ATM mutant cellular phenotype in B-cell chronic lymphocytic leukaemia: clinical consequences and therapeutic implications

by

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ABSTRACT

ATM germ-line mutations have been identified in a proportion of patients with chronic lymphocytic leukaemia (CLL) but their role in the development of this tumour remains unknown. In the course of this study it was established that the frequency of germ-line *ATM* pathogenic mutations was increased among patients with chromosome 11q deletion/loss of one *ATM* allele when compared to healthy control individuals but not in those who did not acquire this deletion in their leukemic clone. The results indicate that *ATM* germ-line heterozygosity does not play a role in CLL but may influence disease progression through complete *ATM* loss and clonal expansion.

The analysis of the clinical outcome among CLL patients from phase III LRF UK CLL4 trial identified a distinctive subgroup with a particularly poor prognosis. Those patients had bi-allelic inactivation of *ATM* gene and showed significantly reduced progression-free survival (PFS) compared to those with *ATM* wild-type or mono-allelic *ATM* defects. Furthermore, they had equally poor PFS as those with mono- and bi-allelic *TP53* abnormalities. The clinical implication of this finding might include re-consideration of the treatment strategies for this particular subgroup of patients.

The improved understanding of the biological background of progressive and resistant CLL tumours, such as those with *TP53* or *ATM* defects, results in further development of targeted treatment strategies. The assessment of their efficacy and toxicity could be facilitated by CLL xenograft models. The optimization strategies overcoming the limitations of existing xenograft model were investigated during the course of this study. The results showed that partial depletion of patient CD3+, CD4+ or CD25+ cells prior to injection into NOG mice can prolong the engraftment of CLL cells within xenogenic microenvironment hence, providing expanded period of time for testing new therapeutic agents.

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ABBREVIATIONS

APRIL	a proliferation inducing ligand
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 related protein
BAFF	B cell activating factor
Bcl2	B cell lymphoma 2
BCR	B cell receptor
BLAST	Basic Local Alignment Sequence Tool
BLM	Bloom's syndrome mutated
BRCA1	breast cancer type 1 susceptibility protein
BSA	bovine serum albumin
CD	cluster of differentiation
CDK2	casein kinase 2
CDR3	complementarity determining region 3
CFSE	carboxyfluorescein diacetate succinimidyl ester
Chk1	checkpoint 1
CLL	chronic lymphocytic leukaemia
CMV	cytomegalovirus
CSR	class switch recombination
CtIP	C-terminal binding protein interacting protein
ddNTP	dideoxynucleotide
dL	decilitre
DLBCL	diffuse, large B-cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic sub-unit
dNTP	deoxyribonucleotide triphosphate
DSB	double strand break
dsDNA	double strand DNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FAT	FRAP, ATM and TRRAP
FBS	Fetal bovine serum
FISH	fluorescence in situ hybridization

G1,2	cell cycle gap 1, 2
g	gram
GC	germinal centre
H2AX	histone 2A variant X
γH2AX	phosphorylated histone 2A variant X
HEAT	Huntington, Elongation factor 3, A subunit phosphatase 2A, TOR1
HLA	human leukocyte antigen
HR	homologues recombination
IGHV	immunoglobulin heavy chain variable gene
IR	ionising radiation
IRF4	interferon regulatory factor 4
IV	intravenous injection
KAP-1	KRAB associated protein-1
L	litre
LDT	lymphocyte doubling time
M	mitosis
MACS	Magnetic cell sorting
mTOR	mammalian target of rapamycin (FRAP)
Mcl1	myeloid cell leukemia 1
MDC1	mediator of DNA-damage checkpoint protein-1
MDR	minimally deleted region
miR	microRNA
MRD	minimal residual disease
Mre11	meiotic recombination 11
MRN	Mre11, Rad50 and NBS-1
NBS	Nijmegen breakage syndrome
NCI-WG	National Cancer Institute Working Group
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NHEJ	non-homologues end joining
NK	natural killer cells
NLC	nurse like cells
NOG	NOD/Shi-scid/IL2R gamma null
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PIKK	phosphoinositide 3-kinase-like kinases

PP2A	protein phosphatase 2A
Rad50	DNA repair protein Rad50
RNA	ribonucleic acid
RPA	replication protein A
rpm	rotations per minute
SCID	severe combined immunodeficiency
SDF-1α	stroma derived factor-1α
SHM	somatic hypermutation
SNP	single nucleotide polymorphism
ssDNA	single strand DNA
SMC1	structural maintenance of chromosomes 1,
SMG-1	suppressor of morphogenesis in genitalia-1
TAN	Tel-1/ATM N-terminal
Taq	Thermus aquaticus polymerase
TCL-1	T cell leukaemia/lymphoma 1
TCR	T cell receptor
TEAA	triethylammonium acetate
TK	thymidine kinase
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF	TNF receptor-associated factor
TRRAP	Transactivation-transformation domain associated protein
UV	ultraviolet
WBC	white blood cell
ZAP70	Zeta associated protein 70kDa
(v/v)	volume to volume
β2M	β2-microglobulin
µg	microgram
µl	microliter

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To my parents

Moim rodzicom

CHAPTER I

INTRODUCTION

1.1. Chronic Lymphocytic Leukaemia

Chronic Lymphocytic Leukaemia (CLL) is a malignancy of morphologically mature B cells. The median age at diagnosis is between 65 and 72 years and the yearly incidence is approximately 3-4 patients per 100,000 healthy individuals in USA and Europe which makes it the commonest leukaemia in the western world (Goldin and Slager, 2007; Gribben, 2009; Oscier et al., 2012; Parker and Strout, 2011). It is twice as common in men as in women. The aetiology of CLL remains unknown. However, the family history of CLL and other B-cell malignancies is an important risk factor for the development of this tumour.

The disease results from the expansion of clonal B-cells in the peripheral blood, bone marrow and lymphoid organs. CLL monoclonal cells are restricted to expression of either kappa or lambda immunoglobulin light chain and have a unique immuno-phenotype which distinguishes them from other B-cell lymphoproliferative (LP) disorders. They are positive for surface markers: CD5, CD19 and CD23; express weak surface membrane immunoglobulin (SmIg); and display weak or no expression of CD79b, CD22 and FMC7 (Oscier et al., 2004).

CLL is characterized by marked clinical heterogeneity. Although the diagnosis of CLL requires evidence of at least $5x10^9$ B-lymphocytes/L in peripheral blood, the majority (70-80%) of patients are diagnosed incidentally as a result of a routine full blood check. At diagnosis, most patients present with no symptoms and require no immediate intervention (Hallek et al., 2008; Oscier et al., 2004). So far, no therapy has been proven to be more beneficial for patients with indolent CLL than the ‘’watch and wait’ approach, therefore only patients with progressive or symptomatic disease will qualify for treatment. The symptoms of advanced disease usually include: tiredness, night sweats, weight loss and the history of recurrent or prolonged infections. Patients may present with lymphadenopathy, enlarged spleen or liver. Furthermore, anaemia and/or thrombocytopenia may also be present at diagnosis which may be the result of the replacement of normal bone marrow with leukemic cells (Oscier et al., 2012).

Infections and autoimmune cytopenias are the commonest complications associated

with CLL and are caused by defective immune system and/or immunosuppressive effects of treatment. The incidence of second malignancies is increased in both treated and untreated CLLs when compared to normal population. Approximately 5% of CLL cases will undergo transformation to aggressive lymphoma, which is usually diffuse large B-cell lymphoma (DLBCL). It is called Richter's transformation and is associated with a poorer prognosis (Rossi and Gaidano, 2009).

Response to current treatment is very heterogeneous and, although there is no cure for CLL, adequately timed and individually tailored therapy can delay the natural course of disease and prolong survival.

1.2. Aetiology of CLL- inherited predisposition to CLL.

CLL is a rare disease but approximately 6% of all patients have at least one relative with CLL and they are considered as "familial" cases (Yuille et al., 2000). The phenomenon of "anticipation" has been observed in familial CLL cases whereby in each succeeding generation leukaemia is diagnosed at an earlier age. However, this effect might also be due to the introduction of more sensitive diagnostic methods over the past decades (Goldin et al., 1999).

The concept of genetic predisposition in the development of CLL is strongly supported by the evidence of multiple CLL case families and registry studies which confirm an 8.5 fold increased risk of developing this malignancy by first degree relatives of patients (Goldin et al., 2009; Goldin and Slager, 2007). CLL segregates also with other LP tumours as shown in the analysis from 2007 of family history of cases with Hodgkin Lymphoma (HL), non-Hodgkin Lymphoma (NHL), leukaemia (SLL/CLL) and multiple myeloma (MM) (Wang et al., 2007). The analysis was a pooled result of 17 multinational case-control studies contributing as a part of InterLymph consortium. It showed that not only family history of leukaemia but of occurrence of any LP malignancy was a significant predictor of risk for CLL. Other studies based on linked registry data from Sweden and Denmark reported that CLL and other indolent subtypes of LP disorders such as lymphoplasmatic

lymphoma/Waldenstrom's macroglobulinemia (LPL/WM) and hairy cell leukaemia (HCL) aggregate together in affected families (Goldin et al., 2009). Since no good evidence for the environmental risk factors have been identified so far (Linet et al., 2007), these findings indicate the presence of shared genetic variants with pleiotropic effects predisposing to B-cell malignancies.

Further support for the role of inherited component in the aetiology of CLL is provided by the presence of low-level clones of CD5 and CD23 positive B-lymphocytes within normal individuals. The condition is called monoclonal B-cell lymphocytosis (MBL) and precedes the diagnosis of CLL in virtually all cases (Landgren et al., 2009). The MBL clones share genetic and phenotypic characteristics with indolent CLL tumours but occur over a younger age and are more common amongst first degree relatives of CLL (13-18%) than within general population (3-5%) (Goldin et al., 2010). CLL-like MBL identified in individuals who refer to the clinic with lymphocytosis (B cell count above 1.5-1.9x10^9/L but below 5x10^9/L)) progress to CLL requiring treatment at the rate of 1-2% per year (Rawstron et al., 2008; Shanafelt et al., 2009a).

Various approaches have been utilized in search for the genetic markers predisposing for CLL. In the multi-case families, genome wide linkage studies were aimed to identify shared, dominantly or recessively acting mutations with strong predisposing effects. One such study used over 10 000 genetic markers in a cohort of 206 CLL families and identified 3 susceptibility loci: 2q21.2, 6p22.1 and 18q21.1 (Sellick et al., 2007). However, no common genetic changes associated with increased risk have been identified in those loci. Notably, some deleterious mutations in the *CXCR4* gene at locus 2q21.2 have been found in the germ-line material from a small proportion of CLL patients (Crowther-Swanepoel et al., 2009). Furthermore, locus 6p22.1 is a part of the *HLA* genes coding region which has been repeatedly linked to risk of CLL development (Di Bernardo et al., 2012b; Machulla et al., 2001).

Subsequently, it was reasoned that the power to detect a single disease-causing allele in the familial linkage studies in CLL might have been limited by genetic heterogeneity,

i.e. different families might carry different predisposing alleles. However, the separate analysis of two large multi-case families also did not provide the evidence for a single locus conferring susceptibility to CLL (Raval et al., 2007), (Fuller et al., 2008).

The failure in identifying causative genes through linkage studies led to re-evaluation of the inheritance model for CLL which suggested that a proportion of familial cases were caused by the mutations with high predisposing effect. Instead, a new model was considered in which familial CLL is a consequence of the co-inheritance of the low penetrance loci acting in an additive way to generate high risk. According to this new model low penetrance loci are considered to be common genetic variants such as single nucleotide polymorphisms (SNPs) whose frequency is expected to be slightly but significantly higher in CLL cases than in control population.

Initially, it was reasoned that these common variants would be located within the genes which contribute to the biology of cancer, such as those involved in cellular proliferation, apoptosis, replication and repair, immune response, signal transduction. Therefore, the majority of initial association studies were focusing only on a set of genes predicted to have potential biological roles in CLL aetiology. In a large-scale candidate–gene association study (1467 SNPs in 865 genes, 992 patients and 2707 controls) Rudd and colleagues found non-synonymous SNPs in the *ATM-BRCA2-CHEK2* DNA damage response axis to be in a significant association with risk of CLL (Rudd et al., 2006). Another large association study (used 768 SNPs from 172 genes, 692 patients, 738 controls) identified 6 previously unreported SNPs in the genes related to apoptosis and immunoregulation pathways, to be strongly associated with risk of developing CLL (Enjuanes et al., 2008). The authors suggested, that the different results between these two studies might have been related to the populations investigated (British versus Spanish) and that the findings require further validations in larger, possibly pooled data sets.

Since the biology of predisposition is still unknown, biased selection of loci for candidate gene-association studies carries a risk of missing the important variants in omitted or unknown genes while selecting the markers for genotyping. Genome-wide association

(GWA) studies overcome this problem by looking at the association across the entire genome without any biological assumptions. Three GWA studies were conducted recently and together identified 13 loci associated with the development of CLL (Table 1.1): 2q13, 2q37.1 (*SP140*), 2q37.3 (*FARP2*), 6p21.3 (*HLA-DRB5 and HLADQ1*-in familial CLLs only, HLA-A*0201) 6p25.3 (*IRF4*), 8q24.21, 11q24.1, 15q21.3, 15q23, 15q25.2 (*CPEB1*), 16q24.1 (*IRF8*), 18q21.1 (*CXXC1,MBD1*) and 19q13.32 (*PRKD2*) (Crowther-Swanepoel et al., 2010a; Crowther-Swanepoel et al., 2011; Di Bernardo et al., 2012b; Di Bernardo et al., 2008; Slager et al., 2011). Nine of these loci were verified in independent CLL and control cohorts (Crowther-Swanepoel et al., 2010b; Slager et al., 2010; Slager et al., 2011). Interestingly, 2 CLL susceptibility loci 2q37.1 and 6p21 were also associated with familial MBL risk. Unfortunately, it is not known whether the MBL individuals progressed to CLL (Slager et al., 2011).

Individually, these common alleles confer a small risk of about 1.3 or greater and as a group account for around 5% (up to around 10%) of genetic variation associated with heritability of CLL. Therefore, most of the genetic risk still remains unexplained (Di Bernardo et al., 2012a). However, GWA studies provide further insight into the biology of CLL. One of the most plausible genes linked with CLL risk is interferon regulatory factor 4 (*IRF4*), a regulator of lymphocyte maturation and proliferation. In normal B-lymphocytes expression of IRF4 is indicative of germinal centre origin. Lack of IRF4 expression in CLL cells has been shown to correlate with poor outcome and more advanced disease stage (Chang et al., 2002).

It is still possible that in a small proportion of cases genetic predisposition is conferred by rare deleterious variants rather than common SNPs. These would be difficult to detect by linkage or association studies. One of the possible approaches to identify these variants is a large-scale gene sequencing of constitutive material from CLL samples followed by the functional assays to establish the cellular consequences of germ-line changes. Another method is a case control study focusing on the frequency of germ-line pathogenic mutations within candidate genes known to be involved in the pathogenesis of blood malignancies.

Table1.1. Chromosome loci associated with the development of CLL.

Chromosome locus	Nearest gene(s)	Odds ratio
2q13*	ACOXL, BCL2L11	1.39
2q37.1 *	SP140, SP110	1.41
2q37.3 §	FIR	1.39
6p21.3# ^	HLA-DQA1, HLA-DRB5, HLA-A*0201	1.46-1.87 1.24
6p25. 3*	IRF4	1.54
8q24.21§	–	1.26
11q24.1 *	GRAMD1B	1.45
15q21.3 §	RFX7, NEDD4	1.36
15q23 *	–	1.37
15q25.2+	CPEB1	1.17
16q24.1 §	IRF8	1.22
18q21.1+	CXXC1, MBD1	1.16
19q13.32 *	PRKD2, STRN4	1.35

*(Di Bernardo, Crowther-Swanepoel et al. 2008)
§ (Crowther-Swanepoel, Broderick et al. 2010)
# (Slager, Rabe et al. 2011)
+ (Crowther-Swanepoel, Di Bernardo et al. 2011)
^ (Di Bernardo et al. 2012b)

Overall, there are no major clinical differences between familial and sporadic CLL cases. The younger age at diagnosis in familial cases was reported in one study (Ishibe et al., 2001) but not confirmed in another (Goldin et al., 2009). There is a higher proportion of females than males in familial CLL population and females with familial CLL have been reported to have a higher rate of secondary malignancies compared to females in sporadic cases. This indicates that susceptibility alleles might be associated with the gender and confirms a concept of pleiotropic nature of predisposing genetic variants (Mauro et al., 2006).

Notably, familial cases have been shown to have similar clinical prognosis as sporadic CLLs (Mauro et al., 2006). Therefore it appears that although inherited

predisposition to CLL plays a role in disease development, it is probably not involved in the mechanisms of progression.

1.3. Clinical and biological features of CLL- prognostic implications.

Because of the major clinical and biological heterogeneity in CLL, extensive effort has been undertaken in search of the features which would have a predictive value for the individual patients. Ideal prognostic factors used in the clinical setting for CLL would have three main purposes: 1) Predict the progression of the disease including time to first treatment, in other words, distinguish between the patients who are more likely to have indolent disease and no requirement for treatment from those who will have progressive course; 2) Influence the choice of the treatment by identifying patients with tumours harbouring unique clinical and biological features and assigning them to the therapy from which they would benefit the most; 3) Predict the response to the treatment, the period of treatment free survival and indirectly, overall survival.

The extensive search over the years for clinically useful prognostic factors resulted not only in improved predictions of the outcome but also in an improved understanding of the pathophysiology of CLL including the identification of critical signalling pathways in tumours cells. The advances in understanding the mechanisms of CLL progression are already leading to development of novel, improved and targeted treatments.

Prognostic factors could be classified as clinical or biological markers. Clinical prognostic markers are obtainable from physical examination, medical history investigation and basic laboratory tests. Markers which had been found to be associated with impaired outcome and are routinely used include: older age, male gender, advance stage of the disease, short lymphocyte doubling time. In addition, serum markers are relatively easy to obtain from patients' blood and some of them such as β2-microglibulin (β2M), thymidine kinase (TK) and soluble CD23 have been shown to have prognostic value.

Biological markers tend to utilize the molecular aspects of the CLL cells. Markers which have been well investigated and validated in multiple patient studies include:

immunoglobulin heavy chain variable gene (*IGHV*) mutation status and gene usage, expression of the cell surface marker CD38, the level of expression of Zeta associated protein 70 (Zap70) and the presence of cytogenetic abnormalities (Table 1.2). Furthermore, in recent years Next Generation Sequencing (NGS) approaches have identified a number of recurrently mutated genes in CLL, these include *NOTCH1* and *SF3B1* which have been demonstrated to have an impact on prognosis.

Table 1.2. Clinical and biological prognostic markers in CLL.

Prognostic marker	Better prognosis	Worse prognosis
age	younger	older
gender	female	male
disease stage	A	B/C
LDT	>6 months	<6 months
serum markers β2M TK CD23	low levels	high levels
IGHV status *IGHV* usage	mutated *VH4-34*	unmutated *VH3-21*
CD38	low expression	high expression
ZAP70	low expression	high expression
cytogenetics	13q (sole)	17p deletion 11q deletion 14q32 translocation

LDT- lymphocyte doubling time, β2M- β2-microglobulin, TK- thymidine kinase, *IGHV*- immunoglobulin heavy chain variable gene

1.3.1. Clinical prognostic markers.

1.3.1.1. Age and gender.

Both age and gender have been shown to predict CLL outcome. Sporadic CLL is not only twice as common in men than in it is in women but also disease in males progresses more rapidly and have worse response rates than in females (Catovsky et al., 1989; Molica et al., 2005; Oscier et al., 2010). The mechanisms behind these clinical differences are unknown.

The poor outcome associated with increasing age partly reflects co-morbidity rather than characteristics of the tumour cell population (Shanafelt et al., 2009b). Furthermore, age can play a role in the choice of the treatment as some elderly patients might not be fit enough to tolerate chemotherapy-related toxicity

1.3.1.2. Staging.

Two well validated and widely accepted staging systems are in clinical use: the Rai system (Rai et al., 1975) which is more commonly used in North America and the Binet system (Binet et al., 1981) more common in Europe. They both rely on physical examination and standard laboratory tests and stage patients according to lymph node or organ involvment and peripheral blood cytopenia. In the Binet staging system five potential sites of involvement are taken under consideration: cervical, axillary and inguinal lymph nodes as well as spleen and liver. Patients categorized as stage A have less than three areas involved, stage B patients have more than three lymphoid sites involved and stage C patients present with anaemia (haemoglobin <10g/dL) and/or thrombocytopenia (platelets $100x10^9$/L) regardless of the number of sites involved. Approximately 60% of patients will be stage A at diagnosis, 30% stage B and 10% stage C (Oscier et al., 2004). While assessing patients to stage C the basis of anaemia or thrombocytopenia (autoimmune or infiltrative) should be established as the knowledge about underlying cause influences the choice of the first treatment (Hallek et al., 2008; Oscier et al., 2004). Furthermore, a report by Moreno and colleagues shows that patients with cytopenia originating from defective autoimmune

mechanisms have significantly better survival than those in whom cytopenia was caused by extensive bone marrow infiltration (Moreno et al., 2010). Following The National Cancer Institute –sponsored Working Group (NCI-WG) criteria for treatment initiation, the majority of patients with stage C and B and a fraction of patients with stage A require immediate treatment. The NCI-WG recognizes anaemia and/or thrombocytopenia (infiltrative or autoimmune but not responsive to therapy) as indications for initiation of CLL treatment (Hallek et al., 2008).

It is important to note that staging systems identify patients eligible for treatment but cannot fully predict rate of progression for patients presenting with low tumour burden.

1.3.1.3. Lymphocyte doubling time.

Lymphocyte doubling time (LDT) has been shown to have prognostic value independent of age, sex, anaemia, thrombocytopenia and lymphocyte count (Molica and Alberti, 1987; Montserrat et al., 1986). It is most useful in patients presenting with early stage disease. NCI-WG criteria recognize LDT of less than 6 months as a sign of progressive lymphocytosis and an indication for treatment (assuming that other contributing factors such as infections are excluded) (Hallek et al., 2008).

CLL was initially believed to be a disease resulting from passively accumulating tumour cells due to faulty apoptotic mechanisms. This was supported by the fact that majority of circulating CLL cells are arrested in early G1 phase (Obermann et al., 2005) and overexpress anti-apoptotic proteins such as Bcl-2 (Pepper et al., 1997). However, newer findings revealed that the rapid increase in lymphocyte count might be also due to high proliferation rate of CLL cells and not only due to decrease in their death rate. The evidence for this is provided by the studies on telomeres length and experiments with deuterated water (D_2O).

Shortening of telomeres indicates cellular proliferation. Interestingly, CLL B-cells have the shortest telomeres ever reported in primary human tissue (Lin et al., 2010). Furthermore, erosion of the telomeres in CLL correlates with the advance disease stage and other poor

prognostic markers (Lin et al., 2010).

The kinetics of CLL cell division *in vivo* was measured by Messemer and colleagues in the experiment with deuterated "heavy" water. The authors evaluated the incorporation of non-radioactive deuterium (^2H) in the DNA of the newly generated cells (Messmer et al., 2005). They showed that the daily formation of the new tumour cells varied between 0.1-1.7% of the entire clone and that patients with higher clone birth rates were more likely to have symptomatic disease. Furthermore, some patients with stable white blood cell count (WBC) had high birth rates coupled with equally high death rates. The trend in the lymphocyte cell count is therefore determined by the balance between their birth and death. Furthermore, the authors speculated that rapid turnover of the CLL clone was sufficient to promote appearance of new lesions leading to development of a more aggressive tumour.

These discoveries have supported the two compartment CLL model in which CLL cells are produced in proliferative compartment (lymph node and bone marrow) and are subsequently released into the blood (accumulative compartment) as resting lymphocytes. The proliferation pools are called proliferation centres (PC) or pseudo-follicles. Malignant B cells in PC are positive for Ki-67 (cell proliferation marker), express high level of CD23 (regulator of proliferation and growth), co-express Survivin (inhibitor of apoptosis) and Bcl-2 (anti-apoptotic protein), and are negative for p27 (cycling kinase inhibitor) (Granziero et al., 2001; Lampert et al., 1999).

It became apparent that the PC microenvironment plays a major role in both the survival and progression of CLL by creating a niche for cell-cell interactions to promote pro-survival and anti-apoptotic signalling. This signalling is supported by chemokine receptors and adhesion molecules. Apart from proliferating CLL cells, the PC consists of activated T helper cells, stromal cells and follicular dendritic cells. Interaction of CLL cells with T-cells occurs through CD40 receptor/CD40 ligand signalling, and induces expression of Survivin in CLL cells (Granziero et al., 2001). Stromal cells constitutively express stoma derived factor-1α (SDF-1α/CXCL12) which engages with chemokine receptor CXCR4 on CLL cells and this interaction promotes survival. Nurse-like cells are stromal cells that are present in the PC of

secondary lymphoid organs and express B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) in addition to SDF-1α/CXCL12. Expression of APRIL by NLCs is believed to induce expression of NF-κB and anti-apoptotic protein MCL-1 (myeloid cell leukemia-1) in CLL cells (Nishio et al., 2005). Furthermore, interaction between CD38 on CLL cells and its ligand CD31 on NLCs also promotes homing and survival of CLL cells. In addition, up-regulation of anti-apoptotic protein MCL-1 in tumour cells is induced through interaction with follicular dendritic cells and involves the adhesion molecule CD44 which is expressed on CLL cells (Pedersen et al., 2002b). Another adhesion molecule expressed on CLL cells is the integrin CD49b (VLA-4), an important component of cell-cell and cell-matrix interaction in the lymph nodes and bone marrow which plays a role in CLL homing (Burger, 2011). Recent gene expression profile analysis of CLL cells collected from lymph node and bone marrow biopsies confirmed the activation of proliferation signalling mediated via B-cell receptor and NF-κB (Herishanu et al., 2011). Molecular interactions in CLL PC microenvironment are presented in (Figure 1.1.).

A high activity of proliferation centres manifests as an increase in peripheral lymphocyte count in some cases, although it could be more indicative of nodal involvement and hence, advance disease stage. It has been shown that patients with expanded proliferation centres and high proliferation rate have poor prognostic features and shorter survival (Ciccone et al., 2011; Gine et al., 2010).

1.3.1.4. Serum markers.

Serum markers such as β2-microglobulin (β2M), thymidine kinase (TK) and soluble CD23 have all been described to have independent prognostic value in CLL.

β2M is a component of human leucocyte antigen (HLA) and is present at low levels in serum in healthy individuals (Evrin and Wibell, 1972). Elevated level of β2M in serum of CLL patients is the result of an increased accumulation of malignant cells in blood, and therefore reflects high tumour burden (Simonsson et al., 1980). It has been associated with shorter time to first treatment, poor response to chemotherapy and chemoimmunotherapy as well as

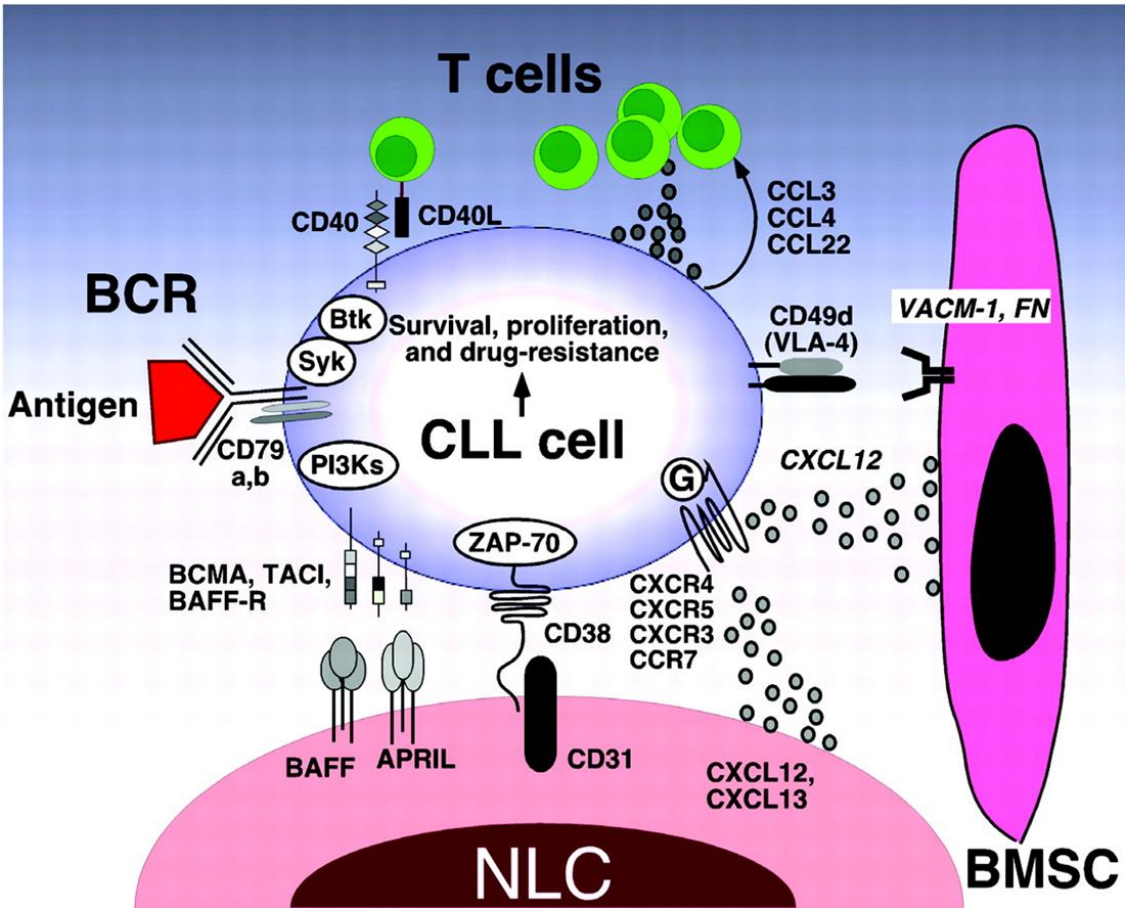


Figure 1.1. Microenvironment in CLL, from Burger, J.A. 2011. Hematology Am Soc Hematol Educ Program. 2011:96-103, see text for details.

shorter progression free survival (PFS) and shorter overall survival (OS) after those therapies (Gentile et al., 2009; Hallek et al., 2010; Oscier et al., 2010).

TK is a phosphotransferase and has essential functions during DNA synthesis. Therefore, high serum levels in CLL patients reflect increased proliferation of tumour cells. Increased TK levels has been shown to be independently associated with short time to progression (Hallek et al., 1999).

CD23 is a transmembrane glycoprotein with affinity for immunoglobulin E (IgE) and is involved in allergy and resistance to parasites. It is also produced by CLL cells and has been shown to be an independent prognostic factor for time to first treatment and overall survival in untreated Binet stage A patients (Meuleman et al., 2008)

In summary, clinical markers provide important prognostic information; can predict disease activity and progression. However, they rely mostly on tumour burden. The factors which have prediction value independent of tumour burden focus mostly on molecular aspects of CLL cells.

1.3.2. Biological prognostic markers.

1.3.2.1. *IGHV* mutation status and *IGH V-D-J* genes usage.

Two independent studies in 1999 reported the prognostic value of mutational status of immunoglobulin variable heavy chain gene (*IGHV*) in CLL patients (Damle et al., 1999; Hamblin et al., 1999). In both reports patients with mutated *IGHV* genes had significantly better survival than patients with unmutated *IGHV.* Mutated cases were defined as those exhibiting *IGHV* genes with less than 98% concordance with the most similar germ-line gene and unmutated cases were those with more than 98% concordance. The cut-off of 98% was chosen to exclude potential, unknown polymorphic variants within *IGHV* gene. Since 1999, unmutated *IGHV* status has been repeatedly confirmed to be associated with progressive disease, chemotherapy resistance and adverse cytogenetic features (Stilgenbauer et al., 2002). Furthermore, *IGHV* mutation status has a prognostic value independent of clinical staging and remains constant during the course of the disease (Chiorazzi and Ferrarini,

2003). Mutated and unmutated cases are represented in approximately equal percentages in CLL cohorts.

In normal B-cells, the presence of somatically mutated *IGHV* genes is a consequence of the antigen stimulation and leads to increased specificity of B-cell receptor (BCR) towards the antigenic epitope. This process is usually T-cell dependent and takes place in germinal centres. Therefore it seemed plausible to speculate that mutated and unmutated subtypes represent different CLL entities originating from the different precursor cells. According to this hypothesis tumours with mutated *IGHV* genes have arisen from the B-cells which have experienced antigen and have undergone a germinal centre reaction, whereas tumours with unmutated *IGHV* genes, would have developed from naïve, pre-germinal B-lymphocytes. This hypothesis explains clinical differences between two subtypes as consequences of distinct biological origins.

Interestingly, these assumptions were not confirmed by gene expression profiling which revealed that all CLL cases share a similar gene expression profiles independent on *IGHV* mutation status, thus indicating a common precursor cell (Klein et al., 2001; Rosenwald et al., 2001). In addition, all CLL cells have genome expression profile and cellular phenotype of antigen-experienced B cells. They have been related to marginal zone, memory B-cells or, as newer findings indicate, to mature peripheral blood CD5 positive B cells (Damle et al., 2002; Klein et al., 2001; Seifert et al., 2012).

Although the debate about the cell (or cells) of origin still continues (Ferrarini, 2009), these findings suggested that CLL is an antigen driven disease regardless whether cells undergo somatic hypermutation (SHM) in germinal centres (mutated *IGHV*), or respond to antigen in the T-cell independent manner (unmutated and mutated *IGHV*).

The discovery of the skewed *IGHV* usage by the proportion of CLL cases supports the notion of antigen stimulation. The most frequent *IGHV* genes include *VH1-69, VH3-21, and VH4-34* (Agathangelidis et al., 2012). In addition, they are often associated with particular *D* and *J* genes on immunoglobulin heavy (IGH) and light (IGL-lambda or IGK-kappa) chains and also with nucleotide-diversity sequences (N-regions) between those

genes created during the process of V-(D)-J recombination via exonuclease trimming and addition of non-template nucleotides. The junctional sequence (V-N1-D-N2-J) is called complementarity-determining region 3 (CDR3) and encodes the binding site for specific antigen. It has been reported that particular CDR3 regions are identical or highly homologous between different CLL clones in distant parts of the world and are specific or significantly more frequent in CLL cohorts than in general population and other B-cell malignancies (Bende et al., 2005; Stamatopoulos et al., 2007).

BCRs carrying one of those overrepresented CDR3 regions are called 'stereotyped' receptors (Messmer et al., 2004). In general, 'stereotypy' is about four times more frequent in unmutated than mutated cases although some stereotyped subsets are almost exclusively associated with only one mutation status (*VH1-69* is mostly found in unmutated cases whereas *VH4-34*, *VH3-23, VH3-7* is most frequent in mutated). Furthermore, particular gene usage like *VH3-21* and *VH4-34* have been shown to have prognostic value irrespective of *IGHV* mutation status with the former being correlated with aggressive disease and latter associated with indolent course (Stamatopoulos et al., 2007; Tobin et al., 2002). Some 30% of CLL cases belong to one of over 100 stereotyped receptors identified to date (Agathangelidis et al., 2012; Murray et al., 2008).

The presence of stereotyped receptors in such a high proportion of CLL cases world-wide strongly indicates the role of a limited set of structurally similar antigenic epitopes in the propagation of the leukemic clone. The nature of the antigens recognized by stereotyped receptors is largely unknown. However, there is some evidence to suggest that they might include auto-antigens on apoptotic cells and/or microbial pathogens (Catera et al., 2008; Chu et al., 2008). It is highly possible that some of the CLL clones could develop from normal counterparts of B cells, upon specific antigenic stimulation during chronic or recurrent infections (Forconi et al., 2010).

Alternative explanation for the existence of stereotyped CDR3 sequences has been recently provided by Dühren-von Minden and colleagues (Duhren-von Minden et al., 2012). Using an *in vitro* model system with engineered BCR receptors, the authors suggested that

activation of BCR pro-survival pathways is the result of the interaction between intrinsic epitopes and HCDR3 on the nearby receptor of the same cell rather than due to extrinsic antigen stimulation and that only certain HCDR3 are capable of binding to those epitopes. Interestingly, the authors did not observe any significant differences in ligand-independent cell-autonomous signalling between *IGHV* mutated and unmutated cases. This is in contrast to previous studies reporting increased BCR signalling in unmutated cases when compared to mutated cases (Guarini et al., 2008; Lanham et al., 2003; Mockridge et al., 2007; Muzio et al., 2008). However, the authors did not rule out the possibility that basal activity of certain CLL BCRs (due to autonomous signalling) can be enhanced by extrinsic antigen stimulation. Therefore, these two hypotheses antigen-dependent and antigen-independent signalling are not mutually exclusive. The differential outcome in patients according to *IGHV* mutation status might result from different affinity to antigen and/or an increased sensitivity to intrinsic epitope.

Interestingly, cell-autonomous signalling seems to be independent of the poly-reactivity of the receptor. Poly-reactive BCRs are more often observed in tumour cells with unmutated *IGHV* gene rather than in *IGHV* mutated tumours which rarely express poly-reactive antibodies (Herve et al., 2005). Therefore, the stimulation of BCR pathways, if it occurs via binding with antigen, is more easily achieved by unmutated *IGHV* receptors. If coupled with enhanced cell-autonomous signalling, it might lead to increased proliferation in *IGHV* unmutated CLL clones promoting acquisition of additional genetic lesions and translating to more progressive clinical course. Indeed, CLL cells with unmutated *IGHV* have shorter telomeres which indicate increased proliferative history (Damle et al., 2004) and are more frequently associated with poor-prognosis genomic aberrations than mutated CLLs (Krober et al., 2002).

The mutation status of the *IGHV* gene remains the ‘gold standard’ among biological prognostic markers although it is not always reliable in predicting outcome for individual patients. This is partially because of misclassification of the subset of patients whose SHM level is at the 98% cut-off point (borderline cases). In addition, clinical predictions based on

the level of SHM may be 'interfered' by the inclusion of cases with the particular antigen-binding sites (stereotyped CDR3s) for which association with disease outcome is independent on *IGHV* mutation status.

1.3.2.2. CD38 expression.

CD38 is a transmembrane glycoprotein with enzyme and receptor capabilities. It is expressed on B-cell precursors, down-regulated in mature, resting lymphocytes and re-expressed by activated B-cells (Malavasi et al., 2011). In CLL, high expression of CD38 was initially found to correlate with unmutated *IGHV* status (Damle et al., 1999). Due to this correlation and feasibility of routine flow cytometry analysis, CD38 was proposed to be a surrogate marker for *IGHV* status.

A minimum of 30% of CLL cells expressing CD38 has been used as a cut-off for positivity in most studies, however lower thresholds such as 20% (Ibrahim et al., 2001) and 7% (Krober et al., 2002) have also been applied. Although discordance between *IGHV* mutation status and CD38 has been observed, expression of CD38 has been confirmed to be associated with shorter time to treatment, poor response to therapy and shorter PFS (Hamblin et al., 2002; Ibrahim et al., 2001; Jelinek et al., 2001). Several studies reported fluctuation in the levels of CD38 during the course of disease raising the suspicion that CD38 might be an unreliable marker. However, this fluctuation applies only to a very small proportion of cases and does not appear to modify the prognostic predictions made at diagnosis (Ghia et al., 2004).

Association of CD38 with progressive disease can be explained by the role this molecule plays in the CLL malignancy. It has been reported that its expression is transient and marks the proliferating fraction of CLL clones with CD38 positive cells having greater capacity for signalling through BCR receptor (Damle et al., 2007; Deaglio et al., 2003) (Calissano et al., 2009; Cutrona et al., 2008). Subsequent studies showed that although CD38 and BCR most probably act in independent pathways, the enhanced signalling which promotes proliferation requires co-localization of CD38 and BCR/CD19 complex in the cell

surface membrane's micro-domains regions termed lipid rafts (Deaglio et al., 2007b). Immunohistochemistry, gene expression profiling and *in vitro* studies provided evidence that binding between CD38 and its ligand CD31, which is expressed on the NLC, promotes CLL cell survival, proliferation and migration *in vivo* (Deaglio et al., 2010; Deaglio et al., 2005; Patten et al., 2008).

These data suggest that antigenic stimulation through the BCR that takes place in lymphoid tissues is supported by molecules such as CD38 which facilitate 'bridging' between the CLL cell and its microenvironment. Therefore, CD38 is not only a marker of activated and dividing CLL cells but also a vital member of the microenvironmental network which supports CLL clone maintenance and progression.

Finally, another report shows that CD38 ligation with CD31, promotes phosphorylation of ZAP-70, another adverse prognostic marker in CLL, hence providing a functional link between the two (Deaglio et al., 2007a).

1.3.2.3. ZAP-70 expression.

Expression of Zeta-associated protein 70 kDa (ZAP-70), intracellular protein kinase in CLL cells was originally discovered by gene expression profiling analysis in which it correlated with unmutated *IGHV* status (Rosenwald et al., 2001). As later studies reported, this correlation is not always absolute, however ZAP-70 was shown to be a strong independent prognostic marker associated with shorter time to treatment and poorer outcome (Del Principe et al., 2006; Rassenti et al., 2004; Wiestner et al., 2003). The level of ZAP-70 expression appears to be stable over time and the threshold of 20% of B cells is used in most studies, if measured by flow cytometry assays (Durig et al., 2003; Rassenti et al., 2004). The standardized flow cytometry method for ZAP-70 expression has been recently determined, hence allowing for the multi-centre assessment. The protocol is available on European Research Initiative on CLL (ERIC) website: http://www.ericll.org/projects/ZAP70_CD38_harmonization_cymbalista.php).

The association of ZAP-70 expression and inferior prognosis in CLL may be related

to its role in BCR signalling pathway. Although the exact molecular mechanisms of this interaction remain unknown, it has been shown that expression of ZAP-70 enhances BCR signalling resulting in induction of an anti-apoptotic phenotype and hence poorer clinical outcome (Chen et al., 2005a).

As mentioned before, phosphorylation of ZAP-70 upon CD38 stimulation has also been reported (Deaglio et al., 2007a). Moreover, the study has shown that CD38 signalling is dependent on ZAP-70 expression and that cells expressing both CD38 and ZAP-70 exhibit enhanced migratory potential towards SDF-1α/CXCL12 and are more often associated with *IGHV* unmutated status.

These findings support clinical observation and suggest that combined *IGHV* mutation status with ZAP-70 and/or CD38 assessment might provide more accurate identification of patients with high-risk CLL (D'Arena et al., 2007; Morabito et al., 2009). Evaluation of these three prognostic markers is currently obligatory at the entry of the clinical trials but not in the general practise (Hallek et al., 2008).

1.3.2.4. Cytogenetic and genetic abnormalities.

Genetic abnormalities are present in over 80% of CLL cases. They have prognostic value and can be present at the diagnosis or acquired at the later stage of disease contributing to clonal evolution which correlates with progressive disease (Stilgenbauer et al., 2002).

In 2000 Döhner and colleagues proposed the hierarchical cytogenetic classification of CLL, based on the presence of cytogenetic abnormalities and its correlation with survival (Dohner et al., 2000). Five cytogenetic groups were defined including the most frequent changes, such as deletions at chromosome 13q (as sole abnormality), 11q, 17p, trisomy 12q and normal karyotype (Figure 1.2.). Other recurrent cytogenetic changes are deletion at chromosome 6q (Stilgenbauer et al., 1999) and translocation of chromosome 14q32 involving the immunoglobulin gene locus (Cavazzini et al., 2008).

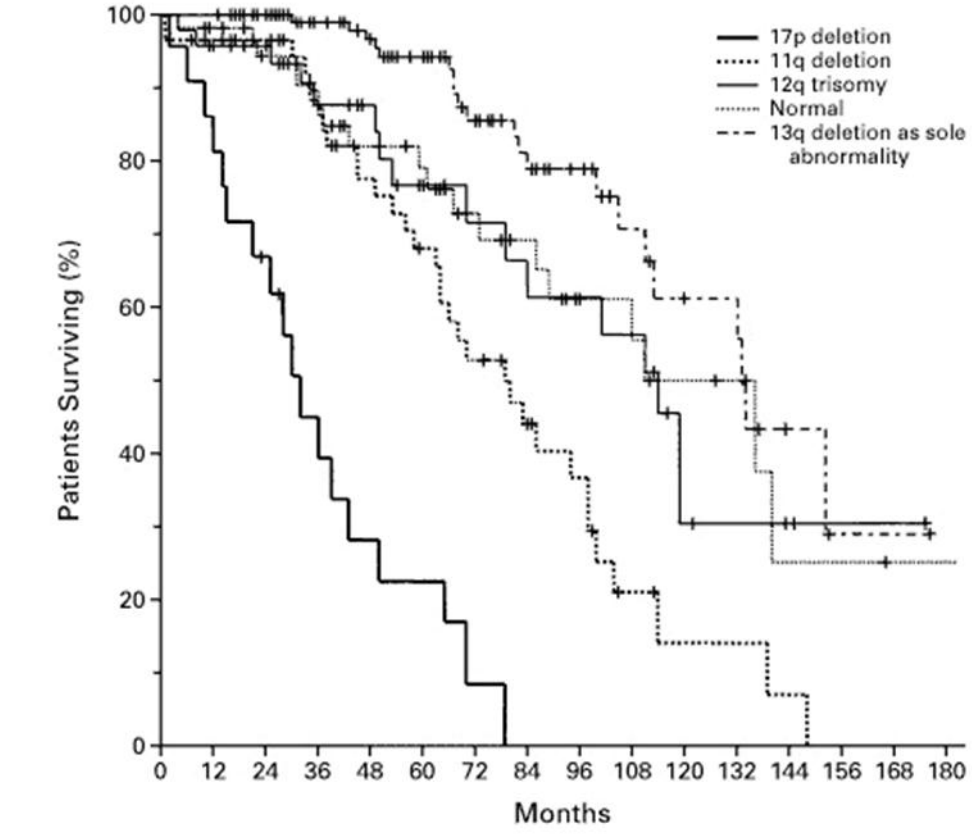


Figure 1.2. The hierarchical cytogenetic classification of CLL.

(from Döhner et al. N Engl J Med 2000; 343:1910-1916)

Patients with a deletion of chromosome 13q as a sole abnormality have been shown to have better outcome compared to patients with other aberrations or normal karyotype. The shortest survival has been seen in patients with a 17p deletion. Patients with an 11q deletion have a survival that is intermediate between those with a 17p deletion and those with a normal karyotype. The outcome of patients with trisomy 12q does not differ from those who have normal karyotype.

Interphase fluorescence in situ hybridization (iFISH) is a current standard procedure which allows detection of specific genetic abnormalities with the probes against the chromosomal regions of interests. Detection of chromosome aberrations is currently obligatory for clinical trial entry, but apart from the screen for the presence of a *TP53* abnormality, not recommended in general practice (Oscier et al., 2012).

Deletion of 13q14 as a sole abnormality is detected in approximately 36% of CLL patients and is associated with better outcome (median survival of 133 months) as compared to other aberrations or to normal karyotype (median survival 111 months) (Dohner et al., 2000). A minimal deleted region (MDR) at chromosome 13q14 contains two microRNAs (*miRs*): *miR-15a* and *miR-16-1* expressed as a cluster (Calin et al., 2002). *MiRs* are the small non-coding molecules which have the capacity to repress the expression of multiple genes through messenger RNA (mRNA) degradation or to prevent mRNA from being translated. A recent mouse model with targeted deletion of *miR15a/16-1* recapitulated features of indolent CLL disease and suggested the role of these *miRs* in regulating the expression of genes involved in proliferation (cyclins - *CCND1, CCND2,CCND3, CCNE1* and cyclin dependent kinases and phosphatases -*CDK4, CDK6, CHK1, MCM5 ,CDC25A*) and possibly apoptosis (*BCL-2*) (Klein et al., 2010).

If cases with various abnormalities are considered, the chromosome 13q14 deletion is present in about 50% of CLL cohorts (Dohner et al., 2000). The similar frequency (48%) is observed in MBL individuals (Rawstron et al., 2008). This implies a role for this abnormality in early clonal evolution by promoting acquisition of additional genetic changes (Klein et al., 2010).

Trisomy 12q and deletion of chromosome 6q are present in approximately 16% and 7% of patients, respectively. No inferior overall survival has been related to these abnormalities. However, it has been reported that early stage CLL patients with trisomy 12 have shorter time to progression (Dohner et al., 2000; Zenz et al., 2007) and that deletion of chromosome 6q is associated with higher white blood cell count and more extensive lymphadenopathy at diagnosis (Stilgenbauer et al., 1999). The molecular consequences of

these abnormalities remain unknown.

Translocation of chromosome 14q32 involving the immunoglobulin gene locus is detected in approximately 7% of CLL patients (as a sole abnormality or in combination with 13q14 deletion) and is associated with inferior treatment free survival and overall survival, comparable to those patients who had unfavourable cytogenetic abnormalities, such as deletions of chromosome 11q and 17p (Cavazzini et al., 2008). Partner chromosomal translocations include genes such as *BCL2* (18q21), *BCL11A* (2p12), *CCND3* (6p21), and *CDK6* (7q21). In a recent report, 14q32/IGH translocation has been shown to be one of the most frequent aberrations acquired during the natural history of CLL (Cavazzini et al., 2012).

The frequency of deletion of chromosome 17p is around 7% across all stages with the median overall survival estimated in one of studies at 32 months (Dohner et al., 2000). This aberration has been associated with inferior survival, shorter PFS, advanced stage of disease and unmutated *IGHV* status. It also acts as an independent negative prognostic factor (Dohner et al., 2000; Krober et al., 2002; Oscier et al., 2002).

Deletion at chromosome 17p13 always results in the loss of one allele of *TP53*. This gene encodes a transcription factor that is involved in cell cycle arrest and apoptosis. Since many cytotoxic treatments utilize p53-dependent apoptotic pathway to kill the malignant cells, tumours with this abnormality are usually resistant to these therapies and their prevalence is increased in chemo-refractory cohorts (Stilgenbauer, 2006; Catovsky et al., 2007; Sturm et al., 2003).

Deletion of 17p13 is frequently accompanied with the inactivating mutation in *TP53* gene on the remaining allele (80-90%), hence causing a complete loss of a normal p53 protein. *TP53* mutations can be also present in some 5% of cases without 17p13 deletion (Zenz et al., 2008). Nevertheless, loss of at least one functional *TP53* allele confers poor outcome (17p deletion only and *TP53* mutation only) and chemo-refractoriness (*TP53* mutation only) comparable with a bi-allelic defect (Rossi et al., 2009; Zenz et al., 2008).

The clinical observation is supported by *in vitro* studies showing that CLL cells with either 17p13 deletions and/or *TP53* mutations are defective in activating pro-apoptotic

responses upon treatment with DNA damage inducing agents such as gamma irradiation and anticancer chemotherapeutic drugs (chlorambucil and fludarabine) (Stankovic et al., 2004; Sturm et al., 2003).

Under normal physiological conditions p53 protein is present as a tetramer. Therefore, the poor clinical outcome in patients with *TP53* mutation but without 17p deletion could possibly result from a dominant negative effect that these mutations may have on interactions with wild-type monomers and the stability of binding with DNA. This gain-of-function mechanism might be also explained by the ability of p53 mutant to bind and inactivate the homologue proteins such as p63 and p73, hence altering their downstream signalling (Oren and Roer, 2011).

Chromosome 17p13 deletion is affecting induction treatment approaches whereby patients with this abnormality are offered chemotherapy-independent options and these include often administration of monoclonal antibodies that bind to epitopes expressed on the surface of CLL cells with or without steroids (Lozanski et al., 2004; Pettitt et al., 2012) or allogeneic stem-cell transplantation for fitter patients (Oscier et al., 2012).

In recent years NGS technology has been used to identify other recurrently mutated genes in CLL aside from *TP53* (Puente et al., 2011; Quesada et al., 2012; Wang et al., 2011). Some of these genes such as *NOTCH1* and *SF3B1* have been demonstrated to impact on prognosis.

NOTCH1 encodes a ligand-dependent transcription factor that regulates transcription of genes such as *MYC*. Almost all detected *NOTCH1* mutations are predicted to affect a functional PEST domain involved in protein degradation of NOTCH1. *NOTCH1* mutations are present in approximately 10% of patients at diagnosis, in 20% tumours refractory to chemotherapy, and in 30% of patients with transformed aggressive clinical phase. They are associated with aggressive disease, unmutated *IGHV* status, trisomy 12, high level of CD38/ZAP70 expression, shorter time to treatment and inferior survival (Fabbri et al., 2011; Mansouri et al., 2013; Oscier et al., 2013; Puente et al., 2011; Rossi et al., 2012b)

SF3B1 is a critical component of spliceosome (Cazzola et al., 2013). It is mutated in

4-17% of CLL tumours and is associated with progressive and fludarabine-refractory disease, high CD38 expression and shorter overall survival (Mansouri et al., 2013; Oscier et al., 2013; Quesada et al., 2012; Rossi et al., 2011).

1.3.2.4.1. Chromosome 11q deletion – mono-allelic loss of *ATM* gene.

Chromosome 11q22 deletion is detected in approximately 18% of all CLL patients at diagnosis and it has been observed that these patients have the median survival of approximately 79 months that is intermediate between those with normal karyotype and those with 17p13 deletion (Dohner et al., 2000).

The characteristic features of tumours with 11q deletion are marked lymphadenopathy and progressive disease especially in younger patients (Dickinson et al., 2006; Dohner et al., 1997; Fegan et al., 1995; Neilson et al., 1997). Chromosome 11q deletion is more frequent among patients with advanced stage and chemorefractory disease (Stilgenbauer, 2006). Chromosome 11q deletion has been associated with increased genomic complexity (Ouillette et al., 2010), *IGHV* unmutated status (Krober et al., 2002; Oscier et al., 2002; Trbusek et al., 2006) and ZAP-70 expression (Dickinson et al., 2006). It can also act as an independent negative prognostic factor for time to first treatment (Wierda et al., 2011) and PFS after chemotherapy with alkylating agents and purine analogues (Grever et al., 2007; Oscier et al., 2010).

Interestingly, the size of an 11q deletion clone also seems to have prognostic impact. Marasca and colleagues showed that the presence of 11q deleted clone larger than 25% shortens the time to treatment, whereas the presence of a small clone with less than 25% of cells with 11q deletion does not have the same influence (Marasca et al., 2012)

In the majority of 11q deleted cases the deleted region is longer than 20 megabase (Mb) and includes hundreds of genes (Gunn et al., 2009; Gunnarsson et al., 2011). MDR on chromosome 11 might confine to only 2-3 megabase (Stilgenbauer et al., 1996) or according to newer reports even less than that, and almost always encompasses *ATM* gene which encodes ATM protein kinase, one of the main mediators of DNA damage responses

(Gardiner et al., 2012; Gunnarsson et al., 2011). Therefore, *ATM* specific FISH probes are commonly used to detect 11q deletion in CLL patients.

The residual *ATM* allele can be mutated in up to 36% of all cases with 11q deletion and *ATM* mutations can also occur in a smaller proportion of CLL tumours without 11q deletion (Austen et al., 2005; Austen et al., 2007; Bullrich et al., 1999; Navrkalova et al., 2013; Stankovic et al., 2002b). Notably, bi-allelic defects of the *ATM* locus have been reported to confer a worse prognosis than sole 11q deletion therefore promoting the *ATM* gene as a key target in 11q deletion (Austen et al., 2007).

However, because *ATM* mutations do not overlap in 100% with 11q deletion, various studies are aiming to provide the alternative or additional explanation for a cause of clonal selection in this population of patients. For example, 11q deletion status has been linked with elevated expression of insulin receptor which potentially can promote anti-apoptotic and pro-growth stimuli (Saiya-Cork et al., 2011). Secondly, combined defects of multiple genes (as opposed to one target) located at the commonly deleted region of 11q could also provide proliferative advantage and confer poor outcome (Ouillette et al., 2010). Finally, mutations and/or deletions of *BIRC3* gene located on chromosome 11q have been recently reported in a proportion of patients resistant to fludarabine treatment (Rossi et al., 2012a) and associated with poor overall survival independently of chromosome 11q deletion (Rossi et al., 2013).

Nevertheless, apart from *ATM* no other genes located in MDR have been reported to be mutated or linked to poor outcome in the group of patients with chromosome 11q deletion.

1.4. ATM

ATM is a 350kDa protein kinase comprised of 3056 amino acids. It is encoded by the Ataxia Telangiectasia Mutated gene (*ATM)* located on chromosome 11q22-23 and extending over 150kb of genomic DNA with an open reading frame of 9.138 kb. Its genomic sequence contains 62 coding exons and the translational start codon- methionine is located in exon 4.

The ATM protein activates multiple downstream targets and exerts various functions

in regulating cellular processes mainly in the response to DNA double strand breaks. Inheritance of bi-allelic mutations of *ATM* gene leads to disorder named ataxia telangiectasia (AT) which is, among other features, associated with an increased predisposition to lymphoid tumours (Taylor et al., 1996).

1.4.1. Functional domains of the ATM protein.

The ATM protein is a member of phosphoinositide 3-kinase-like kinases (PIKK) family and shares sequence homology with other members of this group that includes DNA protein kinase catalytic sub-unit (DNA-PKcs), Ataxia Telangiectasia and Rad3 related protein (ATR), mammalian target of rapamycin (mTOR/FRAP), suppressor of morphogenesis in genitalia-1 (SMG-1) and the Transactivation-transformation domain associated protein (TRRAP) which retains PI3K homology but lacks kinase activity. The characteristic feature of this family of proteins is their ability to phosphorylate target proteins on serine and threonine residues followed by glutamine, which are recognised as SQ (serine/glutamine) or TQ (threonine/glutamine) motifs (Shiloh Y, 2003). PIKK family proteins share three functional domains: phosphoinositide 3-kinase catalytic domain- PI3K; FAT domain named after FRAP, ATM and TRRAP; and FAT C-terminal domain (shown in Figure 1.3.).

Within the translated sequence of the ATM protein, between amino acid residues 2715 to 3011, a PI3K catalytic domain is encoded. It includes ATP binding site and catalytic site and is highly conserved between species and between related family members (Lavin et al., 2004). The FAT domain is located between the residues 1966 to 2566. At the distant C terminal region of the protein a FATC domain is located extending from residues 3034 to 3056. FAT and FATC domains contain several conserved residues and occur only in combination, hence it has been suggested that they might fold together in a configuration which facilitates proper function of the kinase domain (Bosotti et al., 2000). In addition, one of the ATM autophosphorylation sites serine 1981 is located within the FAT domain. The FATC domain contains an acetylation site (lysine 3016) for the histone acetyltransferase Tip60. It has been reported that acetylation of this residue following DNA damage is an important

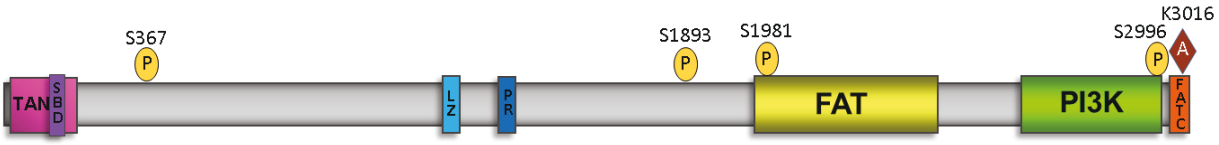


Figure 1.3. Schematic representation of the ATM protein.

Distribution of functional domains, auto-phosphorylation sites (P) and acetylation site (A) are shown. TAN- Tel-1/ATM N-terminal, SBD-substrate binding domain; LZ-leucine zipper; PR-proline rich motif; FAT-FRAP, ATM, TRRAP; PI3K -kinase domain; FATC-FAT Carboxy-terminal domain.

event in the activation of ATM protein (Sun et al., 2007).

The N-terminal region of the ATM protein has been shown to be important in regulating the response to DNA damage by mediating interaction with substrate proteins such as p53, BRCA1, LKB1, BLM and NBS1 (Beamish et al., 2002; Falck et al., 2005; Fernandes et al., 2005). Moreover, it is required for ATM nuclear localization and chromatin association (Young et al., 2005). The N-terminal region also contains TAN domain (Tel-1/ATM N-terminal). The TAN domain has been reported in a study on the ATM ortholog in Yeast - Tel-1 (Seidel et al., 2008). It is located within the first 30 amino-acids of *ATM/Tel-1* genes, contains a consensus motif of 13 amino acids and has been shown to be essential for maintenance of the telomere length and DNA damage response in yeast (Seidel et al., 2008). Evolutionary conservation of the TAN motif sequence indicates that it also has important functions in the human ATM protein.

Another two domains have been identified towards the middle of the ATM protein. Firstly, the amino acid sequence located between residues 1373-1382 and described as a proline rich region which mediates the constitutive binding between ATM and c-Abl tyrosine kinase, a repair protein (Shafman et al., 1997). Secondly, an incomplete leucine zipper from residues 1217 to 1238, which theoretically could facilitate protein-protein interactions or dimer formation but neither of these functions has been identified (Chen et al., 2003).

Finally, numerous HEAT (*H*untington, *E*longation factor 3, *A* subunit of protein phosphatase 2A, *T*OR1) repeats have been identified upstream of the PI3K catalytic domain. HEAT motifs are predicted to allow the ATM protein to fold into superhelical conformation creating two structural regions which facilitate association with double-stranded DNA and interaction with other proteins (Llorca et al., 2003).

1.4.2. Role of ATM in DNA damage response (DDR).

ATM regulates the response to DNA double strand breaks (DSB) through triggering signalling which synchronises DNA repair, cell-cycle arrest and apoptosis.

DSBs are normally created during meiotic recombination and VDJ gene

recombination in B and T-cells. They can be also caused by exposure of the genome to free radicals, ionizing irradiation (IR) and some chemotherapeutic agents. Moreover, they can arise from two single strand breaks during replication. DSBs are the most dangerous types of DNA damage which, if unrepaired, can contribute to cell death or chromosomal translocations and carcinogenesis (Jeggo and Lobrich, 2007).

There are two main mechanisms involved in the repair of DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is a DSB repair mechanism during which two broken strands are joined in a rapid but error-prone manner with no requirement for the sequence homology. Studies on DDR induced by IR suggest that NHEJ is a major DSB repair mechanism in mammalian cells throughout the whole cell cycle (it repairs ~85% of DSBs). Therefore, only around 15% of DSBs are predicted to be repaired exclusively by HR (Beucher et al., 2009; Goodarzi et al., 2008). HR is a high-fidelity process based on the recombination between sister chromatids. Since they exist together in the cell only after DNA replication, HR repair is restricted to S and G2 stages of the cell cycle (Jeggo and Lobrich, 2007). The main activator of downstream signalling pathways during NHEJ repair is DNA-PKcs, whereas HR is mediated mainly by ATM and ATR (Jazayeri et al., 2006).

Importantly, repair of IR-induced DSBs localized in a highly condensed heterochromatin requires functional ATM protein and is characterized by slow kinetics (Goodarzi et al., 2008; Riballo et al., 2004). Therefore, ATM is involved in G1 phase NHEJ repair if it takes place in heterochromatin by enabling the NHEJ proteins to reach and resolve the damage and is always present during G2-phase HR repair of this highly condensed regions (Beucher et al., 2009). Furthermore, ATM is an important component of HR-directed repair during replication in S phase (Kocher et al., 2012).

Overall, the lack of functional ATM results in sustained unrepaired lesions in G1, S and G2 phases of the cell cycle (Goodarzi et al., 2010; Kocher et al., 2012). When coupled with defective checkpoint activation, these lesions are passed on to daughter cells contributing to genomic instability and tumorigenesis (Bartkova et al., 2005).

Existing compensation mechanisms such as subsets of common downstream targets allow for a certain degree of redundancy between all PI3 kinases involved in DSB responses (Tomimatsu et al., 2009). However, they do not sufficiently prevent the development of defective cellular phenotypes associated with the inherited defects of each of these important proteins. Loss of fully functional ATM or DNA-PK leads to development of AT or SCID (severe combined immunodeficiency) syndromes respectively, which are characterized by increased radiosensitivity and immunodeficiency (Savitsky et al., 1995; van der Burg et al., 2009). ATR is essential for the early embryonic development and its disruption is lethal in mice and most probably in humans because no human disease has been linked with a complete ATR loss, whereas inheritance of bi-allelic splice site mutation causes Seckel disorder - microcephalic dwarfism (Brown and Baltimore, 2000; O'Driscoll et al., 2003).

1.4.2.1. Activation of ATM dependent DNA damage responses (DDR).

The main cellular localization of ATM is in the nucleus from where it can quickly respond to DNA damage. In undamaged cells ATM forms inactive dimers or even higher-order multimers (Bakkenist and Kastan, 2003). Young and co-workers proposed a model in which, inactive ATM dimer remains associated with chromatin until the occurrence of DSBs which changes the DNA helix conformation and promotes ATM activation (Young et al., 2005). The exact activation mechanisms appear to be very complex and not fully understood yet, and Young's model is continuously being updated (Bhatti et al., 2011).

It is believed that auto-phosphorylation on serine 1981 (and possibly serines 367, 1893 and 2996) is required for ATM activation and leads to dimer dissociation and initiation of the ATM cellular kinase activity (Bakkenist and Kastan, 2003; Kozlov et al., 2011). This event is proceeded by acetylation of lysine 3016 by Tip60 (Sun et al., 2007). Moreover, there is some evidence suggesting that protein phosphatases might participate in the activation of ATM. The example is PP2A (protein phosphatase 2A) which has been shown to be constitutively associated with ATM by maintaining its basal state until DNA damage abrogates this interactions and leads to ATM auto-phosphorylation (Goodarzi et al., 2004).

An important event in the initiation of the ATM-mediated DDR in heterochromatin is phosphorylation of the KAP-1 (KRAB associated protein-1). This protein is constitutively bound with heterochromatin, and its phosphorylation causes chromatin relaxation and enables the sequential assembly of the members of the response machinery to the sites of DNA damage (Goodarzi et al., 2008).

Recruitment of ATM to DNA DSBs sites is facilitated by NBS-1 protein (Nijmegen breakage syndrome 1) through interaction with its C-terminal domain (Falck et al., 2005).

NBS-1 protein is a part of the MRN complex (Mre11, Rad50 and NBS-1) which acts both upstream and downstream of ATM protein. MRN senses DSBs and binds to the broken ends of DNA via Mre11 and Rad50, while NBS1 initially acts as an anchor for ATM (Lee and Paull, 2005; Paull and Lee, 2005). Formation of DNA-MRN-ATM complex increases the affinity of ATM for its substrates, amplifies downstream signalling and facilitates retention of ATM at the DSB sites.

An important event in the activation of the DDR is phosphorylation of histone H2AX on serine 139 by ATM and by other members of PI3K family. Phosphorylated H2AX, termed γH2AX, localizes at the sites of DSBs and can be visualized as nuclear foci which spread along the chromatin over several megabases away from the breaks (Pilch et al., 2003). γH2AX serves as an assembly platform for DNA damage repair complexes such as MRN and BRCA1. One of the proteins which recognize γH2AX is MDC1 (mediator of DNA-damage checkpoint protein-1). MDC1 is involved in binding to NBS1 hence attracting the MRN complex to DNA DSBs and, through this, the recruitment of ATM. The mediating role of MDC1 at the sites of DNA DSBs is dependent on constitutive phosphorylation by CK2 (casein kinase 2) (Spycher et al., 2008). 53BP1 (p53 binding protein-1) is another protein which has been shown to co-localize with γH2AX, MDC1 and MRN complex after induction of DNA DSBs and, similar to MDC1, serves as a co-activator of ATM signalling (Lee et al., 2010).

Therefore, the current model of ATM-dependent activation in response to DNA DSB suggests a positive feedback loop: DNA DSBs cause chromatin alteration leading to post-

translational modification of ATM, auto-activation and phosphorylation of H2AX (and KAP-1 if DSBs are located in heterochromatin), this results in the assembly of the MRN complex which is required for the recruitment of ATM to the breaks and downstream signalling including sustained phosphorylation of H2AX and auto-phosphorylation. This feedback loop remains active until the completion of the repair (Kinner et al., 2008).

Activation of the ATM following DNA damage leads to phosphorylation of substrates which are involved in cell-cycle arrest, DNA repair or if the damage is too severe in the transduction of apoptotic signals. These responses are tightly regulated and several proteins activated by ATM proteins exert their functions in more than one cellular pathway. The spectrum of ATM targets is shown in Figure 1.4.

1.4.2.2. ATM and cell-cycle arrest.

By interacting with multiple cell cycle-regulators, ATM can induce cell arrest in G1, S or G2/M checkpoints. Regulating the activation of p53 protein, one of its main downstream targets, can lead to G1/S phase arrest. P53 is activated and stabilized by ATM through several mechanisms. Firstly, it is directly phosphorylated on serine 15, which subsequently facilitates its ability to induce expression of $p21^{Cip1/Waf1}$protein, an inhibitor of the CDK2 (cycle dependent kinase 2). Secondly, ATM phosphorylates a negative regulator of p53, MDM2 (mouse double minute2), on serine 395 marking it for degradation. Finally, it phosphorylates checkpoint protein kinase Chk2 on threonine 68 which phosphorylates p53 on serine 20 and contributes to its stabilization. Chk2 phosphorylation also contributes to Cdc25 inactivation and G1-S, intra-S or G2 arrest (Bartek and Lukas, 2003).

Moreover, phosphorylation of NBS1 on serine 343 and serine 278 inhibits transition through S-phase either directly or via facilitating the ATM-dependent phosphorylation of other checkpoint proteins such as SMC1 (structural maintenance of chromosomes-1, serines 966 and 957), Chk2 (threonine 68) and FANCD2 (Fanconi anaemia complementation group D2, serine 222) (Buscemi et al., 2001; Falck et al., 2002; Taniguchi et al., 2002; Yazdi et al., 2002),

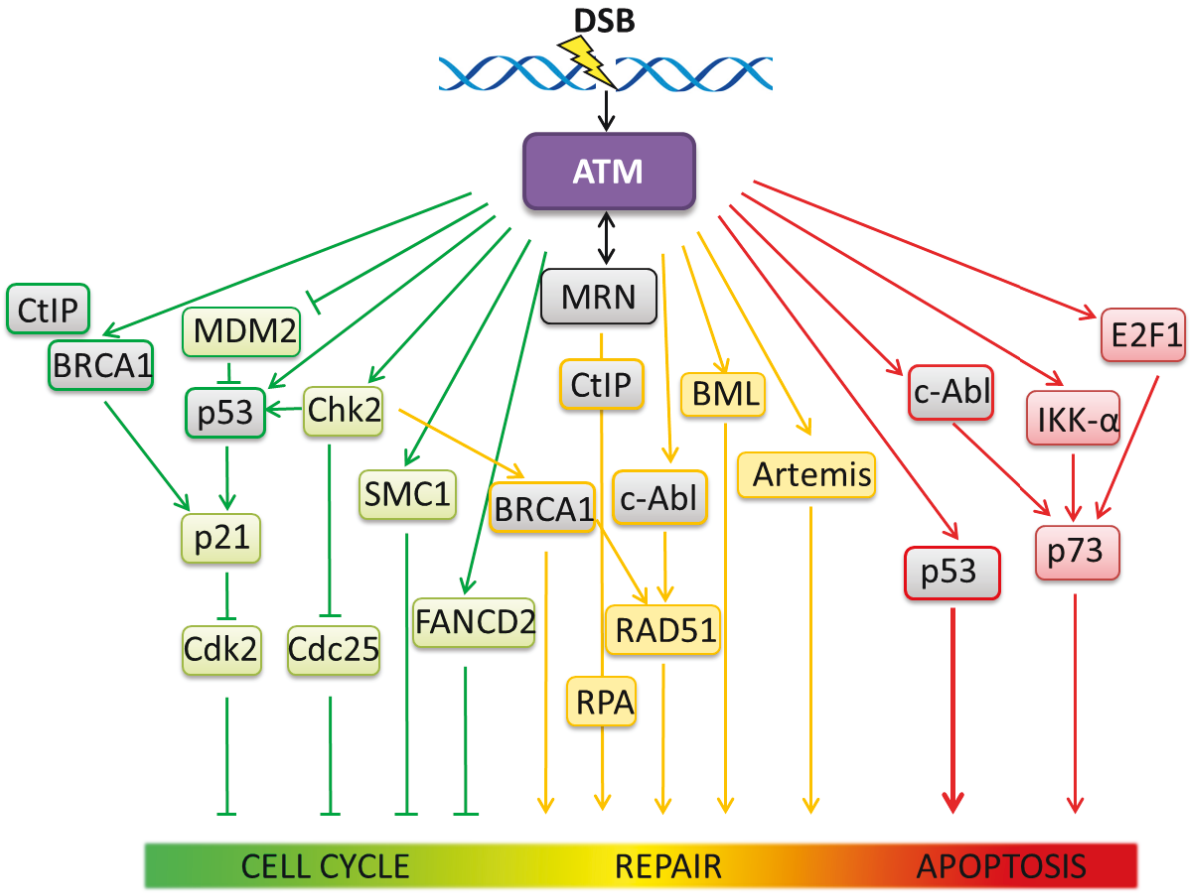


Figure 1.4. Spectrum of phosphorylated by ATM proteins during the response to DNA double strand breaks (DSBs).

The figure shows major target proteins involved in the cell cycle regulation, DNA repair and apoptosis. Grey boxes indicate the proteins which take part in more than one cellular pathway. Arrows indicate activation, blunt T-ends indicate inhibition. See text for details.

Phosphorylation of BRCA1 protein (breast cancer susceptibility protein-1) on several sites causes cell-cycle arrest either in S-phase (if serine 1387 is targeted) or in G2-M checkpoint (serine1423) (Wang et al., 2000). ATM regulates BRCA1-dependent cell-cycle arrest also by phosphorylating BRCA1-associated protein CtIP (on serines 664 and 745) which then dissociates from BRCA1 allowing it to induce the expression of the cell cycle inhibitors p21 and Gadd45a (Li et al., 2000).

1.4.2.3. ATM and DNA double-strand breaks repair.

Cell cycle arrest after DNA damage allows for the induction of DNA repair mechanisms. ATM contributes to DNA damage repair by phosphorylating some of the proteins involved directly or indirectly in this process. As mentioned earlier, KAP-1 protein is phosphorylated on serine 824 and although not directly involved in the repair, this event enables the DSB repair to proceed (Ziv et al., 2006).

MRN complex proteins are also substrates for ATM. Although the exact role of this phosphorylation for MRN mediated DNA repair activities is still not clear, it is important to mention that the MRN complex serves not only as a scaffold for ATM and other proteins involved in the DNA damage response but most likely carries out the initial processing of DNA broken ends and is involved in DNA recombination (Hopfner et al., 2001; Williams et al., 2008).

Other repair proteins activated by ATM include: endonuclease Artemis (serine 645) ; BML- Bloom syndrome protein (threonine 99); c-Abl kinase (serine 465) which then activates RAD51 (tyrosine 315); and BRCA1 (serine 988) whose activity in DNA repair is mediated indirectly via ATM-dependent Chk2 phosphorylation (threonine 68) (Beamish et al., 2002; Chen et al., 1999; Chen et al., 2005b; Riballo et al., 2004; Zhang et al., 2004). It has been also reported that phosphorylation of MDC1 by ATM at the early stage of the DNA damage response leads to recruitment of the ubiquitin ligases RNF8 and RNF168 (RING-finger protein-8 and -168) which promotes ubiquitylation of H2AX, facilitating the accumulation of BRCA1 and 53BP1 and other repair proteins at the DNA DSB sites (Mailand et al., 2007;

Stewart et al., 2009) .

Moreover, ATM is required for the recruitment of CtIP protein to DNA damage sites. It is unknown however, if this occurs via direct phosphorylation. CtIP in a collaboration with MRN complex and independently from its function in cell-cycle regulation, plays an important role in the DNA resection, recruitment of RPA (replication protein A) to single stranded DNA and activation of ATR kinase which continues to facilitate the repair of DNA breaks (You et al., 2009).

1.4.2.4. ATM and apoptosis.

In the presence of severe or persistent DNA damage, cells may activate apoptotic mechanisms. Unrepaired DSBs are very dangerous lesions, especially if the cell is attempting DNA replication, as this can result in chromosomal rearrangements, genomic instability and carcinogenesis (Jeggo and Lobrich, 2007).

The major target in apoptotic pathways activated by ATM is p53 protein which plays a critical role in determining the final cell fate after DNA damage by regulating the balance between cell cycle arrest and the induction of apoptosis (Bree et al., 2004). Once activated, it can interact directly or influence the expression of a variety of proteins, including pro-apoptotic proteins such as Puma, Noxa, Bax, Bid, and anti-apoptotic proteins such as Bcl-2, Mcl-1 and Bcl-xl. All of them belong to the Bcl-2 family of proteins. They are involved in the so-called intrinsic apoptotic pathway which relies on cytochrome c release from mitochondrial inter-membrane space into the cytoplasm and subsequent activation of caspase cascade resulting in a cleavage of a large number of downstream targets and ultimately, cell death (Bai and Zhu, 2006).

ATM can also induce apoptosis by interacting with transcription factor E2F1 (Lin et al., 2001). Subsequent activation of E2F1 might contribute to accumulation of p53 and/or expression of p73 protein (functional homologue of p53). Both scenarios lead to cellular death. Furthermore, ATM phosphorylates IKK-α (Inhibitor of nuclear factor kappa-B kinase subunit alpha) in response to cisplatin (DNA crosslinker and chemo-therapeutic agent)

(Yoshida et al., 2008). This interaction leads to accumulation of nuclear IKK-α, which in turn stabilizes p73 protein, prolongs its half-life and enhances its pro-apoptotic activity (Furuya et al., 2007). P73 protein can also be activated by c-Abl protein tyrosine kinase. This event is dependent on ATM phosphorylation of c-Abl during the response to DNA damage (Wang, 2000).

1.4.3. Role of ATM in other cellular processes.

Apart from involvement in the DDR, ATM has been shown to participate in oxidative stress responses, telomere capping during replication and lymphocyte development.

Oxidative stress conditions have been reported to induce ATM kinase activity in mammalian cells. For example, during hypoxia ATM phosphorylates HIF1-α (hypoxia inducible factor 1α) which in turn facilitates down-regulation of the pro-survival signals (Cam et al., 2010). Importantly, ATM activation under oxidative stress is DSB- and MRN complex-independent. Moreover, ATM deficient cells have reduced levels of antioxidants and are more sensitive to oxidative stress than normal cells, which suggests that ATM acts as a redox sensor and response regulator (Guo et al., 2010).

ATM, along with ATR, plays an important role in activating G2/M cell cycle arrest during replication of the telomeres. This checkpoint activation is distinct from IR-induced DNA damage response and prevents progression into mitosis with uncapped telomeres which otherwise would lead to telomere fusion and genomic instability (Thanasoula et al., 2012).

ATM is involved in T and B-lymphocyte development and maturation by taking part in two processes, *V(D)J* genes rearrangement and class switch recombination (CSR). Both of these processes generate DSBs and attract ATM protein to the sites of DNA breaks (Pan et al., 2002; Perkins et al., 2002).

VDJ recombination is initiated by RAG1 and RAG2 proteins (recombinase activating gene-1 and -2 protein) which induce DSBs, these are subsequently processed and joined by proteins involved in NHEJ repair pathway including DNA-PK, Ku70/Ku80, Artemis, XRCC4,

DNA-Lig4 (Gapud and Sleckman, 2011).

Bredemayer and co-workers used *in vitro* studies with murine pre-B cell lines to show that ATM deficiency results in the accumulation of unrepaired coding ends. They speculated that these free DNA ends may participate in aberrant chromosomal translocations involving antigen receptor gene loci during both T-cell receptor (TCR) and immunoglobulin gene assembly (Bredemeyer et al., 2006).

CSR is defective in ATM deficient human and mouse cells. Lumsden and colleagues reported that in AT mouse models, production of certain types of immunoglobulin was significantly decreased upon stimulation with antigens and that switch regions were characterized with greater microhomology than in control animals (Lumsden et al., 2004). In addition to a decreased number of mutations around the breakpoints, the same phenomenon was observed in B-lymphocytes from AT patients. These observations led to the hypothesis that ATM is involved in classical c-NHEJ pathway in CSR and when ATM functions are impaired (i.e. in AT patients), the less-efficient alternative end joining (A-EJ) mechanism is responsible for joining S regions in B-cells which leads to abnormal levels of certain Ig classes (Pan et al., 2002).

1.4.4. Ataxia Telangiectasia (AT) – a syndrome associated with inherited bi-allelic inactivation of *ATM*.

AT is a rare autosomal recessive disorder characterized by neuronal degeneration, immunodeficiency, genomic instability, radiosensitivity, premature aging, oculocutaneous telangiectasia and increased risk of developing cancer, particularly of lymphoid origin (Meyn, 1999; Peterson et al., 1992; Taylor et al., 1996). The observation of the clinical features of AT patients, development of ATM deficient mice models and *in vitro* studies have been providing the clues to understanding the multiple functions of ATM protein in sustaining cellular homeostasis and preventing these physiological defects. Much of AT clinical features are explained by known cellular functions of ATM protein.

Patients with ‘classical’ AT have no ATM protein in their cells, they present with

progressive ataxia by the age of 2 years with progressive loss of capability to walk to such an extent that they require a wheelchair by the time they reach their early teens (Meyn, 1999). These neurologic abnormalities are related to increased cell death of Purkinje cells in the cerebellum. The exact mechanism by which ATM protein protects against neurodegeneration is unknown. However, in neuronal cells ATM has mainly cytoplasmic localization suggesting that it can take part in the processes which might be distant from DNA damage response activation (Barlow et al., 2000).

In a recent study using a Drosophila model of human AT, Petersen and co-workers suggested that neuronal loss in AT may be due to neurotoxic effects of an increased innate immune response exerted by glia cells in the absence of functional ATM protein (Petersen et al., 2012). The authors pointed at the similar phenotype observed in other neurodegenerative diseases such as Alzheimer's and Parkinson where prolonged activation of microglia is thought to cause neurotoxicity.

Given the possible role of ATM in oxidative stress responses, another explanation for the increased death of the neurons in AT patients is the defective activation of antioxidant systems. This in turn, could result in accumulation of toxic reactive oxygen species (ROS) and cellular death. Neuronal cells are likely to be particularly sensitive to these defects as they have to maintain an extraordinarily high metabolism (this includes mitochondrial production of ATP and ROS) in order to control ionic gradients across cellular membranes and neurotransmission (Guo et al., 2010).

When considering the role of ATM protein in lymphocyte development it may come as no surprise that AT patients exhibit primary immunodeficiency resulting in an increased likelihood for the development of recurrent or severe infections. The majority of AT patients have lymphopenia affecting B and T-cells (resulting from insufficient VDJ rearrangement), functional impartment of T-cell mediated immunity (reduced activity of CD4 and CD8 cells) and/or reduced level of immunoglobulin IgA, IgE, IgG2 and IgG4 (defects in the CSR process). Immune defects also include thymic hypoplasia (Nowak-Wegrzyn et al., 2004; Waldmann et al., 1983).

The role of ATM in preventing telomeric fusions and resolving DNA breaks during spontaneous and programmed DSBs is reflected in an increased frequency of chromosomal aberrations in the cells of AT patients. These include chromosome breaks, acentric fragments, dicentric chromosomes, aneuploidy, telomere fusions and structural rearrangements (Meyn, 1999). In T-lymphocytes the inversions and translocations frequently involve loci on chromosomes 7 and 14 interrupting TCR genes, and in certain cases involve oncogenes. This results in the proliferation of T-cell clones which then might develop into T-cell malignancies, including T-cell acute lymphocytic leukaemia (T-ALL) and T-cell prolymphocytic leukaemia (T-PLL) (Taylor, 1992).

Overall, AT patients have an increased predisposition for developing cancer. The most frequent are tumours of lymphoid system (approximately 200-fold higher than in the general population) which constitute for over two thirds of all types of tumours observed in AT patients. The most common types are T cell tumours including T-PLL, T-ALL and T-lymphomas although there is also a clear increase in the frequency of B-cell lymphomas (Taylor et al., 1996). The incidence of breast cancer is also markedly increased among AT patients (Reiman et al., 2011). The strong predisposition for cancer development is a result of genomic instability generated from defective DNA repair and cell cycle control mechanisms.

AT is characterized by a clinical and cellular radiosensitivity, a demonstration of which is required in order to diagnose this disorder. It is now believed that radiosensitivity is a result of the combined defects in the cell-cycle checkpoint regulation and in DSB repair in the absence of functional ATM protein (Goodarzi et al., 2010).

1.4.5. AT heterozygotes- clinical phenotype and predisposition to cancer.

The frequency of AT heterozygotes in the general population of the UK is estimated to be around 0.5% (Thompson et al., 2005). Importantly, AT carrier status has been shown to cause a small but significant risk (relative risk 2.37) for development of breast cancer (Renwick et al., 2006). Interestingly, only *ATM* mutations which had been previously reported

in AT patients as causative alleles or strongly predicted to be pathogenic (truncating, splice site mutations, affecting functional domains) are associated with the risk of breast cancer (Goldgar et al., 2011; Tavtigian et al., 2009). Furthermore, using a whole-genome sequencing approach, AT carrier status has been identified as a risk factor for developing familial pancreatic ductal adenocarcinoma (Roberts et al., 2012b).

There is no conclusive evidence regarding predisposition to other tumour types among AT heterozygotes, although increased incidences of various cancers have been reported in family and population studies (Olsen et al., 2001; Swift et al., 1991; Thompson et al., 2005).

AT carriers have normal clinical phenotype (Taylor et al., 1996) and mild cellular radiosensitivity which can be demonstrated by assessing the level of chromosomal aberrations induced by ionizing radiation in peripheral lymphocytes or lymphoblastoid cell lines using three colour FISH chromosome painting metaphase analysis methods (Neubauer et al., 2002).

1.4.6. Nature and distribution of *ATM* mutations in AT patients.

Around 80% of the mutations which cause ataxia telangiectasia are truncating or splice site defects and the remaining are missense changes or short in-frame deletions. Truncating mutations are usually unstable and rapidly degrade, whereas missense mutations are often associated with the expression of mutant protein (Lakin et al., 1996; Stankovic et al., 1998). Overall, mutations in AT patients are distributed across the entire coding sequence (Li and Swift, 2000; Stankovic et al., 1998). However, missense mutations which result in the complete loss of kinase activity on downstream targets tend to localise at the C-terminus of *ATM* coding region which includes the FAT, PI3K and FATC domains (Barone et al., 2009).

The majority of AT patients are compound homozygotes with two different inherited sequence changes. The great neurological and immunological variability among AT patients is most probably determined by the type of inherited mutations. For example, patients who

inherit bi-allelic truncating mutations have a complete loss of ATM function and most severe clinical phenotype (Lakin et al., 1996). In contrast, milder AT phenotype is characteristic for patients who carry at least one *ATM* mutation with reduced kinase activity (Stewart et al., 2001). Importantly, patients with missense variants which cause complete loss of kinase activity might have the same clinical phenotype as patients with two truncating mutations (Barone et al., 2009). Furthermore, Reiman and co-authors reported that mutations with residual kinase activity or leaky mutations of splice site defects protect from childhood cancer in AT patients (Reiman et al., 2011). On the other hand, some mutations might be preferentially associated with an increased risk for developing cancer. For example, one of the missense changes, a founder mutation c.7271T>G is present in a significantly high frequency among breast cancer patients in both AT homozygotes and heterozygotes (Goldgar et al., 2011; Stankovic et al., 1998).

1.5. ATM status in CLL.

1.5.1. Frequency, nature and distribution of *ATM* mutations in CLL and other lymphoid tumours.

ATM mutations have also been detected in sporadic lymphoid tumours as acquired genomic changes and are proposed to play a role in the pathogenesis of these malignancies. This suggests a tumour–suppressor function for ATM in sporadic malignancies.

As mentioned before, T-PLL is a rare lymphoid tumour which occurs at a much higher frequency in individuals with AT compared to the general population. Interestingly, it has been reported that *ATM* mutations are common in sporadic T-PLL where they are often accompanied by the loss of the other copy of *ATM* through deletion of chromosome 11q (Stilgenbauer et al., 1997; Stoppa-Lyonnet et al., 1998; Vorechovsky et al., 1997). These findings suggest that the loss of functional ATM protein is an important factor in the development of T-PLL. However, in contrast to AT patients, mutations in sporadic T-PLL are mostly missense changes clustered in the C-terminal part of the *ATM* coding sequence.

ATM mutations in CLL cases were first described in 1999 by three independent

groups (Bullrich et al., 1999; Schaffner et al., 1999; Stankovic et al., 1999). Similarly to T-PLL cases, chromosome 11q deletion is a frequent genetic event in CLL and *ATM* mutations might be even 3-times as common in a cohort of patients with tumours which carry this chromosomal aberration (Austen et al., 2007). The majority of reported mutations in CLL are predicted to result in an amino-acid substitution in the translated protein, although there is also a substantial proportion of truncating mutations (Austen et al., 2005; Stankovic et al., 2002a). Distribution of missense changes in unselected CLL cohorts does not appear to have any obvious clustering pattern, however in tumours with chromosome 11q deletion they seem to localize to the region of the gene encoding C-terminal functional domains (Austen et al., 2007).

Frequent bi-allelic inactivation of *ATM* has also been reported in sporadic mantle cell lymphoma (MCL), where a mixture of missense and truncating mutations are distributed across the whole coding sequence of the *ATM* gene (Schaffner et al., 2000).

ATM mutations occur with much lower frequencies in other haematological malignancies such as B-cell non-Hodgkin's lymphoma (B-NHL), (Vorechovsky et al., 1997), diffuse large B-cell lymphoma (DLBCL) (Gronbaek et al., 2002) and multiple myloma (MM) (Austen et al., 2008).

1.5.2. Germ-line and acquired *ATM* mutations in CLL.

The majority of *ATM* mutations described in CLL tumours are acquired within the leukemic clone at different time points during the course of the disease but in a small proportion of cases they were also discovered in a patient's germ-line (Bullrich et al., 1999; Stankovic et al., 2002b). These findings and the fact that lymphoid tumours are present at increased frequency among AT patients prompted the speculation that AT carriers might be prone to develop CLL. The lack of the reports of CLL developing in AT patients could be due to usually short life span of AT patients who do not survive long enough to develop CLL, a malignancy of middle and old age. The frequency of CLL tumours in AT heterozygotes remains controversial. CLL cases have been reported in blood relatives of AT patients by

some investigators (Swift et al., 1987). However, another report did not confirm these findings (Thompson et al., 2005).

The possibility of contribution of germ-line *ATM* mutations to the development of CLL has been investigated through family linkage studies, candidate gene association studies and genome-wide association studies. The studies on CLL family pedigrees did not find evidence to link CLL development and *ATM* gene (Bevan et al., 1999), nor have they shown the segregation of *ATM* mutations between affected family members (Yuille et al., 2002). However, these results do not exclude the possibility that *ATM* acts as a low penetrance tumour suppressor gene and confers risk for CLL without apparent familial clustering. Interestingly, a candidate gene association study found evidence linking certain *ATM* polymorphisms with CLL patients when compared to controls (Rudd et al., 2006). Nevertheless, these results were not confirmed in another genome wide association study (Enjuanes et al., 2008).

The low number of multiple-case families of CLL, genetic heterogeneity, low penetrance nature of the *ATM* gene and the great variety in functional consequences of different *ATM* sequence variants make the evaluation of the role of *ATM* in the predisposition to CLL very difficult. These difficulties are reminiscent of the efforts to establish the role of germ-line *ATM* mutations in the development of breast cancer. They were finally resolved by a large case-control study approach which focused on sequence changes with known pathogenic effects and confirmed the link between AT carrier status and predisposition to breast cancer (Renwick et al., 2006).

With regards to somatic sequence changes, the time of acquisition of *ATM* mutations has been addressed in two studies. Stankovic and co-workers reported two mutations which were absent from the patients' germ-line but present not only in tumour cells, but also in T-cells and monocyte populations of the same individuals suggesting their origin in early haematopoietic progenitors (Stankovic et al., 2002b). Furthermore, in one of these cases, the monocyte cell population was lacking the wild-type allele suggesting that deletion of chromosome 11q was not restricted only to tumour cells. The consequences of the *ATM*

mutations in non tumour cells remain unknown. However, regarding the functions of ATM in DNA damage repair and class switch recombination an early acquisition of *ATM* defects at the time before clonal transformation suggested a potential causative role.

The second study provided the evidence that development of *ATM* mutations can also be a late genetic event occurring on the background of chromosome 11q deletion and leading to a complete loss of functional ATM protein and more aggressive disease (Austen et al., 2007). The loss of one *ATM* allele through chromosome 11q deletion is generally considered to be a later event in CLL pathogenesis (Cuneo et al., 2002; Fegan et al., 1995) (Rossi et al., 2013). These results support the model in which the loss of ATM function occurs in a step-wise manner and contributes to disease progression (Austen et al., 2007). However, the validation of this model has never been addressed in a large patient series. Moreover, although it is highly possible that germ-line *ATM* mutation could equally account for this phenomenon; the role of mono-allelic constitutive defects in promoting loss of another *ATM* allele has not yet been demonstrated.

1.5.3. Disease outcome of the *ATM* mutant CLL patients under conventional chemotherapy.

In vitro studies reported defective responses to DNA damage in CLL cells with *ATM* abnormalities. This included reduced ability to repair chromosomal breaks, reduced expression of p53 and p21 proteins following ionising radiation (IR) and reduced induction of p53 mediated apoptosis (Austen et al., 2005; Pettitt et al., 2001; Stankovic et al., 2002b) (Navrkalova et al., 2013). In addition, defects in p53 activity in the cells with *ATM* bi-allelic inactivation were observed when they were exposed to cytotoxic agents such as fludarabine, chlorambucil, cyclophosphamide (Austen et al., 2007) and doxorubicin (Navrkalova et al., 2013). In contrast, CLL cells with wild-type ATM protein were capable of inducing DSBs responses and showed phosphorylation of ATM downstream targets under the exposure to these drugs. Subsequently, cellular impairment of responses after IR and/or chemotherapeutics was correlated with clinical outcome and showed that bi-allelic *ATM*

inactivation was translated into shorter patient survival (Austen et al., 2005; Austen et al., 2007). Therefore, the authors suggested that patients with these defects might benefit more from the treatment which is independent of ATM function.

Importantly, these studies established that mono-allelic loss of *ATM* might be sufficient for its normal kinase activity. Furthermore, *ATM* mutations can act as an independent prognostic factor in the tumours with 11q deletion, and were also associated with unmutated *IGHV* status in an unselected cohort.

Undoubtedly, these findings significantly contributed to the understanding of the biology of the CLL tumours with *ATM* defects and if confirmed in randomized clinical trials, could have an important impact on the decision making regarding treatment choice.

Notably, cellular and clinical phenotype of *ATM* mutant tumours is less marked than in tumours with impaired p53 function. Stankovic and co-workers investigated gene expression profile in *ATM* mutant and *TP53* mutant CLL tumours and observed failure in activating pro-apoptotic responses in both groups (Stankovic et al., 2004). However, *ATM* mutated tumours also failed to activate p53-independent pro-survival transcriptional responses, thus counteracting IR-induced apoptotic resistance to some extent. This dual ATM function explains observed differences between *ATM*- and *TP53*-mutated tumours but also emphasizes the molecular complexity involving ATM protein in CLL and challenges in developments of optimal treatment strategies.

Interestingly, although tumours with only mono-allelic loss of *ATM* due to chromosome 11q deletion do not exhibit defective responses to damaging agents *in vitro*, many of them have poor outcome which indicates a possibility that factors other than *ATM* defects contribute to this clinical phenotype (Malek, 2012).

1.5.4. *ATM* mutation as a therapeutic target in CLL

ATM inactivation in CLL cells is not only a valuable prognostic marker which further reveals the heterogeneity of the disease but may also act as a molecular therapeutic target. *ATM* mutant cells exhibit defective responses to DNA DSBs generated by currently used

cytotoxic agents. These defects include impaired activation of apoptosis and therefore contribute to chemo-refractoriness. Therefore these tumour cells could be expected to be selectively sensitized for modifications in other DNA repair pathway which possibly compensates for dysfunctional ATM protein. This concept of 'synthetic lethality' has already been explored in an *in vitro* study (Weston et al., 2010). In this study the authors showed that *ATM* mutant lymphoid cells were preferentially sensitized to killing by inhibition of poly ADP ribose polymerase (PARP), a component of single strand break repair pathway. The cells died due to accumulation of unrepaired DNA breaks resulting in mitotic catastrophe, independent of p53 mediated apoptosis. PARP inhibition also reduced the tumour volume in the mice engrafted with an *ATM* mutant MCL cell line. Therefore PARP inhibition was proposed to be a viable strategy to treat refractory *ATM* mutant lymphoid malignancies and is currently under further investigation in undergoing clinical trial phase I/II (registered as an International Standard Randomised Controlled Trial, number 34386131).

The pleiotropic nature of ATM protein, its involvement in multiple cellular processes (of which some are not fully understood) and the trend towards personalized therapeutic approaches, make the development of a CLL xenograft model highly desirable. This tool would contribute hugely to the understanding of the biology of this tumour sub-population and facilitate the development of targeted treatments.

1.6. Treatment of CLL patients and drug testing tools- animal models.

1.6.1. Initiation and first-line treatment.

Current guidelines for diagnosis and treatment of CLL recommend initiating the therapy only in patients with symptomatic or progressive disease (Hallek et al., 2008). This recommendation is based on the findings from the studies which compared the outcome of patients with indolent disease either untreated for their CLL or treated with chemotherapeutic agents such as chlorambucil and fludarabine (CLL Trialists' Collaborative Group, 1999), (Bergmann et al., 2007). Those studies suggest that early treatment does not have a beneficial effect on patients' overall survival. Moreover, it has been observed that acquisition

of secondary genetic changes is typically the result of chemotherapy (Cavazzini et al., 2012; Schuh et al., 2012). Therefore, avoiding treatment until the symptoms that require an intervention appear, can be an advantageous approach. On the other hand, it is possible that administrating newer and more effective therapies in patients with an early stage disease but with the poor prognostic markers might slow down or even stop the progression. This hypothesis is currently addressed in German CLL Study Group CLL7 trial which randomizes asymptomatic, high risk, Binet stage A patients to chemo-immunotherapy versus observation.

Currently, the first-line treatment for CLL patients usually includes chemotherapy or chemo-immunotherapy. The most common drugs used in the UK and already approved for previously untreated patients include alkylating agents (such as chlorambucil, cyclophosphamide), purine analogues (e.g. fludarabine), monoclonal antibodies (e.g. rituximab and alemtuzumab). Apart from these, there are a number of therapeutic agents which have been shown to have anti-leukaemic activity in CLL and the evaluation of their efficacy either as mono-therapy or in combination is under the investigation in clinical trials.

Chlorambucil and cyclophosphamide belong to the class of drugs known as nitrogen mustards. They bind to DNA strands at guanine N-7 position creating cross-links between and within double helixes so-called intrastrand cross-links (ICLs). As binding is irreversible, it interferes with replication and transcription. Furthermore, these cellular processes generate DNA breaks, which if not properly repaired, lead to cell death.

Chlorambucil was one of the first effective alkylating agents which has been used from 1950s in the treatment of CLL. It is still considered as a potent drug and due to its less toxic side effects when compared to more aggressive chemotherapeutics it is recommended as a first-line treatment for elderly and unfit patients (Catovsky et al., 2011; Eichhorst et al., 2009). Cyclophosphamide is administrated as a prodrug and is oxidized in the liver to produce its active form- phosphoramide mustard. It is used usually in combination with other agents, such as Fludarabine.

Fludarabine is a purine analogue, in which one carbon atom has been replaced by

fluorine. Fludarabine is converted within the cell into its 5'-triphosphate derivative which exerts its cytotoxic effect by directly interfering with ribonucleotide reductase and by incorporating into DNA it stalls polymerisation, therefore blocking DNA synthesis during replication and DNA damage repair. Since the majority of CLL cells are arrested in G1 phase, the inhibitory effect of purine analogues in these cells most probably occurs during repair of DNA damage. In the presence of purine analogues a substantial number of DNA lesions remain unrepaired which in turn, leads to activation of apoptosis (Pettitt, 2003). It has been shown that treatment with fludarabine triggers expression of multiple genes involved mainly in the p53-dependent response to DNA damage and activation of cellular death (Rosenwald et al., 2004). This explains why patients with p53 defects exhibit profound resistance to this type of treatment.

It had been suggested that combination of alkylating agent (to induce DNA damage) with purine analogue (to inhibit DNA repair) would have greater cytotoxic effect than either of the drug alone. Indeed, combination of fludarabine and cyclophosphamide (FC) has been proven to have higher efficacy than fludarabine or chlorambucil as a monotherapy in clinical settings and when assessed in randomized trials (Catovsky et al., 2007; Eichhorst et al., 2006; Flinn et al., 2007).

The comparison between induction regimens of chlorambucil alone, fludarabine alone and fludarabine combined with cyclophosphamide has been the aim of the recent United Kingdom Leukaemia Research Fund Chronic Lymphocytic Leukaemia 4 trial (UK LRF CLL4) (Catovsky et al., 2007). The assessment of the clinical consequences of *ATM* abnormalities in the context of UK LRF CLL4 trial is also a part of this thesis. Therefore the overall trial setting, main objectives and results are summarized in the greater detail in the next section - 1.6.1.1.

An improvement of the FC regimen with better overall and progression free survival can be achieved with the addition of monoclonal antibody- Rituximab (R) (Hallek et al., 2010). This new strategy is called the FCR regimen. A randomized German CLL Study Group (GCLLSG) CLL8 trial showed a supremacy of this treatment when compared to FC.

Since then, FCR became recommended as a first-line treatment choice for fit CLL patients. Importantly, the FCR regimen significantly improves the outcome in patients with 11q deletion, previously associated with poor prognosis (Hallek et al., 2010; Tsimberidou et al., 2009). Unfortunately, patients with high risk genomic features like 17p deletion do not greatly benefit from either FC or FCR (Gonzalez et al., 2011; Zenz et al., 2010).

Rituximab is a monoclonal antibody which binds to CD20 phosphoprotein expressed on all B-cells apart from early-pro B-cells and plasma cells targeting them for destruction by the immune system and inducing apoptosis (Jaglowski et al., 2010). The predominant cytotoxic mechanism is cell type specific and it seems that in CLL cells the major apoptotic pathway involves p38 MAPK (mitogen activated protein kinase), p53 protein and caspase-9 (Pedersen et al., 2002a).

Another monoclonal antibody used in CLL treatment is Alemtuzumab which binds to CD52, a transmembrane glycoprotein expressed on CLL cells, healthy mature B-cells, T-cells, NK cells, granulocytes, macrophages, monocytes, eosinophils and dendritic cells (Rao et al., 2012). The mechanism of killing in CLL cells, although not fully understood, seems to be independent of p53 status and caspase activity (Mone et al., 2006). Probably, this is why the therapy with Alemtuzumab as a first-line treatment has been shown to have some activity in tumours with *TP53* abnormalities (Hillmen et al., 2007). However, treatment with alemtuzumab is associated with increased immune suppression leading to cytopenias and infections including cytomegalovirus (CMV) reactivation. Combinations of alemtuzumab with FC and FCR regimens do not appear to provide obvious clinical benefits and again, might be associated with high infectious toxicity (Lepretre et al., 2009; Parikh et al., 2009). Therefore, alemtuzumab therapy is usually limited to patients in whom predicted benefit gain is outweighed by potential side effects. These are usually patients with *TP53* abnormalities not eligible or awaiting for allogeneic stem cell transplantation or/and refractory to other treatments (Gribben and O'Brien, 2011).

Importantly, combination of alemtuzumab with high dose glucocorticoid (methylprednisolone) has been recently documented in a phase II of National Cancer

Research Institute CLL206 trial, as an effective induction treatment strategy for CLL patients with *TP53* deletions (Pettitt et al., 2012). Complete remission-CR (65% patients), OS and PFS (median 38.9 months and 18.3 months respectively) were all superior when compared to outcome of patients treated with FCR in the GCLLSG CLL8 trail (CR-5%, OS-28.8 months and PFS-11 months) (Hallek et al., 2010), or with alemtuzumab monotherapy (CAM307 trail, CR-24%, median PFS-11 months) (Hillmen et al., 2007). These results emphasised the benefits of stratified medicine approach, which takes into account molecular basis of the disease and mechanisms of drug resistance, in the first line treatment.

1.6.1.1. UK LRF CLL4 trial.

UK CLL4 trial randomly assigned 777 patients to first line treatment in a 2:1:1 ratio with chlorambucil, fludarabine alone or fludarabine plus cyclophosphamide between 1st February 1999 and 31st October 2004. Patients were recruited among those who needed treatment and were at the Binet stage A-progressive, B or C. All ages were eligible, the median age at diagnosis was 65 and 30% of patients were 70 years and older. Patients' clinical characteristics like gender, age, disease stage did not differ between treatment arms. In total, 136 centres participated and 86% of patients were from the UK. The endpoints were OS, response to treatment, PFS, toxic effects and quality of life. The follow up for analysis was 68 months. The response was assessed following criteria of National Cancer Institute proposals from 1996 (Cheson et al., 1996) and was classified either as complete remission, nodular partial response, partial response, no response or progressive disease. The panel of clinical and laboratory prognostic markers was measured at trial entry and assessed with respect to OS, PFS and response to treatment. Laboratory markers included β-2 microglobulin; *IGHV* mutation status and usage; cytogenetics status for chromosomes 17p13 deletion, 11q deletion, trisomy 12, 13q14 deletion, 6q21 deletion; CD38 expression; ZAP70 expression (Catovsky et al., 2007; Oscier et al., 2010).

Overall, there was no difference in OS between treatment arms, however PFS, complete remission and overall response (OR) were significantly better in patients treated

with FC than with fludarabine alone or chlorambucil alone and this was irrespective of age and disease stage. The lack of the differences in OS between treatment arms could be explained by the poor second-line responses among the patients who were initially treated with fludarabine plus cyclophosphamide and better second-line responses for those initially treated with chlorambucil or fludarabine alone (Catovsky et al., 2007). The survival of patients with stage A-progressive disease was similar to stage B and both were significantly better than for stage C. The response to chlorambucil was the worst but this treatment was associated with fewer days in a hospital and less neutropenia than the other two regimens.

Quality of life was better for those who responded to therapy, and there was no significant difference between treatment arms either during treatment nor up to 5 years after its completion (Else et al., 2011).

This trial emphasized the role of biological prognostic factors in predicting the outcome of the patients requiring chemotherapy as the first line treatment. Chromosome 17p deletion, *TP53* mutation, *IGHV* gene mutational status and/or *IGHV3-21* usage, β-2-microglobulin level and 11q deletion were all identified as independent prognostic markers. Three risk groups were identified according to the presence of negative prognostic features: poor risk group (presence of 17 deletion and/or *TP53* mutation), intermediate risk group (presence of at least one of the following features, *IGHV* unmutated, *IGHV3-21* usage, β-2-microglobulin level above 4mg/L, 11q deletion), and good risk group (none of the features from above). These risk groups had significantly different response rate, PFS and OS even within each of the Binet stage category and regardless of treatment allocation.

Importantly, although the FC regimen had the highest response rates when compared to Fludarabine or Chlorambucil alone, independent of prognostic factors (clinical and biological), it did not benefit patients with 17p deletion and/or *TP53* mutations (Gonzalez et al., 2011; Oscier et al., 2010).

Furthermore, some of the biological prognostic markers identified in CLL 4 trial as independent predictors of poor prognosis might not hold their strong prognostic value if applied to different regimens. For example, it has been shown that FCR therapy significantly

improves the outcome for patients with 11q deletion and this marker is no longer an independent predictor of worse prognosis (Hallek et al., 2010).

1.6.2. Treatment of relapsed CLL and novel approaches.

Despite the improvements in first-line treatment, CLL remains an incurable disease for the majority of CLL patients. Drug resistance in tumour cells is often coupled with an increased capability to repair DNA damage or with an inactivation of apoptotic pathways. This means that relapsed disease might become even more aggressive and therapeutic options become more limited.

The performance status, the type of the previous therapy and duration of response to that therapy determine the choice of secondary treatment. Patients who responded well to the first-line treatment with alkylating agent such as chlorambucil might be offered the same drug when the disease relapses. However, the duration of responses is shorter than after initial treatment and multiple courses eventually lead to drug resistance (Oscier et al., 2004). Interestingly, relapse after chlorambucil does not exclude the response to second-line purine analogue-based therapies. This has been shown in CALGB 9011 randomized trial which allowed for crossover between chlorambucil and fludarabine arm after unsuccessful initial treatment with one of these drugs. In this study, patients treated with fludarabine after chlorambucil had good overall response rate (ORR 46%). However, the response to chlorambucil in patients resistant to fludarabine was low (7%) suggesting little role of alkylating agents in fludarabine resistant tumours (Rai et al., 2000).

FCR combination has been shown to be an effective therapy for previously treated patients in both phase II and phase III trials, where ORR was 73% and 70% respectively (Robak et al., 2010; Wierda et al., 2005). The same randomized phase III study also reported improved complete remission rate (CR) and PFS when compared to FC regimen (Robak et al., 2010).

In patients with *TP53* abnormalities and resistant to chemotherapy Alemtuzumab has shown to have some activity overcoming defects in p53-dependent apoptosis (Lozanski et

al., 2004; Stilgenbauer et al., 2009). However, for this group of patients, allogeneic stem cell transplantation remains the most promising option providing they are fit enough to receive it.

Most of the potent chemo- and immuno-therapeutics are efficient in decreasing the load of CLL in peripheral blood compartment. However, they are less efficient in accessing the lymph nodes and bone marrow, where proliferation of CLL cells takes place. Therefore, aggressive disease might relapse even after initial, short response to those agents. Some of the newer therapeutic approaches focus on inhibition of proliferation and pro-survival signalling in CLL either by interfering with cellular pathways or disturbing CLL-microenvironment interactions. These include BCR signalling pathways inhibitors such as Ibrutinib (Btk inhibitor), Dasatinib (Lyn inhibitor), Fostamatinib (Syk inhibitor), CAL101 (PI3K inhibitor) (Advani et al., 2013; Woyach et al., 2012); Bcl-2 inhibitors (Roberts et al., 2012a); immune-modulatory agents like lenalidomide which have the ability to induce CD154 (CD40L) expression on CLL cells thus overcoming immune defects (Lapalombella et al., 2010); as well as chemokine receptors inhibitors (Burger et al., 2005).

Worth mentioning are novel immunotherapy approaches which utilize engineered autologous T-cells which engage in CLL recognition and killing. The recognition occurs via chimeric antigen receptors (CAR) on T-cells and epitopes expressed on tumour cells. Very promising results have been reported in recent studies which used autologous T-cells which target CD19 or ROR1 (tyrosine-like orphan receptor 1) expressing cells in CLL patients (Hudecek et al., 2010; Porter et al., 2011). However, as those cell markers are not exclusively expressed by CLL cells, side effects can occur and the technique requires further optimization before it can be considered for general use.

The development of new treatment requires extensive laboratory-based research and adequate tools to assess efficacy and toxicity before it is introduced into the clinic. Animal models of CLL disease provide such tool. A brief overview of transgenic and xenograft models of CLL disease is given below.

1.6.3. Animal models for CLL as tools to test new treatments and study the biology of the disease.

Animal models are used to elucidate the biology of the disease, demonstrate the activity of the potential therapeutic agents in a living organism and provide necessary data for clinical study design. Although *in vitro* studies inform about drug's potency, they cannot predict unfavourable/undesirable interactions with other molecules in the body, distribution in organs or cytotoxic effects to the whole organism.

1.6.3.1. Transgenic mice.

CLL is a slow growing tumour which requires adequate support from the microenvironment and these characteristic have made the engraftment of tumour cells into mice very problematic. In contrary, there are few genetically engineered mice models which develop lymphoproliferative disorders resembling human B-CLL. These include Traf2DN/Bcl2 transgenic mice model of chemoresistant CLL (Zapata et al., 2004), Eμ-miR29-overexpressing model of indolent CLL (Santanam et al., 2010), 13q14deletion model of indolent CLL (Klein et al., 2010), IgH.TEμ mouse CLL model (ter Brugge et al., 2009) and Eμ-TCL1-overexpressing model of aggressive CLL (Bichi et al., 2002).

Traf2DN/Bcl2 transgenic murine model has been developed by overexpressing two proteins, Traf2DN and Bcl2. Traf2DN protein is a double mutant of the molecule belonging to the Traf-family proteins. Traf (TNF receptor-associated factors) molecules are recruited to the activated TNF (tumour necrosis factor) receptors and take part in signalling cascade involving cell proliferation, gene expression and control of apoptosis. Traf2DN mimics Traf1, a protein which is overexpressed in many human CLLs and other hematologic malignancies whereas Bcl-2 is a pro-survival protein commonly overexpressed in CLL cells. The proportion of transgenic mice expressing both Bcl2 and Traf2DN develop an age-dependent B-cell leukaemia and lymphoma of a slowly proliferating clonal cells characterized by the presence of chemotherapy resistance and CD5 positivity (Zapata et al., 2004).

Del13q14 and Mir29 CLL murine models have been generated based on the

suggestion that miRs play an important role in the biology of CLL (Calin et al., 2002; Calin et al., 2004). Minimally deleted region (MDR) in 13q14 contains *DLEU2/miR15a/16-1* cluster. Some 26-42% of transgenic mice with this MDR, depending on the size of the deleted region, develop CD5 positive clonal lymphoproliferative disorders including MBL, CLL and aggressive lymphoma (Klein et al., 2010).

Mir29 has been discovered to be overexpressed in indolent human CLL when compared to aggressive CLL and to normal B cells. In one study, overexpression of miR29 in transgenic mice led to development of low-grade lymphoma and 20% of animals died of this disease in old age (Santanam et al., 2010).

By introducing simian virus 40 (SV40) large T antigen under Eμ enhancer into mice genome Ter-Brugge and collegues (ter Brugge et al., 2009) generated mice which developed clonal expansion of mature B cells in blood, lymph nodes, spleen and bone marrow by the age of 10 months. Expanded B cells were CD5 positive, and either *IGHV* unmutated with predominant *VH11* gene usage or with extensive SHM.

The Eμ-TCL1 model has been the most extensively investigated and also validated as a useful preclinical tool in the drug development. *TCL1* (T cell leukaemia/lymphoma 1) gene is expressed in immature normal T-cells, pre-B cells, T–cell prolymphocytic leukaemia and B-cell malignancies including CLL (Narducci et al., 2000). High expression of TCL-1 in CLL patients correlates with unmutated *IGHV* status, ZAP70 expression and deletion of chromosome 11q22-23 (Herling et al., 2006). Moreover, CLL cells that show high TCL-1 expression have also higher proliferation rates upon BCR engagement and this is, at least partially, due to increased co-recruitment and phospho-activation of anti-apoptotic Akt protein (Herling et al., 2009).

Overexpression of human *TCL1* gene in mice under the B-cell specific VH promoter and IgH-Eμ enhancer leads to development of lymphadenopathy and splenomegaly associated with a high number of clonal CD5 positive cells mostly arrested in G0/G1 phase. This phenotype is evident and defined as CLL in all adult mice from around 12 months of age. Furthermore, clonal leukeamic cells in this model have unmutated *IGHV* and T-cell

immune defects similar to those observed in human CLL (Gorgun et al., 2009; Yan et al., 2006). Finally, the responses to the therapeutic agents in Eμ-TCL1 model have been shown to be similar to CLL patients, which confirms its utility in the preclinical drug development (Johnson et al., 2006; Ramsay et al., 2008).

Therefore, transgenic mice can serve as a tool for drug testing and help to verify the involvement of specific genes in CLL pathogenesis. One caveat is a low penetrance for CLL disease in transgenic mouse models, where only a proportion of animals develop CLL at an old age. Furthermore, they do not recapitulate the very specific clinical subgroups of CLL disease such as those with high-risk prognostic markers, deletion of chromosome 17p or 11q. Finally, the resistance mechanism in CLL has not been fully understood and probably results from the dysfunction of multiple factors rather than from the deregulation of a single gene. Therefore, transgenic mouse models are unlikely to provide an accurate insight into the natural history of human disease including clonal evolution that is characteristic for CLL.

1.6.3.2. CLL Xenograft human-mouse models

The disease aspects noted above might be better addressed if the patients' cells were transplanted into the adoptive mice. However, this approach has encountered serious obstacles due to the nature of CLL circulating cells which are non-proliferative and the fact that their longer term survival seems to be completely dependent on the microenvironment. In addition, transplanting cells between different species requires a highly immunodeficient recipient. Therefore, the first attempts to engraft human cells in mice suffered from rejection by xenogeneic mice or in the best scenarios, from inefficient or organ restricted proliferation as well as short lasting engraftments.

In 1992 Kobayashi and co-workers reported CLL transplantation into SCID mice (Kobayashi et al., 1992). Animals were injected intraperitoneally with CLL peripheral blood mononuclear cells (PBMC) and developed a B-cell LP disorder which histologically resembled aggressive lymphoma. However, these tumours were composed of oligoclonal cell populations which did not migrate to other organs, they were clonally distinct from the

injected CLL, did not express CD5 marker and were Ebstein-Barr virus positive which suggested that they originated from the normal B-cell compartment.

To avoid EBV transformation resulting in tumour development from normal B cells, Shimonni and colleagues used lethally irradiated normal strains of mice radioprotected with SCID bone marrow. They injected CLL PBMCs into the peritoneal cavity and observed a marked engraftment of T-cells or combined T and CLL cells when transplanting early-stage CLL PBMCs, and predominant engraftment of CLL cells when transplanting high-stage CLL PBMCs (Shimoni et al., 1997). Subsequently, the same authors investigated the role of autologous T-cells on growth and expansion of transplanted CLL cells by depleting T-cells from PBMC using anti-T-cells antibody, or by manipulating the T-cell number in PBMC (depleting or enriching) prior the injection (Shimoni et al., 1999). Elimination of T–cells resulted in a good level of engraftment of low-stage CLLs, however was not essential for leukemic cells engraftment of high-stage disease. Furthermore, the T-cell enrichment before adaptive transfer from advanced-stage patients led to a significant reduction of CLL engraftment. Therefore, the authors concluded that autologous T-cells can actively suppress the expansion of CLL in murine recipients and suggested that their model could provide a useful tool for the investigation of pathogenesis and progression in CLL. Importantly, in all cases, engraftment was restricted to the peritoneal cavity and was followed only for relatively short period of 2 weeks which was a great limitation of this study.

The similar results were obtained by Durig and co-workers (Durig et al., 2007), who used sub-lethally irradiated NOD/SCID (nonobese diabetic) mice as recipients for CLL xenotransplantation. They obtained a stable splenic and peritoneal engraftment over a time span of 4 to 8 weeks. However, the recovery of leukemic cells from murine bone marrow and peripheral blood was relatively low. Furthermore, mice transplanted with PBMCs of low stage disease favoured T cell engraftment over CLLs. In contrast, predominant engraftment of CLLs was observed in mice injected with the cells from advanced-stage patients. Therefore, the authors confirmed the previous findings of Shimon's study regarding a suppressive role of T-cells in CLL engraftment.

Recently, a novel adaptive transfer model has been described that allowed not only for reproducible proliferation of leukemic cells in mice but also provided further explanation for the role of autologous T-cells in CLL engraftment and growth. Bagnara and co-authors (Bagnara et al., 2011) used sub-lethally irradiated highly immunodeficient mice strain NSG (NOD/SCID/IL2Rγ^{null}). Deletion of interleukin-2 family common cytokine receptor gamma chain (IL2Rγ) renders mice completely deficient in lymphocytes including NK cells which significantly reduces the chances of tumour cell rejection after transplantation (Cao et al., 1995). The authors discovered that such a modified murine microenvironment was sufficient to support CLL survival and that activated autologous T-cells were essential for engraftment and proliferation of leukemic cells. However, within 12 weeks all human CLL cells disappeared and the animals died of suspected graft versus host disease which coincided with the expansion of autologous T-cells. Despite serious limitations, this model for the first time allowed for measurement of CLL cell kinetics and represents an important step forward in pre-clinical studies for novel, personalised therapies.

Finally, another model has been established by introducing CLL cell line Mec-1 into mice strain Rag2^{null}/γ_c^{null} (Bertilaccio et al., 2010). Mec-1 cells injected subcutaneously or intravenously rapidly created solid tumours at the injection sites and/or populated multiple organs resembling aggressive human disease. This model however, suffers from the lack of full representation of primary CLL. Furthermore, CLL cell line was derived via EBV immortalization and represents only a single patient with *TP53* abnormalities.

1.7. Aims.

(1). Population-based and genome wide association studies indicate the presence of predisposing genetic variants in the development of CLL. However, the molecular basis underlying the genetic predisposition in CLL remains largely unknown. The *ATM* germ-line mutations have been previously identified in CLL tumours but their role in the disease initiation has not been established. I have therefore aimed to compare the frequency of germ-line *ATM* pathogenic mutations between the CLL patients' cohort and a control population in order to address whether they can predispose to the development of CLL.

(2). *ATM* mutations have been previously shown to be associated with shorter overall survival and treatment free survival. Chromosome 11q deletion is a recurrent genetic aberration in CLL that leads to the loss of one copy of the *ATM* gene and is associated with the poor prognosis. There is a partial correlation between the presence of *ATM* mutation and the loss of chromosome 11q. Furthermore, some evidence from unselected CLL cohort suggest that bi-allelic inactivation of *ATM* may confer inferior outcome compared with mono-allelic loss or mutation. I have therefore aimed to verify these findings by assessing the role of *ATM* mutation, either as sole *ATM* abnormality or in combination with chromosome 11q deletion on the treatment responses and patients' survival in the context of randomized clinical trial.

(3). CLL remains mainly an incurable disease and many novel therapeutic agents would ideally require assessment of their efficacy and toxicity in CLL xenograft models before they are introduced into the clinic. Despite the advances in recent years to establish such a model the current limitations impose a need for further optimization. Therefore, I have aimed to further optimize the existing CLL xenograft model with the emphasis on obtaining the prolonged engraftment of CLL cells in murine blood and organs.

CHAPTER II

MATERIALS AND METHODS

2.1. Samples from CLL patients and control individuals

2.1.1. CLL samples

Three cohorts of CLL patients were studied. The first consisted of 318 patients diagnosed and managed in two local Birmingham haematology departments in Queen Elizabeth and Heartlands Hospitals (n=94); also patients referred from Bournemouth and Leicester Hospitals (n=61) and patients that had been treated on the UK CLL4 Trial (n=163). The material for the study was available in the form of genomic DNA extracted from tumours cells and in some cases from patient granulocytes.

The second studied cohort (224 patients) consisted entirely of patients treated on the UK LRF CLL4 Trial. For 80 cases material for the study was available as frozen tumour peripheral blood mononuclear cells (PBMCs), and for the remaining 144 samples, as genomic DNA extracted from tumour cells at the hospitals participating in the CLL4 Trial.

The third, small cohort included 7 consecutive patients that attended the haematology outpatient clinics at Queen Elizabeth and Heartlands Hospitals in Birmingham. On the day of their clinic visit, fourteen millilitres of peripheral blood was collected from each patient put into heparinised tubes. Subsequently, tumour cells were extracted from the blood samples and stored accordingly to protocols described below.

The studies were performed according to local ethical guidelines and written informed consent was obtained from all patients.

2.1.2. Control cohort

The control cohort (n=281) consisted of a local volunteer research subgroup (n=71) (One Thousand Elders Group, Birmingham University) and a subgroup of anonymous blood donors (n=210). Ethical approval for the study was obtained from South Birmingham Ethics Committee (Ref. O4/Q2709/25).

2.1.3. Cord blood samples

Six to sixty millilitres of anonymous, fresh cord blood samples were obtained from

Women Hospital in Birmingham. The reference number of the ethical approval for the study is: 09/H1010/75. Cord blood samples were used for mice humanization in CLL xenograft model.

2.1.4. Isolation of peripheral blood mononuclear cells (PBMC) from CLL and cord blood samples

Whole blood samples were diluted with RPMI medium (Sigma-Aldrich Ltd, Dorset, UK) in a ratio 1:1 in 50 ml tubes and layered on 13 ml of Lymphoprep solution (Axis-Shield, Cheshire, UK). Samples were centrifuged in a swing-out rotor at 1600 rpm for 30 min with the breaks off. Mononuclear cells containing lymphocytes were collected from the interface and transferred to another 50 ml tube and washed twice with RPMI medium. Cells were counted and stored at concentration of up to $5x10^7$ cells/ml in 90% fetal calf serum (Sigma-Aldrich) and 10% Dimethyl Sulphoxide (DMSO, Sigma-Aldrich). Cryovials with cells were initially stored in insulated boxes at -80°C and after 24 hours transferred to liquid nitrogen.

2.1.5. Germ-line material from CLL patients

Germ-line material of CLL patients was available either as a red blood cells pellet (containing granulocytes) collected at the time of mononuclear cell separation and stored at -80°C until the time of this study when genomic DNA was extracted, or as already extracted genomic DNA from patients granulocytes (CLL4 trial samples).

2.1.6. Genomic DNA extraction

Genomic DNA from frozen tumour PBMCs, tumour red blood cells pellets and from the control blood samples was extracted using FlexiGen extraction kit (QIAGEN, Crawely, UK). Extraction of genomic DNA from tumour PBMCs was performed following the manufacturer protocol. Briefly, the cells were thawed, washed with RPMI and counted. Up to $2x10^6$ cells were resuspended in 300µl lysis buffer. 300µl denaturation buffer containing 3µl of protease (reconstituted in hydration buffer according to manufacturer's specifications) was

added to each sample which was mixed and incubated at 65°C for 10 minutes. 600µl of 100% (v/v) isopropanol was added in order to precipitate the DNA. The samples were well mixed and micro-centrifuged at 13,000 rpm for 5 minutes. The DNA pellets were washed with 70% (v/v) ethanol, air-dried and dissolved in hydration buffer by incubation at 65°C for 30 minutes.

DNA from patient red blood cell pellet and from the control whole blood samples was extracted using FlexiGen extraction kit (QIAGEN) and following the protocol- 'Isolation of DNA from Whole Blood'. 7.5 ml of lysis buffer was added per 3ml of blood. Samples were mixed and centrifuged in a swing-out rotor for 5 minutes at 3000 rpm. After discarding supernatant, 1.5 ml denaturation buffer containing 15µl of protease was added to each pellet. Samples were mixed and incubated at 65°C for 10 minutes. The DNA was precipitated by adding 1.5ml 100% (v/v) isopropanol, washed in 70% (v/v) ethanol, air-dried and dissolved in hydration buffer. All DNA samples were diluted in hydration buffer to achieve the concentration of approximately 30 ng/µl.

2.2. Screening for *ATM* mutation

2.2.1. Polymerase Chain Reaction (PCR) for *ATM* gene

PCR reactions were done for each of the 62 *ATM* coding exons (4-65) using 60 primer pairs. Exons 4 and 5 and, exon 43 and 44 were each expanded in a single PCR reaction using primers located outside both exon coding sequences, as these pairs of exons are positioned close together within the gene sequence. The PCR reactions for each exon were performed in 96-well plates on up to 96 tumour samples at a time. Each reaction used 30ng of genomic DNA (1ul), 0.4µl of dNTPs (deoxyribonucleotide triphosphates) (0.2µM) (Invitrogen, Paisley, UK), 1ul of each primer (0.2µM) (Alta Bioscience, Birmingham, UK), 10ul of 5x PCR buffer containing 1.5mM of $MgCl_2$ at final reaction concentration and 1 unit of High-Fidelity DNA polymerase, Phusion (Thermo Scientific, Loughborough, UK). These were mixed with sterile water to form a total reaction volume of 50µl. PCR conditions included 30 seconds at 98°C, followed by 36 cycles of 10 seconds at 98°C for denaturation of dsDNA,

30 seconds at 53°-58°C for primer annealing and 30 seconds at 72°C for polymerase dependent DNA extension, with a final extension of 7 minutes at 72°C. Primer sequences and PCR annealing temperatures are provided in Table 2.1.

Table 2.1. PCR and DHPLC conditions for exons 4-65 of *ATM* gene.

Exon	Primer sequence	PCR annealing temp (°C)	DHPLC melting temp (°C)
4/5	Forward – CACACCTCTTTCTCTCTATATATGC Reverse- AAGCAAAGATAAATGTTAAGACTTACAC	59	53.5
6	Forward-ATTGTTCTTGTAGGAGTTAGGCCTTG Reverse-AAAAACTCACGCGACAGTAATCTG	58	54.0
7	Forward-TAAATAGTTGCCATTCCAAGTGTC Reverse-TGGTGAAGTTTCATTTCATGAGG	58	54.7
8	Forward-CCTTTTTCTGTATGGGATTATGGA Reverse-TACTGAGTCTAAAACATGGTCTTGC	58	54.8
9	Forward-TTCTTTCAGCATACCACTTCATAAC Reverse-TGAATGAAGAAGCAAATTCAAAACAG	58	55.0
10	Forward-TGGGAGCTAGCAGTGTAAACAGAG Reverse-CAGGAAATTTCTAAATGTGACATGAC	58	54.0
11	Forward-GCTCAAAAAAAAAAAAAAGAAAAAAGTGG Reverse-AAATGACTTAGTTCTGGTTGAGATG	56	54.7
12	Forward-TCCTTTAGTTTGTTAATGTGATGG Reverse-ACTATGAAAATGATCAGGGATATG	56	56.0
13	Forward-CCTCCAATAGCTTGCTTTTCAC Reverse-AAACAGCAGCATGCTAATGAAC	58	55.8
14	Forward-GTATTCTTTACATGGCTTTTGGTC Reverse-TACTACCCAGCTAAAATTATCATC	56	53.9
15	Forward-CATATAAGGCAAAGCATTAGGT Reverse-CCTATTTCTCCTTCCTAACAGT	56	55.5
16	Forward-GAATTTGTTCTTACAAAAGATAGAG Reverse-GAATACATTTCATTCAAATTTATCCGA	55	56.0
17	Forward-GTATGTCCAAGATCAAAGTACACTG Reverse-GGGTGACAGAGAAAGATCCTATC	58	55.5
18	Forward-GTTTTTATTTCTTTGTTGCTTGGTTCT Reverse-CAAATATGATAGCAAAACAGGAAGC	58	55.9
19	Forward-CTCCCAAATTGCTGAGATTACAGATG Reverse-ATGAGGCCTCTTATACTGCCAAATC	58	56.4
20	Forward-ATATATGGCTGTTGTGCCCTTCTC Reverse-CATCAGATAAAATCCAAGAGCTTC	58	56.5
21	Forward-AAACCTGATTTTTTTCCCTCCTAC Reverse-TTTATAAGCTTAACAGAACACATCAGT	58	56.0
22	Forward-AATAACTGATGTGTTCTGTTAAGC Reverse-AAACTTGCATTCGTATCCACAGAT	56	55.9

23	Forward-GTAACTTATTAATAACCTTTAAGTGAG Reverse-ACTCATTAACAAAACAAAGACTGCT	56	54.6
24	Forward-CTATTTCATATTTAACCACAGTTC Reverse-TATGTAAGACATTCTACTGCCATC	56	56.7
25	Forward-TTGTTTGTTTGTTTGCTTGCTTGTTT Reverse-CATATGATAACAGCAAATACATGTTAC	56	57.4
26	Forward-GTCAAAAAATCTGGAGTTCAGTTG Reverse-GGAAGCTTCTAATAAAATACTCATC	56	55.0
27	Forward-GAATGTTGTTTCTAGGTCCTACTC Reverse-GTGAGGGGGACTTGCTAAGTATTG	58	53.0
28	Forward-CTTGGAAAAGTTATATATAACCTG Reverse-AACTTAAAGGTTATATCTCATATC	53	55.0
29	Forward-TTTGAGCTGTCTTGACGTTCACAG Reverse-TTGAAATAGACATTGAAGGTGTCAAC	58	55.7
30	Forward-TTTTCATTTTGGAAGTTCACTGGTC Reverse-GGAATGTTCTATTATTAAACTCATC	56	55.0
31	Forward-GTGTATTTATTGTAGCCGAGTATC Reverse-GGAAGAACAGGATAGAAAGACTGC	56	54.5
32	Forward-GACTTGTGAATGAATTTATTTCAGAG Reverse-CACTCAAATCCTTCTAACAATAC	56	56.5
33	Forward-TTACAGTAAGTTTTGTTGTCTTAC Reverse-CAGATTTTTGAAAAGTACTACTATG	56	54.0
34	Forward-CCAATACGTGTTAAAAGCAAGTTAC Reverse-AACAGGTAGAAATAGCCCATGTC	58	56.4
35	Forward-AAACAAAAGTGTTGTCTTCATGCT Reverse-TCCTATATGTGATCCGCAGTTGAC	58	57.2
36	Forward-GTTTTATGTATGATCTCTTACCTATG Reverse-GAAGTATCATTCTCCATGAATGTC	58	55.5
37	Forward-CAGTCCACCTTAACATTCATCAAG Reverse-ACAGTCATGACCCACAGCAAACAG	58	55.6
38	Forward-AAGGTACAATGATTTCCACTTCTC Reverse-CAGCACTCTTTAGATAAACAGGTC	58	55.0
39	Forward-AGCAGTATGTTGAGTTTATGGCAG Reverse-GGATTCCATCTTAAATCCATCTTTC	58	55.2
40	Forward-CCTTATAGCATAGTGGGAGACAG Reverse-CAAGTTACACTCTAGATCCTAAACG	58	57.6
41	Forward-TAAGCAGTCACTACCATTGTATTC Reverse-TACCCTTATTGAGACAATGCCAAC	58	54.0
42	Forward-CAGGAGCTTCCAAATAGTATGTTC Reverse-CACATGGCATCTGTACAGTGTCT	58	54.5
43/44	Forward-TAGAGTTGGGAGTTACATATTGGT Reverse-GCACTACACTAGTGATGGCTTTAC	58	55.4
45	Forward-GTTTTTCTGTTGATATCTTTGATTAC Reverse-GAGAGGCAAAAAAAAAAAAATCAAGTC	56	55.7
46	Forward-GTCCTTTGGTGAAGCTATTTATAC Reverse-CTCAAGTTTTCAGAAAAGAAGCCA	58	54.2

47	Forward-CCTCTTCTTTATTTTCAGAGTGTC Reverse-GTCACTATTGGTAACAGAAAAGC	58	58.0
48	Forward-CATTTCTCTTTGCTTACATGAACTC Reverse-TAGAGATCTCTATCTCTTAATGAC	53	57.0
49	Forward-CATGGTAGTAGTATCAGTAGTAAAAG Reverse-CAGTAAAACACTAATCCAGCCAAT	58	57.5
50	Forward-AGTTGGGTACAGTCATGGTAATGC Reverse-CTAAGTAACTATCTTAAGGGTTGCTC	58	57.0
51	Forward-GTGTATTACCTTAATTTGAGTGATTC Reverse-AAGACCAAGTCACTCTTTCTATGC	58	56.1
52	Forward-ATCATGTGTGATTTTGTAGTTCTG Reverse-TTCAAGCACAGGGTAGAATATTGG	56	56.8
53	Forward-TTGTGCTAATAGAGGAGCACTGTC Reverse-GTATTTCCATTTCTTAGAGGGAATG	58	54.4
54	Forward-TCTACCCACTGCAGTATCTAGAC Reverse-CAGCCTTGAACCGATTTTAGATG	56	54.3
55	Forward-TGTTGGGTAGTTCCTTATGTAATG Reverse-GGATTACGTTTGTGATTTTAAGCAG	56	55.6
56	Forward-ACTATTCCTGCTTGACCTTCAATG Reverse-GCCAATATTTAACCAATTTTGACC	56	54.7
57	Forward-TAAGTGCAAATAGTGTATCTGACC Reverse-CATCACTAAAACTCTAAGGGCTAAG	56	57.3
58	Forward-TTGCTATTCTCAGATGACTCTGTG Reverse-GCCTCCCAAAGCATTATGAATATG	56	55.9
59	Forward -CATCAAATGCTCTTTAATGGCCTT Reverse-TGCCAAACAAACAAAGTGCTCAATC	58	57.3
60	Forward-CATCTTTATTGCCCCTATATCTGTC Reverse-TGCCAAACAACAAAGTGCTCAATC	58	56.0
61	Forward-AAGAGATGGAATCAGTGATTTCAG Reverse-AGGCAAACAACATTCCATGATGAC	58	54.3
62	Forward-TTAGCTGTCAAACCTCCTAACTTC Reverse-TTGAGTAGCGGGATTACAGGTG	58	57.2
63	Forward-AGATATGTTGACAACATTGGTGTG Reverse-GAGATACACAGTCTACCTGGTAAG	58	55.0
64	Forward-GATACTGGTTCTACTGTTTCTAAG Reverse-AAAGGTTTCAGTGAGGTGAACAG	58	56.3
65	Forward-GGTGAGCAGTATTTTAAGAAGGTC Reverse-TCCCTACTTAAAGTAGTTGGCAG	58	56.8

2.2.2. Denaturing High Performance Liquid Chromatography (DHPLC)

Screening for *ATM* sequence changes was performed using denaturing high performance liquid chromatography (DHPLC) approach by the WAVE® DNA fragment analysis system (Transgenomic, Glasgow, UK). Prior to analysing the PCR products on the

DHPLC machine, double stranded DNA was denatured at 95°C for 5 minutes and slowly hybridized back by decreasing the temperature by 1°C per minute, from 95°C to 65°C using PCR machine. If the individual has one allele mutated the hybridized DNA form a mixture of heterodupexes (two alleles are mis-matched) and homoduplexes (two alleles are perfectly matched). Wild-type samples would always form only homoduplexes.

Samples with 11q deletion in their leukemic clone would have only one *ATM* allele remaining therefore a potential sequence change might not be detected by DHPLC, as there would be no wild type allele to form a heteroduplex. Therefore, to analyse 11q deleted samples slightly modified protocol was applied. 10µl of the final product for each exon was mixed with 10ul of the product from a sample that was known to have both *ATM* wild-type alleles present. Subsequently both the mixed and unmixed products were analysed (Xiao and Oefner, 2001).

2.2.2.1. DHPLC principles

Distinction between homo- and heteroduplexes is possible due to their different properties, which is exploited by DHPLC to screen for potential mutation. WAVE® System separation principle is based on two important factors: an ion-pairing reagent which acts as a "bridging" molecule and DNASep cartridge matrix which is a stationary phase. DNASep cartridge matrix is electronically neutral and hydrophobic. The bridging molecules (triethylammonium acetate –TEAA) are positively charged and allow the interaction between negatively charged DNA fragments and the cartridge matrix. The concentration of TEAA and the temperature of the DNASep cartridge matrix are programmed to alter during the analysis. At a given cartridge temperature and TEAA concentration, the heteroduplexes are less stable (due to miss-match) and denature into single strand DNA (ssDNA) faster than homoduplexes. ssDNA has reduced negative charged and less affinity to the stationary phase than dsDNA therefore elutes from the cartridge earlier. The ideal temperature will be one where the degree of denaturation is predicted to be the greatest between a homoduplex and a heteroduplex. This can be identified using the Wavemaker software.

The choice of temperature is not always straightforward due to variations in the denaturing characteristics across any given exon. However, there is typically a range of temperatures over which it is theoretically possible to resolve the heteroduplexes from the homoduplexes. Occasionally, a few different melting temperatures have to be applied to the same exon to ensure that the whole fragment melts in the optimum conditions so no potential heteroduplex is missed. The temperatures for the DHPLC for each *ATM* exon are shown in Table 2.1.

Hetero- and homoduplexes eluted from the cartridge matrix are detected using a UV lamp and presented as peaks on so-called chromatographs. A single peak pattern is present if there are only homoduplexes in the sample. Any alteration in this single peak pattern is indicative of the presence of heteroduplexes in the sample. A schematic description of homo- and heteroduplex formation and an example of a chromatograph revealing the presence of a mutation are shown in Figure 2.1.

In this study, all chromatographs were analysed visually and compared across large numbers of samples for each exon. The PCR product was subsequently sequenced for all cases where a variant pattern of the peak was detected.

2.2.3. PCR product purification

Prior to sequencing PCR products were purified using ExoSAP-IT PCR Cleanup kit (GEhealthcare Life Sciences, Little Chalfont, UK). ExoSAP-IT consists of two enzymes, Exonuclease I (Exo) which removes residual single-stranded primers and Shrimp Alkaline Phosphatase (SAP) which removes remaining, unincorporated dNTPs. 2.5µl of each PCR product was mixed with 1µl ExoSAP-IT in PCR tube and incubated for 15 minutes at 37°C followed by further 15 minutes incubation at 80°C to inactivate the enzymes. The tubes were placed on ice ready to be used in sequencing reaction or frozen at -20C for later analysis.

2.2.4. Labelling of DNA and precipitation of products

Purified PCR products were fluorescently labelled using Big Dye Terminator v3.1

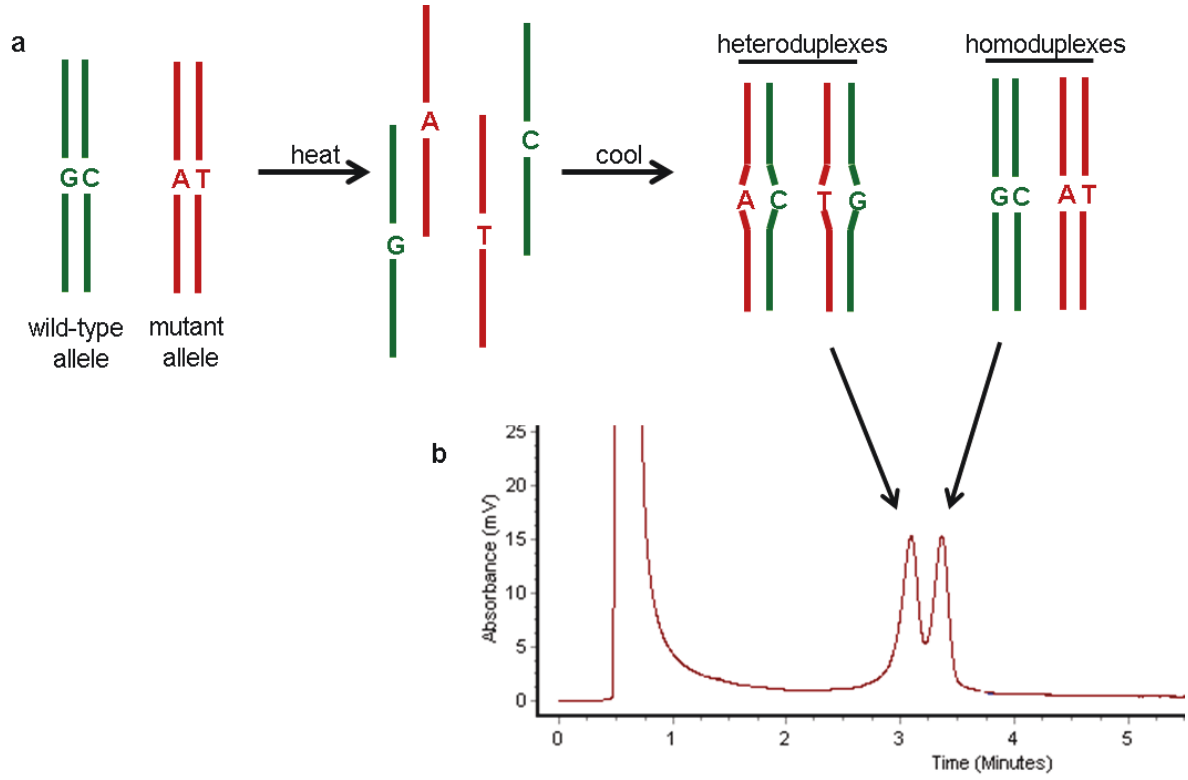


Figure 2.1. The principle of mutation detection by DHPLC.

(a) Formation of the homo- and heteroduplexes in an amplified sample containing mutated allele. PCR product is briefly heated which results in denaturation of the double stranded DNA and subsequently slowly cooled down allowing the formation of a mixture of matched (homoduplexes) and mis-matched (hetroduplexes) alleles' pairs. (b) Hetero – and homoduplexes are visualized as peaks on chromatograph. Since mis-matched heteroduplexes are less stable they elute from the column earlier than homoduplexes.

Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK). The reactions were set up with 1µl of Big Dye mix, 4 µl of the 5x sequencing buffer, 0.3µl of primer (0.06uM) and up to 14.7µl of DNA in sterile water to make a total reaction volume of 20µl. Big Dye mix contains ddNTPs (dideoxynuclotides, which lack 3'-hydroxyl group, labelled with four different fluorescent dyes), unlabelled dNTPs and Taq Polymerase. The sequencing reactions were set up separately for forward and/or reverse primers for each exon. The labelling PCR was carried out for 29 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 64°C for 4 minutes.

The labelled products were precipitated with 50µl of 100% (v/v) ethanol and 2µl of 3M sodium acetate (pH 4.6) in PCR 96 well plates at room temperatures for 30 minutes. The plates with the samples were then centrifuged at 2000 rpm for 20 minutes. The supernatant was removed and pellets were washed twice in 100µl of 70% ethanol (v/v) by centrifugation for 10 minutes. The supernatant was removed and DNA pellets allowed to air dry for approximately 2 hours.

2.2.5. The 3100 ABI prism™ DNA sequencer

Prior to loading in the capillary sequencer, 10µl of HiDi-Formamide™ (PE Applied Biosystems) was added to the DNA pellet. Samples were denatured by heating at 95°C for 5 minutes, cooled to 4°C in PCR machine and loaded on to MicroAmp™ optical 96-well reaction plates (PE Applied Biosystems). Before starting the run the buffer chambers were filled with 1x EDTA buffer (PE Applied Biosystem). POP-7™ polymer was used to fill the sequencer capillary array. The 3100 data collection software version 3.0 was set for standard mode. The results were analysed using the ABI sequencing analysis version 3.6.1.

2.3. Analysis of DNA sequences across immunoglobulin heavy chain rearranged VDJ regions and recombined switch regions

2.3.1. PCR reaction for IGH VDJ regions

Semi-nested PCR approach was adopted to analyse IGH VDJ regions in selected CLL tumours. Primers used in this analysis were design to correspond to consensus regions

flanking *IGHV* and *IGHJ* genes (Fr1 forward primer and JH6ex, JH6in reverse primers). This design allowed for the amplification of the full length of VDJ rearranged region. Semi-nested PCR consisted of two rounds of DNA amplification. In the first round 30ng of genomic DNA was mixed with 0.2μM of dNTPs (Invitrogen), 0.2μM of forward (Fr1) and reverse (JH6ex) primer each (Alta Bioscience), 10x Buffer1 containing 1.75mM $MgCl_2$ (at final concentration) and 3.75 units of Expand Long Template polymerase (Roche), and sterile water to form the total reaction volume of 50 μl. PCR conditions were: 4 minutes at 94°C, followed by 20 cycles of 20 seconds at 94°C for denaturation of dsDNA, 20 seconds at 60°C for primer annealing and 2 minutes and 30 seconds at 68°C for polymerase dependent DNA extension, and final extension of 7 minutes at 68°C. For the second round, 1μl of the PCR reaction product was transferred to the new PCR tube containing the reaction mix similar to the mix for the first round, but JH6in reverse primer was used instead of JH6ex. This time, 35 cycles were applied and annealing temperature was set at 62°C. Primer sequences are provided in Table 2.2. The semi-nested PCR conditions were previously optimised to provide the highest DNA yield.

Table 2.2. The list of primers used in the amplification and sequencing of rearranged IGH VDJ regions.

Primer name	Primer sequence
Fr1 forward	5'-SAGGTRCAGCTGBWGSAGTCNG-3'
JH6ex reverse	5'-GAAACCCCACAGGCAGTAGCAG-3'
JH6in reverse	5'-CAAAGGCCCTAGAGTGGCCATTC-3'
JH5 reverse	5'-AGAGAGGGGGTGGTGAGGACT-3'
JH4 reverse	5'-CAGAGTTAAAGCAGGAGAGAGGTTGT-3'
JH3 reverse	5'-AGGCAGAAGGAAAGCCATCTTAC-3'
JH2 reverse	5'-GGTGCCTGGACAGAGAAGACT-3'

2.3.2. Gel excisions and DNA purification

20µl of the second round PCR reaction was loaded on 1% agarose gel made up by dissolving 1g of agarose in 100ml of TBE buffer (45mM Tris, 45mM Boric acid, 1mM EDTA). To visualise DNA products on the gel 1µl of Ethidium Bromide was added to the mix. PCR products and 100bp molecular weight marker were mixed with DNA loading buffer (30% (v/v) Glycerol and 0.25% (w/v) Bromophenol Blue in sterile water). The PCR products were run at 100V for around 1hour 30 minutes and visualised by UV transilumination.

Amplification of VDJ rearranged regions of CLL PBMC samples was expected to yield not only a monoclonal, dominant product (from tumour cells) but also polyclonal products amplified from the normal B-cells compartment visualised as multiple fade bands or a smear. In addition, some CLL tumours might undergo bi-allelic rearrangements resulting in two dominant products. Therefore, in order to analyse clonal VDJ rearrangements, only dominant bands were excised from gel with a clean, sharp scalpel and stored in a separate 0.5ml tubes.

DNA was extracted from excised gel bands using QIAquick Gel Extraction kit protocol (QIAGEN). Briefly, to each tube, three volumes of QG Buffer were added to one volume of gel (e.g. 300µl of QG for 100mg of gel) and incubated at 50°C using vortexing every 3 minutes until the gel dissolved. One volume of 100% (v/v) isopropanol was added to tube and mixed. The mixture was transferred to a QIAquick column and microcentrifuged at 13,000 rpm for 1 minute to bind the DNA. The flow-through was discarded and 0.5ml QG Buffer was applied to the column and centrifuged to remove all traces of agarose. To wash, 0.75ml of PE buffer was added, centrifuged, discarded and an additional 1 minute of centrifugation was applied to fully dry the DNA. The QIAquick columns were placed into clean 1.5ml tubes and 30µl of EB Buffer was applied to the centre of the column, and microcentrifuged for 1 minute to elute the bound DNA.

2.3.3. Sequencing of VDJ regions

The excised from gel and purified PCR products were sequenced in both directions

(approximately 50 ng per reaction). Fr1 primer was used to sequence in forward direction and the range of primers was available to sequence in reverse Table 2.2. The choice of which reverse primer should be used for sequencing reaction was based on the PCR product size as visualized on an agarose gel. Since the amplification of rearranged VDJ region was performed on genomic DNA and the reverse primer in PCR reaction was binding at the consensus region flanking *IGHJ* genes, all *IGHJ* genes downstream from the one which was rearranged were still present in the DNA sequence. For example, if *IGHJ4* gene was rearranged, genes *IGHJ5* and *IGHJ6* would be amplified as well and PCR product size would be longer than if *IGHJ6* gene was rearranged, since only this *JH6* gene will be present in genomic DNA sequence (and thus amplified) Figure 2.2.

Labelling (BigDye) reactions, product precipitation and sequencer analysis was performed as outlined in sections 2.2.4. and 2.2.5.

2.3.4. Cloning and transformation of chemically Competent *E. Coli* cells

One of the analysed tumour samples underwent bi-allelic VDJ rearrangement. The amplified products were almost the same size and it was not possible to excise them separately from the gel. To isolate individual rearrangements TOPO TA Cloning® Kit (Invitrogen) was used. Approximately 40ng (4ul) of VDJ PCR product was transferred into 0.2ml tube and following reagents were added: 0.5µl of salt solution, 0.5µl of pCR™4-TOPO® linearized vector and 1ul of sterile water. The reagents were mixed gently and incubated at room temperature for 10 minutes to allow the ligation of the insert and the vector. Subsequently, 2µl of the cloning reaction was added to 25µl of defrosted One Shot®Top10 chemically Competent *Escherichia Coli* cells (Invitrogen) and very gently mixed. An additional tube containing only chemically competent cells was set as negative control. The tubes were left on ice for 5 minutes to allow E.Coli and ligated vectors to associate. The tubes were then heat-shocked for 30 seconds at 42°C in water bath, returned on ice, diluted with 75µl of pre-warmed S.O.C. medium, capped and shaken horizontally at 200rpm and 37°C for 1 hour. The E.Coli mixtures were spread on pre-made ampicillin (100µg/ml) LB agar

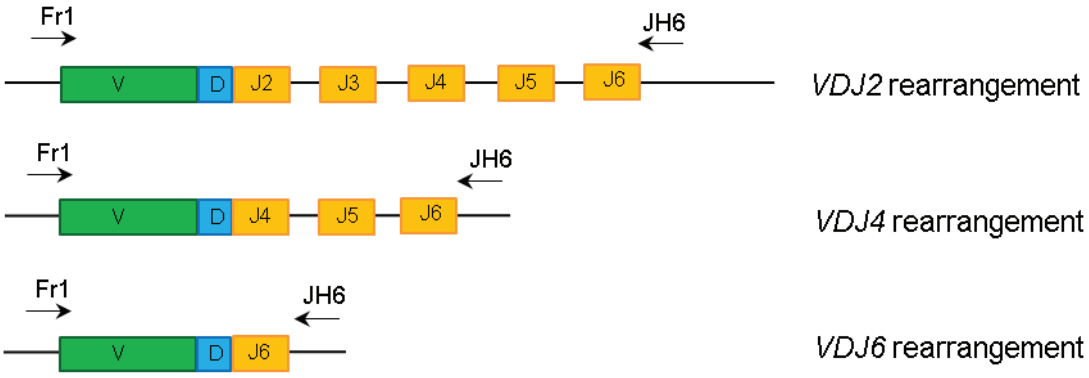


Figure 2.2. The amplification of the rearranged IGH VDJ regions from genomic DNA. The rearranged IGH VDJ regions were amplified from genomic DNA using primers Fr1 and JH6 which bind at 5'-end of rearranged *V* gene and at 3'-end of *J* gene, respectively. If *J2* gene was rearranged the PCR product will be longer than if *J4* or *J6* gene were rearranged. This is because all *J* genes downstream from the one which was rearranged are still present on the genomic DNA.

plates and incubated overnight at 37°C. Colonies were picked from the agar plate with the pipette tip and transferred to 0.2ml tube containing 10µl of sterile water. These were heated at 95°C for 5 minutes to lyse E.Coli and 5µl was amplified by PCR using TrueStart *Taq* polymerase (Invitrogen) with the primers provided in the kit (T3, T7). 2.5µl of the products were cleaned with ExoSap IT enzyme mix (as described in section 2.2.3.) and sequenced with Fr1 and Jh6in primers (as described in section 2.2.4.).

2.3.5. BLAST sequence alignment

To establish level of SHM and the usage of IGH *V D J* genes the sequences of rearranged VDJ regions were aligned to germ-line genes using IgBlast (Immunoglobulin Basic Local Alignment Sequence Tool) on the NCBI (National Centre for Biotechnology Information) website http://www.ncbi.nlm.nih.gov/igblast/.

2.4. Analysis of DNA sequences across immunoglobulin recombined switch junctions

2.4.1. PCR reaction for the recombined switch junctions

Genomic DNA was the only available material for CSR analysis. Therefore, in order to detect which tumours underwent CSR and to analyse clonal switch junctions, a single-round PCR approach was applied. The PCR reagents apart from primers were the same as for amplification of IGH VDJ regions (section 2.3.1.). Two PCR reactions with two different primers pairs were set up for each tumour sample. To identify the clones which have undergone CSR to IgA, Sµ1 and Sα-Common1 primers were used; to identify the clones which has undergone CSR to IgG, Sµ1 and Sγ-Common primers were used (primer' sequences are provided in Table 2.3.). These consensus primers correspond to conserved and unique regions flanking more polymorphic switch regions. Although these primers are usually used in nested PCR technique to analyse switch junctions in normal polyclonal B-cells (Pan et al., 2001; Pan et al., 1997), it was reasoned that amplification of switch regions in monoclonal population using one-round approach should yield sufficient amount of DNA products, considering that the CLL clones underwent CSR. Two additional CLL clones were

previously identified as those which switched to either IgA or IgG and were used as positive controls in this analysis.

Table 2.3. The list of primers used in the amplification and sequencing of Sμ-Sα and Sμ-Sγ class switch regions.

Primer name	Primer sequence
Sμ1	5'-GACCATGGGGACCTGCTCATTTTTATC-3'
Sα-Common1	5'-ACGTCGACGCCCCTCAGAACCCCTAAGAA-3'
Sγ-Common	5'-GTCTGCAGTGCCCCTGCCTGAGAGC-3'

PCR conditions were: 4 minutes at 94°C, followed by 37 cycles of 1 minute at 94°C for denaturation of dsDNA, 1minute at 65°C for primer annealing and 1 minute and 30 seconds at 68°C for polymerase dependent DNA extension, with a final extension of 7 minutes at 68°C. 20μl of each PCR product was run on the agarose gel. Gel excision of the strongest band, labelling reactions, product precipitation and sequencing analysis were performed as outlined in sections 2.3.2., 2.2.4. and 2.2.5.

2.4.2. BLAST sequence alignment.

In order to analyse the switch junctions including the degree of microhomology, the sequences were aligned to unrecombined germ-line DNA using online BLAST database http://blast.ncbi.nlm.nih.gov/Blast.cgi. The search option was selected for 'somewhat similar sequences', and the algorithm parameters were set for a word size 7 with the low complexity filter disabled.

2.5. Preparation of the human cells prior the injection into mice.

Prior to injection into mice, specific type and number of human cells were prepared. This preparation involved isolation of CD14 positive ($^{+}$) cells from whole blood, isolation of

$CD34^+$ cells from cord blood samples and depletion of different cell subsets from CLL PBMCs.

2.5.1. Isolation of human mature CD14 positive cells

$CD14^+$ cells were negatively selected from healthy volunteers bloods (School of Cancer Sciences, University of Birmingham) using RosetteSep™ Human Monocyte Enrichment Cocktail (STEMCELL, Manchester, UK) containing antibody against CD2, CD3, CD8, CD19, CD56, CD66b, CD123 present on human hematopoietic cells and against glycophorin A present on red blood cells bound in Tetrameric Antibodiy Complexes (TAC). Ten ml of fresh blood was mixed with EDTA to a final concentration of 1mM (200ul of 50mM EDTA was added into 10ml of blood) and with 500ul of antibodies cocktail and incubated at room temperature for 20 minutes. The blood sample was diluted with 10ml of PBS + 2% FBS and 1mM EDTA, mixed gently, layered on 13ml of lymphoprep solution, and spun at 1200xg for 20 minutes at 20°C with the break off. The plasma interface enriched with $CD14^+$ cells was transfer to a new 50ml tube and washed twice with PBS plus 2% FBS and 1mM EDTA. Purity of monocytes was measured by FACS after staining with FITC conjugated anti-CD14 antibody (Beckman Coulter, High Wycombe, UK)

2.5.2. Magnetic cell sorting (MACS) of different cell subtypes.

To sort out $CD34^+$ cells from cord blood PBMCs and to deplete CLL PBMCs of $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$ and $CD56^+$ cells, magnetic cell sorting (MACS) system was utilized (Miltenyi Biotec, Bisley, UK). The following kits were used: human CD34 MicroBead Kit, human CD3 MicroBeads, human CD4 MicroBeads, human CD8 MicroBeds, human CD25 MicroBeads II and human CD56 MicroBeads.

Frozen CLL PBMC and cord blood PBMC samples were thawed, washed twice in RPMI medium and one time in MACS buffer containing PBS, 0.5% BSA and 2mM EDTA. Up to $1x10^8$ cells were suspended in 800ul of the buffer and mixed with 200µl of Micro Beads conjugated to monoclonal anti-human antibodies. The samples were incubated at 4°C for 15

minutes, washed in cold MACS buffer and centrifuged at 1600rpm for 10 minutes. After complete removal of the supernatant the cells were suspended in 500ul of MACS buffer and loaded onto LS column which was previously assembled on magnetic separator and rinsed with 3ml MACS buffer. The column was washed 3 times, each time with 3ml of the buffer applied once the column reservoir was empty. Total fraction of the unlabelled cells which passed through the column was collected in the universal tube. The column was then removed from the separator and placed on the new collection tube. 5ml of the buffer was added into the column and the magnetically labelled cells were flashed out by firmly applying the plunger. Both fractions were placed on ice. The depletion and the purity of the unlabelled and labelled fractions respectively, were assessed by FACS analysis by single antibody staining. Antibodies used for immuno-staining recognizing the same cell markers as during the depletion procedure and are presented in Table 2.4.

The above protocol was applied to all sort types with small modifications following manufacturer's instructions. CD34 labelled cells or CD3, CD4, CD8, CD25, CD56 depleted cell populations were injected into mice later on the same day. The CD34 negative ($^-$) cell fraction was refrozen in liquid nitrogen for further use in microsatellite analysis (section 3.7.).

2.5.3. Gradual depletion of autologous T-cells.

Some experiments involved a partial depletion of autologous T-cells from CLL PBMCs prior to the injection into the mice. Using magnetic cell sorting beads with conjugated CD3 antibodies three different fractions of the same CLL PBMC sample were prepared to be injected: 1) PBMC fraction which contained all autologous T-cells and where depletion procedure has not been performed; 2) PBMC fraction in which the frequency of T-cells was between 10-25% of the original T-cell number; 3) PBMC fraction in which the level of autologous T-cells was below 5% of the original T-cell number before depletion. To obtain the T-cell depletion level of 10-25% of the original T-cell number the magnetically labelled $CD3^+$ cells were put back together with the unlabelled, $CD3^-$ fraction of CLL PBMC in the adequate proportions. This was calculated based on the cell count in both fractions and on

the cell purity assessed by FACS analysis after depletion procedure. Depletion of T-cells down to the level of 1.5-5% was the most achievable depletion and might have been a result of insufficient binding of those cells to magnetic column during separation. No mixing of the fractions was performed to achieve this depletion level. Equal numbers of PBMC cells for each depletion fractions were injected into mice on the same day following CD3 magnetic sorting.

2.5.4. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling of CLL PBMC.

CFSE Is a fluorescent dye which covalently couples with intracellular macromolecules and is not transferred to adjacent cells in a population. The dye-protein adducts are inherited by the daughter cells and the intensity of CFSE is progressively halving following each cell division. CFSE can be used for the cell tracking in animals injected with labelled cells and to measure proliferation and cell division *in vivo* and *in vitro* using flow cytometry.

In order to track CLL cells migration, homing and proliferation in mice blood and organs over a period of engraftment, tumour PBMC samples were labelled with CFSE prior to the injection into the animals. Viable cells were suspended in PBS at the concentration of $2x10^7$cells / 1ml of PBS and incubated with 10μM CFSE (Sigma-Aldrich) for 15 minutes at 37°C, in the dark. They were washed twice with cold RPMI with 10% FSC to stop the binding reaction and to remove the access of the dye. Finally, they were suspended in 100μl of PBS, ready to be injected into mice.

2.6. Xenogenic mouse transplantation.

2.6.1. NOG mice.

All injections were performed using NOG (NOD/Shi-*scid/IL-2Rγ^{null}*) mice (Central Institute for Experimental Animals, CIEA, Japan). The strain was derived by cross breeding of three main mouse strains: 1) non-obese diabetic - NOD/Shi; 2) severe combined immune deficiency – SCID; 3) interleukin 2 receptor-gamma-null - IL-2Rγ$^{null.}$ The NOG mice have a multiple immuno-deficiencies due to combined genetic backgrounds. NOD/Shi background is

responsible for macrophage dysfunction and reduced hemolytic complement activity (complement play role in recognition and destruction of red blood cells either in ABO non-matched transfusions or during bacterial and viral infections) (Shultz et al., 1995). NOG mice lack functional B and T-cells mainly due to SCID background which is a result of nonsense mutation in Tyr-4046 of the gene encoding DNA-dependent protein kinase catalytic subunit (DNA-PKcs)- *Prkdc* (Araki et al., 1997). This mutation affects DNA-PKcs role in DNA repair pathways leading to aberrant processing of V(D)J recombination in lymphocytes, aberration in T- and B-cell receptors (TCR, BCR) rearrangements and a high sensitivity to ionizing radiation (Danska et al., 1996). Finally, truncating mutation in interleukin-2 gamma chain gene *IL-2Rγ* is causing severe defects in NK cells activity and dendritic cell dysfunction (Ohbo et al., 1996), (Ito et al., 2002).

All mice were bred in the Biomedical Services Unit (BMSU) at the University of Birmingham and housed in ventilated micro-isolator cages with autoclaved water and irradiated food under specific pathogen–free conditions.

2.6.2. Humanization of NOG mice with allogeneic monocytes.

Four to nine weeks old NOG mice were gamma irradiated 2.5 Gy and within 3 hours intravenously (iv) co-injected with human allogeneic monocytes (10^5 in 100µl of PBS per injection) derived from healthy individuals and CFSE labelled CLL PBMC cells ($0.5x10^8$ cells suspended in 100µl PBS per injection). Mice were maintained on antibiotic Baytril in the drinking water at 25.5 mg/kg for 1 week prior injection. Starting one week post cell transfer, a 50µl of peripheral blood samples (from the tail) was collected into a 1.5ml tube containing sodium heparin and analysed by FACS to access the engraftment kinetics. Tail bleeding was performed on a weekly basis.

2.6.3. Humanization of NOG mice with cord blood CD34-positive stem cells.

Four to nine week old NOG mice were gamma irradiated (1.5-2.5 Gy) and iv injected (into the tail) within 3 hours with human cord blood CD34 positive cells -10^5/100µl PBS.

Gamma irradiation was decreased during this study period from 2.5 Gy to 1.5 Gy to prevent animal death from irradiation. Mice were maintained on Baytril in the drinking water at 25.5 mg/kg for 1 week prior to the injection. Four weeks later 50µl of peripheral blood was taken from mouse tail to assess human haematopoietic engraftment. Engraftment was considered adequate if 1-10% of human CD45 positive cells were detected in the blood as measured by FACS analysis. Within the next couple of days mice were iv injected with CFSE labelled tumour PBMC cells (0.13-1x10^8 cells suspended in 100µl PBS per injection). Weekly or every few weeks approximately 50µl of peripheral blood (from tail) was collected into a 1.5ml tube containing sodium heparin and analysed by FACS to access the engraftment kinetics. At a fixed time point or when the sign of illness occurred, animals were culled by cervical dislocation and organs were harvested for analysis. The symptoms which were indication for culling included: paleness, lethargy, hunched posture, ruffled fur and weight loss. Xenogenic mouse transplantation procedure is based on the method developed by Bagnara and colleagues (Bagnara et al., 2011).

2.6.4. Extraction of the cells from mouse bloods.

Murine peripheral blood samples were diluted with 10x volume of lysis buffer (BD Pharm Lyse™, BD Biosciences, Oxford UK) in FACS tubes and incubated at room temperature for 15 minutes to remove red blood cells. The samples were centrifuged in a swing-out rotor at 1500rpm for 5 minutes and the supernatants were removed. Prior to staining for FACS analysis, 10µl from each sample was taken and combined in a separate tube to form an unstained control.

2.6.5. Extraction of the cells from murine organs.

Murine organs analysed by FACS included: spleen, bone marrow and peritoneum. Half of the spleen and peritoneum were mashed through sterile tea strainer in 10ml of RPMI on 90mm Petri dish. The cell clumps were filtered through 70µm cell strainer (BD Biosciences) and 10ml of the tissue samples were layered over 10ml of lymphoprep solution

(Axis-Shield). Samples were centrifuged at 1600rpm in a swing-out rotor for 20 minutes with the brakes off. Meantime, bone marrow from one femur was flushed out using 25 gauge needle into 5ml of RPMI on a 90mm petri dish. All tissues samples were transferred to Falcon FACS tubes and washed 3 times with PBS. At this point, at least $2x10^6$ cells (when possible) from spleen or bone marrow sample was transferred to another tube and saved for DNA extraction.

2.6.6. Fluorescence activated cell sorting (FACS) analysis of the human cells engraftment.

FACS analysis was performed on LSRII (BD Biosciences) supported by BD FACSDiva™ software v.6.1.2. All antibodies were titrated to obtain the concentrations which allowed optimal separation between cell populations with the minimum use of the reagent. The samples were incubated with antibodies for 30 minutes at 4°C, washed by centrifugation with cold PBS and resuspended either in 200µl PBS for the assessment of the purity after $CD14^+$ isolation and microbeads depletion; or 200µl of 1% Formaldehyde (mice blood samples) and 500 µl of 1% Formaldehyde (mice organ samples) to asses the engraftment. Samples stained for isolation purity assessments were analysed on the same day whereas mice bloods and organs samples were usually stored at 4°C for up to a few days before FACS analysis.

All blood and organ samples were stained with a cocktail of fluorochromes conjugated antibodies which recognize different cell markers. The antibodies which were used to detect cells with particular cell markers are provided in Table 2.4.

The emission spectras of different fluorochromes overlap resulting in the detection of more than one fluorochrome when measured with a particular filter. To correct for this spectral overlap, compensation beads (BD Biosciences) stained with a single fluorochrome were prepared. Compensation beads are the small polysteryne microspheres that are either coated with IgG mouse or rat (positive beads), or uncoated (negative beads). A mix of 1 drop of positive and 1 drop of negative beads with 100µl of PBS was prepared in a test FACS

tube. Each of the antibodies was added to a separate test tube and mixed with compensation beads. The volumes of antibodies being added to compensation beads were the same as for cell staining (Table 2.4). The beads with antibodies were incubated for 15 minutes at room temperature, in the dark, washed with 2 ml of PBS and centrifuged at 1500 rpm for 5 minutes. The pellets were re-suspended in 500µl of PBS, stored at 4°C and used within 2 weeks. These compensation beads were used every time the samples were analysed and they provided a separate positive and negative peaks for each fluorochrome used. Based on their emission spectras, the compensation was applied automatically using FACSDiva™ software.

Table.2.4. Antibodies used in FACS analysis.

Antibody	µl per 100µl of sample	Company
Used for labelling murine blood and organs		
anti-mouse CD45 Alexa Fluor 700	0.5	eBioscience
anti-human CD45-V450	3	eBioscience
anti-human CD3-APC-Cy7	2.5	BD Biosciences
anti-human CD19-PeCy7	1.25	eBioscience
anti-human CD5-APC	1.25	eBioscience
Used to assess the purity of the cell fractions after separation		
anti-human CD14-FITC	10	Beckman coulter
anti-human CD34-FITC	20	BD Biosciences
anti-human CD3-FITC	3	Beckman coulter
anti-human CD4-APC-Cy7	2	BD Biosciences
anti-human CD8-APC	2	BD Biosciences
anti-human CD25-APC	2	BD Biosciences
anti-human CD56-PE	2	BD Biosciences
anti-human CD19-PerCP	20	Beckman coulter

At least $10x10^4$ cells of each murine blood and organ sample were analysed by FACS. To assess the engraftment of total human cells and $CFSE^+$ cells, the gates were set on mononuclear live cell populations. Human B-cells were defined as mouseCD45/human$CD45^+$/$hCD5^+$/$hCD3^-$/$hCD19^+$. Human T-cells were defined as $mCD45/hCD45^+/hCD5^+/hCD3^+$. $CFSE^+$ B-cells (CLL cells) were defined as $CFSE^+/hCD5^+/hCD3^-/hCD19^+$ and $CFSE^+$ T-cells were defined as $CFSE^+/hCD5^+/hCD3^+/hCD19^-$.

2.7. Immunohistochemistry.

Immunohistochemistry analysis was performed by Dr Zbigniew Rudzki and Mr Gavin Rock in Pathology Department, Heartlands Hospital, Birmingham, UK. Upon culling, the animal organs (spleen, femoral bone marrow, peritoneum, and in some cases kidney, liver, brain, skin, lungs) were fixed in 1% formalin and stored at 4°C until analysis. Organs were paraffin-embedded, cut at 5μm–thick sections, and stained with hematoxylin and eosin. Antigen retrieval was performed in 10mM citrate buffer with a microwave oven. Immunophenotyping was carried out using anti–human antibodies desribed in Table 2.5. A standard set of human tissue sections positive for the respective antigens, mounted on the same slide, was used as positive controls; each antibody was also tested on murine tissues to assure lack of cross-reactivity with mouse antigens).

Table 2.5. Antibodies used in immunohistochemistry.

Antibody	Company	Dilution
ATM	In-house	prediluted
Pax5	ThermoScientific	1:50
CD5	Leica	1:100
CD3	Leica	1:25
Ki67	Dako	1:100
EBV	Roche	prediluted

2.8. Microsatellite DNA analysis.

Microsatellite analysis was performed in Molecular Genetics Laboratory, Birmingham Women's Hospital, UK. Three different genomic DNA samples were prepared for each microsatellite analysis, including 1)DNA from murine spleens infiltrated by human cells, 2) cord blood $CD34^-$ cells DNA and 3)DNA from CLL cells saved from the PBMC sample before the injection to animals. In addition, murine DNA was used as the negative control for species specificity of the microsatellites markers.

Genomic DNA from frozen cells of murine spleens and from cord blood CD34 depleted PBMCs was extracted using Qiagen FlexiGen kit following the manufacturer protocol -'Extraction of DNA from cultured cells', and performed as described in section 2.1.6. To sort out human $CD3^+$ T-cells from murine spleens, MACS protocol with anti-human CD3 Microbeads was performed (as described in section 2.5.2) using the column with a smaller capacity (MS column).

Sixteen different markers, specific to particular regions on various chromosomes were used. The procedure was performed following the protocol applied normally to analyse chimaerism after bone marrow transplantation (BMT) in humans. The typical sensitivity of BMT monitoring calculations is established to be 1–2% level. Each DNA sample was tested by multiplex PCR using 16 fluorescently labelled primers sets. Fluorescently labelled products with size standards were run on ABI 3130 (PE Applied Biosystems) where they were size-separated by the capillary electrophoresis. ABI data was then analysed using GeneMapper software with appropriate settings. Two markers were selected based on variation between CLL and cord blood DNA and ease of PCR amplification. The prevalence of these two markers was used to quantify the proportion of cord blood and CLL DNA in murine spleen.

2.9. Statistical analysis.

In the Result 1 chapter the Kaplan Meier method was used to calculate overall survival, and log rank test was used to compare the significance of any differences of

survival between patients' subgroups. Chi-square test and Fisher exact t-test were used to compare clinical parameters between different CLL subgroups and frequencies of *ATM* mutations between different cohorts.

In the Results 2 chapter Fisher's Exact t-test was used to compare clinical and biological characteristic between the CLL sub-groups with and without *ATM* mutation. To compare the three age groups Chi-square test was used. Logistic regression analysis was applied to test probability of involvement of multiple tissue sites when *ATM* mutation, 11q deletion, and anaemia and thrombocytopenia were also taken under consideration. Response to therapy was primary tested by Fisher Exact test and responses to Chlorambucil were tested using logistic regression model. OS and PFS comparison between *ATM* mutation and 11q deletion groups was initially conducted using Kaplan-Meier survival analysis and log rank test, and subsequently multivariable analysis using Cox's proportional hazards regression. All statistical analysis was performed using two-sided 5% significance level.

In the Results 3 chapter, Kaplan Meier analysis and log rank test was used to compare survival of different subgroups of mice, the time to onset of T- cell outgrowth in murine blood, and the time from the first bleed to the final bleed before cull in different animal groups. To compare the human cells engraftment levels between different groups of animals and different organs, Mann-Whitney U test (when comparing two groups) or 2way-ANOVA (when comparing more than two groups) were used. To compare the relative engraftment of $hCD45^+$ cells that are B- or T-cells, between the first bleed and the final bleed before cull, the Wilcoxon test for matched-paired samples was used. All statistical analysis was performed using two-sided 5% significance level. Data were presented as the mean ± SEM unless otherwise specified.

CHAPTER III

RESULTS I

The role of *ATM* germ-line pathogenic mutations in initiation and progression of CLL.

3.1. Introduction

ATM mutations are considered to be late events in CLL tumorogensis, but in a small proportion of cases they have been also detected in a patient's germline (Bullrich et al., 1999; Stankovic et al., 2002b). In these early studies germ-line changes were found to be more frequent in the CLL patients than had been previously reported for general population, suggesting the possibility of a causative role for *ATM* mutations in the development of CLL (Stankovic et al., 2002b). However, due to small CLL cohorts' sizes in those studies, valid comparison was not possible.

Another *ATM* aberration detected in CLL tumours is loss of one *ATM* allele through deletion of long arm of chromosome 11. This chromosomal abnormality is a well established marker of poor prognosis and is frequently associated with the presence of an *ATM* mutation on the remaining allele (Austen et al., 2007; Navrkalova et al., 2013). Cases with bi-allelic *ATM* inactivation have been shown to have a poorer prognosis than those with mono-allelic loss or mutation supporting the concept of a stepwise acquisition of genetic defects in tumour development (Austen et al., 2007). The exact proportions of germ-line *ATM* mutations in CLL cases with and without chromosome 11q deletion are unknown. Furthermore, the impact of constitutional *ATM* mutations on CLL initiation and progression (via promoting bi-allelic *ATM* inactivation) has never been established.

Therefore, the aim of the study described in this chapter has been to establish whether germ-line *ATM* mutations contribute to initiation and progression of CLL and whether in this respect, there is a difference between tumours that carry 11q deletion and those that do not.

I have addressed this by:

1) Determining and comparing the frequency of germ-line pathogenic *ATM* sequence alterations between a cohort of CLL patients (including aggressive tumours with 11q deletion and a more indolent group without 11q deletion) and a control population.

2) Establishing the usage of *VDJ* genes, *IGHV* mutation status and efficiency of CSR processes occurring in tumours of *ATM* mutation carriers.

3) Comparing the clinical features of *ATM* carriers to the rest of the CLL cohort.

In addition, I have investigated the nature, distribution and frequency of all other *ATM* sequence changes detected in both CLL and control cohorts.

3.2. Characteristic of the cohorts

3.2.1. Cohort of CLL patients

The frequency of pathogenic germ-line *ATM* mutations in CLL patients was assessed in a cohort of 318 CLL patients for whom 11q status was known. 140 CLL patients had deletion of 11q in their tumour clone. The remaining 178 patients did not have 11q deletion. In total, the CLL cohort included patients from Birmingham Hospitals (n=94), Bournemouth and Leicester Hospitals (n=61) as well as patients treated on the LRF supported UK CLL4 Trial (n=163, 100 with and 63 without chromosome 11q deletion). Thus, the cohort was enriched for aggressive CLL tumours by inclusion of cases from the LRF UK CLL4 Trial who required treatment.

The choice of CLL cases used here was determined by material availability and power of the study was calculated by following analysis. Assuming that *ATM* germ-line mutations occur in 0.4% of the general population in UK (Thompson et al., 2005), and *ATM* germ-line mutations have been reported to be associated with the risk of breast cancer in the study where 2.7% of cases were AT carriers (Renwick et al., 2006), a study with 281 controls and 318 CLL cases would have predicted 80% power of detecting 8-fold increase in CLL risk at the significance level of 5%.

In order to compare the clinical features of *ATM* carriers with the rest of the CLL cohort I have collected available information including: age and stage at diagnosis, *IGHV* mutation status and date of death. In total, the mean age at diagnosis for the cohort of 318 patients was 63.7 years. Information about disease stage was available for 301 patients; 137 patients were diagnosed with Binet stage A disease and 164 patients with stage B or C. The *IGHV* status had been determined for 284 patients, 119 patients had mutated *IGHV* genes and 165 had unmutated *IGHV* status (Table 3.1).

Table 3.1. Clinical characteristics of 318 CLL patients.

Clinical feature	Total n=318
mean age at diagnosis	63.7
stage at diagnosis A B/C	(n=301) 137 164
IGHV status mutated unmutated	(n=284) 119 165
11q deletion No Yes	 178 140

IGHV - Immunoglobulin heavy chain variable gene

3.2.2. Cohort of control individuals

The control cohort was determined by material availability. It consisted of 71 Caucasian healthy volunteers over 65 years old plus a further 210 samples from anonymous blood donors making a total of 281 individuals. It was not possible to use age matched control and CLL cohorts because the median age of the control population was unknown. However, it is expected that the control group was enriched for healthy young individuals.

If controls were indeed younger than patients, then there is a possibility some of them could develop CLL later in life as the median age of diagnosis is 65-70. Therefore these individuals would be misclassified as controls.

On the other hand, recent studies show that the accumulation of single nucleotide variants (SNVs) in hematopoietic stem/progenitor cells (HSC) of healthy individuals with normal blood counts is a function of age (Welch et al., 2012). Accordingly, the presence of an *ATM* mutation is more probable in older individual. Theoretically, HSCs with an *ATM* mutation could give rise to a small, non-tumorous population of B-cells which could be detected by the sensitive DHPLC technique (described in Material and Methods chapter, section 2.2.2). Therefore, it remains possible that the incidence of *ATM* pathogenic mutations in younger controls would be lower than in age-matched individuals. This could potentially confound the results and is therefore considered to be a limitation of this study.

3.3. Criteria for *ATM* pathogenic mutations

ATM is a highly polymorphic gene and many previously unreported sequence variants with unknown functional consequences are frequently detected in CLL tumours. The majority of sequence changes identified in CLL samples are single nucleotide substitutions which lead to amino acid substitution in the translated protein. Some of those missense changes have been reported to be causative in AT patients and some to be polymorphic variants (common in the general population with no or unproven impact on protein function). However, many of them are novel changes and their classification is difficult. A strong indication that a missense change is pathogenic is its localization within a region encoding functional domain in the translated protein and/or if it is predicted to affect an amino acid residue which is conserved between species. However, the only way to prove functional consequences of those novel sequence changes is by mutation modelling.

If a single nucleotide substitution occurs near an intron/exon boundary it is likely to interfere with the splicing process. It is particularly deleterious, if it alters conserved GT and AG dinucleotide at the donor acceptor splicing sites. In AT cell lines this usually results in skipping of an entire exon or in activation of the cryptic splice sites and loss of ATM protein expression and/or activity (Teraoka et al., 1999).

Short in-frame deletions/insertions have also been reported in CLL tumours. In AT patients they may result in the absence of kinase activity and therefore are likely to be pathogenic (Stewart et al., 2001).

Other sequence changes present in CLL tumour cells include truncating mutations. These are either the result of nucleotide substitutions that directly lead to formation of a stop codon or out of frame deletions or insertions which cause a translational frame shift leading to the generation of a premature stop codon. Truncating mutations result in the protein instability. Consequently, western blot analysis of AT cell lines containing truncating mutations shows no expression of ATM protein (Reiman et al., 2011).

The crucial challenge in this study was to define criteria for the identification of sequence changes with pathologic consequences. A sequence change was classified as

pathogenic if it met at least one of the following criteria:

1) Previously reported as causative mutations in Ataxia Telangiectasia (AT) patient;

2) Predicted to alter conserved GT and AG dinucleotide at the donor acceptor splicing sites, likely interfering with splicing process;

3) Predicted to cause protein truncation;

4) Was short in frame deletion or insertion.

All remaining sequence changes which did not fall into this category were classified either as: *missense variants,* if they were predicted to cause amino acid substitution in the translated protein but had neither been previously reported as causative in AT patients, nor found to represent a polymorphic variant; or *neutral sequence changes* if they had been previously reported as polymorphisms or were not predicted to cause an amino-acid substitution (silent or intronic changes) and their potential impact on the splicing process has not been investigated or proven.

Each detected sequence change was assigned to one of the above category after it was compared to previously published studies and to Leiden Open Variation Database (http://chromium.liacs.nl/LOVD2/home.php?select_db=ATM).

3.4. *ATM* sequence changes in the CLL patients and controls

Of 318 CLL patients 155 have been previously analysed for *ATM* mutations and the results have been reported (Austen et al., 2005; Austen et al., 2007). The remaining 163 CLL cases were analysed in this study.

Altogether, using DHPLC and Sanger sequencing approach, 85 different sequence changes were detected in 318 CLL patients, which included 19 changes also present in control individuals and 66 changes observed solely within the CLL cohort. Seventy five of the changes were present within the coding exonic regions and 10 within flanking intronic sequences. Sixty seven changes were nucleotide substitutions, 16 were nucleotide deletions (deletions ranged from 1 to 34 nucleotides) and 2 changes were single nucleotide insertions. Sixty seven sequence changes were identified in just one CLL patient and 18 were identified

in more than one CLL case. Using the criteria mentioned above, 31 changes were classified as *pathogenic ATM mutations*, 26 were *missense variants* and the remaining 28 were *neutral sequence changes*.

Of 281 controls, 171 samples had been previously analysed for *ATM* mutations. The remaining 110 controls were analysed during this study. Thirty five different sequence changes were detected in 281 controls. Twenty six of them were present within the coding exonic regions and 9 within flanking intronic sequences. Thirty two changes were nucleotide substitutions, one was a single nucleotide deletion and 2 changes were single nucleotide insertions. No change fulfilled the criteria for a *pathogenic ATM mutation*; 5 were classified as *missense variants* and 30 as *neutral sequence changes,* including known polymorphisms.

3.4.1. *ATM* pathogenic mutations in CLL patients.

Thirty two CLL patients had pathogenic *ATM* mutations: 24 CLLs with 11q deletion and 8 CLLs without 11q deletion. One pathogenic mutation (c.5228 C>T) occurred twice: in a patient with 11q deletion and in a patient without 11q deletion. In total, 4 mutations were predicted to cause amino-acid substitutions, 4 were splice site defects, 20 were predicted to cause protein truncation and 3 were short in-frame deletions. Seventeen of the 31 pathogenic mutations have previously been reported as causative in AT patients (Table 3.2.).

3.4.1.1. CLL patients with 11q deletion.

In 11q deleted cases three pathogenic mutations were predicted to cause amino-acid substitutions and had been previously detected in AT patients: (c.5228C>T [p.T1743I], c.8672G>A [p.Gly2891Asp], c.9023G>A [p.Arg3008His]) (Byrd et al., 2011; Stankovic et al., 1998) and unpublished data. Missense mutation c.9023G>A has been also reported in the DLBCL tumour (Gronbaek et al., 2002), in MCL tumour cells with a low ATM protein level (Camacho et al., 2002) and in another CLL sample (Schaffner et al., 1999).

Three mutations were predicted to alter splicing: (c.1066-6T>G [splice site exon 11], c.5006-2A>G [splice site exon 36], c.8787-1G>T [splice site exon 63]).

Table 3.2. Pathogenic *ATM* mutations in CLL cohort.

Sequence change	Amino Acid change	Mutation type	Previously observed in AT patients
Pathogenic *ATM* mutations in tumours with 11q deletion			
c.478_482delTCTCA	p.(Ser160fs)	truncating	no
c.1066-6T>G	splicing site exon 11	splice defect	yes
c.1120C>T	p.(Gln374X)	truncating	yes
c.1402delAA	p.(Lys468fs)	truncating	yes
c.2308G>T	p.(Glu770X)	truncating	no
c.2720_2723delGTGT	p.(Cys907fs)	truncating	yes
c.3651delG	p.(Leu1217fs)	truncating	no
c.3712_3716delTTATT	p.(Leu1238fs)	truncating	yes
c.3720_3736del17	p.(Asn1240fs)	truncating	no
c.3883_3885delCTT	p.(Lys1295del)	in-frame deletion	no
c.4591C>T	p.(Gln1531X)	truncating	no
c.5006-2A>G	splicing site exon 36	splice defect	no
c.5228C>T **	p.(Thr17431Ile)	missense	yes
c.6375insT	p.(Glu2126fs)	truncating	yes
c.6815delA	p.(Glu2272fs)	truncating	no
c.6989_6995del7	p.(Leu2330fs)	truncating	no
c.7638_7646del9	p.(Arg2547_Ser2549del)	in-frame deletion	yes
c.7883_7887del5	p.(Ile2628fs)	truncating	yes
c.8246_8252del7insT	p.(Lys2749_Thr2751delinsIle)	in-frame deletion	no
c.8672G>A	p.(Gly2891Asp)	missense	yes
c.8787-1G>T	splicing site exon 63	splice defect	yes
c.8977C>T	p.(Arg2993X)	truncating	yes
c.9023G>A	p.(Arg3008His)	missense	yes
c.9139C>T	p.(Arg3047X)	truncating	yes
Pathogenic *ATM* mutations in tumours without 11q deletion			
c.1058_1059delGT	p.(Cys353fs)	truncating	no
c.2193delC	p.(Tyr731fs)	truncating	no
c.2466+2T>G	splicing site exon 18	splice defect	no
c.4095_4109+4del19	p.(Lys1365fs)	truncating	no
c.5228C>T	p.(Thr17431Ile)	missense	yes
c.8266A>T	p.(Lys2756X)	truncating	yes
c.8834_8867del34	p.(Lys2945fs)	truncating	no
c.9022C>T	p.(Arg3008Cys)	missense	yes

fs - frameshift

The mutation c.1066-6T>G has previously been reported in AT patients and in breast cancer patients with AT carrier status (Dork et al., 2001; Thompson et al., 2005).

Fifteen mutations were predicted to lead to premature termination of the protein. These included: (c.478_482delTCTCA [p.Ser160fs], c.1120C>T [p.Gln374X], c.1402delAA [p.Lys468fs], c.2308G>T [p.Glu770X], c.2720_2723delGTGT [p.Cys907fs], c.3651delG [p.Leu1217fs], c.3712_3716delTTATT [p.Leu1238fs], c.3720_3736del17 [p.Asn1240fs], c.4591C>T [p.Gln1531X], c.6375insT [p.Glu2126fs], c.6815delA [p.Glu2272fs], c.6989_6995del7 [p.Leu2330fs], c.7883_7887del5 [p.Ile2628fs], c.8977C>T [p.Arg2993X]), c.9139C>T [p.Arg3047X]. Mutations: c.1120C>T, c.1402delAA, c.2720_2723delGTGT, c.3712_3716delTTATT, c.6375insT, c.7883_7887del5, c.8977C>T and c.9139C>T have been previously reported in AT patients (Broeks et al., 1998; Castellvi-Bel et al., 1999; Ejima and Sasaki, 1998; Li and Swift, 2000; Stankovic et al., 1998). Mutation c.9139C>T was reported in another CLL patient as somatic (Schaffner et al., 1999), as well as in an MCL tumour and a T-PLL patient with unknown germ-line status (Greiner et al., 2006; Vorechovsky et al., 1997).

Two mutations were short in-frame deletions (c.7638_7646del9 [p.R2547_S2549del] (c.3883_3885delCTT [p.Lys1295del]) and one was a combined short deletion-insertion (c.8246_8252del7insT [p.8246_8252del7insT]) which was not predicted to cause a frame-shift. Of these, mutation c.7638_7646del9, had been previously reported in AT patients (Gilad et al., 1996).

3.4.1.2. CLL patients without 11q deletion.

Of 8 pathogenic mutations found in non 11q deleted cases 2 were predicted to cause amino-acid substitutions and had been previously reported in AT patients: c.5228C>T [p.Thr1743Ile] and c.9022C>T [p.Arg3008Cys] (Li and Swift, 2000; Stankovic et al., 1998). The c.9022C>T mutations has also been reported in other haematological tumours including T-PLL and MCL (Schaffner et al., 2000; Stilgenbauer et al., 1997).

One pathogenic mutation was predicted to alter exon splicing (c.2466+2T>G [splicing site exon18]).

The remaining 5 mutations were predicted to lead to premature termination of the

protein: (c.1058_1059delGT [p.Cys353fs], c.2193delC [p.Tyr731fs]), c.4095_4109+4del19 [p.Lys1365fs], c.8266A>T [p.Lys2756X], c.8834_8867del34 [p.Lys2945fs]). Of these, mutation c.8266A>T has been reported in AT patients (Stankovic et al., 1998). Importantly, the patient with c.1058_1059delGT mutation also acquired an additional sequence change (c.5224G>C) classified as a missense variant in this study.

3.4.1.3 Frequency and distribution of all pathogenic *ATM* mutations

Pathogenic mutations accounted for 36.5% (31 of 85) of the overall sequence changes detected in this CLL cohort. Most of the pathogenic mutations 64.5% (20 of 31) were truncating; 12.9% (4 of 31) were splice defects and 9.7% (3 of 31) were short in-frame deletions. Only 4 (12.9%) pathogenic mutations were missense mutations and 3 of them were localized within the C-terminal functional domains of the ATM protein (Figure 3.1). All 4 missense mutations involved a conserved amino acid residue and have been reported to affect ATM kinase activity (Barone et al., 2009; Byrd et al., 2011; Camacho et al., 2002). Remaining mutations were scattered across the whole coding region of the *ATM* gene.

Notably, there was a significantly higher proportion of 11q deleted CLL tumours affected by pathogenic mutations (24 of 140) compared to non 11q deleted tumours (8 of 178) (p=0.0003).

3.5. Determination of the germ-line genetic status of pathogenic *ATM* mutations in CLL patients.

For 11 out of 32 CLL cases with *ATM* pathogenic changes the germ-line origin of the mutation was previously established using buccal cell material. Of these, 10 were classified as acquired (c.1120C>T, c.2308G>T, c.3651delG, c.6815delA, c.7883_7887del5, c.8787-1G>T, c.9023G>A, c.5228C>T, c.8834_8867del34, c.9022C>T) and one as germ-line (c.1058_1059delGT). For an additional 16 patients with pathogenic mutations the germ-line material was obtained from patients' peripheral blood granulocyte cells fraction. For the remaining 5 tumour samples no germ-line material was available.

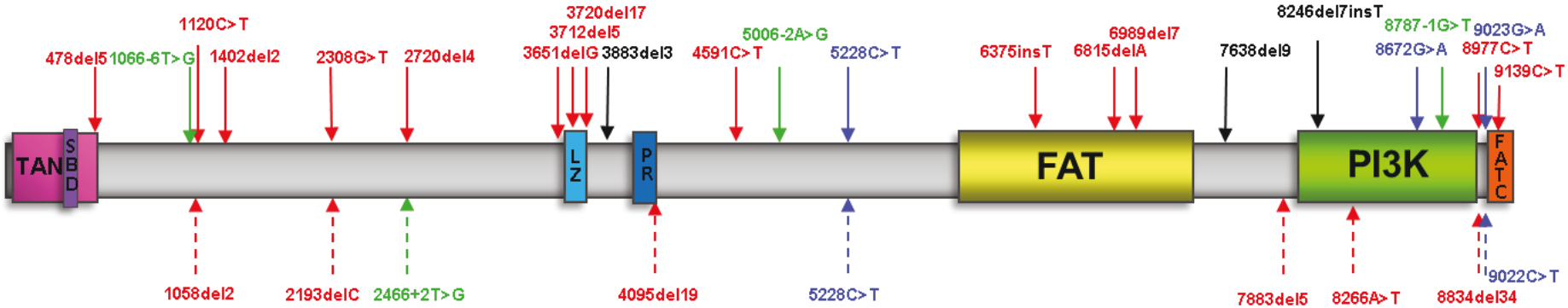


Figure 3.1. Distribution of the pathogenic *ATM* mutations across the coding region of *ATM* gene.

Truncating mutations were the most frequent type of pathogenic sequence changes. Most of the mutations were distributed evenly across the whole coding region of *ATM* gene apart from the missense mutations which tended to localise within the region encoding C-terminal domains of ATM protein. There was a significantly higher proportion of 11q deleted CLL tumours affected by pathogenic mutations compared to non 11q deleted tumours ($p \leq 0.001$). Solid line arrows indicate mutations detected in tumours with 11q deletion, dashed line arrows indicate mutations detected in tumours without 11q deletion. Font colours indicate different types of mutations: red are truncating mutations, blue-missense mutations, green-splice defect mutations, black-short in-frame deletions.

The analysis on the granulocyte DNA was performed using DHPLC technique and initially revealed the presence of pathogenic mutations in a surprisingly high proportion of samples. The DHPLC method is very sensitive and capable of detecting a sequence change present in only 5% of the cell population (Bernstein et al., 2003; Xiao and Oefner, 2001). Therefore, it was reasoned that in some cases the granulocyte fraction might have been contaminated with tumour B-cells and therefore the results that were obtained did not represent the real frequency of constitutive changes present in the CLL cohort.

3.5.1. 'Allele quantification' approach to establish the origin of pathogenic *ATM* mutations

To distinguish genuine germ-line changes from those that were a consequence of granulocyte contamination with tumour DNA, a strategy called 'allele quantification' using the semi-quantitive features of DHPLC was developed. As described in the Material and Methods chapter (section 2.2.2., Figure 2.1.) the presence of a mutation is visualized as the additional peak on the chromatograph and represents the DNA duplex which is a result of mismatched (mutated and wild-type) alleles. Therefore, the area under the mismatched (heteroduplex) peak corresponds to the amount of mutated allele present in the analysed sample. Theoretically, if an *ATM* mutation is present on 1 allele in 100% of tumour cells the areas under the heteroduplex peak (mismatched alleles) and homoduplex peak (two wild-type or two mutant alleles) should be the same. Accordingly, if the mutation is of germ-line origin, areas under those two peaks should also be equal, because 1 inherited allele is mutated. Therefore, comparison of the hetero- to homoduplex peaks sizes in germ-line samples should provide an indication as to whether it represents a true germ-line change (the heteroduplex peak is equal to homoduplex peak) or whether it is the result of contamination with small number of tumour cells (the heteroduplex peak is smaller than homoduplex peak). However in practice, the interpretation of hetero- and homo-duplex peaks is not that straight forward, as the sizes and shapes of hetero and homo- duplexes peaks depend on PCR efficiency, mutation type and DHPLC's denaturing conditions. In a CLL

sample they may also depend on the size of the sub-clone with 11q deletion. I have reasoned that by comparing CLL tumour and granulocyte DNA analysed under the same conditions (previously optimized to obtain the best peak separation) and by repeating analysis multiple times I should obtain valid and reliable results. I have calculated the relative size of the heteroduplex peak in the granulocyte fraction as compared to the size of the heteroduplex peak in tumour cells using following formula:

(A/B) / (C/D) x 100%

A - Area under heteroduplex peak in granulocytes

B - Area under homoduplex peak in granulocytes

C – Area under heteroduplex peak in tumour cells

D – Area under homoduplex peak in tumour cells

According to this formula, if the mutation was of germline origin I expected the result to be near 100%, thus representing the high similarity between tumour and garnulocytes fractions of the samples.

To validate this methodology I have analysed both tumour and granulocyte cell samples of various CLL patients which served as controls in the 'allele quantification' analysis. These included:

1) 4 CLL samples with *ATM* sequence changes for which carrier status was previously established using buccal cells. Two of those were acquired pathogenic mutation (c.8834_8867del34, c.8787-1G>T), one was a germ-line pathogenic mutation (c.1058_1059delGT) and one was an acquired missense variant (c.7313C>A).

2) 2 CLL samples with known polymorphisms in tumour cells (c.146C>G, c.2119C>T). These sequence changes were expected to be present in granulocytes' material.

3) 6 CLLs with non-pathogenic *ATM* sequence changes, for which granulocyte DNA was available (c.1048G>A, c.3383A>G, c.5975A>G, c.8249T>C, c.7327C>G, c.8592C>T). The germ-line status of these sequence changes was unknown but by increasing the number of samples in the analysis I was expecting to obtain a more accurate interpretation of the results.

In addition, known *ATM* wild-type DNA was used as a negative control.

Altogether, there were 28 different sequence changes, including 19 pathogenic mutations, in the matched tumour and granulocyte DNA samples (Table 3.3). Twenty five of these were analysed under the same denaturing conditions by DHPLC and repeated at least twice including DNA amplification, 3 were analysed only by sequencing due to a very limited amount of granulocytes' DNA available and PCR amplification was not sufficient for DHPLC

Table 3.3. List of *ATM* sequence changes which were analysed by the 'allele quantification' approach.

No	Sequence change type	Sequence change	11q deletion	Results of the buccal material analysis (A or G)
1	pathogenic	c.1058delGT	no	**G**
2	pathogenic	c.1066-6T>G	yes	n/a
3	pathogenic	c.1402delAA	yes	n/a
4	pathogenic	c.2466+2T>G	no	n/a
5	pathogenic	c.2720_2723delGTGT	yes	n/a
6	pathogenic	c.3712_3716delTTATT	yes	n/a
7	pathogenic	c.3720del17	yes	n/a
8	pathogenic	c.4095-4109+4del19	no	n/a
9	pathogenic	c.4591C>T	yes	n/a
10	pathogenic	c.5006-2A>G	yes	n/a
11	pathogenic	c.5228C>T	yes	n/a
12	pathogenic	c.6375insT	yes	n/a
13	pathogenic	c.6989del7	yes	n/a
14	pathogenic	c.7638_7646del9	yes	n/a
15	pathogenic	c.8246_8252del7insT	yes	n/a
16	pathogenic	c.8266A>T	no	n/a
17	pathogenic	c.8787-1G>T	yes	**A**
18	pathogenic	c.8834del34	no	**A**
19	pathogenic	c.8977C>T	yes	n/a
20	missense variant	c.1048G>A	no	n/a
21	missense variant	c.3383A>G	yes	n/a
22	missense variant	c.5975A>C	yes	n/a
23	missense variant	c.7313C>A	no	**A**
24	missense variant	c.7327C>G	yes	n/a
25	missense variant	c.8249T>C	yes	n/a
26	polymorphism	c.146C>G	yes	n/a
27	polymorphism	c.2119T>C	yes	n/a
28	neutral/ polymorphism	c.8592C>T	no	n/a

n/a - not assessed, A - acquired, G - germ-line

analysis. All samples were subsequently sequenced to confirm the presence or absence of a particular mutation in the granulocyte fraction.

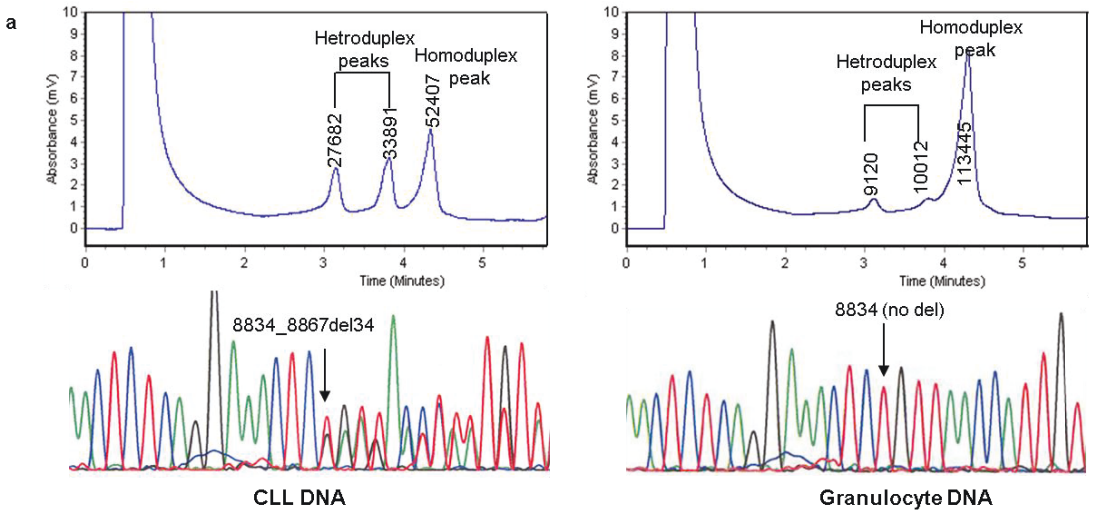
The examples of 'allele quantification' analysis and determination of the origin (germ-line or acquired) of *ATM* sequence changes are shown in Figure 3.2. and 3.3.

3.5.2. 'Allele quantification' results

Theoretically, the results were expected to fall into two categories: 1) a group of the mutations for which the heteroduplex peak area in the granulocyte fraction represented nearly 100% of the peak area observed in the corresponding tumour DNA, representing germ-line changes; and 2) those with acquired mutations where the heteroduplex peak area in the granulocyte fraction was significantly reduced than heteroduplex peak area observed in corresponding tumour DNA

In practice, the actual results for the 'allele quantification' analysis ranged from 0 to 184% and could be grouped into two distinct populations. (Table 3.4**.** and Figure 3.4). The first included acquired sequence changes for which heteroduplex peak area was either absent from the granulocyte DNA or accounted for less than 40% of the peak area observed in the corresponding tumour DNA (range 0-37% and an average of 12%). The results for the majority of sequence changes in the second group clustered around 100% (with an average of 121%) and only three sequence changes had peak areas below 95% (c.2119T>C, c.8977C>T and c.8266A>T). One of these changes (c.2119T>C) with a peak area of 75.6% was previously reported as a polymorphism and hence it was expected to be a truly germ-line change. For that reason, other mutations with a peak area higher than 75% were also classified as germ-line.

With regards to pathogenic mutations, 8 out of 19 analysed in this study were classified as germ-line changes (c.1058delGT, c.1066-6T>G, c.2717delTGTG, c.3705delTTTA, c.5228C>T, c.7638_7646del9, c.8266A>T, c.8977C>T) (Figures 3.3 and 3.5.). These included the change c.1058delGT which was also detected in buccal cell

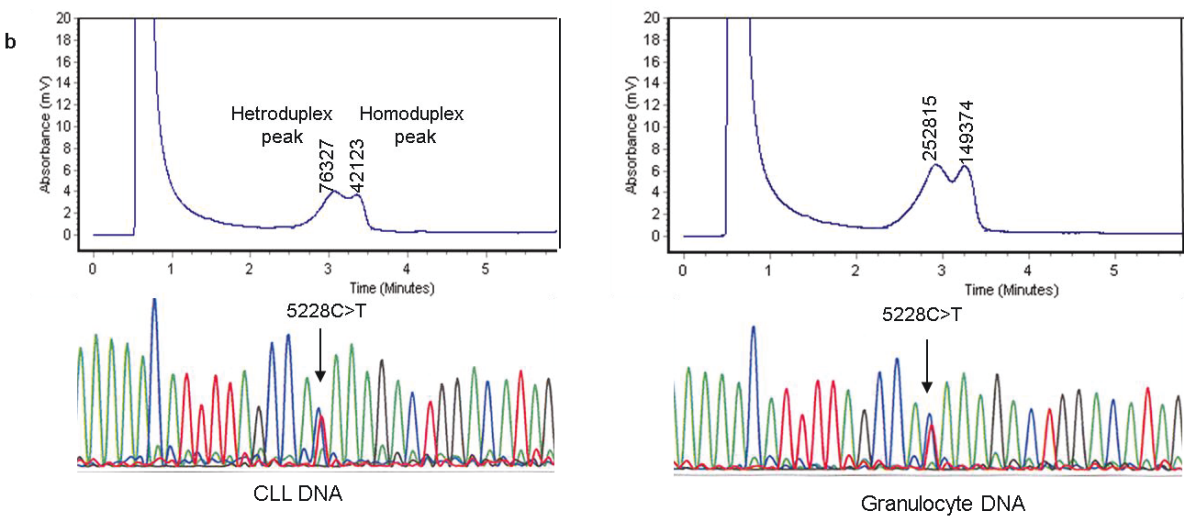


$$\frac{\text{Area under heteroduplex peak in granulocytes/ Area under homoduplex peak in granulocytes}}{\text{Area under heteroduplex peak in CLL lymphocytes/ Area under homoduplex peak in CLL lymphocytes}}$$

$$\frac{(9120+10012)/113445}{(27682+33891)/52407} = 0.14 \quad \textbf{(14\%)}$$

Figure 3.2. Detection of acquired *ATM* sequence change using combined DHPLC-based 'allele quantification' approach and sequencing.

An example of an acquired mutation (8834_8867del34) in exon 63 is shown. The values on DHPLC chromatographs indicate the area under heteroduplex and homoduplex peaks. In this example a multiple peak pattern, which represents mismatch sequence and the presence of mutation, was much more prominent in CLL tumour cells than in granulocytes and the relative proportion of heteroduplex peaks to homoduplex was 14% indicating an acquired change. This result was confirmed by sequencing of CLL and granulocyte DNAs.



$$\frac{(25281/149374)}{(76327/42123)} = 0.934 \quad (\mathbf{93.4\%})$$

Figure 3.3. Detection of germ-line *ATM* sequence change using combined DHPLC-based 'allele quantification' approach and sequencing.

An example of a germ-line mutation (c.5228C>T) is shown. Amplification of *ATM* exon 37 produced a double peak pattern in both CLL tumour DNA and granulocyte DNA fractions in this patient. The ratio of the heteroduplex to homoduplex peak in the granulocyte DNA was 93.4% of the ratio of these peaks in the CLL DNA, indicating that the mutation was a germ-line sequence change. This result was confirmed by sequencing of CLL and granulocyte DNA.

material previously, and change c.7638_7646del9 which was detected in the granulocyte fraction by sequencing only (PCR product from granulocytes fraction from this case showed a high level of mutated sequence) and represented a well-known AT mutation.

Table 3.4. The results of the 'allele quantification' analysis.

No	Sequence change type	Sequence change	11q deletion	Results of the buccal material analysis (A or G)	Results of the granulocytes fraction analysis- average value (A or G)	
1	Pathogenic	c.1058delGT	no	**G**	127.2	**G**
2	pathogenic	c.1066-6T>G	yes	n/a	184.3	**G**
3	pathogenic	c.1402delAA	yes	n/a	0.0	**A**
4	pathogenic	c.2466+2T>G	no	n/a	37.3	**A**
5	pathogenic	c.2720_2723delGTGT	yes	n/a	121.5	**G**
6	pathogenic	c.3712_3716delTTATT	yes	n/a	110.3	**G**
7	pathogenic	c.3720del17	yes	n/a	n/a	**A**
8	pathogenic	c.4095-4109+4del19	no	n/a	5.6	**A**
9	pathogenic	c.4591C>T	yes	n/a	0.0	**A**
10	pathogenic	c.5006-2A>G	yes	n/a	0.0	**A**
11	pathogenic	c.5228C>T	yes	n/a	113.9	**G**
12	pathogenic	c.6375insT	yes	n/a	0.2	**A**
13	pathogenic	c.6989del7	yes	n/a	0.0	**A**
14	pathogenic	c.7638_7646del9	yes	n/a	n/a	**G**
15	pathogenic	c.8246_8252del7insT	yes	n/a	n/a	**A**
16	pathogenic	c.8266A>T	no	n/a	88.6	**G**
17	pathogenic	c.8787-1G>T	yes	**A**	0.0	**A**
18	pathogenic	c.8834del34	no	**A**	17.4	**A**
19	pathogenic	c.8977C>T	yes	n/a	76.7	**G**
20	missense variant	c.1048G>A	no	n/a	95.0	**G**
21	missense variant	c.3383A>G	yes	n/a	138.4	**G**
22	missense variant	c.5975A>C	yes	n/a	178.6	**G**
23	missense variant	c.7313C>A	no	**A**	22.3	**A**
24	missense variant	c.7327C>G	yes	n/a	30.1	**A**
25	missense variant	c.8249T>C	yes	n/a	32.9	**A**
26	polymorphism	c.146C>G	yes	n/a	116.37	**G**
27	polymorphism	c.2119T>C	yes	n/a	75.61	**G**
28	neutral/ polymorphism	c.8592C>T	no	n/a	148.0	**G**

n/a - not assessed, A - acquired, G - germ-line

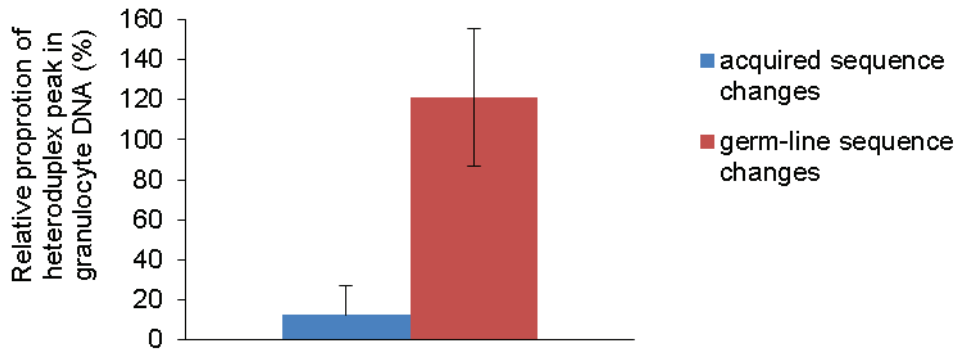


Figure 3.4. The DHPLC-based 'allele quantification' analysis reveals two distinct populations of *ATM* sequence changes.

The sequence changes for which the relative proportion of heteroduplex peak in granulocyte fraction were within a range of 0% to 37% (average of 12%) were defined as acquired sequence changes and those for which this proportion was within a range of 76% to 184% (average of 121%) were defined as germ-line sequence changes.

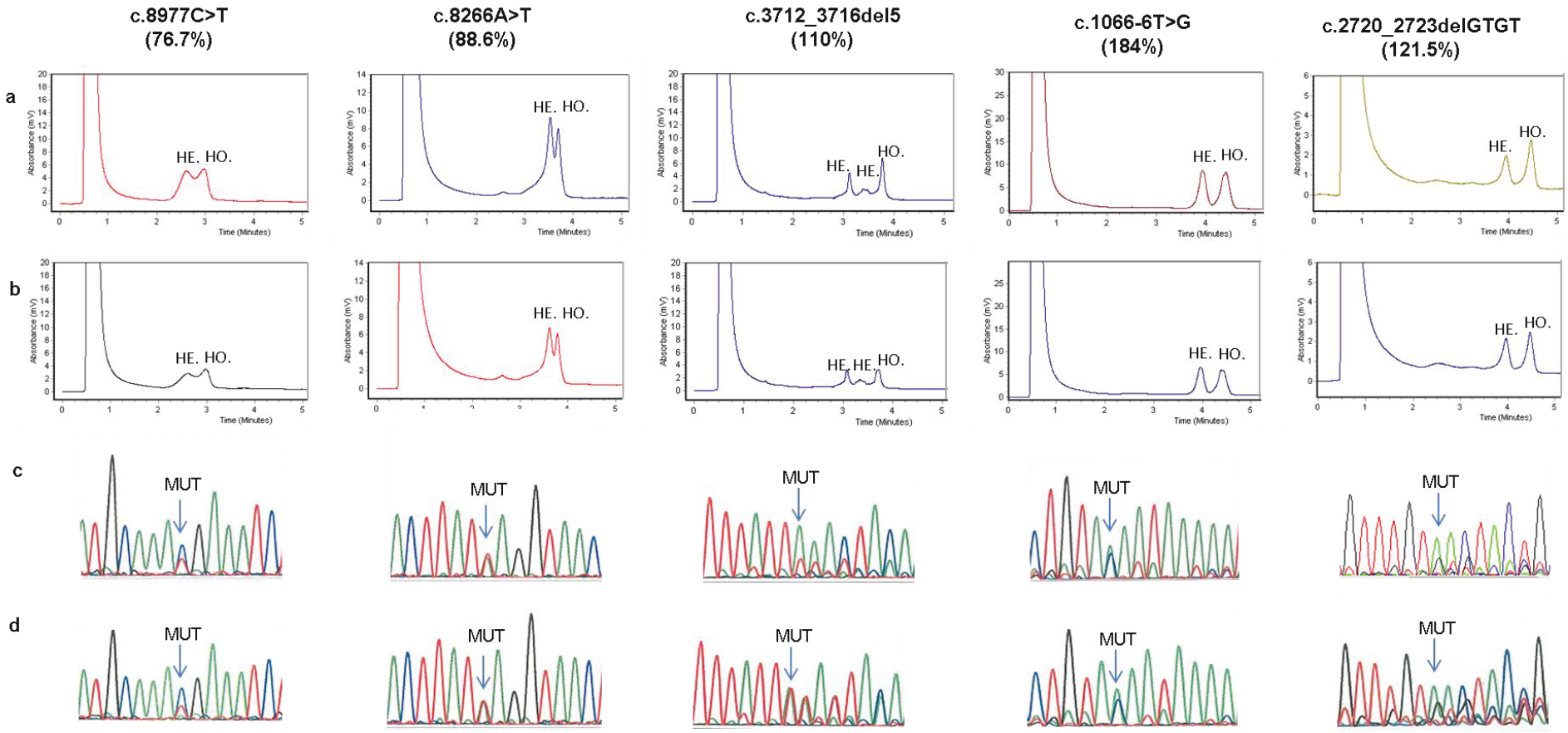


Figure 3.5. Pathogenic *ATM* mutations for which 'the germ-line' status was confirmed by DHPLC and sequencing.

(**a**) DHPLC analysis of CLL tumour DNA, HE indicates heteroduplex peak, HO indicates homoduplex peak. (**b**) DHPLC analysis of granulocyte DNA. (**c**) Sequencing confirms the presence of *ATM* mutation in tumour DNA (**d**) and in granulocyte DNA of the same patient. The average relative proportion of heteroduplex peak in granulocyte fraction for each mutation is shown in brackets.

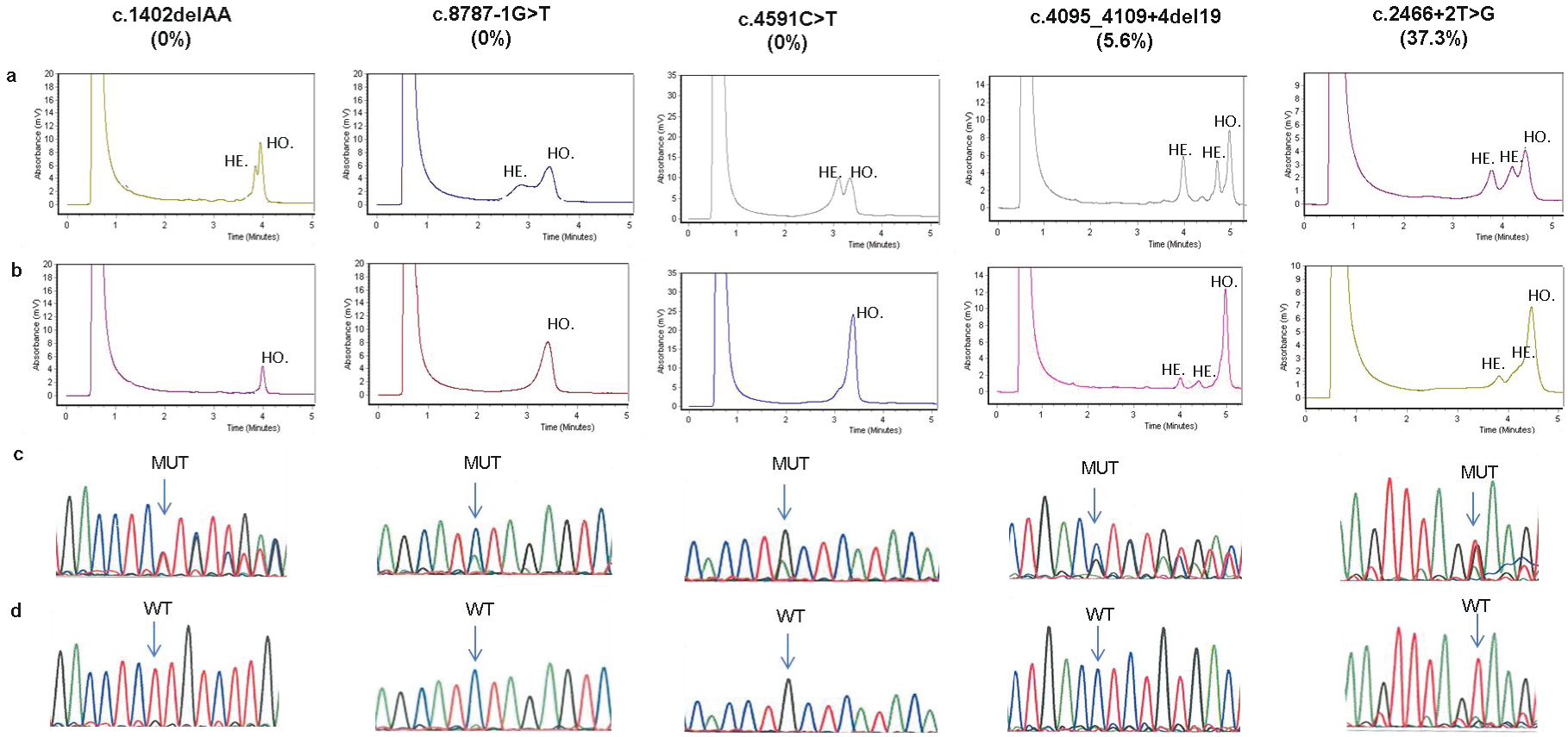


Figure 3.6. Pathogenic *ATM* mutations for which 'the acquired' status was confirmed by DHPLC and sequencing.

(**a**) DHPLC analysis of CLL tumour DNA, HE indicates heteroduplex peak, HO indicates homoduplex peak. (**b**) DHPLC analysis of granulocyte DNA. (**c**) Sequencing confirms the presence of *ATM* mutation in tumour DNA (**d**) and its absence in granulocyte DNA of the same patient. The average relative proportion of heteroduplex peak in granulocyte fraction for each mutation is shown in brackets.

Eleven out of 19 pathogenic mutations which were analysed in this study were classified as acquired changes (c.1402delAA, c.2466+2T>G, c.3720del17 c.4591C>T, c.del4095_4139+4del19, c.5006-2A>G, c.6375insT, c.6989del7, c.8246_8252del7insT, c.8787-1G>T, c.8834del34) (Figures 3.2 and 3.6). These included 2 sequence changes (c.8787-1G>T, c.8834del34) for which the 'acquired' status was previously established using buccal cells, and 2 pathogenic mutations (c.3720del17, c.8246_8252del7insT) which were analysed only by sequencing and could not be detected in the granulocyte DNA. They were classified as acquired because the analysis of the PCR products from the granulocyte' fraction for these 2 patients revealed no presence of mutated DNA.

To establish the total numbers of all germ-line and acquired pathogenic sequence changes in the CLL cohort from this study, I have combined results from the previous (buccal cells material) and current (granulocyte material) analysis. Overall, there were 8 germ-line pathogenic mutations (6 in 11q deleted and 2 in non 11q deleted cases); 19 acquired mutations (14 in 11q deleted and 5 in non 11q deleted tumours); and 5 remained of unknown origin due to the lack of germ-line material (4 in 11q deleted and 1 in non 11q deleted tumours) (Table 3.5.)

3.6. Constitutional pathogenic *ATM* mutations - frequency comparison and cellular and clinical consequences.

Having identified a total number of pathogenic germ-line *ATM* mutations, I have compared their frequencies between the CLL patients and control individuals. I have analysed the biological characteristic of *ATM* carrier clones and compared their clinical features with the rest of CLL cohort.

3.6.1 Frequency of pathogenic germ-line mutations in CLL and controls.

The main aim of this study has been to establish whether there is a higher frequency of pathogenic germ-line mutations in CLL patients when compared to control cohort.

Table 3.5. Pathogenic *ATM* mutations of germ-line, acquired or unknown origin.

No.	Sequence change	Amino Acid change	Chromosome 11q deletion	Previously observed in A-T patients
Pathogenic germ-line *ATM* mutations				
1	c.1066-6T>G	splicing site exon 11	yes	yes
2	c.2720_2723delGTGT	p.(Cys907fs)	yes	yes
3	c.3712_3716delTTATT	p.(Leu1238fs)	yes	yes
4	c.5228C>T	p.(Thr17431Ile)	yes	yes
5	c.7638_7646del9	p.(Arg2547_Ser2549del)	yes	yes
6	c.8977C>T	p.(Arg2993X)	yes	yes
7	c.1058_1059delGT	p.(Cys353fs)	no	no
8	c.8266A>T	p.(Lys2756X)	no	yes
Pathogenic acquired *ATM* mutations				
1	c.1120C>T	p.(Gln374X)	yes	yes
2	c.1402delAA	p.(Lys468fs)	yes	yes
3	c.2308G>T	p.(Glu770X)	yes	no
4	c.3651delG	p.(Leu1217fs)	yes	no
5	c.3720_3736del17	p.(Asn1240fs)	yes	no
6	c.4591C>T	p.(Gln1531X)	yes	no
7	c.5006-2A>G	splicing site exon 36	yes	no
8	c.6375insT	p.(Glu2126fs)	yes	yes
9	c.6815delA	p.(Glu2272fs)	yes	no
10	c.6989_6995del7	p.(Leu2330fs)	yes	no
11	c.7883_7887del5	p.(Ile2628fs)	yes	yes
12	c.8246_8252del7insT	p.(Lys2749_Thr2751delinsIle)	yes	no
13	c.8787-1G>T	splicing site exon 62	yes	yes
14	c.9023G>A	p.(Arg3008His)	yes	yes
15	c.2466+2T>G	splicing site exon 18	no	no
16	c.4095_4109+4del19	p.(Lys1365fs)	no	no
17	c.5228C>T	p.(Thr17431Ile)	no	yes
18	c.8834_8867del34	p.(Lys2945fs)	no	no
19	c.9022C>T	p.(Arg3008Cys)	no	yes
Pathogenic *ATM* mutations with unknown germ-line status				
1	c.478_482delTCTCA	p.(Ser160fs)	yes	no
2	c.3883_3885delCTT	p.(Lys1295del)	yes	no
3	c.8672G>A	p.(Gly2891Asp)	yes	yes
4	c.9139C>T	p.(Arg3047X)	yes	yes
5	c.2193delC	p.(Tyr731fs)	no	no

I have identified 8 germ-line pathogenic ATM mutation carriers among 318 CLL cases (2.5%) and 0 among 281 controls (0%). Therefore, the frequency of these *ATM s*equence changes was significantly higher in CLL cohort than in controls (p=0.008). However, consideration of two separate CLL cohorts (with 11q deletion and without 11q deletion) revealed that constitutional pathogenic *ATM* mutations were significantly more common only in CLL patients who developed a chromosome 11q deletion (6 of 140 versus 0 of 281, p=0.001) but not in those who did not acquire this deletion in their leukemic clone (2 of 178 versus 0 of 281, p=0.15).

As the frequency of *ATM* germ-line mutations was not increased in all CLL patients, irrespective of 11q deletion, I concluded that constitutional pathogenic *ATM* mutations did not contribute to CLL initiation. However, the increased incidence of germ-line *ATM* mutations in tumours with 11q deletion could suggest that in a small subgroup of CLL cases these mutations may play a role in clonal evolution by contributing to loss of another *ATM* allele.'

3.6.2. Phenotypic characteristic of CLL tumours in patients that are mono-allelic *ATM* mutations carriers.

The mechanism by which mono-allelic *ATM* mutation carrier status could impact on the development of a CLL clone is not known. Clonal development is probably an antigen driven process which, in a proportion of cases, can involve use of stereotypic VDJ segment combinations (Murray et al., 2008; Stamatopoulos et al., 2007), somatic hypermutation of *IGHV* genes and CSR. ATM ensures fidelity of CSR and prevents utilization of non-classical non homologous end joining (NHEJ) resulting in excessive sequence homology across switch junctions (Pan-Hammarstrom et al., 2006). It is unknown whether constitutional mono-allelic loss of *ATM* could disturb these processes and contribute to clonal development.

I have investigated these processes in CLL clones of carriers of pathogenic *ATM* mutations. Two patients revealed a CLL clone carrying the previously described stereotypic VDJ combinations V1-69/D3-3/J6 (Stamatopoulos et al., 2007) and the remaining cases showed a range of VDJ segments (Table 3.6).

Table 3.6. Clinical and biological characteristic of CLL in *ATM* mutation carriers.

Germline mutation	Patients age at diagnosis (years)	Stage at diagnosis	Chromosome 11q deletion	VDJ profile	CSR region profile
c.1058_1059delGT	61	B	no	V1-2/D21-9/J4 UM	n/a
c.1066-6T>G	42	B	yes	V1-69/D3-3/J6 UM	n/a
c.2720_2723delGTGT	65	B	yes	V1-69/D3-3/J6 UM V3-13/D3-10/J6 UM	n/a
c.3712_3716delTTATT	71	B	yes	V3-30/D3-3/J6 UM	n/a
c.5228C>T	47	A	yes	VH3-11/D3-10/J4 UM	cNHEJ
c.7638_7646del9	68	C	yes	V4-4/D3-9/J6 UM	n/a
c.8266A>T	52	B	no	V3-15/D4-17/J3 M	n/a
c.8977C>T	51	B	yes	V1-18/D3-3/J4 M	cNHEJ

UM – unmutated *IGHV* gene, M – mutated *IGHV* gene, CSR- class switch recombination; n/a - CSR region not amplified; cNHEJ- classical non homologous end joining

Amplification of switch regions was successful for two patients' clones. One clone showed switching to IgG1 and another to IgG3. Analysis of CSR breakpoints across the Sμ-Sγ sequences revealed normal switch junctions with no utilization of excessive microhomology (Figure 3.7.).

The normal Sμ-Sγ junctions and no excessive use of stereotyped BCRs among *ATM* mutation carriers suggested that mono-allelic germ-line defects of *ATM* have no effect on these processes. Therefore, they would not have contributed to CLL clonal transformation.

3.6.3. Clinical characteristics of *ATM* carriers and comparison to the rest of the CLL cohort.

ATM mutations and chromosome 11q deletions have previously been associated with more aggressive disease, defective responses to chemotherapeutic agents, and un-mutated

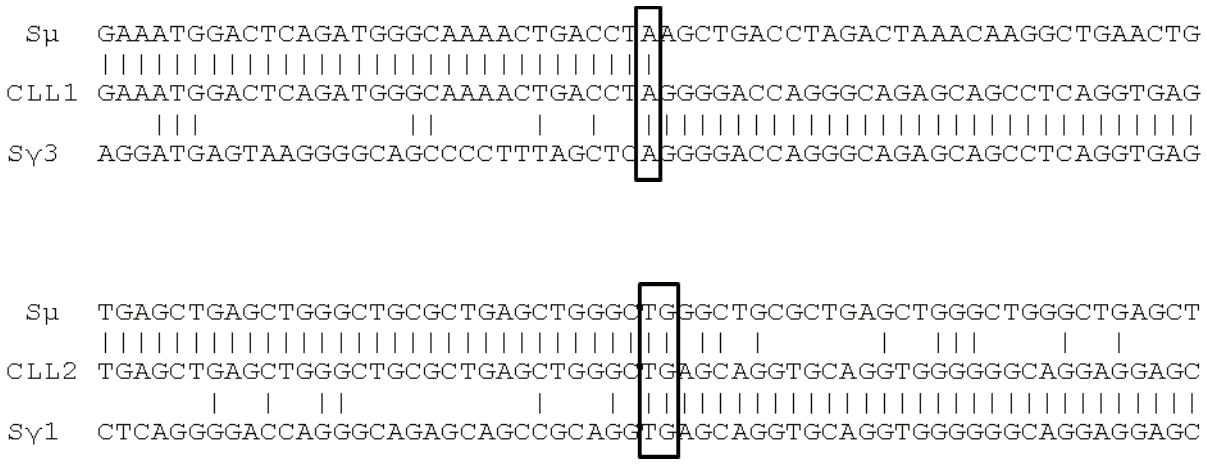


Figure 3.7. Monoallelic germ-line *ATM* defects have no effect on CSR process.

Amplification of Sμ-Sγ switch regions in two *ATM* mutation carriers revealed normal switch junctions with no utilization of excessive microhomology. Sμ-Sγ joint region in CLL1 tumour with c.5228C>T germ-line mutation had one base pair homology (A). Two base pair homology (TG) across Sμ-Sγ joint region was detected in tumour CLL2 with c.8977C>T *ATM* germ-line mutation.

IGHV status -which itself is a poor prognostic marker (Austen et al., 2005; Dohner et al., 2000; Krober et al., 2002). However, the impact of constitutional *ATM* mutations on clinical prognosis has never been established. The increased frequency of germ-line pathogenic changes among patients with chromosome 11q deletion indicates their role in disease progression, possibly through accelerating the complete loss of ATM function. To further investigate this hypothesis clinical characteristic were compared between four groups of patients: those with constitutional pathogenic *ATM* mutations, with acquired pathogenic *ATM* mutations, *ATM* missense variants (with no additional *ATM* mutation) and those with *ATM* wild-type, or neutral sequence changes.

One-way ANOVA and t-test were used to calculate the difference in age between the 4 groups. There was no difference in age at diagnosis between patients with pathogenic *ATM* mutations (germ-line or acquired) and patients with wild-type *ATM* gene. The only statistically significant difference was observed between the group with *ATM* missense variants (mean 69.2 years) and the *ATM* wild-type (mean 62.9, p=0.012) (Table.3.7).

Overall, there was no difference in stage at diagnosis or *IGHV* mutation status between the 4 categories (Chi-square test), but notably among patients with constitutional *ATM* mutations, 7 of 8 exhibited an advanced stage at diagnosis and 6 of 8 had unmutated *IGHV* genes (Table 3.7 and 3.6.).

All three groups with *ATM* sequence changes were enriched for tumours with 11q deletion when compared to the wild-type *ATM* CLLs. The group with *ATM* acquired pathogenic mutations and group with *ATM* missense variants had a significantly higher proportion of tumours with 11q deletion when compared to the group with no *ATM* mutations (p=0.034 and p=0.011, respectively, Chi-square and Fisher exact test). The lack of statistical difference between groups with *ATM* germ-line pathogenic mutations and wild-type *ATM* gene could have been caused by the small sample size of the former group (only 8 cases).

A Kaplan-Meier survival analysis and log-rank test revealed that there was a significant difference in the overall survival between the 4 groups (p=0.004). Patients with constitutional or acquired *ATM* pathogenic mutations as well as those with *ATM* missense

variants all had inferior survival compared to patients with wild-type *ATM* gene (p=0.005, p=0.014, p=0.0047 respectively) (Table 3.7 and Figure 3.8)

Therefore, the results of this analysis confirmed the role of germ-line *ATM* mutations in progression but not initiation of CLL disease.

Table 3.7. Comparative clinical data for CLL patients.

Clinical feature	Pathogenic germline *ATM* mutations (n=8)	Pathogenic acquired *ATM* mutations (n=19)	*ATM* missense variants^ (n=26)	*ATM* wild-type# (n=260)	p value
mean age at diagnosis	61.8	66.7	69.2*	62.9	0.044
stage of disease A B/C	(n=8) 1 7	(n=19) 9 10	(n=26) 13 13	(n=248) 114 134	0.287
IGHV status UM M	(n=8) 6 2	(n=18) 12 6	(n=26) 19 7	(n=232) 128 104	0.196
chromosome 11q deletion (%)	(n=8) 6 75.0	(n=19) 14* 73.4	(n=26) 17* 65.4	(n=260) 100 38.4	0.0004
overall survival (median,months) 95% CI	(n=8) 90* (43.1-97.0)	(n=19) 68.7* (52.5-117.9)	(n=26) 57.7* (46.6-88.5)	(n=249) 105 (99.6-117.2)	0.004

IGHV - Immunoglobulin heavy chain variable gene, M-mutated, UM-unmutated, CI-confidence interval,
^ this group included patients with *ATM* missense variants and no additional *ATM* pathogenic mutations, # this group included patients with wild-type *ATM* gene or the presence of neutral sequence change. The 5 patients with pathogenic mutations of unknown origin were not included in this comparative analysis.
* Indicates statistically significant difference when compared to the group of patients with wild-type *ATM* gene.

3.7. Other *ATM* sequence changes

In addition to investigating the role of constitutional pathogenic *ATM* mutations in the development of CLL I have also characterized all other detected sequence changes and compared their frequencies between CLL and control cohorts. Interestingly, specific polymorphic variants have been linked with CLL disease in one of the large case-control

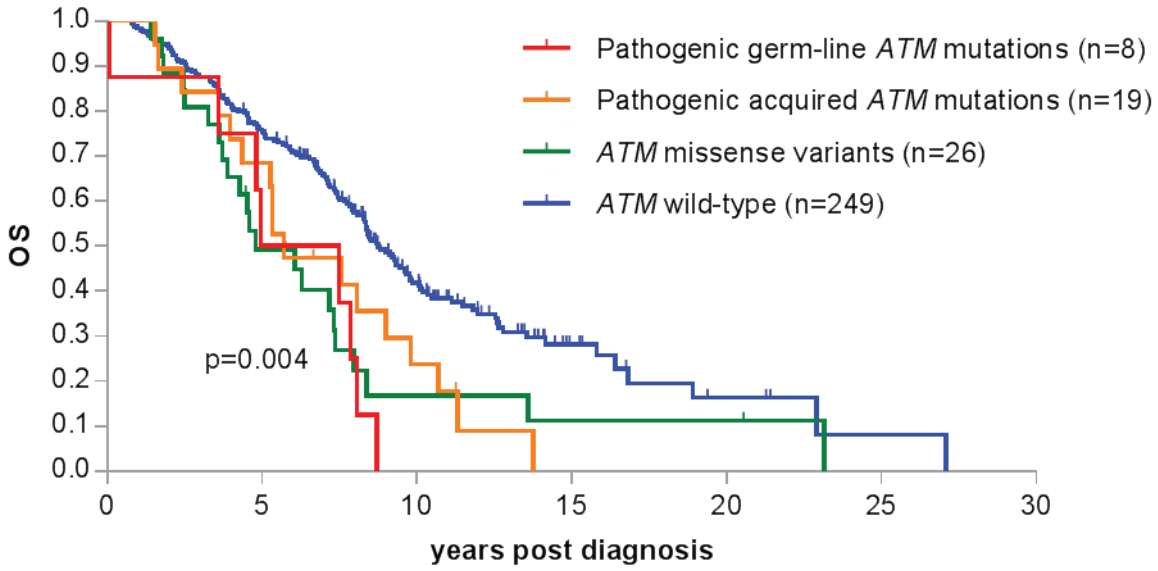


Figure 3.8. Impact of *ATM* sequence change on overall survival (OS) in studied CLL cohort of 318 patients.

OS in patients with germ-line pathogenic *ATM* mutations, acquired pathogenic *ATM* mutations and *ATM* missense variants was significantly shorter than in the *ATM* wild-type group ($p \leq 0.01$, $p \leq 0.05$ and $p \leq 0.01$ respectively).

studies (Rudd et al., 2006). Although assessing the role of sequence variants was not the main aim of this project I have used the opportunity to establish a possible association.

3.7.1. *ATM* missense variants in CLL and control cohorts

Twenty six different missense variants were found in the CLL cohort and 5 in the control group (Table 3.8). Since some changes were present in more than 1 individual 29 CLL patients were affected (18 with 11q deletion and 11 without 11q deletion).

In 11q deleted tumours, 13 missense variants were identified once (c.2417T>G [p.Leu806Trp], c.3383A>G [p.Gln1128Arg], c.5882A>G [p.Tyr1961Cys], c.5975A>C [p.Lys1992Thr] c.5980A>G [p.Lys1994Glu], c.6106T>A [p.Tyr2036Asn], c.7047C>G [p.Cys2349Trp], c.7327C>G [p.Arg2443Gly], c.8056T>C [p.Phe2686Leu], c.8161G>A [p.Asp2721Asn], c.8600G>A [p.Gly2867Glu], c.8839A>T [p.Thr2946ser], c.8861A>G [p.Tyr2954Cys]), one missense variant (c.6067G>A [p.Gly2023Arg]) was found in 2 different CLLs and one (c.8249T>C [p.Leu2750Ser]) in 3 different CLLs. None of 11q deleted patients had more than one missense variant.

In non 11q deleted cases, 10 missense variants were identified once (c.1009C>A [p.Arg337Ser], c.1048G>A [p.Ala350Thr], c.1229T>C [p.Val410Ala] c.5224G>C [p.Ala1742Pro], c.5857A>G [p.Thr1953Ala], c.7313C>A [p.Thr2438Lys], c.7438C>T [p.His2480Tyr], c.8095C>T [p.Pro2699Ser], c.8663T>C [p.Ile2888ThrThr], c.9032T>A [p.Met3011Lys]); and one (c.5821G>C [p.Val1941Leu]) was found in 2 different CLL samples. One of those two patients had 2 different missense variants (c.5821G>C, c.7313C>A). Notably the same patient also had acquired a pathogenic mutation (c.8834_8867del34) in the tumour clone.

None of the missense variants was common for both 11q and non11q deleted patients' groups.

In total, 8 of the missense variants identified here have previously been reported in various other cancers: c.1009C>A in adenoid cystic carcinoma (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=sample&id=1090027);

Table 3.8. Missense variants found in CLL patients and controls

No.	Sequence change	Amino Acid change	No. of 11q CLL affected (n=140)	No. of non11q CLL affected (n=178)	Control affected (n=281)	Amino Acid conserved between mouse and human	Domain affected	Germ -line
Missense variants found in CLL patients only								
1	c.2417T>G	p.(Leu806Trp)	1	0	0	yes	none	no
2	c.3383A>G*	p.(Gln1128Arg)	1	0	0	no	none	yes
3	c.5882A>G	p.(Tyr1961Cys)	1	0	0	yes	none	yes
4	c.5980A>G	p.(Lys1994Glu)	1	0	0	yes	none	no
5	c.6067G>A*	p.(Gly2023Arg)	2	0	0	yes	none	yes x1 nk x1
6	c.6106T>A	p.(Tyr2036Asn)	1	0	0	yes	none	no
7	c.7047C>G	p.(Cys2349Trp)	1	0	0	yes	FAT	no
8	c.7327C>G	p.(Arg2443Gly)	1	0	0	yes	FAT	nk
9	c.8056T>C	p.(Phe2686Leu)	1	0	0	yes	none	no
10	c.8161G>A	p.(Asp2721Asn)	1	0	0	yes	PI3Kc	nk
11	c.8249T>C	p.(Leu2750Ser)	3	0	0	yes	PI3Kc	yes x2 no x1
12	c.8600G>A	p.(Gly2867Glu)	1	0	0	yes	PI3Kc	no
13	c.8839A>T	p.(Thr2946Ser)	1	0	0	yes	PI3Kc	nk
14	c.8861A>G*	p.(Tyr2954Cys)	1	0	0	yes	PI3Kc	no
15	c.1009C>A*	p.(Arg337Ser)	0	1	0	yes	none	no
16	c.1048G>A*	p.(Ala350Thr)	0	1	0	yes	none	nk
17	c.1229T>C*	p.(Val410Ala)	0	1	0	yes	none	nk
18	c.5224G>C	p.(Ala1742Pro)	0	1	0	yes	none*	no
19	c.5857A>G	p.(Thr1953Ala)	0	1	0	yes	none	nk
20	c.7313C>A	p.(Thr2438Lys)	0	1	0	yes	FAT	no
21	c.7438C>T	p.(His2480Tyr)	0	1	0	yes	FAT	nk
22	c.8095C>T*	p.(Pro2699Ser)	0	1	0	yes	none	nk
23	c.8663T>C*	p.(Ile2888Thr)	0	1	0	yes	PI3Kc	nk
24	c.9032T>A	p.(Met3011Lys)	0	1	0	yes	none	nk
Missense variants found in controls only								
25	c.1727T>C	p.(Ile576Thr)	0	0	1	yes	none	yes
26	c.3419A>G	p.(Asn1140Ser)	0	0	1	yes	none	yes
27	c.7390T>C*	p.(Cys2464Arg)	0	0	1	yes	FAT	yes
Missense variants common to CLL and control cohorts								
28	c.5821G>C	p. (Val1941Leu)	0	2	2	yes	none	yes x2 yes x2
29	c.5975A>C	p.(Lys1992Thr)	1	0	1	yes	none	yesx1 yes x1

* Indicates missense variants detected also in various other tumours, see text for details.

c.1048G>A in another CLL patient (Stankovic et al., 1999); c.1229T>C in Burkitt lymphoma (Gumy-Pause et al., 2006), breast cancer (Thorstenson et al., 2003), follicular lymphoma (Fang et al., 2003) and in T-ALL patient (Liberzon et al., 2004). The sequence variant c.3383A>G was reported in breast cancer (Sommer et al., 2003) and recently in an AT patient (Jacquemin et al., 2012) with a very mild phenotype. The sequence variant c.6067G>A was reported in follicular lymphoma (Gronbaek et al., 2002) and breast cancer (Teraoka et al., 2001); c.8095C>T in another CLL patient (Guarini et al., 2012); c.8663T>C in mantle cell lymphoma (Fang et al., 2003); and c.8861A>G in ovarian carcinoma (Cancer Genome Atlas Research Network (C.G.A.R.N, 2011). The remaining 18 missense variants were found only in the patients included in this study. All but one missense variant (c.3383A>G) involves a residue that is conserved between mouse and human which might further indicate their potential pathogenic character.

Two sequence changes (c.5882A>G, c.5821G>C) have previously been introduced into ATM-null lympoblastoid cell lines and were shown to have a slightly reduced kinase activity (Barone et al., 2009). In addition, some of the tumour samples included in this study have previously been analysed by Western Blotting to measure ATM-dependent responses to IR or the cytotoxic drug –Fludarabine (Austen et al., 2007). These included tumours with following missense variants: c.1009C>T, c.5224G>C, c.5980A>G, c.7047C>G, c.7313C>A, c.8249T>C, c.8839A>T, c.8600G>A. All but one (c. 1009C>T) have a loss of another *ATM* allele, either through chromosome 11q deletion or a truncating mutation, and all showed impaired activation of ATM downstream targets. For the remaining missense variants the exact impact on protein function has not been investigated in functional assays.

Of the 5 different missense variants which were present in the controls (c.1727T>C [p.Ile576Thr], c.3419A>G [p.Asn1140ser], c.5821G>C [p.Val1941Leu], c.5975A>C [p.Lys1992Thr], c.7390T>C [p.cys2464Arg]), 2 changes (c.5821G>C, c.5975A>C) were also identified in 3 CLL cases. The remaining 3 missense variants were detected in a single control case. Of note, the variant c.5821G>C was found in two controls and in two non 11q CLLs. This sequence change results in amino-acid substitution of a conserved residue in the

translated protein and, as mentioned before, has been shown to have reduced kinase activity when expressed in the ATM-null LCL cell line. One missense variant c.7390T>C had been previously reported as a mutation in breast cancer patient (Dork et al., 2001).

3.7.1.1 Frequency and distribution of missense variants in the CLL cohort and controls.

Missense variants accounted for 30.6% (26 of 85) of all sequence changes detected in the CLL cohort and for 14% (5 out of 35) of all sequence changes detected in controls. Thus, 9.1% (29 of 318) of CLL patients and 1.8% (5 of 281) of control individuals were affected by these changes ($p<0.001$). Missense variants were significantly more frequent in chromosome 11q deleted tumours (18 of 140) when compared to non 11q deleted tumours (11 of 178) (12.8% versus 6.2%, $p=0.049$).

Interestingly, missense variants identified in CLL patients tend to localize near the regions encoding functional domains: PI3kinase, FAT and FATC domains (14 of 26, 54%) (Figure 3.9.). In contrast, only one missense variant detected in control cohort was predicted to affect amino-acid residue in the FAT domain (1 of 5, 20%) (Figure 3.10.). Notably, it has been reported that most of the neutral sequence changes in the *ATM* gene are found in the regions that do not encode functional domains (Thorstenson et al., 2001). This further indicates that a subset of the missense variants (at least those affecting functional domains) in CLL cohort might have some pathogenic effect.

However, it is well established that some sequence changes can affect the functions of the protein even if they do not involve the amino acid residue located in the conserved domains. Barone and colleagues modelled various missense changes found in the *ATM* gene and some of them (of which two: c.5821G>C, c.5882A.G were detected in this study), decreased the kinase activity of the ATM protein although they were localized outside the region encoding the PI3Kinase domain (Barone et al., 2009). Furthermore, one of the pathogenic mutations (c.5228C>T) reported in AT patients and in the germ-line of a CLL patient in this study is also localized outside this domain.

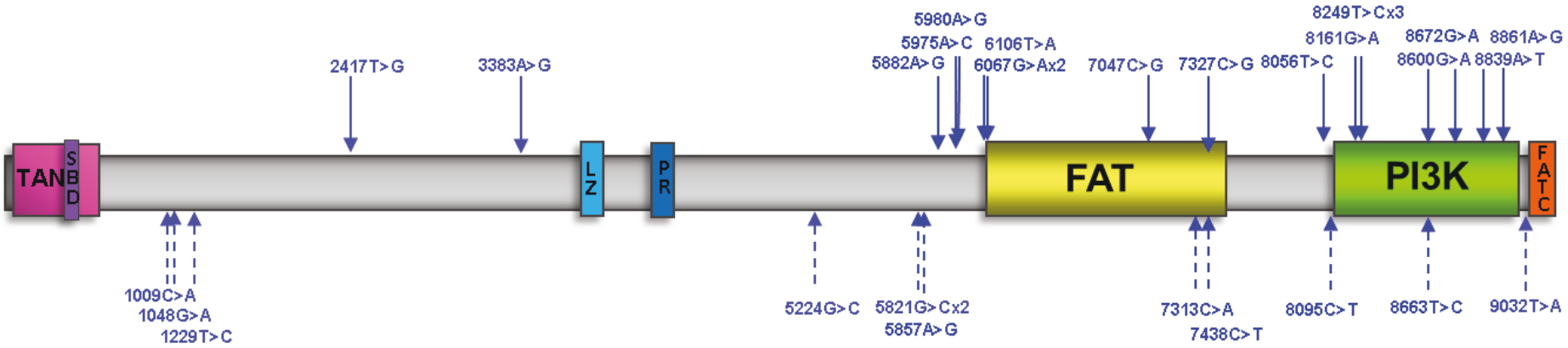


Figure 3.9. Distribution of the *ATM* missense variants across the coding region of *ATM* gene in the CLL cohort.

Missense variants tended to localise in the regions encoding functional domains of ATM protein: PI3kinase, FAT and FATC domains. There was a significantly higher proportion of 11q deleted CLL tumours affected by missense variants compared to non 11q deleted tumours (p=0.049). Solid line arrows indicate missense variants detected in tumours with 11q deletion, dashed line arrows indicate missense variants detected in tumours without 11q deletion.

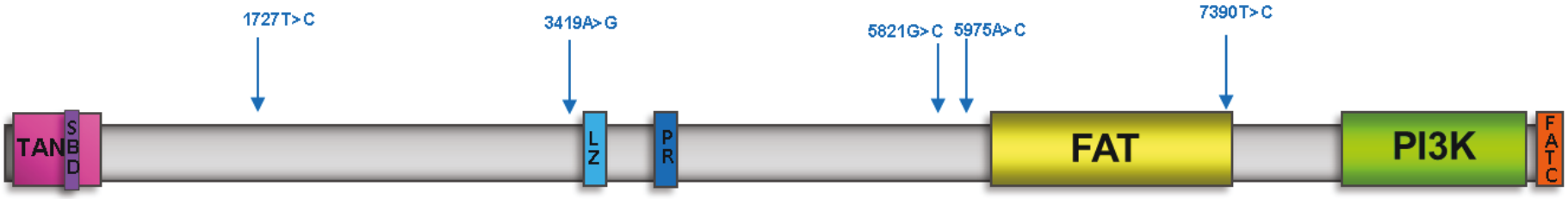


Figure 3.10. Distribution of missense variants across the coding region of *ATM* gene in the control individuals.

Missense variants detected in controls were distributed evenly across the whole coding region of *ATM* gene. Only one change (c.7390T>C) was predicted to affect functional domain - FAT.

The germ-line status was known for 17 out of 30 missense variants in different CLL cases (Table 3.8.). Six different missense variants (c.3383A>G, c.5882A>G, c.6067G>A, c.8249T>C, c.5821G>C, c.5975A>C) in 8 different patients were present in germ-line. Six of these were found in patients with 11q deletion. One missense variant (c.5821G>C) was found in the germ-line material of 2 patients without 11q deletion one of whom also had another 2 acquired sequence changes (c.7313C>A and c.8834_8867del34).

In conclusion, *ATM* missense variants were more frequently detected in CLL tumours than in controls and many of them could be predicted to impact on protein function. However, due to the limited information about their functional consequences and the need for stringent criteria for *ATM* germ-line mutations they were not classified in this study as pathogenic mutations.

3.7.2. *ATM* neutral sequence changes in the CLL cohort and controls.

There were 41 different sequence changes detected in CLL cohort and controls classified as *neutral sequence changes* for the purpose of this study (Table 3.9). Twenty eight were found in CLL individuals and 30 in controls; 25 of them were known polymorphisms, whereas 16 were previously unreported; 17 changes were common for both CLL and control cohorts, 11 were found only in CLL cases and 13 only in controls.

Twenty three changes previously reported as polymorphisms were detected in CLL cases. These included 13 missense changes (c.998C>T c.146C>G, c.378T>A, c.1744T>C, c.2119T>C, c.2572T>C, c.3161C>G, c.4138C>T, c.4258C>T, c.4802G>A, c.5071A>C, c.5557G>A, c.6820G>A); 6 silent changes in the coding sequence (c.735C>T, c.795T>C, c.1986T>C, c.2805G>C, c.4578C>T, c.5793T>C) and 4 single nucleotide substitutions within an intron not predicted to affect splicing (c.3403-12insA, c.5497-15G>C, c.5497-8T>C, c.8786+8A>C).

Three silent changes (c.4947C>T, c.5352C>T, c.8592C>T) and two intronic changes (c.3284+6G>A, c.8671+9T>G) had never been reported as polymorphisms and were absent

Table 3.9. *ATM* neutral sequence changes detected in CLL and control cohorts.

No.	Sequence change	Amino Acid change	Reported as polymorphism	No. of 11q CLL affected (n=140)	No. of non11q CLL affected (n=178)	Controls affected (n=281)
Found in CLL patients only						
1	c.378T>A	p.(Asp126Glu)	yes	1	1	0
2	c.795T>C	No AA change	yes	2	0	0
3	c.998C>T	p.(Ser333Phe)	yes	1	1	0
4	c.1986T>C	No AA change	yes	1	0	0
5	c.3284+6G>A	Non coding	no	0	1	0
6	c.4802G>A	p.(Ser1601Asn)	yes	1	0	0
7	c.4947C>T	No AA change	no	1	0	0
8	c.5352C>T	No AA change	no	0	1	0
9	c.6820G>A	p.(Ala2274Thr)	yes	0	1	0
10	c.8592C>T	No AA change	no	0	1	0
11	c.8671+9T>G	Non coding	no	1	0	0
Found in controls only						
12	c.162T>C	No AA change	no	0	0	2
13	c.609C>T	No AA change	no	0	0	1
14	c.2250+22A>C	Non coding	no	0	0	1
15	c.2922-22del T	Non coding	yes	0	0	4
16	c.4119T>C	No AA change	no	0	0	1
17	c.4167A>G	No AA change	no	0	0	1
18	c.4473C>T	No AA change	no	0	0	2
19	c.4724G>A	p.(Arg1575His)	yes	0	0	1
20	c.4980C>T	No AA change	no	0	0	1
21	c.6975+13insT	Non coding	no	0	0	1
22	c.7788+8G>T	Non coding	no	0	0	2
23	c.8987+50A>T	Non coding	no	0	0	1
24	c.9200C>G	Non coding	no	0	0	2
Common for CLL and control cohorts						
25	c.146C>G	p.(Ser49Cys)	yes	1	1	4
26	c.735C>T	No AA change	yes	1	0	4
27	c.1744T>C	p.(Phe582Leu)	yes	1	0	2
28	c.2119T>C	p.(Ser707Pro)	yes	2	2	9
29	c.2572T>C	p.(Phe858Leu)	yes	3	10	14
30	c.2805G>C	No AA change	yes	0	1	1
31	c.3161C>G	p.(Prp1054Arg)	yes	0	12	19
32	c.3403-12insA	Non coding	yes	34	74	97
33	c.4138C>T	p.(His1380Tyr)	yes	1	0	1
34	c.4258C>T	p.(Leu1420Phe)	yes	4	9	12
35	c.4578C>T	No AA change	yes	3	19	24
36	c.5071A>C	p.(Ser1691Arg)	yes	1	0	2
37	c.5497-15G>C	Non coding	yes	1	0	1
38	c.5497-8T>C	Non coding	yes	5	4	16
39	c.5557G>A	p.(Asp1853Asn)	yes	27	57	101
40	c.5793T>C	No AA change	yes	1	4	3
41	c.8786+8A>C	Non coding	yes	5	17	7

from the control group. The silent changes are unlikely to impact on the protein since they would not result in a change of amino acid in the translated protein. However, sequence change c.8592C>T was proposed by Thorstenson and colleagues to have a potential effect on splicing, but this has not been proven or investigated further (Thorstenson et al., 2003). The impact of the two intronic changes had also not been investigated and their potential deleterious effect on the splicing process cannot be completely excluded. However, due to the criteria established for this study these changes were not classified as mutations.

There were 19 known *ATM* polymorphisms detected in the control population: 10 were missense changes (c.146C>G, c.1744T>C, c.2119T>C, c.2572T>C, c.3161C>G, c.4138C>T, c.4258C>T, c.4724G>A, c.5071A>C, c.5557G>A), 4 were single nucleotide substitutions not resulting in amino acid change (c.735C>T, c.2805G>C, c.4578C>T, c.5793T>C) and 5 were intronic changes (c.2922-22delT, c.3403-12insA, c.5497-15G>C, c.5497-8T>C, c.8786+8A>C).

Six silent changes (c.162T>C, 609C>T, c.4119T>C, c.4167A>G, c.4473C>T, c.4980C>T) and 5 intronic changes (c.2250+22A>C, c.6975+13insT, c.7788+8G>T, c.8987+50A>T, c.9200C>G) had not been previously reported and all of them were detected only in control samples.

Importantly, some of the polymorphisms and other sequence variants of unknown significance might have some functional consequences, albeit unproven. The example is a silent change c.735C>T which has been reported as a neutral variant (Hacia et al., 1998) but was also shown to coincide with deletion of exon 9 in 3 Nordic AT families. Since no other mutations were detected in two of those three families, it was suggestive that this sequence change might affect splicing (Laake et al., 2000a).

3.7.2.1 Frequency comparison of known *ATM* polymorphisms.

The majority of these sequence changes were rare, present in less than 5 individuals in total (Table 3.9). The most common changes were: c.2119T>C, c.2572T>C, c. 3161C>G,

c.4258C>T, c.4578C>T, c.5557G>A, c.5793T>C, c.3403-12insA, c. 5497-8T>C and c.8786+8A>C.

When considering two CLL subgroups (with and without 11q deletion) the frequency of 4 common polymorphisms: c.4578C>T, c.3403-12insA, c.3161C>G, c.5557G>A was higher in controls than in 11q deleted patients but this was not the case when comparing the non 11q deleted group to controls. This finding is not surprising as a reduced frequency of polymorphisms was expected among patients with 11q deletion which results in the loss of one *ATM* allele. Only one change 8786+8A>C was more frequent in the non 11q deletion group when compared to controls.

3.8. Discussion

The molecular basis underlying the genetic predisposition in CLL has not been established yet. The *ATM* gene has been considered as a potential predisposing candidate due to the following reasons: it is frequently mutated in CLL tumours and some of the mutations are present in the patients' germ-line; AT patients have an increased incidence of lymphoid malignancies including those of B-cell origin; certain polymorphisms have been associated with CLL; AT heterozygotes have a small but significantly increased risk of developing breast cancer (relative risk 2.37) and familial pancreatic cancer (odds ratio 10.5) indicating that carrier status can be a predisposing factor for tumour development in general (Renwick et al., 2006; Roberts et al., 2012b; Rudd et al., 2006; Stankovic et al., 2002b; Taylor et al., 1996).

Many of the *ATM* sequence changes detected in CLL tumours are novel, and apart from those predicted to cause protein truncation, the assessment of their functional consequences and potential causative influence is problematic.

I have investigated the role of germ-line *ATM* mutations in the pathogenesis of CLL by comparing their frequencies between cohorts of CLL patients and control individuals. To avoid false positive results and to distinguish truly pathogenic germ-line mutations from rare

polymorphisms I have used highly stringent criteria. In consequence, the majority of the identified germ-line pathogenic mutations were truncating and were scattered across the whole coding region of the *ATM* gene. This particular distribution and nature of the mutations were the same as for the remaining pathogenic changes which were either acquired or remained of unknown origin. The distribution and frequency of particular types of the pathogenic mutations were similar to those observed in AT patients and this was mostly due to criteria created for this study. Notably, more than 70% of reported mutations in AT patients are truncating leading to low or no expression of the protein and loss of its kinase activity (Concannon and Gatti, 1997; Stankovic et al., 1998).

I found that incidence of the *ATM* mutation carrier status in the CLL cohort was significantly higher than in the control cohort (2.5% and 0% respectively) p=0.008. However, closer analysis revealed that this finding could be confirmed only for the subgroup of patients with chromosome 11q deletion (6 in 140 versus 0 in 281, p=0.001) but not for those who did not acquire this deletion in their leukemic clone (2 in 178 versus 0 in 281, p=0.15).
I concluded that germ-line *ATM* pathogenic mutations are not involved in the development of CLL, otherwise they would be present at significantly increased frequencies not only in chromosome 11q deleted tumours but also in non 11q deleted cases; instead they may predispose to a rapid disease progression through acquired chromosome 11q deletion, loss of ATM activity and clonal expansion.

As I have not identified a single pathogenic germ-line *ATM* mutation among 281 control individuals, the frequency of these changes detected in controls was lower than the previously estimated level of *ATM* heterozygotes carriers in the UK population of 0.4% (Thompson et al., 2005). However, even if the incidence of AT carriers among controls was 0.7% which relates to 2 affected individuals within 281 cohort, the difference between controls and tumours with 11q deletion would be still significant (6 in 140 versus 2 in 281, p=0.0185).

It is also possible that the number of pathogenic germ-line *ATM* mutations was higher

among CLL patients than I was able to demonstrate. There were 5 truly pathogenic mutations in the CLL cohort for which germ-line status could not have been established due to a lack of constitutional material (in 4 patients with and in 1 patient without chromosome 11q deletion). If they were to represent germ-line changes, the frequency of *ATM* mutation carriers in CLL patients could have been underestimated but this would not change the general findings of this study (10 in 140 and 3 in 178 versus 0 in 281, $p<0.0001$ and $p=0.06$ respectively).

Although chromosome 11q deletion in CLL is present usually at diagnosis, it might also be a later event in tumour progression. In both scenarios it probably follows initial clonal proliferation. This is indicated by the fact, that often less than 100% of the tumour cells from one individual carry this abnormality. In addition, its acquisition has been demonstrated by the analysis of serial post-diagnostic samples from individual patients (Cuneo et al., 2002; Fegan et al., 1995). Recent studies suggest the presence of extensive clonal heterogeneity during progression of CLL (Landau et al., 2013; Schuh et al., 2012). The emergence of a dominant sub-clone depends on selective pressures (one of which is treatment). The high incidence of germ-line *ATM* mutations among the tumours with 11q deletion suggests that they can exert some selective pressure towards the loss of another *ATM* allele. This sub-clone would have a survival advantage caused by its bi-allelic *ATM* inactivation and by defects in the apoptotic pathway.

The explanation of the results is supported by the analysis of clinical features and outcome of *ATM* mutation carriers. I have not observed any significant differences in age at diagnosis between *ATM* mutation carriers and the rest of the CLL cohort, indicating that the complete loss of ATM function, clonal expansion and disease progression occur after initial proliferation and do not result in the early onset of disease. However, most patients with pathogenic germ-line mutations presented with advanced stage and unmutated *VH* genes (although these findings did not reach significance), which implies that bi-allelic *ATM* loss, once it occurs, promotes a very rapid progression. Importantly, the whole CLL cohort was

biased for tumours with advanced stage of disease because the large proportion of samples from the LRF UK CLL 4 trial was included in this study. Therefore, it would be expected that the *ATM* wild-type group would also be enriched for progressive disease due to factors independent of *ATM* inactivation. However, the inferior survival was observed not only among *ATM* pathogenic mutations carriers but also among patients with acquired pathogenic mutations as well as those with missense variants when compared to *ATM* wild-type individuals. Furthermore, all these three groups were enriched for tumours with chromosome 11q deletion when compared to *ATM* wild-type subgroup. These observations suggest that some missense variants might represent pathogenic mutations that have stayed unclassified in this study due to the stringent criteria used; and also strengthen the role of *ATM* aberrations in progression of CLL via promoting a multi-step acquisition of a more aggressive phenotype.

Nevertheless, it is important to emphasize the limitation of this clinical correlation. The CLL cohort analysed was heterogeneous both in terms of treatment as well as biologically. Time to first treatment and response to treatment are two important aspects to be examined when evaluating the progression of the CLL disease. However, the assessment of these clinical features was not performed in this study, due to very heterogeneous nature of CLL cohort. Therefore, the purpose of the clinical comparison was to only indicate the preliminary observations.

The mechanism by which *ATM* sequence changes could potentially contribute to the development of CLL is unknown. ATM protein has been reported to be involved in VDJ recombination and CSR and both these processes are important for normal B cell development (Pan et al., 2002; Perkins et al., 2002). Furthermore, CLL is believed to be an antigen driven disease which is reflected in increased frequency of certain *VDJ* genes usage (Agathangelidis et al., 2012). Therefore, the observation of aberrant *VDJ* rearrangement or CSR, as well as overrepresentation of stereotyped BCRs among the CLL clones in *ATM* mutation carriers could indicate the potential involvement of monoclonal *ATM* inactivation in

tumorigenesis through these processes.

Nevertheless, the switch junctions analysed in tumour cells from patients did not reveal any abnormalities. Furthermore, there were only two patients with stereotyped BCRs in their tumour clones which constituted 25% (2 of 8) of this small sub-group and this frequency did not strikingly differ from estimated 30% within the general CLL population (Agathangelidis et al., 2012). Therefore, the analysis of *VDJ* genes usage and switch joints generated during CSR in the clones of *ATM* mutation carriers does not imply the role of pathogenic germ-line *ATM* mutations in the initiation of CLL. It also suggests that the loss of a second *ATM* allele was most likely acquired after the tumour cell underwent CSR.

Interestingly, one of the patients carried a tumour which had un-mutated *IGHV* gene but also switched isotype. Normally, the encounter of antigen by B-cells results in the induction of SHM and CSR and both these processes take place in germinal centres (GCs). However, it has been also shown that they are not spatially and temporally linked. In addition, both of them can happen independently of T-cell help and outside of GCs (Bergqvist et al., 2006; Klein and Dalla-Favera, 2008). Therefore, it is possible that in this case the original clone underwent antigenic stimulation outside a GC.

The interpretation of the results is further supported by the lack of an overrepresentation of CLL cases among AT family members (AT carriers), and the non-clustering pattern of *ATM* mutations among CLL families (Bevan et al., 1999; Thompson et al., 2005; Yuille et al., 2002). Both of these would be expected to occur if *ATM* mutation was a strong predisposing factor. In addition, CLL has never been reported in AT patients. This latter observation could possibly be explained by the fact that AT patients have a short life expectancy and do not live long enough to develop this malignancy. However, other adult haematological malignancies such as T-PLL and MCL, which normally occur at an older age, are seen in increased frequencies among AT patients (Taylor et al., 1996). Possibly the molecular mechanism underlying the development of these tumours differs from CLL. While both T-PLL and MCL are believed to be induced by the specific chromosomal translocations involving T-cell

receptor or the immunoglobulin genes, respectively (Klein and Dalla-Favera, 2005; Taylor, 1992), chromosomal translocations involving immune genes in CLL are a much less common genetic event and its role in initiation of clonal transformation is unknown. Since AT cells have increased levels of chromosomal translocations, which are the consequences of the defective repair of DNA DSBs during VDJ recombination, AT patients are predisposed to the development of lymphoid tumours such as T-PLL and MCL but not CLL.

In addition to the pathogenic *ATM* mutations, I observed a substantial number of missense variants. They were significantly more frequent in the CLL cohort than in the control and more frequent in tumours that had an 11q deletion than those that did not. Missense variants in CLL were clustered in the C-terminal region of the gene which encodes the functional domains of the ATM protein (this was particularly apparent in the chromosome 11q deleted group), whereas in control individuals, they were scattered across the whole coding region.

It is likely that the missense variants within the *ATM* gene may exert deleterious effects on protein function and therefore on the cellular and clinical phenotype. This is because there was an increased frequency compared to the controls and there is a high probability that most of them were acquired by the tumour clone. Importantly, in this study, the group of patients with missense variants had a inferior overall survival when compared to patients with wild-type *ATM*. In addition, some missense variants included in this study, have been either previously tested in *in vitro* assays and shown to have defective ATM-dependent responses to DNA damaging agents (Austen et al., 2007), or were previously reported in other cancers indicating that they indeed might represent a hot spots of *ATM* inactivation.

The current model for predisposition to CLL considers co-inheritance of multiple low penetrance loci in generating higher risk. Following this model, the potential role of certain *ATM* polymorphisms in predisposition to CLL has been previously indicated by a large candidate-gene association study including 1467 non-synonymous SNP markers in selected genes in 992 CLL cases and 2707 controls from British Islands (Rudd et al., 2006). The *ATM*

gene along with other members of DNA damage response pathways - *Chk2* and *BRCA1,* were the only three loci associated with CLL in that study. The two polymorphisms identified in *ATM* gene were 2572T>C and 3161C>G.

I have not observed an increased frequency of these polymorphisms in the *ATM* gene when I compared CLL patients and controls. Instead I have identified another known polymorphism c.8786+8A>C to be more represented in tumours without 11q deletion than in controls. Interestingly, other big association studies (a candidate-gene study in Spanish population and a genome-wide study including British residents) also did not find any convincing evidence of the link between SNPs in the *ATM* gene and CLL (Di Bernardo et al., 2008; Enjuanes et al., 2008). These contradictions might be a consequence of differences in methodologies used and/or in the studied populations.

In summary, the results from this study suggest that *ATM* germ-line mutations do not contribute to CLL initiation but may rather predispose to rapid disease progression through acquired chromosome 11q deletion, loss of ATM protein activity and clonal expansion. High prevalence of all non polymorphic *ATM* sequence variants in the cohort of CLL patients and especially in the subgroup of tumours with 11q deletion, when compared to the general population, supports the model in which inactivation of *ATM* occurs in a step-wise manner and further strengthens the role of this tumour suppressor gene in preventing the aggressive phenotype in CLL affected individuals.

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CHAPTER IV

RESULTS

Clinical consequences of *ATM* mutations in patients from UK LRF CLL4 Trial.

4.1. Introduction

ATM mutations have been previously shown to be associated with shorter overall survival and treatment free survival in an unselected CLL cohort (Austen et al., 2005). Furthermore, 11q deletion which results in mono-allelic loss of *ATM,* is a well established prognostic marker and distinguishes the group with more advance disease and poorer prognosis (Dohner et al., 2000). There is only a partial correlation between CLL tumours carrying an *ATM* mutation and those with 11q deletion, although bi-allelic loss of *ATM* gene appears to be even three times more frequent in 11q deleted group than in unselected cohort (Austen et al., 2007). There is also evidence from a retrospective study of 72 CLL patients with 11q deletion treated with DNA damage inducing agents suggesting that bi-allelic inactivation of *ATM* may confer inferior outcome compared with mono-allelic loss or mutation (Austen et al., 2007). That study showed that activation of the ATM-dependent DNA damage response differs among tumours with 11q deletion depending on the status of the remaining *ATM* allele and that the presence of one wild-type *ATM* allele might be sufficient for normal ATM function. Shorter overall survival from diagnosis for patients with *ATM* mutation plus 11q deletion compared to those with residual wild type allele suggested that in CLL tumours with 11q deletion *ATM* mutation status have further clinical significance. However, those findings have not been verified in the context of a clinical trial where patients with similar stage of disease are treated under the uniformed protocol.

The recently conducted United Kingdom Leukaemia Research Fund CLL4 trial (UK LRF CLL4) recruited previously untreated patients with advance stage of disease and randomized them to different treatment arms: alkylating agents (Chlorambucil and Cyclophosphamide) and/or purine analogue (Fludarabine). On this trial patients with 11q deletion had significantly worse overall response rate, shorter progression free survival and overall survival when compared to patients without 11q deletion or *TP53* abnormalities (chromosome 17 deletion and/or *TP53* mutations) (Oscier et al., 2010). However, the clinical impact of the *ATM* mutations has not been investigated in the context of this trial.

Therefore the aim of the study described in this chapter was to establish whether the presence of *ATM* mutation, either as sole *ATM* abnormality or in combination with 11q deletion, influences the response to treatment and survival of the patients enrolled on the UK LRF CLL4 trail. I have done this by performing *ATM* status analysis on patients' tumour samples taken at the time of trail entry and, subsequently by investigating the relationship of *ATM* mutations to other clinical and biologic parameters; and the impact of *ATM* abnormalities on response to treatments, progression free survival (PFS) and overall survival (OS).

4.2. Characteristic of LRF CLL4 trial cohort

The characteristics of UK LRF CLL4 trial cohort have been described in details by Catovsky and colleagues (Catovsky et al., 2007) and were summarized in the 'Introduction' chapter of this thesis (section 1.6.1.1). Briefly, the UK CLL4 trial randomly assigned 777 patients to first line treatment in a ratio 2:1:1 with Chlorambucil (Chl), Fludarabine alone (Flu) or Fludarabine plus Cyclophosphamide (FC) between 1st February 1999 and 31st of October 2004. The biological prognostic markers including cytogenetics, *IGHV* mutation status and *VDJ* genes usage, *TP53* mutation status, level of CD38 and ZAP-70, were measured in peripheral blood samples taken at the trial entry. The analysis of these biomarkers was performed by the centralized biomedical institutions (Oscier et al., 2010) and the results were available for the purpose of this study. The *TP53* gene regions examined for mutations were exons 5-9 (Gonzalez et al., 2011).

The cohort analysed in this chapter was restricted to 224 trial patients due to availability of material. Although the DHPLC technique made the analysis of the large cohorts more feasible, screening large genes like *ATM* still remains a challenge. Out of the original cohort of 777 patients, 67 cases with chromosome 11q deletion/loss of *ATM* allele for which sufficient material and clinical information were available were included in this study. Patients without chromosome 11q deletion comprised the remaining 157 cases analysed. This

approach facilitated the identification of patients with an *ATM* mutation, but enriched for samples with chromosome 11q deletion, which constitutes 29.9% of the selected cohort. Patients' characteristics are included in Table 4.1.

Table 4.1. Clinical and biological characteristics of 224 CLL patients.

Variable	Total n=224
Gender	
Female	54
Male	170
Treatment allocation	
Chlorambucil	104
Fludarabine	52
Fludarabine+cyclophosphamide	68
Age group, years	
<60	76
60-70	84
>70	64
Binet stage	
A	56
B	94
C	74
IGHV mutation status (98% cutoff)	
Mutated	78
Unmutated	144
Number of tissue sites	
<3	87
≥3	137
17p deletion (10% cut off)	
No	213
Yes	11
TP53 mutation	
No	207
Yes	17
11q deletion (5% cut off)	
No	157
Yes	67
Trisomy 12 (3%cut off)	
No	199
Yes	24
13q deletion (5% cut off)	
No	89
Yes	134
CD38 positive (7% cut off)	
No	70
Yes	123
ZAP-70 positive (10% cut off)	
No	95
Yes	86

IGHV- immunoglobulin heavy chain variable gene

The cohort of 224 patients consisted of 170 men and 54 women. One hundred four

patients were assigned to Chl, 52 to Flu and 68 to FC treatment groups. Seventy six patients were under 60 years of age at the time or randomization, 84 were between 60 and 70 years old and 64 patients were older than 70 years. Fifty six patients had Binet stage A-progressive, 94 had stage B and 74 stage C.

The *IGHV* status and usage had been determined for 222 patients; 78 patients had mutated *IGHV* genes and 144 had unmutated or stereotype *VH3-21* gene. Chromosomal abnormalities of 11q deletion, 17p deletion and trisomy 12 were assessed in 224 patients and 13q deletion in 223 patients. The cut-offs for positivity for all these abnormalities were based on the Dohner's hierarchical model (Dohner et al., 2000) and normalized to the signal levels in negative controls (Dohner et al., 1995) and patient outcomes (Oscier et al. 2010). According to these criteria, the sample was assessed positive for 11q deletion if greater than 5% of clone cells carried this abnormality. Accordingly, the cut-off for 17 deletion was 10%, for trisomy 12 was 3%, and for 13q deletion was 5%. Using these cut-off values, in the cohort of 224 patients, 67 patients had 11q deletion, 11 patients had 17 deletions, 24 patients had trisomy 12 and 134 patients had 13q deletion (80 patients had it as a sole abnormality). *TP53* mutation was detected in 17 patients and 9 of them also had chromosome 17p deletion, 8 patients had only *TP53* mutation and 2 patients had only 17p deletion.

The results of CD38 (cut-off 7%) and ZAP-70 (cut-off 10%) positivity were available for 193 and 171 patients respectively. Of those, 123 patients were positive for CD38 and 86 patients for ZAP-70.

Involvement of multiple (≥3) lymphoid tissue sites by tumour cells occurred in 137 patients out of 224.

Only a proportion of cases from the selected cohort of 224 patients were tested for Beta2-Microglobulin (β2-M). β-2M has been shown to be important, independent prognostic factor in the cohort of LRF CLL4 trial (Oscier et al., 2010). Therefore, it seemed desirable to investigate the correlation between *ATM* mutations and β2M. However, the inclusion of the limited test results in the initial calculations significantly weakened the statistical model,

subsequently leading to exclusion of β2M from the analysis of this study (communication with the biostatistician).

The latest follow-up was to 31st October 2009, with the median follow-up for survivors of 92 months. The records of the treatment responses were available for 212 patients and the survivals data were know for 224 patients.

4.3. *ATM* mutations in the cohort of 224 CLL4 trial patients.

The entire coding region of *ATM* gene consisting of 62 exons and flanking intronic regions was screened using the DHPLC technique and sequencing approach. From this, criteria for *ATM* mutations and their frequency and distribution in the cohort of 224 trial tumours was established.

4.3.1. Criteria for *ATM* mutation.

In this study *ATM* sequence change was classified as a mutation if it was:

a) predicted to cause premature termination of the protein either due to nucleotide substitution creating a stop codon or out of frame deletion or insertion;

b) short in frame deletion;

c) predicted to affect splicing;

d) a missense mutation reported in AT patients or predicted to cause an amino acid substitution in the residue located within the region encoding conserved domain of ATM or/and conserved between man and mouse and was not previously reported as polymorphism.

Sequence changes which did not meet any of these criteria or have previously been reported as polymorphisms were not classified as mutation in this study.

These criteria for *ATM* mutation differ from those previously described in Chapter 3, which were established to avoid classifying rare, novel polymorphisms as germ-line causative mutations. Using criteria from Chapter 3, non-synonymous substitutions might

have been classified as missense variants rather than pathogenic mutations. Current criteria, although less stringent, was carefully designed to minimise misclassification of the sequence changes and was broadened because it was no longer necessary to identify the origin of a mutation (germ-line or somatic). Furthermore, most of the mutations detected in CLL tumours are acquired and only a fraction is expected to be present in the patient's germ-line. The acquisition and maintenance of *ATM* mutation within a tumour clone already indicates its likely pathogenic character.

4.3.2. Nature of *ATM* mutations in 224 CLL4 trial tumours

Altogether, 36 *ATM* sequence changes in 33 of 224 patients (16%) that fulfilled the criteria for mutations have been identified (Table 4.2). Seventeen patients without 11q deletion (17/157, 11%) and 16 patients with 11q deletions (16/67, 24%) were affected by *ATM* mutations. Overall:

a) 41.7% (15/36) of all mutations were predicted to cause premature termination (c.217_218del2 [p.Glu73fs], c.478_482del5 [p.Ser160fs], c.617delA [p.Asn206fs] , c.1402_1403del2 [p.Lys468fs], c.2193delC [p.Tyr731fs], c.2720_2723del4 [p.Cys907fs], c.3712_3716del5 [p.Leu1238fs], c.3720_3736del17 [p.Asn1240fs], c.4095_4109+4del19 [p.Lys1365fs], c.6375insT [p.Glu2126X], c.6989_6995del7 [p.Leu2330fs], c.8266A>T [p.Lys2756X], c.8428_8450del23 [p.Lys2810fs], c.8965C>T [p.Gln2990X], c.9139C>T [p.Arg3047X]);

b) 5.6% (2/36) were short in-frame deletions (c.1006_1020del15 [p.Phe336_Ala340del5] , c.7638_7646del9 [p.Arg2547_Ser2549del3]);

c) 5.6% (2/36) were splice site mutations (c.1066-6T>G [splice site exon 11], c.5006-2A>G) [splice site exon 36];

d) and 44.4% (16/36) were missense mutations (c.1048G>A [p.Ala350Thr], c.1229T>C [p.Val410Ala] c.5857A>G [p.Thr1953Ala], c.6067G>A [p.Gly2023Arg], c.7327C>G [p.Arg2443Gly], c.7390T>C [p.Cys2464Arg] c.7438C>T [p.His2480Tyr], c.7474C>T

Table 4.2. *ATM* mutations detected in 224 CLL tumours with and without 11q deletion.

Patient	Nucleotide Change	Protein Change	AA residue	Protein Domain	Reported Previously
CLL tumours without chromosome 11q deletion					
CT29	c.217_218del2	p.(Glu73frameshift)	NA	TAN	AT
CT23*	c.617delA	p.(Asn206frameshift)	NA	none	AT
CT24*	c.1006_1020del15	p.(Phe336_Ala340del5)	NA	none	No
CT16	c.1048G>A	p.(Ala350Thr)	Conserved	none	CLL[#]
CT14	c.1229T>C	p.(Val410Ala)	Conserved	none	BC,BL,FL, TALL
CT2	c.2193delC	p.(Tyr731frameshift)	NA	none	No
CT12	c.4095_4109+4del1	p.(Lys1365frameshift)	NA	none	No
CT1	c.5857A>G	p.(Thr1953Ala)	Conserved	none	No
CT31	c.7390T>C	p.(Cys2464Arg)	Conserved	FAT	No
CT4	c.7438C>T	p.(His2480Tyr)	Conserved	FAT	No
CT18	c.7474C>T	p.(Leu2492Phe)	Conserved	edge of FAT	No
CT24*	c.7570G>A	p.(Ala2524Thr)	Conserved	none	No
CT5	c.8095C>T	p.(Pro2699Ser)	Conserved	none	CLL[#]
CT15	c.8266A>T	p.(Lys2756X)	NA	PI3Kc	AT
CT27	c.8428_8450del23	p.(Lys2810frameshift)	NA	PI3Kc	No
CT23*	c.8494C>T	p.(Arg2832Cys)	Conserved	PI3Kc	AT,B-NHL
CT8	c.8663T>C	p.(Ile2888Thr)	Conserved	PI3Kc	MCL
CT10*	c.8668C>G	p.(Leu2890Val)	Conserved	PI3Kc	MCL,T-PLL
CT10*	c.8965C>T	p.(Gln2990X)	NA	PI3Kc	No
CT7	c.9032T>A	p.(Met3011Lys)	Conserved	none	No
CLL tumours with chromosome 11q deletion					
CT6	c.478_482del5	p.(Ser160frameshift)	NA	TAN	AT
CT26	c.1066-6T>G	Splice defect -termination	NA	none	AT,MM,BC
CT11	c.1402_1403del2	p.(Lys468frameshift)	NA	none	AT
CT33	c.1402_1403del2	p.(Lys468frameshift)	NA	none	AT
CT17	c.2720_2723del4	p.(Cys907frameshift)	NA	none	AT
CT13	c.3712_3716del5	p.(Leu1238frameshift)	NA	none	AT
CT21	c.3720_3736del17	p.(Asn1240frameshift)	NA	none	No
CT22	c.5006-2A>G	Splice defect- termination	NA	none	No
CT19	c.6067G>A	p.(Gly2023Arg)	Conserved	none	BC,FL
CT32	c.6375insT	p.(Glu2126X)	NA	FAT	AT
CT20	c.6989_6995del7	p.(Leu2330frameshift)	NA	FAT	No
CT28	c.7327C>G	p.(Arg2443Gly)	Conserved	FAT	No
CT30	c.7638_7646del9	p.(Arg2547_Ser2549del3	NA	none	AT
CT3	c.8249T>C	p.(Leu2750Ser)	Conserved	PI3Kc	CLL[#]
CT25	c.8672G>A	p.(Gly2891Asp)	Conserved	PI3Kc	AT
CT9	c.9139C>T	p.(Arg3047X)	NA	FATC	AT,CLL[#],MCL, TPLL

AA-amino acid; AT-ataxia telangiectasia; BC-breast cancer ; BL-Burkitt lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; CLL-chronic lymphocytic leukemia; FL-follicular lymphoma; MCL-mantle cell lymphoma; MM-multiple myeloma; T-ALL,-T-cell acute lymphoblastic leukemia; T-PLL,-T-cell prolymhocytic leukemia; NA,-not applicable, * patients with two *ATM* mutations; # previously reported in another CLL patient

[p.Leu2492Phe] c.7570G>A [p.Ala2524Thr], c.8095C>T [p.Pro2699Ser], c.8249T>C [p.Leu2750Ser], c.8494C>T [p.Arg2832Cys], c.8663T>C [p.Ile2888ThrThr], c.8668C>G [p.leu2890Val], c.8672G>A [p.Gly2891Asp], c.9032T>A [p.Met3011Lys]).

Three patients without 11q deletion had 2 different mutations in their tumour cells: patients CT10 and CT23 had one truncating and one missense mutation (c.8965C>T, c.8668C>G and c.617delA, c.8494C>T respectively) and patient CT24 had one short in-frame deletion and one missense mutation (c.1006_1020del15 and c.7570G>A). One truncating mutation c.1402_1403del2 was detected in two different 11q deleted tumour samples (CT11 and CT33).

Twenty-four of 36 mutations included in this cohort have been already reported in the previous chapter. These included 15 mutations identified in tumours with chromosome 11q deletion (patients: CT6, CT26, CT11, CT17, CT13, CT21, CT22, CT19, CT32, CT20, CT28, CT30, CT3, CT25, CT9) and 9 mutations identified in tumours without chromosome 11q deletion (patients CT16, CT14, CT2, CT12, CT1, CT4, CT5, CT15, CT7). Twenty mutations were previously reported in AT patients and/or various tumours which further confirm their pathogenic character (Table 4.2). Of the mutations not included in the previous chapter, changes c.217_218del2, c.617delA, c.8494C>T have been previously observed in AT patients (Li and Swift, 2000), and change c.8494C>T was also reported in B-NHL (Jadayel et al., 1997). Furthermore, change c.8663T>C was reported in MCL (Fang et al., 2003) and change c.8668C>G in MCL and T-PLL (Greiner et al., 2006; Vorechovsky et al., 1997).

4.3.3. Frequency and distribution of *ATM* mutations in 224 CLL4 trial tumours.

ATM mutations affected significantly more patients with 11q deletion (16 patients of 67, 24%, 16 mutations), than without 11q deletions (17 patients of 157, 11%, 20 mutations), p=0.022. The mutations causing premature termination, in-frame deletions and splice site defects combined together were more frequent in the tumours with 11q deletion than in tumours without 11q deletion. Seventy five percent (12/16) of all mutations detected in 11q

deleted tumours and 40% (8/20) detected in non 11q deleted tumours belonged to this combined group (p=0.049). On the contrary, missense mutations were more frequent in tumours without 11q deletion (60%, 12 of 20) than in those with 11q deletion (25%, 4 of 16) p=0.049. The frequencies of different mutation types and comparison between patient groups with and without 11q deletion are illustrated in Figure 4.1.

Consistent with previous observation and similar to the distribution observed in sporadic T-PLL tumours (Vorechovsky et al., 1997) missense mutations clustered in the 3' terminal region of the gene encoding FAT, PI3kinase and FATC functional domains, whereas other mutations were scattered across the whole coding region of the gene (Figure 4.2.).

4.3.4. *ATM* neutral sequence changes in 224 CLL4 trial tumours.

There were also 18 different sequence changes in the cohort of 224 CLL tumours (Table 4.3). Two of them (c.2494C>T [p.Arg832Cys] and c.4947C>T [p.Val1649Val]) had not been previously reported as polymorphisms but they did not meet the criteria for mutations due to following reasons. None of them have been ever reported as causative mutation in AT patient. Missense variant c.2494C>T affects the amino-acid residue which is not conserved between mouse and human and is not located within any conserved domain and in addition, it was reported in a non-breast cancer control (Tavtigian et al., 2009). Change c.4947C>T does not result in amino acid substitution. Therefore, I concluded that both of these sequence variants are unlikely to be pathogenic.

4.4. Association between *ATM* mutations and other clinical and biological features.

As noted in the introduction, some of the clinical and biological characteristics of CLL are known to be associated with poorer outcome in patients. These include clinical prognostic markers such as increased age, male sex, advanced clinical stage, involvement of multiple tissue sites, as well as and biological markers such as unmutated *IGHV* status or *IGHV3-21* usage, high expression of ZAP-70 and CD38, 17p deletion, *TP53* mutation and

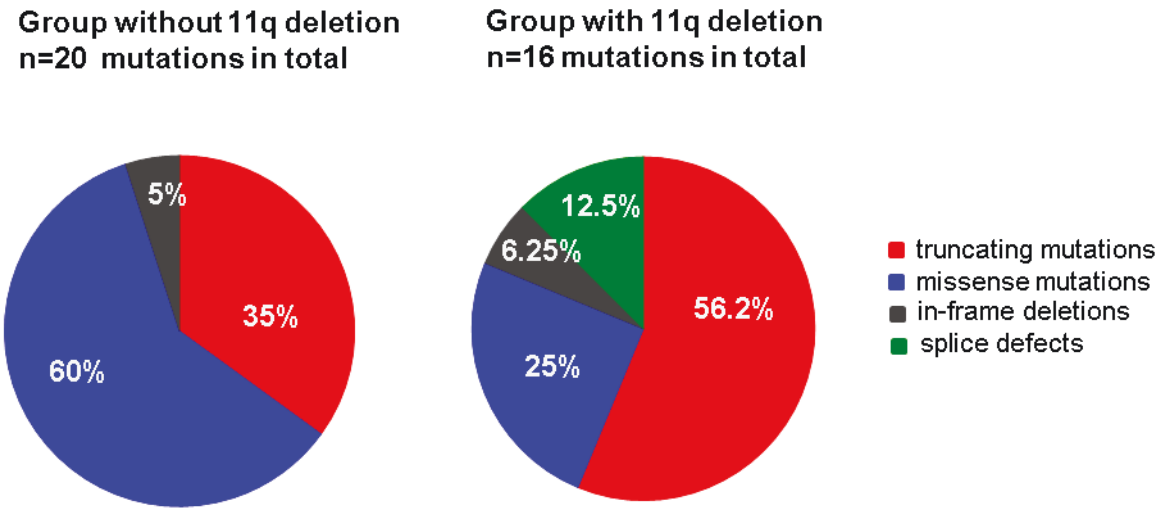


Figure 4.1. The frequencies of different type of *ATM* mutations in CLL tumours with and without chromosome 11q deletion.

ATM missense mutations were significantly more frequent in patients without 11q deletion (p=0.049), whereas other mutation types were more frequent in patients with 11q deletion. In general, *ATM* mutations (all types) were significantly more frequent among patients with 11q deletion (16/67), than among those without 11q deletion (20/157) p=0.047.

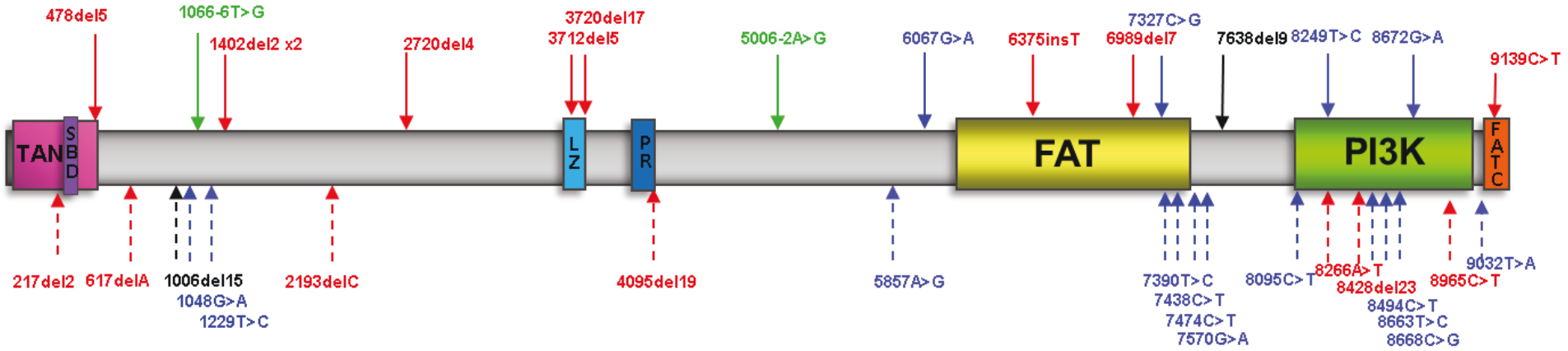


Figure 4.2. Distribution of the *ATM* mutations across the coding region of *ATM* gene in the CLL4 trial cohort.

Solid line arrows indicate mutations detected in 11q deleted tumours, dashed line arrows indicate mutations detected in non 11q deleted tumours. Font colours indicate different types of mutations: red are truncating mutations, blue-missense mutations, green- splice site mutations, black-short in-frame deletions. Missense mutations clustered in the 3' terminal region of the gene encoding FAT, PI3kinase and FATC functional domains, whereas other mutations were scattered across the whole coding region of the gene.

Table 4.3. *ATM* neutral sequence changes in 224 CLL4 trial tumours.

Sequence change	AA change	Previously reported as polymorphism	CLL affected (n=224)
c.146C>G	p.(Ser49Cys)	yes	1
c.735C>T	No AA change	yes	2
c.998C>T	p.(Ser333Phe)	yes	1
c.1744T>C	p.(Phe582Leu)	yes	1
c.2119T>C	p.(Ser707Pro)	yes	4
c.2494 C>T	p.(Arg832Cys)	no	1
c.2572T>C	p.(Phe858Leu)	yes	5
c.3161C>G	p.(Pro1054Arg)	yes	12
c.4258C>T	p.(Leu1420Phe)	yes	7
c.4578C>T	No AA change	yes	23
c.4947C>T	No AA change	no	1
c.5071A>C	p.(Ser1691Arg)	yes	1
c.5557G>A	p.(Asp1853Asn)	yes	63
c.5793T>C	No AA change	yes	2
c.3403-12insA	Non coding	yes	138
c.5497-8T>C	Non coding	yes	9
c.5497-15G>C	Non coding	yes	2
c.8786+8A>C	Non coding	yes	9

11q deletion. *ATM* mutations have previously been shown to be associated with unmutated *IGHV* gene (Austen et al., 2005; Stankovic et al., 2002b), whereas 11q deletion, which harbours mono-allelic loss of *ATM*, identifies patients with marked lymphadenopathy and advanced disease stage (Dohner et al., 1997).

To investigate the relationship between *ATM* mutations with clinical and biological prognostic factors in a cohort of 224 CLL patients, a comparison between patient groups with and without *ATM* mutations was performed using Fisher exact test, except for age and treatment allocation analysis where Chi-square test was used. The results are summarised in Table 4.4.

Altogether, there was no difference in the male to female ratio or age distribution between cases with *ATM* mutations and those without. However, *ATM* mutations were significantly more frequent in patients with Binet disease stage B/C than stage A (30/33,

Table 4.4. Patients' characteristic according to the mutation status of the *ATM* gene.

Prognostic markers	Patients without *ATM* mutation	Patients with *ATM* mutation	p value
Gender			
Female	50	4	0.121
Male	141	29	
Age group, years			
<60	67	9	0.367
60-70	68	16	
>70	56	8	
Binet stage			
A	53	3	**0.027**
B/C	138	30	
IGHV mutation status (98% cut off)			
Mutated	70	8	0.155
Unmutated	119	25	
Number of tissue sites			
<3	80	7	**0.032**
≥3	111	26	
17p deletion (10% cut off)			
No	181	32	1.00
Yes	10	1	
TP53 mutation			
No	174	33	0.084
Yes	17	0	
11q deletion (5% cut off)			
No	140	17	**0.022**
Yes	51	16	
Trisomy 12 (3%cut off)			
No	169	30	0.542
Yes	22	2	
13q deletion (5% cut off)			
No	81	8	0.079
Yes	110	24	
CD38 positive (7% cut off)			
No	62	8	0.402
Yes	102	21	
ZAP-70 positive (10% cut off)			
No	83	12	0.408
Yes	71	15	
Treatment allocation			
Chlorambucil	89	15	0.987
Fludarabine	44	8	
Fludarabine+cyclophosphamide	58	10	

IGHV-immunoglobulin heavy chain variable gene

90.9% of *ATM* mutated cases versus 168/191, 72.2% of not *ATM* mutated cases were stage B or C, p=0.027). They were also more frequent in cases with 3 or more enlarged tissue sites (26/33, 78.8%, versus 111/191, 58.1%, p=0.032).

As expected, *ATM* mutations were associated with the presence of an 11q deletion (16 of 33, 48.5% cases with *ATM* mutation and 51 of 191, 26.7% patients without *ATM* mutation had 11q deletion, p=0.02). Overall, 7.6% (17/224) patients had an *ATM* mutation alone, 22.8% (51/224) had an 11q deletion alone, 7.1% (16/224) had both *ATM* mutation plus an 11q deletion, and both *ATM* alleles were wild type in the remaining 62.5% (140/224) patients. There was no association between *ATM* mutations and 17p or *TP53* mutations. In fact, only one patient with *ATM* mutation carried also 17p deletion (patient CT1). This finding is not surprising, since according to previous studies, *ATM* and *TP53* mutations seem to occur usually in distinct CLL tumours subgroups (Austen et al., 2005; Stankovic et al., 2002b). Interestingly, *ATM* mutation status was not significantly associated with other biological markers including *IGHV* status, ZAP-70 or CD38.

Marked lymphadenopathy is the feature of tumours with 11q deletion. Involvement of multiple tissue sites, 11q deletion and advance disease stage were all associated with *ATM* mutations in this study. Therefore, further analysis was performed to investigate the relationship between those variables. Since diagnosis of advance disease stage requires in most cases the involvement of 3 or more tissue sites, these two variables were highly correlated and could not be used in the same statistical model. Applied logistic regression analysis tested correlation between involvement of multiple tissue sites and *ATM* mutation, 11q deletion, and also anaemia and thrombocytopenia (as these two features are implicated in Binet staging). This analysis revealed that association between *ATM* mutations and involvement of 3 or more tissue sites was independent on 11q deletion, thrombocytopenia or anaemia (p=0.014, OR=3.13, 95% CI 1.26-7.80).

Importantly, there was no difference in treatment allocation between cases with *ATM* mutations and those without (p=0.987).

4.5. *ATM* mutations and response to treatment.

To investigate the consequences of *ATM* mutations on response to treatment cohort of 224 patients was stratified into 4 hierarchical groups based on the presence of *ATM* mutation and 11q deletion (*ATM* mutation alone, 11q deletion alone, *ATM* mutation plus 11q deletion and *ATM* wild-type with no abnormalities)

Although the impact of 11q deletion on response to treatment has been already assessed in CLL4 trial cohort, cases with chromosome 11q deletion had not been stratified according to presence of *ATM* mutation, hence potential differences in outcomes between tumours with 11q deletion only and 11q deletion plus *ATM* mutation have not been determined (Catovsky et al., 2007; Oscier et al., 2010). None of the cases with *ATM* mutation also had *TP53* mutation and only one case with an *ATM* mutation carried a deletion of chromosome 17p. Therefore, negative influence of *TP53* abnormality on response to treatment was not expected to play a major role in the group with *ATM* mutations.

The criteria for response were set by the trial committee and the response was recorded at the end of the treatment (Catovsky et al., 2007). In this study, the overall response (OR) to treatment was defined either as complete remission, nodular partial response or partial remission. The response data was available for 65 patients treated with FC, for 47 patients treated with Fludarabine and for 100 patients treated with Chlorambucil (Table 4.5.). No response data was available for 3 patients treated with FC, 1 patient had *ATM* mutation (patient CT18), 1 had 11q deletion only and 1 had no *ATM* abnormalities. Five patients treated with Fludarabine had no available response data, including 1 patient with *ATM* mutation and chromosome 11q deletion (CT30), 1 patient with chromosome 11q deletion only and 3 patients with no *ATM* abnormalities. All 4 patients treated with Chlorambucil and missing response records had wild- type *ATM* status.

Firstly, overall responses to therapy were compared between 4 groups which were stratified according to the presence of *ATM* abnormality. The OR was observed in: 87.5% (14/16) of cases with *ATM* mutation alone, 72.9% (35/48) cases with 11q deletion alone,

46.7% (7/15) cases with *ATM* mutation plus 11q deletion and 84.2% (112/133) cases with *ATM* wild-type. Only patients carrying *ATM* mutations combined with the 11q deletions showed significantly worse response when compared to *ATM* wild-type group (p=0.002). And in fact, this was the only significant difference in response between those 4 groups (Figure 4.3.).

Table 4.5. *ATM* abnormalities and response to treatment.

Treatment arm	11q/*ATM* status	Total	Response	
			No	Yes
Fludarabine plus cyclophosphamide (n=65)	*ATM* mutation alone	6	0 (0%)	6 (100%)
	11q deletion alone	16	0 (0%)	16 (100%)
	ATM mutation and 11q deletion	3	0 (0%)	3 (100%)
	ATM wild-type	40	2*(5%)	38(95%)
Fludarabine (n=47)	*ATM* mutation alone	4	1 (25%)	3 (75%)
	11q deletion alone	11	3 (27%)	8 (73%)
	ATM mutation and 11q deletion	3	2 (67%)	1 (33%)
	ATM wild-type	29	5[€] (17%)	24 (83%)
Chlorambucil (n=100)	*ATM* mutation, no 11q deletion	6	1 (17%)	5 (83%)
	11q deletion, no *ATM* mutation	21	10 (48%)	11 (52%)
	ATM mutation and 11q deletion	9	6 (67%)	3 (33%)
	ATM wild-type	64	14[¥] (22%)	50 (78%)

*both patients had *TP53* abnormality, [€] three patients had *TP53* abnormality, [¥] six patients had *TP53* abnormality

Responses differed between three treatment arms. Patients treated with FC responded significantly better than those treated with Fludarabine or Chlorambucil. In total, there were 44 non responders: 2 in FC arm (3.1%, 2/65), 11 in Fludarabine arm (23.4%, 11/47), 31 in Chlorambucil arm (31%, 31/100). Subsequently, the responses to different treatment types between each of the four groups stratified according to the presence of *ATM*

abnormality were investigated.

In the FC treatment arm 6 patients had *ATM* mutation alone, 16 patients had 11q deletion alone and 3 patients had bi-allelic *ATM* abnormality. Remaining 40 patients had *ATM* wild-type CLL. Response to FC was good among all 4 groups and no significant difference was observed. There were only 2 non-responders; both of them had *ATM* wild-type CLL with *TP53* abnormalities.

In Fludarabine treatment arm 4 patients had *ATM* mutation alone, 11 patients had 11q deletion alone, 3 patients had *ATM* mutation plus 11q deletion and 29 patients had wild-type *ATM*. There were 11 non-responders: 4 patients with *ATM* mutation alone, 11 patients with 11q deletion alone, 2 patients with *ATM* mutation plus 11q deletion and 5 patients with *ATM* wild-type CLL. Notably, 2 of 3 patients with bi-allelic *ATM* abnormalities were non responsive to Fludarabine treatment. However, there were no significant differences observed between 4 groups.

The responses to Chlorambucil were the worst of all three treatments types and the impact of *ATM* abnormalities was the most pronounced within this group. Of 6 patients with *ATM* mutation alone, 1 did not respond to treatment. Ten of 21 patients with 11q deletion alone and 6 of 9 patients with *ATM* mutation plus 11q deletion were also non-responders. Fourteen of 64 *ATM* wild-type patients also did not respond to treatment; however 6 of them had *TP53* abnormalities. Altogether, patients with *ATM* mutation plus 11q deletion as well as patients with 11q deletion alone had significantly worse response to this treatment when compared to *ATM* wild-type group (p=0.01 and p=0.047 respectively), whereas patients with *ATM* mutation alone did not differ from *ATM* wild-type group (Figure 4.3).

The response to Chlorambucil was subjected to a detailed analysis to investigate possibility that the poor response in the patients with *ATM* mutation plus 11q deletion or 11q deletion alone could be due to positive association with other poor prognostic factors. Logistic regression was applied and association between *ATM*/11q status and other prognostic factors like age, *IGHV* status, gender, *TP53* mutation/17 deletion and Binet stage

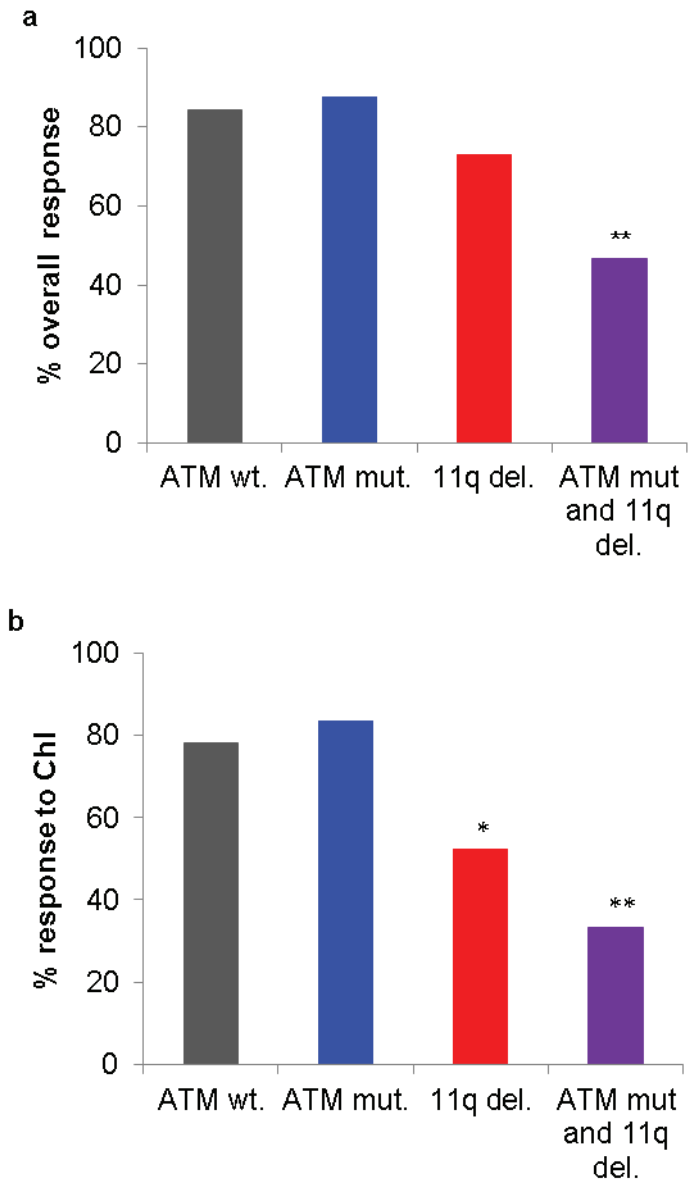


Figure 4.3. Overall response (a) and response to Chlorambucil only (b) among 4 hierarchical groups of CLL4 trial patients.

(**a**) The significantly worse overall response was observed within the group of patients with *ATM* mutation and 11q deletion when compared to patients with *ATM* wild-type ($p \leq 0.01$).

(**b**) The responses to Chlorambucil were significantly worse in 11q deletion only and *ATM* mutation plus 11q deletion groups of CLL patients when compared to patients with wild-type *ATM* ($p \leq 0.05$ and $p \leq 0.001$ respectively).

was tested. This approach confirmed that the poorer responses to Chlorambucil among patients with *ATM* mutation plus 11q deletion (OR=0.09, 95% CI 0.02-0.50, p=0.003) or patients with 11q deletion alone (OR=0.23, 95% CI 0.07-0.69, p=0.009) were not resulting from the influence of other known prognostic factors. Treatment allocation and response to treatments among all patients with *ATM* mutation are presented in Table 4.6.

4.6. *ATM* mutations and survival.

To investigate the impact of *ATM* mutations on PFS and OS, the Kaplan Meier survival analysis and log rank test were initially applied and all four groups stratified by the presence of *ATM* abnormalities were analysed. PFS was expressed as time from randomization to relapse needing therapy, progression or death from any cause and OS was calculated from randomization to death from any cause. PFS and OS for all patients with *ATM* mutation are presented in Table 4.6.

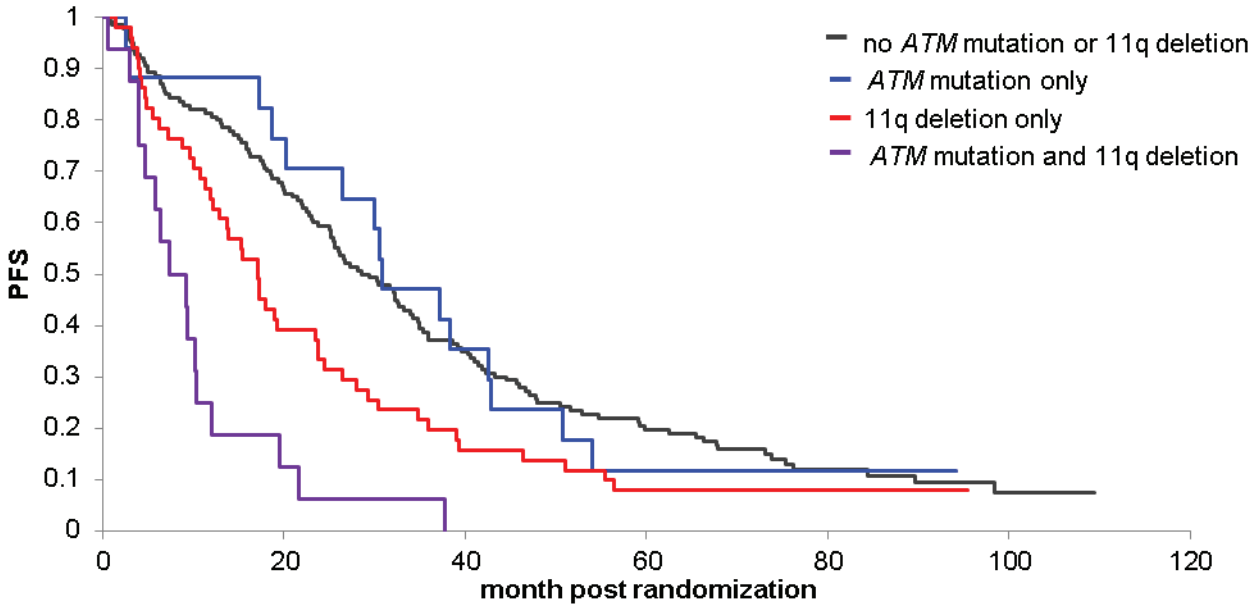
The median PFS of patients with *ATM* mutation plus 11q deletion (7.4 months, 95% CI 1.9-12.9) or patients with 11q deletion alone (17.1 months, 95% CI 13.0-21.1) was significantly shorter when compared to patients with *ATM* wild-type (28.6 months 95% CI 23.1-34.2) (7.4 versus 28.6 p<0.001 and 17.1 versus 28.6 p=0.007 respectively). Furthermore, patients with bi-allelic *ATM* abnormalities had shorter PFS than patients with 11q deletion only (7.4 vs 17.1 p=0.002) or patients with *ATM* mutation only (median 30.8 months 95% CI 21.1-40.5) (7.4 versus 30.8 p<0.001). However, there was no significant difference in median PFS between patients with *ATM* mutation only and those with *ATM* wild-type (30.8 versus 28.6, p=0.983) (Figure 4.4.). Notably, regardless of the initial positive response to therapy in majority of patients with *ATM* mutation only, only two eventually did not progress (patients CT31 and CT15).

The median OS was also inferior in patients with *ATM* mutation plus 11q deletion (42.2 months, 95% CI 18.1-66.2, p=0.002) and in patients with 11q deletion only (54.4 months 95% CI 45-63.8, p=0.001) when compared to cases with *ATM* wild-type (85.5 months

Table 4.6. Treatment allocation and clinical outcome of patients with *ATM* mutation.

Ptn.	Nucleotide Change	Mutation type	Age	Treatment allocation Flu,Ch,FC	Response	PFS	OS
CLL tumours without chromosome 11q deletion							
CT29	c.217_218del2	truncating	61	Chl	Yes	29.9	68.7+
CT23	c.617delA c.8494C>T	truncating missense	74	Chl	No	2.5	19.1
CT24	c.1006_1020del15 c.7570G>A	in-frame deletion missense	65	Flu	No	2.9	17.5
CT16	c.1048G>A	missense	57	FC	Yes	50.8	93.7+
CT14	c.1229T>C	missense	57	Flu	Yes	38.3	88.9
CT2	c.2193delC	truncating	64	Flu	Yes	20.2	77.6
CT12	c.4095_4109+4del19	truncating	63	Chl	Yes	30.8	67.8
CT1	c.5857A>G	missense	69	Chl	Yes	17.2	29.6
CT31	c.7390T>C	missense	63	FC	Yes	56.9	56.9+
CT4	c.7438C>T	missense	77	Chl	Yes	53.9	119.2+
CT18	c.7474C>T	missense	49	FC	n/a	30.5	83.1+
CT5	c.8095C>T	missense	75	FC	Yes	42.9	47.7
CT15	c.8266A>T	truncating	52	FC	Yes	94.2	94.2+
CT27	c.8428_8450del23	truncating	58	FC	Yes	26.4	50.5
CT8	c.8663T>C	missense	66	Flu	Yes	42.5	45.5
CT10	c.8668C>G c.8965C>T	missense trucating	59	Chl	Yes	18.6	102.3
CT7	c.9032T>A	missense	65	FC	Yes	37.2	50.1
CLL tumours with chromosome 11q deletion							
CT6	c.478_482del5	truncating	59	Chl	No	5.7	32.1
CT26	c.1066-6T>G	splice defect	60	Flu	No	3.9	35.3
CT11	c.1402_1403del2	truncating	73	Chl	Yes	9.2	51.8
CT33	c.1402_1403del2	truncating	72	FC	Yes	12.0	12.0
CT17	c.2720_2723del4	truncating	65	Chl	Yes	19.5	93.2+
CT13	c.3712_3716del5	truncating	71	Flu	Yes	37.7	42.1
CT21	c.3720_3736del17	truncating	67	Chl	No	6.4	51.1
CT22	c.5006-2A>G	splice defect	54	Flu	No	3.0	19.2
CT19	c.6067G>A	missense	69	Chl	Yes	9.3	72.0
CT32	c.6375insT	truncating	69	Chl	No	7.4	68.2
CT20	c.6989_6995del7	truncating	56	Chl	No	10.1	80.7
CT28	c.7327C>G	missense	63	Chl	No	4.6	22.3
CT30	c.7638_7646del9	In-frame deletion	68	Flu	n/a	0.6	0.6
CT3	c.8249T>C	missense	75	FC	Yes	21.6	21.6
CT25	c.8672G>A	missense	71	Chl	No	3.9	71.3+
CT9	c.9139C>T	truncating	65	FC	yes	10.3	10.8

Flu- fludarabine, Chl- Chlorambucil, FC- Fludarabine plus cyclophoshamide, + censored patient at time of the analysis.



Group	Median PFS (months)	Comparison group, p value		
		ATM mut. only	11q del only	*ATM* mut. + 11q del
ATM wt. (n=140)	28.6	0.98	**0.007**	**<0.001**
ATM mut. only (n=17)	30.8	NA	0.095	**<0.001**
11q del only (n=51)	17.1	0.095	NA	**0.002**
ATM mut .+ 11q del (n=16)	7.4	**<0.001**	**0.002**	NA

Figure 4.4. Impact of *ATM* mutation and 11q deletion on progression free survival (PFS) in CLL4 trial patient cohort.

PFS in patients with *ATM* mutation plus 11q deletion and in patients with 11q deletion only was significantly shorter than in *ATM* wild-type patient group ($p \leq 0.001$, $p \leq 0.01$, respectively). Significant difference was also observed between *ATM* mutation only or 11q deletion only groups and *ATM* mutation plus 11q deletion group ($p \leq 0.001$ and $p \leq 0.01$ respectively).

95% CI 69.9-101.0), but not in patients with *ATM* mutation only (77.6 months, 29.9-125.4, p=0.433). There was no difference in OS between patients with bi-allelic and mono-allelic abnormalities (*ATM* mutation plus 11q deletion versus 11q deletion only, p=0.28; and *ATM* mutation plus 11q deletion versus *ATM* mutation only p=0.092); or between 2 groups with mono-allelic *ATM* abnormalities (11q deletion only versus *ATM* mutation only, p=0.153) (Figure 4.5).

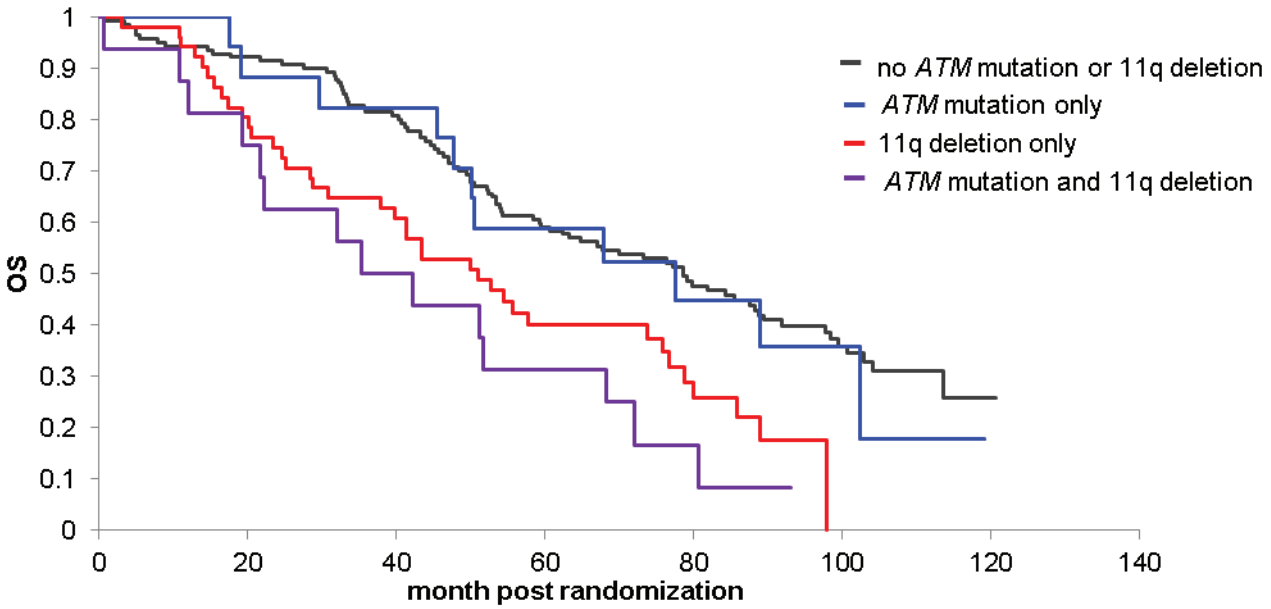
Since shorter progression free survival and overall survival in patients with *ATM* mutation plus 11q deletion or with 11q deletion alone could be due to positive association between *ATM* abnormalities and other prognostic factors, survival data was subjected to multivariate analysis using Cox's proportional hazards regression test. The other prognostic factors taken under consideration in this analysis included gender, age, Binet stage, treatment, chromosome 17p deletion, *TP53* mutation and *IGHV* status or *IGHV3-21* usage. Multivariate analysis confirmed that compared to *ATM* wild-type cases, the presence of *ATM* mutation plus 11q deletion or 11q deletion alone were independently associated with shorter PFS (p<0.001, and p=0.001 respectively) and shorter OS (p=0.009 and p=0.003 respectively).

Further analysis of this model confirmed that bi-allelic *ATM* defects were associated with significantly higher risk of progression when compared to *ATM* mutation alone or 11q deletion alone (p<0.001, p=0.004 respectively) but there were no significant differences in OS between mono- and bi-allelic *ATM* abnormalities (p=0.123, p=0.154).

4.6.1. Prognostic effect of *ATM* abnormalities when compared to *TP53* defects.

The presence of a *TP53* abnormality (mutation and/or deletion) is now a well-established negative prognostic factor in CLL (Oscier et al., 2012). To investigate whether *ATM* defects have similar effect on the outcome, the additional analysis was conducted concurrently to this study.

The analysed cohort consisted of 501 CLL 4 trial patients and included the cases with



Group	Median OS (months)	Comparison group, p value		
		ATM mut. only	11q del only	*ATM* mut. + 11q del
ATM wt. (n=140)	85.5	0.433	**0.001**	**0.002**
ATM mut. only (n=17)	77.6	NA	0.153	0.092
11q del only (n=51)	54.4	0.153	NA	0.28
ATM mut .+ 11q del (n=16)	42.2	0.092	0.28	NA

Figure 4.5. Impact of *ATM* mutation and 11q deletion on overall survival (OS) in CLL4 trial patient cohort.

OS in patients with 11q deletion and in patients with *ATM* mutation plus 11q deletion was significantly shorter than in *ATM* wild-type patient group (p=0.01, p=0.002 respectively). No significant difference was observed among any other comparison groups.

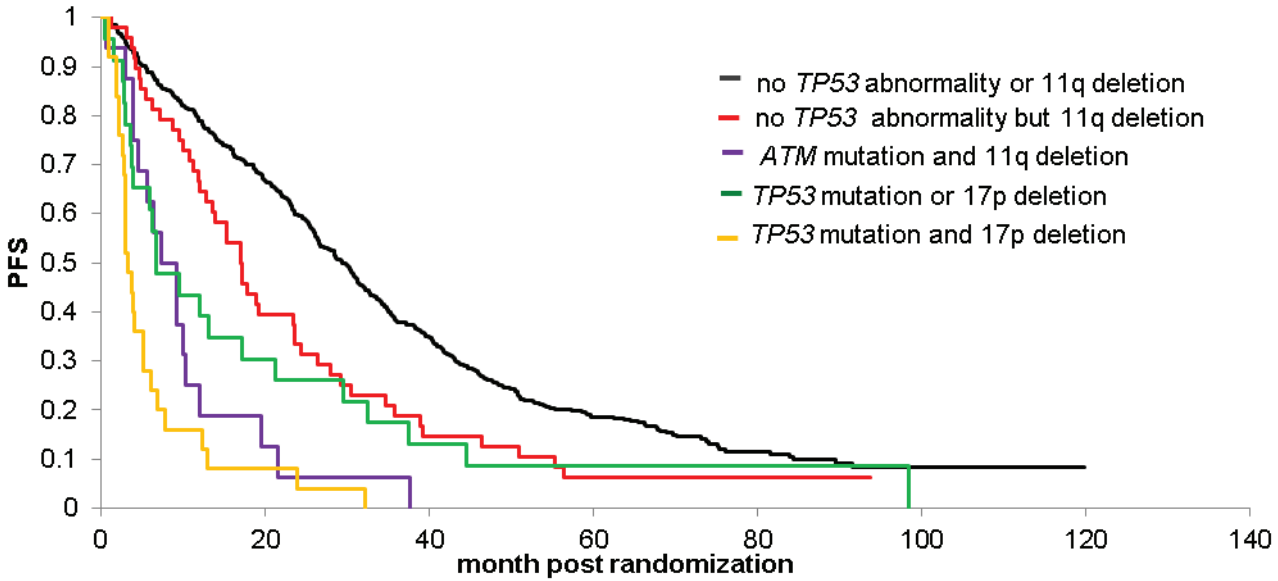
11q deletion and wild-type *TP53* gene (48 patients); with 11q deletion combined with *ATM* mutation and wild-type TP53 (16 patients); with mono-allelic or bi-allelic *TP53* abnormalities (23 and 25 patients, respectively) ; and cases with no *TP53* abnormality or 11q deletion (389 patients).

This additional analysis revealed that all four groups (11q deletion, *ATM* mutation plus 11q deletion, *TP53* mono-allelic and *TP53* bi-allelic abnormalities) had inferior PFS compared to cases with no *TP53* abnormalities or 11q deletion (17.1 months, 7.4 months, 6.8 months, 3.4 months respectively compared to 29.5 months, $p<0.003$ for each). However, only patients with bi-allelic *ATM* or bi-allelic *TP53* abnormalities had inferior outcome below that observed in cases with 11q deletion alone ($p=0.001$, $p<0.001$ respectively) (Figure 4.6.). Furthermore, there was no significant difference in median PFS between cases with bi-allelic *ATM* abnormalities and mono- or bi-allelic *TP53* abnormalities ($p=0.3$ and $p=0.07$, respectively) Also median OS was inferior in those four groups when compared to cases with wild-type *TP53* and no 11q deletion (54.4 months, 42.2 months, 43.2 months, 13.9 months compared to 89 months, $p<0.001$) (Figure 4.7.)

Therefore, patients with bi-allelic *ATM* alterations represent a distinctive cohort with a particularly poor response to DNA damaging agents and survival comparable to patients with *TP53* defects.

4.7. Discussion

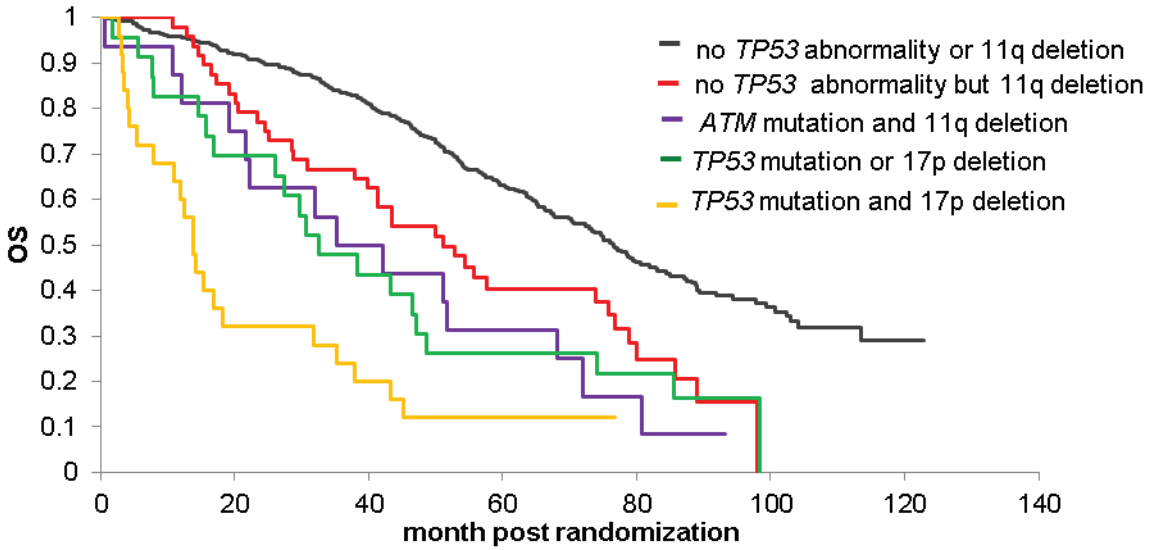
I had the opportunity to investigate the clinical consequences of mono- and bi-allelic *ATM* defects among the patients with advanced stage CLL disease enrolled on the phase III UK CLL trial (UK LRF CLL4). All of the trial patients were randomized to the first time therapy with DNA damage-inducing chemotherapeutics conventionally applied to treat this disease. After screening the *ATM* gene in 224 cases and identifying the patients with potentially deleterious mutations, I stratified a selected CLL cohort according to presence or absence of



Group	Median PFS (months)	Comparison group, p value			
		no *TP53* abn. but 11q del	*ATM* mut .+ 11q del	*TP53* mut. or 17p del	*TP53* mut. + 17p del
no *TP53* abn. or 11q del (n=389)	29.5	**0.002**	**<0.001**	**0.001**	**<0.001**
no *TP53* abn. but 11q del (n=48)	17.1	NA	**0.001**	0.3	**<0.001**
ATM mut .+ 11q del (n=16)	7.4	**0.001**	NA	0.3	0.07
TP53 mut. or 17p del (n=23)	6.8	0.3	0.3	NA	**0.006**
TP53 mut. + 17p del (n=25)	3.4	**<0.001**	0.07	**0.006**	NA

Figure 4.6. Hierarchical model of the impact of *TP53* and *ATM* abnormalities on PFS in CLL4 trial patient cohort.

All four groups with *TP53* or *ATM* abnormalities had inferior PFS compared to patients without these defects. Only patients with bi-allelic *ATM* or bi-allelic *TP53* abnormalities showed inferior PFS when compared with patients who had 11q deletion alone (p≤0.001 for both).



Group	Median OS (months)	Comparison group, p value			
		no *TP53* abn. but 11q del	*ATM* mut .+ 11q del	*TP53* mut. or 17p del	*TP53* mut. + 17p del
no *TP53* abn. or 11q del (n=389)	89	**<0.001**	**<0.001**	**<0.001**	**<0.001**
no *TP53* abn. but 11q del (n=48)	54.4	NA	0.26	0.32	**<0.001**
ATM mut .+ 11q del (n=16)	42.2	0.26	NA	0.87	**0.03**
TP53 mut. or 17p del (n=23)	43.2	0.32	0.87	NA	**0.043**
TP53 mut. + 17p del (n=25)	13.9	**<0.001**	**0.03**	**0.043**	NA

Figure 4.7. Hierarchical model of the impact of *TP53* and *ATM* abnormalities on OS in CLL4 trial patient cohort.

All four groups with *TP53* or *ATM* abnormalities had inferior OS compared to patients without these defects p<0.001. Patients with bi-allelic *TP53* abnormalities had significantly the worst survival among all compared subgroups.

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ATM abnormalities such as mutation and 11q deletion. This approach allowed me to characterize the subgroup of CLL patients with *ATM* mutation and/or 11q deletion in respect to other biological and clinical features, to responses to unified treatment schedules and to disease progression and overall survival.

The classification of detected sequence changes is not always straightforward. The *ATM* gene is highly polymorphic and lacks obvious mutational hotspots. In addition, different mutation types confer different levels of functional defects. Particularly problematic is classification of novel missense variants, whose consequences on protein activity have not been investigated. Therefore, in this study a novel sequence change had to meet a minimum criterion of affecting conserved amino-acid residue, to be classified as mutation. Importantly, majority of missense mutations were also located in the region encoding functional domains of ATM protein.

A number of online tools are available to help prediction of functional consequences of the novel non-synonymous sequence variants. I have also used two of them to evaluate the group of missense mutations I have detected in this study, namely: *Sorting Intolerant From Tolerant* (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html) and *Polymorphism Phenotyping* (Polyphen2) (http://genetics.bwh.harvard.edu/pph2/). Notably, It was not my intention to include the outcome of the *in silico* analysis into the mutation criteria I have initially established. However, I have reasoned that the additional estimation could potentially contribute to the interpretation of the results.

According to *in silico* tools SIFT and PolyPhen-2, 4 missense mutations (c.7390T>C, c.7438C>T, c.7474C>T, c.9032T>A) were not predicted to have harmful functional consequences and remaining 12 were classified as damaging by at least one of these algorithms. Interestingly, all of these four mutations were detected in the cases with non chromosome 11q deletion. None of them has been previously detected in AT patient or tumour cells. Nevertheless, they have been classified here as missense mutations because all of these changes were affecting conserved amino-acid residues located within functional domains of ATM protein and I reasoned that these features can potentially

impact on the protein function.

Following the mutation criteria established in this study I have detected 36 different mutations in 33 patients. In agreement with previous observations (Austen et al., 2007) I have shown that *ATM* mutations are more frequent in the group of tumours with 11q deletion (24%) than in those without this chromosomal abnormality (11%). Moreover, mutations which were predicted to result in protein truncation or short in frame deletions were also more frequent in the tumours with 11q deletion than in tumours without 11q deletion.

Possible explanation for this phenomenon could lie in the micro-environmental selective pressure imposed on tumour clone and in functional consequences of different mutation types. While protein translated from an allele bearing truncating mutation is unstable and promptly degraded, the missense mutation may retain residual kinase activity (Barone et al., 2009; Stankovic et al., 1998). Both, 11q deletion and *ATM* mutation can be late tumorigenic events and possibly precede each other (Austen et al., 2005; Cuneo et al., 2002). Therefore, it is probable that the sub-clone with *ATM* mono-allelic loss (11q deletion or truncating mutation) is under selective pressure to acquire complete *ATM* inactivation leading to more rapid expansion, whereas clones which are *ATM* wild-type could acquire mutations in the more random manner. Some of them will be truncating, some will be missense. Those mutations are the first potentially deleterious changes affecting *ATM* gene and subsequently some of them might acquire additional defect on another allele. Interestingly, three tumours with two different mutations and non chromosome 11q deletion have been observed in this study (Table 4.6, CT23, CT24 CT10). All of them carried one missense mutation and one truncating or short in-frame deletion mutation. Although these three cases have been classified as tumours with mono-allelic *ATM* defects, it is possible that these sequence changes are located on different alleles leading to bi-allelic *ATM* loss. It is intriguing, that all three patients had relatively poor outcome when compared to other cases with *ATM* mutation alone.

One of the features that characterize clinically useful biomarker is the ability to

provide an insight into the biology of disease. I demonstrated here that *ATM* mutations are associated with involvement of multiple tissue sites independent on the presence of 11q deletion. Importantly, marked lymphadenopathy is the feature of tumours with 11q deletion (Dohner et al., 1997). However, it has been also shown to be associated with ATM deficiency irrespective of the presence of chromosomal aberrations (Joshi et al., 2007a; Starostik et al., 1998). Therefore, the results from this study imply that the criteria I have imposed for *ATM* mutations were correct and reflected pathogenic character of these sequence changes.

Increased involvement of multiple tissue sites might be a combined result of defective activation of apoptosis and increased rate of proliferation. Extensive telomere shortening observed in CLL cells carrying defects in *ATM* gene indicate increased proliferation rate (Britt-Compton et al., 2012). Furthermore, *ATM* mutations/deficiency could impact on motility and homing of CLL tumour cells, although the exact mechanism remains unknown.

Interestingly, it has been reported that the levels of ATM transcripts can be significantly lower in CLL cells with high CD38 levels (Joshi et al., 2007b). Importantly, CD38 itself is involved in enhanced BCR signalling and acts as an adhesion molecule. However, no functional link between CD38 and *ATM* has been provided so far. In addition, I have not observed any significant correlation between *ATM* mutation status and CD38 expression level in this study.

Importantly, *ATM* mutation status was not significantly associated with other biological markers including *IGHV* status, ZAP-70 or already mentioned CD38. Previous study reported positive association between *ATM* mutations and unmutated *IGHV* (Austen et al., 2005). Notably, unmutated *IGHV* is often associated with 11q deletion (Krober et al., 2002). The cohort analysed by Austen and colleagues were not stratified according to the presence of 11q deletion. Therefore, considering a small group size, reported correlation between *ATM* mutations and *IGHV* status, could potentially reflect the influence of 11q deletion and its association with *IGHV* status. Furthermore, another study also failed to

detect significant association between *ATM* sequence variants and *IGHV* status (Lozanski et al., 2012).

Tumours with *ATM* mutation appear to be refractory to chemotherapeutic agents *in vitro* and *in vivo* as shown in a unselected cohort of 155 CLL patients (Austen et al., 2005). Here I investigated the impact of *ATM* mutations on response to the therapy which included Fludarabine, Chlorambucil, or Fludarabine plus Cyclophosphamide. These therapeutic agents are exerting their cytotoxic activity via inducing DNA damage in exposed cells. Since ATM is one of the main activator of DNA damage responses, defects/deficiencies in ATM signalling could explain the refractoriness to these agents.

I have demonstrated that overall response among the tumours with bi-allelic *ATM* defects (*ATM* mutation plus 11q deletion) is significantly worse compared to cases with *ATM* wild-type. Interestingly, study by Oscier and colleagues, which reported the responses of the entire CLL4 trial cohort (777 patients) showed that 11q deletion was also associated with lower overall response rate (Oscier et al., 2010), whereas I observed this association only in tumours with 11q deletion plus *ATM* mutations. This discrepancy might be due to the fact that in the previous analysis 11q deleted tumours have not been stratified according to *ATM* mutation status and poor response within this group could be, at least partially, the result of bi-allelic inactivation of *ATM* gene. Alternatively, it is possible that my study was under-powered due to smaller sizes of selected cohorts to detect difference between 11q deletion only and *ATM* wild-type subgroups.

The impact of *ATM* defects on response to treatment was the most pronounced within the group of patients treated with Chlorambucil. Patients with *ATM* mutation plus 11q deletion or with 11q deletion alone had significantly worse overall response than those with no *ATM* abnormalities. This was confirmed in multivariate analysis and was independent on other poor prognostic factors. Since mechanism of killing by Chlorambucil is mainly based on the induction of p53-dependent apoptosis, patients with abnormalities in the p53 pathway are profoundly resistant to this therapy (Sturm et al., 2003). I have shown here that defects in *ATM* gene which acts upstream of p53 also confer

refractoriness to this treatment.

Therefore, poor response to geno-toxic treatment among patients with *ATM* mutation combined with 11q deletion strengthens the role of *ATM* bi-allelic loss as powerful prognostic factor. These results are clinically important and strongly suggest that these patients are unlikely to benefit from this treatment and should be offered alternative therapy. For example, addition of monoclonal antibody Rituximab to FC schedule (FCR) could be a potentially good choice since it was shown to improve the outcome in patients with 11q deletion (Hallek et al., 2010; Tsimberidou et al., 2009). In the CLL8 trial, the 3 year PFS for cases with 11q deletion was 64% in the FCR arm compared to 32% in the FC arm (univariate analysis). However, it is still unknown if this regimen could overcome the adverse effect of bi-allelic *ATM* loss as it does not seem to benefit patients with *TP53* defects (Hallek et al., 2010; Zenz et al., 2010).

I have shown here that PFS in patients with bi-allelic abnormalities is shorter than in patients with mono-allelic loss and in patients with no *ATM* abnormalities. In addition, patients with an 11q deletion/loss of one *ATM* allele had also shorter PFS than those with *ATM* wild-type. This was independent of other prognostic markers suggesting that *ATM* genetic alterations affect patients' responses to DNA damaging agents in a stepwise manner.

Notably, PFS in patients with an isolated 11q deletion was significantly shorter than in those with an isolated *ATM* mutation. In addition, the latter group did not show any inferior PFS when compared to cases with no *ATM* abnormalities. Possible explanation for this difference between tumours with mono-allelic *ATM* defects might be due to the fact that 11q deletion encompasses the loss of a number of genes including those implicated in DNA damage responses. These comprise *MRE11*, a component of the MRN complex that participates in sensing and processing DNA DSBs and directly interacts with ATM, as well as *H2AX*, a histone subtype that is directly involved in chromatin relaxation. Other genes within the 11q deleted region, such as *MLL*, *NPAT, CUL5* and *PP2R1*B might also be indirectly involved in DNA damage response through their roles in the regulation of

replication, cell cycle or apoptosis (Gardiner et al., 2012; Guarini et al., 2012; Kalla et al., 2007; Ouillette et al., 2010). Thus, the collective impact of haploinsufficiency of a number of functionally related genes might exceed the impact of the loss of a single *ATM* allele.

Notably, recent studies have identified *BIRC3* gene, a negative regulator of non-canonical NFκB signalling located on chromosome 11q22, to be frequently disrupted (deleted or mutated) in fludarabine refractory CLL tumours (24% of the studied cohort) but not in patients at diagnosis (4% of the cohort) (Rossi et al., 2012a). Disruption of *BIRC3* has also been associated with poor overall survival indicating that the inferior prognosis of patients with chromosome 11q deletion could be at least partially explained by the aberration in this gene (Rossi et al., 2013). However, another recent study showed no difference in either the OS or PFS between cases with 11q deletion which included *BIRC3* locus and cases where 11q deletion did not encompass BIRC3 locus (Rose-Zerilli et al., 2013). Furthermore, when both *BIRC3* and *ATM* mutation status was incorporated into the analysis it was *ATM* mutation rather than *BIRC3* disruption that was associated with poor response to chemotherapy. Therefore, the role of *BIRC3* loss in pathogenesis of CLL remains unclear.

Another explanation for clinical variation among tumours with mono-allelic *ATM* defects considers possible difference in the molecular background of cells with isolated 11q deletion or *ATM* mutations. Both abnormalities are often later events in pathogenesis of CLL and molecular events that precede 11q deletion or *ATM* mutation may cause differences in clinical course between the two subgroups. This is supported by findings of a different gene expression profile in tumours with *ATM* mutations and those with isolated 11q deletion (Guarini et al., 2012).

Furthermore, observed mild clinical phenotype within the group with *ATM* mutations and no 11q deletions might be the consequences of residual ATM function retained by some of the *ATM* missense changes, which in fact, constitute majority of *ATM* mutations detected in this group of patients. This, and intact second *ATM* allele might be sufficient to act efficiently in DNA damage responses and sustain milder cellular

phenotype.

Finally, presence of *ATM* mutation only could be associated with a small size sub-clone, where majority of tumour cells still have capacity to respond to treatment.

Overall survival of patients with *ATM* mutation plus 11q deletion and those with chromosome 11q deletion alone was significantly worse than in cases with *ATM* wild-type tumours. However, groups with mono- and bi-allelic *ATM* defects did not differ between each other. Possible explanation for the lack of survival differences between the groups with *ATM* abnormalities (the differences were present in PFS) may reflect the influence of secondary treatment which might have positively influence the survival at least in a proportion of cases (Catovsky et al., 2007). Nevertheless, the salvage protocols were not sufficient enough to overcome the adverse effect of neither bi-allelic *ATM* defect nor 11q loss since patients with these abnormalities still exhibited inferior survival.

One of the important features of any clinically useful biomarker is the ability to provide independent prognostic information. *ATM* mutations have been previously indicated to possess this attribute (Austen et al., 2005). I was not able to confirm that result in the context of prospective randomised UK LRF CLL4 trial. The results from OR, PFS and OS analysis did not implicate strong prognostic value of the *ATM* mutations as sole abnormality in this group of patients. However, I have demonstrated a clear significance of bi-allelic *ATM* loss which is associated with the loss of *ATM* function. Therefore, the results of this study support a need for addressing the *ATM* integrity in a clinically applicable functional test and support its role as an important biological prognostic factor in CLL.

Importantly, another two smaller studies which were assessing the clinical impact of *ATM* mutations did not recognize the defects in this gene as negative prognostic factor (Lozanski et al., 2012; Ouillette et al., 2012). However, none of these studies differentiated between patients with mono- and bi-allelic abnormalities. Moreover, Lozanski and colleagues included in the group of patients with *ATM* defects also those carrying *ATM* polymorphisms, which most likely confounded the impact of truly pathogenic *ATM*

mutations on patient survival. Ouillette and colleagues assessed the function of ATM protein relying only on the phosphorylation of the single ATM target- serine 1981. Assessing ATM activity on the basis of a single ATM target activation might be misleading due to complexity of ATM activation mechanisms, redundancy of different kinases phosphorylating the same targets and unpredicted functional consequences of ATM mutated proteins (Bhatti et al., 2011; Kozlov et al., 2011; Pellegrini et al., 2006).

A very important finding in this study resulted from the analysis of the outcome among the patients with *ATM* abnormalities and those with *TP53* abnormalities. Bi-allelic defects of any of these genes conferred significantly shorter PFS and OS compared to *ATM* and *TP53* wild-type cases. Furthermore, only those two groups had inferior PFS when compared to patients with 11q deletion only and there was no significant difference in PFS between cases with bi-allelic *ATM* abnormalities and mono- or bi-allelic *TP53* abnormalities. This is the first study which in a comprehensive and reliable manner assesses the impact of complete inactivation of *ATM* gene on the clinical outcome among patients enrolled into phase III trial.

In summary, the results from this study strongly indicate that bi-allelic inactivation of *ATM* gene is associated with inferior overall response rate and significantly shorter PFS as well as OS following the first-line CLL treatment with alkylating agents and purine analogues. Moreover, PFS in patients with bi-allelic abnormalities is increased above the risk caused by mono-allelic loss and is similar to those with *TP53* loss and/or mutation. These findings may be relevant for the clinical management of CLL because they could influence the choice of a therapy. Current diagnostic procedures do not include assessment of *ATM* mutation status in patients with 11q deletion. Therefore the additional impact of inactivation of the second allele is not recognized.

The work described in this chapter has been summarized and published as - Skowronska, A., A. Parker, G. Ahmed, C. Oldreive, Z. Davis, S. Richards, M. Dyer, E.

Matutes, D. Gonzalez, A.M. Taylor, P. Moss, P. Thomas, D. Oscier, and T. Stankovic., 2012. *Biallelic ATM Inactivation Significantly Reduces Survival in Patients Treated on the United Kingdom Leukemia Research Fund Chronic Lymphocytic Leukemia 4 Trial*. J Clin Oncol, 20;30(36):4524-32 (included at the end of this thesis).

CHAPTER V

RESULTS III

Optimization of a primary CLL xenograft model in NOG mice - establishing the conditions for prolonged CLL engraftment.

5.1. Introduction

In the two previous chapters I was investigating the role of *ATM* mutations in the development and/or progression of CLL. Based on the results obtained from two large CLL cohorts I have concluded that a mono-allelic *ATM* defect does not contribute to CLL initiation but promotes development of a more aggressive clone via facilitating the loss of the second *ATM* allele resulting in complete inactivation of the ATM protein. These findings emphasize the urgency to develop new treatment strategies for *ATM* deficient CLL tumours.

Pre-clinical testing of new therapeutic agents include *in vitro* studies and utilization of animal and xenograft models. Xenograft models do not only enable investigation of the responses of human disease to novel treatments but also provide an opportunity to obtain an insight into the biology of the disease *in situ* and recapitulate the phenotype of particular subgroups of patients. However, attempts to engraft primary CLL cells into mice were hampered for many years by two major obstacles: human cells were either rejected by insufficiently immune-deficient recipients (Kobayashi et al., 1992) or only attained a low level of proliferation once engrafted in murine organs (Durig et al., 2007). Recently, Bagnara and colleagues seemed to resolve these problems in their newly developed CLL xenograft model by using highly immune-deficient NSG mice (NOD/Lt-*scid/IL2Rγ*^{null}) in combination with the provision of a human allogeneic microenvironment for engrafting CLL cells (Bagnara et al., 2011). The authors have demonstrated that in this setting the main factor enabling CLL cells to engraft and proliferate were activated autologous CD4 expressing T-cells. However, this novel model has serious limitations. Proliferating CLL cells gradually disappeared and were substituted by autologous T-cells. All mice died with suspected graf-versus-host disease (GvHD) within 12 weeks post injection. Hence, autologous T-cells seem to possess a dual role: they facilitate CLL development in mice but also promote termination of the graft.

Despite the obvious potential of this model to provide a pre-clinical testing tool, its limitations impose a need for further optimization. Therefore, the aim of the study

described in this chapter has been to further optimize the existing CLL xenograft model and obtain prolonged engraftment of CLL cells with the emphasis on tumours harbouring *ATM* defects. To achieve this aim I have investigated:

1) Whether ATM null cells can be engrafted at sufficient levels into immune-deficient mice.

2) Whether CLL engraftment and its duration can be modified by manipulating T-cell numbers.

3) Whether depletion of various T-cell subsets can prolong CLL engraftment.

5.2. NOG (NOD/Shi-scid/IL2Rγnull) mice.

Xenogenic mouse transplantation procedures performed in this study were based on the protocols developed by Bagnara and colleagues (Bagnara et al., 2011). The Biomedical Services Unit (BMSU) at the University of Birmingham was not able to provide NSG mice (as suggested in the protocol), offering instead the NOG (NOD/Shi-*scid/IL2Rγ*^{null}) strain of mice.

In these circumstances, the NOG strain was accepted as an adequate standard for further optimizing the CLL xenograft model. This decision was based on the fact that both strains have very similar genetic backgrounds regarding a highly deficient immune system lacking functional B, T and NK cells. The differences refer mainly to the way in which the *interleukin-2 receptor gamma chain (IL-2Rγc)* gene was disrupted in each strain. IL-2Rγc is an essential component of cytokine signalling and its abrogation leads to the loss of functional T and NK cells in humans and T, B and NK cells in mice. In NSG mice the *IL-2Rγc* gene has been knocked out resulting in complete loss of expression of the entire receptor gamma chain including the extracellular, transmembrane and cytoplasmic domains (Cao et al., 1995). In NOG mice, only the cytoplasmic domain was deleted which did not affect ligand binding but completely abrogated signal transduction (Ohbo et al., 1996). In addition, engraftment of human CD34 positive hematopoietic stem cells in both NSG and NOG mice has been shown to be equally efficient and comparable (Ito et al., 2002; McDermott et al., 2010).

5.3. Engraftment of *ATM* mutant CLL cells in humanized NOG mice.

To test whether *ATM* mutant CLL cells can be engrafted using the current CLL xenograft model, a single CLL sample (CLL-1, Table 5.1) was injected into 8 NOG mice. The selected CLL tumour harboured two *ATM* mutations (one germ-line truncating mutation c.1058_1059delGT [p.Cys353fs], and one acquired missense mutation c.5224G>C [p.Ala1742Pro]) resulting in complete loss and therefore function of the ATM protein (Stankovic et al., 1999, patient B-CLL-1). It also had an unmutated *IGHV* status and followed progressive clinical course.

Table 5.1. Clinical and biological characteristics of CLL samples used for transplantation in NOG mice.

CLL sample	Binet stage	Clinical course	*ATM* status	*TP53* status	*IGHV* mutation status	% of T-cells in PBMC	CD4:CD8 ratio
CLL-1	B	progressive	MUT	WT	99% UM	n/a	n/a
CLL-2	A	stable	WT	WT	92.3% M	4.6	3.5
CLL-3	A	progressive	WT	WT	98% UM	4.2	0.9
CLL-4	A	stable	WT	WT	98% UM	3.3	3.4
CLL-5	A	progressive	WT	WT	98% UM	2.6	n/a
CLL-6	A	stable	WT	WT	M	12.1	2.8
CLL-7	A	stable	WT	WT	90.7% M	9.2	1.3

IGHV- immunoglobulin heavy chain variable gene, n/a- not assessed,
PBMC-peripheral blood mononuclear cells

Following Bagnara's protocol, two approaches to provide a humanized microenvironment to support CLL cells were utilized. The first approach involved injection of 4 NOG mice with umbilical cord blood derived CD34 positive cells 4 weeks prior to adoptive transfer of CFSE stained CLL PBMC. This allowed the establishment of a human hematopoietic niche from their engraftment and subsequent differentiation in murine organs (Figure 5.1a). In another approach, 4 mice were intravenously co-injected with human allogeneic CD14+ monocytes and CFSE-labelled CLL PBMCs (Figure 5.1b).

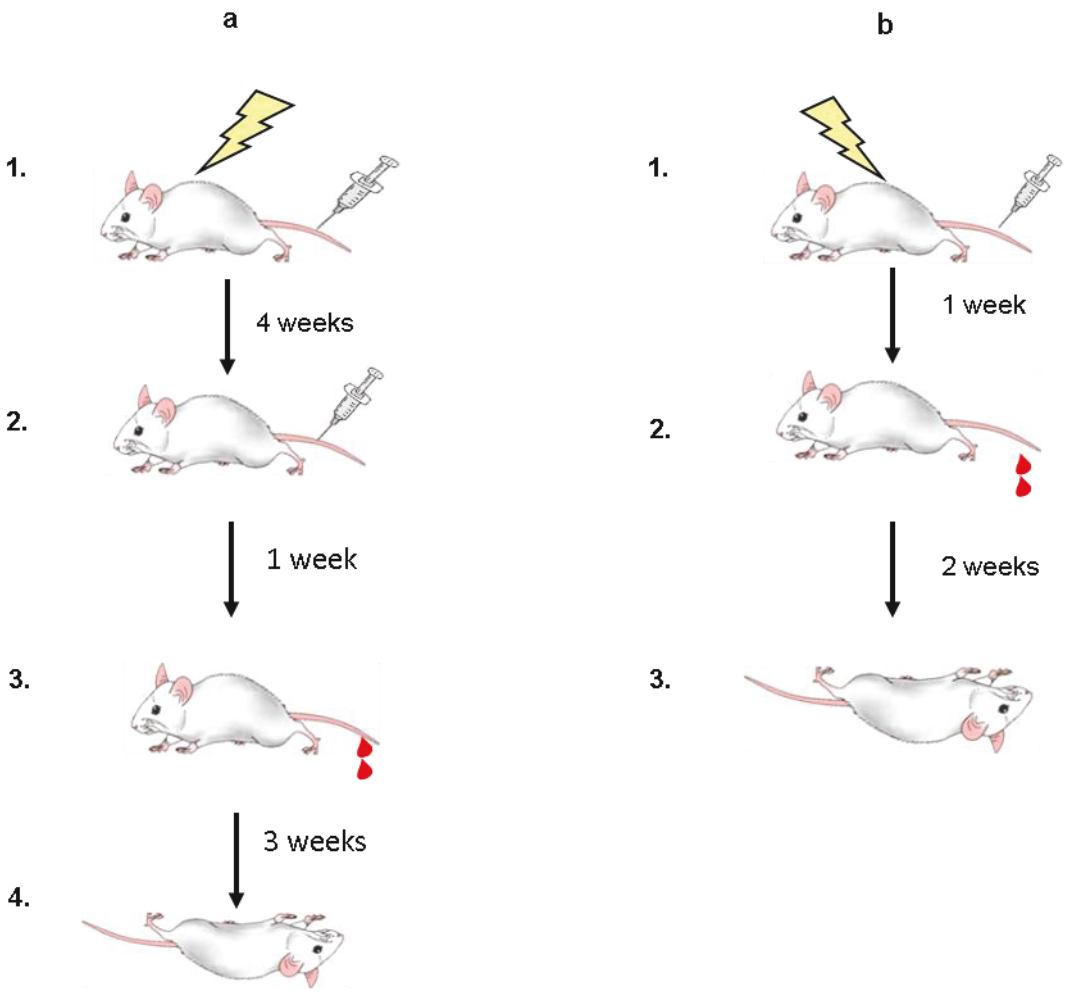

Figure 5.1 Transplantation of *ATM* mutant CLL cells in humanized NOG mice.

(**a**) Humanization with $CD34^+$ hematopoietic stem cells and adoptive transfer of CLL PBMC. 1. sub-lethal irradiation (2x1.25Gy) and intravenous (iv) injection of umbilical cord blood $CD34^+$ cells, 2. iv injection of CLL PBMC labelled with CFSE, 3. weekly tail bleeds, 4. cull and harvest the organs.

(**b**) Humanization with $CD14^+$ cells and adoptive transfer of CLL PBMC. 1. sub-lethal irradiation and simultaneous iv co-injection of $CD14^+$ monocytes with CFSE labelled CLL PBMC, 2. weekly tail bleeds, 3. cull and harvest the organs.

Starting one week post tumour cell injection, peripheral blood samples were taken on weekly basis for flow cytometry analysis (Materials and Methods section 2.6.6)

The point at which T-cells began to increase in numbers was detected via weekly monitoring of human cells in peripheral blood, at this point mice were sacrificed and the spleen, bone marrow and peritoneum were collected for analysis. The first sign of CLL patients' T-cell outgrowth in mice humanized by monocytes occurred at week 3 post CLL injections whereas in animals humanized with $CD34^+$ umbilical cord cells it occurred at week 4 post CLL transfer (Figure 5.2).

In general, murine spleens and bone marrows were predominantly engrafted by human B-cells whereas the peritoneum contained a greater population of human T-cells (Figure 5.3). The majority of all engrafted human $CD45^+$ cells in murine organs were $CFSE^-$ which could be attributed to the high proliferation rate of CLL cells (Figure 5.4). On average, spleens of mice humanized with $CD34^+$ cells were significantly more infiltrated with human cells than of mice humanized with $CD14^+$ monocytes at this time-point ($p \leq 0.001$). The discrepancies in the level of engraftment observed between the two approaches could be due to cord blood derived hematopoietic cells contributing to the detected load in former method. To discount this and further confirm the engraftment of CLL cells and investigate their proliferation capability, murine spleen samples (only those humanized with $CD34^+$ cells) were analysed by immunohistochemistry.

Immunohistochemistry staining showed engraftment of human B-cells visualized by a Pax5 antibody (a marker of mature B-cells) and very low level or lack of ATM protein in those cells confirming origination from the patient CLL sample. Furthermore, CLL cells were positive for Ki67 (proliferation marker) and their distribution was resembling proliferation centres in human lymph nodes of CLL patients (Figure 5.5) (Ciccone et al., 2011). To ensure that engrafted and proliferating B-cells do not originate from normal patients B-cells latently infected with EBV, histology staining for EBV mRNA (EBER) has been performed and revealed minimal or no infection level, therefore ruling out the possibility of EBV-driven proliferation.

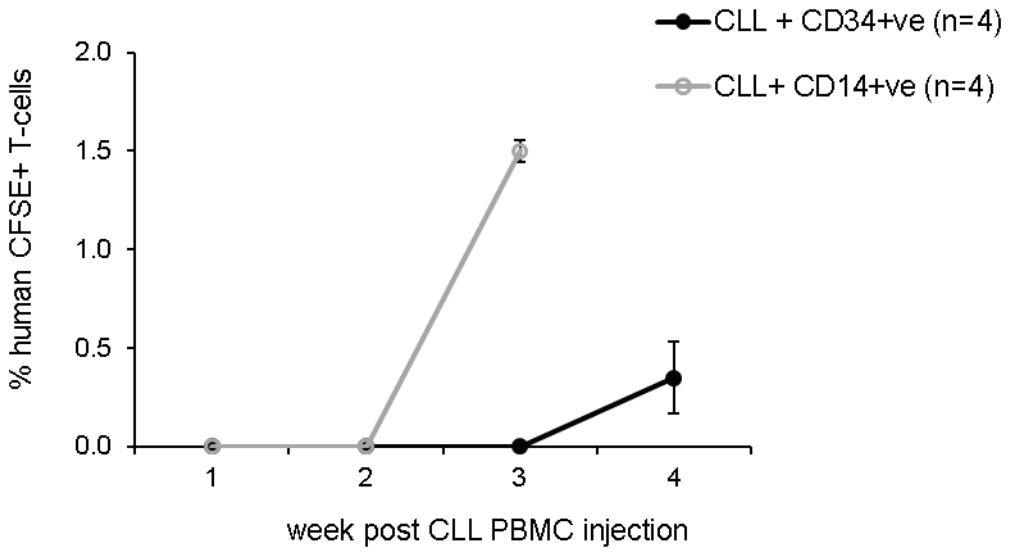

Figure 5.2. Engraftment of patients' T-cells in blood of NOG mice humanized with either CD34$^+$ stem cells or CD14$^+$ monocytes

The onset of CFSE$^+$ T-cells was observed earlier in animals humanized with CD14$^+$ monocytes.

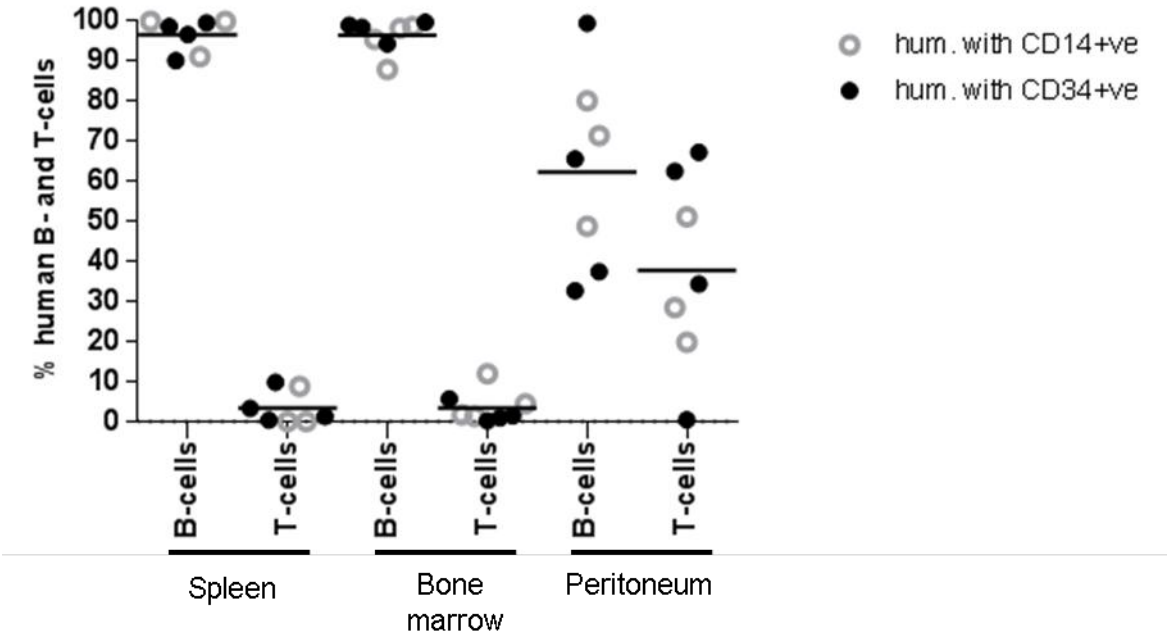

Figure 5.3. Engraftment of human B and T-cells in murine organs.

Engraftment levels were similar between mice humanized with either $CD14^+$ monocytes or $CD34^+$ stem cells. The spleen and bone marrow were mostly engrafted with human B cells, whereas the peritoneum harboured a proportion of human T-cells.

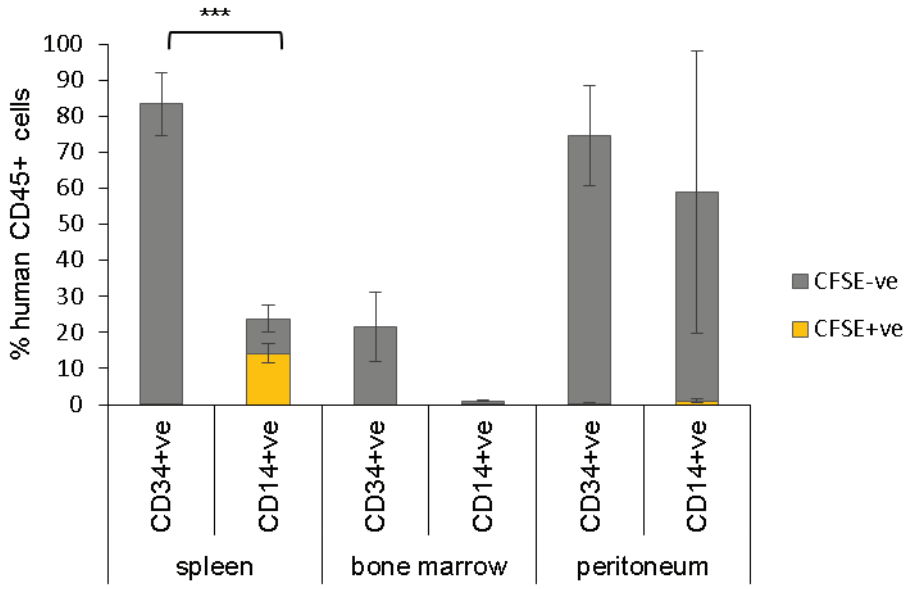

Figure 5.4. Engraftment of human CD45^{+} cells in organs of NOG mice injected with an *ATM* mutant CLL.

The majority of all engrafted human CD45^{+} cells in murine organs were CFSE^{-ve}. The spleens of mice humanized with CD34^{+} stem cells were significantly more infiltrated with human cells than those of mice humanized with CD14^{+} monocytes ($p \leq 0.001$).

Both the spleen and peritoneum of mice humanized with CD34^{+} stem cells were highly infiltrated with human cells, whereas in mice humanized with CD14^{+} monocytes the peritoneum was the most infiltrated organ.

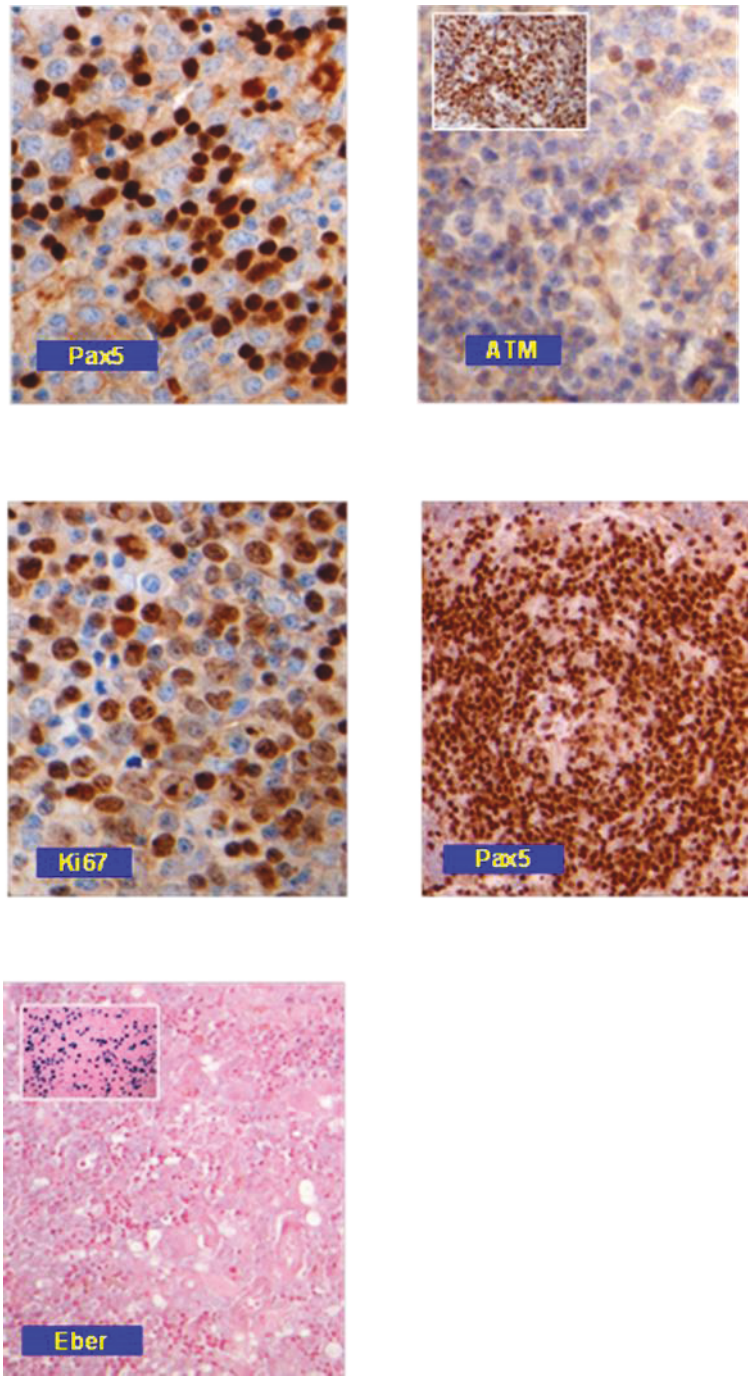

Figure 5.5. Localization of *ATM* mutant CLL cells in NOG mice spleen.

Cells populating the infiltrated murine spleen were Pax5^{+} (mature B-cell marker), ATM negative (confirming CLL origin), expressed the proliferation marker Ki67, and their distribution resembled proliferation centers in human lymph nodes. They were also negative for Eber (EBV mRNA marker). Inserted smaller photos are positive controls for the respective antibodies, ATM and EBER.

Therefore, this part of the study confirmed that ATM null CLL cells can be engrafted in immuno-deficient NOG mice and that the use of umbilical cord blood $CD34^+$ stem cells might be the preferential choice for murine humanization because it seemed capable of not only providing a microenvironment for CLL engraftment and proliferation but also of promoting delayed onset of human T-cells outgrowth in blood. Consequently, this method of humanization was used in all later transplantations in this study.

5.4. Depletion of autologous T-cells in CLL xenograft.

Eliminating the factors which hinder CLL growth in a murine xenograft model is likely to be the most effective approach in prolonging CLL engraftment. Bagnara et al (2011) suggested that autologous T-cells might be the main culprits of graft termination. However, their complete depletion abrogated the proliferation and survival of leukemic cells providing evidence that they are also the key mediators of leukemic cell growth. Hence, we hypothesized that decreasing (rather than complete elimination) the number of T-cells down to a minimal level, still sufficient to provide growth stimuli for CLL cells could delay the onset of T-cell outgrowth and consequently, prolong CLL engraftment. To test this hypothesis gradual depletion of autologous T-cells from patients PBMC prior injection into mice was performed (Figure 5.6., Materials and Methods section 2.5.3).

In total, 26 NOG mice were humanized with CD34 positive cells and subsequently injected with 4 different patient samples (CLL-2, CLL-3, CLL-4, CLL-5) labelled with CFSE. Patients' characteristics and proportions of T and B-cells in PBMC samples are provided in Table 5.1. Two CLL patients had a stable clinical course and had not received treatment or were responding well to treatments and 2 patients had a progressive course of disease. None of the patients had an *ATM* or *TP53* mutation whereas 3 had an unmutated and 1 a mutated *IGHV* status.

CLL samples were injected either as non T-cell depleted PBMC (9 mice); intermediate T-cell depleted PBMC consisting of 10-25% of the original number of T-cells with $0.1\text{-}0.28 \times 10^6$ CD3 positive cells per mouse (7 mice); or T-cell depleted to 1.5-5% of

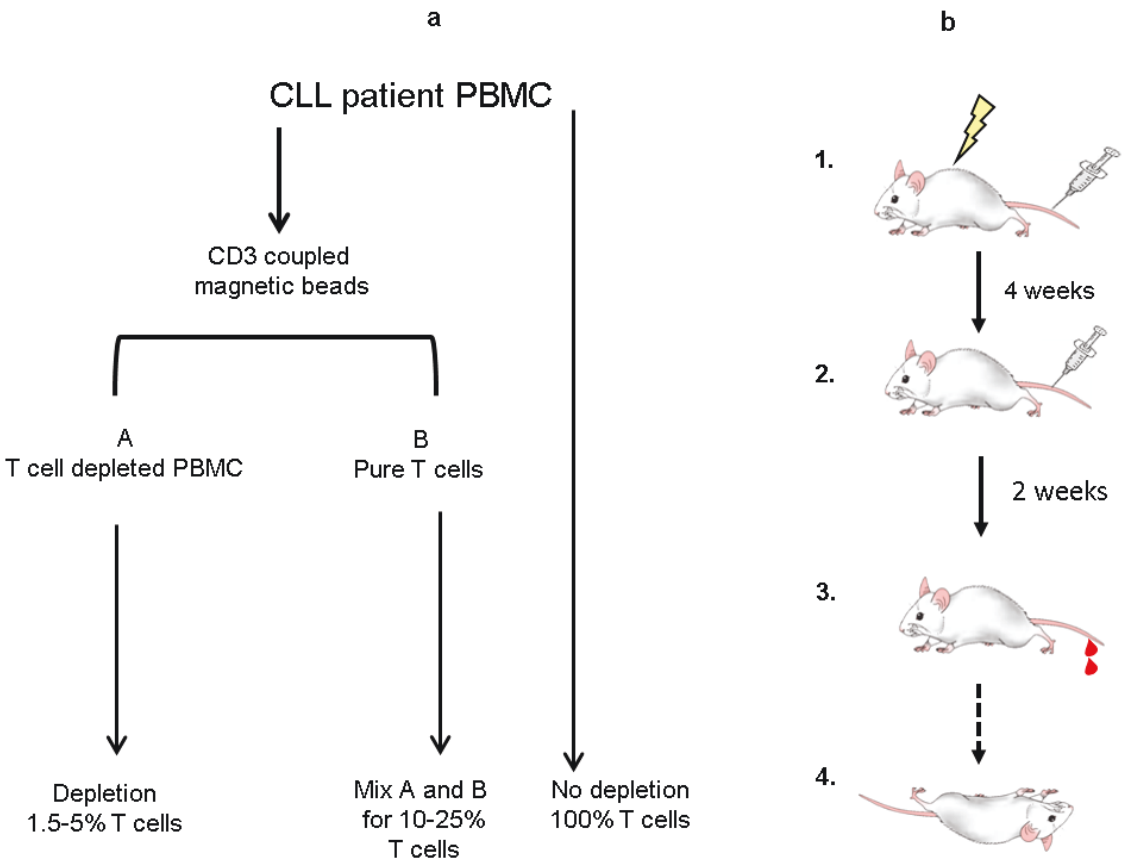

Figure 5.6. Schematic presentation of the T-cell depletion procedure (a) and transplantation of resulting CLL PBMC into NOG mice (b).

(**a**) Three fractions of CLL PBMCs were prepared for injection: non T-cell depleted, T-cell depleted down to 1.5-5% of the original number or down to 10-25% of the original T-cell number.

(**b**) 1. Sub-lethal irradiation (1.5-2.5Gy) and iv injection of $CD34^+$ umbilical cord blood hematopoietic stem cells, 2. iv injection of CFSE-labelled CLL PBMC with various degrees of T-cell depletion, 3. weekly tail bleeds, 4. cull at signs of illness and harvest the organs for analysis.

original T-cell number and 0.006-0.05x10^6 CD3 positive cells per mouse (9 mice). The total number of PBMCs injected into $CD34^+$ humanized mice differed between CLL samples and was determined by availability of the tumour material (**Table 5.2).**

Table 5.2. NOG mice transplantation experiments.

CLL sample	Transplantation experiment	PBMC depletion	No. of NOG mice injected	No. of total PBMC cells per injection	No. of CD3+ cells per injection
CLL-1	*ATM* null cell engraftment	no depletion, humanization by UCB CD34+ cells	4	0.5x10^8	n/a
		no depletion, humanization by CD14+ monocytes	4	0.5x10^8	n/a
CLL-2	T-cell depletion	non depleted	1	0.5x10^8	2.3x10^6
		10% CD3	1	0.18x10^8	0.1x10^6
		2% CD3	1	0.18x10^8	0.02x10^6
CLL-3	T-cell depletion	non depleted	2	0.5x10^8	2.08x10^6
		12% CD3	3	0.5x10^8	0.28x10^6
		2% CD3	3	0.5x10^8	0.05x10^6
CLL-4	T-cell depletion	non depleted	2	0.13x10^8	0.43x10^6
		25% CD3	3	0.13x10^8	0.12x10^6
		5% CD3	3	0.13x10^8	0.023x10^6
CLL-5	T-cell depletion	non depleted	3	0.15x10^8	0.39x10^6
		1.5% CD3	3	0.15x10^8	0.006x10^6
CLL-6	T-cell subset depletion	non depleted	2	0.8x10^8	9.6x10^6
		5% CD3	2	0.8x10^8	0.48x10^6
		24% CD4	2	0.8x10^8	n/a
		3% CD8	2	0.8x10^8	n/a
		0% CD56	2	0.8x10^8	n/a
CLL-7	T-cell subset depletion	non depleted	3	0.8x10^8	7.4x10^6
		48% CD3	3	0.8x10^8	3.5x10^6
		61% CD4	3	0.8x10^8	n/a
		8% CD8	2	0.8x10^8	n/a
		0% CD56	2	0.8x10^8	n/a
		0% CD25	3	0.8x10^8	n/a

% indicate the proportion cells from particular subset remaining in PBMC after depletion procedure , n/a- not assessed, UCB-umbilical cord blood, PBMC- peripheral blood mononuclear cells

5.4.1. Survival and kinetics of engraftment in peripheral blood of CLL with autologous T-cell depletion.

In total, mice injected with highly T-cell depleted PBMCs (1.5-5%) survived significantly longer than mice injected with non T-cell depleted or intermediate T-cell depleted PBMCs (median survival 25, 6 and 6 weeks respectively, p=0.009; Figure 5.7). Importantly, further analysis revealed that prolonged survival of mice injected with T-cell depleted PBMC was not due to delayed engraftment of CLL cells. The presence of $CFSE^+$ B-cells (CLL cells) in mice blood was taken as a sign of engraftment and persistence of tumour cells in animals. All of the mice injected with CLL cells successfully engrafted within 6 weeks post injection. The time to first detection of CFSE-labelled human CLL cells (at least 1% of live cells) differed between tumour samples but not between the animals injected with the same CLL sample. Furthermore, even the most profound depletion of T-cells (1.5-5% of original T-cells number) did not delay or prevent the engraftment of CLL cells (Figure 5.8).

In general, the level of $CFSE^+$ cells in mice bloods was gradually decreasing and the CFSE fluorescence intensity of circulating CLL cells was diminishing indicative of proliferation. However, an interesting phenomenon was observed, every few weeks the level of $CFSE^+$ cells in blood would rise, only to drop again a week or two later. As animals injected with non depleted PBMCs died early in the experiment (between weeks 4 and 8), the reappearance of $CFSE^+$ cells at later time points during the engraftment was observed only in mice which were injected with T-cell depleted PBMCs (Figure 5.8). Interestingly, in some cases the intensity of CFSE fluorescence of these reappearing cells was the same as at the time of injection suggesting the release of non proliferating CLL cells from murine tissue sites into the blood stream (Figure 5.9).

Due to the gradual loss of CFSE signal over time, the kinetics of engraftment in murine blood were also measured by assessing the proportion of total human B and T cells (as combined $CFSE^+$ and $CFSE^-$). This approach was justified by the results of the immunohistochemistry staining described in section 5.3 of this chapter (Figure 5.5) and by

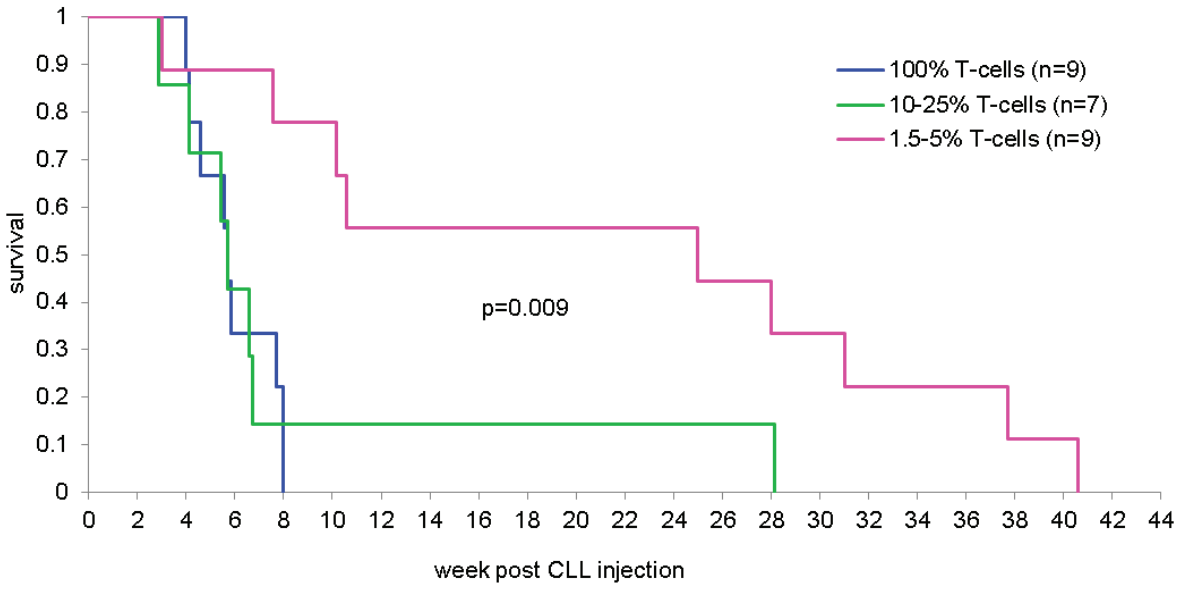

Figure 5.7. Survival of NOG mice engrafted with autologous T-cell depleted CLL PBMC.

Three groups were studied: NOG mice injected with CLL PBMC depleted of T-cells down to 1.5-5% of the original T-cell number, depleted to 10-25% or non depleted of T-cells (100% T-cells). Mice with the most profoundly depleted fraction of T-cells (1.5-5% T-cells) survived significantly longer than the two other groups (p=0.009).

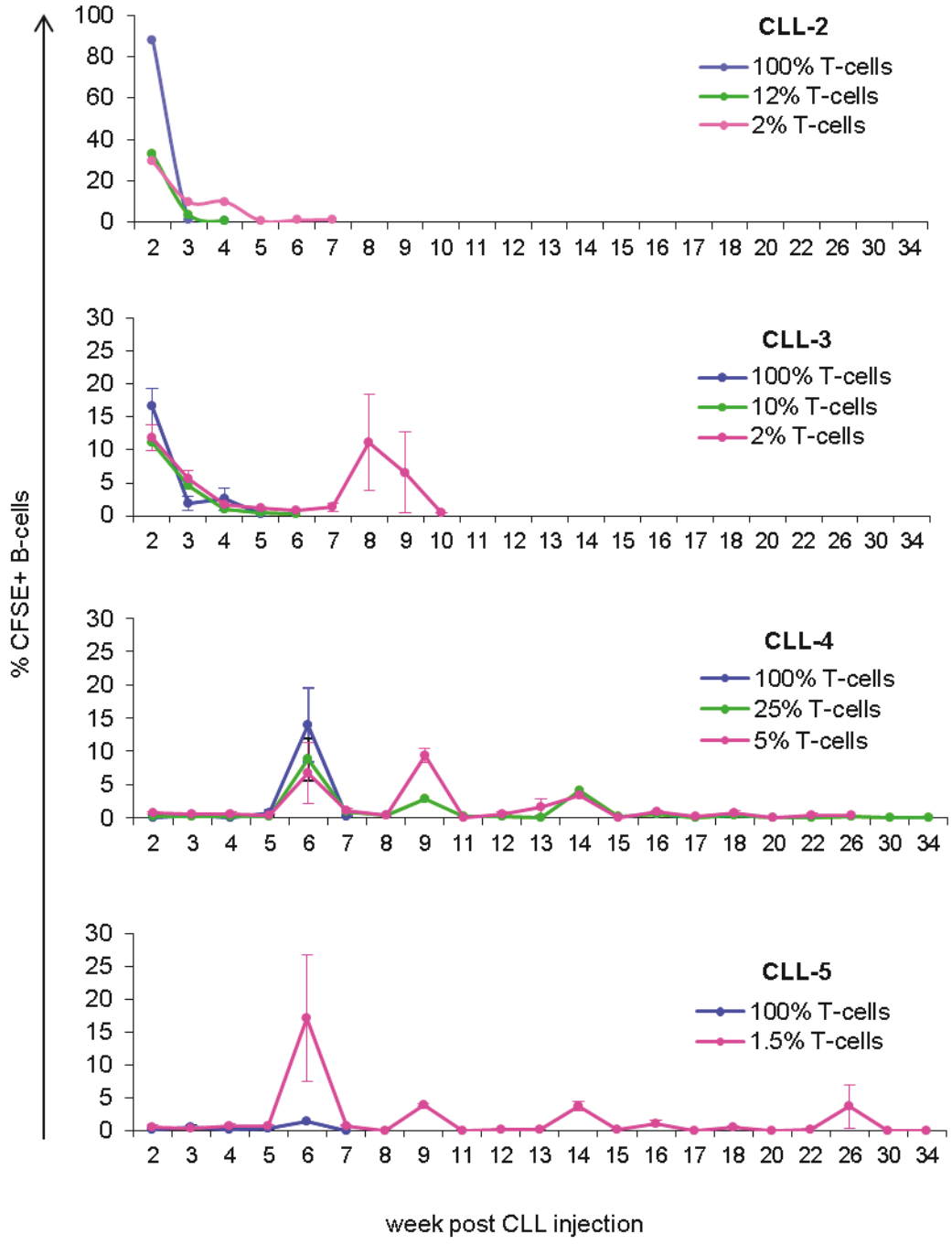

Figure 5.8. Engraftment kinetics of autologous T-cell depleted CLL cells in murine blood.

Four different CLL samples were injected (CLL-2, CLL-3, CLL-4, CLL-5). T cell depletion from CLL PBMC prior to injection into NOG mice did not abrogate CLL engraftment but prolonged its sustained presence in murine peripheral blood. The Y-axes indicate the % of $CFSE^{+}$ B-CLL cells present in the total live cell population.

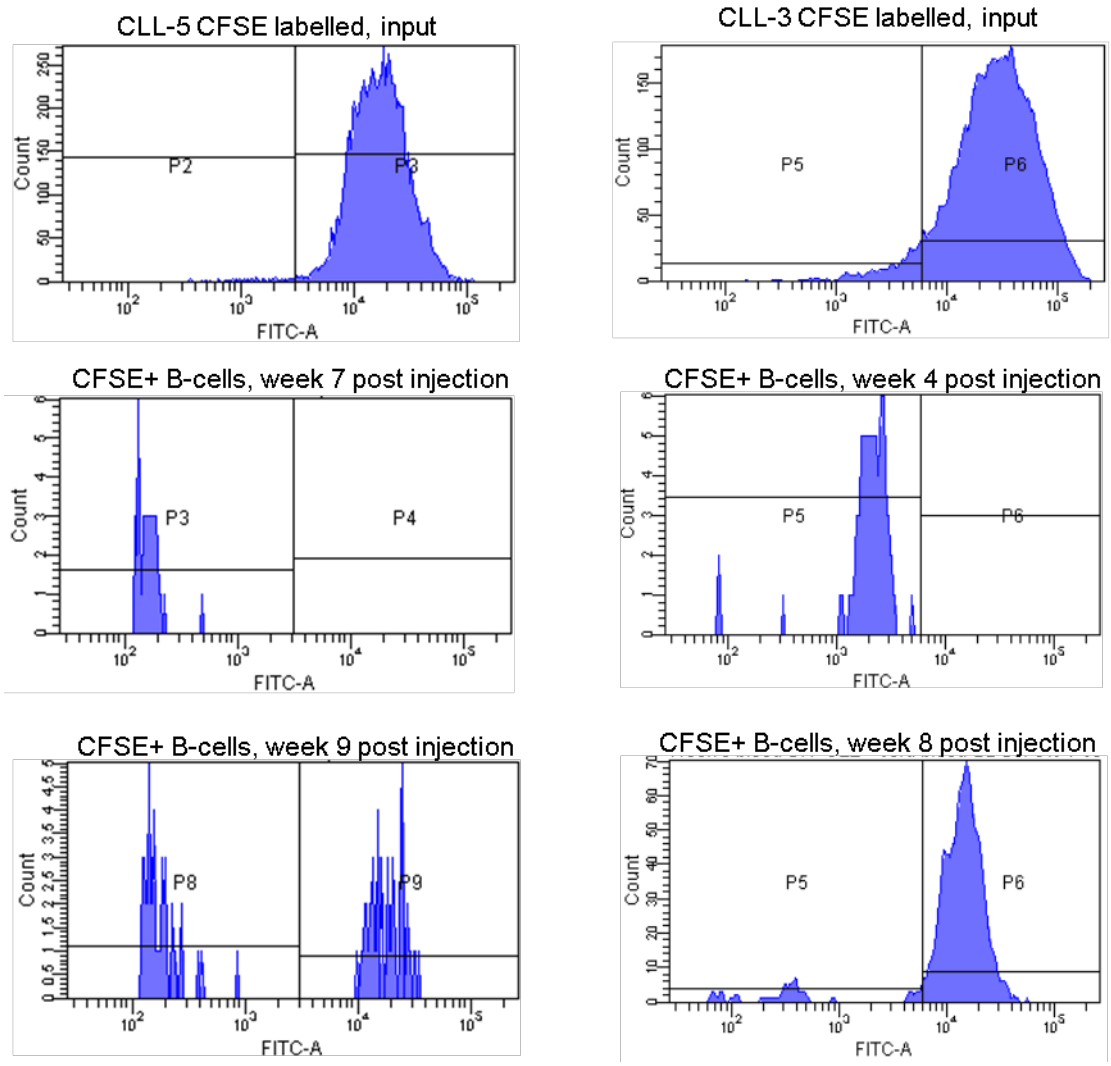

Figure 5.9. Reappearance of a non proliferated fraction of CLL PBMCs in murine blood.

High CFSE expressing, non-proliferated CLL PBMCs reappear in the murine circulation 8-9 weeks post-CLL PBMC injection.

results from Bagnara's study, both demonstrating that human B-cells infiltrating murine organs were of CLL origin. Therefore, we assumed that majority of $CFSE^-$ B-cells should be derived from CLL PBMC.

The level of engraftment of human B and T-cells in murine blood fluctuated considerably between the time of the first bleed (at week 2 post CLL injection) and the last bleed performed before the cull. Nevertheless, in accordance with previous reports, a significant increase in the proportion of T-cell population in most animals was observed (in 100% T-cells PBMC group p=0.004, in 1.5-5% T-cells PBMC group p=0.004, in 10-25% T-cell group p=0.06) coupled with significant decrease in the proportion of the B-cell population ($p \leq 0.03$ for each group) (Figure 5.10). The increase of the proportion of T-cells was rapid in mice injected with non T cell depleted PBMCs and the median time between the first and the last bleed was 3 weeks. In mice injected with T-cell depleted PBMCs, the increase of human T-cells was gradual and delayed in time. The median period between these two time points was 20 weeks and significantly longer than in non T-cell depleted group ($p<0.0025$). For mice with intermediate T-cell depletion, it was 3.5 weeks.

The increase in the proportion of circulating T-cells was also manifested in the time to onset of T-cell outgrowth. The time of onset was recorded as the week that the proportion of circulating T-cells exceeded 15% for the first time. The reason behind this cut-off was to find the most generic value which could be applied to any CLL sample utilized in this xenograft model and 15% seems to represent the average proportion of $CD3^+$ cells in CLL PBMC (D'Arena et al., 2011). Notably, there were no significant differences between depletion groups in respect to the proportion of T-cells in murine blood when the animals were bled for the first time, 2 weeks after CLL injection (the median for all groups combined was 2%).

The outgrowth of circulating T-cells in the 100% T-cell group started at week 4 post CLL injection and it was significantly earlier than in 1.5-5% T-cell depletion group which began on week 8 (p=0.0007) (Figure 5.11). Moreover, the proportion of human T-cells at

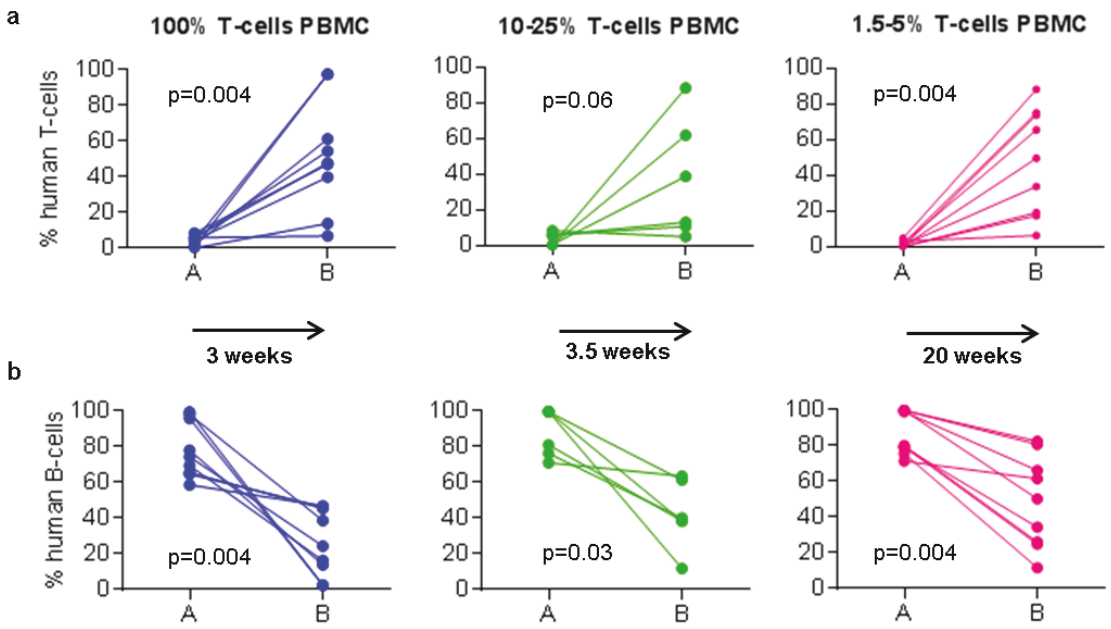

Figure 5.10. The relative T and B-cell proportions of engrafted human CD45+ cells in murine blood.

Each graph depicts the comparison of the proportion of engrafted hCD45+ cells that are B- or T-cells between the first bleed at week 2 (A) and the final bleed before cull (B).

(**a**) The increase of human T-cells was observed in all three groups of animals but took significantly longer in mice injected with PBMC highly depleted of T-cells (1-5-5% of T cells) when compared to the other two groups ($p<0.01$). (**b**) The relative proportion of human B-cells decreased significantly in all three groups of animals during the time of engraftment.

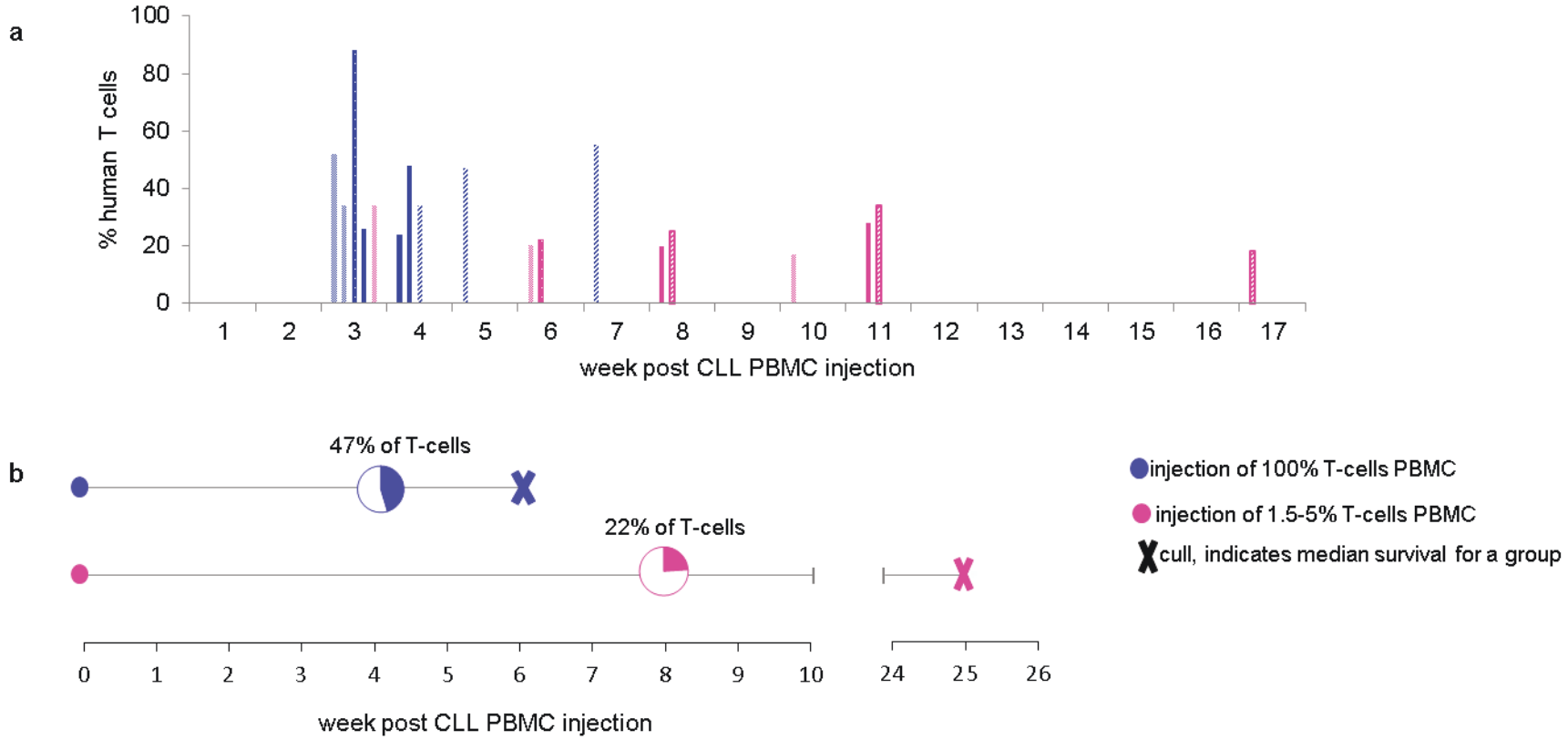

Figure 5.11. Effect of T-cell depletion upon the onset of human T-cell outgrowth in murine blood.

(**a**) T-cell proportion of the human CD45+ cell population in murine blood at the time of T cell onset. Each bar indicates a different mouse. Pink bars indicate mice injected with 1.5-5% T-cell PBMC, blue bars indicate mice injected with 100% T-cell PBMC. Fill patterns indicate different CLL samples injected. (**b**) The median time before human T cell onset in murine blood was shorter in mice injected with 100% T-cell PBMC compared to mice injected with 1.5-5% T-cell PBMC (p=0.007), and the proportion of human T-cells at the time of onset was higher in the 100% T-cell group (47%) than the 1.5-5% T-cell group (22%, p=0.0035).

onset was more than 2-fold higher in the 100% T cell group than the 1.5-5% T cell group (47% vs 22%, p=0.003). Aki to the 100% T-cell group, time of T-cell onset also occurred at week 4 in the 10-25% T-cell group, however, the median proportion of T-cells (29.5%) did not differ from the other two groups.

In conclusion, depletion of autologous T-cells to less than 5% of their initial level prior to the adoptive transfer of patient PBMCs into recipient mice did not abrogate the engraftment of CLL cells but did delay the outgrowth of T-cells and prolong survival of engrafted animals. On the contrary, intermediate depletion of T-cells to 10-25% of their original level did not confer any advantageous prolongation of murine survival or delay in the outgrowth of human T-cells.

5.4.2. Engraftment of patient B and T-cells in murine organs upon T-cell depletion.

The engraftment of patient cells in murine spleen, bone marrow and peritoneum was analysed by FACS at the time when animals succumbed to illness. Due to the loss of CFSE signal, detection of $CFSE^+$ cells in organs and comparison between different depletion groups was only possible in mice injected with 2 of the CLL samples, CLL-2 and CLL-3 (Figure 5.12.). In these animals, a larger proportion of total CFSE positive cells and higher accumulation of CFSE positive T-cells was observed in the peritoneum than in other organs at the time of culling. In addition, the spleens of mice injected with non T-cell depleted PMBCs seemed to be predominantly infiltrated with CFSE positive T-cells in comparison to spleens of mice which received T-cell depleted PBMCs. Furthermore, the highest accumulation of $CFSE^+$ B-cells was observed in bone marrows regardless of the level of T-cell depletion. Despite the initial observations, they did not reach significance when statistical analysis was applied to test the differences between the groups.

The engraftment analysis of human $CD45^+$ cells (encompassing $CFSE^+$ and $CFSE^-$ cells) in murine organs was performed in 24 animals, injected with one of 4 CLL samples (CLL-2, CLL-3, CLL-4, CLL-5). This analysis showed that human mononuclear cells

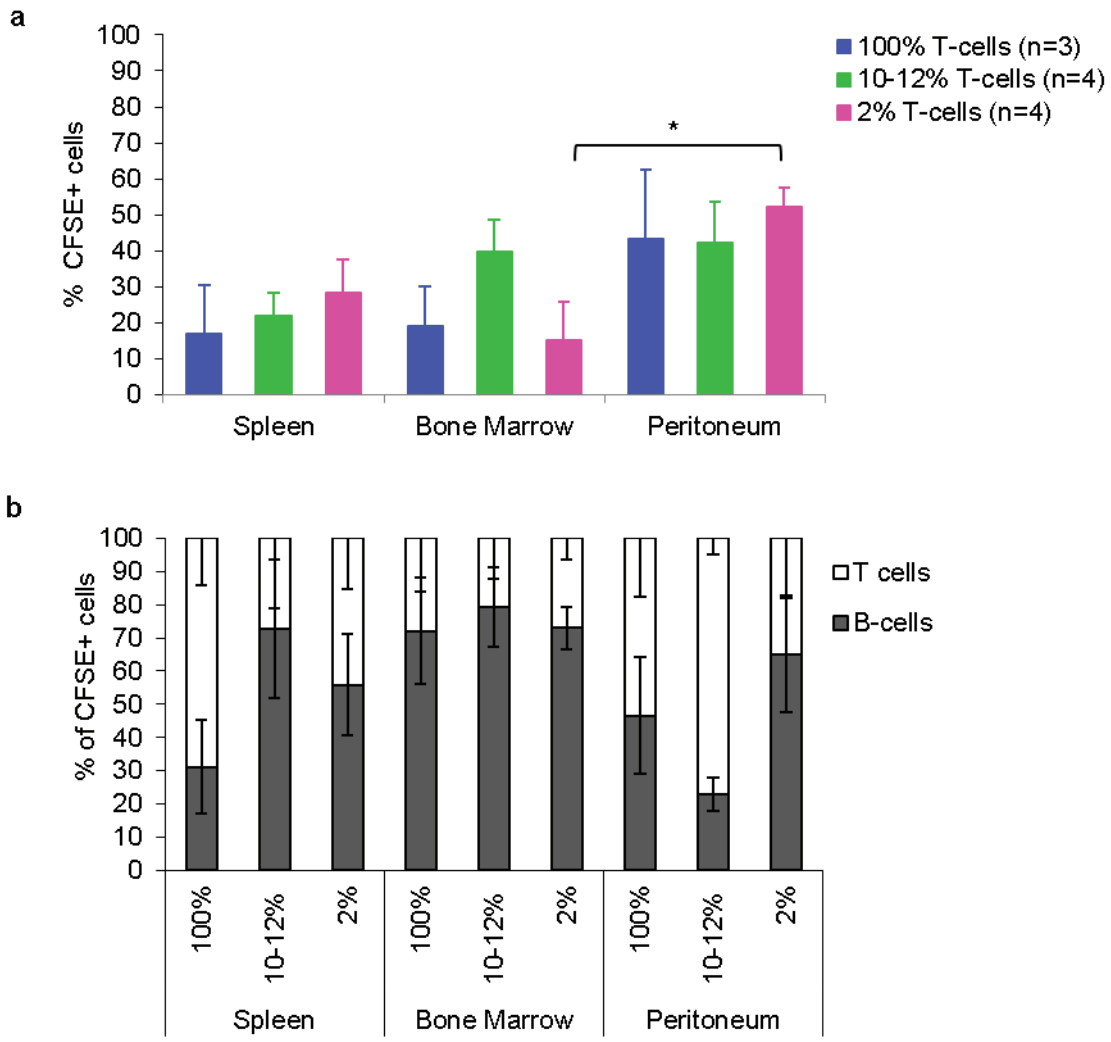

Figure 5.12. Minimal effect of T-cell depletion upon engraftment of CFSE$^+$ CLL PBMC in murine organs.

(**a**) In general, an increased accumulation of CFSE$^+$ cells was observed in the murine peritoneum compared to other organs. However, the only confirmed, significant difference was between the bone marrow and peritoneum in the 2% T-cell group ($p<0.05$).

(**b**) The relative proportions of CFSE$^+$ B-CLL and T-cells did not differ significantly between different organs or depletion groups. However, spleen from mice injected with non T-cell depleted PBMC seemed to contain a higher proportion of T-cells than the other two groups.

localized predominantly to the murine spleen contributing on average >50% of total live cells in this tissue site regardless of T-cell depletion (Figure 5.13a) The predominant infiltration of spleen by human $CD45^+$ cells was significant in animals engrafted with non T cell depleted PBMC (spleen vs bone marrow and spleen vs peritoneum, both $p \leq 0.001$) and in animals engrafted with 1.5-5% T-cells PBMC (spleen vs bone marrow $p \leq 0.01$ and spleen vs peritoneum $p \leq 0.05$). However, depletion of $CD3^+$ cells from injected CLL PBMCs to ≤ 5% of initial levels resulted in significantly lower accumulation of total human cells in the spleen (100% T-cells versus 1.5-5% T-cells, $p<0.05$) and bone marrow (10-25% T-cells versus 5% -cells, $p<0.05$).

With respect to the relative proportion of B- and T-cells, bone marrow seemed to preferentially support the engraftment of B-cells over T-cells, whereas peritoneum appeared to be mostly infiltrated by human T-cells (Figure 5.13b). However, significant differences were only observed between the level of infiltration of different organs by the same T-cell depletion strategy. Depletion of T-cells, to 12% from the injected PBMCs resulted in a significantly higher proportion of B- than T-cells in the bone marrow and spleen than in the peritoneum ($p \leq 0.05$). And depletion of T-cells to 1.5-5% resulted in a significantly higher proportion of B- than T-cells in the bone marrow than in the peritoneum ($p \leq 0.05$). No significant differences in the B and T-cell relative proportions were observed between the various T-cell depletion groups within any particular organ.

To further investigate the localization of patient cells within the murine organs, immunohistochemistry analysis was performed on representative splenic samples from 3 mice injected with either non T-cell depleted CLL-3 PBMC or CLL-3 depleted to 12%, or 2% of the original T cell number (Table 5.1). These mice succumbed to illness at week 4, 7 and 10 post CLL injection, respectively.

Interestingly, this analysis revealed that the spleen from the mouse transplanted with the profoundly depleted T-cell fraction had a strikingly high accumulation of human B-cells in comparison to spleens obtained from animals injected either with patient PBMC containing 100% or 12% of T-cells which were mostly infiltrated with T-cells (Figure 5.14.).

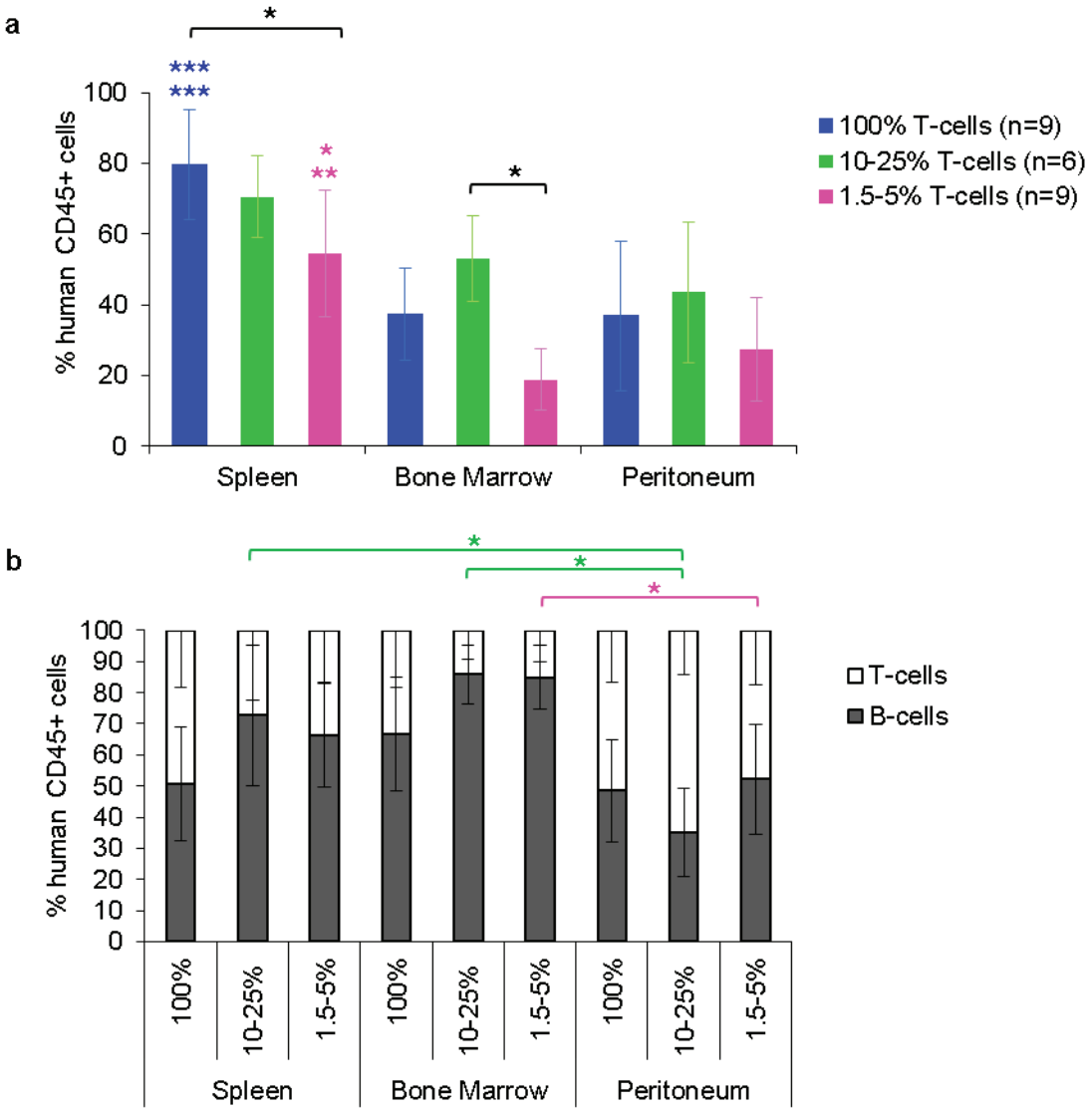

Figure 5.13. Predominant infiltration of murine spleen with human CD45$^+$ cells.

(**a**) An increased accumulation of human PBMCs was observed in the murine spleen compared to bone marrow or peritoneum in 100% T-cell ($p \leq 0.001$ for both) and in 1.5-5% T-cell ($p \leq 0.01$ $p \leq 0.05$) depletion groups. A significant difference was also confirmed between the 100% T-cell and 1.5-5% T-cell depletion groups in the spleen ($p < 0.05$), and between the 10-25% T-cell and 5% T-cell depletion groups in bone marrow ($p<0.05$).

(**b**) In general, the peritoneum seemed to preferentially support engraftment of human T-cells whereas the spleen and bone marrow supported the engraftment of human B cells. There was a significantly higher proportion of B-cells in spleen and bone marrow than in the peritoneum in the 10-25% depletion group and a higher proportion of B-cells in bone marrow than in the peritoneum in the 1.5-5% T-cell depletion group ($p \leq 0.05$, for all). There was no significant difference in engraftment between depletion groups in any organ.

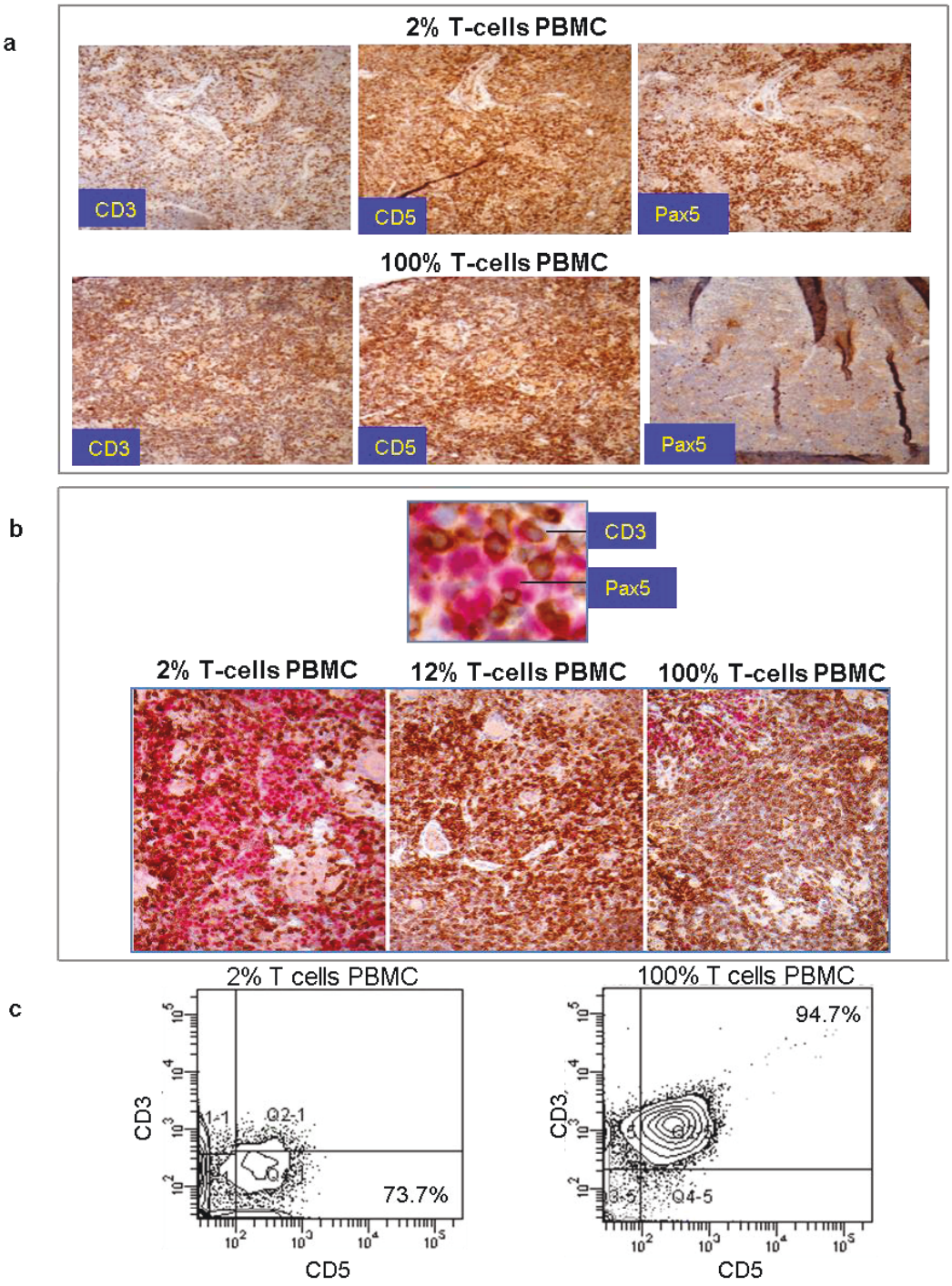

Figure 5.14. T-cell depletion induces preferential engraftment of human B-cells in murine spleen.

(**a**)The spleen from a mouse injected with 2% T-cell PBMC was highly infiltrated with human B cells (Pax5), whereas the spleen from a mouse injected with 100% T-cell PBMC was mostly infiltrated with human T-cells (CD3).(**b**) Confirmation of greater infiltration of murine spleen with B-cells upon maximal T-cell depletion by double staining for B and T-cells in murine spleen in three depletion groups. (**c**) FACS analysis corroboration of the B-cell ($CD3^{-}CD5^{+}$) or T-cell ($CD3^{+}CD5^{+}$) nature of $hCD45^{+}$ PBMC upon T-cell depletion.

The immunohistochemistry results of these splenic samples correlated with FACS analysis which also showed predominant accumulation of human B-cells in the spleen obtained from the mouse transplanted with highly T-cell depleted PBMC (73.7% B-cells of human $CD45^+$ cells) and conversely high accumulation of T-cells in the spleens from mice injected with either non T-cell depleted PBMC (94.7% T-cells of human $CD45^+$ cells) or depleted to intermediate level (87% T-cells).

In conclusion, depletion of autologous T-cells from CLL PBMC prior to adoptive transfer did not abrogate the engraftment of CLL cells in murine organs and in one case resulted in high accumulation of CLL B-cells in the spleen, resembling the phenotype of aggressive B-cell lymphoproliferative disease.

5.5. Partial depletion of different subsets of the autologous T-cell population.

In the next stage of optimization of the CLL xenograft model it became of interest to investigate whether there is a particular T-cell subset which is predominantly responsible for termination of the graft. It was hypothesised that its removal (partial or complete) from CLL PBMC prior to injection could possibly further prolong the CLL engraftment in addition to what was observed with indiscriminate T-cell depletion.

Therefore, the next experiment was designed to deplete each of the main T-cell subsets prior to injection into mice and subsequently assess the engraftment kinetics and survival. Twenty six mice were injected with one of 2 different CLL PBMCs (CLL-6 and CLL-7, Table 5.1). Samples of these 2 PBMCs were depleted of one of each of the following subsets: CD4 positive cells (including helper T-cells), CD8 positive cells (including cytotoxic T-cells), CD25 positive cells (including regulatory and activated T-cells), CD56 positive cells (including NK cells), CD3 positive T-cells; or non-depleted. The depletion level for each subset presented in Table 5.2 was determined by the efficiency of the magnetic cell sorting (MACS) technique (described in Material and Methods Chapter, section 2.5.2.). The first bleed was performed on week 4 post CLL injection.

5.5.1. The effect of depletion of different T-cell subsets on survival, the onset of T-cells outgrowth and the engraftment kinetics in murine blood.

It was observed that mice engrafted with non-depleted PBMC, with moderate $CD3^+$ T-cell depletion or specifically depleted of $CD8^+$ or $CD56^+$ T-cells individually fared worse than mice engrafted with either CD4+, CD25+ or CD3+ depleted CLL PBMCs (Figure 5.15a). The survival difference revealed to be significant upon pooling of the depletion sets into 2 groups. Group I included mice engrafted with non-depleted PBMC, moderate $CD3^+$ T-cell depleted and specifically depleted of $CD8^+$ or $CD56^+$ T-cells. Group II included mice engrafted with CD4+, CD25+ or CD3+ depleted PBMCs. Median survivals of those two Groups was 12 weeks and 34 weeks, respectively (p=0.007) (Figure 5.15b).

Engraftment of CLL cells in the majority of animals was confirmed by the presence of $CFSE^+$ B cells in the blood sample taken 4 weeks post CLL injection. However, after this period, the CFSE signal disappeared in the majority of murine bloods, hence, the kinetics of engraftment were assessed by following the presence of human $CD45^+$ cells. This was regarded as acceptable due to evidence published by Bagnara and our previous observations (Figure 5.5.) corroborating that the majority of engrafted $hCD45^+$ cells were of CLL sample origin.

Outgrowth of human T-cells in murine blood was observed in all cases but the time of onset differed between the various depletion groups (Figure 5.16a). It appeared significantly earlier in Group I than in Group II (week 4 versus week 15, respectively; p=0.003).

The level of engraftment of human B-cells gradually decreased in murine blood for all the T-cell subset depletion sub-groups. In general, the peak accumulation of circulating human B-cells was observed on week 10 recurring at week 14 post CLL injection (Figure 5.17). There was however, a significantly higher proportion of circulating B-cells present at week 10 in Group I than in Group II (p=0.015).

In summary, these observations lead to two conclusions. Firstly, partial depletion of $CD4^+$ cells and complete depletion of $CD25^+$ cells resulted in delayed onset of T-cells

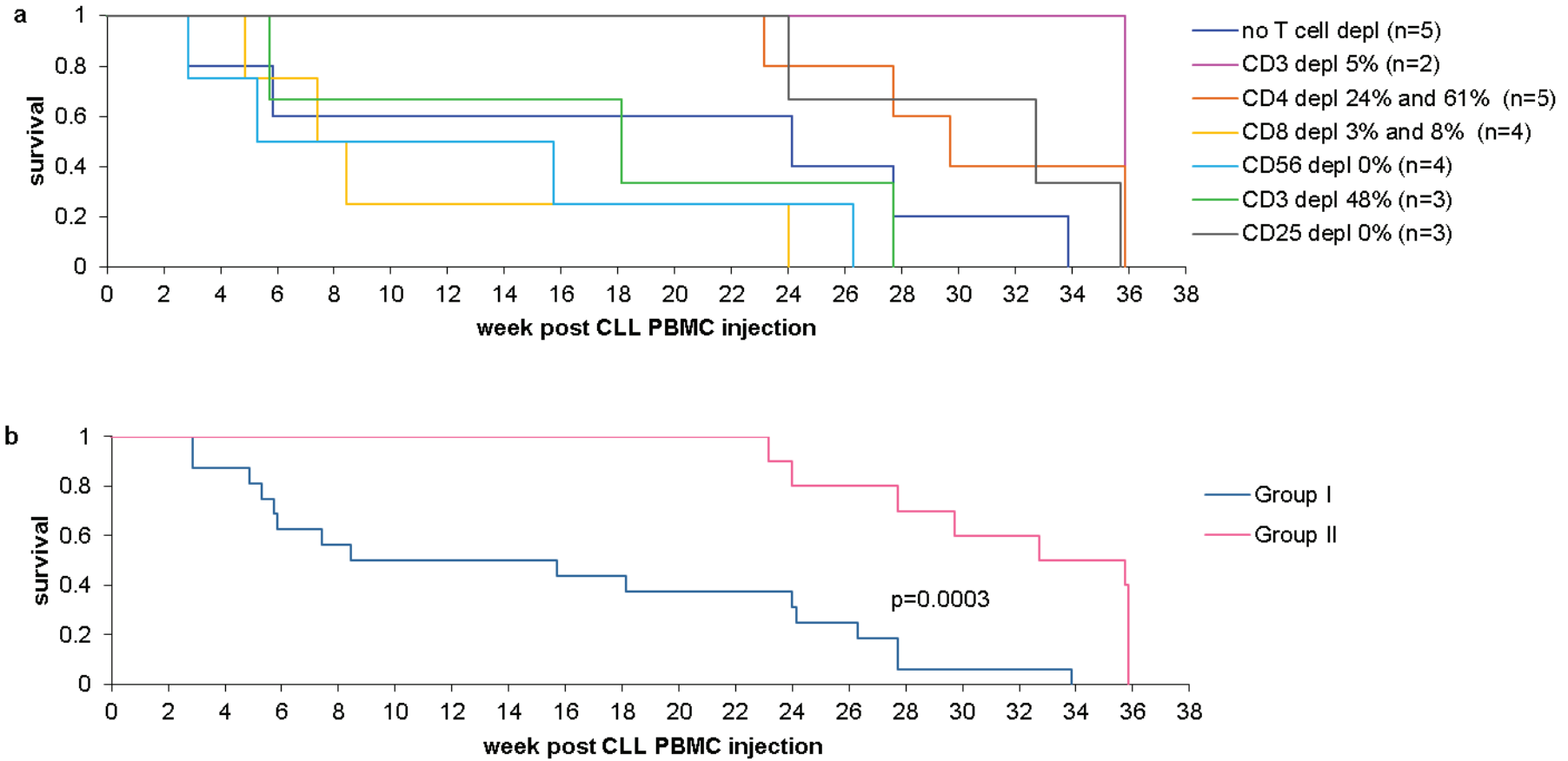

Figure 5.15. Differential survival of NOG mice injected with various T-cell subset depleted PBMCs.

(a) Kaplan-Meier curve depicting a survival advantage conferred upon depletion of specific T-cell subsets prior to CLL PBMC administration.

(b) Pooling of subgroups highlights that mice with depletion of CD3, CD4, CD25 from injected CLL PMBCs (Group I) survived significantly longer than those with non T cell depleted or CD8,CD56 and CD3-48% depleted PBMCs (Group II).

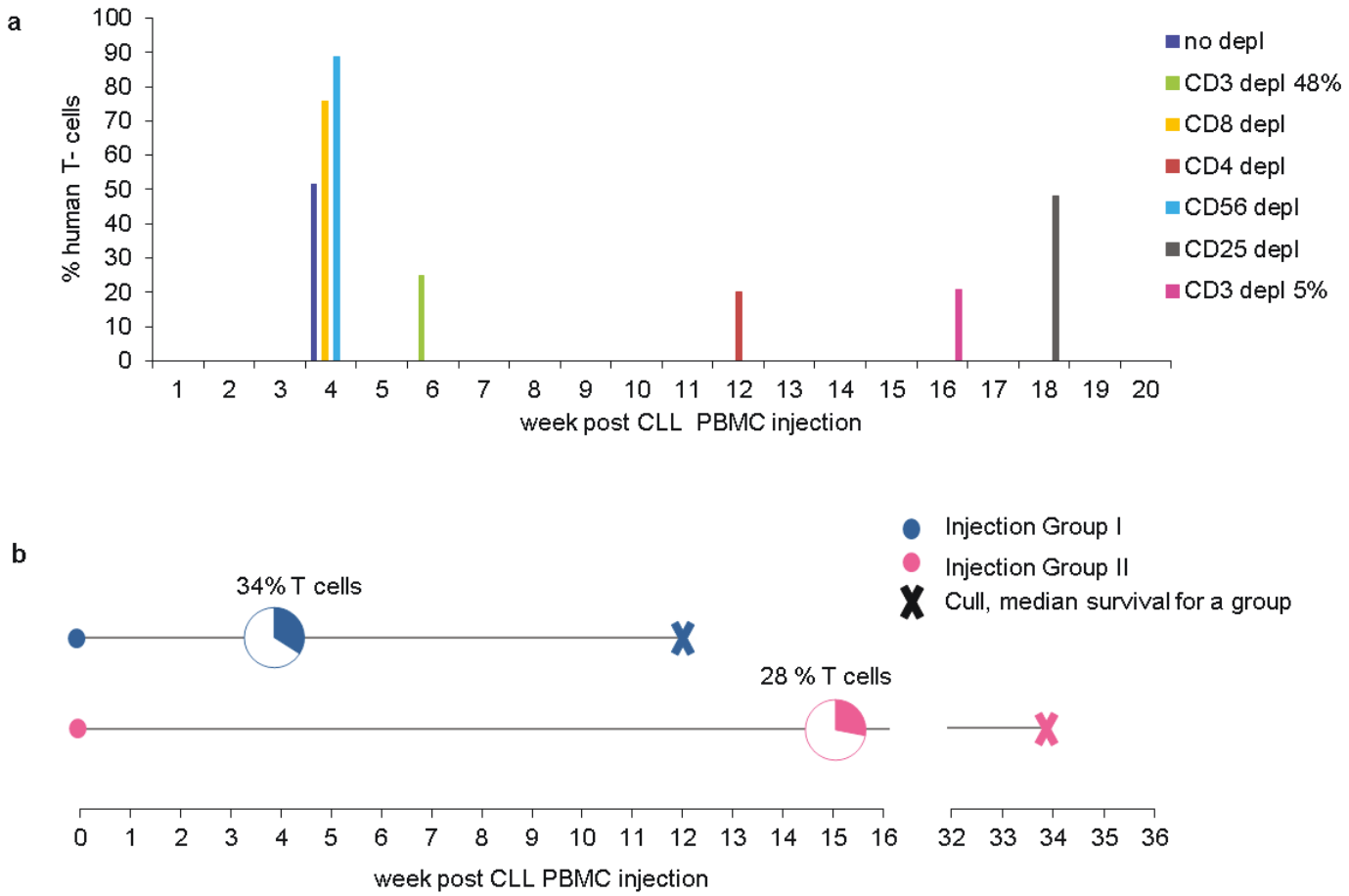

Figure 5.16. Effect of T-cell subset depletion upon the onset of human T cell outgrowth in murine blood.

(**a**) Median time of onset of T cell outgrowth in different T-cell subset depleted subgroups of animals. (**b**) The median time before onset of T cell outgrowth was significantly longer (p=0.008) in mice from Group I (non T-cell depleted PBMC, depleted of CD56, CD8, CD3-48% cells) than in Group II (PBMC depleted of CD4, CD25, CD3 cells). The relative proportion of T-cells at the time of onset did not differ between two groups.

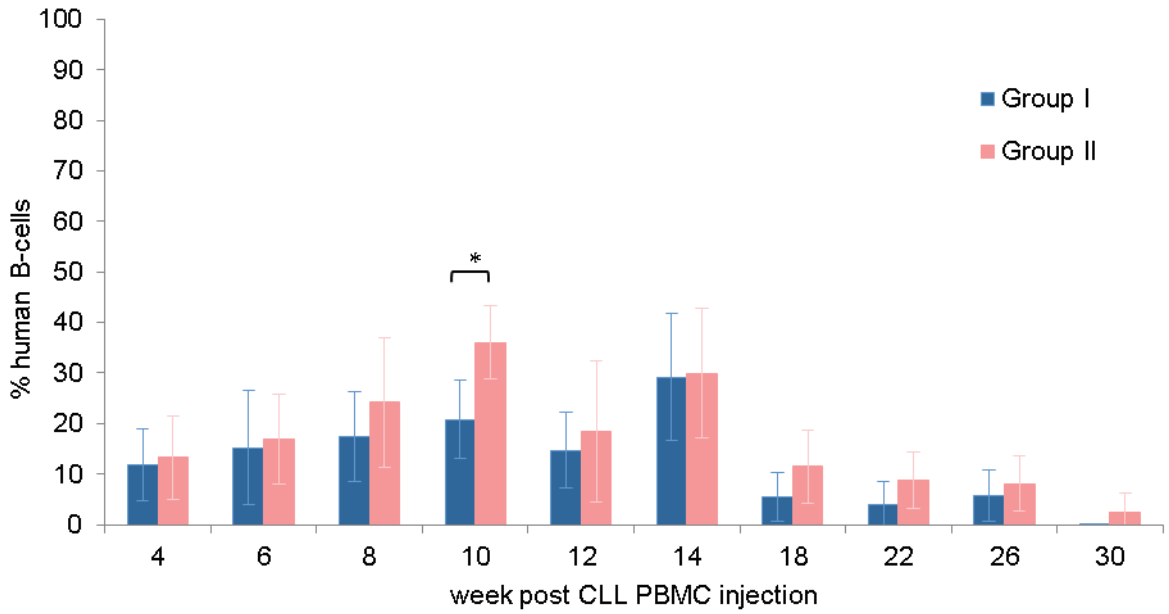

Figure 5.17. Effect of CLL PBMC T-cell subset depletion upon engraftment of human B cells in murine blood.

A significant difference (p=0.015) between the two combined Groups was observed at week 10, when the proportion of circulating B cells was higher in Group I (non T cell depleted PBMC, depleted of CD56, CD8, CD3-48% positive cells) than in Group II (PBMC depleted of CD4, CD25, CD3).

outgrowth but did not abrogate the engraftment of B-cells in murine blood. Therefore, these depletion strategies provided a prolonged CLL engraftment period during which potential therapeutic drug testing could take place. However, they did not prolong CLL cells engraftment beyond of what could be achieved by high depletion of $CD3^+$ cells.

Secondly, the delayed onset of human T-cells outgrowth in CD4 and CD25 depletion sub-groups indicated that $CD4^+$ cells and possibly its subset $CD4^+/CD25^+$ double positive cells might be involved in termination of the graft.

5.6. Microsatellite analysis reveals the origin of human T-cells in murine spleen.

This study is consistent with the findings of Bagnara and colleagues in that, CLL engraftment and death of animals injected with non T-cell depleted PBMC coincided in most cases with outgrowth of human T-cells. This outgrowth was also observed in murine blood and organs in this study, irrespective of T-cell manipulation.

As described earlier, the CLL xenograft protocol required humanization of recipient mice with $CD34^+$ hematopoietic stem cells prior to injection of CLL. This approach has been shown to facilitate the engraftment of CLL cells via providing a supportive human micro-environment. Yet, use of allogeneic $CD34^+$ cells was predicted to result in development of a hematopoietic system which could co-exist with engrafted CLL PBMCs in the murine body for at least a short period of time. Identification of patient cells was initially based on the CFSE signal. However, due to gradual loss of CFSE dye in highly proliferating cells (usually the signal vanishes after 6 cell divisions) and the lack of patient-specific cell markers, accurate identification of CLL derived cells by FACS analysis in murine organs at the time of cull was very problematic.

To investigate the origin of outgrowing T-cells in murine organs (allogeneic or autologous) genomic DNA was extracted from 4 representative murine spleens (mice 4-2, 3-1, 1-4, C4-1) and analysed by microsatellite technique. Three NOG mice had been injected with CLL-4 or -5 PBMCs with their full T-cell complement (mice 4-2, 3-1, 1-4) and one mouse had been engrafted with CD56+ depleted CLL-6 PBMC (Table 5.3).

Table 5.3. Microsatellite analysis of human T-cells engrafted in murine spleen

mouse	CLL PBMC sample injected	Engraftment of human T-cells in spleen (of live cells) %	DNA from sorted or unsorted spleen cells	Result, (patient's DNA) %
4-2	CLL-5 non depleted	66	sorted T-cells only	100
3-1	CLL-5 non depleted	11	sorted T-cells only	16
1-4	CLL-4 non depleted	44	total splenic cells	34
C4-1	CLL-6 CD56+ depleted	82	total splenic cells	100

Histological evaluation of various organs from two of these mice suggested bone marrow insufficiency, which might have caused anaemia, as a cause of death (mice 4-2 and 1-4, communication with pathologist who performed the immunohistochemistry staining).

Mouse 4-2 was injected with non T-cell depleted PBMC, and was culled due to illness at week 8 post CLL cells injection. According to FACS analysis, the spleen was highly infiltrated with human T-cells at the time of death (66% of all live cells). DNA was isolated from isolated human T-cells and microsatellite analysis revealed that 100% of it originated from CLL patient cells (Figure 5.18).

Mouse 3-1 was culled on week 6 post injection. DNA for analysis was extracted from sorted human T-cells which constituted 11% of all live cells. According to microsatellite analysis, 16% of the DNA originated from CLL patient T-cells and 84% from T-cells which had differentiated from CD34+ allogeneic stem cells used for humanization.

Mouse 1-4 was culled on week 5 post CLL injections and DNA was extracted from total, unsorted splenic cells. In this case, microsatellite analysis revealed that 34% of the human DNA from this sample was of patient origin. Human T-cells constituted 44% of all live cells in this spleen.

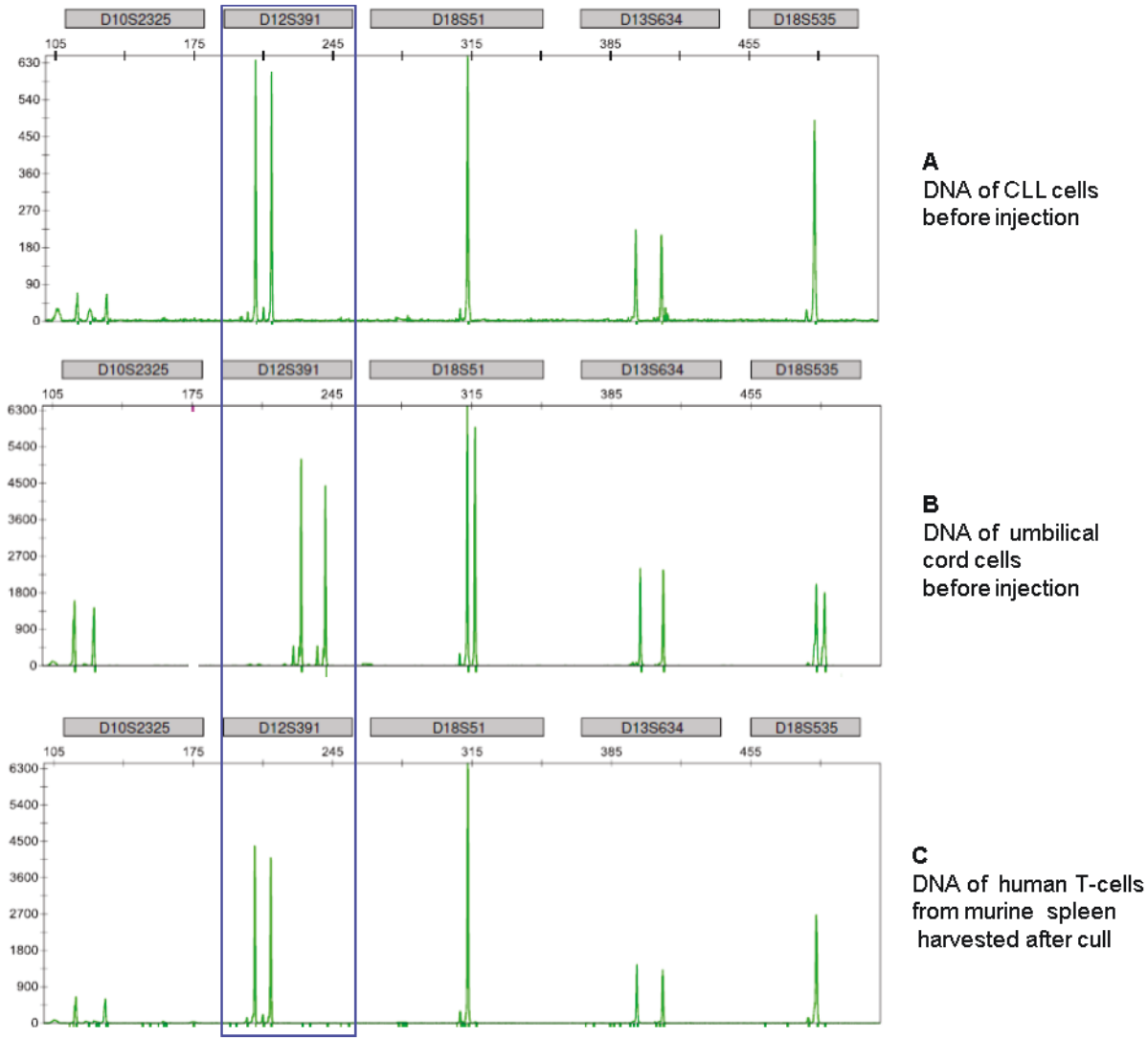

Figure 5.18. Microsatellite analysis of T-cells derived from an infiltrated murine spleen. T-cells which infiltrated the spleen of this particular mouse originated from CLL PBMC as indicated by the identical microsatellite amplification (bordered in blue) in sample A and C

Finally, mouse C4-1 was culled on week 6 post injection when 82% of all live cells in the spleen were human T-cells. DNA extracted from total, unsorted splenic cells, according to microsatellite amplification was all (100%) of patient origin.

The number of samples analysed was small but the results may imply couple conclusions. Firstly, the very high accumulation of human T-cells in murine spleen appears to correlate with a greater proportion of autologous T-cells in this organ. Secondly, injection of two individual human cell populations into NOG mice can result in a 'mixed origin/lineage T-cell chimeric engraftment' which is still present in various proportions at the time of animal cull.

5.7. Discussion

This study aimed to further optimise an existing CLL xenograft model, relying on the previously developed protocol of adoptive transfer of CLL cells into highly immune-deficient recipient mice. The findings of the experiments were expected to contribute towards establishing the optimal conditions for prolonged engraftment of CLL cells and ultimately a reproducible pre-clinical drug testing platform.

The development of an adequate and reproducible CLL xenograft model is of crucial importance considering the fact that the tumour population, which is believed to drive disease progression, resides in proliferation centres in lymph nodes and bone marrow, and usually not accessible to the majority of standard chemotherapeutics. This supporting micro-environment consists of antigen presenting cells and activated T-lymphocytes and is difficult to reconstitute *in vitro.* Culture systems using peripheral blood cells provide only limited support for CLL proliferation and fail to fully recapitulate CLL biology (Dadmarz et al., 1990; Decker et al., 2000; Larson and Yachnin, 1983; Weston et al., 2010).

In this study, the engraftment and growth of *ATM* mutant CLL cells (as shown by FACS analysis and immunohistochemistry staining) were achieved by following the previously established protocol for this CLL xenograft model (Bagnara et al., 2011) confirming its utility in recapitulating the CLL disease *in vivo.*

According to Bagnara's report, humanization of recipients' mice (with $CD34^+$ stem cells or $CD14^+$ monocytes) provides superior stimulation (compared to mice not receiving allogeneic transfer) for autologous T-cells which are an important factor required for CLL growth. However, although activation of T-cells and CLL growth stimulation can be obtained utilizing any of the humanization protocols, in both scenarios CLL engraftment terminates within weeks of CLL transfer due to the outgrowth of these necessary human T-cells (Bagnara et al., 2011).

Accordingly, upon adoptive transfer of an *ATM* mutant CLL tumour, an increased number of T-cells in murine blood was also observed. However, the rise in human T-cells occurred earlier in mice which received allogeneic monocytes than in those receiving umbilical cord blood $CD34^+$ cells. This observation guided the decision to use cord blood-based humanization in later transplantations. However, because it was based on a single experiment, further investigation and comparison of the two systems are currently under study.

The role of activated T-cells in promoting CLL cell growth is documented in studies of proliferation centres in CLL patients (Ghia et al., 2002; Granziero et al., 2001) and in CLL xenograft models (Bagnara et al., 2011; Chen et al., 2012). In Bagnara's report, complete elimination of human T-cells by an anti–CD3 monoclonal antibody in the humanized recipient mice abrogated the CLL proliferation in most cases.

Notably however, it has been reported that engraftment of CLL cells without the support of autologous T-cells is possible (Shimoni et al., 1999; Durig et al., 2007). Contrary to these reports, Bagnara et al, found that CLL proliferation was only detected in mice which also had T-cell expansion. Furthermore, only these authors provided detailed, quantitative analysis of CLL growth kinetics and their findings have been supported by consecutive studies (Chen et al., 2012). Discrepancies amongst these reports may relate to differences in detection techniques, experimental conditions and proliferation capacities of the injected CLL cells.

The results of the T-cell depletion experiment reported here show, that depletion of

autologous T-cells to a minimal level (1.5-5% of original number) prior to transfer into the recipient mice is sufficient for CLL engraftment. The level of CLL cells circulating in murine blood was very similar between animals injected with the same CLL clone, regardless of the level of T-cell depletion. Furthermore, the CLL cells which engrafted in the murine body not only survived but actively proliferated as shown by the gradual loss of CFSE dye from the CLL cell population in blood and organs (the exact numbers of cell division was not assessed in this study). Therefore, our experiments showed that 0.006-0.05x10^6 of autologous T-cells is sufficient to induce the growth of CLL cells in NOG mice.

It seems that the optimal period for drug efficacy assessment is between the initiation of CLL growth and the beginning of expansion of autologous T-cells. The results from this study indicate that this period can be prolonged by profound depletion of autologous T-cells. Although there is variability between CLL samples the outgrowth of T-cells in mice injected with CD3+ depleted PBMC was on average delayed by 4 weeks (Figure 5.11) but might be even longer, up to 11 weeks (Figure 5.16)

Interestingly, the effect of prolonged CLL engraftment and delayed T-cell outgrowth may depend more upon the proportion of T-cells injected rather than on their total numbers, which also varied greatly between different CLL PBMCs (Table 5.2). This could be caused by an inhibiting influence of the co-injected CLL B-cells which make up the dominant and enriched cell population of PBMC. It is feasible that, slowly reconstituting T- cells can escape the control of these CLL B-cells only when they reach sufficient cell number which we propose to be 15% of the total human $CD45^+$ cells in murine blood in this study.

The time of the first appearance of CLL cells in blood can differ between animals injected with different CLL samples. According to report by Chen and colleagues, the engraftment and growth of CLL cells depends on the expression of CXCR4 cell marker (Chen et al., 2012). Cells which highly express CXCR4 can migrate better to tissues to receive survival signals and when injected into mice have better long-term CLL engraftment capacity than cells which have very low level of CXCR4 expression. However, the former have greater ability to activate autologous T-cells. Together, these cell fractions constitute

only 1-10% of CLL clone, and the majority of cells express an intermediate level of CXCR4 (Chen et al., 2012). Although expression of CXCR4 was not assessed in this study, it could be speculated that the observed variances in engraftment kinetics between CLL samples reflected specific homing and proliferation capacity of different CLL clones and their different abilities to respond to microenvironment signals.

The actual level of CLL engraftment in organs in T cell depletion experiment was not assessed until mice succumbed to illness, which in most cases was correlated with expansion of T-cells rather than B cells. Therefore, the analysis of the level and type of the human cells engrafted at the time of culling was not expected to confirm the infiltration of organs with CLL cells but rather to provide the additional information regarding the cause of animal death and engraftment kinetics at this final stage. This explains why there were no significant differences observed between depletion groups in respect to relative proportion of B and T-cells in organs. Nevertheless, the histo-pathological analysis of 3 randomly selected mice revealed that the depletion of T-cells could in some cases lead to a high level of CLL B-cell engraftment in the spleen, possibly contributing to animal death. Further insight into the engraftment kinetics within organs could be obtained through a sequential cull experiment when multiple animals are injected with the same CLL sample and sacrificed at regular intervals.

Since the xenograft model utilized required the injection of the cell population from two different individuals which were both expected to expand, the origin of outgrowing T-cells was investigated by microsatellite amplification. This analysis confirmed that in two of four analysed murine spleens outgrowing T-cells were of patient origin. In another two, however, the engraftment at the final stage, represented a chimeric population of autologous (CLL patient) and allogeneic (healthy individual) T-cells. This chimeric engraftment was not reported in the previous xenograft model (Bagnara et al., 2011). We were also unable to confirm autologous (GvHD) as the cause of animal death, although the symptoms of illness in the majority of culled mice were the same as described by other authors. Apart from murine spleen no other organs such as liver, brain, skin or kidney were infiltrated with T-cells.

Observed discrepancies between the two studies may be the result of the subtle differences between two murine strains, NSG and NOG.

In order to investigate whether there is any particular T-cell subset which hinders prolonged engraftment of CLL cells, the complete or partial depletion of major T-cell subsets from two CLL PBMCs prior the transfer into animals was performed, followed by the analysis of the engraftment kinetics and mice survival. Identification of such a subset could be important for a couple of reasons. Firstly, it would allow the establishment of a depletion procedure to prolong the engraftment window of CLL B-cells. Secondly, it could potentially indicate the target for immunotherapeutic intervention in human CLL. Importantly, the T-cell population from CLL patients is characterized by compromised anti-tumour immune responses, abnormal levels of various subsets and altered expression of genes involved in cell differentiation and cytoskeletal formation when compared to age-matched healthy donors T-cells (Cerutti et al., 2001; D'Arena et al., 2011; Görgün et al., 2005; Riches et al., 2010). Hence, not surprisingly, there is extensive research currently being undertaken, aiming to further understand T-cell defects in CLL and to utilize this knowledge to restore normal T-cell function in order to suppress the expansion of tumour cells. Our xenograft model could potentially aid this research direction.

In this study we have observed that partial depletion of $CD4^+$ T-cells (down to 24% and 61% of the original number) seemed to temporarily supress T-cell expansion in murine blood. A similar result was observed in animals injected with CLL PBMC which were completely depleted of CD25 positive cells. On the contrary, depletion of $CD8^+$ or $CD56^+$ cells resulted in early T-cell outgrowth and animal illness, the outcome which was very similar to the non T-cell depleted group.

These results confirmed and complemented a previous study which has shown that complete elimination of $CD4^+$ cells by injection of anti-CD4 antibody, at the time of CLL PBMC transfer hinders CLL proliferation in murine blood, whereas depletion of $CD8^+$ cells does not have this effect (Bagnara et al., 2011).

Notably, depletion of $CD4^+$ or $CD25^+$ cells subsets did not delay the onset of T-cells

outgrowth nor prolong the engraftment of CLL cells beyond that observed with profound CD3 depletion (5% of the original number). This could be partially explained by the fact that $CD4^+$ cells comprise the majority of the CD3 population and therefore depletion of $CD3^+$ cells results mostly in a reduction of $CD4^+$ cells in the two CLL PBMCs employed in these studies.

Cell markers CD4 and CD25 are normally co-expressed on activated T-cells and on regulatory T-cells. Partial depletion of $CD4^+$ or $CD25^+$ cells could have affected both of those fractions. However, it has been previously suggested that expanding T-cell population in CLL xenograft model constitute of activated T-cells (Bagnara et al., 2011).

It is therefore possible, that in our study decreased fraction of activated T-cells (due to depletion) was still sufficient in number to stimulate the growth of CLL cells (as this growth was observed in this study) but took longer to expand.

It is not clear what was the exact role of regulatory T-cells in this model and whether/how their depletion affected the engraftment kinetics. Notably, T-regulatory cells are in general involved in suppression of T-cell immune responses (Fehervari and Sakaguchi, 2004) and elevated numbers of T-regulatory cells have been reported in CLL, particularly in patients with advanced disease (D'Arena et al., 2011; Piper et al., 2011). Depletion experiment in which pure fractions of $CD4^+/CD25^+/FoxP3^+$ T-regulatory cells is removed from PBMC prior injection could possibly help to answer this question.

Importantly, CD25 is also expressed on activated B cells including B-CLL (Damle et al., 2002). Although depletion method utilised in this study did not discriminate between B and T-cells, a high number of CLL cells were recovered after CD25 magnetic sorting indicating that the B-cell fraction was not hugely affected.

One of the interesting observations in this study was the reappearance of leukemic cells in mice blood stream every few weeks. In some cases the reappearing cells had the same CFSE intensity as prior to injection. In others, CFSE was lower than at the time of transfer. There are a few possible scenarios to explain this phenomenon. In the former situation, these cells might have survived somewhere in tissues without significant cell division. Peritoneum cavities could be a possible residing sites for those cells since

accumulation of high CFSE intensity CLL cells was detected in this organ at the time of animal cull. In the latter situation, released CLL cells might have undergone several rounds of division in infiltrated organs and entered the blood as resting lymphocytes. This would recapitulate one of the main characteristics of CLL and should correlate with the changing expression of cell markers such as CD38, CD5 and CXCR4 as observed in CLL patients (Calissano et al., 2009). Unfortunately, co-expression of these cell markers was not investigated in this study. An alternative scenario suggests that there is a coexistence of both of the cell fractions: one resides in tissues and does not proliferate and another one, extensively dividing and circulating between blood and lymphoid organs such as spleen. Finally, it is also possible that the diminished CFSE intensity could be caused by spontaneous loss of fluorescence in prolonged 'incubation' time in murine tissues.

In summary, this study confirmed that the current xenograft CLL model can be used to successfully engraft and induce the growth of primary CLL cells including clones with *ATM* defects. In addition, partial depletion of $CD3^+$, $CD4^+$ or $CD25^+$ cells from patient PMBC prior the transfer into mice can delay the onset of T-cell outgrowth and prolong the engraftment of CLL cells hence, allowing a sufficient period of time required for testing novel therapeutic agents.

The assessment of the treatment efficacy could be monitored in murine blood and spleen from engrafted animals. The spleen seems to provide the most sufficient support for CLL growth but further studies are needed to correlate engraftment levels between the two organs and to determine the optimal time to harvest.

Despite initial corroboration via histology of Bagnara's results that the resultant engrafted cells are derived from the patient CLL tumour cells and not the humanization, we have since discovered by microsatellite analysis that this is not always the case. Thus, a limiting factor in the current xenograft model is 'mixed human cell chimerism'. The discovery of co-engraftment of autologous and allogeneic T-cells in murine spleen suggests that a similar situation may exist in respect to the B-cell population. Notably, B-cells which

differentiate from umbilical cord $CD34^+$ cells have a similar cell phenotype to CLL cells, they are $CD5^+$ and $CD19^+$ positive (Matsumura et al., 2003). CFSE dye can be used for detection of B-CLL cells at early stages of engraftment, but it is no longer present in cells which undergo extensive cell division. Therefore, current investigations are aiming to evaluate whether the CLL specific ROR1 cell marker (Baskar et al., 2008) and/or HLA phenotyping could be used to discriminate between allogeneic and autologous B-cells in this xenograft model. An alternative approach is to utilise the monocytes as a humanization method or establish a condition for CLL activation enabling the omission of humanization altogether. Nevertheless, the presence of a healthy human hematopoietic system in this CLL xenograft model comes as advantage whilst assessing the toxic effect of novel therapeutic agents not only on tumour but also on normal human cells.

CHAPTER VI

FINAL CONCLUSIONS AND FUTURE PERSPECTIVE

The aims of my project were to establish the role of *ATM* germ-line mutation in the development of CLL, investigate the clinical consequences of mono- and bi-allelic inactivation of *ATM* in the context of randomized clinical trial and to investigate the strategies of overcoming the limitations of the existing CLL xenograft model. I have examined the nature, germ-line origin and prevalence of *ATM* mutations in the relevant CLL cohorts and verified the conditions facilitating prolonged engraftment of CLL cells in murine microenvironment. I would like to summarize the obtained results considering the role of *ATM* gene alterations in the initiation and progression of CLL. Furthermore I would like to discuss the future perspective regarding prognostic value of *ATM* genomic alterations and further development and applications of CLL xenograft model.

6.1. *ATM* germ-line mutations do not contribute to initiation of CLL but may play a role in clonal evolution and disease progression.

Alteration of the *ATM* gene is a frequent event in CLL. *ATM* mutations affect approximately 12% of patients from unselected cohorts (Austen et al., 2005) and are even three times more frequent among tumours with 11q deletion which already harbour the loss of one *ATM* allele (Austen et al., 2007; Navrkalova et al., 2013). Although most of the *ATM* mutations are acquired by the tumour clone, a small proportion is present in the germ-line which has been suggestive of the role of these abnormalities in the development of CLL (Bullrich et al., 1999; Stankovic et al., 2002b).

In Chapter 3 of this thesis, I have studied the frequencies of *ATM* pathogenic germ-line mutations in the cohorts of 318 CLL patients and 281 controls. The prevalence of *ATM* pathogenic germ-line mutations in our CLL cohort was 2.5%. The comparison to the control cohort revealed that this frequency was significantly higher than in general population (2.5% versus 0%, p=0.008). The difference remained significant when only subgroup of patients with chromosome 11q deletion was compared to control cohort (4% versus 0%, p=0.001). However, the prevalence of *ATM* germ-line pathogenic mutations in the subgroup of patients without this chromosome abnormality (1%) was not significantly different when compared to

control population (p=0.15). Therefore, the conclusion from this analysis was that *ATM* mutation heterozygote status does not predispose to CLL in general but it might influence clonal evolution by contributing to acquisition of chromosome 11q deletion and hence loss of another *ATM* allele.

This conclusion was supported by the fact that patients with *ATM* germ-line mutations were not diagnosed at earlier age than patients with non germ-line *ATM* mutations or wild-type *ATM* gene. Furthermore, tumours of *ATM* mutation carriers exhibited normal *IGHV* usage and isotype switch regions indicating that even if defects in those processes contributed to CLL development, mono-allelic *ATM* inactivation would not have any significant altering influence. Importantly however, most of CLL carrying germ-line *ATM* mutations were diagnosed with advanced stage of disease and had unmutated *IGHV* gene which itself is an unfavourable prognostic marker.

Taken together, the initiation of CLL is independent on the presence of *ATM* germ-line mutation, however clones with this abnormality appear to be subject to selective pressure leading to bi-allelic *ATM* inactivation, complete loss of ATM protein function and ultimately to the disease progression. This is a reasonable explanation considering the important role of ATM protein in DNA damage responses resulting in induction of p53-dependent apoptotic pathway and protecting genomic stability.

6.2. Bi-allelic *ATM* inactivation significantly reduces overall responses and the survival in patients treated on the UK LRF CLL4 trial.

The important role of *ATM* bi-allelic inactivation in pathology of CLL was demonstrated in Chapter 4 of this thesis, in the analysis of the outcome among patients enrolled on phase 3 randomized UK CLL trial (UK LRF CLL4). Patients from this trial consisted of a homogeneous population exhibiting progressive, previously untreated disease (Catovsky et al., 2007). The offered treatment included standard chemotherapeutic regimens such as Chlorambucil, Fludarabine or Fludarabine plus Cyclophosphamide (FC). The analysis in this study was limited to 224 patients with known 11q deletion status. The

screening of the genomic sequence of *ATM* revealed the presence of *ATM* mutations in 16% of the tumours from this cohort. To assess the impact of *ATM* aberrations on responses to treatment and on PFS and OS, the cohort was divided into 4 hierarchical sub-groups including wild-type *ATM* tumours (62.5% of the cohort), tumours with *ATM* mutation alone (7.6%), tumours with 11q deletion alone (22.8%) and tumours with *ATM* mutation and 11q deletion which constituted 7.1% of the studied cohort.

When compared to *ATM* wild-type subgroup, patients with bi-allelic *ATM* inactivation exhibited significantly worse overall response to treatment ($p=0.002$) which was particularly pronounced in the Chlorambucil treatment arm ($p=0.01$); shorter PFS ($p<0.001$) and OS ($p=0.002$). Moreover, when additional, hierarchical model including also *TP53* defects was analysed, patients with bi-allelic *ATM* alterations had equally poor PFS as those with mono- and bi-allelic *TP53* abnormalities ($p=0.3$ and $p=0.07$, respectively), and only patients with bi-allelic *ATM* or bi-allelic *TP53* abnormalities had inferior outcome below that observed in cases with 11q deletion alone ($p=0.001$ and $p<0.001$ respectively) (all p-values provided here are from univariate analysis).

6.3. The evaluation of the impact of bi-allelic *ATM* inactivation under the alternative treatment strategies.

TP53 gene alteration is a well-recognized poor prognostic marker associated with chemo-refractoriness and short survival, and patients with defects in this gene are offered alternative treatment options (Oscier et al., 2012). Accordingly, the newly identified subpopulation of CLL patients with bi-allelic *ATM* inactivation and comparable bad outcome may also require re-consideration of the treatment strategies.

ATM and TP53 proteins act in the overlapping pathway but their cellular functions are not identical. Patients with *TP53* defects seem not to benefit from the addition of Rituximab into FC regimen (Zenz et al., 2010) whereas this treatment benefits patients with the loss of one *ATM* allele in a form of chromosome 11q deletion (Hallek et al., 2010). Also treatment with lenalidomide has been shown to induce responses in patients with 11q deletion

(Ferrajoli et al., 2008). Nevertheless, it remains unknown if the clinical outcome of patients with bi-allelic *ATM* inactivation can be improved with these treatments and the evaluation of the clinical outcome of this sub-population should be a subject of the future studies. Ideally the assessment should be carried on the samples from the relevant clinical trials.

Alternative options may include the therapies which have shown some efficacy in *TP53* mutation/deletion tumours such as immunotherapy with glucocorticoids, treatment with Flavopiridol (an inhibitor of cyclin-dependent kinases) or with Bcl-2 inhibitor (Lin et al., 2009; Pettitt et al., 2012; Roberts et al., 2012a). Also promising are BCR signalling inhibitors which are currently undergoing evaluation in the CLL trials (Advani et al., 2013; Byrd et al., 2012; Friedberg et al., 2010; O'Brien et al., 2011).

6.4. *ATM* bi-allelic defect as therapeutic target.

An alternative treatment options for patients with bi-allelic *ATM* defects can include synthetic lethality approach. Loss of ATM function results in defective DNA damage repair which can be partially compensated by other cooperative proteins. Therefore potentially, the inhibition of redundant compensating pathways may result in the collapse of the tumour's escape mechanisms and cellular death.

One such approach involves PARP inhibition. ATM null CLL cells exhibit sensitivity to PARP inhibition both *in vitro* and in a xenograft model (Weston et al., 2010). A phase I/II trial is currently on-going to assess the efficacy of this approach in CLL patients with refractory tumours and ATM functional loss. Other DNA repair proteins which could be targeted in ATM null cells include DNA-PK, ATR and Fanconi anemia (FA) complex. The evaluation of the efficacy of these approaches in ATM null CLL still remains the subject of future studies, both *in vitro* and *in vivo*.

6.5. The need for standardization of methods to measure ATM inactivation in CLL.

Detection of functionally relevant *ATM* sequence changes has not been a straightforward task due to the large size of the *ATM* gene and the requirement of a sufficient

clone size. These obstacles can be overcome by the New Generation Sequencing (NGS) approach. However, the absence of the stringent criteria for determining the functional significance of *ATM* mutations might still result in misinterpretation of the novel polymorphisms as pathogenic changes and potentially mask the impact of deleterious *ATM* mutations in clinical studies. Nevertheless, the identification of the poor prognosis subgroup of patients with bi-allelic *ATM* defects which might constitute around 7% of CLL cohort imposes the need for the development of standardized methods to measure ATM inactivation.

Several groups have attempted to develop a functional test which would surrogate mutational screening and could be clinically applicable. These include Western Blotting and FACS assay to test IR-induced up-regulation of ATM/p53 dependent targets (p53 and p21) (Carter et al., 2006; Carter et al., 2004; Mohr et al., 2011; Pettitt et al., 2001), multiplex PCR assay to test DNA damage induced up-regulation of selected number of ATM/p53 target genes (Te Raa et al., 2013) and cytotoxicity assays to measure damage induced apoptosis *in vitro* (Best et al., 2008; Navrkalova et al., 2013).

It is important to note that both cytotoxicity assays and responses of ATM dependent target genes can be affected by mechanisms other than ATM and p53 functional loss (Johnson et al., 2009). Therefore, an alternative approach is to directly measure activation of ATM and its direct targets. This approach involves quantification of IR-induced ATM dependent phosphorylation of ATM, p53, SMC1, Nbs1 and Kap1 (Austen et al., 2007). However, it is crucial to measure the phosphorylation within the first hour of DNA damage induction as many ATM targets can be activated by alternative kinases at later time points. Also important is evaluation of more than one ATM target because of the high complexity of the activation mechanisms involving various posttranslational modifications which could be cell or signal type specific (Bhatti et al., 2011; Kozlov et al., 2011). Assessing ATM activity on the basis of a single ATM target activation might be misleading also due to unpredicted functional consequences of ATM mutant proteins which for example, may retain their normal downstream signalling even in the absence of wild-type auto-phosphorylation site as

observed in mutant ATM mice models (Pellegrini et al., 2006).

Importantly, variations in the size of ATM null clone can greatly affect ability of any functional test to recognize the clone with ATM functional loss. Overall, it seems that at this stage of research combination of mutation analysis and a functional test might be the most reliable approach to address functional ATM status.

6.6. The role of mono-allelic loss or mutation of *ATM* gene in progression of CLL.

The clinical and cellular consequences of mono-allelic defects of *ATM* gene are less unified than bi-allelic inactivation. In the cohort of CLL4 trial patients described in Chapter 4 of this thesis the presence of mono-allelic *ATM* mutation did not predict for inferior treatment responses or shorter survival whereas patients with chromosome 11q deletion exhibited significantly worse response to Chlorambucil (p=0.047), shorter PFS (p=0.007) and shorter OS (p=0.001) when compared to *ATM* wild-type group.

Activation of ATM protein as measured in *in vitro* assays also differs between the two groups of mono-allelic *ATM* alterations. Tumours with a single mutation and the presence of a wild-type allele show reduced ATM activity which might indicate potential dominant-negative effect of at least a proportion of the mutations (Austen et al., 2005; Navrkalova et al., 2013; Stankovic et al., 2002b) On contrary, tumours with 11q deletion and remaining wild-type allele have intact ATM signalling indicating that one *ATM* allele is sufficient for normal enzymatic activity (Austen et al., 2005; Austen et al., 2007; Navrkalova et al., 2013).

Overall, finings from current and previous studies indicate that *ATM* gene might be a key target of chromosome 11q deletion. However, the progressive clinical phenotype of tumours with this chromosomal aberration and without *ATM* mutation could be explained by combined haploinsufficiency of numbers of genes which are usually affected by this deletion (Gunnarsson et al., 2011; Malek, 2012). These genes can be implicated in DNA damage responses and in other CLL-relevant cellular pathways (Guarini et al., 2012; Ouillette et al., 2010)

Interestingly, at the time of diagnosis both *ATM* mutation and 11q deletion can be

independent of each other associated with poor clinical features such as advanced stage of disease (both *ATM* mutation and 11q deletion), involvement of multiple tissue sites or lymphadenopathy (*ATM* mutations and 11q deletion respectively), and unmutated *IGHV* status (11q deletion) (Dohner et al., 1997; Krober et al., 2002; Oscier et al., 2002; Trbusek et al., 2006). These associations indicate the negative influence of mono-allelic *ATM* defects at the early stage of disease.

Moreover, regardless of the lack of impact of mono-allelic *ATM* mutations on treatment responses and survival after treatment (which could result from the small sub-clone size containing mutated ATM protein and/or residual ATM activity of certain types of *ATM* mutations) their presence may impose a selective pressure leading to complete inactivation of ATM protein through loss or mutation of another allele and to aggressive clinical phenotype. This model of step-wise loss of tumour suppressor functions is supported by significantly shorter time to first treatment among patients with mono-allelic *ATM* mutations (Guarini et al., 2012); the significantly increased prevalence of pathogenic germ-line mutations and somatic *ATM* mutations in patients with chromosome 11q deletion (as described in Chapter 3 and Chapter 4 in this thesis); and finally by very poor outcome after therapy among patients who have bi-allelic inactivation of *ATM* gene as shown in Chapter 4 of this study.

6.7. Partial depletion of autologous T-cells can prolong the engraftment of CLL cells in humanized murine xenograft model.

The development of novel therapeutics facilitated by the improved understanding of biological background of progressive and resistant tumours (which has been facilitated by FISH and whole genome sequencing applications) will lead ultimately to more efficient and personalized treatment approaches.

The assessment of the efficacy and toxicity of these new drugs could be greatly aided by well optimized and reproducible CLL xenograft models. The advantages of primary CLL xenografts over transgenic mice models, or cell lines xenografts, include the opportunity to

recapitulate the biology of disease of particular subpopulations of patients and to follow the responses of different sub-clones from the same tumour under various treatment regimens.

In the Chapter 5 in this thesis I have described the results of the primary CLL xenograft model optimization and shown that prolonged engraftment of CLL cells in highly immunosuppressed mice could be achieved by partial depletion of patients' $CD3^+$, $CD4^+$, or $CD25^+$ cells prior the injection into adoptive animals. The depleted cell population most probably represents activated T-cell fraction ($CD3^+/CD4^+/CD25^+$ cells) which has been already implicated in enabling CLL cells engraftment and proliferation but also in terminating the graft (Bagnara et al., 2011). Despite the limitations of 'mixed human cell chimerism' associated with described xenograft, this procedure is an important step forward towards establishing reproducible, long-lasting engraftment of CLL cells to enable testing of the new therapeutic agents for the expanded period of time.

The application of a different primary CLL xenograft model as a drug testing tool has been recently investigated by Hermann and colleagues (Herman et al., 2013). The authors used NSG strain and limited their study to four weeks period during which they measured the CLL proliferation and activation of BCR and NFκB pathways in CLL cells engrafted in murine blood and organs. During this time the animals were treated with Ibrutinib (Btk inhibitor) to test whether utilized xenograft model can recapitulate the effects of the human microenvironment. The xenograft protocol did not involve the provision of humanization or pre-activation of autologous T-cells before the transfer of CLL PBMC to NSG mice. Despite this, authors detected proliferation of CLL cells in murine blood and spleen and observed inhibition of BCR and NFκB pathways by Ibrutinib proving that even simplified xenografting protocol provides a powerful tool for drug testing. It also indicated that different therapeutic strategies may require different xenografting protocols since 4 weeks period during which CLL cell count gradually decreases might not be sufficient for adequate evaluation of all novel treatments.

6.8. Understanding the role of autologous T-cells by studying their behaviour in CLL xenograft model.

The interesting observation from Chapter 5 of this thesis and from previous studies (Bagnara et al., 2011; Herman et al., 2013) was that co-injected autologous T–cells can unleash their proliferative capabilities once transferred into highly immunosuppressive mice. Normally, autologous T-cells in CLL patients are under inhibiting influence or control of tumour CLL cells and/or CLL microenvironment and their anti-tumour functions are compromised (Riches et al., 2010). Therefore, the observation of T-cells behaviour in CLL xenograft studies further confirms the important role of microenvironment in the pathology of CLL disease.

The detailed characterization of these T-cells is currently under scrutiny by our group. These will include the identification of the TCR repertoire to identify the possible antigens which drive T-cell proliferation and the in vitro assessment of cytotoxic capabilities of the T-cells (harvested from the last stage of engraftment) against CLL cells. Those assays might include measurement of the cytokines release such as interferon-gamma and the expression of certain cell markers indicating activation. These investigations might aid the identification of the mechanisms which hinder anti-tumour T-cells activities in CLL patients and in longer term help to restore normal T-cell functions in CLL.

Nevertheless, it is equally possible that the outgrowth of T-cells and disappearance of CLL cells (which initially proliferate and expand) is caused by the different growth capacities of these two cell populations rather than the direct cytotoxic activities. This is supported by the lack of strong evidence of GvH disease in our xenograft model. In addition, transferred CLL cells had mature cells phenotype and the existence of leukemic stem cells encompassing great proliferation capacities is still not confirmed in this disease although one study reported recently that self-renewing hematopoietic stem cells might be involved in pathogenesis of CLL (Kikushige et al., 2011). The future investigations using an early population of CD34+ hematopoietic progenitors in xenograft models obtained from the range of CLL patients will possible shed light on these unresolved questions.

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ATM germline heterozygosity does not play a role in chronic lymphocytic leukemia initiation but influences rapid disease progression through loss of the remaining *ATM* allele

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ABSTRACT

Ataxia telangiectasia patients, with constitutional bi-allelic *ATM* mutations, have a marked risk of lymphoid tumors and *ATM* mutation carriers have a smaller risk of cancer. Sporadic *ATM* mutations occur in 10-20% of chronic lymphocytic leukemia and are often associated with chromosome 11q deletions which cause loss of an *ATM* allele. The role of constitutional *ATM* mutations in the pathogenesis of chronic lymphocytic leukemia is unknown. Here we investigated the frequency of constitutional *ATM* mutations in either of two chronic lymphocytic leukemia cohorts, those with and without a chromosome 11q deletion. We found that in comparison to controls, constitutional pathogenic *ATM* mutations were increased in patients with chromosome 11q deletions (6 of 140 *vs.* 0 of 281, P=0.001) but not in those without 11q deletions (2 of 178 *vs.* 0 of 281, P=0.15). These results suggest that *ATM* germline heterozygosity does not play a role in chronic lymphocytic leukemia initiation but rather influences rapid disease progression through *ATM* loss.

Key words: sickle cell disease, nephropathy, hemolysis, kidney.

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Introduction

Ataxia telangiectasia (AT) results from bi-allelic constitutional mutations in the *Ataxia Telangiectasia Mutated* (*ATM*) gene on chromosome 11q23. AT is characterized by a 200-fold increased risk of lymphoid tumors[1] and carriers are also at an increased risk of cancer development albeit at a much lower level.[2] Sporadic *ATM* mutations have been detected in tumor cells in patients with various lymphoid malignancies including B-cell chronic lymphocytic leukemia (CLL).[3-5] The ATM protein signals cellular responses to DNA damage in the form of DNA double strand breaks (DSBs) and prevents accumulation of potentially tumorigenic cells with unrepaired DNA DSBs.[1]

The diagnosis of CLL requires a B-cell clone of more than 5×10^9 cells/L of blood with characteristic cell surface markers. Individuals with a CLL clone of less than 5×10^9/L and no additional pathological features are classified as having the pre-malignant condition monoclonal B-cell lymphocytosis (MBL).[6,7] CLL shows marked clinical heterogeneity and prognostic markers associated with a poor outcome include deletions of chromosomes 17p and 11q, leading to loss of *TP53* and *ATM* alleles, respectively.[8] Mutations in *ATM* are linked to poor prognosis and are commonly, but not exclusively, associated with a chromosome 11q23 deletion. We found that 36% of CLLs with an 11q deletion carry a mutation in the remaining *ATM* allele resulting in bi-allelic *ATM* defects.[3,4]

Instances of familial occurrence are recognized in CLL[9] and registry studies confirm that first degree relatives of CLL patients have a 7-fold increased risk of developing CLL.[10] Relatives are also at an increased rate of MBL.[11] Within CLL pedigrees there are no consistent patterns of leukemic development suggesting involvement of multiple low risk alleles rather than a single high-risk locus.[9] Recent genome wide association studies provide evidence for low penetrance risk alleles that together may increase an individual's risk of developing CLL.[12,13]

ATM mutations occur at different stages of CLL development and in occasional cases an *ATM* mutation is present in a patient's germline.[3,4,14] In one previous study, *ATM* mutations were not found to be responsible for CLL familial clustering;[15] however, the role of constitutional pathogenic *ATM* muta-

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Biallelic *ATM* Inactivation Significantly Reduces Survival in Patients Treated on the United Kingdom Leukemia Research Fund Chronic Lymphocytic Leukemia 4 Trial

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ABSTRACT

Purpose
The prognostic significance of *ATM* mutations in chronic lymphocytic leukemia (CLL) is unclear. We assessed their impact in the context of a prospective randomized trial.

Patients and Methods
We analyzed the *ATM* gene in 224 patients treated on the Leukemia Research Fund Chronic Lymphocytic Leukemia 4 (LRF-CLL4) trial with chlorambucil or fludarabine with and without cyclophosphamide. *ATM* status was analyzed by denaturing high-performance liquid chromatography and was related to treatment response, survival, and the impact of *TP53* alterations for the same patient cohort.

Results
We identified 36 *ATM* mutations in 33 tumors, 16 with and 17 without 11q deletion. Mutations were associated with advanced disease stage and involvement of multiple lymphoid sites. Patients with both *ATM* mutation and 11q deletion showed significantly reduced progression-free survival (median, 7.4 months) compared with those with *ATM* wild type (28.6 months), 11q deletion alone (17.1 months), or *ATM* mutation alone (30.8 months), but survival was similar to that in patients with monoallelic (6.7 months) or biallelic (3.4 months) *TP53* alterations. This effect was independent of treatment, immunoglobulin heavy chain variable gene (*IGHV*) status, age, sex, or disease stage. Overall survival for patients with biallelic *ATM* alterations was also significantly reduced compared with those with *ATM* wild type or *ATM* mutation alone (median, 42.2 *v* 85.5 *v* 77.6 months, respectively).

Conclusion
The combination of 11q deletion and *ATM* mutation in CLL is associated with significantly shorter progression-free and overall survival following first-line treatment with alkylating agents and purine analogs. Assessment of *ATM* mutation status in patients with 11q deletion may influence the choice of subsequent therapy.

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INTRODUCTION

The clinical course of chronic lymphocytic leukemia (CLL) is extremely variable, ranging from an indolent disease to a rapidly progressive leukemia that is resistant to therapy with standard DNA-damaging agents. Several features of CLL tumor cells influence clinical outcome, including the functional integrity of the DNA damage response (DDR) genes *ATM* and *TP53*.[1-8]

ATM and p53 function may be impaired either through physical deletion of the genes on chromosome 11q and 17p, respectively, or the development of inactivating mutations. *TP53* gene alterations are observed in 3% to 8% patients at diagnosis or during first-line treatment and in up to 30% of patients with refractory CLL.[5] Several recent studies[6-8] confirmed that *TP53* gene alterations represent the single most important parameter of CLL chemoresistance.

ATM is a protein kinase that, following induction of DNA double-strand breaks, synchronizes cellular responses that include DNA repair, activation of cell cycle checkpoints, and induction of apoptosis.[9] The activation of p53 by ATM leads to transcriptional activation of proapoptotic genes and elimination of cells with excessive DNA damage. The inherited inactivation of *ATM* causes the ataxia telangiectasia (AT) syndrome characterized by frequent lymphoid tumors.[9]