between male and female.

Table 7.8 Comparison of hazard ratio for OS, TTFT and LDT between male and female

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Female (n=175)	0.704	0.472	1.05	0.085
Male (n=321)	1			

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Female	0.676	0.488	0.936	0.018
Male	1			

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Female	0.825	0.653	1.043	0.108
Male	1			

Table 7.9 Characteristics of patients with short (≤1 year) and long lymphocytes doubling time (>5 years).

	LDT>5y (n=92)	LDT<1y (n=88)	Statistics
Age median (range)	62 (34; 84)	64 (42; 97)	p=0.12[b]
M:F (n/n)	1.4:1 (53/39)	2:1 (60/28)	p=0.5[a]
WCC median (range)	18.3 (6.3; 92.9)	20.5 (9.3; 255)	p=0.0391[c]
A0 n(%)	76 (83%)	33 (38%)	p=0.0013[a]
An(%)	14 (15%)	29 (33%)	p=0.04[a]
B n(%)	2 (2%)	21 (24%)	p=0.0008[a]
C n(%)	0	5 (6%)	p=0.06[a]
LN=0 n(%) LN>3cm n(%)	0: 80 (87%) >3cm: 1(1%)	0: 35(40%) >3cm: (13%)	p=0.002[a] p=0.005[a]
Treatment n(%)	8 (9%)	62 (70%)	<0.0001[a]
Asians n(%)	3	4	p=0.7[a]
autoimmune n(%)	8 (9%)	16/88	p=0.13[a]
M n(%)	71 (92%)	39 (49%)	p=0.013[a]
UM n(%)	6 (8%)	41 (51%)	
13q n(%)* n available for cytogenetics	28 (51%) 55	15 (22%) 66	p=0.02[a]
normal n(%)	19 (35%)	15 (23%)	p=0.189[a]
t12 n(%)	6 (11%)	18 (27%)	p=0.073[a]
11q n(%)	2 (4%)	18 (27%)	p=0.003[a]
17p n(%)	0	7 (11%)	p=0.017[a]
other(%)	2 (4%)	10 (15%)	p=0.069[a]
β2M median (range) n available	1.9 (1.1; 3.7) n=40	2.5 (1.4; 5.6) n=44	p<0.0001
CD38 median n (% of >30%) n available	3.39; 21 (27%) n=77	9.55; 21 (30%) n=70	p=0.4[c] p=0.4[a]
TTFT	346 months	25 months	p<0.001[d]
OS	undefined	118 months	p<0.001[d]

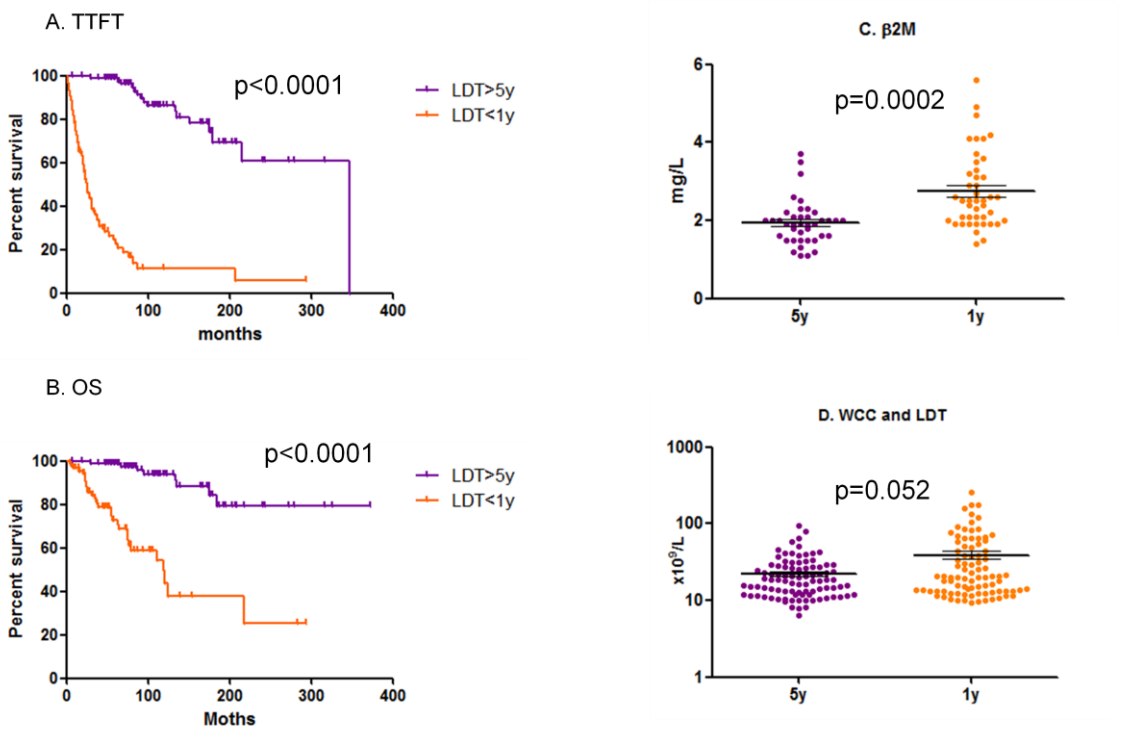


Figure 7.3 Mantel-Cox survival curve Time to first treatment,

B. Overall survival, C. β2M distribution in patients with 1 year doubling time and 5y, bars represent SEM. D. WCC difference between <1year group and >5 year group (Y axis is $\log_{10}$ scale for the purpose of clarity).

Table 7.10 Comparison of hazard ratio for OS and TTFT for each year of LDT

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
LDT	0.718	0.639	0.807	<0.0001

For every year increase in LDT risk of death is reduced by 29.8%: for 100 patients dying if LDT doubled in 1 year, 72 patients died; if LDT was 2 years and 52 died; if it was 3 years, 37 patients etc.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
LDT	0.446	0.371	0.537	<0.0001

For every year increase in LDT chances of treatment are reduced by 55.4%: for 100 patients treated when LDT doubled in 1 year, 45 patients were treated if LDT was 2 years and 20, if it was 3 years: only 9 patients needed treatment.

7.1.5 *IGHV* gene segments usage and mutational status

The characterisation of the B cell receptor in CLL cells became a mainstay of prognostic analysis, in particular the presence of *IGHV* mutations and type of gene segments used. Unmutated *IGHV* status and specific segment usage (i.e. *IGHV*1-69 (if unmutated) and 3-21) are well documented poor prognostic factors. Here, I performed characterisation and comparison of each of the group. The *IGHV* mutation can be analysed using the genomic DNA or RNA (cDNA). Our laboratory used the genomic DNA based method.

The majority (84%, 418 out of 496) of the patients had their *IGHV* mutation status tested. The 16% of missing results constituted pending results, lack of stored material or rare cases were the amplification of PCR product was not possible, three patients who had both a mutated and unmutated clones were also excluded. The data is presented in Figure 7.4-7.10 and Table 7.11. The frequencies of *IGHV* gene segments observed in our cohort were shown in Figure 7.4.A. The ten most common utilised segments were grouped in Figure 7.4.B and compared to the *IGHV* distribution in Mediterranean cohort (Ghia *et al.*, 2005b). The three most common gene segments were 1-69, 4-34 and 3-23 with similar frequency in both groups. There were certain *IGHV* families which were preferentially mutated and unmutated, those genes are presented in Figure 7.4.C and D and show that the most commonly mutated *IGHV* is 4-34 and the most common unmutated was *IGHV*1-69.

The comparison between mutated and unmutated patients irrespective of *IGHV* is shown in Table 7.11 and Figure 7.5 and 7.6, WCC at the diagnosis p=0.052 (Figure 7.5.B), the statistically significant differences found were: the proportion of 11q (p=0.0002), but 17p deletion (p=0.007) in unmutated cases, normal cytogenetics (p=0.02), β2M (p=0.0002) (Figure 7.5.A), LDT (p<0.0001), treatment requirement (p=0.004), and lack of lymphadenopathy (p=0.01) presentation with lymph nodes greater than 3 cm in diameter (p=0.025). There was a significant difference in OS and TTFT between the two groups (both p<0.0001).

There was no significant difference in the proportion of trisomy 12, 13q deletion, stage at presentation or proportion of patients with CD38>30%. Unlike other analyses I found that by comparing CD38 positive (i.e.>30%) and negative cases (i.e. <30%) using the Fisher's exact test, the difference was not significant, however when the means were compared using Mann Whitney test, the difference was significant (Table 7.11).

Not statistically significant variables were 13q and 'other' abnormalities and stage A0 and stage B at the diagnosis.

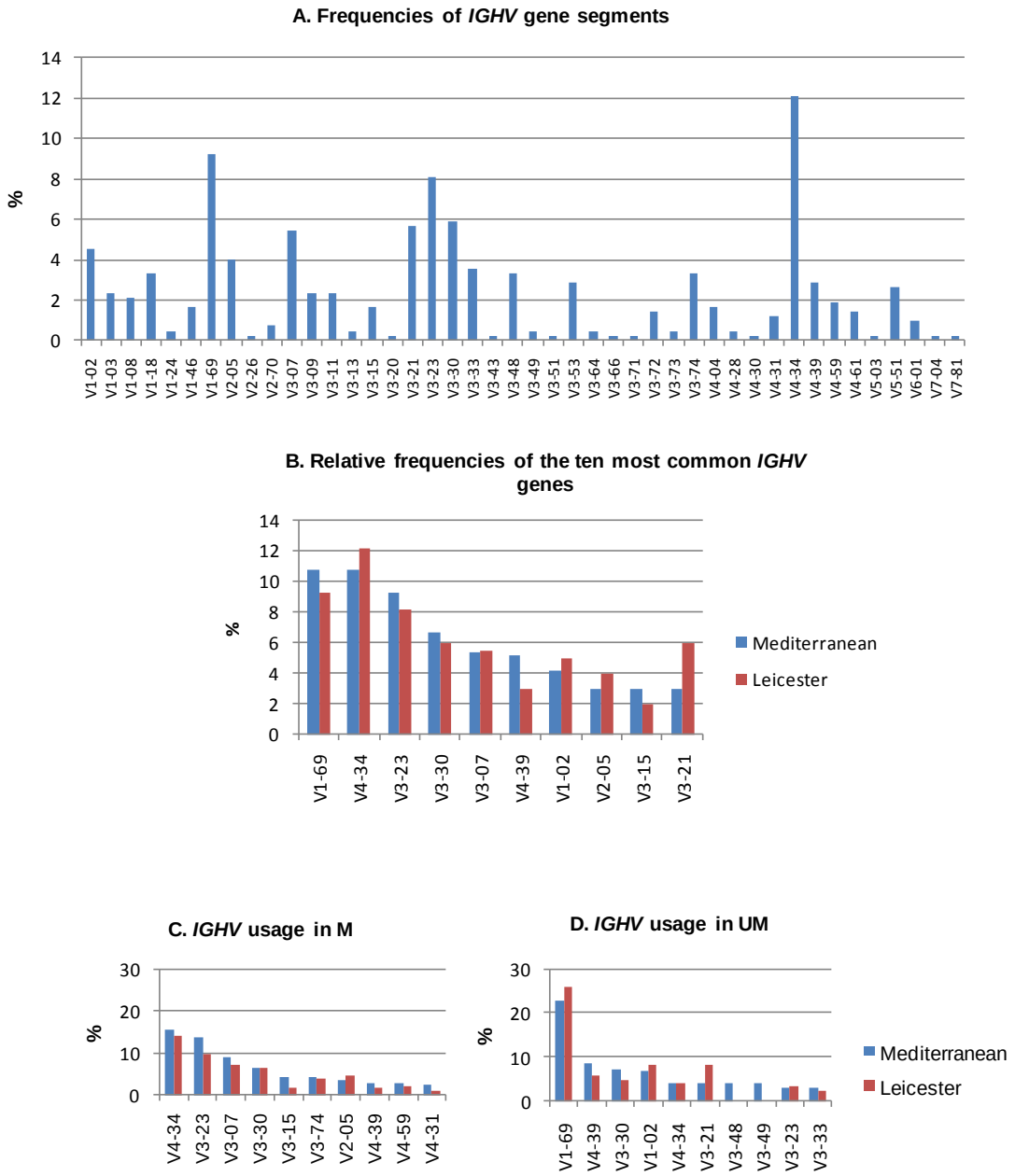


Figure 7.4 *IGHV* families in the Leicester cohort

A. Frequencies of all *IGHV* gene segments. B. Ten of the most common *IGHV* genes in comparison to the Mediterranean cohort (Ghia, 2004). C. Ten most common mutated *IGHV*. D. Ten most common *IGHV* in unmutated gene segments.

Table 7.11 Characteristics of mutated *IGHV* versus unmutated.

	Mutated (n=297)	Unmutated (n=121)	Statistics
Age median (range) n available	65 (34; 97) n=280	65 (40; 90) n=117	p=0.5[b]
M:F (n:n)	1.8:1 (192:105)	1.95:1 (80:41)	p=0.8[a]
WCC median (range) n available	19.25 (4.5; 419) n=281	21.7 (4.3; 568) n=116	p=0.052[c]
A0 n(%)	167(59%)	50(43%)	p=0.1[a]
A n(%)	64(23%)	33(28%)	p=0.4[a]
B n(%)	35(12%)	24(21%)	p=0.07[a]
C n(%)	18(6%)	10(9%)	p=0.5[a]
LN=0 n(%) LN>3cm n(%)	0: 183(70%) >3cm: 14(5%)	0= 52(43%) >3cm=16(13%)	p=0.01[a] P=0.025[a]
Treatment n(%)	92(31%)	75(62%)	p=0.004[a]
Asians n(%)	11(4%)	3(2%)	p=0.5[a]
autoimmune n(%)	27(9%)	17(14%)	p=0.22[a]
13q n(%)* n available for cytogenetics	81(43%) 190	24(27%)	p=0.1[a]
normal n(%)	58(31%)	12(13%)	p=0.02[a]
t12 n(%)	30(16%)	21(24%)	p=0.3[a]
11q n(%)	12(6%)	26(29%)	p=0.0002[a]
17p n(%)	1(0.5%)**	7(8%)	p=0.007[a]
other n(%)	19(1%)	16(18%)	p=0.1[a]
β2Mmedian (range) n available	2 (1.1; 5.6) n=131	2.5 (1.3; 7.6) n=56	p=0.0002[c]
CD38 median n (% of >30%) n available	2 >30%: 39(17%) n=226	9.72 >30%: 22 (23%) n=95	p=0.0216[c] p=0.7[a]
LDT n available	62 months n=227	52 months n=89	0.0005[d]
TTFT	173 months	37 months	p<0.0001[d]
OS	216 months	111 months	p<0.0001[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, β2M , OS and PFS are shown as median values
CD38: top value, median, bottom number of positive cases (i.e. >30%)
*some patients had more than one cytogenetic abnormality, % calculated from number of patients
**this patient had CLL with prolymphocytic transformation

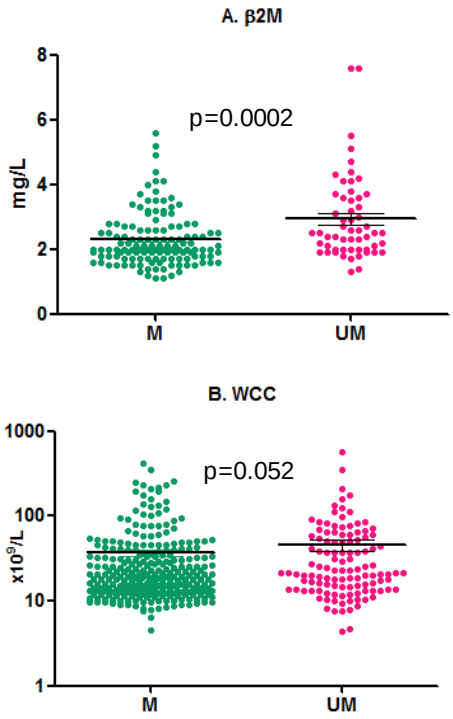


Figure 7.5 β2M na mutation status
A. β2M distribution in patients with mutated (M) and unmutated (U)*IGHV* gene segments, bars represent SEM. B. WCC difference between M group and UM group (Y axis is $\log_{10}$ scale for the purpose of clarity).

Table 7.12 Comparison of hazard ratio for OS , TTFT and LDT between M and UM

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
M	0.417	0.279	0.622	<0.0001
UM	1			

Patients with mutated *IGHV* (M) gene segments have risk of death reduced by 58.3% than with unmutated I*GHV* (UM), e.g. for 100 UM patients who die, 42 M patients die.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
M	0.353	0.258	0.484	<0.0001
UM	1			

Patients with mutated *IGHV* (M) gene segments have risk of death reduced by 64.7% than with unmutated I*GHV* (UM), e.g. for 100 UM patients who receive treatment, 35 M patients are treated.

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
M	0.771	0.590	1.008	0.058
UM	1			

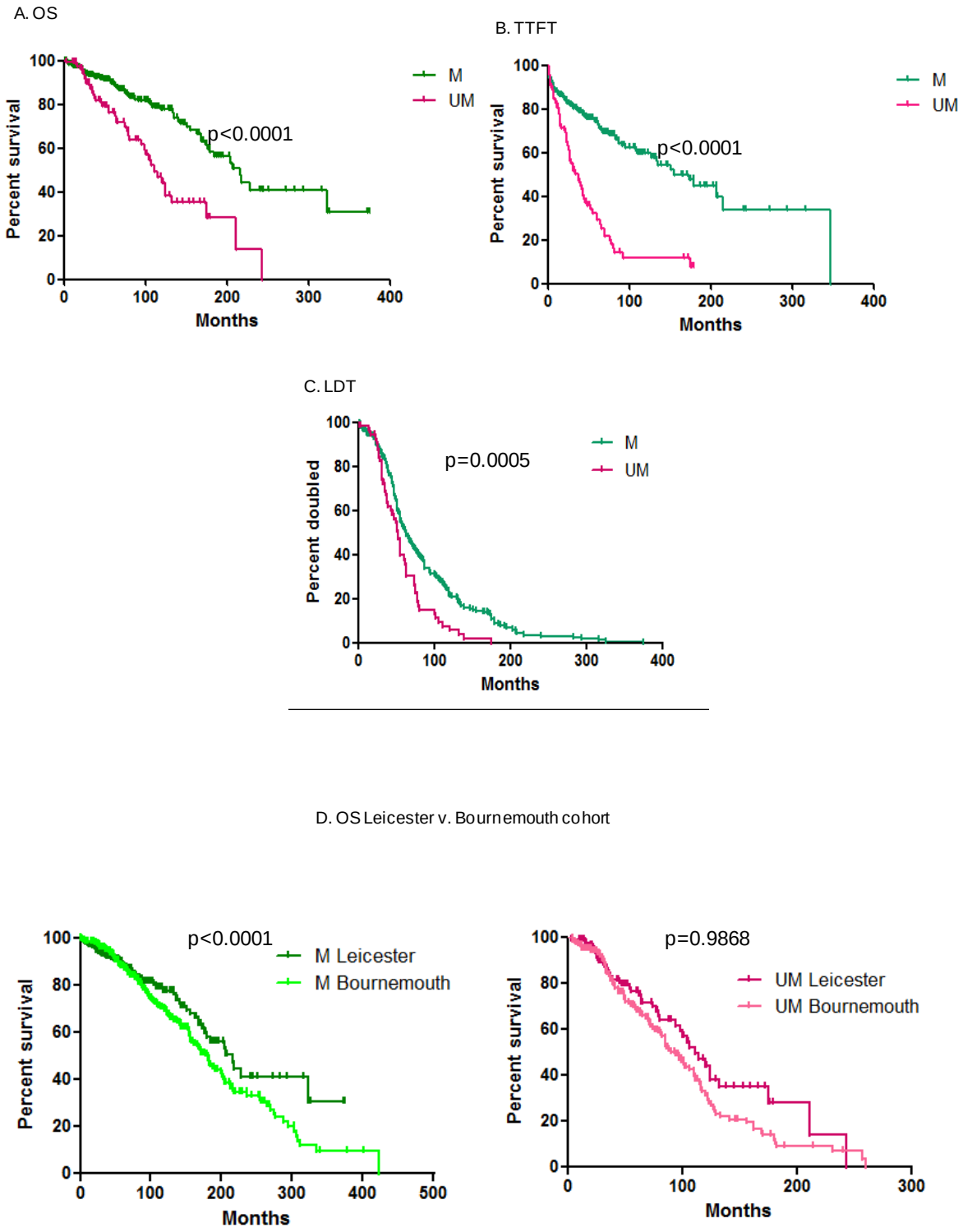


Figure 7.6 Survival curves in the mutated and unmutated.

Mantel-Cox survival curve Overall survival (A) and time to first treatment(B), LDT (C) between mutated (M) and unmutated (UM), , D. comparison between OS mutated patients from Bournemouth and Leicester (Bournemouth data courtesy of Z. Davis, M: n=172 median OS:216 and 94months, UM: n=339, median OS 111 v 98 months).

The distinct differences observed within the mutated group were between treated and untreated patients (Table 7.13 and Figure 7.7).

The significant differences were in age (p=0.046) and WCC (p<0.0001) at the diagnosis, Binet stage (A0, p<0.001; A, p=0.0326; B, p<0.0001; C, p<0.0001), absence of lymphadenopathy (p<0.0001) and lymph nodes greater than 3cm (p=0.0008), LDT (p<0.0001), proportion of co-existing autoimmune conditions (p=0.0486), β2M level (p<0.0001), 13q deletion (p<0.0001) and trisomy 12 (p=0.0012), other cytogenetic abnormalities were not statistically significant. There was a marginally statistical difference in OS. There was a reduced risk for the patients with M *IGHV* for both dying (HR 0.417; 95%CI 0.279-0.622) and treatment (HR 0.353; 95%CI 0.258-0.484) (Table 7.12).

Below I present detailed analysis of two *IGHV* segments: 1-69 and 3-21. *IGHV*1-69 presents with dichotomy where mutated *1-69* had good prognosis disease, and unmutated poor prognostic disease, similar to 3-21, which was known for the bad prognostic characteristic regardless of the mutation status. I found that *IGHV*1-69 in our cohort showed male preponderance, this observation was published as a letter in Leukemia (Walewska *et al.*, 2007).

7.1.5.1 IGHV1-69

*IGHV*1-69 was the second most commonly found *IGHV* segment after 4-34 (Figure 7.4.B) and the most common amongst unmutated *IGHV* (Figure 7.4.D): *IGHV*1-69 is 9.6% of all *IGHV* segments (40/418), 26% (32/121) amongst unmutated *IGHV* and 2.7% (8/297) of mutated. I observed that in our cohort *IGHV*1-69 occurs predominantly in men. I compared our data with data from Bournemouth (n=307) and the CLL4 trial (n= 540, both kindly provided by Prof D.G. Oscier). Figure 7.8 shows numbers of males and females in each gene segment. In all three cohorts (total 1,239) there were 133 men and 28 women (ratio 4.75:1). I calculated the difference between male to female proportions using the two-tail probability test between the two independent proportions, p<0.0002.

The minority of *IGHV*1-69 was mutated, in CLL4: there were eight mutated cases out of 92 (8.7%); in the Leicester cohort 8 out of 31 (26%). There were no females amongst mutated patients. All mutated cases presented at stage A0 and their lymphocyte doubling time was very long (Figure 7.9.A). The unmutated *IGHV* cases had long periods where the disease was stable before progression (Figure 7.9.B). These two figures (3.8.A and 3.8.B) were presented in Figure 7.9.C, where the Y axis represents rates of WCC increase calculated as counts per day calculated by the difference of

Table 7.13 Characteristics of patients with M genes treated v untreated

	M treated n=91	M not-treated n=192	Statistics
Age median (range)	62(34; 91)	66 (41; 97)	p=0.046[b]
M:F (n:n)	2:1(62:29)	1.7:1(122:70)	p=0.506[a]
WCC median (range)	34.9 (4.5; 419)	17.04(7.8; 193)	p<0.0001[c]
A0 n(%)	16(17%)	150(78%)	p<0.0001[a]
A n(%)	28(31%)	36(19%)	p=0.0326[a]
B n(%)	30(33%)	5(2%)	p<0.0001[a]
C n(%)	17(19%)	1(1%)	p<0.0001[a]
LN=0 n(%) LN>3cm n(%)	0: 27(36%) 3cm:10(14%)	0: 155(84%) 3cm=4(2%)	p<0.0001[a] P=0.0008[a]
Asians n(%)	4(4%)	7(4%)	p=0.749[a]
autoimmune n(%)	13(14%)	13(7%)	p=0.0486[a]
13q n(%)* n available for cytogenetics	11(20%) 55	70(53%) 131	p<0.0001[a]
normal n(%)	18(33%)	38(29%)	p=0.605[a]
t12 n(%)	16(29%)	12(9%)	p=0.0012[a]
11q n(%)	6(11%)	6(5%)	p=0.1865[a]
17p n(%)	1(2%)	0	p=0.223[a]
other n(%)	8(15%)	10(8%)	p=0.298[a]
β2M n(%)	2.6(1.6; 5.2)	2.0 (1.1; 5.6)	p<0.0001[c]
CD38 median n (% of >30%) n available	3.8 17 (29%) 58	1.8 36(22%) 161	p=0.124[c]
LDT median	61 months	48.5 months	p=0.1946[c]
TTFT	n/a	n/a	
OS median	184 months	undefined	p=0.0556[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, OS, β2M, CD38 and PFS are shown as median values
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

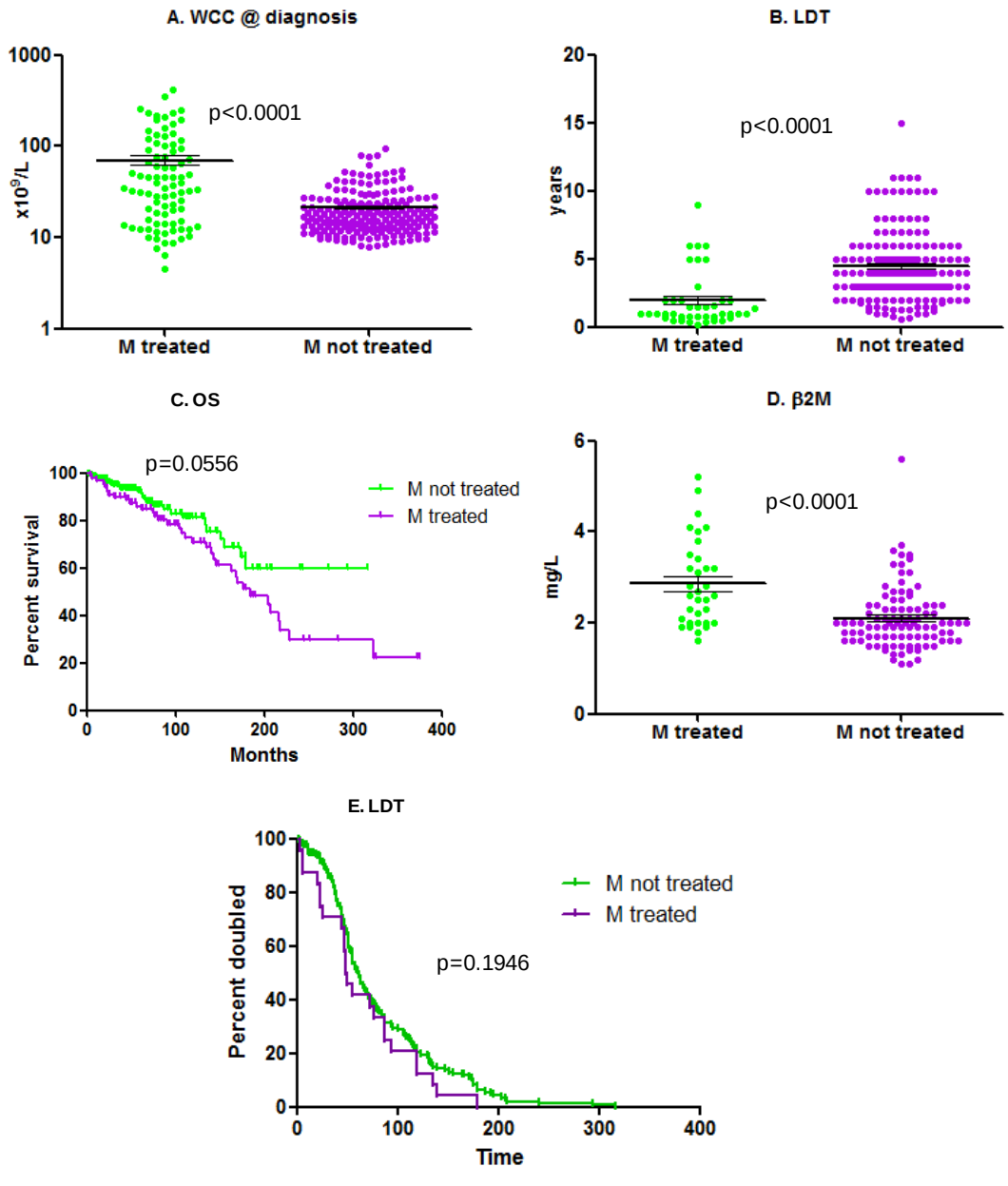


Figure 7.7 Comparison of patients treated and untreated with mutated *IGHV*.

A. WCC at diagnosis; B. Lymphocyte doubling time; C. Overall survival; D. β2M, E. LDT.

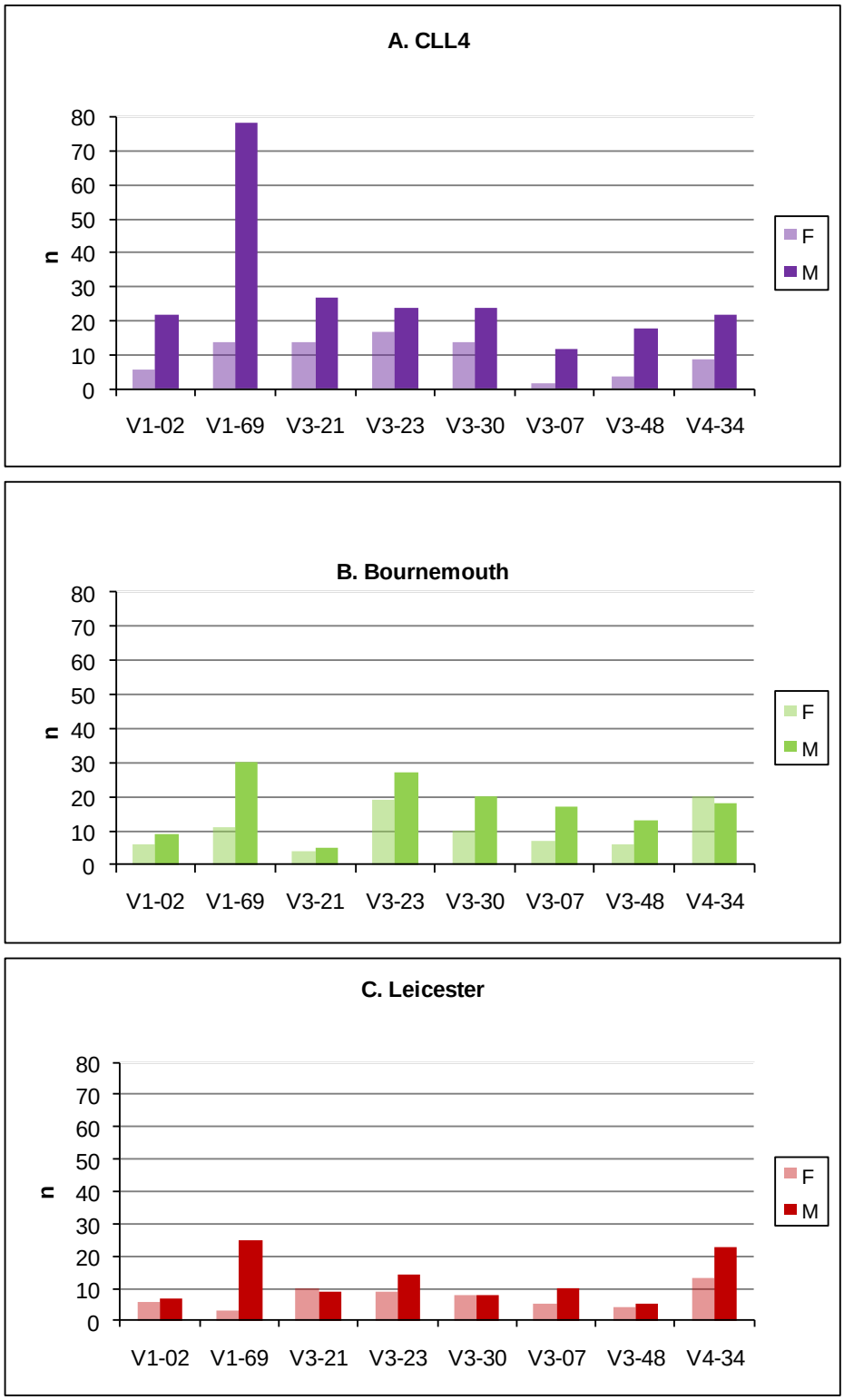
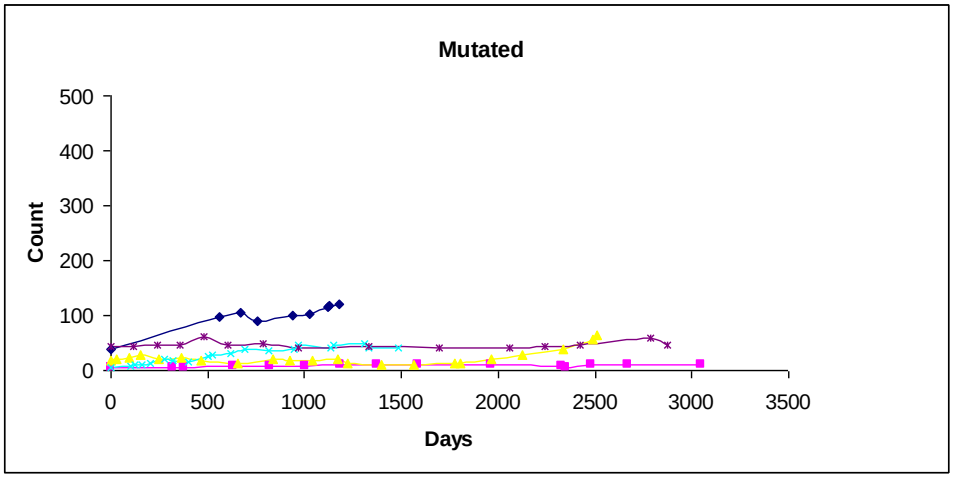
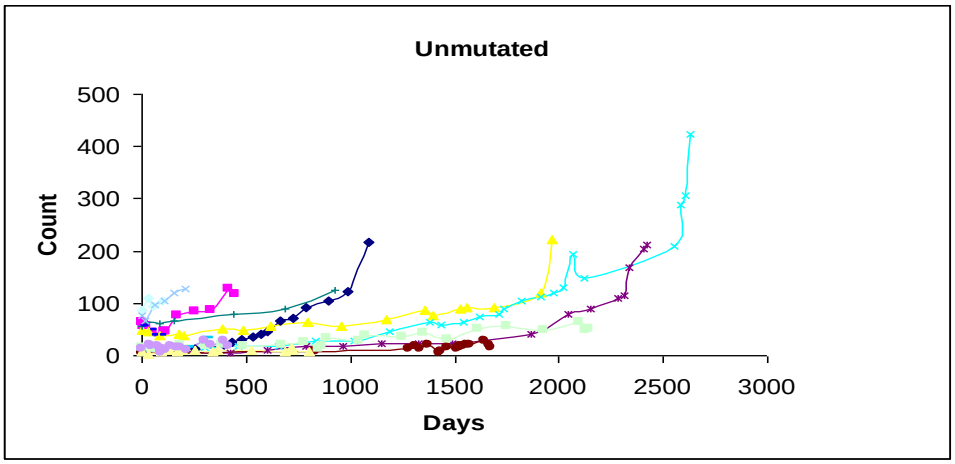


Figure 7.8 Comparison of male, female and their distribution across *IGHV* gene segments in three cohorts.

A.



B.



C.

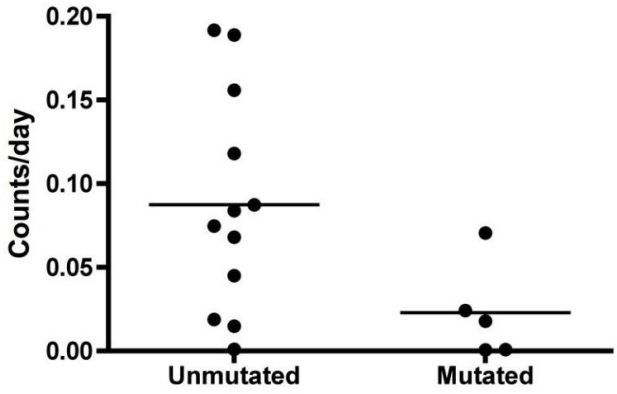


Figure 7.9 Rates of peripheral blood accumulation in *IGHV 1-69*

A-Mutated and **B**-unmutated rates of peripheral blood accumulation **C.** Rates of counts per day for unmutated and mutated *IGHV 1-69*. A and B Lymphocytes counts plotted on the linear scale , different colours represent different patients. Mutated patients have different lymphocyte count's trends. C. The counts/ day were calculated by the difference of WCC taken at the diagnosis and at the last follow up divided by number of follow up days.

WCC taken at the diagnosis and at the last follow-up divided by number of follow up days. However there was no significant difference in OS between mutated and

unmutated *IGHV*1-69 (the numbers in mutated group were small, n=8) (Figure 7.10.B). There was a significant difference in TTFT, p=0.0047 (Figure 7.10.A).

7.1.5.2 IGHV3-21

*IGHV*3-21 is an interesting gene segment, it is 4th commonest in the Leicester cohort and 10th in the Mediterranean cohort (Figure 7.4). It was shown that patients with this gene segments have poor prognosis regardless of the mutation status (Tobin *et al.*, 2002b). Comparison between unmutated *IGHV* and *IGHV*3-21 is presented in Figure 7.11 and Table 7.14. There were no significant differences between unmutated *IGHV* group and *IGHV*3-21 (both mutated and unmutated), there were no cases of *IGHV*3-21 in the unmutated cohort. The overall survival and time-to-first-treatment between the two groups were very similar.

7.1.6 CD38 as a prognostic marker

Analysis of *IGHV* mutation was considered unfeasible for daily clinical practice and therefore CD38 was proposed as a surrogate marker. CD38 positivity, as has been previously published (Damle *et al.*, 1999), was found to be associated with unmutated *IGHV* genes (p=0.0001). CD38 as a surrogate marker and the cut-off used remains a controversial issue. CD38 however emerged as an important independent proliferation marker and showed values of inverse prognostic characteristics. Because CD38 is a surface marker, therefore is easily accessible for the surface staining by flow cytometry, easily reproducible, inexpensive and suitable for quality control.

The summary of results is shown in Table 7.15 and Figure 7.12.

There were 25% (n=92) CD38 positive patients. The median age was not statistically significant between the two groups (p=0.089), but CD38 positivity was seen in older patients. Male to female ratios were similar in positive and negative sets. There were also no differences in representation of stage A and B in both groups. There was a weak statistical significance of the distribution in stage C, this may be due to the small numbers in each set. There was no difference between overall survival between CD38+ and CD38- groups (p=0.146) but was a significant difference in time to first treatment (p=0.045).

In order to see if CD38 has discriminatory value in all stage A patients (including A0) I re-analysed overall survival data in CD38 positive and negative stage A patients. There was no significant difference in OS of stage A CD38 positive and negative patients (p=0.087), however when stage A0 was excluded, the significance was marginally improved: p=0.07, but did not reached statistical significance.

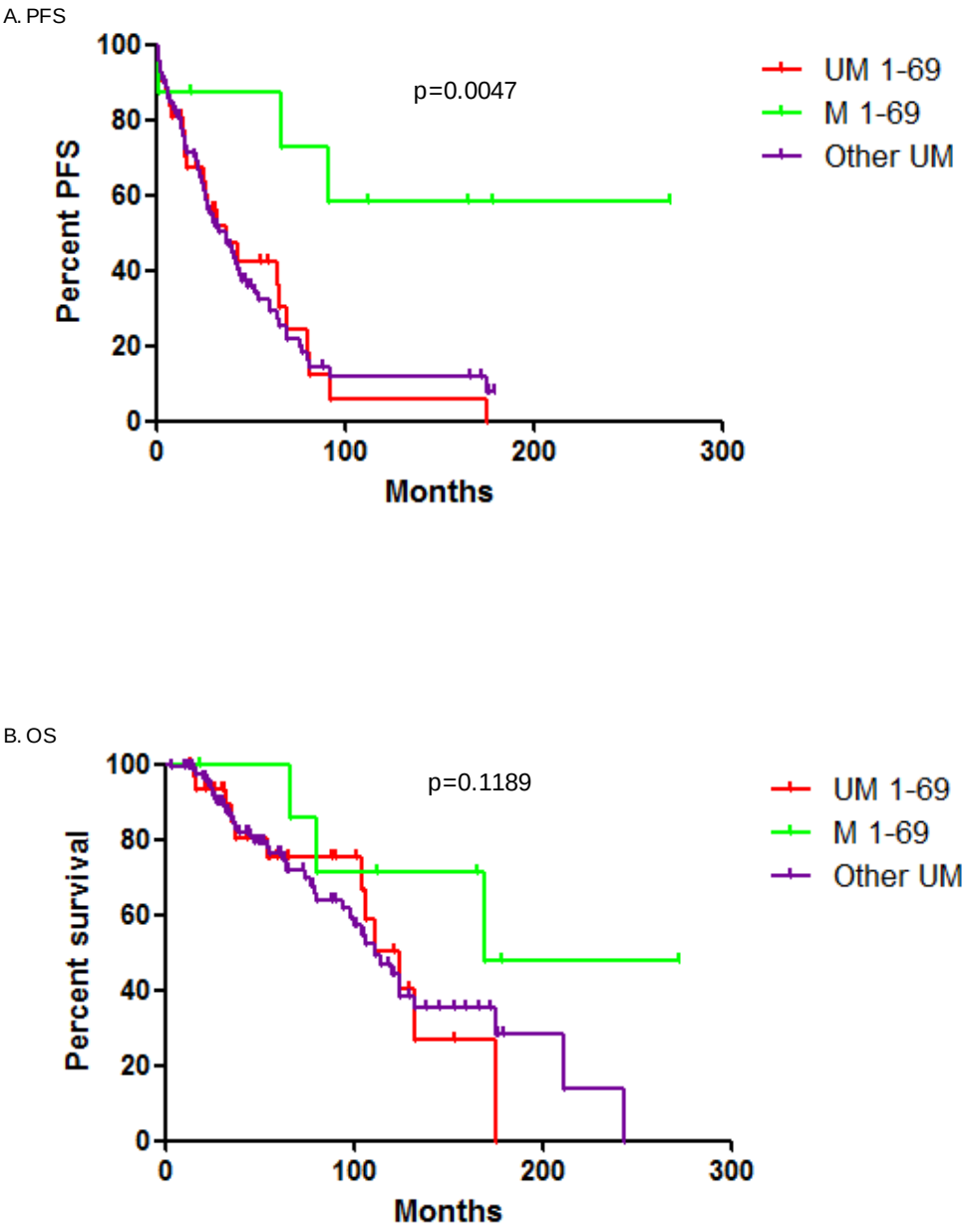


Figure 7.10 Mantel-Cox survival curve of *IGHV 1-69*

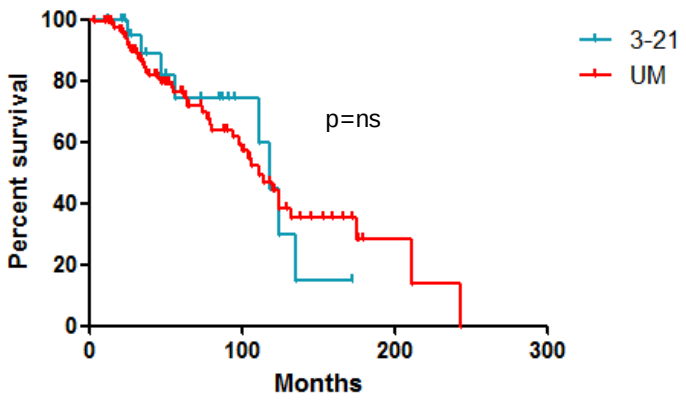
OS and PFS for Leicester cohort with *IGHV1-69* mutated (M) and unmutated (UM).PFS M=undefined, UM=37 months; OS M=169, UM 124 months, p values correspond to the difference between *IGHV 1-69* mutated and unmutated. PFS and OS of other unmutated IGHV is shown for comparison.

Table 7.14 Characteristics of *IGHV 3-21* versus unmutated group

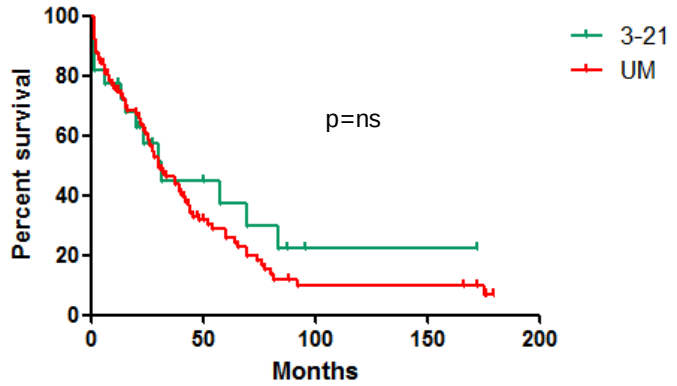
	IGHV 3-21 (n=24)	All UM* N=121	Statistics
Age median (range)	65 (50; 80)	65 (40; 90) n=121	p=0.822[c]
M:F (n:n)	1.3:1 ()	1.95:1 (80:41)	p=0.7[a]
WCC median (range)	20.5 (7.5; 174) n=22	21.7 (4.3; 568) n=115	p=0.5363[c]
A0 n(%)	10(45%)	50(43%)	p=1[a]
A n(%)	5(23%)	33(28%)	p=0.6[a]
B n(%)	3(14%)	24(21%)	p=0.6[a]
C n(%)	4(18%)	10(8%)	p=0.3[a]
Treatment n(%)	16(67%)	77(64%)	p=0.8[a]
autoimmune n(%)	1(4%)	17(14%)	p=0.3[a]
M n(%)	13 (57%)	n/a	
UM n(%)	10(43%)	n/a	
13q n(%)** n available for cytogenetics	7(50%) 14	24(27%)	p=0.3[a]
normal n(%)	4(29%)	12(13%)	p=0.3[a]
11q n(%)	1(7%)	26(29%)	p=0.19[a]
t12 n(%)	0	21(24%)	p=0.12[a]
17p n(%)	0	7(8%)	p=0.6[a]
other n(%)	2(14%)	16(18%)	p=1[a]
β2M median (range)	2.2 (1.6; 7.6) n=13	2.5 (1.3; 7.6) n=55	p=0.215[c]
CD38 median n (% of >30%) n available	6.92 9(43%) n=21	9.72 24(24%) n=98	p=0.38[c] p=0.3[a]
LDT median n available	85 months n=17	52 months n=89	p=0.0351[d]
TTFT median	31 months	37 months	ns[d]
OS median	118 months	111 months	ns[d]

[a]Fisher's exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance;
WCC, age, β2M, OS and PFS are shown as median values; CD38: top value, median, bottom number of positive cases (i.e. >30%)
*None of UM include 3-21 gene segment
**some patients had more than one cytogenetic abnormality, % calculated from number of patients

A. OS



B.TTFT



C.LDT

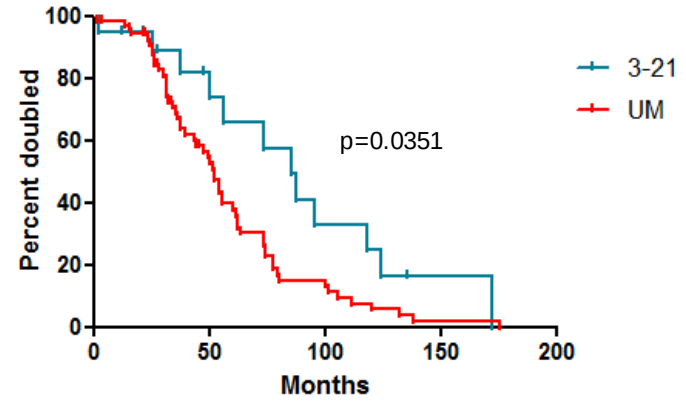


Figure 7.11 Mantel-Cox survival curve *IGHV 3-21* compared to UM *IGHV* in Leicester cohort;

A. overall survival; B: time to first treatment. There is no difference in OS and PFS between UM and *IGHV* 3-21 (both M and UM); C. LDT.

Table 7.15 Characteristics of CD38+ (>30%) versus CD38- (<30%) group

	CD38+ (n=92)	CD38- (n=283)	Statistics
Age median (range) n available	67 (35; 87) n=92	64 (34; 92) n=265	p=0.089[b]
M:F (n:n)	1.8:1 (59: 33)	1.6:1 (176:106)	p=0.8[a]
WCC median (range) n available	18.9 (9.1; 178) n=90	20 (7.9; 255) n=258	p=0.3169[c]
A0 n(%)	56 (61%)	167 (61%)	p=1[a]
A n(%)	22 (24%)	68 (25%)	p=1[a]
B n(%)	7 (8%)	27 (10%)	p=0.68[a]
C n(%)	7 (7%)	10 (4%)	p=0.145[a]
Treatment n(%)	41(45%)	89(32%)	p=0.0026[a]
autoimmune n(%)	17 (18%)	23 (8%)	p=0.03[a]
M n(%)	55 (70%);	171(71%)	p=1[a]
UM n(%)	23 (30%)	70 (29%)	p=1[a]
13q n(%)* n available cytogenetics	21 (27%)	76 (37%)	p=0.2[a]
normal n(%)	19 (25%)	54 (26%)	p=1[a]
t12 n(%)	16 (21%)	27 (13%)	p=0.009[a]
11q n(%)	9/75 (12%)	23(11%)	p=0.8[a]
17p n(%)	3 (4%)	7 (3%)	p=1[a]
other n(%)	9 (12%)	19 (9%)	p=0.5[a]
β2M median (range)	2.3 (1.1; 4.7) n=42	2.1 (1.1; 5.5) n=125	p=0.0803[c]
LDT median n available	49 months n=79	62 months n=225	p=0.0007[d]
TTFT median	65 m	91 m	p=0.045[d]
OS median	151 m	216 m	0.146[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank);
green: marked values with marginal significance, red: statistical significance;
WCC, age, LDT, β2M , OS and PFS are shown as median values
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

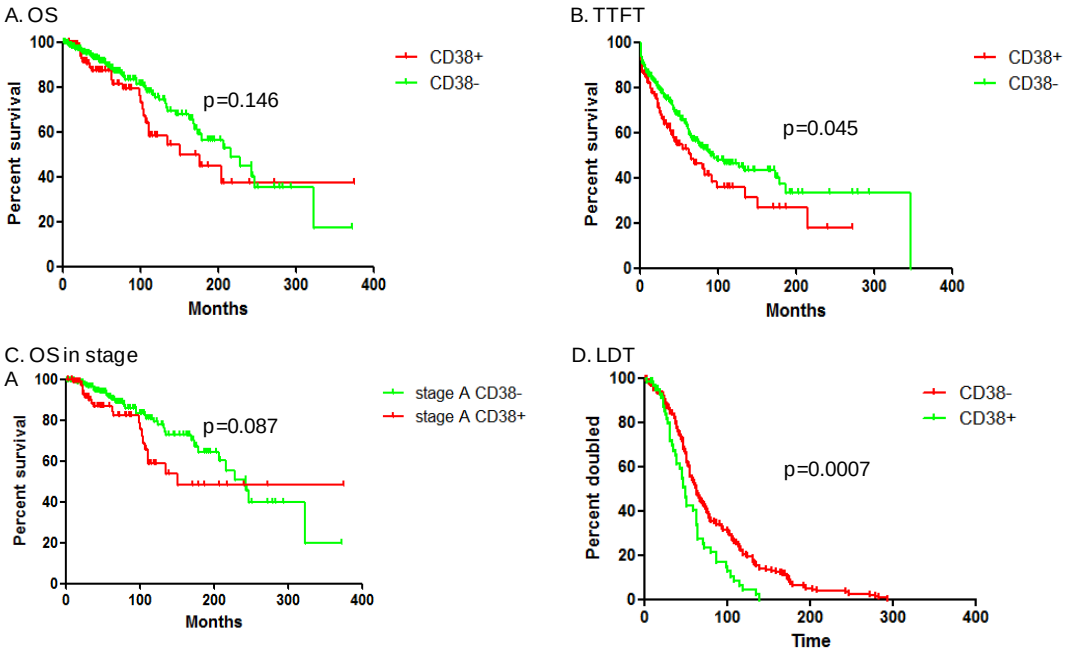


Figure 7.12 Mantel-Cox survival curve for CD38 as a prognostic marker,

A. OS in all patients. B. TTFT in all patients, C.OS in stage A patients. D. LDT.

Table 7.16 Comparison of hazard ratio for OS ,TTFT and LDT for CD38 expression

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
CD38	1.007	0.999	1.014	0.068

For every 1% increase in CD38 there is a 1.007 times more chance of dying: e.g. for 100 patients who die, with 1% increase: 101 patients die, p=0.068.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
CD38	1.006	1.0	1.012	0.037

For every 1% increase in CD38 there is a 1.006 times more chance of receiving treatments: e.g. for 100 patients who are treated, with 1% increase: 101 are treated.

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
CD38	1.003	0.998	1.007	0.243

The significant differences between CD38+ and CD38- patients were the proportion of autoimmune conditions, t12, number of treated patients, TTFT and LDT. The distribution of *IGHV* unmutated cases was similar in both groups.

The TTFT between CD38+ and CD38- patients was statistically significant, p=0.045 (median TTFT 65 versus 91 months respectively) and significance for LDT was p=0.0007; the single cytogenetic abnormality which showed a significant distribution was trisomy 12: in the CD38+ group there were 21%, whereas in the CD38- group: 13% (p=0.009). There were also more patients presenting with autoimmune conditions in the CD38 positive group (p=0.03). In the CD38 positive group there were 7 (8%) cases of AIHA (autoimmune haemolytic anaemia), 7 (8%) with ITP (immune thrombocytopenic purpura), 3 (3%) with RA (rheumatoid arthritis), 1 (1%) with autoimmune rash; in the CD38 negative group: 15 (5%) - AIHA, 8 (3%) - ITP, 2 (0.7%) cases of RA (significance for all autoimmune abnormalities was p=0.03). There were no significant differences in the frequency of ITP, p=0.062, and RA, p=0.0985 (two-tailed Fisher exact test). CD38 positivity had an increased risk for treatment requirement (HR 1.006; 95%CI 1.0-1.012) but not for OS (Table 7.16).

7.1.7 Beta$_2$-microglobulin (β2M) as a prognostic marker

β2M is a 12kDa non-glycosylated protein encoded on the chromosome 15 expressed by all nucleated cells, which forms a invariable light chain subunit of MHC class I antigen. It is a soluble protein, found in body fluids, as result of shedding from cell surfaces (Shi *et al.*, 2009). It is a wildly used, inexpensive serum marker; is one of the oldest prognostic markers in solid tumours and haematological malignancies, including CLL and multiple myeloma. β2M may mark an important biological process, tumour load, immune response or combination to all these factors (Albitar *et al.*, 2007).

The data are presented in Table 7.17-7.19 and Fig 3.13-14. β2M was determined by an absorption immunoassay, a service provided by the biochemistry department. The normal range was 0.7-3.5 mg/L.

Forty two percent (n=212/496) of our patients had β2M measurements available at diagnosis. I included patients only with normal creatinine levels, as the increase in β2M is due to abnormal glomerular filtration rate. I used two cut-offs: 2.5mg/L and a cut-off of 4mg/L (the reasons for this were discussed in 3.2.8). Analysis with the cut-off of 4mg/L is shown in the Table 7.17. Only 17 (8%) patients had values above the chosen cut-off. Despite small numbers, I found significant differences in OS (p<0.0001) and TTFT (p<0.0001) and the proportion of treated patients (p=0.0026), the group with the

Table 7.17 Characteristics of patients with β2M >4mg/l and <4mg/L

	β2M>4mg/L (n=17)	β2M<4mg/L (n=195)	Statistics
Age median (range)	74 (48; 97)	63 (34; 91)	p=0.0019[a]
M:F	2.4:1 (12:5)	1.9:1 (128:64)	ns
WCC median (range)	64.59 (8; 419)	18.8 (4.5; 354)	p=0.0015[c]
A0 n(%)	0	126(65%)	p=0.001[a]
A n(%)	5 (29%)	41 (21%)	p=0.5[a]
B n(%)	9 (53%)	21 (11%)	p=0.0015[a]
C n(%)	3 (18%)	7 (3%)	p=0.05[a]
LN=0 n(%) LN≤1cm n(%) LN>3cm n(%)	0: 3(18%) 1: 9 (53%) 3: 4(24%)	0: 137 (75%) 1: 39 (21%) 3: 6 (3%)	p=0.0043[a] p=0.0255[a] p=0.0045[a]
Treatment n(%)	16(94%)	62(32%)	p=0.0026[a]
autoimmune n(%)	3 (18%)	21 (11%)	ns[a]
M n(%)	7 (41%)	124 (73%)	p=0.086[a]
UM n(%)	10 (59%)	45 (27%)	
13q n(%)* n available for cytogenetics	4(31%) 133	64(43%) 149	ns[a]
normal n(%)	1(8%)	39(26%)	0.189[a]
t12 n(%)	3(23%)	27(18%)	p=0.71[a]
17p n(%)	0	1(1%)	ns[a]
11q n(%)	5(38%)	17(11%)	p=0.046[a]
other n(%)	1(8%)	18/149(12%)	ns[a]
CD38 median n (% of >30%) n available	7.67 3 (38%) n=8	3.05 39 (25%) n=158	p=0.155[c]
LDT median n available	48 months n=10	60 months n=175	p=0.0607[d]
TTFT median	8m	109m	p<0.0001[d]
OS median	48m	Undefined	p <0.0001[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significance; WCC, age, LDT, OS and PFS are shown as median values
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

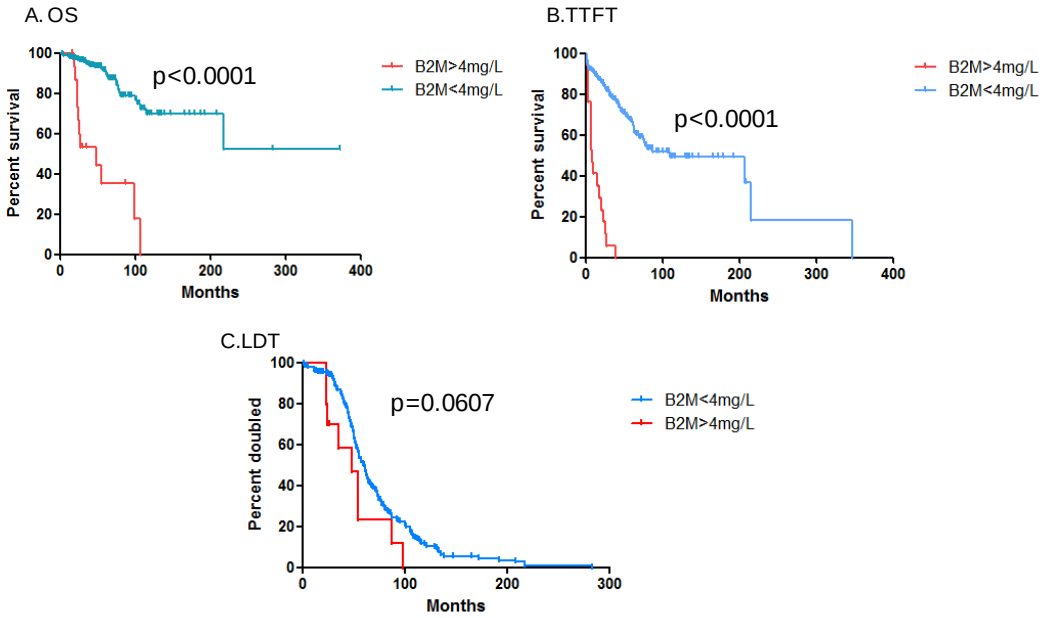


Figure 7.13 Mantel-Cox survival curve analysis in group of high and low β2M.

A. Overall survival; B. Time to first treatment C. Lymphocyte doubling time..

Table 7.18 Comparison of hazard ratio for OS , TTFT and LDT for β2M

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
β2M	2.247	1.809	2.792	<0.0001

For every unit increase in β2M there is a 2.247x more chance of dying: e.g. for 100 patients who die with the level of 2mg/L, at 3mg/L there will be 225 patients who will die.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
β2M	2.067	1.753	2.437	<0.0001

For every unit increase in β2M there is a 2.067x more chance of treatment: e.g. for 100 patients treated who have level of 2mg/L, at 3mg/L there will be 207 patients requiring treatment

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
β2M	1.222	0.987	1.513	0.065

For every unit increase in β2M there is a 1.222x more chance of treatment: e.g. for 100 patients treated who have level of 2mg/L, at 3mg/L there will be 122 patients who double lymphocytes.

Table 7.19 Characteristics of patients with β2M >2.5 mg/l and <2.5mg/L

	β2M>2.5mg/L (n=69)	β2M<2.5mg/L (n=143)	Statistics
Age median (range)	70 (44; 97)	61 (34; 84)	p<0.0001[b]
M:F (n:n)	3:1 (52:17)	1.6:1 (88:55)	p=0.0628[a]
WCC median (range)	36 (4.5; 354)	17.9 (8; 419)	p=0.0003[c]
A0 n(%)	25(36%)	101(71%)	p<0.0001[a]
A n(%)	17(25%)	29(20%)	p=0.4812
B n(%)	19(28%)	11(8%)	p=0.0002[a]
C n(%)	8(11%)	2(1%)	p=0.0023[a]
LN=0 n(%) LN≤1cm n(%) LN>3cm n(%)	0=33 (53%) 1cm=21(34%) 3cm=8 (13%)	0=106 (78%) 1cm=27 (20%) 3cm=3 (2%)	p=0.0007[a] p=0.0483[a] p=0.0046[a]
Treatment n(%)	44 (67%) (8 refractory)	31 (22%) (6 refractory)	p<0.0001[a]
autoimmune n(%)	10(14%)	14(10%)	p=0.3567
M n(%)	36(56%)	95(79%)	p=0.005[a]
UM n(%)	26(44%)	25(21%)	
13q n(%) n available	11(21%) 52	57(67%) 85	p<0.0001[a]
normal n(%)	12(23%)	28(33%)	p=0.249[a]
t12 n(%)	14(27%)	11(13%)	p=0.0116[a]
17p n(%)	1(2%)	0	p=0.3796[a]
11q n(%)	13(25%)	9(11%)	p=0.1024[a]
other n(%)	7(13%)	12(14%)	p=0.2867[a]
CD38 median n (% of >30%) n available	7.83 16 (29%) 55	1.82 26 (23%) 111	p=0.006[c] p=0.452[a]
LDT median	61months	57 months	p=0.6861[d]
TTFT median	24 months	346 months	p<0.0001[d]
OS median	98 months	undefined	p<0.0001[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significance; WCC, age, LDT, OS and PFS are shown as median values

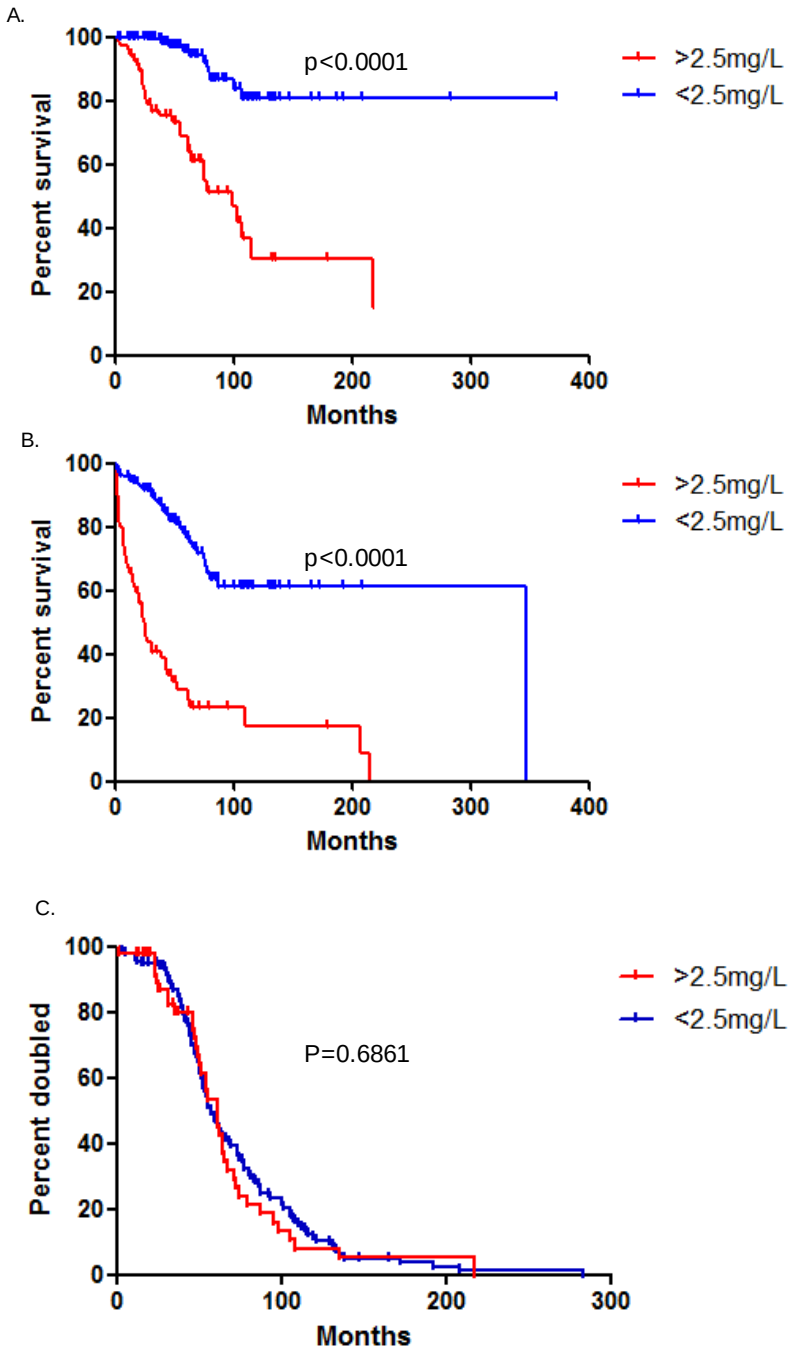


Figure 7.14 Mantel-Cox survival curve analysis in group of β2M >2.5mg/L and <2.5mg/L.

A. Overall survival; B. Time to first treatment; C. Lymphocyte doubling time.

high β2M was older (74 v. 63, p=0.0019), presents with higher WCC at diagnosis (p=0.0015). There were significant differences between stage A0 (p=0.001, but not A), B (p=0.0015), C (p=0.05) and therefore closely associated the presence and size of lymphadenopathy. The only significant cytogenetic difference was 11q, there was a higher proportion of patients with 11q deletion in >4mg/L group (p=0.046).

There were no significant differences in the proportion of mutated *IGHV* segments and CD38. The lymphocyte doubling time was shorter (48 v 60 months) although p value did not reach statistical significance (p=0.0607).

Table 7.19 shows the analysis using a cut-off of 2.5 mg/L, there were 69 patients with high β2M and 143 with low. Comparing Table 7.17 to the Table 7.19 shows that there were additional significant differences using a cut-off 2.5mg/L in the following variables: gender, mutation status, 13q and trisomy 12. The proportion of 11q deletion cases in the 2.5mg/L cut-off group lost statistical significance. The variables which were consistently statistically insignificant in both cut-offs were: stage A, proportion of autoimmune cases, normal and 'other' cytogenetics.

In the univariate analysis the risk of death was doubled with increasing β2M (HR 2.247; 95%CI 1.809-2.792), similarly to treatment requirement (HR 2.067; 95%CI 1.753-2.437) (Table 7.18). The risk of doubling lymphocytes was increased with higher β2M, although it did not reach statistical significance (p=0.065).

7.1.8 Cytogenetics

Cytogenetic abnormalities are rare in the early stages of disease, therefore they are unlikely to be the original abnormality leading to the development of CLL (the first (Knudson, 1971). The most common identified abnormalities are del 13q, del 11q, del 17p and trisomy 12. The gene involved in del11q is thought to be ATM gene and p53 in 17p deletion. The target for 13q deletion is *DLEU2/miR-15a/16-1* cluster and trisomy 12 is still unknown.

The data is presented in Table 7.20-7.21 and Figure 7.15-19. The FISH and conventional cytogenetics were performed by Prof R Siebert's group at the University of Kiel, Germany. The used mitogens were immunostimulatory CPG oligonucleotide and IL2 (Decker *et al.*, 2000). There were 330 patients (66%) available for analysis. The most common cytogenetic abnormality (124/330, 38%, Figure 7.15.A and Table 7.20) observed in our cohort was an isolated 13q deletion (cases who had 13q deletion with other cytogenetic abnormalities were excluded from the analysis). There were 86/330 (26%) cases with the normal cytogenetics, 60/330 (18%) with 11q deletion, 42/330 (13%) with trisomy 12 (t12) and 11/330 (3%) with 17p deletion.

Table 7.20 Summary of cytogenetic abnormalities and median survival (Mantel-Cox)

	n= 330	medianPFS (months)	medianOS (months)
13q	124	undefined	undefined
normal	86	346	211
t12	60	30	168
11q	42	26	120
17p	11	27	37
t(14;18)	8	87	undefined
others	30	23	204

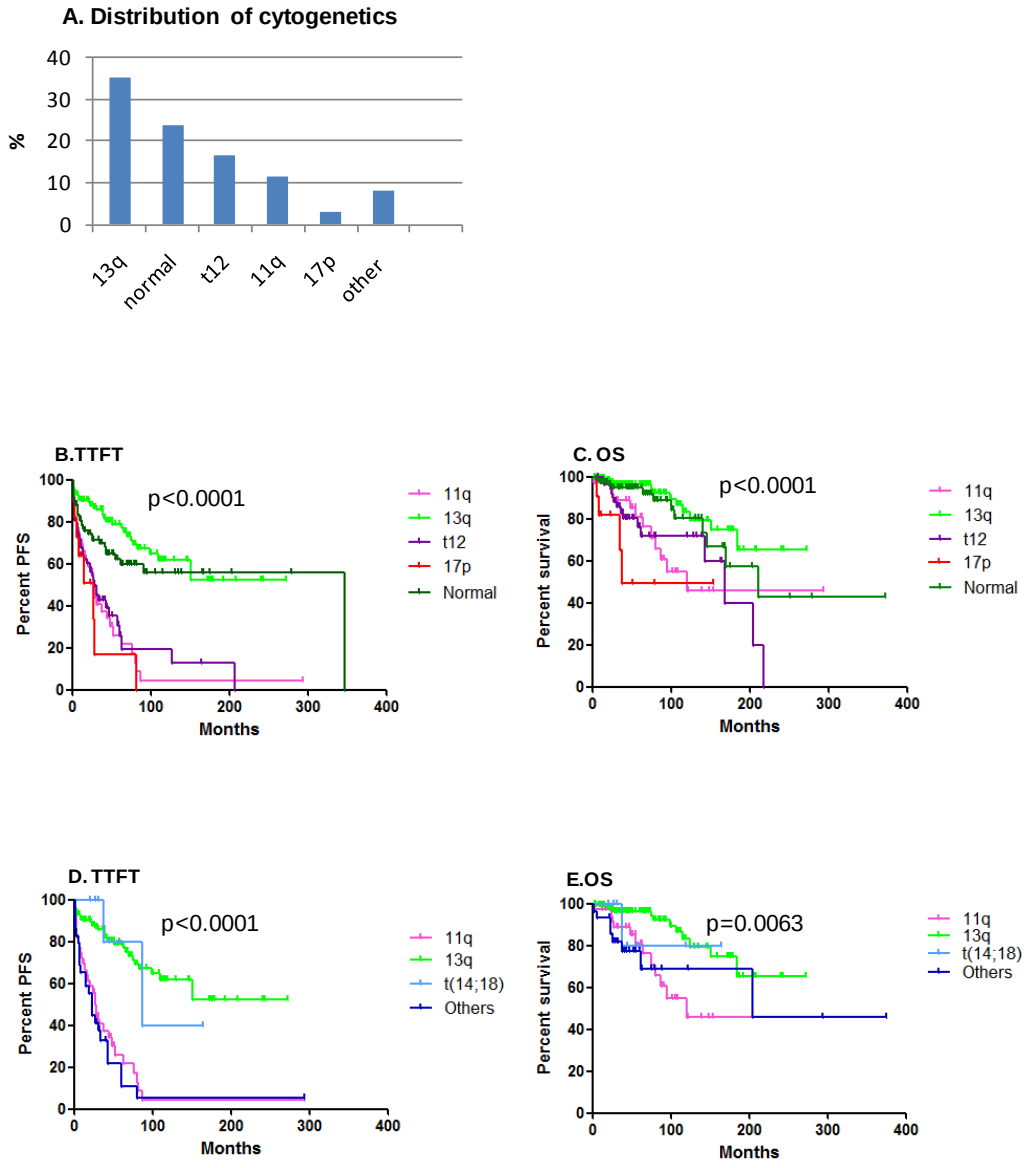


Figure 7.15 Cytogenetic abnormalities.

A. Distribution of abnormalities in the Leicester cohort. **B.** Mantel-Cox survival curves for time to first treatment for 11q deletion, 13q deletion, trisomy 12, 17p and normal cytogenetics. **C.** Overall survival for 11q deletion, 13q deletion, trisomy 12, 17p and normal cytogenetics. **D.** time to first treatment for t(14; 18) and other abnormalities compared to 11q and 13q deletions. **E.** Overall survival for t(14; 18) and other abnormalities compared to 11q and 13q deletions. LDT doubling time not shown (not statistically significant, p=0.4668).

Other chromosomal abnormalities (30/330, 9%) included, trisomies of 5, 7, 8, 18, 19; deletions of 2, 5q, 6q, 14q, 18q, translocations: t(3;8), t(7;15), t(5;10), t(x;19), monosomy X, 17q gain. They had similar OS to normal cytogenetics. Translocation t(14;18) although represented by small number of patients (8/330, 2.4%) had not reached median OS, similar to the 13q deleted group.

I compared OS to Döhner's data (Dohner *et al.*, 2000) in Figure 7.16. The Ulm CLL cohort presented by Döhner had only 23.7% treated patients (77/325), which was significantly less than in our cohort (38%, 179/469, p<0.0001, two-tailed Fisher's exact test). Despite this, median OS was significantly better in the Leicester cohort in all cytogenetic groups (13q, undefined survival (Leicester cohort) v. 133 months (in Dohner's cohort); normal cytogenetic 211 v. 111 months; trisomy 12 (168 v. 114 months), 11q deletion, 120 v. 79 months; 17p deletion, 37 v. 32 months).

There were significant differences in the proportion of cytogenetic abnormalities distributed across the Binet stages (Table 7.2), except for trisomy 12 and 'other' abnormalities: 13q deletion (p=0.052) and normal cytogenetics (p=0.044) were mostly seen in stage A0, stage B had the highest proportion of 11q deletion (p=0.0116) and 17p deletion (p=0.0104). Interestingly there were no cases with 17p deletion in stage C.

Patients with long doubling time (>5 years) tend to have 13q deletion, p=0.02 (Table 7.9), whereas patients with a short doubling time had more 11q deletion (p=0.003), 17p deletion (p=0.017) and trisomy 12, although the latter did not reached statistical significance (p=0.073).

There were significant differences when comparing cytogenetic abnormalities and mutation status (Table 7.11). There were more cases with normal cytogenetic (p=0.02) in patients with mutated *IGHV* gene segments and less with 11q (p=0.0002) and 17p (p=0.007). 13q deletion was not statistically significant. However, when the group with mutated *IGHV* was further divided into treated and untreated subgroup (Table 7.13), 13q deletion was detected mainly in the mutated untreated group with high statistical significance, p<0.0001.Trisomy 12 was similarly distributed between the mutated and unmutated group (p=0.3), but was seen in a high proportion in the mutated treated subgroup (Table 7.13), p=0.0012.

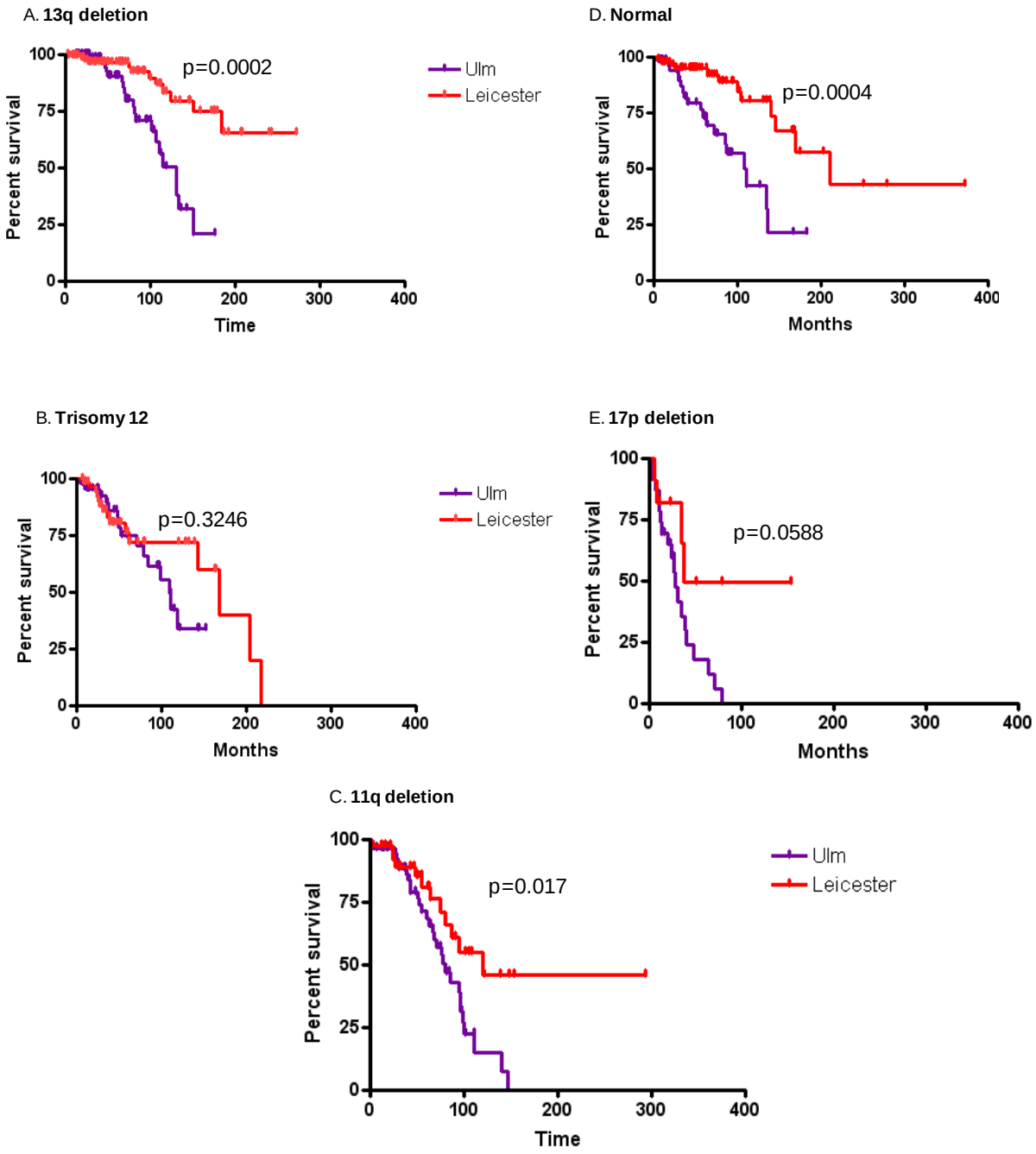


Figure 7.16 Cytogenetic abnormalities,

Mantel-Cox survival curves for OS comparing the Ulm cohort (Döhner, 2000) A. 13 q deletion; B. trisomy 12; C: 11q deletion; D. normal cytogenetics; E: 17p deletion

Trisomy 12 was present at higher proportion in CD38+ patients (Table 7.17). The significance of cytogenetics and β2M was variable depending on the cut-off level: at g/L cut-off (Table 7.17) the 11q deletion was significant, whereas at 2.5mg/L cut-off significance of 11q was lost and trisomy 12 became significant (Table 7.19).

17p deletion is a well documented cytogenetic abnormality associated with a poor prognosis, therefore we compared this group to another poor prognosis cohort, stage C patients (Table 7.22 and Figure 7.17). The numbers in the 17p deleted group were small, n=11. In our cohort there were no stage C patients with 17p deletion. The significant differences were observed in proportion of mutated and unmutated patients (p=0.0093), proportion of treated patients (p=0.0152) and TTFT (p=0.0012). Only one 17p deleted patient had mutated *IGHV* gene segments (*IGHV* 4-04), this patient had a prolymphocytic transformation of CLL. There were no statistical significance between the two groups in age, WCC (p=0.0715) at the diagnosis, gender, lymphadenopathy, autoimmune conditions, additional cytogenetic abnormalities (13q, 11q, 'other'), β2M and LDT (p=0.0853).

Univariate analysis of each chromosomal abnormality did not identify increased risk in OS, TTFT or LDT. When chromosomal abnormalities were analysed together as multivariate analyses (Table 7.21) 13q deletion and t12 appeared to promote survival: 13q deletion patients had better chances of survival (HR 3.11; 95%CI 1.156-8.365) than patients without 13q deletion, similarly to t12 (HR 2.99; 95%CI 1.34-8.644). The risk of treatment was not significantly increased with chromosomal aberrations.

7.1.9 Analysis of 13q deletion, what matters: hemi-, homo and mosaic deletions or percentage of cells affected?

13q deletion was a good prognostic factor if found as an isolated abnormality. 13q can present as a single allele deletion (heterozygous, 13q-x1), both alleles (homozygous, 13q-x2) and the mixture of the two (mosaic, 13q-x1/13q-x2). There were single reports in the literature attempting to understand the significance of these differences (Van Dyke *et al.*, 2008).

The data is presented in Table 7.25 and Figure 7.18.

In our cohort there were a total of 127 cases with the isolated 13q deletion, out of which 87 (68%) were hemizygous (13q-x1), 25 (20%) were homozygous (13q-x2) and 15 (12%) mosaic (13q-x1/13q-x2). The significant difference (p=0.0009) was in the presence of lymphadenopathy at diagnosis. There was a marginally significant

Table 7.21 Comparison of hazard ratio for OS, TTFT and LDT for cytogenetic abnormalities
(univariate analysis were not significant therefore multivariate analysis for cytogenetics abnormalities).

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
13q	3.11	1.156	8.365	0.025
Normal cyto	2.606	0.854	7.957	0.093
11q	0.904	0.429	1.903	0.79
t12	2.99	1.034	8.644	0.043
17p	0.701	0.246	2.002	0.507
Other cyto	1.419	0.555	3.631	0.465

None of cytogenetic abnormalities when analysed separately were significant. When analysed together HR is significant for 13 q deletion cases and t12, with marginal significance in normal cytogenetic cases. For 100 patients with 13q who die: 311 patients will die who do not have 13q and for 100 patients with t12 who die: 299 will die who had other abnormalities. To combine risks for100 patients with 13q and t12 who die: 930(3.11x2.99) patients die who are negative for 13q and t12

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
13q	1.865	0.899	3.872	0.94
Normal cyto	1.524	0.664	3.496	0.32
11q	0.881	0.485	1.601	0.678
t12	1.995	0.936	4.251	0.074
17p	1.061	0.383	2.942	0.91
Other cyto	1.412	0.692	2.881	0.343

None of cytogenetic abnormalities when analysed separately were significant. When analysed together HR is borderline significant for t12. For 100 patients with t12 who received treatment: 200 of t12 negative patients will receive treatment.

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
13q	2.630	1.500	4.611	0.001
Normal cyto	2.457	1.294	4.665	0.006
11q	1.296	0.820	2.049	0.267
t12	1.543	0.896	2.658	0.118
17p	1.154	0.558	2.386	0.7
Other cyto	1.311	0.796	2.160	0.288

Only the13q deletion was a statistically significant cytogenetic abnormality when analysed separately (HR1.376, 95%CI 1.044, 1.814; p=0.023).When analysed together HR is statistically significant for 13q and normal cytogenetic. For 100 patients with 13q who double their lymphocytes: 263 of 13q negative patients double their lymphocytes. For 100 patients with Normal cytogenetics, 246 patients (who have other cytogenetic abnormalities) will their double lymphocytes

Table 7.22 Characteristics of 17p deleted group and stage C

	17p deletion n=11	Stage C n=29	Statistics
Age median (range)	62 (46; 91)	70.5 (44; 87)	p=0.61[b]
M:F (n:n)	4.5:1(9:2)	1.9:1(19:10)	p=0.451[a]
WCC median (range)	15.1 (10.8; 255)	45.5 (4.7; 354)	p=0.0715[c]
Stage n(%)	A0=3(27%), A=2(18%), B=6(55%), C=0	n/a	
LN n(%)	6(55%)	16(55%)	p=1[a]
Treatment n(%)	7(64%)	28(97%)	p=0.0152[a]
Autoimmune n(%)	3(27%)	4(14%)	p=0.369[a]
M n(%)	1(12%)*	13(72%)	p=0.0093[a]
UM n(%)	7(88%)	5(28%)	
13q n(%)** n available for cytogenetics	6(55%) 11	6(33%) 18	p=0.438[a]
normal n(%)	n/a	6(33%)	
t12 n(%)	3(27%)	5(28%)	p=1[a]
11q n(%)	2(18%)	11(61%)	p=0.0524[a]
17p n(%)	n/a	0	
other n(%)	3(27%)	2(11%)	p=0.3386[a]
β2M median (range)	2.15 (1.4; 4.3) n=4	3.6(1.8;5.5) n=10	p=0.233[c]
CD38 median n (% of >30%) n available	2 0 1	6.18 7 (41%) 17	small sample
LDT median	83 months	68 months	p=0.0853[d]
TTFT median	27 months	1 months	p=0.0012[d]
OS median	37 months	74 months	p=0.832[d]

[a]Fisher's exact test; [b]unpaired t test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, LDT, OS, PFS, CD38 are shown as median values. β2M was not analysed because there was only one value in 17p deletion group.
*this patient had CLL with prolymphocytic transformation
**some patients had more than one cytogenetic abnormality, % calculated from number of patients

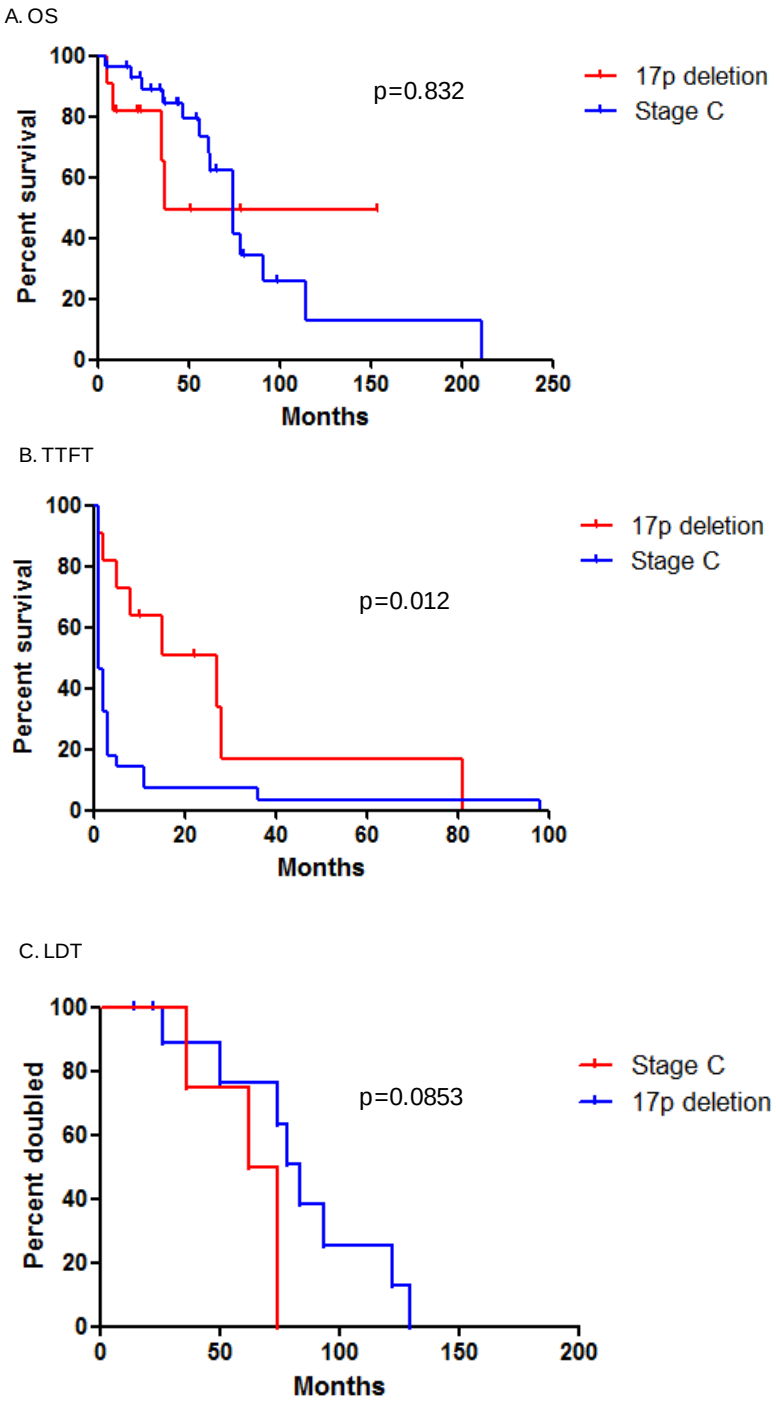


Figure 7.17 Mantel-Cox survival curve analysis in group of 17p deletion and stage C.
A. Overall survival; B. Time to first treatment; C. Lymphocyte doubling time.

Table 7.23 Characteristics between hemi-homo- and mosaic deletions of 13q

	Hemizygous n=87 (68%)	Homozygous n=25 (20%)	Mosaic n=15 (12%)	Statistics
Age median (range)	64 (42; 83)	70 (49; 97)	61 (46; 91)	p=0.049[b]
M:F (n:n)	1.4:1 (51:36)	2.6:1 (18:7)	1.1:1 (8:7)	p=0.24[a]
WCC median (range)	18.45 (7.5; 568)	18.5 (10; 419)	17.7 (12; 61.5)	p=0.98[c]
A0 n(%)	55 (65%)	14(88%)	12(100%)	p=0.015[a]
A n(%)	20(24%)	0	0	p=0.0173[a]
B n(%)	4(5%)	1(6%)	0	p=0.698[a]
C n(%)	5(6%)	1(6%)	0	p=0.683[a]
LN=0 n(%)	0: 58(82%)	0: 25(100%)	0:15(100%)	p=0.0009[a]
Treatment n(%)	20(23%)	6(24%)	3(20%)	p=0.957[a]
M n(%)	51(72%)	17(81%)	10(83%)	p=0.542[a]
UM n(%)	20(28%)	4(19%)	2(27%)	
β2M median (range)	2 (1.3; 4.1)	2.3 (1.5; 5.6)	2.1 (1.1; 3.3)	p=0.205[c]
CD38 median n (% of >30%) n available	1.68 7 (21%) 33	2.44 9 (43%) 21	1.07 3 (23%) 13	p=0.3184[c]
LDT	73 months	63 months	73 months	p=0.2469[d]
TTFT	186 months	undefined	undefined	p=0.2[d]
OS	184 moths	undefined	undefined	p=0.14[d]

[a]chi-square; [b]one-way ANOVA; [c]Kruskal Wallis; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, LDT, β2M, CD38 ,OS and PFS are shown as median values .

difference in the OS between groups, hemizygous was 184 months, homozygous and mosaic did not reach median survival. The mosaic group had the best overall survival and was represented only by patients in stage A0 and A only. However, these data have to be taken with caution because of the small numbers of patients involved. Our data differed significantly when compared to OS of the Mayo cohort (Van Dyke *et al.*, 2008) for the mosaic group (Figure 7.18.E). The hazard risk for treatment and survival was not significant between these groups (Table 7.24).

I investigated if the percentage of cells with 13q deletion had influenced patients' outcome (Table 7.25 and Figure 7.19). If the patient had a mosaic defect, total percentage is the sum of both cells with heterozygous deletion and homozygous deletion were added. The initial analysis was done separating patients who had >50% and <50% affected cells, there were no significant differences, therefore a 80% cut-off was chosen.

Similarly to the analysis above, there were no differences in OS, however there was a significant difference in the TTFT (p=0.005). Patients with 13q with >80% affected cells required more therapy (p=0.002), more had lymphadenopathy (p=0.0149) and had higher WCC at the diagnosis (Figure 7.19.C). There was increased risk of treatment (Table 7.26) with increased number of affected cells (HR 1.016; 95%CI 1.002-1.032). Univariate analysis was not significant for TTFT, LDT or OS.

7.1.10 CLL in the Asian population

CLL is rare in the Asian population and Leicester is one of the few areas in the UK with a substantial proportion of Asians therefore it created an opportunity to analyse CLL in this group. A significant problem with this analysis was the small number of Asian patients.

The data is presented in Table 7.27 and Figure 7.20.

Amongst 496 CLL patients I recorded 19 Asian patients. I compared the Asian group to the patients with stage A0. The median age of diagnosis was younger (58) than in the Caucasian population (66) (p=0.0024). There was also a higher male to female ratio, this however was not statistically significant (p=0.144). Half of the cases present with good prognostic markers (4: normal FISH, 6: 13q deletion), the proportions of cytogenetic abnormalities were similar to stage A0 patients; there was only a weakly significant difference in t12 proportions. There was a median of 2 years lymphocyte doubling time and overall survival was similar to western population with normal cytogenetic (216 months, p=0.54) or with stage A0 (243 months, p=0.7723). The hazard ratio was not altered for OS, TTFT or LDT (Table 7.28).

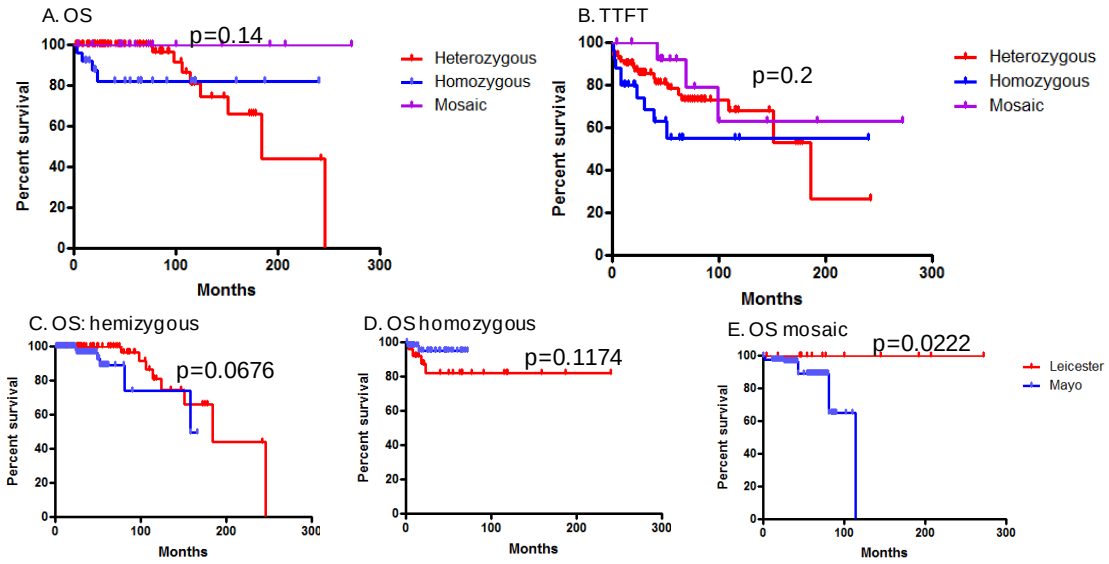


Figure 7.18 Mantel-Cox survival curves in group 13q hemi, homo and mosaic.

A. Overall survival; B. Time to first treatment; Comparison of overall survival between Mayo cohort (Van Dyke, 2008) and Leicester C: OS for hemizygous, D: OS for homozygous, E: OS for mosaic. LDT doubling time not shown, not significant, p=0.2469

Table 7.24 Comparison of hazard ratio (HR) for OS, TTFT and LDT for mosaic, hetero- and homozygous deletions

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
mosaic	0	0		0.977
heterozygous	0.398	0.121	1.317	0.131
homozygous	1			

HR compares to homozygous deletions, for every 100 dead patients with homozygous deletion there are 40 dead with heterozygous (p=0.131).

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
mosaic	0.486	1.25	1.898	0.299
heterozygous	0.844	0.355	2.006	0.7
homozygous	1			

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
mosaic	0.497	0.237	1.044	0.065
heterozygous	0.759	0.457	1.259	0.286
homozygous	1			

HR compares to homozygous deletions, for every 100 homozygous patients who doubled their lymphocytes only 50 mosaic patients will .

Table 7.25 Characteristics of patients with <80% 13q deletion and >80% 13q deletion

	13q>80% (n=46)	13q<80% (n=85)	Statistics
Age median (range) n available	64 (49; 87) n=43	62 (35; 97) n=81	p=0.402[b]
M:F (n:n)	29:17(1.7:1)	50:35 (1.4:1)	p=0.71[a]
WCC median (range)	23.1(7.5; 568)	15.9 (7.5; 90.8)	p=0.0073[c]
A0 n(%)	24 (55%)	35 (70%)	p=0.271[a]
A n(%)	11 (25%)	8 (16%)	p=0.036[a]
B n(%)	5 (11%)	5 (10%)	p=0.32[a]
C n(%)	4 (9%)	2 (4%)	p=0.183[a]
LN=0 n(%)	0=30 (65%)	0=72 (84%)	p=0.0149[a]
Treatment	18 (39%)	12 (14%)	p=0.002[a]
autoimmune	5 (11%)	7 (8%)	p=0.753[a]
M n(%)	25 (68%)	56 (80%)	p=0.164[a]
UM n(%)	12 (32%)	14 (20%)	
β2M median (range)	2.1 (1.3; 4.)	2 (1.1; 5.6)	p=0.534[c]
CD38 median n (% of >30%) n available	1.12 8(25%) 32	1.68 12(17%) 70	p=0.584[c]
LDT median	60 months	74 months	p=0.8972[d]
TTFT median	77 months	undefined	p=0.005[d]
OS median	246 months	undefined	p=0.201[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; in red are marked statistically significant differences, green: marginal significance;
WCC, age, β2M , OS and PFS are shown as median values

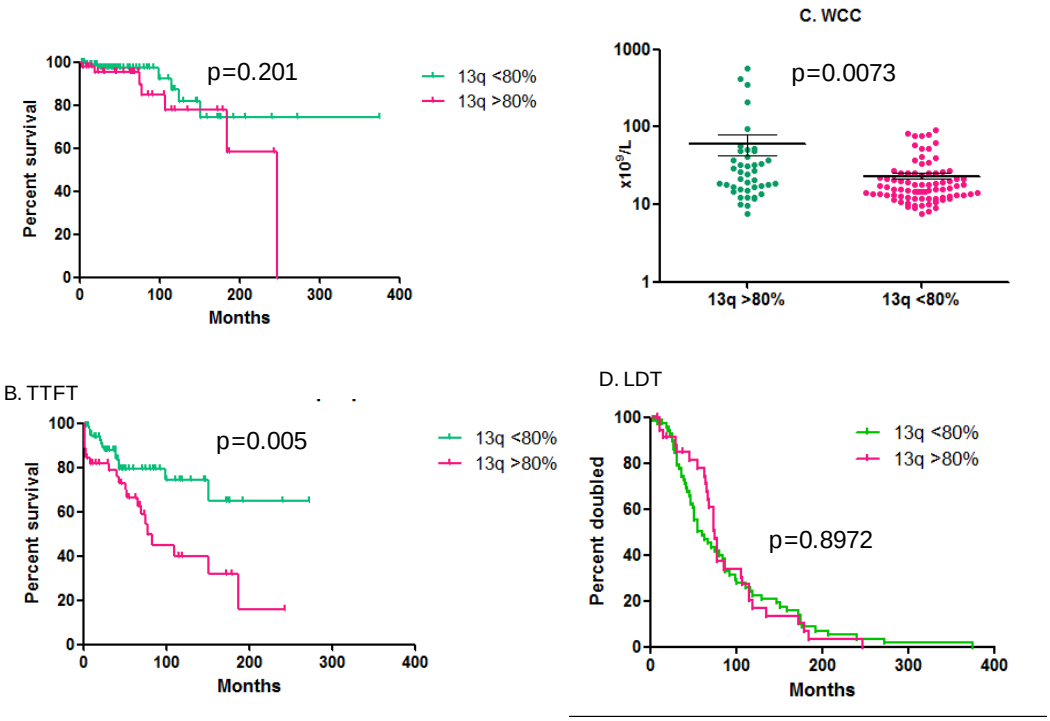


Figure 7.19 Mantel-Cox survival curves in group containing >80% cells with 13q deletion and <80%

A. Overall survival, B. Time to first treatment, C. Scatterplot of differences between WCC at the diagnosis. (Y axis is $\log_{10}$ scale for the purpose of clarity).

Table 7.26 Comparison of hazard ratio for OS , TTFT and LDT for percentage of 13q deletion affected cells

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
13q %	1.006	0.984	1.028	0.583

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
13q%	1.016	1.002	1.032	0.029

For every 1% increase in 13q there is a 1.016 more chance of receiving treatment: e.g. for 100 patients treated there will be 102 patients requiring treatment for each percent, increase by10% mean that 1016 patients will require treatment

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
13q%	0.999	0.993	1.1007	0.854

Table 7.27 Characteristics of Asian patients

	Asian n=19	all stage A n=114	Statistics
Age median (range) n available	58 (6.8; 195) n=19	64 (34;92) n=107	p=0.0574[b]
M:F (n:n)	3.75:1(15:4)	2.3:1(63:27)	p=0.579[a]
WCC median (range)	15.7 (6.8;195)	19.1 (4;251)	p=0.287[c]
A0 n(%)	13 (68%)	n/a	
A n(%)	4 (21%)	n/a	
B n(%)	1 (5%)	n/a	
C n(%)	1 (5%)	n/a	
LN n(%)	0=13(68%) >3:2 (11%)	n/a* >3: 9 (8%)	p=0.999
Treatment n(%)	7(37%)	59(52%)	p=0.322[a]
autoimmune n(%)	1(5%)	16(14%)	p=0.465[a]
M n(%)	11(79%)	64(66%)	p=0.543[a]
UM n(%)	3(21%)	33(34%)	
13q n(%)** n available for cytogenetics	6 (43%) 14	27(36%) 76	p=0.764[a]
normal n(%)	4(29%)	13(17%)	p=0.456[a]
t12 n(%)	4(29%)	18(24%)	p=0.269[a]
11q n(%)	0	13(17%)	p=0.207[a]
17p n(%)	1(7%)	2(12%)	p=0.402[a]
Other n(%)	1(7%)***	12(16%)	p=0.683[a]
β2M median (range)	2.55 (1.7; 3.8)	2.15 (1.4; 4.7)	p=0.15
CD38 median n (% of >30%) n available	2 2(12%) 17	7 22 (24%) 92	p=0.794[c]
LDT median	42 months	72 months	p=0.9473[d]
TTFT median	207 months	45 months	p=0.139[d]
OS median	217 month	216 months	p=0.67[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significances; WCC, age, LDT, β2M , OS and PFS are shown as median values
*All stage A will have LN present, **some patients had more than one cytogenetic abnormality, % calculated from number of patients; ***'other' abnormality was 14q24.

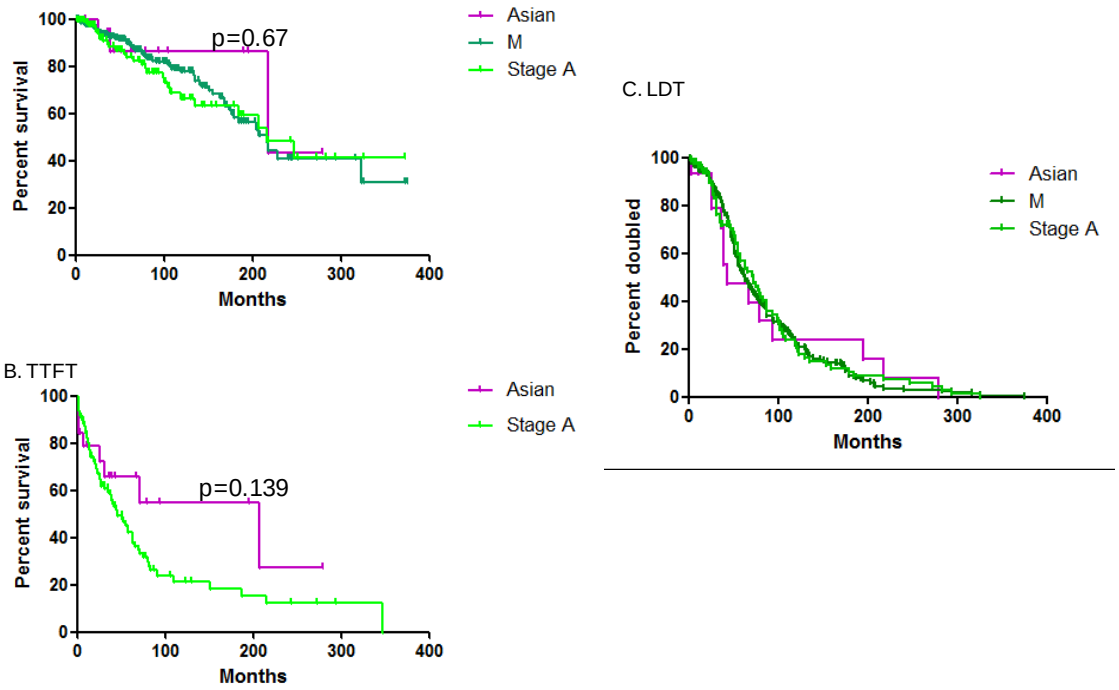


Figure 7.20 Mantel-Cox survival curve in Asian population.

A. OS in Asian population compared to OS and TTFT of mutated and stage A cases. B. TTFT compared to stage A; C.LDT

Table 7.28 Comparison of hazard ratio for OS , TTFT and for Asians

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Asians	0.537	0.170	1.696	0.289
White	1			
Variables	**Hazard Ratio (TTFT)**	**95% CI for HR**		**p value**
		Lower	Upper	
Asians	0.905	0.424	1.932	0.797
White	1			
Variables	**Hazard Ratio (LDT)**	**95% CI for HR**		**p value**
		Lower	Upper	
Asians	0.885	0.506	1.550	0.670
White	1			

7.1.11 Incidence of other cancers in CLL population

Increased incidence of cancers in CLL is well documented and it is thought to be caused mainly by disease induced immunosuppression rather than therapy-induced (Cheson *et al.*, 1999). Skin cancers are particularly prevalent in this group. In this section I examined the frequency of different cancers in our CLL cohort and compared it to UK cancer prevalence (Forman *et al.*, 2003) and a recently published report from MD Anderson (Tsimberidou *et al.*, 2009).

A summary of the data is shown in Table 7.29-31 and Figure 7.21.

The incidence of all other cancers in our cohort was 18% (87/496). The median age of the group of patients with other cancers was 69.5 years (48-92) and the male:female ratio in this group was 2.3:1.

I compared the prevalence of cancers in CLL to the prevalence in ≥65 years of age group in the UK published by Forman (Forman *et al.*, 2003).The prevalence of cancers in the UK was 15,086 per 100,000, cf. in Leicester CLL cohort 17,540 per 100,000 ($p=0.1865$, chi-squared test), therefore cancer prevalence in the Leicester CLL cohort was very similar to that seen in the normal general population. Patients who were diagnosed with a second cancer were older ($p<0.0001$) (Table 7.30) and were more likely to be at stage A ($p=0.0666$), although it was not statistically significant. I did not observed any cancers in the Asian cohort ($p=0.0322$). There was a significant difference in OS between CLL with (135 months) and without cancer (184 months), $p=0.0031$.

There was a significant increase in prevalence of non-melanoma skin cancers (NMSC) in the Leicester CLL cohort (Table 7.29): there were 27 observed cases, against expected 7 (NMSC prevalence in was UK: 8/560, (Harvey *et al.*, 1996). I did not see an increased frequency of melanoma. There was a marginal significance in the prevalence of lung cancers.

Comparing frequency of other cancers to the MD Anderson (MDA) cohort, I observed fewer other cancers in our cohort: Leicester: 87 cases in 496 (18%) versus MDA: 551 in 2028 (27.5%, $p<0.0001$, chi-squared test). I have observed higher proportions in NMSC ($p=0.0068$), urinary cancers ($p=0.0245$) and lymphoma ($p=0.0033$) with statistical significance.

The univariate analysis failed to show an increased risk for TTFT for patients with other cancer (Table 7.31), however there was increased risk of dying for patients with other cancers: for every 100 with other cancers, 53 patients die who do not have other cancers (HR 0.534; 95%CI 0.355-0.802).

Table 7.29 Secondary cancers in the Leicester cohorts *v.* data from MDA and compared to prevalence of cancers in the UK population

Cancer	n =87 (18%)	MDA n=551** (27.5%)	Statistics (with MDA data)	Expected in UK per 498 (≥65)	Statistics (with UK data)
Non-melanoma skin cancer	27 (31)	187 (30)	p=0.0068[b]	7	0.0005[b]
GI	17(20)	56 (9)	p=0.6295[a]	14.8	p=0.67[b]
urinary	12 (14)	23 (3.7)	p=0.0245[a]		
prostate	10 (11)	80 (12.8)	p=0.0256[a]	7.4	p=0.4348[b]
lung	9(10)	38 (6.1)	p=0.93[a]	5.1	p=0.13[b]
breast	7 (8)	58 (9.3)	p=0.11[a*]	14.4	p=0.4[b*]
melanoma	4 (5)	53 (8.4)	p=0.0148[a]	1.8	p=0.22[b]
myeloid	3 (3)	16 (2.6)	p=0.67[a]	-	-
larynx	3 (3)	-	-	-	-
lymphoma	2 (2)	51 (8.1)	p=0.0033[a]	-	-
ovary	2 (2)	-	-	-	-
tonsil	2 (2)	-	-	-	-
Genital (female)	2 (2)	23 (3.7)	p=0.1814[a*]	4.2	0.6809[b*]
pharynx	1 (1)	-	-	-	-
sarcoma	1 (1)	6 (1)	p=0.7205[a]	-	-
thyroid	1 (1)	19 (3)	p=0.0978[a]	-	-
brain	1 (1)	6 (1)	p=0.7205[a]	-	-
Unknown adenocarcinoma	1 (1)	-	-	-	-

[a]Fisher exact test; [b]Chi square; green: marked values with marginal significance, red: statistical significances;
* Corrected for sex ratio.
**MDA population: total 2028 (male=1240, female=788)

Table 7.30 Characteristics of patients with secondary cancers

	Second Cancers n=87	No Cancers n=395	Statistics
Age median (range)	69.5 (48; 92)	64 (34; 97)	p<0.0001[bv]
M:F (n:n)	2.3:1(61:27)	1.8:1(252:143)	p=0.3878[a]
WCC median (range)	18.9 (4; 345)	19.3 (4.3; 568)	P=0.977[c]
A0 n(%)	43 (49%)	225 (59%)	p=0.2334[a]
A n(%)	27 (30%)	84 (22%)	p=0.0666[a]
B n(%)	12 (14%)	52 (13%)	p=0.8622[a]
C n(%)	6 (7%)	23 (6%)	p=0.6264
LN=0 n(%) LN<1 n(%) LN>3 n(%) n availble	50 (63%) 24 (30%) 5 (6%) 79	234 (68%) 81 (24%) 27 (8%) 342	p=0.22 p=0.248 p=0.656
Treatment n(%)	34 (%)	144 (%)	p=0.713[a]
Asian n(%)	0	19 (5%)	p=0.0322[a]
autoimmune n(%)	5 (6%)	46 (12%)	p=0.1244[a]
M n(%)	48 (64%)	243 (71%)	p=0.2114[a]
UM n(%)	27 (36%)	97 (29%)	p=0.2114[a]
13q n(%)* n available for cytogenetics	23 (38%) 60	101 (11%) 269	p=0.8925[a]
normal	14 (23%)	72 (38%)	p=0.7574[a]
t12	9 (15%)	36 (13%)	p=0.6868[a]
11q	11(18%)	31(27%)	p=0.1968[a]
17p	1 (1.6%)	9 (3%)	p=1[a]
Other	7 (12%)	30 (11%)	p=0.8264[a]
CD38 median n (% of >30%) n available	5.03 17 (26%) 66	3.04 74 (25%) 291	p=0.44[c]
β2M median (range)	2.3 (1.5; 7)	2.1 (1.1; 7.6)	p=0.52[c]
LDT median	59 months	62 months	p=0.9681[d]
OS	135	184	p=0.0031[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significances;
WCC, age, LDT, OS and PFS are shown as median values;
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

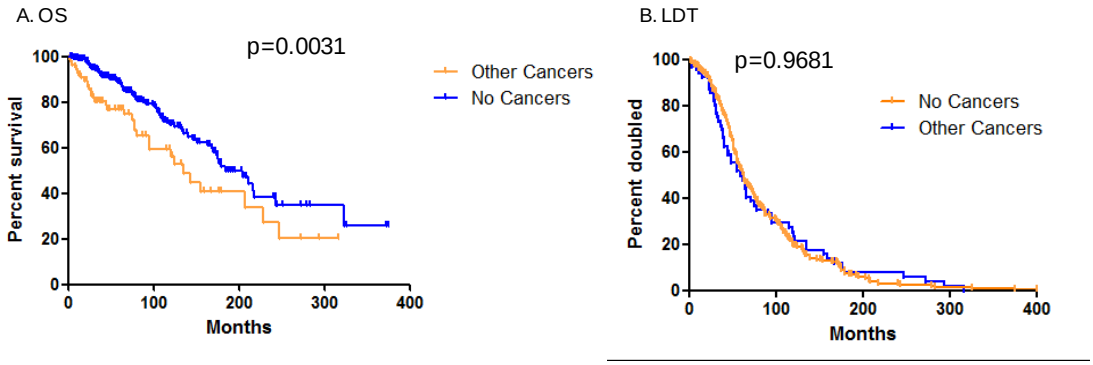


Figure 7.21 Mantel-Cox survival curve of patients with and without cancers.
(A) p=0.0031, median OS 135m (with other cancers) v 184 m; (B) LDT.

Table 7.31 Comparison of hazard ratio for OS , TTFT and LDT for patients with other cancers

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Other Cancers	0.534	0.355	0.802	0.003

Comparison to patients with other cancers, e.g. for every 100 with other cancers, 53 patients die who do not have other cancers.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Other Cancers	0.903	0.62	1.314	0.594

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Other Cancers	1.003	0.751	1.340	0.984

7.1.12 Overview of treatment used in Leicester CLL cohort

The treatment for CLL has evolved over last few decades' chlorambucil became available in 1960s, purine analogues in 1990s, monoclonal therapy with anti-CD20 and anti-CD52 antibodies in 2000s. Currently there are many new agents available as part of clinical trials, e.g. GA101, Lenolidomide, BCL2, PARP and PI3K inhibitors. The choice of treatment depends on fitness and biological age of the patient and access to clinical trials.

I analysed the current treatments for CLL in the Leicester cohort covering the period of 2001-2009. Survival graphs are presented in Figure 7.22.

A total of 179 out of 469 (38%) patients were treated, 110 (61% treated) of these patients had more than one type of regimen; 69 patients were treated with the Fludarabine cyclophosphamide (FC) regimen, 95 with Chlorambucil (CLB) (52 were treated before 2001) and 42 with Fludarabine (F) alone. Out of FC treated patients 59% (41/69) were treatment naïve, 23% (16/69) had one previous course of chemotherapy; 13% (9/69) had had two; 3% (2/69) three and 1.4% (1/69) four courses prior to this treatment.

Doses and form of administration of Chlorambucil and Fludarabine changed in the studied period. The dose of Chlorambucil (as per MRC CLL4 trial, January 2000) was 10mg/m^2 for 7 days every 28 days until maximum response was achieved or up to one year. The currently recommended regime by British National Formulary (http://bnf.org/bnf/) is 150micrograms/kg daily for 14 days every 28 days. Few patients received small daily dose of 2mg.

Prior to September 2001 (when NICE approved oral preparation) Fludarabine was administered in intravenous form: 25mg/m^2 for five days every 28 days, minimum of three and maximum of six courses. Fludarabine and Cyclophosphamide (FC) combination constituted of Fludarabine 25mg/m^2 and cyclophosphamide 250mg/m^2 for three days minimum of three and maximum of six courses. Since introduction of oral form the FC combination is Fludarabine 24 mg/m^2 and Cyclophosphamide 150mg/m^2 for five days for six cycles.

(http://www.lnrcancernetwork.nhs.uk/healthcareprofessionals/medicinesmanagement/protocols/haem/default.aspx).

Because many patients in our cohort had more than one treatment regime, therefore for the analysis of overall survival, I selected patients who received single treatments and did not use those who had multiple therapies, i.e. the patients were not repeated in other curves. For the PFS I analysed all the patients.

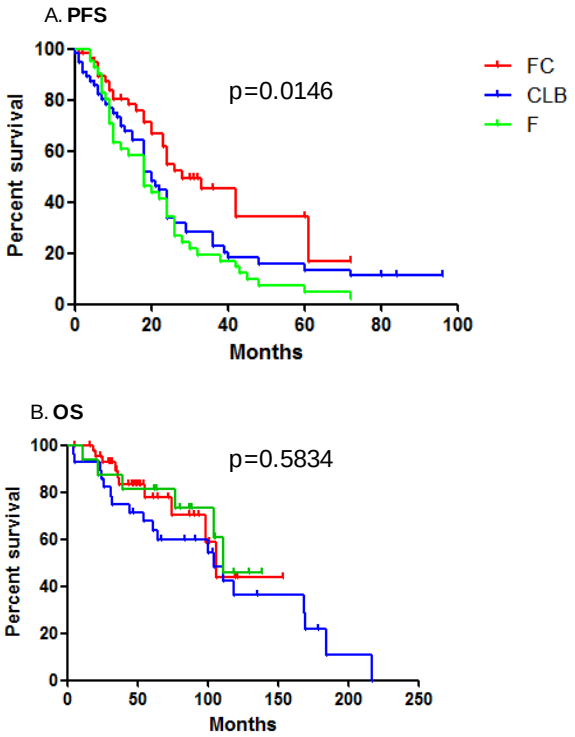


Figure 7.22 Mantel-Cox survival curve for different therapies
A. Progression free survival from time of treatment and B. Overall survival of patients treated with Fludarabine and Cyclophosphamide (FC), chlorambucil (CLB) and Fludarabine (F).

Progression free survival (PFS) was the time between 1st and second treatment. However, PFS for the FC combination was better than F alone or CLB (p=0.0146, Figure 7.23). PFS in our cohort was shorter than published by Catovsky for FC Leicester: 28 months *vs*. 44 months (CLL4), for F Leicester 18 months *vs.* 20 months and for CLB Leicester: 20 months vs. 25 months.

I did not observe any difference in OS between Chlorambucil, Fludarabine alone and the Fludarabine Cyclophosphamide combination within the Leicester cohort. There is a difference between OS of the Leicester cohort and the CLL4 trial data: the 5 year-survival Leicester cohort was 80.9%, CLL4: 56%, with a significance of p=0.0368.

The response to Fludarabine in itself appears to be an important prognostic factor; therefore I also looked at the characteristics of the Fludarabine refractory group versus Fludarabine with good response. The data is presented in Table 7.32 and Figure 7.23.
In the analysis I included patients treated both with Fludarabine alone and a Fludarabine Cyclophophamide combination. Patients who had achieved good response were mainly treated with the FC combination (80% of all F treated patients). The

majority of patients treated with Fludarabine monotherapy (57%) became) became Fludarabine refractory patients.

The significant differences between refractory and non-refractory were observed in WCC at diagnosis (p=0.043), the F refractory patients more often present with a high WCC (45.5 *v.* 19.4). There was no statistical significance in the proportions of patients with mutated and unmutated *IGHV* genes (p=0.13). There were no differences between the two groups in male to female ratio, type of cytogenetic abnormality, stage, LDT, β2M levels, CD38 and lymphadenopathy at presentation.

There was no difference in TTFT. Prior to treatment both groups of patients had similar characteristics, yet the response to Fludarabine had a significant impact on OS and PFS, with median OS of 79 months *vs.* undefined and PFS 10 *vs.* 45 months. In the univariate analysis there was a significantly (p<0.0001) increased risk (78.9%) of dying if the patient was F refractory (HR 0.211; 95%CI 0.094-0.475) (Table 3.18.a).

Table 7.32 Characteristics of Fludarabine refractory v. Fludarabine responsive patients

	F refractory (n=32)	F good response (n=35)	Statistics
Age median (range)	59 (44; 84)	60 (34; 84)	p=0.49[b]
M: F (n:n)	4.3: 1	2.5:1	p=0.4[a]
WCC median (range)	45.5 (4; 568)	19.4 (4.5; 354)	p=0.043[c]
A0 n(%)	8(25%)	4(11%)	p=0.2[a]
A n(%)	9(28%)	15(43%)	p=0.3[a]
B n(%)	7(22%)	10(29%)	p=0.6[a]
C n(%)	8(25%)	6(17%)	p=0.6[a]
LN=0 n(%) LN>3 n(%)	0: 7(22%) >3cm: 3(9%)	0: 9(26%); >3cm :6(17%)	p=0.78[a]
PFS months	10	45	p<0.0001[d]
Treatment n(%)	FC 43%vs. F 57%	FC 80% vs. F 20%	p=0.0007[a]
Asians n(%)	2(6%)	2(6%)	p=1
autoimmune n(%)	3(9%)	6(17%)	p=0.5[a]
M n(%)	12(38%)	19(59%)	p=0.13[a]
UM n(%)	20(62%)	13(41%)	p=0.13[a]
13q n(%)* n available for cytogenetics	3(17%) 18	3(9%) 32	p=0.66[a]
normal n(%)	2(11%)	8(25%)	p=0.47[a]
t12 n(%)	6(33%)	7(22%)	p=0.53[a]
11q n(%)	5(28%)	10(31%)	p=1[a]
17p n(%)	2(11%)	1(3%)	p=0.55[a]
other n(%)	5(28%)	8(25%)	p=1[a]
β2M median (range)	2.7 (1.3; 7.6)	2.55 (1.4; 5.5)	p=0.69[c]
CD38 median n (% of >30%) n available	16 >30%, 8 (40%) 20	2.55 >30%8 (30%) 27	p=0.448[c] p=0.54[a]
LDT median	79 months	64 months	p=0.6189[d]
TTFT months	7.5 months	16.5 months	p=0.26[d]
OS months	74 months	undefined	p=0.0001[d]

[a]Fisher exact test; [b]unpaired t-test; [c]Mann Whitney; [d]Mantel-Cox (log rank) ; green: marked values with marginal significance, red: statistical significances;
WCC, age, LDT, β2M , OS and PFS are shown as median values;
*some patients had more than one cytogenetic abnormality, % calculated from number of patients

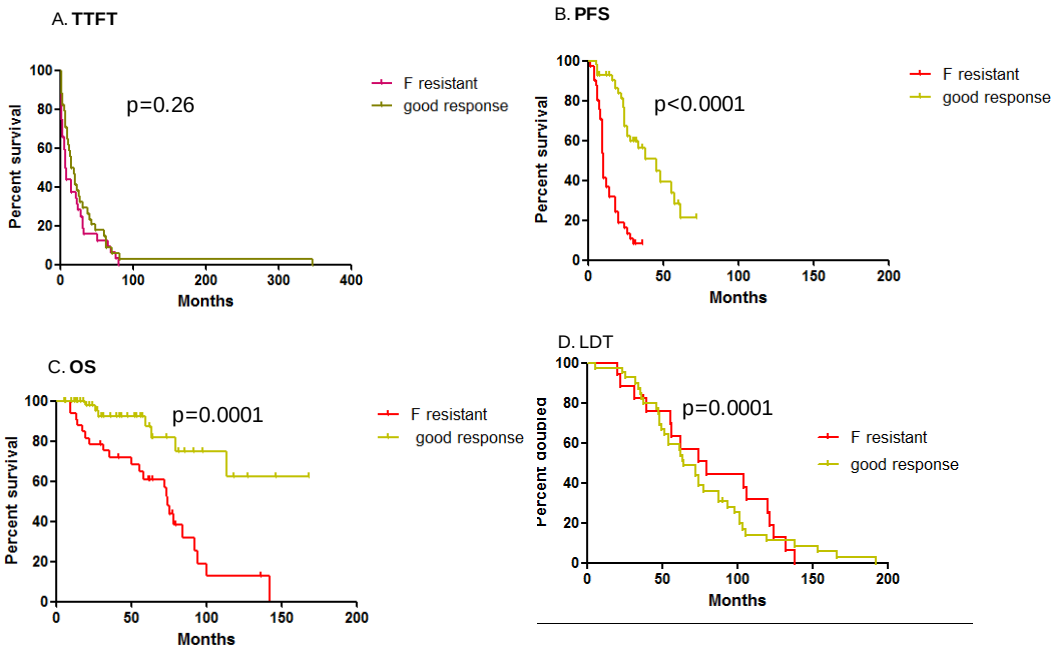


Figure 7.23 Mantel-Cox survival curve of Fludarabine responsive and unresponsive

A. TTFT time to first treatment from diagnosis; **B:** Progression free survival from the fludarabine treatment, **C.** OS from Fludarabine treatment; between Fludarabine refractory and responsive patients; **D.** LDT.

Table 7.33 Comparison of hazard ratios for OS, TTFT and LDT for patients who are refractory and who respond well to Fludarabine.

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
F response	0.211	0.094	0.475	<0.0001

Comparison to patients who are refractory, e.g. for every 100 refractory patients, 21 patients die who are good responders.

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
F response	0.704	0.453	1.093	0.118

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
F response	1.163	0.638	2.119	0.623

7.1.13 The univariate and multivariate analysis

In the univariate analysis using Cox regression analysis the variables which produced a significant hazard risk of shortened OS in advanced stage, age, higher WCC, presence of LN, shorter TTFT, unmutated status, presence of other cancers, poor F response, high β2M. The significant hazard ratio for shortened TTFT was produced by advanced stage, male gender, higher WCC, presence of LN, co-existent autoimmunity, short LDT, unmutated status, high CD38, β2M and clone size of 13q deletion. The variables which affected HR for both OS and TTFT were: stage, WCC, LN, mutation status and β2M.

Univariate analysis short LDT were advanced age of first treatment and age at diagnosis, higher β2M, lack of 13q deletion, bulky LN, unmutated *IGHV* and OS (collated in Table 3.34-37 and Figure 7.24).

The multivariate analysis using Cox regression was used to investigate relationships among variables and to identify independent risk factors. The multivariate analysis requires complete data set and therefore it was difficult to execute in the Leicester cohort because of the number of missing data. Therefore a model was run only on the patients with complete covariate information (for OS, n=182; for TTFT, n=182; for LDT, n=164). The multivariate analysis was done for OS, TTFT, and LDT for the variables which had statistically significant hazard ratios in the univariate analysis.

Multivariate analysis of OS was significant for age at diagnosis, stage, β2M, mutation and WCC.

Independent prognostic factors adversely effecting OS were advanced age ($p<0.0001$), unmutated *IGHV* ($p=0.0132$) and high levels of β2M ($p=0.0134$), for shortening TTFT the independent factors were advanced stage ($p<0.0001$ for stage A and A0) and also unmutated *IGHV* ($p<0.0001$), for shortening LDT was advanced age ($p=0.0004$) and high WCC ($p=0.0142$) (Table 7.37 and Figure 7.25).

Table 7.34 The univariate analysis for OS (summarised statistically significant only).

Variables	Hazard Ratio (OS)	95% CI for HR		p value
		Lower	Upper	
Age at Δ (numeric)	1.11	1.088	1.133	<0.0001
Age at Rx (numeric)	1.075	1.05	1.101	<0.0001
WCC	1.005	1.003	1.007	<0.0001
LN=0	0.412	0.208	0.815	0.011
LN>3	1			
Stage A	0.261	0.137	0.497	<0.0001
Stage A0	0.191	0.105	0.350	<0.0001
Stage C	1			
LDT	0.718	0.639	0.807	<0.0001
β2M	2.247	1.809	2.792	<0.0001
M	0.417	0.279	0.622	<0.0001
UM	1			
Other cancers	0.534	0.355	0.802	0.003
F response	0.211	0.094	0.472	<0.0001
TTFT	0.985	0.981	0.989	<0.0001

Table 7.35 The univariate analysis for TTFT (statistically significant only).

Variables	Hazard Ratio (TTFT)	95% CI for HR		p value
		Lower	Upper	
Female	0.676	0.488	0.936	0.018
WCC	1.009	1.008	1.011	<0.0001
LN=0	0.086	0.052	0.142	<0.0001
LN=1cm	0.496	0.31	0.794	0.003
LN>3	1			
Stage A	0.144	0.09	0.232	<0.0001
Stage A0	0.032	0.019	0.053	<0.0001
Stage B	0.427	0.266	0.686	<0.0001
Stage C	1			
LDT	0.446	0.371	0.537	<0.0001
Autoimmune	0.562	0.377	0.837	0.005
β2M	2.067	1.753	2.437	<0.0001
M	0.353	0.258	0.484	<0.0001
UM	1			
CD38	1.006	1.0	1.012	0.037
13q%	1.016	1.002	1.032	0.029

Table 7.36 The univariate analysis for LDT (statistically significant only).

Variables	Hazard Ratio (LDT)	95% CI for HR		p value
		Lower	Upper	
Age numeric	1.036	1.024	1.048	<0.0001
Age 1st Rx	1.022	1.002	1.042	0.031
LN=0	0.453	0.266	0.769	0.003
LN=1	0.465	0.262	0.823	0.009
LN>3	1			
β2M	1.222	0.987	1.513	0.065
M	0.771	0.59	1.008	0.058
UM	1			
Cyto 13q-no	1.376	1.044	1.814	0.023
Cyto 13q-yes	1			
TTFT	0.986	0.980	0.993	<0.0001
OS	0.818	0.798	0.838	<0.0001

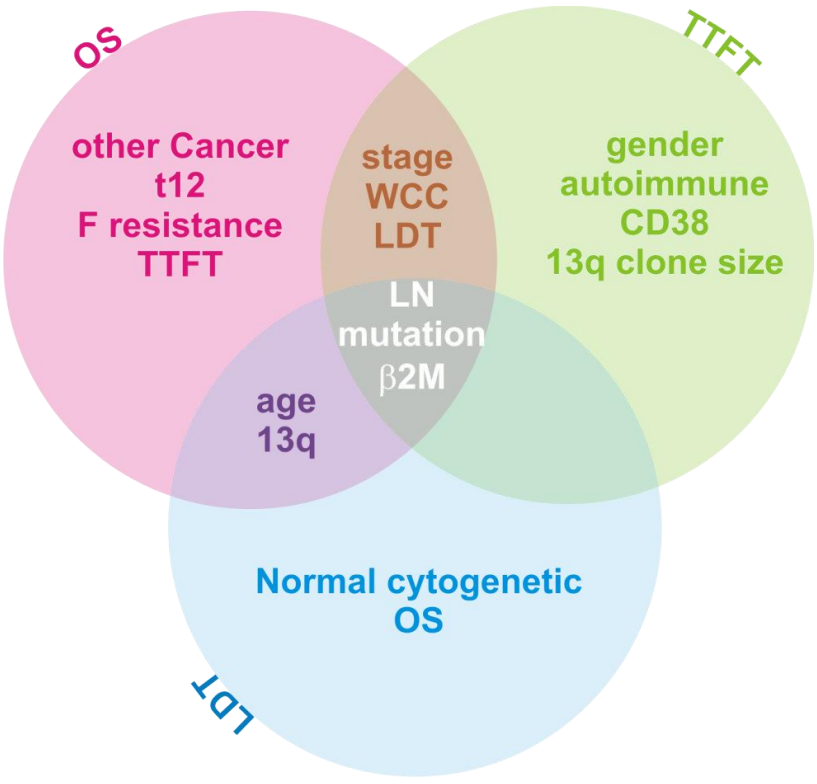


Figure 7.24 Schematic representation of univariate hazard ratio as presented in Table 3.19a.
The variables carrying hazard risk common to OS, TTFT and LDT were lymphoadenopathy, IGHV mutation and β2M. There were no common statistically significant variables between LDT and TTFT.

Table 7.37 The multivariate analysis for OS, TTFT and LDT.

Variables	Hazard Ratio (OS) n=182	95% CI for HR		p value
		Lower	Upper	
age	1.11	1.06	1.15	<0.0001
Mutation M	0.41	0.2	0.83	0.0132
Mutation U	1			
β2M High	2.84	1.24	6.48	0.0134
β2M Low	1			

Variables	Hazard Ratio (TTFT) n=182	95% CI for HR		p value
		Lower	Upper	
Stage A	0.162	0.067	0.393	<0.0001
Stage A0	0.039	0.015	0.099	<0.0001
Stage B	0.289	0.121	0.69	0.0052
Stage C	1			
Mutation M	0.351	0.213	0.577	<0.0001
Mutation U	1			

Variables	Hazard Ratio (LDT) n=164	95% CI for HR		p value
		Lower	Upper	
Age	1.044	1.019	1.068	0.0004
WCC	1.010	1.002	1.018	0.0142

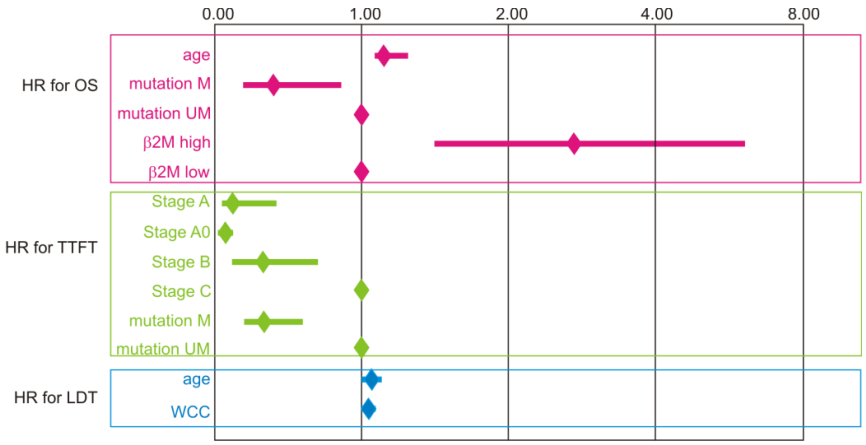


Figure 7.25 Graphical representation of hazard ratio calculated for multivariate analysis (as presented in Table 7.37).

Chapter 8: Use of the Leicester CLL database: expression of single-nucleotide polymorphism (-938C>A) in the BCL2 promoter in CLL and its significance

8.1 Introduction

The Leicester CLL database allowed access to a number of CLL patients with biological and clinical prognostic data and therefore it became an excellent tool to test a number of hypotheses in B cell biology and pathogenesis in CLL. In 2007 the German group (Nuckel *et al.*, 2007) proposed that single nucleotide polymorphism (SNP) in the promoter of *BCL2* has adverse effect on the patients' outcome.

This was an intriguing hypothesis for two reasons: firstly the BCL2 family has an important role in B cell homeostasis. Unlike many proto-oncogenes, BCL2 does not trigger cell proliferation but promotes survival in suboptimal growth conditions (Vaux *et al.*, 1988). Secondly, *BCL2* is overexpressed and appears to have a fundamental defect in CLL cells. *BCL2* overexpression is not driven by the t(14;18) as only very small minority of CLL patients (2% of cases) have this translocation (Dyer *et al.*, 1994). In addition *BCL2* expression in CLL cells is much higher than the expression of any other BCL2 family members. E.g. BAX (BCL2-associated X protein) is a proapoptotic BCL2 protein promoting apoptosis by competing with BCL2. An increased BCL-2/BAX ratio (favouring cell survival) was shown to be associated with treatment response, but not Rai staging (Pepper *et al.*, 1997; Saxena *et al.*, 2004). There was no proven correlation between the worse prognosis in BCL2 positive patients and resistance to chemotherapy (Kitada *et al.*, 1998). There is increasing evidence that BCL2 expression depends on the microenviroment (Buggins & Pepper, 2010). BCL2 is induced by the *in vitro* microenviroment models (e.g. (Willimott *et al.*, 2007)) and is higher in the lymph nodes of CLL patients (Smit *et al.*, 2007). The regulatory mechanism of *BCL2* expression is still unknown in CLL.

BCL2 has two promoters: P1 and P2; P2 decreases the activity of the P1 promoter (Seto *et al.*, 1988; Young & Korsmeyer, 1993). Park *et al.* identified six single nucleotide polymorphisms (SNPs) in the *BCL2* gene by sequencing DNA samples from a White population (Park *et al.*, 2004). One of these SNPs (-938C>A) located in the

negative regulatory promoter P2 was proposed to effect *BCL2* transcription (Nuckel *et al.*, 2007).

Nückel *et al.* tested 123 CLL patients (42AA, 55AC and 26CC). They suggested that the *BCL2*-938AA genotype is associated with increased *BCL2* expression compared to the CC genotype and therefore speculated that it may be used as a novel, unfavourable genetic marker in CLL patients. Our preliminary observation did not support Nückel observation therefore we set out to check the Nückel hypothesis in our Leicester CLL cohort using the database. This work was done together with Dr A. Majid (a postdoctoral researcher) and Olga Tsoulakis (an MSc student) and published in Blood (Majid *et al.*, 2008).

8.2 Results

8.2.1 *BCL2* polymorphism and prognostic parameters

We have genotyped 276 CLL patients (97 patients were AA genotype, 127- AC and 52-CC, the patients characteristics are presented in the Table 8.1. The mean age at diagnosis was 64 (range 30-97). Male predominance was similar to what we observed in the Leicester database (2:1). The *IGHV* mutation data were available for 221/276 (80%) patients. There were 69% mutated and 29% unmutated patients. The interphase FISH data was available for 152/276 (55%) patients. The majority of patients in each group were in stage A0, the most common cytogenetic abnormality was the 13q deletion and in all three groups a mutated *IGHV* gene segment was the commonest abnormality. Untreated patients were the majority in all three groups, as presented in the Table 8.1. There were no significant correlations with patients' clinical characteristics and laboratory prognostic markers (*IGHV* mutation and cytogenetic abnormalities).

The SNP(-938C>A) was identified by amplification and sequence of the *BCL2* promoter. We also checked the sequence of the *BCL2* gene coding the peptide where the antibody binds (41-54aa) to rule out the presence of mutation of the BCL2 antibody epitope.

Table 8.1 Clinical and laboratory data of CLL patients according to *BCL2* polymorphism -938>A

	Whole group	AA	AC	CC	p
No of cases	276	97	127	52	
Mean age at diagnosis (range)	64 (30-97)	63 (34-85)	65 (35-89)	64 (39-97)	.6 [b]
Sex (n= 272)					
Male	179 (66%)	59 (61%)	86 (69%)	34 (67%)	.5 [a]
Female	93 (34%)	37 (39%)	39 (31%)	17 (33%)	—
Male/female ratio	1.9:1	1.6:1	2.2:1	2.0:1	—
Stage (n = 251)					
A0	121 (48%)	48 (55%)	54 (45%)	19 (44%)	.1 [a]
A	67 (27%)	25 (28%)	31 (26%)	11 (26%)	—
B	51 (20%)	14 (16%)	25 (21%)	12 (28%)	—
C	12 (5%)	1 (1%)	10 (8%)	1 (2%)	—
Genomic aberrations (n= 152)					
Deletion 13q14	50	15	25	10	0.8 [a]
Trisomy 12	20	6	12	2	—
Deletion 11q23	18	5	9	4	—
Deletion 17p13	1	1	0	0	—
T(14;18)/IGH-BCL2fusion	5	2	1	1	—
***IGHV* mutational status (n= 225)**					
Mutated (<98% homology to germline)	155 (69%)	54 (71%)	76 (70%)	25 (63%)	.4[a]
Unmutated	66 (29%)	19 (25%)	32 (29%)	15 (37%)	—
Mean WCC at diagnosis (n=249)	41 (4.3-554)	37.9 (5.5-234)	51.4 (4.5-554)	33.8 (4.3-254)	.3[b]
Treatment status (n= 250)					
Treated	98 (39%)	35 (40%)	46 (38%)	17 (40%)	.9[a]
Untreated	152 (61%)	52 (60%)	74 (62%)	26 (60%)	—
Mean time from diagnosis to first treatment (n=98), mo	28.5	28	25.6	32	.3[b]
Survival (n = 258)					
Dead	34 (13%)	14 (16%)	16 (13%)	4 (9%)	.5 [a]
Alive	224 (87%)	76 (84%)	106 (87%)	42 (91%)	—
BCL2 expression (n = 100)					
High (BCL2:actin ration/SC-1>0.48)	80 (80%)	31 (89%)	32 (73%)	17 (81%)	.7[b]
Low (BCL2:actin ration/SC-1<0.48)	20 (20%)	4 (11%)	12 (27%)	4 (19%)	—

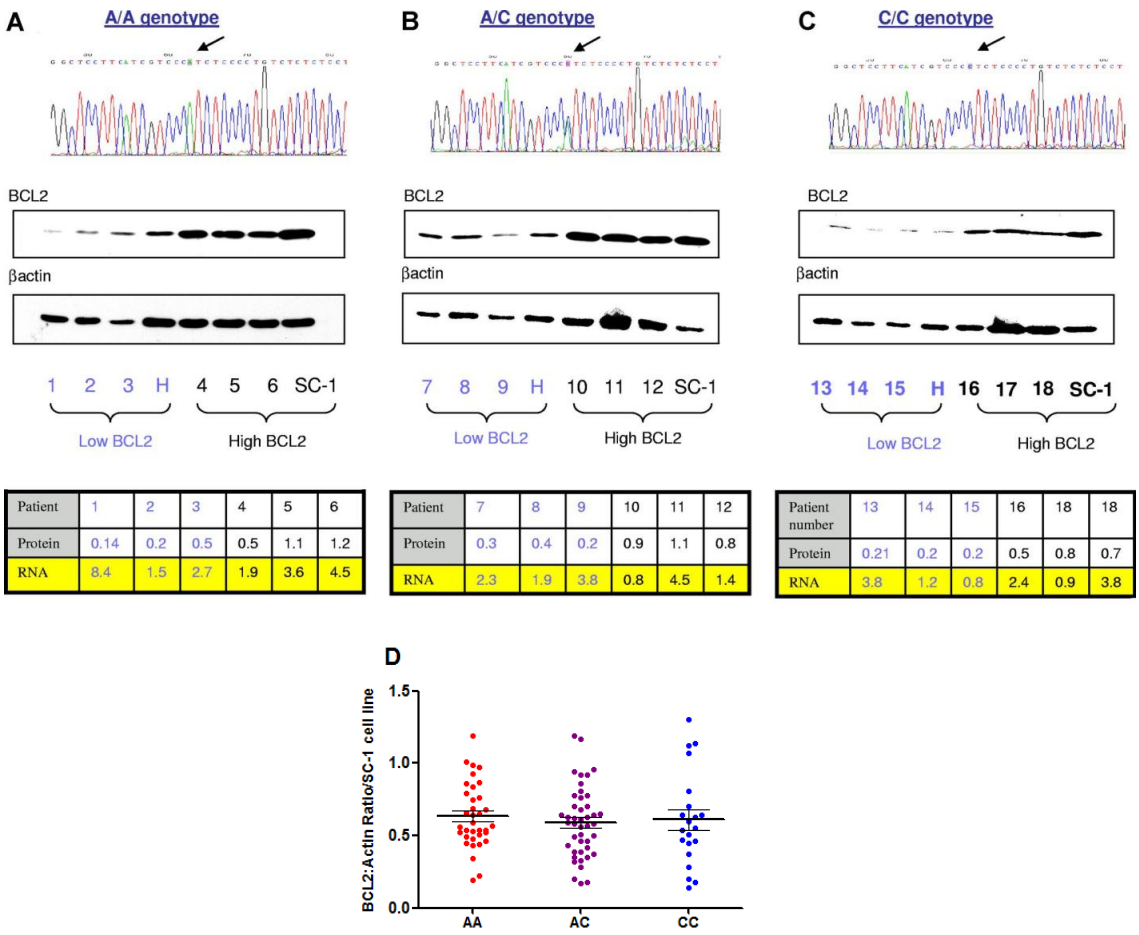
[a]Fisher's exact test;
[b]Kruskal Wallis
No significant association of the *BCL2* promoter SNP was seen in this series with any variable assessed.
WCC: white cell count; - not applicable

I analysed mRNA expression of *BCL2* with quantitative reverse transcription RT-PCR with SYBR Green (method described in Chapter 2.5.8). The endogenous house-keeping gene, TATA Box protein (*TBP*) gene was used to normalise gene expression. From the normalised gene expression the ratio was calculated using gene expression of a follicular lymphoma cell line, SC-1. This was performed to standardise the BCL2 expression to a cell line with high BCL2 expression, therefore to obtain comparable results. SC-1 cell line carries the t(8;14;18) (q24;q32;q21), where the translocation causes the *BCL2* gene (on chromosome 18) to be regulated by the immunoglobulin heavy chain enhancer (chromosome 14) and therefore is excessively overexpressed.

Similarly, BCL2 protein expression was normalised first to β-actin and then compared to SC-1. The controls for high protein expression of BCL2 was the SC-1 cell line and for low BCL2 expression, the HRC-57 cell line. HRC-57 is an EBV immortalised cell line of normal B cells and was used as a normal reference. BCL2 protein expression analysis was performed on 100 CLL patients. Within the tested patients there were 64 treatment-naïve and 36 treated patients. All 100 patients tested expressed BCL2 protein at variable levels and there was no association of BCL2 protein levels and SNP genotype.

Figure 8.1.A-C shows representative examples of protein expression and mRNA levels in each SNP genotype group. Each group (A/A, A/C and C/C genotype) had both low- and high-protein expressing examples. 'H' on the protein blots marks SC1 cell line as a control for high BCL2 protein expression. Many patients with low BCL2 protein expressed large amounts of *BCL2* mRNA (please see the tables in Figure 8.1.A-C). Figure 8.1.D shows a scatter plot of protein expression in each group with mean values represented by horizontal bars. There were no significant differences in protein expression in tested 100 CLL patients.

Figure 8.2 which shows analysis of overall survival (OS) and time to first treatment (TTFT), these analyses were performed in 2009, two years since publication of the *Blood* letter and therefore with longer follow-up data. There was no difference TTFT (p=0.39) or OS (p=0.085) for the 252 analysed patients with AA *v.* CC *v*.CA genotype. In addition there were no significant correlations of the SNP genotype with clinical or biological prognostic parameters (Table 8.1).



Patient	1	2	3	4	5	6
Protein	0.14	0.2	0.5	0.5	1.1	1.2
RNA	8.4	1.5	2.7	1.9	3.6	4.5

Patient	7	8	9	10	11	12
Protein	0.3	0.4	0.2	0.9	1.1	0.8
RNA	2.3	1.9	3.8	0.8	4.5	1.4

Patient number	13	14	15	16	18	18
Protein	0.21	0.2	0.2	0.5	0.8	0.7
RNA	3.8	1.2	0.8	2.4	0.9	3.8

Figure 8.1 Lack of correlation of *BCL2* SNP –938A>C with either BCL2 protein or mRNA levels in CLL.

(A-C) Comparison of levels of BCL2 expression with the–938A>C SNP. All 3 genotypes showed both low and high levels of BCL2 protein. β-actin was used as a standard for normalization of BCL2 expression, and then all samples were compared with levels observed in the cell line SC-1.
For real time PCR, the samples were normalized to SC-1 RNA. The values represent the ratios of CT SC-1 cell line to CT patient RNA.
Note that many of the patients with CLL with low BCL2 expression nevertheless expressed large amounts of *BCL2* RNA. Arrows in top panels denote SNP –938C>A.
(D) Range of levels of expression of BCL2 protein in 100 patients with CLL according to *BCL2* promoter SNP –938 A>C. Horizontal bar denotes mean with SEM values for each group. There was no significant change in the levels of BCL2 protein expression with the SNP genotype.

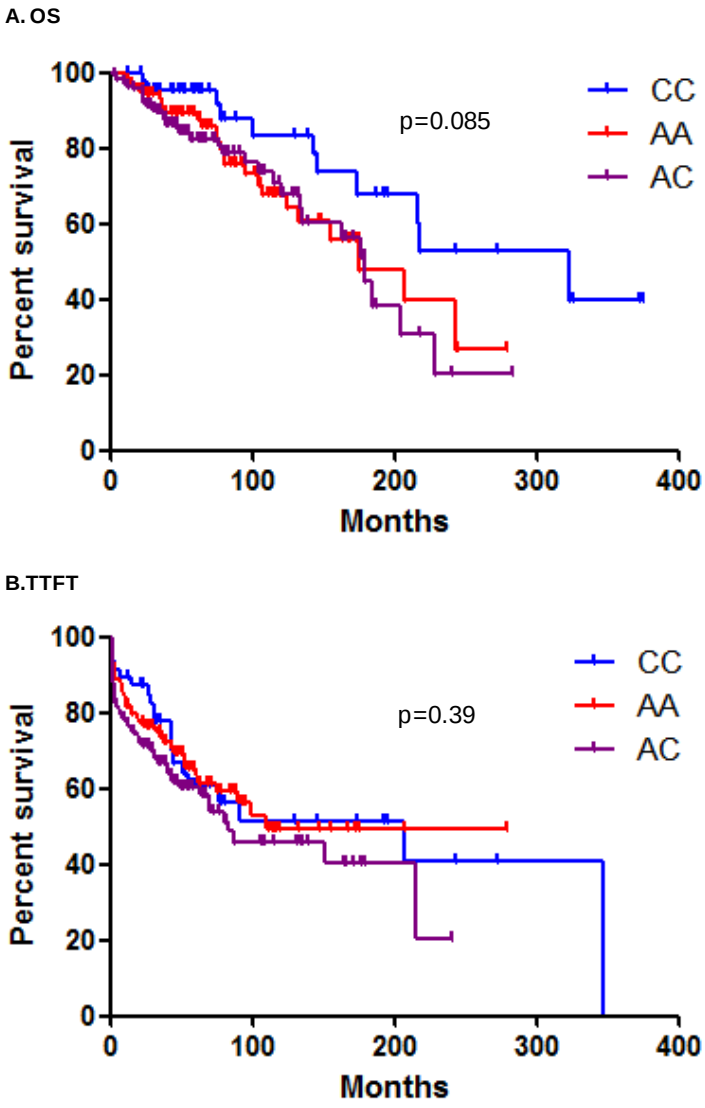


Figure 8.2 Overall survival and time to first treatment .

A. Overall survival for 253 patients. B. Time to first treatment in the 252 patients from this cohort treated to date according to the *BCL2* promoter SNP –938 A>C, showing lack of influence of the SNP in this series.

8.2.2 Levels of BCL2 protein did not vary with therapy

For ten patients we were able to test sequential samples of BCL2 protein expression over the course of the disease, particularly pre- and post-therapy (please see Figure 8.3). The BCL2 expression appears to be constant, we did not observe any changes in BCL2 protein expression between samples taken before and after treatment.

8.2.3 Characteristics of patients expressing low protein levels of BCL2

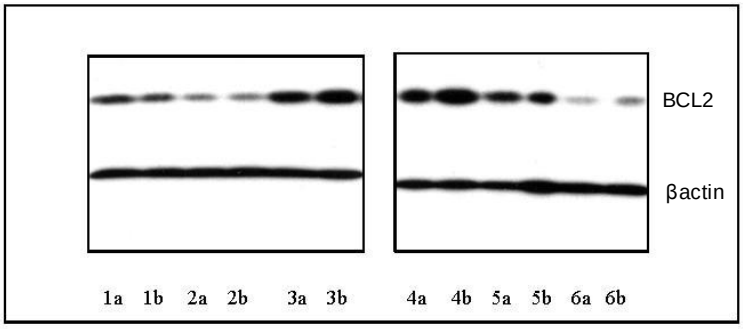
In our tested cohort there were 20% (n=20) of patients with low BCL2 protein levels, Table 8.2 summarises the characteristics of low-expressing patients. The levels were comparable to the levels seen in the HRC-57 cell line. To confirm the validity of this finding we checked the coding sequence of the *BCL2* gene where the antibody binds (41-54aa) to rule out the presence of mutation of the BCL2 antibody epitope. We excluded possibility of conformation change of the epitope caused by mutation and therefore potentially effecting epitope-antibody binding.

The range of protein expression in these 20 patients was 0.16-0.48 (the mean level for HRC-57 was 0.34, a control cell line for low-expression). There were four patients with AA genotype (20%), four with CC (20%) and 12 with CA (60%, p=0.0031, two-tailed chi-squared). Although the proportions were significant, there was no specific correlation between clinical parameters including survival and the genotype in the low expressing BCL2 protein. There was no preferential expression of MCL1 in these patients (data not shown). RT-qPCR showed heterogenous mRNA expression levels within the low BCL2 subgroup: five had RNA levels comparable to the levels of the SC-1 cell line. Four patients in this category had a progressive disease.

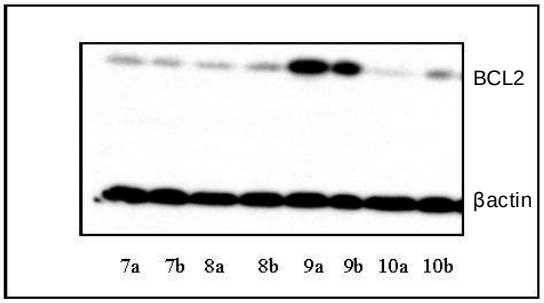
8.2.4 Characteristics of patients with the 13q deletion and t(14;18)

The BCL2 chromosomal translocation t(14;18)(q32;q21) is rare in CLL (Dyer *et al.*, 1994). We have analysed all five patients with the t(14;18) which were identified in the CLL Leicester database (1%, n=496), all showed high levels of BCL2 protein, the median protein level was 0.8 (0.7;1.1, n=5). Figure 8.4.A shows a representative example of BCL2 levels in this group.

The majority (15/18, 83%) of patients with the 13q deletion had high levels of BCL2 (Figure 8.4.B). The median protein level was 0.9 (0.15-1.9).



Patient	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b
Protein	0.9	0.9	0.8	0.8	0.9	0.9	0.9	0.9	0.8	1.0	0.8	0.9

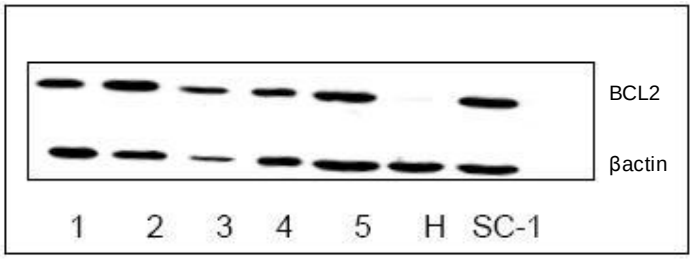


Patient	7a	7b	8a	8b	8a	9b	10a	10b
Protein	0.6	0.6	1.0	1.1	1.1	1.2	1.1	1.3

Figure 8.3 Levels of BCL2 protein are relatively constant in CLL at different points before and after therapy.

Sequential patient samples were taken before (a) and after (b) treatment. The levels of BCL2 remain relatively constant.

A.



Patient	1	2	3	4	5
Protein	0.8	1.1	1.1	0.7	0.8
RNA	0.3	0.8	1.2	1.2	2.4

B.

Patient	1	2	3	4	5	6
Protein	0.7	1.1	1.1	0.7	1.3	1.3
RNA	2	4.4	1.7	n/d	n/d	5
%13 del	75	80	44	53	26	96

Figure 8.4 CLL with t(14;18)(q32;q21) showing expression of BCL2 comparable to that of the SC-1 cell line.

Apart from exhibiting the t(14;18)(q32;q21) by interphase FISH, these five cases were otherwise typical CLL. B. 13q homozygous deletion samples showing expression of BCL2 comparable to that of the SC-1 cell line.
H denotes HRC-57 cell line.

8.3 Discussion

In Leicester cohort we did not confirm Nückel's observation that SNP genotype AA is associated with poor prognosis. Our data are likely to be representative as we investigated a large cohort of patients: 276 versus 123 in Nückel's study. We analysed 100 patients for protein expression of BCL2 versus 9 by Nückel. The distribution of genotypes across both cohorts was very similar (this study AA 35%, AC 46%, CC 19% *vs.* Nückel's: AA: 34.1%, AC: 44.7%, CC:21.1%; p=0.87, two-tailed Chi-squared).
A potential discrepancy between the two cohorts was the overrepresentation of stage B and C in AA genotype. In Nückel's cohort there were 45.9% (17/37) patients with stage B and C, whereas in our study only 17% were stage B and C (15/88). The difference between these proportions in the two groups was highly significant (p=0.0007, Fisher's exact test) and caused a bias in Nückel's AA group. Two years later the same group reanalysed *BCL2*-938C>A polymorphism on the different cohort to the original study (n=271) and did not associate genotypes with the significantly different time to first treatment or overall survival, confirming our findings (Zenz *et al.*, 2009). The second cohort was the group with a more aggressive disease than the original cohort. There was no difference in TTFT and OS when patients with stage A were analysed. In addition, another study by an Italian group confirmed our results on 182 patients (Rossi *et al.*, 2008b).

An interesting observation in Leicester study was a group of patients with low BCL2 protein levels. The group was very heterogenous (Table 8.2). There was a significant difference in the distribution between the genotypes (p=0.0031) and it was mainly represented by the AC genotype. We rule out the possibility of reduced anti-BCL2 antibody affinity in this group by checking for mutations in the sequence of the BCL2 epitope. The RNA levels in this group were varied, with a median of 0.4(0.1;1.3). This suggests translational and post translational mechanisms might control BCL2 protein expression in CLL. The BCL2 levels in our CLL patients appeared to be constant throughout the course of the disease and did not change after patients were treated.
The drawback of the Leicester study was a lack of genotype analysis on an age- and gender- matched group of healthy volunteers. Following our publication Nückel found similar distribution of -938C>A polymorphism in the normal donors, suggesting that this polymorphism did not increase susceptibility for CLL.

The prognostic importance of the BCL2 in CLL is controversial and this study we refuted hypothesis of any prognostic significance of the *BCL2*-938C>A SNP. There

Table 8.2 Clinical characteristics of low BCL2-protein expressing CLL

	All	AA genotype	AC genotype	CC genotype	p
Low BCL2 patients (n)	20	4	12	4	0.0031[a]
Median age at diagnosis (range)	62 (35; 89)	63 (50; 82)	63 (44; 79)	62 (46; 77)	0.9[b]
Gender (n=19) Male:Female	11:8	1:3	6:5	4:0	0.1[a]
Stage (n=18) A0 A B C	 5 5 5 3	 3 1 0 0	 2 3 3 3	 0 1 2 0	0.4[a]
IGHV mutational status n=16 Mutated Unmutated	 10 6	 2 2	 6 3	 2 1	0.8[a]
Median WCC at diagnosis n=18 (range)	23(8;419)	14.9(10;23)	49.5 (8; 419)	15.8 (8.3; 24)	0.14[b]
Treatment (n=19) Yes No	 9 10	 0 4	 6 5	 3 1	0.08[a]
Survival (n=19) Yes No	 3 16	 0 4	 2 9	 1 3	0.6[a]
BCL2 RNA	0.4 (0.1;1.3)	0.5 (0.4;0.6)	0.3 (0.1;1.2)	1.1 (0.8;1.3)	0.13[b]
BCL2 Protein	0.4 (0.2;0.5)	0.39 (0.19; 0.46)	0.34 (0.17; 0.43)	0.18 (0.14;0.37)	0.23[b]

[a]Fisher's exact test;
[b]Kruskal Wallis
WCC denotes WCC at diagnosis; Rx number of different chemotherapy/antibody regimens. Note that despite expressing lower amounts of BCL2 protein, four cases were nonetheless resistant to fludarabine, and five showed high-level of *BCL2* RNA, comparable to that observed in the lymphoma cell line SC-1 without comparable level of protein expression (shaded boxes).
Modified with permission from O. Tsoulaki (MSc Thesis, University of Leicester, 2008)

was a previous report of increased BCL2 protein or BCL2/BAX ratio with poor outcome in CLL (Molica *et al.*, 1998). More recently Grever prospectively analysed 235 patients with Fludarabine and Fludarabine Cyclophosphamide treatment and investigated association of response to treatment and progression free survival with *IGHV* mutation, cytogenetics abnormalities, ZAP70 and BCL2 family proteins. There was a lack of prognostic significance for the BCL2, MCL1 and BAX protein levels (Grever *et al.*, 2007).

Publications arising from this thesis:

1. **Walewska R**, Boyd RS, Majid A, Jukes-Jones R, Cain K, Dyer MJ (2009) FAM129C a novel B cell protein is downregulated during CD40cell activation but not BCR stimulation. Poster accepted for presentation at the BSH 49th Annual Scientific Meeting, Brighton, April 2009.
2. Baou M, Kohlhaas SL, Butterworth M, Vogler M, Dinsdale D, **Walewska R**, Majid A, Eldering E, Dyer MJ, Cohen GM. Role of NOXA and its ubiquitination in proteasome inhibitor-induced apoptosis in chronic lymphocytic leukemia cells. *Haematologica*. 2010 Apr 7
3. Inoue S, Harper N, **Walewska R**, Dyer MJ, Cohen GM Enhanced Fas-associated death domain recruitment by histone deacetylase inhibitors is critical for the sensitization of chronic lymphocytic leukemia cells to TRAIL-induced apoptosis.*Mol Cancer Ther.* 2009 Nov;8(11):3088-97.
4. Boyd RS**, Jukes-Jo**nes R, **Walewska R**, Brown D, Dyer MJ, Cain K (2009) Protein profiling of plasma membranes defines aberrant signalling pathways in mantle *cell lymphoma. Mol Ce**ll Proteom**ics*. 20**09 Jul;8(7**):1501-15.
5. Vogler M, Butte**rworth M,** Majid A, **Walewska R**, Sun XM, Dyer MJ, Cohen GM. (2008) Concurrent upregulation of BCL-XL and BCL2A1 induces~1000-fold resistance to ABT-737 in chronic lymp*hocytic* leukaemia. *Blood,* 2008 Nov 13.
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14. **Walewska R,** Teobald I, Dunnion D, Abdulmajed H, Aldred M, Sadler J, Chapman C & Browning M (2007) Preclinical development of hybrid cell vaccines for multiple myeloma. *European Journal of Haematology,* **78,** 11-20.
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20. **Walewska R**, Boyd RS, Majid A, Jukes-Jones R, Cain K, Dyer MJ (2009) FAM129C a novel B cell protein is downregulated during CD40cell activation but not BCR stimulation. Poster accepted for presentation at the BSH 49th Annual Scientific Meeting, Brighton, April 2009.
21. **Walewska R**, Buckby E, Farnham P, Majid A, Kennedy DB, Dyer MJS (2007) The Cam1515 Study: a Phase I/II Study to Determine the Safety, Toxicity and Pharmacokinetics of Daily Subcutaneous Alemtuzumab in Patients with CLL. XII International Workshop on CLL, London, Sept 2007
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