THE ROLE OF TELOMERE DYSFUNCTION IN MODULATING DISEASE PROGRESSION AND RESPONSE TO TREATMENT IN CHRONIC LYMPHOCYTIC LEUKAEMIA

Ceri Hopcyn Jones

A thesis submitted for the degree of Doctor of Philosophy

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Institute of Cancer and Genetics
School of Medicine
Cardiff University

Funded by Bloodwise and Cardiff University
Cancer begins and ends with people.
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Finally, to all the CLL patients who donated their samples – without you none of this would have been possible.
SUMMARY

In Chronic lymphocytic leukaemia (CLL), short telomere length (TL) and dysfunction and is a powerful predictor of patient survival. The aim of this project was to understand the dynamics of TL in CLL and the relationship between TL in CLL B-cells and autologous T-cells. Normal B-cells from CLL patients showed TL erosion that was indistinguishable from normal ageing. In contrast, T-cells showed rapid TL shortening. Using serial sampling and long-term follow-up, the change in CLL B-cell TL correlated strongly with starting TL. Patients with CLL B-cell TL above the fusogenic threshold displayed TL erosion, whereas patients below the threshold showed no change. There was no lower limit to the mean TL in CLL B-cells suggesting a dysfunctional DNA-damage response allowing on-going cell division and stabilisation of TL. T-cell TL demonstrated marked erosion over the same timescale and the degree of erosion correlated with longevity of diagnosis. Mean T-cell TL did not reach the same degree of shortening as CLL B-cells, consistent with the induction of replicative senescence. T-cell TL was associated with immunophenotypic changes indicating T-cell exhaustion. Furthermore, TCR repertoires showed evidence of clonality and a skewed reliance on certain V gene segments. Long-term co-culture of PBMC from CLL patients, showed survival and proliferation for up to 154 days in-vitro. Despite proliferation, little change in CLL B-cell TL was observed. Surprisingly T-cell TL increased probably due to the selective loss of shorter TL T-cells during the culture. This study shows that the telomeres of CLL B-cells show distinct dynamics over the course of the disease, suggesting that the TL may be pre-determined at the initiation of disease. In contrast, the TL of T-cells showed significant erosion, evidence of replicative senescence, and a phenotype that, together with the associated T-cell exhaustion, may have consequences for the therapeutic use of autologous T-cells.
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<tr>
<td>AIHA</td>
<td>Autoimmune haemolytic anaemia</td>
</tr>
<tr>
<td>Allo-SCT</td>
<td>Allogeneic stem cell transplant</td>
</tr>
<tr>
<td>Alt-NHEJ</td>
<td>Alternate non homologous end joining</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia related protein</td>
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<tr>
<td>B-ALL</td>
<td>B acute lymphoblastic leukaemia</td>
</tr>
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<td>BAFF</td>
<td>B-cell activating factor</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2 protein</td>
</tr>
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<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
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<tr>
<td>BFB</td>
<td>Breakage-fusion-bridge</td>
</tr>
<tr>
<td>BIRC3</td>
<td>Baculoviral IAP repeat-containing protein3</td>
</tr>
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<td>BP</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer Type 1 susceptibility</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer Type 2 susceptibility</td>
</tr>
<tr>
<td>CAR T</td>
<td>Chimeric antigen receptor therapy</td>
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<td>CBS</td>
<td>Central Biotechnology Services</td>
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<td>CCL3</td>
<td>Chemokine (C-C motif) ligand 3</td>
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<td>CLL</td>
<td>Chronic Lymphocytic Leukaemia</td>
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<tr>
<td>CLL-IPI</td>
<td>Chronic lymphocytic leukaemia international prognostic index</td>
</tr>
<tr>
<td>CRS</td>
<td>Cytokine release syndrome</td>
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<tr>
<td>CSR</td>
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<td>C-X-C motif chemokine 12</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DDX3X</td>
<td>ATP-dependent RNA helicase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FBXW7</td>
<td>F-box/WD repeat-containing protein 7</td>
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<td>FC</td>
<td>Fludarabine and cyclophosphamide</td>
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<td>FCR</td>
<td>Fludarabine, cyclophosphamide and rituximab</td>
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<td>Foetal Calf Serum</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in-situ hybridisation</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>HCL</td>
<td>Hydrogen cholride</td>
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<tr>
<td>HR</td>
<td>Homologous recombination</td>
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<td>hTERC</td>
<td>Telomerase reverse transcriptase</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
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<td>IgH</td>
<td>Immunoglobulin heavy chain</td>
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<tr>
<td>IGVH</td>
<td>Immunoglobulin variable-region heavy chain</td>
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<td>Interleukin-4</td>
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<td>ITP</td>
<td>Immune thrombocytopenia</td>
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<td>KBP</td>
<td>Kilo base pairs</td>
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<td>LYN</td>
<td>Tyrosine-protein kinase Lyn</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCL-1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
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<td>MBL</td>
<td>Monoclonal B-cell lymphocytosis</td>
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<td>MYD88</td>
<td>Myeloid differentiation primary response 88</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NFkB</td>
<td>Nuclear factor kappa β</td>
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<tr>
<td>NHEJ</td>
<td>Nonhomologous DNA end joining</td>
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<td>NK</td>
<td>Natural Killer</td>
</tr>
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<td>NOTCH1</td>
<td>Notch homolog 1</td>
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<td>OS</td>
<td>Overall survival</td>
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<td>P21CKI</td>
<td>p21 Cyclin dependent kinase inhibitor</td>
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<td>PARP-1</td>
<td>Poly(ADP-ribose) polymerase-1</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDL-1</td>
<td>Programmed death-ligand 1</td>
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<td>PE</td>
<td>Phycoerythrin</td>
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<td>PFS</td>
<td>Progression free survival</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>Q-FISH</td>
<td>Quantitative fluorescence in situ hybridisation</td>
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<td>RAP1</td>
<td>Repressor/activator protein 1</td>
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<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
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<td>RPMI</td>
<td>Roswell park memorial institute</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodeyl sulphate</td>
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<td>SF3B1</td>
<td>Splicing factor 3B subunit 1</td>
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<td>SLL</td>
<td>Small lymphocytic lymphoma</td>
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<td>SS</td>
<td>Single-stranded</td>
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<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
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<td>STELA</td>
<td>Single telomere length analysis</td>
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<td>SYK</td>
<td>Spleen tyrosine kinase</td>
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<td>TAE</td>
<td>Tris base, ascetic acid, EDTA buffer</td>
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<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TEL2</td>
<td>Telorette</td>
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<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
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<td>TH2</td>
<td>Th2 helper T-cells</td>
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<td>TIF</td>
<td>Telomere dysfunction-induced focus</td>
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<td>TIN2</td>
<td>TRF-1 interacting nuclear protein-2</td>
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<td>TL</td>
<td>Telomere length</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor- α</td>
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<td>TP53</td>
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<td>TPP1</td>
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<td>TRF1</td>
<td>Telomeric repeat binding factor 1</td>
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<tr>
<td>TRF2</td>
<td>Telomeric repeat binding factor 2</td>
</tr>
<tr>
<td>TTFT</td>
<td>Time to first treatment</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
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<td>XLF</td>
<td>XRCC4-like factor</td>
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<td>Exportin 1</td>
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Chapter 1: Introduction

1.1 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is characterised by the clonal expansion of malignant mature CD19+/CD5+/CD23+ B-cells. The accumulation of these cells in the marrow, lymph nodes and secondary lymph organs lead to multi-lineage cytopenias, lymphadenopathy and hepatosplenomegaly. The clinical course is heterogeneous with about 30% of patients presenting due to symptoms/signs related to CLL but about 70% are found by chance following a routine full blood count taken for an alternative purpose. Of the patients found by chance, a significant number will never require clinical intervention even after decades. However, some patients will have progressive and aggressive disease that will respond poorly to therapy and result in significant morbidity and early mortality (Guarini et al., 2003, Molica and Levato, 2001, Oscier et al., 1990).

1.1.1 Epidemiology

CLL is the most common leukaemia diagnosed in the western world and accounts for 40% of all of the leukaemias in individuals over the age of 65 years (Oscier et al., 2004). The age-adjusted incidence rate of CLL in the UK and USA is 4.5 -19 per 100,000 per year (Blum et al., 2018, Cheson et al., 1996). The incidence of CLL increases with age with the median age at presentation being 72 years, with only 11% of patients being diagnosed under the age of 55 years. The male/female ratio of affected patients is approximately 2:1 although the cause of this has not been identified. There is widespread national variation in the incidence of CLL with it being rare in people of Japanese origin but common
in black people. Recent studies have shown that around 5% of CLL patients have a family member with CLL or a similar lymphoproliferative disorder in keeping with an inherited genetic predisposition, which is supported by the fact that Japanese people who move to the USA retain their low incidence of CLL (Ishibe et al., 2001, Tamura et al., 2001). There have been many studies, mostly from Europe, which have now identified over 40 inherited genes pre-disposing patients to CLL (Crowther-Swanepoel et al., 2010, Di Bernardo et al., 2008, Kleinstern et al., 2018, Law et al., 2017, Speedy et al., 2014, Speedy et al., 2016). The genes identified are thought to be important in cell survival pathways (including \(NFkB\)), active chromatin (including \(POT1\)) and transcription factor binding for the key determinants of B-cell development and immune response.

### 1.1.2 Pathogenesis

There is ongoing debate about the origin of the leukaemic B-cell. However, the CLL cell as well as expressing the classical B-cell markers of CD19 and CD20 also express the classical T-cell marker CD5. There have been a few reports of normal B-cells which express CD5 with the most likely candidate being the B1 lymphocyte although others have identified a CD5⁻/CD27⁺ post germinal centre B-cell subset (Caligaris-Cappio et al., 1982, Caligaris-Cappio, 1996, Ghia and Caligaris-Cappio, 2006, Seifert et al., 2012). Many studies have looked at the antigen recognition of the B-cell receptors (BCR) used by CLL cells and showed that these were not randomly rearranged. The discovery of almost identical or 'stereotyped' BCR immunoglobulins among unrelated CLL patients suggests that antigen selection is important in disease ontogeny and evolution (Ghiotto et al., 2004, Stamatopoulos et al., 2017). This model hypothesises that antigens could be involved in CLL development by triggering proliferation of normal B-cells with specific BCRs leading to an increased risk of transforming events. Interestingly, recombinant antibodies produced from these restricted CLL subsets showed similar cytoplasmatic reactivity within each group, suggesting
recognition of a limited number of bacterial or viral antigens or autoantigens (Hadzidimitriou et al., 2009, Murray et al., 2008, Tobin et al., 2006).

The development of next generation sequencing has enabled a very deep examination of molecular genetic changes that occur in CLL due to its ability to examine very low number clones. This has identified many common mutations in ATM, NOTCH1, BIRC3, TP53, MYD88, XPO1, POT1, CHD2, XPO1, FBXW7 DDX3X and SF3B1 amongst many others genes (Malcikova et al., 2015, Puente et al., 2015, Quesada et al., 2011, Wang et al., 2011, Weissmann et al., 2013, Landau et al., 2013). Further studies have identified recurrently mutated driver genes such as RPS15 and IKZF3 with many altering RNA processing and export, MYC activity and survival signalling pathways such as MAPK (Landau et al., 2015).

Thus the present hypothesis is that a normal CD5-expressing B-cell undergoes antigen-driven proliferation as a consequence of encountering viral, bacterial or autoantigen and those proliferating cells then undergo a mutagenic event leading to uncontrolled B-cell proliferation. The proliferative rate in CLL is estimated to be 0.1-1% per day - but the majority of circulating CLL cells are arrested in G0/G1 of the cell cycle (Messmer et al., 2005). Therefore one of the main mechanisms for CLL progression is reduced apoptosis with over expression of the anti-apoptotic BCL-2 family of proteins including BCL-2 and MCL-1 (Fegan and Pepper, 2013, Kitada et al., 1998, McConkey et al., 1996, Paterson et al., 2012, Thomas et al., 1996). These proteins also determine the speed of progression of CLL, drug sensitivity and ultimately overall survival (OS) (Pepper et al., 1996, Pepper et al., 1997, Pepper et al., 2008).

CLL is now viewed as one disease but with two separate entities (Damle et al., 1999, Fegan, 2002, Hamblin et al., 1999) based on whether the CLL cells have rearranged their IGHV genes with 2% or less being regarded as germline (unmutated) whilst greater than 2% indicating somatically hypermutated (mutated) IGHV genes. The presence of somatic mutations of IGHV gene is in
keeping that at least a proportion of CLL cases having encountered antigen during the natural history of the disease and have subsequently transited through the germinal centre (Walsh and Rosenquist, 2005). Furthermore, gene expression profiling has shown that both types of CLL are most similar to memory B-cells with microarray studies also showing that CLL cells located in lymphoid organs display an up-regulation of genes belonging to the BCR signalling pathway (Herishanu et al., 2011, Klein et al., 2001, Rosenwald et al., 2001). Since 1999 very many studies have confirmed the IGHV status has a marked bearing on the clinical progression of CLL, response to treatment and ultimately final outcome with patients with unmutated IGHV genes having the worst outlook (Le Bris et al., 2017, Oscier et al., 2002, Trojani et al., 2010). Indeed IGHV status is one of the most robust prognostic markers yet identified.

CLL cells are found throughout the lymphoid tissues, bone marrow and of course the blood with proliferating CLL cells predominantly found in lymphoid tissue. The CLL microenvironment in lymphoid organs is created and maintained through a dynamic, interactive co-evolution and location between leukemic and normal bystander cells including nurse like cells, follicular dendritic cells and T lymphocytes. Proliferation centres in the spleen and lymphoid tissues of CLL patients contain nurse-like cells, which actively protect CLL cells from spontaneous apoptosis by producing CXCL12 and BAFF (B-cell-activating factor of the tumour necrosis factor family) (Burger et al., 2000, Nishio et al., 2005, Tsukada et al., 2002). Nurse-like cells are attracted to CLL by antigen triggering of the B-cell receptor inducing secretion of CCL3 and CCL4 (Burger et al., 2009). BCR signalling also leads to activation of the pro-survival Nuclear Factor-κB (NF-κB)/STAT3 pathway (Rozovski et al., 2017). CLL cells can express CD38 which when bound to CD31 leads to activation of CD68+ macrophages, which secrete tumour necrosis factor-α (TNF-α), in turn up-regulating the expression of vascular cell adhesion protein 1 (VCAM-1) by endothelial cells (Zucchetto et al., 2009). In lymphoid tissues and bone marrow CLL cells are typically found in close proximity to stromal follicular dendritic cells which upregulate the anti-apoptotic protein MCL-1 via CD44 ligation (Pedersen et al., 2002). Contact
between CLL cells and stromal cells, including fibroblasts, results in the upregulation of the pro-survival phosphatidylinositol 3-kinase (PI3K) and NF-κB pathways (Cuni et al., 2004, Edelmann et al., 2008). Proliferation centres also contain activated CD4+ T-cells again adjacent to leukaemic CLL cells suggest adhesion and possibly bi-directional signalling (Patten et al., 2008). CLL cells also secrete CCL22 in addition to CCL3 and CCL4 leading to T-cell recruitment into lymph nodes. This suggests that CLL cells themselves play an active role in the accumulation of T lymphocytes. Many of the T-cells in proliferation centers express CD40L, a member of the TNF superfamily that mediates interactions with CD40+ CLL cells, rescuing them from apoptosis via NF-κB pathway activation and survivin upregulation (Granziero et al., 2001, Plander et al., 2009). CD40L/CD40 interactions also leads to upregulation of the co-stimulatory molecules CD80 and CD86 by CLL cells (Van den Hove et al., 1997, Yellin et al., 1994). Functionally, both CD4+ and CD8+ T-cells from patients with CLL have been shown to secrete increased amounts of the prototypical Th2 cytokine interleukin-4 (IL-4) which protects CLL cells from apoptosis by upregulating expression of the anti-apoptotic molecule BCL-2 (Ahearne et al., 2013, Dancescu et al., 1992, Kay et al., 2001, Mu et al., 1997, Panayiotidis et al., 1993).

1.1.3 Immunological defects in Chronic Lymphocytic Leukaemia

Although CLL is a malignancy of B lymphocytes for several decades it has been known that the T lymphocytes are quantitatively and qualitatively abnormal. The first observations of T-cell dysfunction were the clinical observations that up to 50% of CLL patients develop hypogammaglobulinaemia, autoimmune haemolytic anaemia (AIHA), immune thrombocytopenia (ITP) and/or red cell aplasia (Ben-Bassat et al., 1979, Rozman et al., 1988). Early studies identified many T-cell abnormalities including an expansion of T suppressor cells and reduced alloreactivity to normal B-cells, NK cell activity and response to T-cell mitogens (Fernandez et al., 1983, Herrmann et al., 1983, Whelan et al., 1983). Furthermore, CLL cells were able to suppress immunoglobulin production by
normal B lymphocytes (Kunicka and Platsoucas, 1988). Increased knowledge and access to better reagents allowed later researchers to refine these abnormalities and identified a decrease in some CD4 T-cell sub-populations but overall both CD4+ and CD8+ T-cells are both expanded in CLL with some patients having an inverted CD4/CD8 ratio with the latter leading to an inferior clinical course with shorter lymphocyte doubling time, shorter time to treatment and progression-free survival (Crockard et al., 1990, Mackus et al., 2003, Nunes et al., 2012). Some early studies suggested that the expanded CD8+ cell population were positive for CD45RA and CD57 but negative for CD27 suggesting they have a cytotoxic effector function but later the CD8+ cells were also shown to express CD28 and PD1 indicating an exhaustive phenotype. Furthermore, there is a reduction in both naive CD4+ and CD8+ T-cells in CLL patients (Nunes et al., 2012). Most of the expanded CD4+ cells are effector memory cells and in the inverted CD4/CD8 patients there is a combined expansion of CD8+ effector memory cells and senescent/exhausted CD8+ cells.

The adaptive immune system relies on the capacity for extensive cell division and clonal expansion of lymphocytes. Effective immune response may require repeated expansion of a limited number of antigen-specific T lymphocytes and limitations in the replicative capacity of such cells could eventually compromise immune function (Rufer et al., 1999, Son et al., 2000). However, in CLL there is downregulation of the co-stimulatory molecule CD28 and overexpression of the T-cell downregulating molecule CD152 in both CD4+ and CD8+ cells which results in T-cell hypo-responsiveness or even anergy, although not all studies have found CD152 overexpression (Frydecka et al., 2004, Motta et al., 2005, Riches et al., 2013). In the TCL1 transgenic CLL mouse model, CLL cells express high levels of PD-L1 which induces T-cell exhaustion as demonstrated by their expression of PD1 and LAG3 which can be reversed by PD-L1 blockade (Gassner et al., 2015, Kater and van der Windt, 2015, McClanahan et al., 2015b, McClanahan et al., 2015a). In human studies other exhaustion markers, notably CD244, CD160, and PD1, have been identified in the T-cells of CLL patients. So although some of
the likely mechanisms of why T-cells in CLL become exhausted have been elucidated there are probably other factors playing pivotal roles including:

- The T-cells could be responding to the same bacterial/viral or auto-antigens that it appears the CLL were/still are responding to
- The T-cells are responding to antigens being expressed by the malignant CLL cells
- The T-cells are simply proliferating due to stimuli/cytokine release either from CLL cells or possibly normal cells the CLL cells are interacting with

It has been shown that the T-cells are clonally restricted with skewed expression of the TCR repertoire suggesting they are responding to a single or at least a limited number of antigens (Scrivener et al., 2003). In the TCL1 transgenic CLL mouse model monocytes aberrantly expressed high levels of PD-L1 and secretion of multiple inflammatory and immunosuppressive cytokines including interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α) and CXCL9 (Hanna et al., 2016). However, when myeloid cells were depleted the CLL-associated skewing of T-cells toward antigen-experienced phenotypes was repaired. However, on direct contact CLL cells are able to inhibit actin polymerisation in even normal CD4+ and CD8+ T cells leading to defective immunological synapse formation with antigen presenting cells via a CD11a/CD18 integrin dependent mechanism (Ramsay et al., 2008). Interestingly this immune synapse dysfunction can be reversed by lenalidomide (Ramsay et al., 2008, Ramsay et al., 2012). CLL cells secrete IL-10 which has been shown via CXCR-4 and STAT3 to reduce effector CD4+ and CD8+ T-cells (Alhakeem et al., 2018). However, lenalidomide suppresses IL-10-induced STAT3 phosphorylation in healthy T-cells, thus reversing CLL-induced T-cell dysfunction (Shaim et al., 2017). Many other cytokines are dysregulated in CLL patients including IL-2, TNF-α, IL-4, IL-6, IL-17, IL-21 and CD40L many of which alter many immunological functions/interactions (Browning et al., 2016, De Cecco et al., 2015, Lapalombella, 2015, Riches et al., 2013, Taghiloo et al., 2017).
Infection is a common cause of CLL patient death (50 to 80% of patients) as a consequence of neutropenia (disease and therapy induced), hypogammaglobulinaemia, reduced normal B-cell numbers and the T-cell dysfunction outlined above (Dearden, 2008, Francis et al., 2006, Molteni et al., 2005, Morrison, 2010). The commonest infections are chest infections typically caused by Streptococcus pneumoniae and Haemophilus influenza and urinary tract infections due to Escherichia coli with one study showing an inverse correlation between the IgG levels and the risk of infection (Griffiths et al., 1992). One of the major problems CLL induces is a failure to respond appropriately to infective agents and in particular to mount an antibody response. Several studies have shown that CLL patients respond sub-optimally to many vaccines including Streptococcus pneumonia, Haemophilus influenza, Influenza A and Influenza B. Vaccination with the 23 valent unconjugated pneumococcal vaccine showed only a 50% response to vaccination but slightly better responses (58%) were seen using the 13 valent conjugated vaccine (Hartkamp et al., 2001, Pasiarski et al., 2014). Depending on the serotype, the percentage of the CLL patients with antibody levels suggested to provide protection against invasive pneumococcal disease varied from 29 to 71% five years after vaccination (Lindstrom et al., 2018). In a recent randomised study the conjugated 13 valent vaccine was shown to provide a superior immune response with at 1 month post vaccination 25/63 CLL patients responded compared to only 14/63 with the unconjugated 13 valent vaccine (Svensson et al., 2018). Furthermore, the recipients of the conjugated vaccine responded to 10/12 pneumococcal serotypes compared to 5/12 responses observed with the 23 valent unconjugated vaccine with the response at 6 months again superior in the patients receiving the conjugated vaccine. These results are similar to an earlier study in which a 7 valent conjugated vaccine was compared with the 23 unconjugated pneumococcal vaccine (Sinisalo et al., 2007). Conjugation of a polysaccharide with a protein conjugate (protein-conjugate vaccine) renders a T-cell dependent memory inducing vaccine and it is thought CLL patients can respond better to these types of antigens as highlighted by the observation that there is an expanded CD4+ memory pool in CLL patients (Baxendale et al., 2000,
Chapter 1: Introduction

de Roux et al., 2008, Nunes et al., 2012). Similarly, sub-optimal responses are seen with the conjugated *Haemophilus Influenza* vaccine with 21-43% of patients responding but only 50% of those achieving protective antibody levels (Jurlander et al., 1995, Sinisalo et al., 2001). Even worse responses are seen with Influenza vaccination with response rates of only 5% for *influenza A* and 15% for *Influenza B* after the single vaccination rising to 15% for *Influenza A* and 30% for *Influenza B* after the booster vaccination (van der Velden et al., 2001). Protection rates were 0% for *Influenza A* and 25% for *Influenza B* after the single vaccination; they were 5% (H1N1) and 10% (H3N2) for *Influenza A* and 30% for *Influenza B* after the booster. Lower age, earlier stage, normal IgG levels have been in some studies to be associated with improved vaccine responses and hence vaccination is recommended as soon as possible after diagnosis of CLL (Sinisalo et al., 2001, Sinsalo et al., 2002).

1.1.4 Clinical presentation

As outlined above around 70% of CLL patients are diagnosed when still asymptomatic via a routine blood test. Of the other 30% the commonest presentation is lymphadenopathy usually noted first in the neck. However, especially with more advanced stage disease patients may present with so-called constitutional symptoms notably weight loss of >10% over 6 months, drenching night sweats, fever and excessive tiredness. More rarely patients may present with abdominal discomfort due to hepatomegaly or splenomegaly with the latter sometimes causing a bloating feeling after eating. In advanced disease patients may experience symptoms secondary to cytopenia’s, which result from marrow infiltration, hypersplenism or a combination of the two. Other causes of anaemia seen in CLL patients are folate deficiency due to increased consumption and secondary AIHA – see below.
1.1.5 Diagnosis and staging

The diagnosis of CLL is based upon a combination of blood film lymphocyte morphology, the presence of greater than $5 \times 10^9/L$ circulating clonal B-cells persisting for more than 3 months, and a characteristic immunophenotype. This immunophenotype includes the expression of CD5 and CD23 with weak surface immunoglobulin expression and, absent or low expression of CD79b and FMC7. A scoring system has been proposed which has become adopted worldwide and confirms that more than 90% of CLL cases having 4 or 5 of these immunophenotyping characteristics (Matutes et al., 1994, Moreau et al., 1997).

The importance of a uniformly agreed scoring system for CLL is because there are many other types of lymphoproliferative disorders which have both clinical, phenotypic and genetic similarities to CLL most notably Marginal Zone Lymphoma, Mantle Cell Lymphoma, Waldenstroms Macroglobulinaemia, Follicular Lymphoma in Leukaemic phase, Hairy Cell Leukaemia and Splenic Lymphoma with Villous Lymphocytes (Jayaswal et al., 1977, Matutes et al., 1994, Matutes et al., 2004, Nelson et al., 2002, Sweet et al., 1977).

Care must also be taken to differentiate CLL from the very closely related CD5+ related disorders notably, CD5+ monoclonal B-cell lymphocytosis (MBL) and small lymphocytic lymphoma (SLL) that share the above immunophenotype, lymphocyte morphology and histology, although MBL and SLL do not feature clonal B-cells at greater than $5 \times 10^9/L$. MBL does not typically require any treatment as only about 1% per annum progress to CLL and even then it is usually the less aggressive CLL with long term survival (Marti et al., 2005, Rawstron et al., 2008, Strati and Shanafelt, 2015). SLL however, should really be regarded as CLL except there is no peripheral blood lymphocytosis. Although the reason why two identical conditions in terms of phenotype, genetics and clinical behavior should only be different in terms of CLL having peripheral blood lymphocytosis is unknown but it has been suggested that there may be different adhesion molecule expression which enables CLL cells to migrate from...
the lymphoid tissues to the blood whereas SLL cells lack these (Pinto et al., 1993, Csanaky et al., 1997). Neither MBL, nor SLL are included for the remainder of this thesis.

### 1.1.6 Staging

It was evident from the first recognition of CLL as a distinct illness that there was a very variable clinical outcome. Although patients with bulky disease, severe cytopenias and severe constitutional symptoms self-evidently did worse, how to predict what happened to the majority of patients who had asymptomatic presentations was more challenging. In 1975 Rai and colleagues reported a staging system based on blood tests and physical examination (Rai et al., 1975).

The Rai system divides CLL into 5 stages based on the results of blood tests and a physical exam (Table 1.1)

<table>
<thead>
<tr>
<th>Table 1.1. Rai staging system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rai stage 0</strong></td>
</tr>
<tr>
<td>Lymphocytosis; no enlargement of the lymph nodes, spleen, or liver; red blood cell and platelet counts are near normal</td>
</tr>
<tr>
<td><strong>Rai stage I</strong></td>
</tr>
<tr>
<td>Lymphocytosis; enlarged lymph nodes; spleen and liver are not enlarged; red blood cell and platelet counts are near normal</td>
</tr>
<tr>
<td><strong>Rai stage II</strong></td>
</tr>
<tr>
<td>Lymphocytosis; enlarged spleen (and maybe an enlarged liver); lymph nodes may or may not be enlarged; red blood cell and platelet counts are near normal</td>
</tr>
<tr>
<td><strong>Rai stage III</strong></td>
</tr>
<tr>
<td>Lymphocytosis; lymph nodes, spleen, or liver may or may not be enlarged; Hb &lt;11g/dL and platelet count &gt;100x10^9/L</td>
</tr>
<tr>
<td><strong>Rai stage IV</strong></td>
</tr>
<tr>
<td>Lymphocytosis; enlarged lymph nodes, spleen, or liver; red blood cell counts may be low or near normal; platelet count &lt;100x10^9/L</td>
</tr>
</tbody>
</table>
Although 5 stages were originally proposed clinically they fall into three prognostic groups of Rai 0/1, Rai II and Rai III/IV (Wierda et al., 2007)(Figure 1.1).

**Figure 1.1. Overall survival of Rai stages 0-IV.** (From Weirda et al 2007).

In 1981 a simplified 3 stage staging system using the same parameters was proposed by a French group (Binet et al., 1981)(Table 1.2 and Figure 1.2):

**Table 1.2. Binet staging system**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage A</td>
<td>Fewer than three areas of enlarged lymph nodes</td>
</tr>
<tr>
<td>Stage B</td>
<td>Three or more areas of enlarged lymph nodes</td>
</tr>
<tr>
<td>Stage C</td>
<td>Anaemia (Hb&lt;10g/dL) and/or Thrombocytopenia (platelets &lt;100x10⁹/L)</td>
</tr>
</tbody>
</table>
Both staging systems are in widespread use with North America preferring the Rai staging system and Europe the Binet system.

1.1.7 Prognostic markers

Both the Rai and Binet staging systems are extremely important in terms of predicting disease progression, time to first treatment (TTFT) and ultimately OS. However, with 70% of patients presenting with early stage disease identifying which of these patients will progress and require treatment from those who will not is a very important clinical problem. This has led to a very large number of laboratory tests being assessed including morphology (percentage of pro-lymphocytes), lymphocyte doubling time, bone marrow histology (diffuse infiltration), β-2 microglobulin, thymidine kinase, CD38 expression, CD49d, ZAP 70 (a component of the T-cell receptor) expression, IGHV mutation status, FISH cytogenetics, karyotype, whole genome sequencing and telomere length (Bulian et al., 2014, Crespo et al., 2003, Damle et al., 1999, Dohner et al., 2000, Hallek et al., 1996, Hamblin et al., 1999, Herling et al., 2016, Krober et al., 2002, Lin et al., 2014, Pangalis et al., 1984, Rassenti et al., 2004, Rozman et al., 1981,
Fluorescence in-situ hybridisation (FISH) cytogenetics has revealed that deleted 13q14 occurs early in the course of the disease either as a single abnormality or part of a more complex genetic profile and is by far the commonest abnormality being present in about 50-60% of patients. The deleted region contains the *miR15* and *miR16* genes, which are thought to be pivotal in the pathogenesis of CLL and occasionally RB1 (Calin et al., 2002, Zenz et al., 2011). Unlike 13q deletions, the 11q23 deletion results in an inferior prognosis albeit it’s frequency is less: 12-30% (Fegan et al., 1995, Zenz et al., 2011). There are several potential genes of importance in the deleted area including *ATM* and *BIRC3* both of which have been associated with a worse outcome (Rossi et al., 2012, Stankovic et al., 1999). Typically, there is a deletion of one arm of 11q with mutation in the *ATM* gene on the other allele (Austen et al., 2005). 17p deletion is the least frequent genetic abnormality being 3-8% at presentation but rising to up to 50% in relapsed/refractory patients (Zenz et al., 2011). However, it is the most serious genetic abnormality as it involves the *TP53* gene which leads to disease progression, drug resistance and poor survival with conventional therapies (Dohner et al., 2000). About 80-90% of cases with a deletion of one copy of the *TP53* locus will have a *TP53* mutation on the remaining copy leading to a dysfunctional p53 pathway (Zenz et al., 2010)(Figure 1.3).
Figure 1.3. Overall survival of CLL patients based on cytogenetic subgroups. (From Dohner et al 2000).
The ATM/p53-dependent DNA damage response (DDR) pathway plays an important role in the progression of many tumours. Although the ATM and p53 pathways overlap, they are not congruent but it appears that after exposure to DNA damage transcriptional responses are entirely dependent on ATM function in CLL (Stankovic et al., 2004). The p53 pro-apoptotic responses comprise only a part of ATM-regulated transcription with the ATM pro-survival responses in CLL being independent of p53. Consequently, the better survival of CLL patients with 11q deletion compared to 17p deletions is thought to be the additive effect of defective apoptotic and elevated survival responses after DNA damage in 11q deleted patients.

No single laboratory test defines the prognosis perfectly and several attempts have been made to combine these various tests and improve the ability to predict the outcome for individual patients. The most recent iteration was developed by an international working group which used patients from many different clinical trials and several validation cohorts and a multivariate Cox model to produce the CLL International Prognostic Index (CLL-IPI working group -International, 2016). This uses five differently weighted independent prognostic factors shown in table 1.3.

### Table 1.3. – CLL-IPI scoring system

<table>
<thead>
<tr>
<th>Score 4</th>
<th>TP53 status (no abnormalities vs del[17p] or TP53 mutation or both)</th>
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<tbody>
<tr>
<td>Score 2</td>
<td>IGHV mutational status (mutated vs unmutated)</td>
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<tr>
<td>Score 2</td>
<td>Serum β 2-microglobulin concentration (≤3·5 mg/L vs &gt;3·5 mg/L)</td>
</tr>
<tr>
<td>Score 1</td>
<td>Clinical stage (Binet A or Rai 0 vs Binet B–C or Rai I–IV)</td>
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<tr>
<td>Score 1</td>
<td>Age (≤65 years vs &gt;65 years)</td>
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</table>
Many of the prognostic markers in common use proved not to be independent and fell out in this model notably expression of ZAP-70 and CD38, serum lactate dehydrogenase and thymidine kinase, the FISH abnormalities del11q, trisomy 12, del13q, and del6q and NOTCH1 and SF3B1 mutations. The CLL-IPI splits CLL patients into 4 prognostic risk groups (Figure 1.4):

- Low score 0-1
- Intermediate score 2-3
- High score 4-6
- Very high 7-10

**Figure 1.4.** CLL IPI prognostic groups. A - showing time to first treatment in watch-and-wait patients and B - the MAYO patient cohort, from International CLL-IPI working group, 2016.
1.1.8 Treatment for CLL

As about 70% of patients present by chance and when still asymptomatic so not all patients need immediate treatment as survival without treatment can be very long for some. Therefore, a watch-and-wait approach is often adopted.

1.1.8.1 Indications for treatment

The International Workshop in CLL has recommended criteria for commencing treatment in CLL patients (Hallek et al., 2008, Hallek et al., 2018). Active disease should be clearly documented and at least one of the following criteria should be met – see table 1.4.

Over the last 60 years very many treatments have been tried in CLL most notably chemotherapy, monoclonal antibodies, small molecule inhibitors, allogeneic stem cell transplantation and Chimeric Antigen Receptor T-cell therapy (CAR-T). Although many of these therapies lead to symptomatic improvement and improved survival, CLL is presently rarely curable and new, more effective and safer therapies are still required.
Table 1.4. Criteria for commencing treatment in CLL patients.

<table>
<thead>
<tr>
<th>Criteria</th>
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<tr>
<td>Evidence of progressive marrow failure as manifested by the development of, or worsening of anaemia and/or thrombocytopenia</td>
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<tr>
<td>Massive (i.e. &gt; 6 cm below the left costal margin) or progressive or symptomatic splenomegaly</td>
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<tr>
<td>Massive nodes (i.e. &gt; 10 cm in longest diameter) or progressive or symptomatic lymphadenopathy</td>
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<tr>
<td>Progressive lymphocytosis with an increase of more than 50% over a 2-month period</td>
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<tr>
<td>Lymphocyte doubling time (LDT) of less than 6 months</td>
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<tr>
<td>Autoimmune anaemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapy</td>
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<tr>
<td>A minimum of any one of the following disease-related symptoms must be present:</td>
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<tr>
<td>• Unintentional weight loss more than or equal to 10% within the previous 6 months</td>
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<tr>
<td>• Significant fatigue (i.e. Eastern Cooperative Oncology Group Score 2 or worse; cannot work or unable to perform usual activities)</td>
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<tr>
<td>• Fevers of greater than 100.5°F or 38.0°C for 2 or more weeks without other evidence of infection.</td>
<td></td>
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<tr>
<td>• Night sweats for more than 1 month without evidence of infection</td>
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</table>
1.1.8.2 Chemotherapy

The first therapy shown to improve symptoms and signs in CLL were glucocorticosteroids typically prednisolone. However, as responses were usually very short and as the alkylating agents including melphalan, cyclophosphamide and chlorambucil were being developed across a range of tumours, studies using chlorambucil either as a single agent or in combination were undertaken in CLL (Catovsky et al., 1991, French Cooperative CLL group Group, 1990, Kempin et al., 1982, Montserrat et al., 1985, Montserrat et al., 1988). Responses were 50-75% but the duration of response was typically less than 2 years with no combination proving superior to chlorambucil monotherapy.

In the late 1980s a new class of chemotherapy agent – purine analogues – were introduced into clinical practice which showed early promise as a monotherapy with overall and complete response rates in previously-treated patients of 52% and 37% which rose to 74% and 63%, respectively, in Rai stage 0-II patients and declined to 64% and 46%, respectively, in Rai III-IV disease. The results in previously untreated patients were even more impressive with an overall response and complete response rate of 79% and 63% respectively. These rose to 85% and 70%, respectively, in Rai 0-II patients (O'Brien et al., 1993). In the first randomised US based study directly comparing chlorambucil with fludarabine monotherapy, fludarabine showed a markedly superior complete and partial response rate of 20% and 43% respectively compared to 4% and 33% for chlorambucil treated patients. Furthermore, the median duration of remission and the median progression-free survival (PFS) in the fludarabine treated group were 25 months and 20 months, respectively, whereas both values were 14 months in the chlorambucil group (P<0.001 for both comparisons (Rai et al., 2000)). Although similar superiority of fludarabine over chlorambucil was confirmed in the UK CLL 4 study, a German study although showing a significant improvement in overall and complete response rate (72% v 51%, P = .003; 7% v 0%, P = .011 respectively) very surprisingly there was no significant improvement in PFS (19 months v 18 months) and again surprisingly,
although not significant, fludarabine-treated patients had a shorter OS (46 months v 64 months) (Catovsky et al., 2007, Eichhorst et al., 2009).

Given the obvious clinical activity of purine analogues in CLL studies followed comparing a combination of fludarabine with an alkylating agent (typically cyclophosphamide) with fludarabine monotherapy including the UK CLL 4 study. Complete and overall response rates were significantly superior with the fludarabine /cyclophosphamide (FC) combination than with fludarabine monotherapy (complete response rate 38% v 15%, respectively; overall response rate 94% v 80%, respectively; p<0.0001 for both comparisons). PFS at 5 years was also significantly improved with the fludarabine/cyclophosphamide combination therapy than with fludarabine monotherapy (36% v 10% p<0.00005)(Catovsky et al., 2007). These results were confirmed in another German study where the FC combination chemotherapy resulted in significantly higher complete remission rate (24% v 7% P < .001) and overall response rate (94% v 83% P = .001) compared with fludarabine monotherapy. The FC combination therapy also resulted in longer median PFS (48 months v 20 months; P = .001) and longer treatment-free survival (37 months v 25 months; P < .001) although there was no difference in OS between the two regimens (Eichhorst et al., 2009).

**1.1.8.3 Monoclonal antibody therapy**

Further attempt to improve the response and survival rates by the addition of a further chemotherapy agent Mitoxantrone to the fludarabine and cyclophosphamide combination proved unsuccessful with a disappointing overall response rate of only 58% in a UK study (Hillmen et al., 2011). Although the anti-CD52 monoclonal antibody alemtuzumab had shown activity in CLL especially p53 deleted CLL it was too toxic for combination with chemotherapy (Moreton et al., 2005, Wendtner et al., 2004). However, in the early 1990s studies reporting that a new anti-CD20 monoclonal antibody therapy exhibited
activity in non-Hodgkin lymphoma subsequently led to its use in CLL (Byrd et al., 2001, Huhn et al., 2001, Maloney et al., 1994, Maloney et al., 1997, O'Brien et al., 2001). The results with rituximab monotherapy were disappointing with only a 25%-45% overall response rate and typical duration of response less than 12 months (Huhn et al., 2001, Byrd et al., 2001).

The MD Anderson group pioneered the combination of FC with rituximab (FCR) and showed promising responses with an overall response of 95% in previously untreated patients including 70% complete response which are still some of the highest responses ever reported (Keating et al., 2005, Wierda et al., 2005). In 2010 the German CLL group reported the superiority of the FCR over FC in terms of overall response (90% v 80% p<0.001), complete response (44% v 22% p<0.0001) and at 3 years 65% progression-free compared with 45% (p<0.0001) (Hallek et al., 2010). Further long-term follow up of the German and MD Anderson cohorts showed very long-term survival of up to 10 years and beyond especially in CLL patients with mutated IGHV genes (Fischer et al., 2016, Thompson et al., 2016). However, secondary malignancies were 17% in the German study. Similar, efficacy has been shown when rituximab is combined with another purine analogue bendamustine (Eichhorst et al., 2016).

However, not all patients are able to tolerate purine analogue based regimens. Two newer antiCD20 monoclonal antibodies obinutuzumab and ofatumumab are licensed in CLL. A study comparing chlorambucil monotherapy, with chlorambucil/rituximab combination therapy and a chlorambucil/obinutuzumab showed superior PFS for chlorambucil/obinutuzumab of 26.7 months compared to 16.3 months for chlorambucil/rituximab and only 11.1 months for chlorambucil monotherapy (P<0.0001 for both comparisons)(Goede et al 2014). A similar superior PFS was observed with chlorambucil combined with ofatumumab compared to chlorambucil monotherapy (22.4 months v 13.1 months p<0.0001) (Hillmen et al., 2015).
Antigenic stimulation through the B-cell antigen receptor (BCR) promotes the expansion, migration and survival of CLL B-cells (Quiroga et al., 2009). BCR stimulation can lead to survival signal via phosphoinositide 3–kinase alpha (PI3Kα) and phosphoinositide 3-kinase delta (PI3Kδ) (Figure 1.5).

Figure 1.5. B-cell receptor signaling. A – BCR signaling in the absence of antigen binding provides a survival signal dependent on PI3K. B – BCR signaling in response to antigen leads to LYN and SYK dependent phosphorylation of tyrosine motifs on CD79A and CD79B (shown as red boxes in the transmembrane proteins). A number of protein kinases (red symbols) and the lipid kinase PI3Kδ transmit survival, cell growth and proliferation signals as well as regulating cell migration. Small molecule inhibitors of selected kinases in the BCR pathway are indicated in boxes. (From Wiestner, 2012).

Antigen stimulation leads to triggering of the BCR and a multi kinase system ultimately resulting in protein kinase B (AKT), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and nuclear factor of activated T-cells (NFAT) activation leading to pro-survival signals. Various agents were developed to specifically inhibit this pro-survival pathway including the spleen tyrosine kinase (Syk) inhibitor fostamatinib, the BTK inhibitor ibrutinib and the PI-3k inhibitor idelalisib (GS1101) (Wiestner, 2012). Although fostmatinib showed an initial 55% response rate in CLL, the median PFS was only 4.2 months (Friedberg et al., 2010). Initial results using ibrutinib however were much more promising with an overall response rate of 71% in a mixed treatment naïve and
relapsed/refractory cohort study. Very impressively, the PFS in the relapsed/refractory cohort was 75% at 26 months and 96% in the untreated cohort at 22 months (Byrd et al., 2013, O'Brien et al., 2014). The relapse rate was higher in those patients with 17p deletion however, with 57% PFS at 26 months. Ibrutinib was well-tolerated with the primary potential safety concern being non-thrombocytopenic bleeding. A randomised study comparing ibrutinib with ofatumumab was stopped prematurely following an interim analysis as at a median follow up of only 9.4 months ibrutinib had already significantly improved the overall response rate (42.6% v 4.1%, P<0.001), progression-free survival (not reached v 8.1 months, p<0.001) and overall survival (90% v 81% at 12 months, p=0.005) (Byrd et al., 2014). Likewise, in previously untreated patients, who were deemed unsuitable for purine analogue therapy, ibrutinib proved superior to chlorambucil monotherapy with regards to overall response (86% v 35%, p<0.001), PFS (median, not reached v 18.9 months) and an estimated OS rate at 24 months (98% v 85%) (Burger et al., 2015). Overall the relative risk of death that was 84% lower in the ibrutinib group than in the chlorambucil group (p=0.001). Ibrutinib toxicity was generally mild but included diarrhoea, fatigue, cough and nausea.

Similarly, idelalisib showed good clinical activity in early phase studies in relapsed/ refractory CLL with a 72% overall response rate (Brown et al., 2014). In a randomised study idelalisib proved superior to rituximab monotherapy in relapsed/refractory CLL with an improved overall response (81% v 13% P<0.001), PFS not reached versus 5.5 months (P<0.001) and OS at 12 months (92% v 80% P=0.02) (Furman et al., 2014). Serious adverse events occurred in 40% of the patients receiving idelalisib and rituximab and in 35% of those receiving placebo and rituximab. However, post marketing surveillance studies showed that idelalisib was associated with autoimmune phenomena including severe diarrhoea or colitis, hepatotoxicity, pneumonitis and increased opportunistic fungal infections (Coutre et al., 2015).
A completely new class of small molecule inhibitors targeting the BCL-2 apoptotic proteins has been developed, the most clinically active of which is the BCL-2 inhibitor venetoclax (Vogler et al., 2017). In the initial phase 1 study in relapsed/refractory CLL venetoclax showed excellent activity with tumour lysis occurring even in patients with poor prognostic markers including 17p deleted patients (Roberts et al., 2016). The overall response rate was 79% with complete remission achieved in 20% of patients and minimal residual disease negativity in 5%. The 15 month PFS was 69%. In a randomised trial comparing venetoclax/rituximab combination therapy with bendamustine/rituximab combination therapy the former was superior with a 2 year PFS of 84.9% v 36.3% (P<0.001) in the whole cohort and 81.5% v 27.8% in 17p deleted patients although there was more neutropenia in the venetoclax-treated patients (Seymour et al., 2018).

1.1.8.5 Allogeneic haematopoietic stem cell transplantation (allo-SCT)

Despite the progress made with therapies in CLL it still remains a largely incurable disease. Allo-SCT has the ability to cure many different types of haematological malignancy including CLL (Passweg et al., 2016). However, although high initial response rates are often seen the results in CLL patients are not as good as many other tumours due to apparent higher rates of transplant related mortality including graft versus host disease and infective deaths (30-40%) and relapse providing an overall survival of 28-83% (Brown et al., 2013, Delgado et al., 2006, Dreger and Montserrat, 2002, Dreger et al., 2003, Machaczka et al., 2013, Pavletic et al., 2000, Schetelig et al., 2017, Sorror et al., 2008, Toze et al., 2012, van Gelder et al., 2017, van Gorkom et al., 2018). However, several studies have shown that there is a graft versus leukaemia effect in CLL (Ben-Bassat et al., 2007). Given the plethora of other therapies now available to CLL patients the role of allo-SCT in CLL is less clear than before, although the European Society for Blood and Marrow Transplant have recommended (Dreger et al., 2015) that allo-SCT should be considered in:
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- CLL patients with relapsed 17p-/TP53mut even if responding to small molecule inhibitors
- CLL patients with high risk features:
  - Refractory to purine analogues
  - Relapsing within short response <24 months following purine analogue therapy
  - Progression whilst receiving small molecule inhibitors

1.1.8.6 Chimeric antigen receptor therapy (CAR T) in CLL

The observation of a graft-versus-leukaemia effect in CLL showed that CLL cells can be killed by T lymphocytes. CAR-T uses autologous T-cells taken from the patients following leukopheresis, which are then transfected with a viral construct to increase antigenic recognition to a tumour antigen target e.g. CD19 in CLL and also to enhance T-cell tumour interactions. Typically, this uses a CD3ζ signalling element, which when combined with an anti-CD19 single chain variable fragment enhances CLL cell killing. However, by adding accessory molecules e.g. CD28 and/or 41BB further enhancement of tumour cell killing is achieved (Bouhassira et al., 2015, Davila and Brentjens, 2013).

Although initial results reported in the original studies showed good early activity in CLL including complete remissions, long term survival was not usual which was disappointing given the excellent results obtained with similar or even identical CAR-T in B acute lymphoblastic leukaemia (B-ALL) (Davila et al., 2014, Fraietta et al., 2018, Geyer et al., 2018, Porter et al., 2015). The reasons for the limited clinical efficacy of CAR-T T cells in patients with CLL are presently unknown but it appears that CAR-T T-cells have limited survivability in CLL possibly due to the fact the T-cells from CLL patients are inherently dysfunctional either in quantity (low number of naïve T-cells) or quality (reduced STAT3-related cytokines and serum IL-6 production), the immuno-inhibitory tumour microenvironment of CLL and higher tumour burden at time.
of treatment in patients with CLL compared to B-ALL (Fraietta et al., 2018, Hoffmann et al., 2017). Also, CAR-T is associated with life threatening complications including neurological syndrome and cytokine release syndrome (CRS) which occurs in almost 40% of patients. The aetiology of neurological syndrome is not fully elucidated but CRS appears to be due to the release of monocyte cytokines (especially IL-1 and IL-6) induced by the rapidly in-vivo expansion of the CAR-T T-cells (Chen et al., 2016, Giavridis et al., 2018, Jin et al., 2018, Norelli et al., 2018).
1.2 DNA damage

Environmental agents (including chemotherapy and irradiation), reactive metabolites and inherent defects in DNA damage repair may trigger a complex series of reactions – so called DNA damage response (DDR) mechanisms which can halt cell-cycle progression, induce DNA-repair mechanisms or trigger programmed cell death when DNA damage is irreparable. Cell cycle arrest is induced by two DNA damage checkpoint sensor kinases, ATM and ATR. ATM responds to DNA double-strand breaks (DSB) and disruptions in chromatin structure whereas ATR primarily responds to stalled replication forks (Shiloh, 2003). To date 3 DNA repair mechanisms have been identified notably a) nonhomologous DNA end joining (NHEJ), b) alternate non homologous end joining (alt-NHEJ) and c) homologous recombination (HR). Programmed cell death (apoptosis) or senescence is induced via the p53 apoptotic pathway (Li et al., 2012).

1.2.1 Cell Cycle arrest

In response to DSB the MRN complex (Mre11, RAD-50, NBS1) binds to the exposed DNA ends attracting and then activating ATM (D’Amours and Jackson, 2002, Uziel et al., 2003). This leads to hundreds of proteins being phosphorylated at Ser/Thr-Glu motifs including Chk1, Chk2, MK2, Brca1, H2AX, Mdm2 and p53, mediating the effects of ATM on DNA repair, cell-cycle arrest, apoptosis, and other downstream processes (Lavin, 2008, Liu et al., 2000, Matsuoka et al., 1998, Matsuoka et al., 2007, Reinhardt et al., 2007, Shiloh, 2003). DSBs not only activate ATM, but also the ATR kinase. Both ATM and ATR are critical for DSB-induced checkpoint responses and DSB repair but unlike ATM, ATR is activated by a broad spectrum of DNA damage in addition to DSBs (Brown and Baltimore, 2003). H2AX is essential for the accumulation of numerous DNA repair proteins and chromatin-remodeling complexes around DSBs and triggering ubiquitylation and SUMOylation cascades to promote
recruitment of Brca1 and 53BP1 (Fernandez-Capetillo et al., 2002, Kang et al., 2005, Mattioli et al., 2012, Meerang et al., 2011). 53BP1 binds to wild type p53 further activating p53 (Iwabuchi et al., 1994). The p53 tumor suppressor protein plays a central role in the decision of a cell to undergo either cell-cycle arrest or apoptosis. The amount and transcriptional activity of p53 is regulated by post-translational modification, such as phosphorylation and sumorylation (Appella and Anderson, 2001). Upon DSB, p53 is phosphorylated at several sites in its transactivation domain, including at Ser15 (ATM and ATR dependent) and Ser20 (Muller et al., 1998, Chehab et al., 1999). In normal cells, p53 protein levels are low owing to Mdm2-mediated ubiquitylation and degradation through the proteasome pathway. However, Mdm2 phosphorylation following ATM activation inhibits the interaction of p53 with Mdm2 resulting in reduced p53 degradation and p53 stabilization (Maya et al., 2001, Shieh et al., 1997). p53 is essential for G1 arrest in response to DNA damage. The key transcriptional target of p53 is the p21 Cdk inhibitor (p21CKI) which not only inhibits cyclin E–Cdk2 activity, thereby inhibiting G1/S transition but p21CKI also binds to the cyclin D–Cdk4 complex preventing phosphorylation of Retinoblastoma (RB) and thereby suppressing the RB/E2F pathway (el-Deiry et al., 1993, Harper et al., 1993).

1.2.2 DNA Damage Repair Mechanisms

Which of the 3 DNA damage repair pathways are initiated is partly dependent on the phase of cell cycle in which the DSB occurs. If accurate DSB repair does not take place widespread genetic lesions can ensue including DNA cross-linking, anaphase bridging, mutations, deletions, translocations, widespread genomic instability and possibly the development of cancer. HR is particularly adept at accurately restoring the original configuration of the damaged DNA molecule but NEJH and alt-NHEJ are not so accurate and mutations and small deletions may occur which can be mutagenic.
1.2.2.1 Non Homologous DNA End Joining (NHEJ)

When DSBs occurs NHEJ is a quick method to repair DNA ends throughout the cell cycle. The core NHEJ factors are Ku70/80 (Ku), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA Ligase IV, X-ray repair cross-complementing protein 4 (XRCC4), and XRCC4-like factor (XLF). When DSBs occur Ku 70/80 rapidly binds to the DNA ends acting as a scaffold for other NHEJ factors to join to. Ku 70/80 binding recruits DNA-PKcs to those DNA ends to form the active DNA-PK complex, resulting in increased DNA-PKcs kinase activity which in turn activates DNA ligase IV (Davis et al., 2014, Fell and Schild-Poulter, 2015). XRCC4 binds and stabilizes DNA ligase IV protein in cells and may also enhance end joining by promoting DNA end bridging via its ability to form long filaments with XLF (Ellenberger and Tomkinson, 2008, Mahaney et al., 2013). A key step in NHEJ is the juxta-positioning or synapsis of DNA. Although DNA ligase IV-XRCC4 binds to DNA ends it appears that the DNA-PKcs subunit of the DNA-PK appears to be the major factor bridging DNA ends in mammalian cells (Chen et al., 2000). Although DSBs can lead to mutagenesis they are part of the normal physiological function of B lymphocytes. Normal mature B lymphocytes undergo V(D)J recombination and immunoglobulin (Ig) heavy chain (IgH) class switch recombination (CSR) to change the constant region of IgH chains and, thereby, modulate different antibody effector functions. CSR is initiated by DSBs within a donor IgH switch (S) region and a downstream acceptor S region. CSR is completed by fusing donor and acceptor S region DSB ends by NHEJ (Methot and Di Noia, 2017).

1.2.2.2 Alternate Non Homologous End Joining (alt-NHEJ)

Poly(ADP-ribose) polymerase-1 (PARP-1) protein is involved in different cellular processes, including the DNA base excision repair pathway (BER), responsible for the removal of alkylated bases (Bouchard et al., 2003, Burkle, 2001). PARP-1 binds to DSBs and interact with subunits of DNA-PK to form a synaptic complex
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with the XRCC1-DNA ligase III complex leading to DNA repair (Ariumi et al., 1999, Audebert et al., 2004, Galande and Kohwi-Shigematsu, 1999). Although it was initially thought that the alt-NHEJ only occurred in cells unable to undergo NHEJ DSB repair it is now known that both repair mechanisms are activated in many cells with DNA damage (Bernnado et al 2008, Sfeir et al 2015). Both the NHEJ and alt-NHEJ are antagonised by ATM activated BRCA2 (Han et al., 2017).

1.2.2.3 Homologous Recombination (HR)

Unlike NHEJ and Alt-NHEJ, HR requires a homologous sequence as a template for repair (Kowalczykowski, 2015). This allows the recombination machinery to restore any missing genetic information in the vicinity of the break site, and as a result, HR is largely accurate and hence there is less potential for mutagenesis. HR is primarily active in mid-S phase to early G2 phase of the cell cycle, because an accessible homologous template to mediate repair is readily available via a sister chromatid in these cell cycle phases and a key component BRCA1 that flanks DSB sites is suppressed in G1 cells (Orthwein et al., 2015). In HR, BRCA1 forms a stable complex with BARD1, which binds to DNA and promotes DNA end resection to produce the single-stranded (ss) DNA necessary for homology search and strand invasion (Jasin and Rothstein, 2013, Wu et al., 1996). This leads to the recruitment of BRCA2 via interaction with PALB2 DSB sites (Bhattacharyya et al., 2000). The DSB sites then become coated by Replication Protein A (RPA), which is subsequently displaced by the recombinase protein RAD51 to form a nucleoprotein complex termed the presynaptic filament. This is followed by DNA synthesis and resolution of DNA intermediates to complete repair (San Filippo et al., 2008).
1.3 Telomeres

1.3.1 Telomere Structure

Telomeres are the specialised regions at the end of each chromosome. Human telomeres are nucleoprotein structures composed of long arrays of initially double stranded and then single stranded TTAGGG DNA repeats, measuring up to 25kb in length. They terminate in a 3’ single stranded G rich overhang of ~100–200 nucleotides (nt) which in association with six specialist telomeric DNA-binding proteins: Telomeric Repeat Binding Factor 1 (TRF1), Telomeric Repeat Binding Factor 2 (TRF2), TRF-1 Interacting Nuclear Protein-2 (TIN2), Repressor/activator protein 1 (RAP 1), POT1-TIN2 Organizing Protein (TPP1) and Protection of Telomeres Protein-1 (POT1) form the protective ‘shelterin’ complex (de Lange, 2005, Makarov et al., 1997, Wright et al., 1997). The shelterin complex sequesters away the DNA end of the chromosome by forming a loop which prevents the double strand DNA break repair apparatus recognising this as a DSB and inappropriately triggering the 3 DNA repair mechanisms. The 3’ single stranded overhang acts as a substrate for the reverse transcriptase telomerase, which replicates the telomeric sequence by using an internal RNA subunit as a template to direct DNA synthesis and lengthen telomeres (Nandakumar and Cech, 2013). Shelterin proteins also recruits the telomerase complex to chromosomal ends through an interaction with the telomerase protein component telomerase reverse transcriptase (TERT) (Xi et al., 2015). Telomeric DNA from humans can be arranged into T-loops, in which the 3’ overhang invades the upstream double-stranded region (Griffith et al., 1999).

1.3.2 Telomere DNA binding proteins

TRF1 is a 50 kDa protein containing an N-terminal acidic region and a C-terminal Myb domain that targets it to the chromosomal ends (Chong et al., 1995). It has
specific conserved domains which assist in the formation of a stable TRF1-TRF1 homodimeric structure along with the two myb-domains of the homodimer which help in a stable interaction with the duplex DNA by binding to it in a specific bending angle of approximately 120 degrees (Bianchi et al., 1997). TRF1 overexpression results in telomere shortening (van Steensel and de Lange, 1997). TRF1 binds to the structurally related TRF2 via TIN2 leading to assembly of the shelterin complex. TRF1 has been shown to inhibit the DNA repair response by preventing the activation of ATM, ATR, p53, and RB, leading to cellular senescence (Xin et al., 2008, Martinez et al., 2009, Diotti and Loayza, 2011). TRF2 has many functions including specifically targeting and preventing ATM activation at telomere ends, protecting telomeres against NHEJ DNA repair and allowing the telomere end to form the T loop (Celli and de Lange, 2005, de Lange, 2005, Denchi and de Lange, 2007, Okamoto et al., 2013). RAP1 binds to TRF2 to improve binding to TRF1 and also protects against both NEJH and HR (Sarthy et al., 2009, Sfeir et al., 2010). POT1 also has many roles including prevention of ATM activation and Rad3-related kinase to prevent recognition of the 3′ overhang being recognised as a DSB (Denchi and de Lange, 2007). TPP1/POT1 interacts with the single-stranded TTAGGG repeats present in the 3′ overhang (Palm and de Lange, 2008). In addition, POT1 binds to TPP1 to stabilise TRF1 and TRF2 (Lei et al., 2004, Kim et al., 2004, Kim et al., 2008, Ye et al., 2004)(Figure1.6).
Figure 1.6. Schematic representation of telomere structure. Telomeres are at the extremities of chromosomal DNA. The telomeric 3’ end terminates as a single stranded G-rich overhang able to form the T loop in which the overhand invades the telomeric double helix, remodeling the DNA into a circle. Telomeres are capped by at least six proteins collectively know as shelterin that physically shield the DNA. Shelterin allows discrimination of telomeres from double-stranded DNA breaks. (From Calado and Young, 2008).

1.3.3 Telomere function and maintenance

Telomeres form protective caps – the shelterin complex - at the ends of linear chromosomes (Figure 1.6). The replication of linear DNA molecules required during cell division results in the loss of telomeric TTAGGGG repeats. This telomeric loss arises due to the inability of DNA polymerase to fully replicate the extreme termini of linear chromosomes. This ‘end replication problem’ in association with a putative C-strand resection results in the gradual and predictable erosion of telomeres specifically from the terminus (Harley et al., 1990, Levy et al., 1992). This process eventually leads to the loss of telomeric function as telomeres become critically short which triggers either a p53-mediated cell-cycle G1 arrest referred to as replicative senescence or apoptotic cell death or if that system fails triggering of the DDR leading to nucleolytic degradation, DNA end-to-end fusions, irregular recombination, chromosomal instability, mutagenic risk – so called telomere crisis (d’Adda di Fagagna et al., 2003, Meena et al., 2015, Olovnikov, 1971, Saretzki et al., 1999) (Figure 1.7).
Indeed, replicative senescence which usually takes place after about 50 population doublings (Hayflick limit) could even have evolved as a protective mechanism against cancer (Ancelin et al., 2002). One single short telomere (or one unprotected telomere) may be sufficient to induce replicative senescence in normal cells, which consequently blocks their proliferation (Callen and Surralles, 2004). Pioneering observations by McClintock showed that mis-repair of damaged chromosomal ends could generate cycles of chromosomal degeneration termed breakage-fusion-bridge (BFB) cycles (McClintock, 1941, McClintock, 1938). The BFB cycle begins when a telomere breaks off a chromosome. When the damaged chromosome replicates, its sister chromatids fuse and form a bridge during anaphase, with the two centromeres of the fused sister chromatids pulled into opposite poles of the dividing cell. After the bridge breaks, the resulting daughter cells receive defective chromosomes that lack telomeres and can initiate new BFB cycles (Bi et al., 2004, Gisselsson et al., 2000, Thompson and Compton, 2011). Also when telomere dysfunction occurs, inhibition of TRF2 leads to the induction of 53BP1, gamma-H2AX, Rad17, ATM, and Mre11- a phenomenon referred to as telomere Dysfunction-Induced Focus (TIF) (Takai et al., 2003). Very recently such telomere dysfunction has been shown to be able to induce a wider spectrum of genomic rearrangements including chromothripsis (Maciejowski et al., 2015).

To counteract the shortening of telomeres, an enzyme telomerase, is found in stem cell compartments, germinal centres, and also in a wide range of human malignancies (Kolquist et al., 1998). Cells that have plentiful telomerase will maintain their telomere lengths despite cell division whereas those with insufficient telomerase will shorten their telomeres over time following cell division. Telomerase is a ribonucleic acid–protein complex of a single long noncoding RNA (called telomerase RNA or hTERC – AAUCCC to compliment telomeric DNA TTAGGG) and associated proteins, including TERT. Telomerase functions to extend the 3’ends of linear chromosomes by synthesising multiple copies of the specific DNA telomere repeat sequence (G-strand), utilizing a complementary template contained in the telomerase RNA (Blackburn et al.,
hTERC binds to the 3’ overhang of telomeres. The elongation of one DNA strand, via the telomerase reverse transcriptase mechanism is followed by synthesis of the other telomere DNA strand by DNA polymerase. Another mechanism to lengthen telomeres has been described in some types of cancer cells called Alternative Lengthening of Telomeres. Although not fully elucidated this mechanism is dependent on homologous recombination included roles for MRE11, RAD50 and Nijmegen breakage syndrome 1 protein (Cesare and Reddel, 2010).

**Figure 1.7. Classical concept of telomere and telomerase functions in tumour suppression and initiation.** Telomeres shorten during life and short dysfunctional telomeres activate DNA damage response (DDR) mechanisms. After a maximum number of cell replications (Hayflick limit), critically short telomeres induce replicative senescence in cells with a functional DDR serving as a mechanism to protect from tumourigenesis. In DDR deficient cells, short, dysfunctional telomeres can result in genetic instabilities through repeated breakage-fusion-bridge cycles. Transformed cells require telomerase activity to stabilise telomere function and avoid apoptosis as well as for unlimited proliferation. (From Gunes et al 2018).

The length of an individual’s telomeres is determined by the parental telomere length (TL) as well as common polymorphisms (*hTERT, hTERC, POT1*) with females having longer telomeres than males (Aviv, 2012, Broer et al., 2013, Codd et al., 2010, Gardner et al., 2014, Soerensen et al., 2012). Telomerase activity is largely repressed in somatic tissues, including in hematopoietic stem
cells and hence TL varies across somatic tissues in proportion to replicative activity (Morrison et al., 1996). It is estimated that due to the “end replication problem” telomeres shorten at an annual rate of 26-30 base pairs/year and although differing tissues will have differing telomere lengths the rate of telomere loss is thought to be equivalent in somatic cells (Benetos et al., 2013, Daniali et al., 2013, Muezzinler et al., 2013). Telomeres shorten with age and telomere shortening is considered one of the best-characterized mechanisms of cellular and phenotypic ageing with TL predicting the onset of replicative senescence (Allsopp et al., 1992, Armanios, 2013, Harley et al., 1990, Mikhelson and Gamaley, 2012).

1.3.4 Telomeres in health and disease

Telomere shortening could be considered a “normal” part of ageing and probably an anticancer mechanism (Bartkova et al., 2005). However premature telomere shortening blocks the proliferative capacity of stem cells, affecting their potential to regenerate tissues and triggering the development of age-associated diseases. Dyskeratosis congenita (DC) is an inherited disease and was the first condition to have telomeric dysfunction identified as the main underlying pathogenic mechanism (Mitchell et al., 1999, Vulliamy et al., 2001). Many different genetic abnormalities have been described in DC including seven important in telomere maintenance either because they encode components of the telomerase enzyme complex (DKC1, TERC, TERT, NOP10, NHP2, and TCAB1) or the shelterin complex (TINF2) all of which lead to shortened telomeres (Dokal, 2011). Although the clinical phenotype of DC is characterised by the triad of reticulated skin pigmentation, nail dystrophy and white patches (leukoplakia) in the mouth, bone marrow failure, premature ageing and a predisposition to malignancy are also features. These observations led to recognition of an increasing number of inherited conditions – short telomere syndromes- as being caused by telomere dysfunction including familial pulmonary fibrosis (regulator of telomere length 1 gene, RTE1 mutations),

Patients with short telomere syndromes may also exhibit a T-cell immunodeficiency disorder even in the absence of other bone marrow abnormalities (Wagner et al., 2018). These individuals have T-cells with shortened telomeres, reduced T-cell numbers including naïve T-cells, exhibit pre-disposition to T-cell apoptosis, a reduced TCR repertoire and senescent markers, all of which may lead to increased death from infections. These patients were shown to have *DKC1, hTERT, hTERC* and *RTEL1* mutations.

With the wider understanding of the potential pathogenic roles telomeres may play population studies have now been undertaken and reveal that short telomeres are found in many very common diseases including hypertension, diabetes, peripheral vascular disease, smoking and obesity - all conditions widely regarded as causing premature ageing (Butt et al., 2010, Andujar et al., 2018, Liu et al., 2018, Jeanclos et al., 1998, Starr et al., 2007, Wojcicki et al., 2018, Valdes et al., 2005). All these conditions are now widely regarded to have an inflammatory component and it is thought that the oxidative stress associated with these conditions leads to reactive oxygen species (ROS).

Telomeric DNA is G-rich (TTAGGG) and hence more intrinsically sensitive to ROS-induced damage. The G-rich strand of telomeric DNA accumulates 8-oxo-7,8-dihydroguanine (8oxoG) lesions upon exposure to free oxygen radicals and exhibits a high frequency of single-strand break formation in the DNA backbone. Base excision repair (BER) chemically removes altered bases from telomeric DNA. This includes base modifications caused by ROS including 8-oxoG but this frequently miss-pairs with adenine during DNA replication (Jacobs and Schar, 2012). In BER, a DNA glycosylase that recognises a chemically altered base excises the damaged base. The bases in the single-stranded region of telomeres
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are not protected by hydrogen bonding to the other strand, and hence cannot be repaired by BER, which requires a complementary strand to provide a template for the recognition and replacement of damaged nucleotides (Oikawa and Kawanishi, 1999, Ahmed and Lingner, 2018, von Zglinicki, 2000, Serra et al., 2000).

1.3.5 Telomeres in cancer

Carcinogenesis is a multistep process involving multiple mutations and chromosomal aberrations. The observation that telomere shortening can/should lead to replicative senescence/apoptosis suggests that telomeres act as an intracellular ‘timer’ limiting the number of mitotic cycles (Hayflick limit). However, if this checkpoint is bypassed through p53 dysfunction (deletion and/or mutation) the cell can divide further, resulting in extensive telomere attrition and ultimately telomere failure with chromosomal fusions, chromosome imbalance, gene amplification, non-reciprocal translocation (a hallmark feature in solid tumours), altered genetic expression, chromothripsis – telomere crisis. (Maciejowski et al., 2015, Desmaze et al., 2003).

Widespread TL abnormalities occur in the pre-invasive stages of virtually all human epithelial cancers (Meeker et al., 2004). One of the most prevalent aberrations in pre-cancerous lesions is telomere shortening, telomerase activation and in 50% of tumour p53 dysfunction. Indeed, TP53 is the most commonly mutated gene in cancer (Kandoth et al., 2013, Hollstein et al., 1991). Immortality is a key feature of cancer cells and requires unlimited cell division and hence stabilisation of TL. Telomerase activation occurs in about 80% of solid tumours typically by overexpression of hTERT whilst hTERC remains at constitutional levels or less commonly via the Alternate Lengthening of Telomeres mechanism (Shay and Bacchetti, 1997, Cesare and Reddel, 2010).
There are many mechanisms whereby telomerase activity can be altered including both transcriptional and posttranscriptional mechanisms and pre-RNA alternative splicing of the hTERT gene (Liu et al., 2017). Apart from TP53 many other acquired genetic abnormalities have been identified in very many common tumour types either in the DNA damage response genes (including ATM), shelterin complex (including POT1), telomeres (including hTERC) or telomerase (including hTERT) (Chiba et al., 2017, Gaspar et al., 2018, Cong et al., 2002, Gunes et al., 2018, Gu et al., 2017, Stagni et al., 2014, Rampazzo et al., 2010).

Several studies have shown that TL in cancer cells is prognostic across a whole range of common haematological and non-haematological malignancies including breast cancer, colorectal cancer, prostate cancer, CLL, multiple myeloma and myelodysplasia (Heaphy et al., 2013, Adam et al., 2017, Lin et al., 2014, Hyatt et al., 2017, Strefford et al., 2015, Simpson et al., 2015, Zhang et al., 2015, Williams et al., 2017).

### 1.3.6 Telomere length (TL)

#### 1.3.6.1 Telomere length analysis

Increasingly accurate techniques to measure telomere length (TL) in human tissues have provided a greater understanding of the role of telomeres in the progression to malignancy. Several techniques are available but the most commonly used are terminal restriction fragment (TRF) analysis, quantitative fluorescence in situ hybridisation (Q-FISH), flow-FISH, single telomere length analysis (STELA) and telomeric Q-PCR (Aubert et al., 2012b, Baird, 2005). Each of these techniques has its limitations that should be considered when interpreting TL data. TRF and FISH are based upon hybridisation of telomere repeat probes to the telomere repeat arrays and thus a key limitation is that short telomeres will bind fewer probes and thus there is threshold length below
which telomeres cannot be quantified. Q-PCR on the other hand is suitable for large epidemiological studies and has been widely adopted for this type of work, however the linearity of this assay has been called into question and it can suffer from high coefficient of variance that reduces the overall resolution of this technique (Aviv et al., 2011). STELA overcomes these limitations and can detect the presence of the shortest telomeres, however it technically more challenging and not easily adapted for large-scale studies.

1.3.6.2 Single telomere length analysis (STELA)

STELA is a high-resolution long-range PCR-based assay that allows the measurement of individual telomeres at specific chromosome ends (Baird et al., 2003). The method consists of a linker that is complementary to the telomeric 3’ single strand-overhang that is ligated at the 5’ end, introducing a unique sequence that can be targeted with a specific primer. PCR is performed with an oligonucleotide that anneals to the linker, and a chromosome specific primer targeting the sub-telomeric region of the same chromosome arm. STELA can be performed at multiple chromosome ends including the 2p, 9p, 11q, 12q, 16q, 17p, 18q and XpYp chromosome ends (Britt-Compton et al., 2006). Only small amounts of high-quality genomic DNA (picograms) are required. It is possible to detect extremely short telomeres and the sensitivity of the technique allows a very accurate measurement of the TL. Despite being a very accurate technique it may not be suited for high-throughput analysis or for the study of very long telomeres (>20Kb) (Figure 1.8).
The Telorette (Tel2) linker is comprised of a 7bp-sequence at the 3’ end that is complementary to the telomeric repeat sequence; as a consequence, the Tel2 anneals to the terminal 3’ G-rich ss-DNA telomeric overhang. Tel2 is ligated to the 5’ of the C-rich telomeric strand, through a putative ligase activity of Taq polymerase (Baird et al., unpublished data). The 5’ end of the Tel2 linker also contains a unique sequence identical to the Teltail primer. The Teltail primer, together with a Telomere-specific primer which is specific for each chromosome, are utilised to amplify the telomere in subsequent PCR cycles. (Figure adapted from Baird et al., 2003).

1.3.6.2 PBMC, T-cell and B-cells telomere length in healthy donors

Leucocyte TL reflects the TL of the originating haematopoietic stem cell but is obviously shorter due to the very high proliferative nature of most bone marrow and peripheral blood cells (Kimura et al., 2010). Studies have shown that the rate of age-dependent leucocyte TL shortening is rapid early in life but slows down in adulthood (Frenck et al., 1998, Aubert et al., 2012a). Using the TRF method it has been estimated that the rate of telomere erosion in sequential unselected PBMC samples to be 26-30 base pairs (bp)/year (Daniali et al., 2013, Benetos et al., 2013). However, the proliferative history of myeloid cells and lymphocytes is very different with neutrophils surviving only 24 hours whilst lymphocytes live many, many years.

Using flow-FISH TL was estimated to erode at a rate of 39bp/year in neutrophils compared to 59bp/year in unsorted lymphocytes. Looking in more detail within the T-cell subgroups, CD4⁺ naïve T-cells showed a loss of 39bp/ year where as
CD4⁺ memory T-cells showed a loss of 51bp/year. In the CD8⁺ T-cell population, again the naïve subset showed a smaller TL loss at 34bp/ year whilst the memory subset lost 54bp/year (Rufer et al., 1999). Similarly, using TRF, CD4⁺ naïve T-cell were shown to have a mean TL of 9.5kb compared to 8.0kb for CD4⁺ memory T-cells with an erosion rate of 30bp/year and 50-100bp per cell division (Weng et al., 1995). Similar results were shown using a flow FISH method with naïve CD4⁺ T-cells having telomeres 2.5kb longer than CD4⁺ memory T-cells (Rufer et al., 1998). In the same study it was shown that naïve CD8⁺ T-cells had longer telomeres than CD4⁺ naïve T-cells in keeping with a previous study where TRF was used (Palmer et al., 1997). However, a later study failed to confirm these results and suggested that CD4⁺ T-cells had the longest average telomeres at all ages, followed by B cells, with CD8⁺ T-cell telomeres the shortest suggesting that these lymphocyte subpopulations may have different replicative histories in-vivo (Son et al., 2000).

Using telomeric QPCR to estimate TL, Lin analysed 3 subsets of T-cells: CD4⁺ T-cells (CD3⁺CD4⁺), CD8⁺CD28⁺ T-cells (CD3⁺CD8⁺CD28⁺), CD8⁺CD28⁻ T-cells (CD3⁺CD8⁺CD28⁻) and normal B-cells (CD3⁻CD19⁺) (Lin et al., 2010a). Normal B-cells had longer TL than any of the T-cell subsets whereas CD8⁺CD28⁻ had the shortest TL and the other two T-cell subsets similar and in between.

In conclusion, although both T and B lymphocytes are thought to have low but detectable levels of telomerase, it is estimated that the rate of telomere attrition in both T and B lymphocytes ranges from 19 to 60bp/year in human adults. This would total a loss of between 2 and 5 kb over an 80 year life span in addition to the 1-2 kb loss that occurs through the first decade of life (Weng, 2008).
1.3.6.3 Chronic lymphocytic leukaemia and telomere length

In terms of telomere length, one of the most extensively studied haematological malignancies is CLL. It is starting to become apparent that telomere dysfunction may be a key determinant of the pathology of this disease. The story of advances in the telomere biology of CLL mirrors the development of more detailed methods to measure both TL and telomerase.

Telomerase was initially reported as being increased in stage B/C CLL and that it was a highly specific predictor of survival (Bechter et al., 1998). Subsequently, the further development of a more accurate telomerase assays revealed that telomerase is not commonly increased in CLL at early or late stage. Indeed, CLL cells exhibit low levels of telomerase and telomerase is not a predictor of survival in CLL patients although interestingly some studies have shown telomerase was decreased in CLL B-cells with mutated IGHV genes (Verstovsek et al., 2004, Lin et al., 2010b, Damle et al., 2004).

Shortened TL as measured by TRF, was found to be a predictor of survival in CLL with bone marrow samples with TL below 6kbp associated with a worse prognosis (Bechter et al., 1998). Patients with unmutated IGHV genes had shorter telomeres than patients with mutated IGHV genes and both TL and IGVH status were predictive for survival (Hultdin et al., 2003). It is possible, although not formally established, that the IGHV status differentiates the origin of the malignant B-cell clones from pre (unmutated IGHV) and post germinal centre (mutated IGHV); these telomere length differentials may therefore reflect germinal centre experience (Ladetto et al., 2004). These findings were replicated by Damle et al (2004) who, using flow-FISH showed that TL was significantly shorter in patients compared to controls, and that patients with unmutated IGHV genes had shorter TL than patients with mutated IGHV. However, in contrast, TL as measured by flow-FISH was not statistically significantly related to clinical outcome (Damle et al., 2004). Using telomeric Q-PCR, Grabowski et al (2005) also showed that patients with mutated IGHV genes
had longer TL than patients with unmutated IGHV genes, and that both IGHV status and TL were predictive of survival (Grabowski et al 2005). In this study, TL was a better predictor of outcome than IGHV status, with TL being able to provide additional prognostic information within the mutated IGHV groups (Grabowski et al., 2005).

Using TRF analysis, Ricca et al (2007) showed that using a cut off of 4250 bp, TL was a strong predictor of TTFT, PFS and OS. They also showed that in cases where there was discordance between IGHV mutation status, and TL, that TL was the best outcome predictor, i.e. un-mutated IGHV with long telomere and mutated IGHV with short telomeres (Ricca et al., 2007). It seems possible therefore that TL analysis could be used to risk stratify CLL patients resulting in patients who are low risk being spared unnecessary treatment (Terrin et al., 2007). This study also highlighted another consideration for TL as a useful marker in clinical practice for patient prognosis. TL analysis was successful in 100% of patients, compared to only approximately 90% success for IGHV status. A large cohort of 401 CLL patients confirmed that TL was a predictor of survival, time to treatment and also development of Richter’s Syndrome (Rossi et al., 2009). Furthermore, TL accurately identified patients with rapid disease progression and reduced survival despite the presence of other favorable prognostic factors. These data clearly show that TL may provide additional, clinically useful prognostic information in CLL, however in its current form, this will only be when used in conjunction with the other key prognostic markers in this disease (Rossi et al 2009). An updated report with long-term follow up for a median of 44 months (maximum 231 months) showed a telomere attrition rate of 137bp/year (+174bp to minus 1906 bp) (Ghione et al., 2018). This was particularly evident in IGHV-mutated patients, as compared to unmutated. It concluded by suggesting that baseline TL <5000 bp, a loss of >6% TL/year and unmutated IGHV were independently associated with shorter TFS. Using the STELA assay, Lin et al. showed that TLs capable of inducing telomere crisis were associated with poorer survival even in very early stage patients regardless of their IGVH mutation status or cytogenetic risk group (Lin et al., 2014).
These data implicate TL as a potential factor in the progression of the disease; however, the role that telomere biology may play in this process is not entirely clear. A few recent studies have started to examine the underlying biology and the consequences of telomere erosion in CLL including studies looking at both shelterin and non-shelterin telomeric proteins in CLL. In a mixed cohort study of CLL patients, (25 of 42 with untreated stage A disease), the expression of hTERT, Dyskerin, TRF1, HRAP1, POT1, hEST1A, MRE11, RAD50 and KU80 were decreased whilst there was an increase in the expression of TPP1 and RPA1 (Poncet et al., 2008). These findings correlated with very low levels of telomerase, but did not correlated with TL, or clinical outcome. This study raised the possibility that TL may be shortened in CLL due to dysfunction of the shelterin complex which may in turn be due to altered expression of the shelterin and other telomere associated proteins. In a previously untreated cohort of stage A patients telomere-dysfunction-induced (TIF) foci frequency was increased even in early CLL in comparison to normal controls but the presence of TIFs was not associated with decreased TL as measured by TRF (Augereau et al., 2011). Interestingly, they also showed that the TINF1 and TTP1 gene expression was significantly decreased in those stage A CLL patients that had TIF. Other genes including TRF1, TRF2, RAP1 and POT1 were also decreased but did not show statistical significance. They concluded that DNA damage is present in a proportion of early stage CLL patients and that damage is potentially associated with de-regulated shelterin protection of the telomere and this is not associated with telomere shortening (Augereau et al., 2011).

In contrast to this view, several laboratories have provided evidence that short telomeres may indeed be important in CLL, including significant telomere shortening and dysfunction in the early stages. Telomere structure was studied in a mixed cohort of patients that yielded two populations of CLL cells, one resistant (CLL-R) and one sensitive (CLL-S) to DNA damage induced apoptosis in vitro (Brugat et al., 2010b). TL measured by TRF was significantly shortened in the CLL-R group. hTERT expression was assessed and found to be the same in
both patient groups, as was the expression of five telomeric proteins – TRF1, TRF2, TIN2, hRAP1 and POT1. Telomeres of the CLL-R group were associated with elevated levels of histone lysine 9 tri-methylation in comparison to the CLL-S group. This modification leads to chromatin condensation and may interfere with telomerase-mediated elongation. What was not clear in this study was whether the shorter telomeres found in the CLL-R cells were the cause or the consequence of the resistance to DNA damage induced apoptosis in these cells (Brugat et al., 2010b). In a follow up study, again, using CLL-S and CLL-R cells, there was shortening of the telomeric 3’ overhang in CLL-R cells which may lead to their inability to form an enclosed telomeric structure and therefore they are recognised as DSBs by DNA signalling and repair systems (Brugat et al., 2010a). They observed activation of DNA damage signalling, the initialisation of NHEJ, the presence of telomeric deletions and duplications in CLL-R cells with short telomeres. Furthermore, resection of the protective telomeric 3’ single-stranded overhangs and an increased number of telomere-induced foci containing gammaH2AX and 53BP1 were seen especially in those with short telomeres. Chromatin immunoprecipitation and immunofluorescence experiments demonstrated increased levels of telomeric Ku70 and phospho-S2056-DNA-PKcs 2 essential components of the mammalian nonhomologous end-joining DNA repair system. They conclude that in CLL, telomeric uncapping is associated with multiple chromosomal abnormalities and short telomere length (Brugat et al., 2010b).

Consistent with this view an earlier study showed that TL correlates with Binet staging, CD38 status, ZAP-70 expression, IGHV mutation status as well as OS and treatment-free survival. There was also correlation between TL and the number of genetic aberrations present in CLL cells (Roos et al., 2008). Interestingly 17p deletions were exclusively present in the group with shortened telomeres suggesting that subtle genetic alterations allow cells to bypass replicative senescence, leading to further telomere attrition and the accumulation of shortened telomeres. In keeping with this in the UK CLL 4 study short telomeres were associated with NOTCH/SF3B/ATM and TP53 mutations and a worse
clinical outcome observations supported by a Scandinavian study (Strefford et al., 2015, Mansouri et al., 2013).

Despite these correlations between genetic aberrations and TL, until recently it had not been established if telomeres could become short enough to lose their end-capping function leading to fusion events, genomic instability and telomere crisis in CLL. This was addressed in a study published by Lin et al (2010) who made use of the STELA technique which allows for the measurement of very short telomeres including those which lose their end capping function and are subject to telomere fusion (Lin et al., 2010b, Letsolo et al., 2010, Capper et al., 2007, Britt-Compton et al., 2006, Baird et al., 2003). TRF analysis, Q-PCR and quantitative FISH do not have the sensitivity to detect the extent of short telomeres that lead to telomere fusion/crisis (Capper et al., 2007). This study identified TLs that were as short as those found in cells undergoing crisis in culture where telomere dysfunction has been shown to drive large-scale genomic rearrangements (Counter et al., 1992, Capper et al., 2007). Lin showed direct evidence of telomere dysfunction in CLL cells with critically shortened telomeres in the form of telomere fusion events (Lin et al., 2010b)(Lin et al 2010). Indeed, these fusion events were only detected in CLL B-cells that exhibited the shortest telomere lengths. Importantly, telomere fusion events were also seen in early stage patients with telomere shortening, showing that telomere dysfunction and its sequelae are present before clinical disease progression.

It seems highly likely that for CLL cells to undergo such extensive telomere shortening there would have to be loss of cell cycle checkpoint control to allow the cells to avoid apoptosis or replicative senescence. In a recent study, patients with 13q deletions which involved deletion of the RB1 gene had a significantly worse outcome than those patients with 13q deletion and an intact RB1 gene (Ouillette et al., 2011). As 13q is the commonest genetic abnormality identified in CLL and is usually present at diagnosis, this indicates that loss of checkpoint control may be an early driver of proliferation. Indeed, an impaired DDR has
been identified in samples with 11q (ATM), 13q14 involving the RB1 gene and 17p (p53) deletions (Austen et al., 2007, Ouillette et al., 2010). The distinction between TL at the point of p53-dependent replicative senescence and the TL at which fusion starts to occur in post-senescent cells, represents around 5-6 cell divisions of telomere erosion (Letsolo et al., 2010, Capper et al., 2007). Thus subtle deficiencies in the DNA damage checkpoint response could allow for a relatively small amount of additional cell division that brings TLs to within the length range at which telomere fusion can arise. Pepper and Baird (2010) proposed the scenario in which mutations that compromise the ability of CLL cells to sense and/or respond to DSBs, for example via the p53 DNA damage response pathway, allows for additional cell divisions. This could initiate cycles of anaphase-bridging, breakage and fusion, creating large-scale genomic rearrangements that in turn could result in further loss of checkpoint control and the clonal evolution of the tumour (Roos et al., 2008, Pepper and Baird, 2010, Lin et al., 2014, Lin et al., 2010b). Consistent with this view p53 dysfunction was associated with very short telomeres, high hTERT expression, deletions and chromosomal end-to-end fusions in cells with complex karyotypes (Guièze et al 2016). P53 disruption was also characterized by downregulation of shelterin genes. Interestingly, low expression of POT1, TPP1 and TIN2 was also found in some patients with wild-type p53 and had an adverse impact on progression-free survival after standard genotoxic therapy (Guieze et al., 2016).

Likewise, in CLL patients with ATM mutations TLs were significantly shorter than those with wild type ATM genes, even those with stage A disease (Britt-Compton et al 2011). This TL shortening is present in both patients with ATM mutations and 11q deletions and was independent of IGHV gene mutation status, CD38 status and Zap-70 status (Britt-Compton et al., 2012). This provides evidence that defective double-strand break checkpoints, which are known to exist in ATM mutated cells, may lead to extended replication and extreme telomere shortening. It remains to be seen if telomere erosion and dysfunction can drive the specific genomic rearrangements that are important in CLL. Finally, further evidence of the importance of telomere function in CLL comes
from studies assessing POT1 mutations which show they are an independent prognostic factor in chlorambucil based therapies (Herling et al., 2016).

1.4 Overview

Advancements in the understanding of the pathophysiology of CLL have led to significant progress over the last 10 years with the development of multiple effective treatments including combination chemoimmunotherapy, small molecule inhibitor and now cellular therapy in the form of CAR-T (Turtle et al., 2017). Such has been the improvement of clinical responses to these new generation of treatments that minimal residual disease (MRD) negativity has become the desired goal for treatment and the possibility now exists that some patients may even achieve cure with current non-transplant treatments (Thompson and Wierda, 2016). In this context, the possibility that one of the important options in the armory of CLL clinicians - CAR-T’s effectiveness may be limited by significant T-cell abnormalities found in CLL patients means that further work is needed here.

Together with increasing understanding of prognostic factors and understanding how these play a crucially important role in disease progression and also possibly modulating treatment response in CLL patients, the era of personalised medicine in CLL now seems imminent (Ghamlouch et al., 2017).

The above findings highlight the importance of understanding CLL disease pathology, allowing better, more effective prognostication and continuing to add new therapeutic targets and develop more effective treatments. The ultimate goal of achieving the best possible treatment response for the least possible toxic side effects will continue to depend further development of the current understanding of CLL.
1.5 Hypothesis and aims of this project

The principal aim of this Ph.D. thesis was to investigate the telomere dynamics of patients with Chronic Lymphocytic Leukaemia. Our hypothesis was that the telomeres of CLL patients B-cells would progressively shorten during follow up. In particular, the aim was to determine if and when the telomere shortening seen in CLL occurs.

The specific aims were as follows

- To characterise the XpYp telomere profiles of normal B-cells, CLL B-cells and T cells in CLL patients peripheral blood
- To investigate the telomere dynamics of normal B-cells, CLL B-cells and T-cells during follow up and the role of exposure to treatment and disease stage in modulating these dynamics
- To investigate how T-cell subsets alter during long-term follow-up
- To determine the telomere dynamics of CLL B-cells and T-cells during long-term co-culture where they are stimulated to undergo proliferation
- To characterise the T-cell phenotype changes in long-term culture
Chapter 2

Materials and Methods

2.1 Chemicals and reagents

Chemicals and reagents used were obtained from numerous sources including Amersham Biosciences/GE Healthcare, Beckman-Coulter, Bio-Rad, Biolegend, Clontech, Dako, Ebiosciences, Fisher Scientific, Life technologies, Millipore, Miltenyi Biotech Ltd, Promega, Qiagen, Sigma, Starlab, and Thermo Scientific.

2.2 Plastic lab equipment

Plastic and glass equipment used for experiments was obtained from Gilson, Becton Dickinson, Eppendorf, Thermo Scientific and Corning Incorporated.

2.3 Equipment and machinery

Equipment used during experiments was obtained from Amersham Biosciences Bio-Rad, Applied Biosystems, Hybaid, Thermo Scientific, Promega, and Qiagen.

2.4 Blood samples

Peripheral blood samples were obtained from patients with CLL attending clinics at University Hospital of Wales and University Hospital Llandough. Additional samples were obtained from Birmingham Heartlands Hospital via collaboration with Dr Guy Pratt. Informed consent was obtained from all
patients and ethical approval was granted by the South East Wales Research Ethics Committee (02/4806) and (13/WA/0346).

CLL was defined by clinical criteria as well as cellular morphology and the co-expression of CD19 and CD5 in lymphocytes simultaneously displaying restriction of light-chain rearrangement. Clinical information including treatment histories was available for all patients.

2.5 Cell Separation

2.5.1 Isolation of mononuclear cells

Peripheral blood was layered onto lymphoprep and centrifuged at 300xg for 20 minutes. After centrifugation, the opaque interface containing mononuclear cells was aspirated and transferred into a clean sterile centrifuge tube. The mononuclear cells were then washed with phosphate-buffered saline (PBS) (pH 7.2 (Sigma-Aldrich, Dorset, UK)) at 300xg for 5 minutes. After washing, the supernatant was discarded. The pellet containing the mononuclear cells was then washed with 3mls of water to lyse the contaminating remaining erythrocytes. PBS was added after a few seconds and the cells were again washed at 300xg for 5 minutes. The supernatant was again discarded and the mononuclear cells were re-suspended in PBS.

2.5.2. Separation of normal B-cell, T-cell and CLL B-cell fractions

2.5.2.1 Dynabead separation

CD3⁺ T-cell and CD19⁺ B-cells were positively selected by using CD3⁺ and CD19⁺ dynabeads from Life Technologies. 10μL of the desired bead was used for the isolation of 500,000 target cells form isolated mononuclear cells. The beads
were added to a microtube containing 1mL of PBS. The microtube was placed in a magnetic particle concentrator (Life technologies) for 60 seconds. After this time, the fluid was aspirated and the dynabeads were re-suspended in 1mL of PBS. This process was repeated twice. The cell preparation containing the cells of interest was then added to the washed dynabeads and incubated for 20 minutes at 4°C. Cells were isolated by placing the microtube back into the magnetic particle concentrator for 2 minutes. After this time, the supernatant was aspirated and discarded and the microtube was removed from the magnetic particle concentrator. The beads, which were now attached to the cells of interest where then re-suspended in 1mL of PBS.

2.5.2.2 AutoMACS Separation of CD19+ Cells

An AutoMACS Pro Separator (Miltenyi Biotec) was used for positively selecting 1,000,000 or more CD3+ T-cell and CD19+ B-cells from either whole blood or isolated mononuclear cells. 25µl of the desired bead (CD3+ and CD19+ (Miltenyi) was incubated with either patients whole blood or isolated mononuclear cell fraction in a 15mL falcon tube at 4°C for 20 minutes. Following this incubation, the falcon tube was placed in to the holding block of the autoMACS. A positive selection program (pos_sel) was selected and approximately 8 minutes later, a tube containing the positively selected fraction was collected. The unselected cell fraction was discarded.

2.5.2.3 Fluorescence activated cell sorting

For small numbers of target cells (less than 100,000), cells were isolated following the staining of samples with fluorescence-labelled antibodies recognising CD19-APC (Allophycocyanin), CD5-FITC (fluorescein isothiocyanate) and CD3-PE (phycoerythrin) and a BD FACS Aria supplied by the Central Biotechnology Services (CBS) Core Facility in the School of Medicine, Cardiff University (www.cardiff.ac.uk/medic/cbs). Where cell numbers allowed, purity
checks of the isolated target cells were performed and, in every case, >97% purity was achieved.

2.6 Tissue Culture

2.6.1 Cell line culture, trypsinisation and passage

CD40-ligand (CD40L) transfected mouse fibroblasts were kindly donated by Dr Aneela Majid (University of Leicester). This CD40L cell line was cultured in 10mL of fibroblast media (Dulbecco’s Modified Eagle Medium (DMEM, Sigma) was supplemented with 10% Foetal calf serum (FCS), 100units/ml Penicillin and 100g/ml streptomycin, 1mM Sodium Pyruvate (all Life Technologies)) in T75 or T175 flask at 37°C in a 5% CO₂ moist chamber. Approximately 2x10⁶ CD40L cells were used for the initial seeding of the flasks and were allowed to grow until a confluent monolayer was formed.

The media was then removed, and the cells were incubated for 5 min in the presence of 5mL EDTA-Trypsin (Invitrogen). Once the cells had detached from the flask surface, the cells were washed in 15mL of fibroblast media and spun at 300xg for 5 minutes. The supernatant was then discarded and the cells re-suspended in 10mL of fibroblast media. 1mL was then used to re-seed a new flask and the rest of the cells were used for co-culture experiments or stored at -80°C.

2.6.2 Co-culture of cell lines with CLL cells

CD40L cells were suspended in 5mL of fibroblast media and irradiated (8000RADS). 1x10⁶ irradiated cells were then seeded into a 6-well plate in 3mL of fibroblast media. The plate was incubated for 24 hours at 37°C with 5% CO₂ to allow the fibroblast cells to adhere to the well surface.
Duplicate aliquots of 20x10^6 CLL patient’s PBMCs (Peripheral blood mononuclear cells) were taken and re-suspended in 3mL of primary culture medium - Roswell Park Memorial Institute (RPMI) 1640 (Sigma) was supplemented with 2mM L-Glutamine (Life Technologies), 100units/ml Penicillin and 100g/mL streptomycin (Life Technologies) and 10% FCS (Life Technologies) supplemented with 5ng/µL IL-4 (IL-4 was purchased from Biosource and stored as a stock 100nM solution at -20°C). 4µM fludarabine (Fludarabine purchased from Bayer Pharmaceuticals and stored as a stock 100mM solution at -20°C) was added to one of these samples, whilst the other was unmodified as the control. These samples were left to incubate in 15ml falcon tubes at 37°C in a 5% CO₂ moist chamber for 1 hour. The 20x10^6 CLL patient’s PBMCs were then added to the surface of the irradiated cells and incubated for 7 days at 37°C in a 5% CO₂ humidified chamber. On day 3 or 4, 1mL of fresh culture medium was added to each sample well. Figure 2.1 shows a schematic diagram of the co-culture system employed in these experiments.

**Figure 2.1.** Schematic diagram showing the component parts of the co-culture system developed to maintain primary CLL cells in long-term culture.
2.6.3 Cell counting and viability staining

2.6.3.1 Trypan blue staining

Cell viability was assessed using the live cell exclusion dye trepan blue. Dead cells take up the dye giving them a distinct blue color under microscopy. Live cells, which appear white, can be counted allowing the total number of viable cells in suspension to be measured. Cell counting was performed using the Beckman Coulter Vi-cell.

2.6.3.2 Beckman Coulter Vi-cell XR counting

60µL of sample was added to 540µL of PBS (1/10 dilution) in a cell counting container and placed in the Vi-cell XR carousel. The cell type, dilution and name of the sample were then logged into the Vi-cell program. The machine was then run allowing the sample to be mixed with trypan blue and the number of viable cells per µL were calculated.

2.6.4 Cell line and PBMC cryopreservation

Cells to be frozen were pelleted by centrifugation and re-suspended in equal volume of freezing medium RPMI 1640 (Sigma) was supplemented with 2mM L-Glutamine (Life Technologies), 100units/mL Penicillin and 100g/mL streptomycin (Life Technologies) and 10% FCS (Life Technologies)) and freezing mix (40% freezing media and 50% FCS and 10% Dimethyl Sulfoxide (DMSO, Sigma)). The suspension was then transferred to cryo-vials, which were placed in a freezing box containing isopropanol. This method ensures gradual freezing and prevents the formation of ice crystals in the cells. Cells were then frozen at -80°C and kept at this temperature.
2.6.5 Cell thawing

Cells were removed from the -80°C freezer and thawed rapidly in a water bath (Grant) at 37°C. Once thawed, DMSO was removed by adding 4mL of fresh culture medium drop wise as to minimise cell damage through osmotic shock by slow dilution. The cell suspension was then centrifuged at 300xg for 5 minutes and the supernatant was discarded. The pellet was then suspended in an appropriate volume of fresh culture medium or PBS as required.

2.7 Antibody staining and flow cytometric analysis

2.7.1 Flow cytometric compensation set-up

Cells were analysed using a BD FACS Aria or an Accuri C6 flow cytometer. For analysis on the BD FACS Aria compensation between the fluorochrome emission spectra was done automatically using anti-mouse Ig/negative control compensation beads and FACSDiva software. Briefly, 3 drops of anti-mouse immunoglobulin (Ig) and negative control comp beads were added to 700μL of PBS. The beads were vortexed for 3 seconds and then 100μL was added to 1 FACS tube. Fluorochrome conjugated antibodies were then added at optimised concentrations (1 antibody per tube) and incubated for 5 minutes at room temperature. A further 900μL PBS was then added to each tube. These antibodies were used as single-color compensation controls. Each tube was analysed using the BD FACS Aria by gating on the single bead population using forward side scatter, then gating both the positive and negatively stained populations on a histogram plot. These populations were then used to automatically calculate the compensation between antibodies using FACSDiva software.
2.7.2 Multi-colour immunofluorescence staining

Fluorochrome-conjugated antibodies were incubated with 1-10x10^6 PBMC from CLL patients or cells removed from the co-culture systems. When staining directly from whole blood, without first isolating leukocytes as previously described, whole blood was incubated with red blood cell lysis buffer for 15 minutes (Red blood cell lysis buffer (Becton Dickson) was diluted 1/10 in distilled water). The sample was then centrifuged at 300xg for 5 minutes and the supernatant retained whilst the lysed red cell pellet was discarded.

Antibodies were incubated with cells for 15 minutes at 4°C. Cells were then washed twice in PBS by centrifuging at 300xg for 5 minutes and discarding the supernatant. Cell pellets were re-suspended in 100-200μL of PBS buffer and analysed using a BD FACS Aria or and Accuri C6 flow cytometer (BD Accuri).

2.7.3 Ki-67 proliferation assay

Intracellular Ki-67 staining was used to identify cells in active phases of cell cycle. Cells were taken from CLL co-cultures were washed once in 1mL of PBS and surface stained with antibodies recognising CD19-APC and CD3-PE. Cells were incubated at 4°C for 15 minutes before being washed twice in 1mL of PBS. 60μl of Reagent A (Fixation medium, Invitrogen Life Technologies) was added and the cells were incubated again for 15 minutes at 4°C to fix the cells. Following this, cells were again washed twice in 1ml of PBS before 56μL of reagent B (Permeabilisation Medium, Invitrogen Life Technologies) supplemented with 10%v/v NP40 (10g tergitol-type NP40 (nonyl phenoxypolyethoxylethanol) (Sigma) prior to the addition of 5μL of a Ki-67-FITC antibody (Alere). The cells were then incubated for a further 15 minutes before finally being washed twice more with 1mL of PBS and re-suspended in a final volume of 200μL of PBS for flow cytometric analysis.
2.8 Preparation of cell samples for further experiments

Cells to be stored for STELA at a later time were pelleted by centrifugation at 300xg for 5 minutes at room temperature. The supernatant was aspirated and the cell pellet was frozen at -20°C for up to 6 months.

2.9 Cytokine expression in the supernatants of the long-term cultures

1mL aliquots of the supernatants from the long-term culture were harvested and stored at -20°C following centrifugation to remove cellular material (300xg for 5 minutes). Subsequently, the supernatant levels of IFN-γ, IL-2, IL-6, IL-10, and TNFα were measured using the V-plex Proinflammatory Panel 1 (MSD). All assays were performed according to the manufacturer’s instructions. In all cases, the supernatants were analysed without dilution and were run in duplicate. The data were acquired on the V-plex® Sector Imager 2400 plate reader and analyzed using the Discovery Workbench 3.0 software (MSD). The standard curves for each cytokine were generated using the premixed lyophilised standards provided in the kits. Serial 4-fold dilutions of the standards were run to generate a 7-standard concentration set, and the diluent alone was used as a blank. The cytokine concentrations were determined from the standard curve using a 4-parameter logistic fit curve to transform the mean light intensities into concentrations. The lower limit of quantification (LLOQ) was determined for each cytokine and for each plate as the lowest standard for which both duplicate measurements were above the duplicate background values. The upper limit of quantification (ULOQ) was determined for each cytokine and for each plate as the highest standard that did not reach saturation. Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn’s post hoc test was used to determine the significance, and Mann-Whitney U test was used to determine the p-values between the comparators; a p<0.05 was considered to be statistically significant.
2.10 Molecular Biology

2.10.1 DNA extraction

High molecular weight DNA was isolated from the cell pellets using either a Qiagen Mini kit extraction protocol or a Promega Maxwell Blood Kit protocol.

2.10.1.1 QIAamp Micro Kit DNA extraction

For cell samples containing less than $1 \times 10^5$ cells taken from co-culture and for all samples from FACS samples, genomic DNA was extracted immediately after sorting using QIAamp Micro Kit. The kit enables purification of genomic DNA from small sample sizes by binding the DNA to a silica-based membrane. After several washing steps, the DNA was eluted from the membrane with 30-40 10mM Tris-HCL pH 8.0.

2.10.1.2 Blood Kit Maxwell DNA extraction

Genomic DNA was extracted from cell pellets using the Maxwell 16 LEV Blood DNA kit (Promega) along with the Maxwell 16 instrument. Briefly, cell lysis was achieved through incubation of the cell pellet with 300μL lysis buffer and 30μL proteinase K at 56°C for at least one hour. Cell lysates were then loaded into the cartridges and placed in the Maxwell16 instrument. The research mode with LEV hardware was used to extract DNA from the lysates. Extracted DNA was eluted into 50μL elution buffer.
2.10.2 DNA quantification

DNA concentrations were determined in triplicate in a number of ways: either by Hoechst 33258 fluorometry as described previously (Baird et al., 2003), using a Nanodrop ND-100 system (Thermo scientific) supplied by the Central Biotechnology Services Core Facility in the School of Medicine, Cardiff University or by performing a pilot STELA to determine band number, after which relative DNA quantities to add to reactions were subsequently calculated.

2.11 XpYp Single Telomere Length analysis (STELA) assay

DNA was diluted to 250pg/μL in 10mM Tri-HCl (pH 8). 0.9μM Tel2 linker was added to the diluted DNA. Multiple 10μL reactions were set up per sample (typically 6) each containing 250pg DNA/Tel2 mix, 1x Taq buffer (75mM Tris-Hcl (pH8.8), 20M NH₄SO₄, 0.01% Tween-20) (Abgene), 2mM MgCl2, 1.2mM dNTP’s, telomere-specific primer (0.5μM), teltail primer (0.5μM), and 1U Taq/PWO (Abgene/Roche) at a ratio of 10:1. 1uL of DNA/Tel2 mix was added to each reaction. These reactions were then cycled in a Bio-Rad DNA Engine Tetrad® Thermal Cycler using the following conditions: 94°C (20 seconds), 56-65°C depending on primer used (30 seconds), 68°C (10 minutes) for 22 cycles.

2.12 Gel electrophoresis

2.12.1 Gel Electrophoresis for STELA PCR products

DNA fragments were resolved using 40cm long 0.5% Tris-acetate-EDTA agarose gel submerged in 1xTAE cooled to 4°C by a circulating cooling system. 4μL PCR reactions containing 1x Ficoll-based loading dye (5% bromophenol blue, 5% xylene, 15% ficoll) were loaded in the gel and ran through the length of the gel at 120V for 14-16 hours.
2.12.2 Visualisation of PCR products

Bands stained by ethidium bromide were visualised on a UV-transilluminator.

2.12.3 Southern Blotting

The resolved STELA products were depurinated by washing the gel twice in depurination buffer (0.25M HCl) for 6 minutes. After rinsing with de-ionised water, the gel was then washed in denaturation buffer (1.5M NaCl/0.5M NaOH) for 15 minutes. The DNA was then transferred onto a positively charged membrane (Hybond XL, Amersham) by alkaline Southern blotting with denaturation buffer for 4-6 hours.

2.13 Probe labeling and hybridisation

2.13.1 Probe synthesis

25ng probe DNA and ladder (1:1 of 1kbp: 2.5kbp) in TE buffer (10mM Tris-HCl and 1mM EDTA) was labeled using Ready-To-Go DNA labeling beads (GE Healthcare). This kit generates labeled probes using random hexaprime labeling with $[\alpha^{32}\mathrm{P}]\mathrm{dCTP}$.

2.13.2 Southern Hybridisation

After blotting the membranes were rinsed in de-ionised H$_2$O before undergoing pre-hybridisation for 15 minutes in church buffer (0.5M sodium phosphate buffer (1M disodium hydrogen phosphate and 1M sodium dihydrogen phosphate), 1mM EDTA, 1% BSA, 7% SDS, pH 7.2). 25μL of radioactively labeled probe was added to the hybridisation bottles which were then left to hybridise at 60°C overnight.
2.13.3 Removing the unbound probe

To remove unbound probes, the membrane was washed with 0.1x sodium chloride sodium citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) several times at 60°C. The washed blots were then dried in the hybridisation oven at 60°C for approximately 30 minutes.

2.13.4 Visualisation of radiolabelled blots

Radiolabelled southern blots were placed in a cassette with a phosphoimages screen (Amersham) for 24 hours. The phosphoimager screen was then scanned using the Typhoon 9410 biomolecular imager (GE Healthcare).

2.13.5 Gel analysis

Gels scanned using the Typhoon 9410 were subsequently analysed using Molecular Dynamics ImageQuant 5.0 (GE Healthcare). The molecular weights of individual telomeres on each STELA Southern blot were calculated using Phoretrix 1D software (Nonlinear dynamics). Telomere length measurements were then exported to Microsoft Excel where the distance between the telomere-adjacent primer and the telomere repeat array was subtracted giving an accurate measurement of telomere length. Mean telomere length and standard error measurements were then calculated.
2.14 Statistical Analysis

All statistical analysis was performed using Graphpad Prism 6.0 software (Graphpad Software Inc., CA, USA). For variables between independent groups a non-parametric Mann-Whitney U test was used. Student’s t-test was used to compare telomere means when their distribution was found to conform to a Gaussian distribution by performing a D’Agostino and Pearson omnibus normality test. Progression-free survival and overall survival were calculated from the patient’s date of diagnosis and curves constructed using Kaplan and Meier methodology. For all statistical analysis, no difference between the two data sets was postulated to be the null hypothesis. The calculated p-value was the probability of the null hypothesis being true. A p-value of <0.05 was taken as evidence of significant difference and the null hypothesis was rejected.
Chapter 3

Telomere length profiling in CLL patients

3.1 Abstract

CLL is a disease where telomere length is significantly shortened in the CLL B-cells. As yet, the exact mechanism and timing of when this shortening occurs has not been clearly characterised. Furthermore, there is evidence to suggest that T-cells play an active role in CLL disease pathogenesis but again, the telomere length angle has not been fully investigated. Fluorescence activated cell sorting (FACS) is an established tool that allows highly specific identification and isolation of pure cell populations. Methods used to measure telomere length in previous work in this field cannot produce such a high resolution, quantifiable measurement of TL as STELA. Using STELA along with FACS to identify pure cell populations can provide a very accurate tool for discovering telomere length profiles of specific cell types such as CLL B-cells and T-cells.

Telomere length of unsorted PBMCs, normal B-cells, T-cells and CLL B-cells from a total of 86 CLL patients were analysed. Normal B-cells had the longest TL with CLL B-cells having the shortest TL. Significant difference was seen between unsorted PBMC and purified CLL B-cell TL demonstrating the importance of obtaining purified cell populations before analysing TL. There was a significant correlation between normal B-cells and age whilst the CLL B-cell TL and T-cell TL showed no age-related association. Consistent with previously published data, CLL B-cell TL was significantly shorter in patients with advanced stage B/C disease compared to Stage A patient and this disease stage related TL shortening was also demonstrated here in patients T-cells reinforcing the close relationship between these two cell populations.
The data in this chapter demonstrates that different cell populations in CLL patients PBMC possess different telomere length profiles and that CLL B-cell TL is not dynamic suggesting that ongoing erosion is not the key determinant of CLL B-cell TL.
3.2 Introduction

CLL is a disease where there is significant TL shortening in the CLL B-cells found in peripheral blood when compared with age-matched healthy controls (Damle et al., 2004), and this is consistent with a significant number of cancers where TL is shorter in tumour cells compared to normal tissue (Adam et al., 2017). Furthermore, the TL profiles of CLL B-cells were shown to be shorter in advanced stage disease patients (Lin et al., 2010) but importantly, CLL B-cells with very short TL were also identified in a subset of patients with early stage (Binet Stage A) disease. This discovery led to TL being shown to be a highly accurate predictor of TTFT, PFS and OS, especially in early stage CLL (Lin et al., 2014).

To date, the focus of TL studies in CLL patients has been on the malignant B-cells with no systematic analysis of other cell types in the peripheral blood. Given the chronic nature of CLL, and the probability that patients who require treatment will undergo several courses of treatment and experience multiple relapses, there is significant opportunity for TL to be modulated over the course of the disease. This potential change in TL may apply to the CLL B-cell tumour cells as well as other non-malignant cell types important in this disease such as T-cells, and normal healthy B-cells. These non-malignant cells present in the peripheral blood hold an important role in adaptive immunity and would also be exposed to chemo and immunotherapy during treatment of CLL. The effect of cytotoxic and genotoxic chemotherapy on telomere profiles on these cells is currently unknown and therefore this was one of the key questions I set out to address during this study.

There is also growing interest in studying T-cells in CLL due to the increased understanding of how microenvironmental factors, including the interactions between T-cells and CLL cells, play a significant role in pathology of the disease. In addition, it is clear that T-cell based therapies, including CAR-T are showing
potential in CLL (Porter et al., 2011, Geyer et al., 2018, Fraietta et al., 2018, Turtle et al., 2017). However, there is substantial evidence for multiple T-cell abnormalities in CLL patients including an increase in absolute CD4^+ and CD8^+ numbers, inversion of the CD4:CD8 T-cell ratio (Mackus et al., 2003), defects in the formation of the immunological synapse (Ramsay et al., 2008) and increased proportions of T-cells manifesting an exhausted phenotype (Nunes et al., 2012). Importantly, T-cell TL are significantly shorter in advanced stage CLL patients suggesting that the T-cells are proliferating in response to the CLL cells (Roth et al., 2008). In the context of CAR-T, the characterisation of the telomere profiles of T-cells in CLL patients may be crucial in defining which patients are most suitable for this therapeutic approach as the concept of T-cell stamina is gaining acceptance (Eyquem et al., 2017) largely due to the limited duration of response observed in many patients (Gauthier et al., 2018, Hay et al., 2018).

Single telomere length analysis (STELA) is a well-established tool for accurately measuring TL and also providing information on the range and diversity of TL distributions within specific cell populations (Baird et al., 2003). The primary objective of this chapter was to use multi-colour FACS to obtain purified sub-populations of peripheral blood lymphocytes from CLL patients in order to study their telomere profiles. Data generated from the analysis of these 86 patients was analysed and compared with purified sub-populations of lymphocytes derived from four healthy donors.

Our hypothesis is that TL will vary significantly in the separate cell sub-types present in the peripheral blood. Furthermore the TL in peripheral blood CLL B-cells, normal B-cells and T-cells would shorten both as a function of increasing age and possibly also due to exposure to chemotherapy.
3.2.1 Aims of the chapter

In this chapter, an in-depth retrospective analysis was performed on TL profiles from CLL B-cells (CD19+/CD5+), normal B-cells (CD19+/CD5-) and T-cells (CD3+) derived from peripheral blood as well as unsorted PBMC populations using STELA. Data was collected from a total of 86 patients with the aim of understanding how TL varies between patients in these distinct cellular phenotypes. In addition, the effect of chemotherapy on phenotypic subsets and their TL profiles was also compared in samples collected both pre- and post-treatment.
Chapter 3: Telomere profile characterisation in CLL patients

3.3 Results

3.3.1 In-depth telomere analysis in individual CLL patients

In order to study the underlying telomere length distributions of different cells present in each CLL patient’s peripheral blood, immunophenotyping and FACS was used to separate T-cells, normal B-cells and CLL B-cells from CLL patients’ peripheral blood and their TL were determined using STELA of the XpYp telomere.

The main advantage of using the STELA technique is its ability to measure extremely short telomeres and hence the capacity to derive very accurate mean TL measurements. In addition, STELA provides a representation of the TL profile within a given cell population, the heterogeneity of TL profiles reflects the replicative history of the cell population, telomerase expression and the clonal composition. As STELA is a single-molecule technique very small amounts of DNA are required to obtain robust TL profile and thus it is possible to obtain this data in cell populations present at very low frequencies, such as normal B-cells in CLL patients’ peripheral blood.

Figure 3.1 illustrates the gating strategy used to separate the different cell types and their respective telomere profiles. In this example patient, there were differences in TL profiles (Figure 3.2) between the unsorted peripheral blood mononuclear cell fraction (PBMC, mean TL 7.09kbp), T-cells (mean TL 6.25kbp) and normal B-cells (mean TL 7.77kbp). The TL profile of the normal B-cells was derived from just 5000 cells sorted from the sample.

The difference between the mean TL of the PBMC fraction and the purified CLL B-cell fraction was statistically significant (7.09kbp vs. 4.46kbp respectively; P<0.0001). In addition to these significant differences in TL, there was a significant difference in the variation in TL seen in each cell population with the
standard deviation (SD) of TL being 2.86kbp for T-cells, 3.49kbp for normal B-cells and 1.72kbp for CLL B-cells, all of which were statistically significantly different (P<0.01; ANOVA). The reduction in the heterogeneity of the TL profiles observed in CLL B-cell populations is consistent with the expected clonal growth of these cells. This contrasts with the more heterogeneous TL profiles observed in the normal cell populations, that is consistent with the heterogeneous proliferative lifespan of these cell types.

This example patient clearly demonstrates how peripheral blood cellular populations including T-cells, normal B-cells and CLL B-cells have distinct telomere length profiles. Importantly, the purified CLL cell TL profiles were different to the overall unsorted PBMC TL profiles providing evidence for the importance of deriving a pure cell population before analysing and interpreting TL data.
Figure 3.1. Gating strategy utilised for obtaining purified populations of T-cells (CD3⁺), CLL B-cells (CD19⁺/CD5⁺), normal B-cells (CD19⁺/CD5⁻) from a single CLL patient. T-cells, B-cells and CLL B-cells were separated using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 with a FACS Aria high-speed cell sorter.
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Figure 3.2. Telomere length profiles of T-cells (CD3+), CLL B-cells (CD19+/CD5+), normal B-cells (CD19+/CD5-) and unsorted PBMC from a single CLL patient. Following separation, DNA was extracted using Qiagen Micro kit and STELA was performed using XpYp specific probes. Telomere length was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests were used to determine significance between telomere length profiles. (a) gating strategy for the identification of T-cells, B-cells and CLL B-cells (a) XpYp STELA gel showing the telomere profiles of four cell populations from one CLL patient including unsorted PMBC, T-cell, normal B-cell and CLL B-cell fractions (b) TL data plotted as individual TL profiles (c) frequency histogram showing relative frequency of telomeres by length for each cell population.
3.3.2 Telomere length of peripheral blood subsets

In order to determine if the mean TL of T-cells, normal B-cells, CLL B-cells and overall PBMC fractions differed, samples were obtained from a total of 86 CLL patients and discreet populations of T-cells, normal B-cells and CLL B-cells were isolated by FACS, as shown in Figure 3.1a.

The mean TL of the normal B-cells was shown to be the longest at 5.34kb (±1.28) whilst the CLL B-cell fraction had the shortest mean TL (4.15kb (±1.89); Figure 3.3a). Comparison of these mean TL profiles also showed that the CLL B-cell fraction was statistically significantly shorter than the T-cell fraction (P=0.04) and the normal B-cell fraction (P=0.004). The T-cell fraction (4.35kb (±1.01)) was also shorter than the normal B-cell fraction (P=0.03). The purified CLL B-cell fraction was also shorter than the PBMC fraction though this difference did not achieve statistical significance (P=0.07). This is perhaps not surprising given that the CLL B-cell clone dominates the peripheral blood in the majority of CLL patients.

The number of telomere populations from normal B-cells reported here was notably less than of the CLL B-cells, T-cells (n=16 vs. 83 and 86 respectively) due to the difficulty in obtaining sufficient numbers of normal B-cells to allow for successful STELA analysis. This low number was despite the fact that telomere profiles were produced from as few as 5000 cells, often it was not possible to obtain even these small numbers from patients with peripheral blood composition often dominated by the CLL B-cell clone.

The individual TL profiles of cells sampled from the T-cell compartment showed the largest standard deviations with a mean of 2.06kbp compared to 1.99kbp for normal B-cells (P=0.11), and 1.36kbp (P<0.001) for the CLL B-cells (Figure 3.3b). The difference between the unselected PBMC compartment (mean SD 1.84kbp) and the CLL B-cell compartment was also significantly different (P=0.002). This standard deviation data confirmed that each individual CLL B-cell
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TL profile showed much less variance consistent with clonal growth. However, the CLL B-cell fraction showed the widest inter-patient variation in the mean TL suggesting that whilst the distributions were clonal, their lengths were not homogenous with some patients’ CLL B-cells manifesting very short TL and others much longer TL profiles (Figure 3.3).
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Figure 3.3. Individual cell populations from CLL patient peripheral blood, including T-cells, normal B-cells and CLL B-cells, have significantly different mean telomere length profiles. T-cells, normal B-cells and CLL B-cells were separated using a cocktail of CD19-APC, CD5-FITC and CD3-PE antibodies using a FACS Aria. Following separation DNA was extracted from these sorted cell populations as well as an unsorted PBMC sample using Qiagen Micro Kit and STELA was performed at XpYp. TL was determined using Phoretix1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests were used to determine significance between TL profiles. (a) TL from PBMC (n=33), T-cells (n=86), normal B-cells (n=16) and CLL B-cells (n=83) (b) TL SD from the same samples.
3.3.2.1 Telomere length varies significantly in intra-patient comparison

To study in more detail the comparison between the unsorted PBMC and CLL B-cell fractions, mean TL measurements from paired PBMC and sorted CLL B-cell populations from 33 patients were analysed (Figure 3.4). A statistically significant difference was observed between purified CLL B-cells and unsorted PBMC; purified CLL B-cells manifested a shorter mean TL of 4.32kbp (±1.88) vs. 4.67kbp (±1.54) for PBMC (P=0.002). There was also a statistically significant difference when comparing PBMC and CLL B-cell TL standard deviation (1.84kbp vs. 1.32kbp; P<0.001). Furthermore, paired samples were analysed from 9 patients comparing normal B-cell and CLL B-cell mean TL as well as TL SD. These comparisons revealed larger differences in mean TL (5.16kbp vs. 3.5kbp; P=0.003) and SD (2.41kbp vs. 1.33kbp; P<0.001). These findings confirm that the mean TL is significantly different between these cell populations and that sorted cell populations must be obtained to provide an accurate measurement of either. Furthermore, these differences illustrate that TL could be significantly overestimated if unselected PBMC were analysed instead of a purified CLL B-cell population.
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Figure 3.4. Paired analysis of telomere length in CLL patients. Normal B-cells and CLL B-cells were separated and then DNA was extracted from these sorted cell populations as well as from a matched unsorted PBMC sample using Qiagen Micro Kit and STELA was performed using XpYp-specific primers. TL was determined using Phoretrix 1D software and statistical analysis was performed using Graphpad Prism 6. Student’s t-test was used to determine significance between telomere length profiles. (a) Paired analysis from 33 patients showing a significant difference in the mean TL and (b) TL standard deviation between unsorted PBMC and purified CLL B-cell populations. (c) Paired analysis from 9 patients comparing mean TL and (d) TL standard deviation between normal B-cells and CLL B-cells.
3.3.3 Age-related telomere length changes in CLL patients’ peripheral blood lymphocytes

The TL of PBMCs, as well as isolated granulocytes and lymphocyte populations, were shown to shorten with increasing age (Daniali et al., 2013, Rufer et al., 1999). In order to determine if this was also the case for lymphocyte subsets derived from CLL patients, the correlation between age at the time of sample collection and mean TL of CLL B-cells, normal B-cells and T-cells were assessed (Figures 3.5 and 3.6).

The normal B-cells (CD19+/CD5-) derived from CLL patients conformed to the published dogma in that they showed a statistically significant inverse correlation between age and TL ($r^2$=-0.61; $P$=0.01, Figure 3.5a). From this data it was possible to estimate that the mean TL shortening per year was 69bp, which is consistent with the previously published work by Rufer et al (1999). The standard deviation of the mean TL in this subset was not associated with age (Figure 3.5b) implying that normal B-cell clonal diversity was not significantly affected by age. In contrast to the normal B-cell subsets, there was no correlation between malignant CLL B-cell TL and age in the patient cohort ($r^2$=0.01; $P$=0.30, Figure 3.5c) suggesting that the TL of the malignant B-cells was not age-dependent. Intriguingly, neither was there a significant correlation between age and the TL of the T-cells derived from CLL patients ($r^2$=0.03; $P$=0.09, Figure 3.6a) suggesting that the T-cell pool does not age in the same way as healthy age-matched individuals. Furthermore, there was a weak correlation between CLL B-cell TL and T-cell TL in matched samples from individual patients ($r^2$=0.076; $P$=0.045, Figure 3.7a), but this was not apparent when separated into treated or untreated cohorts (Figure 3.7b and 3.7c).

Comparison of Normal B-cell TL to paired patient T-cell TL showed that there was a suggestion of a correlation, but that this did not achieve statistical significance ($r^2$=0.17; $P$=0.16, n=13) (Figure 3.8).
Figure 3.5. Correlations between age and telomere length in CLL B-cells and normal B-cells derived from CLL patients. CLL B-cells and normal B-cells and were isolated using high-speed cell sorting, the DNA was then extracted and XpYp STELA was performed. TL was determined using Phoretrix1D software and analysed using Graphpad Prism 6. Correlation and linear regression analyses were performed to assess the relationship between age and (a) mean TL and (b) the standard deviation of TL in (B-cells (CD19+/CD5-) derived from CLL patients (n=16). Similarly, the relationship between age and (c) mean TL and (d) the standard deviation of TL in CLL B-cells (CD19+/CD5+) from CLL patients was also performed (n=83).
Figure 3.6. Mean telomere length of T-cells derived from CLL patients show no correlation with age. As above, T-cells, normal B-cells and CLL B-cells were isolated using high-speed cell sorting and DNA was extracted. XpYp STELA was then performed. TL was determined using Phoretrix1D software and analysed using Graphpad Prism 6 software. Correlation and linear regression analyses were performed to assess the relationship between (a) TL and (b) TL standard deviation and age (n=86).
Figure 3.7. Mean telomere length of T-cells derived from CLL patients show a strong correlation with CLL B-cell telomere length. As above, T-cells, normal B-cells and CLL B-cells were isolated using high-speed cell sorting and DNA was extracted. XpYp STELA was then performed. TL was determined using Phoretix1D software and analysed using Graphpad Prism 6 software. Correlation and linear regression analyses were performed to assess the relationship between (a) patient matched CLL B-cell TL and T-cell TL in the whole cohort (n=50) (b) in the untreated cohort (n=24) (c) in the treated cohort (n=26).
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Figure 3.8. Mean telomere length of T-cells shows a correlation with normal B-cell telomere length. Correlation and linear regression analyses were performed (a) Shows the correlation between patient matched T-cell TL and normal B-cell TL (n=13).
3.3.4 The proportion of peripheral blood lymphocyte populations differ between CLL patients and are altered by treatment

CLL is a chronic condition and patients are likely to have the disease for many years and often for several decades. During this period many patients are simply monitored and there is no active intervention, whilst others develop symptoms and consequently require treatment. On receiving chemotherapy or chemoimmunotherapy the proportion of malignant and non-malignant lymphocytes present in their peripheral blood may be altered due to differential toxicity and/or the divergent renewal capacity of lymphocyte subsets (Gassner et al., 2011).

To further examine whether the proportion of CLL B-cells and T-cells was different between untreated patients and those who had received chemotherapy, a cohort of 23 untreated and 47 chemotherapy treated patients were studied and the overall percentage of CLL B-cells and T-cells within lymphocyte gate determined. Data was collected using a FACS Aria high-speed cell sorter and an example FACS plot is shown in Figure 3.9.
Figure 3.9. Example FACS plots showing the comparison between untreated and post treatment peripheral blood lymphocyte populations. Fluorescence-labelled antibodies recognising CD19, CD3 and CD5 were incubated with peripheral blood from untreated and post chemotherapy CLL patients’ peripheral blood. These samples were then analysed using a FACS Aria high-speed cell sorter. Data was analysed using FlowJo software.

The percentage of CLL B-cells (CD19+/CD5+) showed a significant difference between untreated and treated patients; the mean percentage in the untreated patients was 82.9% (±16.0) compared to 45.7% (±41.8%) in post-treatment patients (Figure 3.10a; \(P=0.003\)). Within the treated group, 14/47 showed a dramatic reduction in CLL B-cells to less than 5% of the lymphoid cells present. In contrast, 18/47 had greater than 80% CLL B-cells present post treatment.

The percentage of CD3+ T-cells present in the lymphocyte gate differed less dramatically with a mean of 10.6% (±12.96%) in the untreated group compared to 15.6% (±17.33%) in the post treatment group (\(P=0.68\)). This suggests a trend towards a higher percentage of T-cells present in the post treatment group and would be consistent with the preferential reduction in CLL B-cells following successful treatment. Chemotherapies such as fludarabine cause significant cytoreduction in the CLL B-cell tumour mass in both peripheral blood and bone
marrow and this is likely responsible for the above findings (Thompson and Wierda, 2016). However, it is well established that these drugs are also toxic to T-cells (Fenchel et al., 1995, Goodman et al., 1996); the data presented here describe the relative abundance of T-cells pre- and post-therapy rather than the absolute numbers of T-cells.

These data demonstrate the different responses seen in patients treated with chemotherapy with some patients showing objective clinical response and marked clearance of CLL B-cells consistent with ongoing remission. In comparison, some patients showed the persistence of high levels of peripheral blood CLL B-cells consistent with relapse or poor/incomplete response to treatment.

![Figure 3.10. Cell populations differ in untreated and treated CLL patients.](image)

Figure 3.10. Cell populations differ in untreated and treated CLL patients. T-cells, normal B-cells as well as CLL B-cells were identified and sorted using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 with a FACS Aria high speed cell sorter. Flow cytometry data was analysed in FlowJo software. Statistical analysis using Mann-Whitney U test was carried out in Graphpad Prism 6. (a) Gating strategy for the identification of T-cells, normal B-cells and CLL B-cells from CLL patients showing an example untreated patient (left) and post treatment patient (right) (b) The percentage of CLL B-cells measured in untreated (n=23) and treated (n=47) CLL patients (c) The number of T-cells in untreated (n=23) and treated (n=47) CLL patients.
To further analyse the effect of treatment on the percentages of CD19⁺ CLL B-cells and CD3⁺ T-cell, the treated patient group was sub-divided by the type of chemo/chemoimmunotherapy that they had received. Figure 3.11 shows the percentage of CLL B-cells was lowest in the group treated with FCR; consistent with the prolonged responses seen with this treatment and its status as the current gold standard for the treatment of CLL (Hallek and Pflug, 2011). The next lowest percentage of CLL B-cells was seen in the group treated with FC, which was the gold-standard treatment choice until the addition of Rituximab (Catovsky et al., 2007). The highest percentage of CLL B-cells remaining in the post-treatment samples was seen in the group labelled ‘other’ which included both Fludarabine and Chlorambucil monotherapy.

![Figure 3.11. Cell populations differ depending on the type of treatment received CLL. T-cells, normal B-cells as well as CLL B-cells were separated using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 using a FACS Aria high-speed cell sorter and analysed in FlowJo software. Statistical analysis using Mann-Whitney U test was carried out in Graphpad Prism 6. (a) The percentage of CLL B-cells and (b) T-cells present in the lymphocyte gate of treated CLL patients separated by chemotherapy type (FC = Fludarabine and Cyclophosphamide, RFC = Rituximab, Fludarabine, Cyclophosphamide +/- Mitoxantrone, other = all other chemotherapies used including Chlorambucil and Fludarabine monotherapy).]
These findings demonstrate the variable frequency of CLL B-cells and T-cells in the peripheral blood of CLL patients following treatment. Given the different TL profiles demonstrated between the lymphocyte subsets, these data highlight the need to accurately identify and separate these fractions before TL analysis is performed.
3.3.5 Mean telomere length and telomere length standard deviation differ in patients treated with chemotherapy

As previously discussed, CLL patients may receive chemotherapy or chemo-immunotherapy as part of their disease management. In order to determine if mean TL and TL SD varied between untreated or post-treatment patients, mean TL and SD were compared. The untreated group’s mean age was 69.8 years, which was significantly older than the treated group; mean age 61.8 years (P<0.01; Figure 3.12).

![Figure 3.12. CLL Patient age in untreated and treated groups.](image)

CLL B-cells were separated and following separation, DNA was extracted from these sorted cell populations as well as an unsorted PBMC sample using Qiagen Micro Kit and STELA was performed at XpYp. TL was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests were used to determine significance between (a) untreated (n=31) and treated (n=47) CLL patients age and (b) mean CLL B-cell TL by stage of disease (stage A n=31, stage B/C = 28).
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Mean TL distributions were longer in the untreated group vs. the post treatment group (4.85kbp vs. 3.85kbp, P=0.02), though the standard deviation did not show a significant difference (1.42kbp vs. 1.37kbp, P=0.26) (Figure 3.13). Given that normal lymphocyte TL decreases with age, this finding is contrary to what would be expected if TL shortening in CLL were an age-related phenomenon.

Figure 3.13. Telomere profiles in CLL patient CLL B-cells separated depending on previous exposure to chemotherapy. T-cells, normal B-cells and CLL B-cells were separated and DNA was extracted. STELA was performed using XpYp-specific primers. TL was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses including t-tests were used to determine significant differences between the TL profiles. (a) TL and (b) TL SD in CLL B-cells in untreated (n=31) and treated (n=47)

Previous publications, including Lin et al., (2010), have shown that patients with advanced stage disease have shorter TL and this may account for the above findings, as it is patients with Binet stage B and more commonly stage C that are likely to be treated with chemotherapy. These findings are consistent with this published data but do not show whether exposure to chemotherapy itself is responsible for the telomere shortening. The cohort studied here replicated these findings with the patients with stage A disease having a significantly longer TL mean at 4.62kbp vs 3.65kbp for the patients with stage B/C disease (P=0.05; Figure 3.14).
There was no suggestion in this data that exposure to chemotherapy altered the SD, and hence the clonality, of the TL distributions present in the samples suggesting that exposure to chemotherapy does not induce selective pressure based on TL.

The T-cells showed significantly shorter TL in patients with advanced stage, with stage A patients T-cells having a mean TL of 4.676kbp (SD 1.19, n=27) and stage C patients having a mean TL of 3.977kbp (SD 0.89, n=31)(P=0.01). The standard deviation of the T-cells telomeres was also significantly smaller in stage B/C compared to stage A patients (2.16 vs 1.87, P=0.05). This data is consistent with previously published data that showed that patients who were positive for the prognostic marker Zap-70, had naïve and memory T-cells with significantly shorter telomere lengths (Figure 3.15)(Roth et al., 2008).
Figure 3.15. CLL Patient mean T-cell telomere length in different stage of disease. T-cells were separated and following separation, DNA was extracted from these sorted cell populations as well as an unsorted PBMC sample using Qiagen Micro Kit and STELA was performed at XpYp. Telomere length was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests were used to determine significance between mean CLL B-cell TL by stage of disease (stage A n=27, stage B/C = 31).
The T-cell populations showed a different pattern from the CLL B-cells in that their mean TL was not significantly different in the untreated and treated patient groups (4.42kbp vs. 4.32kbp, P=0.53) (Figure 3.16a). This is a different pattern than what was expected given that we previously showed that CLL B-cell TL and T-cell TL were correlated (Figure 3.6, \( r^2 = 0.076 \), P=0.045), and that CLL B-cell TL was shorter in the treated group when compared to the untreated group. The most obvious explanation for this is the frequency of short telomeres was much higher in patients with advanced stage disease (Lin et al., 2010); the majority of these patients would require treatment, whereas patients with indolent disease (and long telomeres) would not require treatment.

There was a significant reduction in standard deviation (2.24kbp vs. 1.96kbp, P=0.02; Figure 3.16b) suggesting that the range of TL present within the T-cell populations were decreased following exposure to chemotherapy. As above however, it is not clear whether it was exposure to chemotherapy itself, or progression to a stage where chemotherapy was required, was the factor responsible for this decreased variance in TL. There was no difference seen between the TL or the standard deviations of the normal B-cell population based on exposure to chemotherapy (Figures 3.16c and 3.16d respectively). This confirms findings previously published that show that TL is different in CLL prognostic subgroups and furthermore has been demonstrated to be a powerful prognostic tool in this disease (Lin et al., 2010, Lin et al., 2014, Pepper et al., 2012).
Figure 3.16. Telomere profiles in CLL patient T-cells and normal B-cells separated depending on previous exposure to chemotherapy. T-cells, normal B-cells and CLL B-cells were separated and DNA was extracted. STELA was performed using XpYp-specific primers. TL was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses including t-tests were used to determine significant differences between the TL profiles. (a) TL and (b) TL SD in T-cells from treated (n=49) and untreated (n=34) patients (c) TL and (d) TL SD in normal B-cells from treated (n=8) and untreated (n=3) patients.
3.3.6 Healthy donor T-cells and B-cells

Damle et al (2004) previously used Flow-FISH to show that the telomere length of B-cells in CLL patients was shorter than those of healthy donor B-cells (Damle et al., 2004). There are no previous studies detailing the STELA telomere profiles of B-cells and T-cells from healthy donors, though B-cell telomere profiles from healthy donors were shown as comparators by Lin et al (2010). To provide context for the CLL patients’ TL data, T-cell and normal B-cells were isolated from a small cohort of healthy donors. To detail the telomere profiles of the healthy donor B-cell and T-cell populations, CD3+ T-cells and CD19+ B-cells were isolated from the peripheral blood of the healthy donors.

The TL profiles of both T-cells and B-cells are shown in Figure 3.17. The mean TL of the T-cells was shorter than the B-cells though this difference was not statistically significant (5.32kbp (±2.51kbp) vs. 7.34kbp (±3.12kbp), P=0.07). The telomere length profiles of both the normal B-cell and T-cell populations displayed heterogeneous TL profiles with the standard deviation of the TL being 2.51kbp and 2.99kbp respectively, consistent with the extensive replicative history expect of these cell types. Though the B-cell population showed a higher overall standard deviation, this was not statistically significant in comparison to the T-cell population.
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Figure 3.17. Telomere profiles of healthy donor T and B-cells. T-cells and B-cells were purified from whole blood using CD19+ and CD3+ beads for positive selection with an AutoMACS Pro-Separator. DNA was extracted using Qiagen Micro Kit and STELA was performed at XpYp. Statistically significant differences in mean TL and TL standard deviation (SD) were determined by t-test. Molecular weight is indicated on the left of the STELA gel and the corrected TL is indicated on the right. The red line on (a) indicates the molecular weight in kilo base pairs (kbp) with the red lines on (b) indicating the TL in kilo base pairs. (a) STELA gel showing TL profiles from T-cells (n=4) and (b) B-cells (=6) from healthy donors. The plotted (c) mean and (d) SD for the healthy donor T-cells and B-cells are shown.
3.3.6.1 Similar mean telomere length and telomere length standard deviation of T-cells and normal B-cells in CLL patients and healthy donors

Normal B-cells derived from CLL patients (n=11) had a shorter mean TL of 5.16kb in comparison with healthy donor normal B-cells (n=6) at 7.34kb, which was statistically significant (P=0.01, Figure 3.18). T-cells from CLL patients (n=86) also shorter mean TL (4.35kb ±2.06kb) than healthy donor T-cells (n=4) (5.23kb ±2.51kb), but this was not statistically significant (P=0.15). The SD of the CLL patient T-cell TL was smaller than that seen in age-matched healthy controls (2.06kb vs. 2.51kb), but again this was not statistically significant (p=0.1). These results show that the mean TL of normal B-cells and T-cells found in CLL patient are shorter than those of healthy donors, though due to the small numbers in this part of the study this difference did not always reach statistical significance.
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Figure 3.18. Mean telomere length and telomere length standard deviation of normal B-cells and T-cells derived from CLL patients and healthy donors. T-cells, B-cells and CLL B-cells were separated using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 using a FACS Aria high-speed cell sorter. Following subset isolation, DNA was extracted using Qiagen Micro kit and STELA was performed on XpYp. TL was determined using Phoretix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests were used to determine significance between TL profiles. (a) Comparison of mean TL between healthy donor B-cells (n = 6) and CLL patient normal B-cells (n=11) (b) comparison of TL SD between healthy donor B-cells and CLL patient normal B-cells (c) comparison of mean TL between healthy donor T-cells (n=4) and CLL patient mean T-cell (n=86) (d) comparison of TL SD between healthy donor T-cells and CLL patient T-cells.
3.4 Discussion

This study was designed to perform an in-depth analysis of TL distributions of peripheral blood lymphocytes from CLL patients with the aim of characterising both their TL and also the variation of TL present. No previous studies have presented detailed TL profiles of lymphocyte subsets in the peripheral blood of CLL patients. This study demonstrates that normal B-cells, T-cells and CLL B-cells all have distinctly different TL profiles. Furthermore all of these individual profiles are distinct from the overall TL profile obtained by analysing unsorted PBMC. Indeed, according to the data presented here, TL analysis of PBMC rather than purified CLL B-cells would result in the over estimation of CLL telomere length by approximately 0.3kb. Given that previous work by our group has shown that both 3.81kb and 2.26kb are prognostically important CLL B-cell TL thresholds (Lin et al., 2010, Lin et al., 2014), assessment of purified CLL B-cells would be essential in order to avoid incorrect allocation of some individuals into the wrong prognostic subset.

One significant challenge encountered during this work was the fragility of the CLL B-cells during the process of high-speed cell sorting. Initial attempts to obtain STELA telomere profiles were unsuccessful despite significant numbers (>300,000 cells) being sorted. Through multiple sequential alterations to the methodology, it was deduced that the process of centrifugation and freezing of CLL cell pellets did not allow successful reclamation of the DNA on thawing. This is likely, at least in part, due to the documented increased cell fragility seen in CLL (Rizzo et al., 2013, Nowakowski et al., 2007) leading to the cells lysing following cell sorting or the freeze/thawing process resulting in the DNA being degraded to an unusable quality. As a consequence, DNA was extracted immediately post sorting and this resulted in significantly improved yields of high-quality DNA.

The data generated in this chapter shows that CLL B-cell and T-cell TL mean are not very dynamic over the with very little erosion seen in these cell populations.
as a function of age. The normal B-cells showed a telomere erosion rate of 69bp/year and this is comparable to the known lymphocyte TL erosion rate in normal healthy adults of a similar age to that of this CLL patient cohort (Rufer et al., 1999).

The limited TL erosion seen in the T-cell population in this study runs contrary to the published data showing that lymphocyte telomere length decreases with age and the cause of this difference is not clear. There are several possible explanations for the lack of on-going TL shortening with age in addition to the data suggesting that T-cell TL is shorter in CLL patients than in normal age matched healthy controls. One possibility is that the lack of evidence of on-going TL loss is due to the TL loss that occurring in the T-cells at an earlier stage in the disease process.

The relative heterogeneities of the TL profiles described in this chapter are consistent with clonal growth of CLL B-cell with their individual TL profiles showing much less variety in comparison to the same patients T-cell and normal B-cells that exhibit natural within population variation in proliferative lifespan. The clonality observed in the CLL B-cells does not appear to alter with age, in comparison there is a suggestion that clonality does increase with age in the T-cell compartment. This could however reflect the presence of a more restricted T-cell repertoire in older patients (Qi et al., 2014, Yager et al., 2008). This progressive decrease in the standard deviation of the T-cell TL could be consistent with the previously published findings that there is an increase in exhausted phenotype T-cells in patients with disease progression (Brusa et al., 2013) though this would also be contrary to the finding that patients who had received treatment and therefore presumably had progressive disease had a younger mean age than the untreated group. One question that could not be answered here was whether the change in mean TL could be attributed to an alteration in the balance of T-cell subsets. For example, an increase in memory EMRA subsets and a decrease in naïve T-cells could be reflected by the decreased mean TL of the overall T-cell compartment. This issue could be
resolved by the further dissection of telomere dynamics within the various T-cell subsets in CLL patients.

The difference in CLL B-cell mean TL demonstrated in this cohort shown between patients with stage A and stage B/C was statistically significant and consistent with previously published data using STELA in CLL cohorts (Lin 2010 and Lin 2014). The difference also confirms that seen between treated and untreated groups. These data highlight that TL is a strong indicator of prognosis and therefore a predictor of the need for treatment in CLL. The cohort studied here was an unselected group of patients that were treated outside of the guidelines of a clinical trial and it supports the real world use of TL as a prognostic factor.

The TL profiles observed in the normal B-cells derived from CLL patients throws the above observations into stark contrast particularly given that a strong correlation between normal B-cell TL and increasing age was observed. Furthermore, normal B-cell TL standard deviation did not appear to decrease with increasing age, differing markedly from the T-cell subset data. This likely represents the different biology of these two cell populations with B-cells being produced throughout life in comparison to T-cells, where the repertoire is largely fixed after puberty due to thymic involution (Murphy K et al, 2008). Numerous publications have demonstrated the significant interaction between T-cells and CLL B-cells in CLL with significant evidence that there is T-cell exhaustion in poor risk CLL (Mackus et al., 2003, Ramsay et al., 2008, Bagnara et al., 2011, Gorgun et al., 2005, Gothert et al., 2013). The above data gives further strength to the belief that T-cell population are intrinsically linked with the CLL B-cells. In contrast, normal B-cells from CLL patients retain an age-related decrease that appears unaffected by the CLL disease process.

TL was comparable in the T-cell compartment between healthy donors and CLL patients. This was not seen in the B-cell compartment where significantly shorter TL was seen in comparison to the healthy donor group. The healthy
control data provided an important comparison and demonstrated that the normal B-cell mean TL in CLL patients is significantly different from that found in healthy adults. The significant drawback of this comparison data however is that the number of healthy controls analysed was limited and they were not age-matched to the mean of the CLL patient group. It is therefore possible that the TL represented in the healthy control cohort is not a true reflection of an age-matched control cohort. It is possible that part of the cause for the overall difference in mean TL between normal B-cells in healthy donors vs. CLL patients (7.339kbp vs. 5.162kbp, P=0.01) described in this study is due to a degree of contamination with CLL cells (which possess overall shorter telomere lengths) in the ‘normal B-cell’ cell fraction obtained from the CLL patients. This may have been caused by the method for separating these two different populations was based on gating on CD19 and CD5 co-expression. Given that CD5 expression can differ between CLL patients and even within a CLL B-cell clone, some of the CLL B-cells expressing lower surface CD5 may have been sorted alongside the normal B-cell population and subsequently included in the normal B-cell STELA TL profile (Morice et al., 2008). Another possibility is that the shorter mean TL seen in both the T-cells and B-cells within the CLL cohort is due to the older mean age of this group in comparison to the healthy donors and that some of the difference is due to the previously described age related shortening in TL.

In summary, the results of this chapter show clearly that the TLs of purified lymphocyte populations in the peripheral blood of CLL patients differ from each other. With normal B-cell populations exhibiting an age-related decrease in TL, in contrast to the clonal CLL B-cells that exhibit no such decrease. Unexpectedly, the T-cell compartments did not show a significant age-related decrease in TL in comparison with the expected age-related TL erosion seen in normal healthy donors of similar age. To further investigate CLL B-cell and T-cell TL dynamics, we proceeded to analyse sequential samples taken from CLL patients and these results are discussed in Chapter 4.
Chapter 4

Longitudinal telomere length profiling in CLL patients

4.1 Abstract

Patients with CLL often live with their disease for many years; some require simple monitoring and observation, whilst others need repeated intervention with treatment such as combination chemo-immunotherapy (Hallek, 2017). The long-term nature of this follow-up allows the opportunity for longitudinal investigation into the modulation of telomere length with both the passing of time and exposure to chemotherapy. The objectives of this chapter were to use FACS and STELA to undertake sequential analysis of the phenotype of CLL B-cells, T-cells and also normal B-cell populations and to examine the telomere lengths from these repeat samples over the course of an individual patient’s disease. The effect of increasing age, as well as disease progression and exposure to chemotherapy, were also investigated. Furthermore, sequential T-cell receptor sequencing was performed to establish whether the diversity of the T-cell pool is altered throughout the course of the disease.

Telomere length of CLL B-cells analysed in of a cohort of 41 patients on a minimum of two occasions over a median follow up time of over 6 years showed TL erosion correlated strongly with starting telomere length. There was significant difference in TL dynamics seen based upon the starting mean TL of the population with the previously determined fusogenic cut-off of 3.81kbp proving useful as a way of demarcating two different TL behaviours.

In contrast to the CLL B-cells, the T-cell TL erosion was more pronounced in the 9 patients in who repeated measurements were done over similar time periods to that of the CLL B-cell data. Consistent with this TL shortening,
immunophenotyping revealed that there was a significant increase in expression of markers of T-cell exhaustion. Significant inverse correlation was demonstrated between time from diagnosis to sample acquisition against T-cell TL suggesting that T-cell TL and TL shortening may be determined by how long the patient has had the disease. T-cell sequencing was performed and suggested increased skewing of the TCR repertoire over the course of the disease.

The data presented in this chapter demonstrates that whilst there is some TL erosion in CLL B-cells, it occurs in those cells that possess TL greater than the fusogenic range of 3.81kbp and is inversely correlated with starting TL. There is significantly more TL loss seen in T-cell however suggesting that the progressive TL shortening and increasing proportions of exhausted phenotype T-cells is a function of the CLL disease process.
Chapter 4: Longitudinal telomere length profiling in CLL

4.2 Introduction

Given the chronic nature of CLL, with median survival ranging from 6 to over 10 years depending on stage of disease (Molica and Levato, 2001), there is considerable opportunity for longitudinal repeat measurement of telomere profiles in this patient group. Patients are reviewed regularly in the hospital outpatient setting and frequently have their symptoms assessed alongside physical examination and monitoring of peripheral blood counts.

Whilst a significant proportion of patients will not need to undergo treatment, more than half of all patients will require therapy at some point in their care pathway, and they may undergo several courses of treatment and experience multiple relapses during their disease course and follow-up. It is well established that the T-cell pool is significantly perturbed during the disease, often with a profound skewing towards memory phenotypes (Forconi and Moss, 2015, Freeman and Gribben, 2016, Hamblin and Hamblin, 2008). In addition, exposure to chemotherapy and chemo-immunotherapy has a cytoreductive effect not only on the tumour cells but also normal lymphocyte subsets (Gassner et al., 2011, Riches and Gribben, 2014). What remains to be established is whether this has any impact on the TL profiles of these various cellular subsets. Having described significant variation within different patients’ CLL B-cell, T-cell and normal B-cell TL profiles in the previous chapter, possible changes in the TL profiles were investigated here by sequential measurement from samples taken from individual patients in a longitudinal fashion. In addition, the T-cell receptor repertoire was investigated in serially-derived samples to establish whether the T-cell pool is altered during the clinical course of the disease, with or without treatment intervention.
4.2.1 Aims of the chapter

In this chapter, an in-depth retrospective analysis was performed in sequential telomere length profiles from CLL B-cells (CD19+/CD5+), normal B-cells (CD19+/CD5−) and T-cells (CD3+) derived from peripheral blood using STELA. Data was collected from a total of 41 patients with the aim of understanding how TL profiles vary within individual patients during the course of their disease in distinct malignant and non-malignant lymphocyte sub-types. In addition, the effect of chemotherapy on phenotypic subsets and their TL profiles was also compared in samples collected from patients who were untreated against those who received treatment. Finally, the TCR repertoire was investigated in paired longitudinal samples to determine whether the T-cell pool was altered over time.
### 4.3 Results

**Table 4.1. Characteristics of the 41 CLL patients**

<table>
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### Chapter 4: Longitudinal telomere length profiling in CLL

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<td>58</td>
<td>A</td>
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<tr>
<td>41</td>
<td>69</td>
<td>C</td>
</tr>
</tbody>
</table>
4.3.1 In-depth sequential telomere length in an individual CLL patient

In order to study the temporal effects on telomere length profile distributions in individual patients’ peripheral blood, immunophenotyping and flow sorting were again used to purify CLL B-cells, T-cells and normal B-cells from patients with CLL. Telomere lengths were determined using STELA with primers specific for the XpYp telomere.

Figure 4.1 illustrates three sequential CLL B-cell TL profiles from a single patient (patient 1) taken over a time period of 68 months (5.6 years). In this example, there was an overall decrease in mean TL from 4.02kbp to 3.66kbp (total decrease of 0.36kbp) equating to an erosion rate of 63bp/year. Consistent with CLL being a clonal disease, the standard deviation of the TL did not significantly change during this follow-up period (1.06kbp at the start to 0.89kbp at the last measurement).
Chapter 4: Longitudinal telomere length profiling in CLL

Figure 4.1. Telomere length profiles of sequential CLL B-cell (CD19+/CD5+) samples from a single CLL patient. T-cells, B-cells and CLL B-cells were separated using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 with a FACS Aria high-speed cell sorter. (a) shows the gating strategy employed. Subsequently, DNA was extracted using Qiagen Micro kit and STELA was performed using XpYp specific probes. (b) shows STELA blots derived from the same patient’s CLL B-cells taken at different time points as detailed above (months). TL was determined using Phoretix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests, were used to determine significance between TL profiles at (c) shows the changes in telomere length profiles at time 0, 10 and 68 months. A normal, age-matched, B-cell control sample is shown for comparison.
Mansouri et al (2013) investigated telomere dynamics in CLL B-cells in a cohort of 119 patients over a period of 5-8 years and demonstrated that there was an overall reduction in TL over time though they did not quantify the degree of loss in bp/year (Mansouri et al., 2013). The above example patient demonstrates that STELA is able to deliver highly reproducible TL profiles that allow for exact measurement of changes in the TL and TL standard deviation.

4.3.2 Sequential CLL B-cell telomere length in CLL patient cohort

4.3.2.1 Longitudinal analysis of CLL B-cell telomere length

The in-vivo telomere length dynamics were investigated in a total cohort of 41 CLL patients who had the TL of their CLL B-cells measured on at least two occasions. The mean follow-up for the cohort of 41 CLL patients was 64 months (5.33 years, range 1-12.8 years) with the first sample taken at median age of 65 years, (range 41-79 years). The mean starting CLL B-cell TL was 4.294kbp and the end mean TL was 3.852kbp with a telomere length erosion rate of 74.2bp/year ($P=0.002$; Figure 4.2a). The mean standard deviation of the TL did not significantly alter over time; the mean standard deviation was 1.51kb in the initial samples and was 1.377kbp in the follow-up samples ($P=0.12$).

Once again, this data demonstrates evidence that there is statistically significant telomere length shortening in CLL patients’ CLL B-cells. Figure 4.3 however demonstrates that whilst there may be significant TL shortening, starting and end mean TL are still strongly correlated ($r^2 = 0.85$, $P<0.0001$).
Figure 4.2. Paired mean telomere length of CLL B-cells derived from CLL patients at the start and end of follow-up are significantly different. Paired $t$-tests were used to determine significance between (a) telomere length profiles and (b) standard deviation of CLL B-cells over a mean follow-up period of 63 months ($n=41$).

Figure 4.3. Temporal evaluation of the mean telomere length of CLL B-cells derived from the same patients showed a strong correlation. Telomere lengths were compared in a longitudinal study ($n = 41$). There was a strong correlation between start and end telomere length after a mean follow-up 63 months (range 12-154).
To further investigate this, the cohort was separated into two groups using the experimentally determined cut-off of 3.81kbp. This cut-off, the so-called ‘fusogenic threshold’ has been shown to be significant in both prognostic and biologically in delineating telomere dysfunction in the form of telomere fusion events. Lin et al (2014) demonstrated that telomere fusion events only occurred when mean CLL B-cell TL fell below 3.81kbp. Figure 4.4 shows that in patients with a starting TL above 3.81kbp, there was a significant decrease in mean TL from 5.80kbp to 4.90kbp (P<0.0001) over a mean follow up of 72 months equating to an telomere erosion rate of 151.8bp/year. However, in the patients with a starting mean TL below 3.81kbp there was no significant change in mean TL (2.707kbp vs. 2.745kbp, P=0.7), with an increase in TL at a rate 8.9bp/year over a mean of 53 months.

![Figure 4.4. Long-term follow-up in CLL patients bifurcated above and below the fusogenic threshold (3.81kbp). Paired mean telomere length show significant difference in patients’ TL when the initial mean was (a) TL >3.81kbp (n = 21). (b) In contrast, those patients with CLL B-cells with a mean TL <3.81kbp (n = 20) show no significant change in telomere length.](image)

It is clear from the above data that there are two very different patterns of TL behavior with significant TL shortening being seen only in CLL B-cells that possess longer telomeres with a mean TL above the previously determined fusogenic threshold. The TL in the group where the first sample was below the fusogenic range of 3.81kb showed no significant change.
Figure 4.5. Correlation between the starting telomere length and telomere length change per year in CLL B-cells derived from CLL patients. CLL B-cells were isolated and DNA was extracted. XpYp STELA was performed and TL determined using Phoretix 1D software and analysed in Graphpad Prism 6 software. Correlation and linear regression analyses were performed to assess the relationship between TL at the start of the observation period and extent of TL erosion expressed as the loss of TL per year (n=41).

Figure 4.5 shows the relationship between starting TL and overall TL change. There was a clear inverse relationship between initial TL and the amount of telomeric erosion i.e. the degree of erosion decreased as the starting TL decreased ($r^2=0.24$, $p=0.001$).

The telomere erosion rates in the above figure were calculated using the difference between the start and end mean TL and dividing it with the length of follow-up. This method gives an overall loss rate over the total follow-up time but it remains uncertain whether TL erosion in individual patients is constant. In order to investigate this further, additional (intermediate time point) samples were available for 10 of the patients. TL analysis was performed on these samples and linear regression performed to obtain a more accurate estimate of the telomere length change, an example of which is shown in Figure 4.6.
CD19+ CD5+ CLL B-cells

Figure 4.6. Telomere length profiles of sequential CLL B-cell (CD19+ /CD5+) samples from a single CLL patient. CLL B-cells were isolated, DNA was extracted and XpYp STELA was as performed as detailed above. A single patient’s TL analysis was repeated on 4 occasions over a period of 154 months. (a) shows the STELA TL profile data over time. (b) shows the mean TL plotted as a function of time. The slope of the linear regression line was used to calculate the telomeric erosion rate per year.

Figure 4.6b shows an example of one of the 10 patients with repeated measures of the TL, the line created by plotting the mean TL for a single patient does not appear to be entirely linear with most of the TL loss appears to occur over the early follow-up period in this patient. Other patients showed a variety of patterns though all telomere length loss decreased as the mean TL approached 3.81kbp. Rufer et al (1999) detailed that lymphocyte TL showed a rapid and significant decline during the first years of life with a bi-segmented line analysis showing increased statistical significance with a cut-off of 1.5 years over that of a simple linear distribution. CLL B-cells have significant opportunity for proliferation with published estimates show that the proliferation rate of CLL cells is approximately 1% of the total clone per day (Messmer et al., 2005). The rapid decline in TL documented by Rufer et al (1999) relates to early life where there is a marked period of unparalleled proliferation. It seems unlikely that this degree of proliferation is occurring in the CLL B-cells. Nonetheless, in all 10 of the CLL B-cell samples, with 3 or more repeated readings where this linear regression analysis was carried out, the calculated change in telomere length...
decreased, although the difference was not significant (figure 4.7, mean TL erosion for the 10 samples was -57.5bp/year vs. -46.9bp/year, P=0.14). It seems likely that the multiple measurements of the CLL B-cell TL gives a more robust estimate of overall telomere length change compared to those patients in which there was only two sampling points.

Figure 4.7. Analysis of CLL B-cell telomere length erosion rates compared by method of calculation (a) TL change over time showing no statistically significant difference between method of calculation (n=10). Standard method of calculation was using the difference between the start and end mean TL and dividing it with the length of follow-up, the calculated method involved linear regression analysis.
4.3.2.2 Follow-up by disease stage

To investigate if disease stage was associated with TL erosion rates, the cohort was sub-divided by using the Binet staging system. Figure 4.8 shows that Stage A patients (n=31) had a CLL B-cell starting mean TL of 4.51kbp (SD 1.49kbp) that was significantly reduced in the follow-up samples (mean TL of 4.03kbp (SD 1.42kbp) (P=0.004)); equating to a mean erosion rate of -81.8bp/year. Stage B and C patients (n=10) had a CLL B-cell starting TL of 3.64kbp (SD 1.59kbp) and an end mean TL of 3.32kbp (SD 1.28kbp) which was not significantly different (P=0.27). This TL change equated to an erosion rate of -47.4bp/year which is just over half of that seen in the stage A patients though this was not statistically significant (p=0.85).

![CD19+CD5+ CLL B-cells](image)

**Figure 4.8. Analysis of CLL B-cell telomere length erosion rates by stage of disease.** Binet stage A versus B/C patients showed differential changes in (a) TL over time showing not statistical difference between Binet stage A and Binet stage B/C patients.

The stage-specific analysis was consistent with the total cohort analysis; patients with longer telomeres showed more marked telomere erosion than those with short telomeres.
4.3.2.3 The effect of treatment on telomere length profiles

To investigate if exposure to chemotherapy influenced TL erosion, the cohort was also sub-divided into treated and untreated subsets. In all, four groups were studied: the total cohort, a group who remained untreated for the duration of sampling, a group who went on to receive treatment after the initial sample was taken and a group that had both of TL measurements carried out after treatment (Figure 4.9).

In the untreated patient group (n=20, mean follow-up 67 months), CLL B-cells had a starting mean TL of 4.03kbp (SD 2.0kbp) and an end mean TL of 3.79kbp (SD 1.7kbp) (P=0.015), in the post treatment follow-up patients (n=13, mean follow-up 66 months) mean starting TL 4.223kbp (SD 1.6kbp) and an end mean TL of 3.78kbp (SD 1.1kbp)(P =0.1), and finally patients treated in-between the samples (n = 13, mean follow-up 60 months) had a starting mean TL 4.44kbp (SD 1.9kbp) and end mean TL of 4.11kbp (SD 1.5kbp)(P=0.21). The TL erosion seen in the four groups is shown in Figure 4.9. No statistically significant difference was observed in any of the groups regardless of treatment history (multiple Mann-Whitney U tests, P=0.28 - 0.9). Figure 4.9 also suggests that the clonality of the tumour cells, as assessed by TL standard deviation, remained largely unchanged within the different groups (multiple Mann-Whitney U tests, P=0.16 - 0.9).
Figure 4.9. Analysis of CLL B-cell telomere length as a function of exposure to treatment. No significant differences in (a) Mean TL change/year in 41 patients with CLL and (b) TL standard deviation were apparent between any of the groups.
4.3.3. Long-term follow-up of T-cell TL

Having established the TL dynamics in CLL B-cells, I next investigated the in-vivo T-cell TL dynamics in a small cohort of 9 CLL patients who had the telomere length of their T-cells measured on a minimum of two occasions. Six patients had three or more measurements of their purified T-cells. The overall follow-up for the cohort of 9 CLL patients was 75.2 months (6.25 years, range 2-12.8 years). The mean start T-cell TL was 4.75kb and the end mean TL was 4.05kb with a telomere length erosion rate of -118.5bp/year ($P=0.02$; Figure 4.10a). This erosion rate is much greater than reported in peripheral blood lymphocytes in healthy people (Rufer et al., 1999, Weng et al., 1995). The mean standard deviation of the TL started as 2.42kb and was 1.91kb after 75 months ($P=0.02$) suggesting that the T-cell pool may be becoming more clonal over the course of the disease.

**Figure 4.10.** Paired mean telomere length of T-cells (CD3+/CD5+) derived from CLL patients at the start and end of follow-up is significantly different. Paired $t$-tests were used to determine significance between (a) the TL profiles and (b) the standard deviation of T-cells derived from patients diagnosed with CLL over a mean follow-up period of 75 months ($n=9$).

Figure 4.11 illustrates two sequential T-cell TL profiles from a single patient taken over a time period of 52 months (4.3 years). In this particular patient, there is evidence of marked TL shortening with the mean decreasing from...
5.38kb to 4.36kb (a total of 1.02kb) equating to an erosion rate of -237 bp/year.

**Figure 4.11.** Telomere profiles of sequential T-cell (CD3⁺) samples from a single CLL patient. As the data was shown to conform to a Gaussian distribution, a paired t-test was used to determine significance between T-cell TL profiles 52 months apart.

The above example of telomere length shortening is consistent with previously published data by Roth et al (2008) in which flow-FISH was used to determine TL and estimated the overall telomere length decrease in T-cells to be at most -94bp/year for naïve T-cells in a cohort of 30 CLL patients followed up for a median of 33 months. However, the data shown above (Figure 4.10) suggests that Roth et al may have significantly underestimated the degree of TL shortening that occurs in CLL patients’ T-cells but this would need to be confirmed in a much larger study (Roth et al., 2008).
As with the CLL B-cells, several of the patients (n=6) had three or more repeated measurements of their T-cell TL. Figure 4.12a shows an example of one of these 6 patients, with Figure 4.12b again demonstrating the line created by calculating linear regression to obtain a more accurate estimate of the overall rate of telomere length change.

Figure 4.12. Telomere length profiles of sequential T-cell (CD3+) samples from a single CLL patient. T-cells were isolated, DNA was extracted and XpYp STELA was as performed as detailed above. A single patient’s TL analysis was repeated on 5 occasions over a period of 154 months. (a) shows the STELA TL profile data over time. (b) shows the mean TL plotted as a function of time. The slope of the linear regression line was used to calculate the telomeric erosion rate per year.

Once again, no significant difference was seen overall between the telomere length change during long-term follow-up using this method (Figure 4.13)
Figure 4.13. Analysis of T-cell telomere length erosion rates as compared by their method of calculation (a) TL change over time showing no statistically significant difference between method of calculation (n=6). Standard method of calculation was using the difference between the start and end mean TL and dividing it with the length of follow-up, the calculated method involved linear regression analysis as described above in section 4.3.2.1.

4.3.3.1. Follow-up by disease stage

To investigate if disease stage was associated with T-cell TL erosion rates, the cohort was sub-divided by using the Binet staging system. Figure 4.14 shows that Stage A patients (n=6) had a T-cell starting mean TL of 4.56kbp (SD 0.81kbp) that was reduced in the follow-up samples (mean TL of 4.21kbp (SD 0.79kbp) (P=0.22)); equating to a mean erosion rate of -53.8bp/year. Stage B and C patients (n=3) had a T-cell starting TL of 5.14kbp (SD 0.55kbp) and an end mean TL of 3.71kbp (SD 0.19kbp), which was not significantly different (p=0.25). This TL change equated to an erosion rate of -247.8bp/year that is markedly higher than in Stage A patients though this difference did not reach statistical significance (P=0.09).
Figure 4.14. Analysis of T-cell telomere length erosion rates by stage of disease. Binet stage A versus B/C patients showed differential changes in (a) TL over time showing not statistical difference between Binet stage A and stage B/C patients.

This stage-specific analysis, despite the small numbers of patients involved demonstrates that patients with advanced stage disease tended to have much higher T-cell telomere length loss during the follow-up period.
4.3.4 T-cell telomere length in CLL patients relates to time since diagnosis

Given that the evidence presented here and elsewhere supports the concept of on-going telomere erosion in the ‘normal’ T-cell pool in CLL, we set out to establish whether this was caused by the preferential expansion of an oligoclonal population of T-cells, in response to a CLL-related antigen. This investigation into the expansion of an oligoclonal population of T-cells was done to identify if there was further evidence of increased clonality suggested by the decrease in T-cell TL SD described above. We addressed this by taking sequential samples from six CLL patients and compared their TCR repertoires over time. TCR sequencing was performed on CD3-purified samples by Adaptive Biotechnologies (Seattle, USA). The median time interval between samples was 76.5 months with a mean telomere erosion of more than 1kb over this timescale. Table 4.2 shows the Binet stage at diagnosis for each patient, the time from diagnosis to first T-cell TL analysis, the mean initial and follow-up TL and the time interval between sample collection.
Table 4.2. Patient information relating to serial T-cell analysis in samples derived from six CLL patients.

<table>
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<th>Binet Stage at diag.</th>
<th>Time to initial sample (months)</th>
<th>Initial mean CD3+ telomere length (±SD)</th>
<th>Follow-up mean CD3+ telomere length (±SD)</th>
<th>Time interval (months)</th>
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<td>Patient 1</td>
<td>C</td>
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<td>5.78kb ±2.63 Post-Tx</td>
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<td>Patient 2</td>
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<td>3.65kb ±2.39 Pre-Tx</td>
<td>3.19kb ±1.75 Post-Tx</td>
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<tr>
<td>Patient 3</td>
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<td>55</td>
<td>3.71kb ±1.85 Pre-Tx</td>
<td>3.49kb ±1.33 Pre-Tx</td>
<td>59</td>
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<tr>
<td>Patient 4</td>
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<td>4.82kb ±2.34 Post-Tx</td>
<td>3.58kb ±1.85 Post-Tx</td>
<td>76</td>
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<tr>
<td>Patient 5</td>
<td>A</td>
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<td>5.38kb ±2.71 Pre-Tx</td>
<td>4.36kb ±1.89 Pre-Tx</td>
<td>54</td>
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<td>Patient 6</td>
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<td>6.31kb ±2.00 Pre-Tx</td>
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<tr>
<td>Median time interval (range)</td>
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<td>Mean telomere length (±SD)</td>
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<td>4.94kb ±2.32 3.93kb ±1.82</td>
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Tx = treatment

As the date of diagnosis and the date when the initial and follow-up samples were known, it was possible to plot TL as a function time since diagnosis. There was a strong inverse correlation between initial T-cell TL and time since diagnosis (Figure 4.15a). The same inverse correlation was also observed when plotting the follow-up T-cell TL as a function time since diagnosis although the relationship was not as strong (Figure 4.15b).
These data suggest that T-cell telomere erosion is an on-going process in CLL and is related to the time since diagnosis. It was also possible to calculate the average T-cell TL erosion rate for each individual. Figure 4.15c shows a clear relationship between initial T-cell TL and the average erosion rate. As was the case for CLL B-cells, the longer the initial TL the greater the telomere erosion rate/year.

Figure 4.15. T-cell telomere length in CLL patients is associated with the duration of the disease. (a) There was a strong inverse correlation between initial TL and the time since diagnosis. (b) this relationship was maintained when plotting follow-up TL against months since diagnosis. (c) As was the case for CLL B-cells, the initial TL was associated with the rate of telomere erosion shown in kpb.
4.3.5 T-cell phenotypes change in CLL patients over the course of the disease

It has long been known that T-cells derived from CLL patients manifest numerous functional defects (Taghiloo et al., 2017, McClanahan et al., 2015, Riches et al., 2013, Nunes et al., 2012, Christopoulos et al., 2011). However, it is still not clear whether these defects appear early in the disease or whether they accrue as a function of disease burden. Here we investigated the phenotype of the T-cell pool in a longitudinal fashion to establish if there were temporal changes in the proportion of CD4+ and CD8+ T-cells and if these subsets exhibited phenotypic signs of memory and exhaustion.

Although no significant perturbance in the proportions of CD4+ and CD8+ T-cells was evident (Figure 4.16), more detailed phenotyping revealed that there was a significant increase in the percentage of both CD4+/PD1+ (P=0.03) and CD8+/PD1+ T-cells (P=0.02; Figure 4.17). It is worthy of note that there was no significant change in the percentage of cells expressing CD27 or CD57 over the same timescale.
Figure 4.16. Longitudinal evaluation of T-cell subsets in CLL patients. In the six sets of paired samples analysed, there was (a) no significant change in the proportion of CD4+ and CD8+ T-cells over the course of the patients’ disease. Although all of the samples showed a marked skewing towards memory subsets, neither the (b) CD4+ or (c) CD8+ compartments showed any significant change in the percentage of naïve, central memory, effector, or terminal effector memory subsets.
Figure 4.17. The percentage of PD1<sup>+</sup> T-cells increased during the disease. Serial analysis of T-cells derived from CLL patients revealed that there was a marked increase in PD1<sup>+</sup> T-cells in both (a) the CD4<sup>+</sup> subset and (b) the CD8<sup>+</sup> subset over time.
4.3.6 Longitudinal analysis of the T-cell receptor (TCR) repertoire in CLL patients

4.3.6.1 T-cell receptor repertoire changes over the course of the disease

Six sets of paired CD3+ T-cell samples were shipped to Adaptive Biotechnologies (Seattle, USA) for TCR analysis. All samples passed their in-house quality control testing and were subsequently sequenced using the ImmunoSEQ platform. All samples yielded >500,000 individual reads made up of at least 2000 unique and productive TCR sequences (Figure 4.18).

Figure 4.18. TCR analysis of T-cells serially derived from six individual CLL patients. The six sets of longitudinal samples yielded (a) at least 500,000 sequences (b) representing >2000 unique and productive TCR sequences.
4.3.6.2 TCR repertoires from CLL patients show increased clonality and longitudinal samples show evidence of clonal selection

The clonality of the T-cell pools derived from each sample were assessed based on the shape of the frequency distribution of all the identified unique clones. Clonality varies from 0-1 with 0 being a flat distribution (polyclonal) and 1 being an entirely oligoclonal sample. All of the samples tested showed some degree of clonality with clonality scores ranging from 0.05-0.45 (Table 4.3). This was further supported by the analysis of the 10 most abundant clones in each of the samples; these represented between 4% and 47% of all the evaluable sequences (Figure 4.19). Not surprisingly, when unsupervised clustering of the data was employed the matched samples from each individual patient clustered together but it is noteworthy that the similarity scores for the paired samples from each patient ranged between 16.6% and 56.2% suggesting that selective clonal expansion and/or deletion was evident over the course of the disease (Table 4.4).

Table 4.3. Clonality of the TCR repertoire in CLL patients.

<table>
<thead>
<tr>
<th>Clonality</th>
<th>start</th>
<th>end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.075</td>
<td>0.045</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.31</td>
<td>0.48</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.20</td>
<td>0.24</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.16</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.21</td>
<td>0.26</td>
</tr>
<tr>
<td>Patient 6</td>
<td>0.12</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Figure 4.19. Frequency of the top 10 most abundant clones in each patient. (a) Graph displaying the % frequency of the most abundant clones in each paired patient sample. All of the samples tested showed a degree of clonality with evidence of skewed oligoclonal expansion increasing with follow up in five of the six cases. Data analysed and graph produced by Adaptive Biotechnologies.

Table 4.4. TCR repertoire appears to be modulated. Serial T-cell analysis in samples derived from six CLL patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% similarity of paired samples (start vs end)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>16.6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>56.2</td>
</tr>
<tr>
<td>Patient 3</td>
<td>39.2</td>
</tr>
<tr>
<td>Patient 4</td>
<td>42.7</td>
</tr>
<tr>
<td>Patient 5</td>
<td>44.8</td>
</tr>
<tr>
<td>Patient 6</td>
<td>24.7</td>
</tr>
</tbody>
</table>
Chapter 4: Longitudinal telomere length profiling in CLL

It is of particular interest that analysis of V gene usage in the six paired samples revealed that the most frequently used V gene segments shared similarities with the previously reported TCR repertoires derived from CLL patients (Vardi et al., 2016, Vardi et al., 2017) (Figure 4.20a). This implies that T-cells utilizing these common V gene segments may be preferentially expanding in response to an auto-antigen or tumour antigen. When tracking the frequency of the 10 most abundant clones in individual patients, there was evidence of clonal expansion over time in 5/6 samples (Figure 4.19).

Figure 4.20. Frequency of V gene usage in the T-cells derived from CLL patients. (a) The heatmap shows the frequency of individual V gene segment usage in the samples and reveals commonality between all of the samples. Data analysed and graph produced by Adaptive Biotechnologies.
4.4 Discussion

CLL B-cells telomere lengths can vary from being in the normal range for non-malignant B-cells, to very short, and this shortening has been demonstrated to be highly prognostic. At the same time, CLL B-cells TL profiles have also been shown to be homogenous, consistent with clonal expansion. Although CLL is a disease that is frequently associated with profound telomere shortening, this chapter revealed that there is only slow TL erosion in CLL B-cells over long-term follow-up. Further, the erosion observed is comparable to that seen in non-leukaemic leukocyte and lymphocyte samples. Given the high level of proliferation that is apparent in CLL, up to 1% turn over of the entire tumour per day (Messmer et al., 2005), it is surprising that TL remains relatively stable over the course of the disease. It is interesting that the CLL cohort appears to manifest two different types of telomere dynamics; CLL tumour cells with mean TL above the fusogenic threshold show more marked telomere erosion than those tumour cells with short, dysfunctional telomeres. This suggests that telomerase may play a preferential role in maintaining TL in tumour cells with short telomeres (Ma et al., 2009, Palma et al., 2013). This may serve as a mechanism for sustained proliferative drive without the risk of catastrophic genomic instability.

The data in chapter 3.1 (Figure 3.2) demonstrated that whilst overall CLL B-cells mean TL was shorter than normal B-cells form CLL patients, the range of TL present in those populations overlapped significantly. Given the relative lack of telomere erosion seen in the CLL B-cells during long-term follow up, this data suggests that the telomere length of the malignant clone may be determined by the telomere length of the originating cell. In this case, a malignant transformation in a previously normal B-cell with long telomeres would result in a CLL clone with long telomeres, a good prognosis and some telomere length erosion would be expected to occur during disease follow up. Concurrently, a similar transformation to malignant disease from a normal B-cell with short
telomeres would result in a clone with short telomeres that in the absence of a functional DDR apparatus would avoid senescence and be prone to telomere dysfunction that would drive genomic instability. A similar model has previously been described by in colorectal adenomas and its association with chromosomal instability (Roger et al., 2013).

The precise temporal pattern of TL shortening seen in CLL remains unknown, with there being several different possibilities including step-wise, linear or episodic erosion. The limited data presented here in cells that start with telomeres longer than the fusogenic range (>3.81kbp) display both steady, linear decline in TL in as well as some that appear to favour of a parabolic curve in which the greatest rate of erosion occurs earliest during follow up when the telomeres are at their longest. However, it should be noted that the TL observed in CLL, particularly in patients with progressive disease, start short and remain so throughout the disease. Furthermore, the TL manifested by patients with high-risk CLL are not in any way analogous to age-related telomere shortening. Indeed, CLL demonstrates some of the shortest TL profiles ever recorded in human cells, analogous to patients with telomeropathies (Gutierrez-Rodrigues et al., 2014, Holohan et al., 2014, Lin et al., 2014, Stella et al., 2016).

Analysis of telomere dynamics as a function of stage of disease confirmed the findings from the total cohort – when telomeres were longer than the fusogenic range there was more marked telomere erosion than when the telomeres were short. Since the majority of stage C patients presented with short telomeres, they showed the reduced telomere erosion rates when compared to stage A patients; many of whom had longer telomeres. Treatment appeared to have little or no effect on TL profiles indicating that tumours cells with long (or short) telomeres were not preferentially targeted by treatment. However, it should be noted that the patient group who receive treatment predominantly have shorter telomere length profiles. It would appear that different treatments studied above in CLL reduces the numbers of CLL cells leading to improvement
in symptoms whilst having no effect on the TL of the CLL B-cell clone. This would suggest there would be enough of the original clone remaining after treatment was completed that the same clonal TL would re-emerge. Treatment does not actively modulate telomere length and furthermore, there was no evidence that the amount of cytoreduction led to a population bottleneck that was enough to change the TL distribution by random drift.

Although it was surprising to find that CLL B-cell TL remained relatively stable over long periods of time, it was perhaps more surprising to observe that T-cell telomere erosion was more pronounced over comparable timescales. The T-cells in the CLL patients are not malignant, but it would appear that they are prone to telomere shortening. The most likely explanation for this is that the T-cells are reacting to an auto-antigen or a tumour-specific antigen and are therefore chronically stimulated to divide. In keeping with this concept, the standard deviations of the T-cell TL profiles measured in this chapter were significantly reduced over time consistent with preferential clonal growth (and telomere shortening) in a subset of T-cells in response to antigenic drive. The erosion rate of the T-cells described here is greater than previously published reports (Rufer et al., 1999, Weng et al., 1995). It is possible therefore to deduce that T-cells in CLL are exhibiting a faster rate of biological age, and given that they are non-malignant and retain their DDR would lead to an increased proportion of senescent T-cells. This increasing proportion of senescent T-cells would likely lead to them being less effective in controlling CLL B-cells, therefore progression of CLL could accelerate due to the removal of T-cell surveillance (D'Arena et al., 2013).

It has long been established that T-cells derived from CLL patients show a skewing towards memory phenotypes and display a number of functional abnormalities (Taghiloo et al., 2017, McClanahan et al., 2015, Riches et al., 2013, Nunes et al., 2012, Christopoulos et al., 2011). It is possible that at least some of these abnormalities are caused by radical TL shortening. In this study,
no significant changes in the percentage of CD4 or CD8 subsets were apparent in the longitudinal samples and neither was there evidence of increased T-cell differentiation over time. However, there was a high percentage of memory T-cells in both the CD4+ and CD8+ compartments in all of the samples tested. Interestingly, there was a significant increase in the expression of the immunosuppressive marker PD1 on both CD4+ and CD8+ cells, perhaps suggesting a degree of T-cell exhaustion through the course of the disease (Rallon et al., 2018, Sponaas et al., 2018). Additional circumstantial evidence for the chronic antigen stimulation of the T-cell pool was derived from the analysis of the longitudinal samples. When plotting the time from diagnosis to sample acquisition against TL there was a remarkable inverse correlation ($r^2 = 0.93$, $P=0.0015$). This suggests that T-cell TL is determined by how long the patient has had their disease, but this would need to be confirmed in a much larger cohort of patients. If proven, this would present a different scenario to that of tumour cell TL, which remain relatively stable and are not associated with the time since disease diagnosis.

In order to investigate the potential for preferential (oligoclonal) T-cell expansion as a mechanism for T-cell telomere shortening, the TCR repertoires of longitudinally collected samples, from six CLL patients, were evaluated. Consistent with previous reports, there appeared to be a skewed reliance on certain V gene segments in the TCR pool and some of these clones were expressed in multiple patients (Vardi et al., 2017, Vardi et al., 2016). Taken together, the results provide strong evidence for increased TCR clonality in CLL patients, which appears to become more pronounced over the course of the disease. When tracking the frequency of the 10 most abundant clones in individual patients, there was evidence of clonal expansion over time in 5/6 samples (Figure 4.19). These data provide a rationale for the marked reduction in the T-cell pool both in terms of TL and standard deviation. However, they should be regarded as preliminary at this stage and would need to be confirmed in a much larger cohort of CLL patients.
Chapter 4

Longitudinal telomere length profiling in CLL patients

4.1 Abstract

Patients with CLL often live with their disease for many years; some require simple monitoring and observation, whilst others need repeated intervention with treatment such as combination chemo-immunotherapy (Hallek, 2017). The long-term nature of this follow-up allows the opportunity for longitudinal investigation into the modulation of telomere length with both the passing of time and exposure to chemotherapy. The objectives of this chapter were to use FACS and STELA to undertake sequential analysis of the phenotype of CLL B-cells, T-cells and also normal B-cell populations and to examine the telomere lengths from these repeat samples over the course of an individual patient’s disease. The effect of increasing age, as well as disease progression and exposure to chemotherapy, were also investigated. Furthermore, sequential T-cell receptor sequencing was performed to establish whether the diversity of the T-cell pool is altered throughout the course of the disease.

Telomere length of CLL B-cells analysed in of a cohort of 41 patients on a minimum of two occasions over a median follow up time of over 6 years showed TL erosion correlated strongly with starting telomere length. There was significant difference in TL dynamics seen based upon the starting mean TL of the population with the previously determined fusogenic cut-off of 3.81kbp proving useful as a way of demarcating two different TL behaviours.

In contrast to the CLL B-cells, the T-cell TL erosion was more pronounced in the 9 patients in who repeated measurements were done over similar time periods to that of the CLL B-cell data. Consistent with this TL shortening,
immunophenotyping revealed that there was a significant increase in expression of markers of T-cell exhaustion. Significant inverse correlation was demonstrated between time from diagnosis to sample acquisition against T-cell TL suggesting that T-cell TL and TL shortening may be determined by how long the patient has had the disease. T-cell sequencing was performed and suggested increased skewing of the TCR repertoire over the course of the disease.

The data presented in this chapter demonstrates that whilst there is some TL erosion in CLL B-cells, it occurs in those cells that possess TL greater than the fusogenic range of 3.81kbp and is inversely correlated with starting TL. There is significantly more TL loss seen in T-cell however suggesting that the progressive TL shortening and increasing proportions of exhausted phenotype T-cells is a function of the CLL disease process.
4.2 Introduction

Given the chronic nature of CLL, with median survival ranging from 6 to over 10 years depending on stage of disease (Molica and Levato, 2001), there is considerable opportunity for longitudinal repeat measurement of telomere profiles in this patient group. Patients are reviewed regularly in the hospital outpatient setting and frequently have their symptoms assessed alongside physical examination and monitoring of peripheral blood counts.

Whilst a significant proportion of patients will not need to undergo treatment, more than half of all patients will require therapy at some point in their care pathway, and they may undergo several courses of treatment and experience multiple relapses during their disease course and follow-up. It is well established that the T-cell pool is significantly perturbed during the disease, often with a profound skewing towards memory phenotypes (Forconi and Moss, 2015, Freeman and Gribben, 2016, Hamblin and Hamblin, 2008). In addition, exposure to chemotherapy and chemo-immunotherapy has a cytoreductive effect not only on the tumour cells but also normal lymphocyte subsets (Gassner et al., 2011, Riches and Gribben, 2014). What remains to be established is whether this has any impact on the TL profiles of these various cellular subsets. Having described significant variation within different patients’ CLL B-cell, T-cell and normal B-cell TL profiles in the previous chapter, possible changes in the TL profiles were investigated here by sequential measurement from samples taken from individual patients in a longitudinal fashion. In addition, the T-cell receptor repertoire was investigated in serially-derived samples to establish whether the T-cell pool is altered during the clinical course of the disease, with or without treatment intervention.
4.2.1 Aims of the chapter

In this chapter, an in-depth retrospective analysis was performed in sequential telomere length profiles from CLL B-cells (CD19⁺/CD5⁺), normal B-cells (CD19⁺/CD5⁻) and T-cells (CD3⁺) derived from peripheral blood using STELA. Data was collected from a total of 41 patients with the aim of understanding how TL profiles vary within individual patients during the course of their disease in distinct malignant and non-malignant lymphocyte sub-types. In addition, the effect of chemotherapy on phenotypic subsets and their TL profiles was also compared in samples collected from patients who were untreated against those who received treatment. Finally, the TCR repertoire was investigated in paired longitudinal samples to determine whether the T-cell pool was altered over time.
4.3 Results

Table 4.1. Characteristics of the 41 CLL patients

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</thead>
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</tr>
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</tr>
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<tr>
<td>41</td>
<td>69</td>
<td>C</td>
</tr>
</tbody>
</table>
4.3.1 In-depth sequential telomere length in an individual CLL patient

In order to study the temporal effects on telomere length profile distributions in individual patients’ peripheral blood, immunophenotyping and flow sorting were again used to purify CLL B-cells, T-cells and normal B-cells from patients with CLL. Telomere lengths were determined using STELA with primers specific for the XpYp telomere.

Figure 4.1 illustrates three sequential CLL B-cell TL profiles from a single patient (patient 1) taken over a time period of 68 months (5.6 years). In this example, there was an overall decrease in mean TL from 4.02kbp to 3.66kbp (total decrease of 0.36kbp) equating to an erosion rate of 63bp/year. Consistent with CLL being a clonal disease, the standard deviation of the TL did not significantly change during this follow-up period (1.06kbp at the start to 0.89kbp at the last measurement).
Figure 4.1. Telomere length profiles of sequential CLL B-cell (CD19⁺ /CD5⁺) samples from a single CLL patient. T-cells, B-cells and CLL B-cells were separated using fluorescence-labelled antibodies recognising CD19, CD3 and CD5 with a FACS Aria high-speed cell sorter. (a) shows the gating strategy employed. Subsequently, DNA was extracted using Qiagen Micro kit and STELA was performed using XpYp specific probes. (b) shows STELA blots derived from the same patient’s CLL B-cells taken at different time points as detailed above (months). TL was determined using Phoretrix 1D software and analysed in Graphpad Prism 6. Statistical analyses, including t-tests, were used to determine significance between TL profiles at (c) shows the changes in telomere length profiles at time 0, 10 and 68 months. A normal, age-matched, B-cell control sample is shown for comparison.
Mansouri et al (2013) investigated telomere dynamics in CLL B-cells in a cohort of 119 patients over a period of 5-8 years and demonstrated that there was an overall reduction in TL over time though they did not quantify the degree of loss in bp/year (Mansouri et al., 2013). The above example patient demonstrates that STELA is able to deliver highly reproducible TL profiles that allow for exact measurement of changes in the TL and TL standard deviation.

### 4.3.2 Sequential CLL B-cell telomere length in CLL patient cohort

#### 4.3.2.1 Longitudinal analysis of CLL B-cell telomere length

The in-vivo telomere length dynamics were investigated in a total cohort of 41 CLL patients who had the TL of their CLL B-cells measured on at least two occasions. The mean follow-up for the cohort of 41 CLL patients was 64 months (5.33 years, range 1-12.8 years) with the first sample taken at median age of 65 years, (range 41-79 years). The mean starting CLL B-cell TL was 4.294kbp and the end mean TL was 3.852kbp with a telomere length erosion rate of 74.2bp/year (P=0.002; Figure 4.2a). The mean standard deviation of the TL did not significantly alter over time; the mean standard deviation was 1.51kbp in the initial samples and was 1.377kbp in the follow-up samples (P=0.12).

Once again, this data demonstrates evidence that there is statistically significant telomere length shortening in CLL patients’ CLL B-cells. Figure 4.3 however demonstrates that whilst there may be significant TL shortening, starting and end mean TL are still strongly correlated ($r^2 = 0.85$, $P<0.0001$).
Chapter 4: Longitudinal telomere length profiling in CLL

Figure 4.2. Paired mean telomere length of CLL B-cells derived from CLL patients at the start and end of follow-up are significantly different. Paired t-tests were used to determine significance between (a) telomere length profiles and (b) standard deviation of CLL B-cells over a mean follow-up period of 63 months (n=41).

<table>
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<tr>
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<th>Start (kbp)</th>
<th>End (kbp)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>3.852</td>
<td>0.002</td>
</tr>
<tr>
<td>b</td>
<td>1.94</td>
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<tr>
<td>Mean (kbp)</td>
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</tr>
<tr>
<td>SD (kbp)</td>
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</table>

Figure 4.3. Temporal evaluation of the mean telomere length of CLL B-cells derived from the same patients showed a strong correlation. Telomere lengths were compared in a longitudinal study (n = 41). There was a strong correlation between start and end telomere length after a mean follow-up 63 months (range 12-154).
To further investigate this, the cohort was separated into two groups using the experimentally determined cut-off of 3.81kbp. This cut-off, the so-called ‘fusogenic threshold’ has been shown to be significant in both prognostic and biologically in delineating telomere dysfunction in the form of telomere fusion events. Lin et al (2014) demonstrated that telomere fusion events only occurred when mean CLL B-cell TL fell below 3.81kbp. Figure 4.4 shows that in patients with a starting TL above 3.81kbp, there was a significant decrease in mean TL from 5.80kbp to 4.90kbp (P<0.0001) over a mean follow up of 72 months equating to an telomere erosion rate of 151.8bp/year. However, in the patients with a starting mean TL below 3.81kbp there was no significant change in mean TL (2.707kbp vs. 2.745kbp, P=0.7), with an increase in TL at a rate 8.9bp/year over a mean of 53 months.

**Figure 4.4.** Long-term follow-up in CLL patients bifurcated above and below the fusogenic threshold (3.81kbp). Paired mean telomere length show significant difference in patients’ TL when the initial mean was (a) TL >3.81kbp (n = 21). (b) In contrast, those patients with CLL B-cells with a mean TL <3.81kbp (n = 20) show no significant change in telomere length.

It is clear from the above data that there are two very different patterns of TL behavior with significant TL shortening being seen only in CLL B-cells that possess longer telomeres with a mean TL above the previously determined fusogenic threshold. The TL in the group where the first sample was below the fusogenic range of 3.81kb showed no significant change.
Figure 4.5. Correlation between the starting telomere length and telomere length change per year in CLL B-cells derived from CLL patients. CLL B-cells were isolated and DNA was extracted. XpYp STELA was performed and TL determined using Phoretrix 1D software and analysed in Graphpad Prism 6 software. Correlation and linear regression analyses were performed to assess the relationship between TL at the start of the observation period and extent of TL erosion expressed as the loss of TL per year (n=41).

Figure 4.5 shows the relationship between starting TL and overall TL change. There was a clear inverse relationship between initial TL and the amount of telomeric erosion i.e. the degree of erosion decreased as the starting TL decreased ($r^2=0.24, P=0.001$).

The telomere erosion rates in the above figure were calculated using the difference between the start and end mean TL and dividing it with the length of follow-up. This method gives an overall loss rate over the total follow-up time but it remains uncertain whether TL erosion in individual patients is constant. In order to investigate this further, additional (intermediate time point) samples were available for 10 of the patients. TL analysis was performed on these samples and linear regression performed to obtain a more accurate estimate of the telomere length change, an example of which is shown in Figure 4.6.
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Figure 4.6. Telomere length profiles of sequential CLL B-cell (CD19⁺/CD5⁺) samples from a single CLL patient. CLL B-cells were isolated, DNA was extracted and XpYp STELA was as performed as detailed above. A single patient’s TL analysis was repeated on 4 occasions over a period of 154 months. (a) shows the STELA TL profile data over time. (b) shows the mean TL plotted as a function of time. The slope of the linear regression line was used to calculate the telomeric erosion rate per year.

Figure 4.6b shows an example of one of the 10 patients with repeated measures of the TL, the line created by plotting the mean TL for a single patient does not appear to be entirely linear with most of the TL loss appears to occur over the early follow-up period in this patient. Other patients showed a variety of patterns though all telomere length loss decreased as the mean TL approached 3.81kbp. Rufer et al (1999) detailed that lymphocyte TL showed a rapid and significant decline during the first years of life with a bi-segmented line analysis showing increased statistical significance with a cut-off of 1.5 years over that of a simple linear distribution. CLL B-cells have significant opportunity for proliferation with published estimates show that the proliferation rate of CLL cells is approximately 1% of the total clone per day (Messmer et al., 2005). The rapid decline in TL documented by Rufer et al (1999) relates to early life where there is a marked period of unparalleled proliferation. It seems unlikely that this degree of proliferation is occurring in the CLL B-cells. Nonetheless, in all 10 of the CLL B-cell samples, with 3 or more repeated readings where this linear regression analysis was carried out, the calculated change in telomere length
decreased, although the difference was not significant (figure 4.7, mean TL erosion for the 10 samples was -57.5bp/year vs. -46.9bp/year, P=0.14). It seems likely that the multiple measurements of the CLL B-cell TL gives a more robust estimate of overall telomere length change compared to those patients in which there was only two sampling points.

**Figure 4.7. Analysis of CLL B-cell telomere length erosion rates compared by method of calculation** (a) TL change over time showing no statistically significant difference between method of calculation (n=10). Standard method of calculation was using the difference between the start and end mean TL and dividing it with the length of follow-up, the calculated method involved linear regression analysis.
4.3.2.2 Follow-up by disease stage

To investigate if disease stage was associated with TL erosion rates, the cohort was sub-divided by using the Binet staging system. Figure 4.8 shows that Stage A patients (n=31) had a CLL B-cell starting mean TL of 4.51kbp (SD 1.49kbp) that was significantly reduced in the follow-up samples (mean TL of 4.03kbp (SD 1.42kbp) (P=0.004)); equating to a mean erosion rate of -81.8bp/year. Stage B and C patients (n=10) had a CLL B-cell starting TL of 3.64kbp (SD 1.59kbp) and an end mean TL of 3.32kbp (SD 1.28kbp) which was not significantly different (P=0.27). This TL change equated to an erosion rate of -47.4bp/year which is just over half of that seen in the stage A patients though this was not statistically significant (p=0.85).

![Figure 4.8](image_url)

**Figure 4.8.** Analysis of CLL B-cell telomere length erosion rates by stage of disease. Binet stage A versus B/C patients showed differential changes in (a) TL over time showing not statistical difference between Binet stage A and Binet stage B/C patients.

The stage-specific analysis was consistent with the total cohort analysis; patients with longer telomeres showed more marked telomere erosion than those with short telomeres.
4.3.2.3 The effect of treatment on telomere length profiles

To investigate if exposure to chemotherapy influenced TL erosion, the cohort was also sub-divided into treated and untreated subsets. In all, four groups were studied: the total cohort, a group who remained untreated for the duration of sampling, a group who went on to receive treatment after the initial sample was taken and a group that had both of TL measurements carried out after treatment (Figure 4.9).

In the untreated patient group (n=20, mean follow-up 67 months), CLL B-cells had a starting mean TL of 4.03kbp (SD 2.0kbp) and an end mean TL of 3.79kbp (SD 1.7kbp) (P=0.015), in the post treatment follow-up patients (n=13, mean follow-up 66 months) mean starting TL 4.223kbp (SD 1.6kbp) and an end mean TL of 3.78kbp (SD 1.1kbp)(P =0.1), and finally patients treated in-between the samples (n = 13, mean follow-up 60 months) had a starting mean TL 4.44kbp (SD 1.9kbp) and end mean TL of 4.11kbp (SD 1.5kbp)(P=0.21). The TL erosion seen in the four groups is shown in Figure 4.9. No statistically significant difference was observed in any of the groups regardless of treatment history (multiple Mann-Whitney U tests, P=0.28 - 0.9). Figure 4.9 also suggests that the clonality of the tumour cells, as assessed by TL standard deviation, remained largely unchanged within the different groups (multiple Mann-Whitney U tests, P=0.16 - 0.9).
Figure 4.9. Analysis of CLL B-cell telomere length as a function of exposure to treatment. No significant differences in (a) Mean TL change/year in 41 patients with CLL and (b) TL standard deviation were apparent between any of the groups.
4.3.3. Long-term follow-up of T-cell TL

Having established the TL dynamics in CLL B-cells, I next investigated the in-vivo T-cell TL dynamics in a small cohort of 9 CLL patients who had the telomere length of their T-cells measured on a minimum of two occasions. Six patients had three or more measurements of their purified T-cells. The overall follow-up for the cohort of 9 CLL patients was 75.2 months (6.25 years, range 2-12.8 years). The mean start T-cell TL was 4.75kbp and the end mean TL was 4.05kbp with a telomere length erosion rate of -118.5bp/year (P=0.02; Figure 4.10a). This erosion rate is much greater than reported in peripheral blood lymphocytes in healthy people (Rufer et al., 1999, Weng et al., 1995). The mean standard deviation of the TL started as 2.42kbp and was 1.91kbp after 75 months (P=0.02) suggesting that the T-cell pool may be becoming more clonal over the course of the disease.

**Figure 4.10.** Paired mean telomere length of T-cells (CD3+/CD5+) derived from CLL patients at the start and end of follow-up is significantly different. Paired t-tests were used to determine significance between (a) the TL profiles and (b) the standard deviation of T-cells derived from patients diagnosed with CLL over a mean follow-up period of 75 months (n=9).

Figure 4.11 illustrates two sequential T-cell TL profiles from a single patient taken over a time period of 52 months (4.3 years). In this particular patient, there is evidence of marked TL shortening with the mean decreasing from...
5.38kb to 4.36kb (a total of 1.02kb) equating to an erosion rate of \(-237\) bp/year.

**Figure 4.11.** Telomere profiles of sequential T-cell (CD3\(^+\)) samples from a single CLL patient. As the data was shown to conform to a Gaussian distribution, a paired \(t\)-test was used to determine significance between T-cell TL profiles 52 months apart.

The above example of telomere length shortening is consistent with previously published data by Roth et al (2008) in which flow-FISH was used to determine TL and estimated the overall telomere length decrease in T-cells to be at most \(-94\)bp/year for naïve T-cells in a cohort of 30 CLL patients followed up for a median of 33 months. However, the data shown above (Figure 4.10) suggests that Roth et al may have significantly underestimated the degree of TL shortening that occurs in CLL patients’ T-cells but this would need to be confirmed in a much larger study (Roth et al., 2008).
As with the CLL B-cells, several of the patients (n=6) had three or more repeated measurements of their T-cell TL. Figure 4.12a shows an example of one of these 6 patients, with Figure 4.12b again demonstrating the line created by calculating linear regression to obtain a more accurate estimate of the overall rate of telomere length change.

Figure 4.12. Telomere length profiles of sequential T-cell (CD3+) samples from a single CLL patient. T-cells were isolated, DNA was extracted and XpYp STELA was as performed as detailed above. A single patient’s TL analysis was repeated on 5 occasions over a period of 154 months. (a) shows the STELA TL profile data over time. (b) shows the mean TL plotted as a function of time. The slope of the linear regression line was used to calculate the telomeric erosion rate per year.

Once again, no significant difference was seen overall between the telomere length change during long-term follow-up using this method (Figure 4.13)
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Figure 4.13. Analysis of T-cell telomere length erosion rates as compared by their method of calculation (a) TL change over time showing no statistically significant difference between method of calculation (n=6). Standard method of calculation was using the difference between the start and end mean TL and dividing it with the length of follow-up, the calculated method involved linear regression analysis as described above in section 4.3.2.1.

4.3.3.1. Follow-up by disease stage

To investigate if disease stage was associated with T-cell TL erosion rates, the cohort was sub-divided by using the Binet staging system. Figure 4.14 shows that Stage A patients (n=6) had a T-cell starting mean TL of 4.56 kbp (SD 0.81 kbp) that was reduced in the follow-up samples (mean TL of 4.21 kbp (SD 0.79 kbp) (P=0.22)); equating to a mean erosion rate of -53.8 bp/year. Stage B and C patients (n=3) had a T-cell starting TL of 5.14 kbp (SD 0.55 kbp) and an end mean TL of 3.71 kbp (SD 0.19 kbp), which was not significantly different (p=0.25). This TL change equated to an erosion rate of -247.8 bp/year that is markedly higher than in Stage A patients though this difference did not reach statistical significance (P=0.09).
Figure 4.14. Analysis of T-cell telomere length erosion rates by stage of disease. Binet stage A versus B/C patients showed differential changes in (a) TL over time showing not statistical difference between Binet stage A and stage B/C patients.

This stage-specific analysis, despite the small numbers of patients involved demonstrates that patients with advanced stage disease tended to have much higher T-cell telomere length loss during the follow-up period.
4.3.4 T-cell telomere length in CLL patients relates to time since diagnosis

Given that the evidence presented here and elsewhere supports the concept of on-going telomere erosion in the ‘normal’ T-cell pool in CLL, we set out to establish whether this was caused by the preferential expansion of an oligoclonal population of T-cells, in response to a CLL-related antigen. This investigation into the expansion of an oligoclonal population of T-cells was done to identify if there was further evidence of increased clonality suggested by the decrease in T-cell TL SD described above. We addressed this by taking sequential samples from six CLL patients and compared their TCR repertoires over time. TCR sequencing was performed on CD3-purified samples by Adaptive Biotechnologies (Seattle, USA). The median time interval between samples was 76.5 months with a mean telomere erosion of more than 1kb over this timescale. Table 4.2 shows the Binet stage at diagnosis for each patient, the time from diagnosis to first T-cell TL analysis, the mean initial and follow-up TL and the time interval between sample collection.
Table 4.2. Patient information relating to serial T-cell analysis in samples derived from six CLL patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Binet Stage at diag.</th>
<th>Time to initial sample (months)</th>
<th>Initial mean CD3+ telomere length (±SD)</th>
<th>Follow-up mean CD3+ telomere length (±SD)</th>
<th>Time interval (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>C</td>
<td>18</td>
<td>5.78kb ±2.63 Post-Tx</td>
<td>3.62kb ±1.56 Post-Tx</td>
<td>70</td>
</tr>
<tr>
<td>Patient 2</td>
<td>A</td>
<td>66</td>
<td>3.65kb ±2.39 Pre-Tx</td>
<td>3.19kb ±1.75 Post-Tx</td>
<td>136</td>
</tr>
<tr>
<td>Patient 3</td>
<td>A</td>
<td>55</td>
<td>3.71kb ±1.85 Pre-Tx</td>
<td>3.49kb ±1.33 Pre-Tx</td>
<td>59</td>
</tr>
<tr>
<td>Patient 4</td>
<td>B</td>
<td>27</td>
<td>4.82kb ±2.34 Post-Tx</td>
<td>3.58kb ±1.85 Post-Tx</td>
<td>76</td>
</tr>
<tr>
<td>Patient 5</td>
<td>A</td>
<td>22</td>
<td>5.38kb ±2.71 Pre-Tx</td>
<td>4.36kb ±1.89 Pre-Tx</td>
<td>54</td>
</tr>
<tr>
<td>Patient 6</td>
<td>A</td>
<td>14</td>
<td>6.31kb ±2.00 Pre-Tx</td>
<td>5.33kb ±2.51 Pre-Tx</td>
<td>64</td>
</tr>
<tr>
<td>Median time interval (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>76.5 (54-136)</td>
</tr>
<tr>
<td>Mean telomere length (±SD)</td>
<td></td>
<td></td>
<td>4.94kb ±2.32</td>
<td>3.93kb ±1.82</td>
<td></td>
</tr>
</tbody>
</table>

Tx = treatment

As the date of diagnosis and the date when the initial and follow-up samples were known, it was possible to plot TL as a function time since diagnosis. There was a strong inverse correlation between initial T-cell TL and time since diagnosis (Figure 4.15a). The same inverse correlation was also observed when plotting the follow-up T-cell TL as a function time since diagnosis although the relationship was not as strong (Figure 4.15b).
These data suggest that T-cell telomere erosion is an on-going process in CLL and is related to the time since diagnosis. It was also possible to calculate the average T-cell TL erosion rate for each individual. Figure 4.15c shows a clear relationship between initial T-cell TL and the average erosion rate. As was the case for CLL B-cells, the longer the initial TL the greater the telomere erosion rate/year.

Figure 4.15. T-cell telomere length in CLL patients is associated with the duration of the disease. (a) There was a strong inverse correlation between initial TL and the time since diagnosis. (b) this relationship was maintained when plotting follow-up TL against months since diagnosis. (c) As was the case for CLL B-cells, the initial TL was associated with the rate of telomere erosion shown in kpb.
4.3.5 T-cell phenotypes change in CLL patients over the course of the disease

It has long been known that T-cells derived from CLL patients manifest numerous functional defects (Taghiloo et al., 2017, McClanahan et al., 2015, Riches et al., 2013, Nunes et al., 2012, Christopoulos et al., 2011). However, it is still not clear whether these defects appear early in the disease or whether they accrue as a function of disease burden. Here we investigated the phenotype of the T-cell pool in a longitudinal fashion to establish if there were temporal changes in the proportion of CD4+ and CD8+ T-cells and if these subsets exhibited phenotypic signs of memory and exhaustion.

Although no significant perturbation in the proportions of CD4+ and CD8+ T-cells was evident (Figure 4.16), more detailed phenotyping revealed that there was a significant increase in the percentage of both CD4+/PD1+ (P=0.03) and CD8+/PD1+ T-cells (P=0.02; Figure 4.17). It is worthy of note that there was no significant change in the percentage of cells expressing CD27 or CD57 over the same timescale.
Figure 4.16. Longitudinal evaluation of T-cell subsets in CLL patients. In the six sets of paired samples analysed, there was (a) no significant change in the proportion of CD4+ and CD8+ T-cells over the course of the patients’ disease. Although all of the samples showed a marked skewing towards memory subsets, neither the (b) CD4+ or (c) CD8+ compartments showed any significant change in the percentage of naïve, central memory, effector, or terminal effector memory subsets.
Figure 4.17. The percentage of PD1+ T-cells increased during the disease. Serial analysis of T-cells derived from CLL patients revealed that there was a marked increase in PD1+ T-cells in both (a) the CD4+ subset and (b) the CD8+ subset over time.
4.3.6 Longitudinal analysis of the T-cell receptor (TCR) repertoire in CLL patients

4.3.6.1 T-cell receptor repertoire changes over the course of the disease

Six sets of paired CD3$^+$ T-cell samples were shipped to Adaptive Biotechnologies (Seattle, USA) for TCR analysis. All samples passed their in-house quality control testing and were subsequently sequenced using the ImmunoSEQ platform. All samples yielded >500,000 individual reads made up of at least 2000 unique and productive TCR sequences (Figure 4.18).

**Figure 4.18.** TCR analysis of T-cells serially derived from six individual CLL patients. The six sets of longitudinal samples yielded (a) at least 500,000 sequences (b) representing >2000 unique and productive TCR sequences.
4.3.6.2 TCR repertoires from CLL patients show increased clonality and longitudinal samples show evidence of clonal selection

The clonality of the T-cell pools derived from each sample were assessed based on the shape of the frequency distribution of all the identified unique clones. Clonality varies from 0-1 with 0 being a flat distribution (polyclonal) and 1 being an entirely oligoclonal sample. All of the samples tested showed some degree of clonality with clonality scores ranging from 0.05-0.45 (Table 4.3). This was further supported by the analysis of the 10 most abundant clones in each of the samples; these represented between 4% and 47% of all the evaluable sequences (Figure 4.19). Not surprisingly, when unsupervised clustering of the data was employed the matched samples from each individual patient clustered together but it is noteworthy that the similarity scores for the paired samples from each patient ranged between 16.6% and 56.2% suggesting that selective clonal expansion and/or deletion was evident over the course of the disease (Table 4.4).

Table 4.3. Clonality of the TCR repertoire in CLL patients.

<table>
<thead>
<tr>
<th>Clonality</th>
<th>start</th>
<th>end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.075</td>
<td>0.045</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.31</td>
<td>0.48</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.20</td>
<td>0.24</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.16</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.21</td>
<td>0.26</td>
</tr>
<tr>
<td>Patient 6</td>
<td>0.12</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Figure 4.19. Frequency of the top 10 most abundant clones in each patient.
(a) Graph displaying the % frequency of the most abundant clones in each paired patient sample. All of the samples tested showed a degree of clonality with evidence of skewed oligoclonal expansion increasing with follow up in five of the six cases. Data analysed and graph produced by Adaptive Biotechnologies.

Table 4.4. TCR repertoire appears to be modulated. Serial T-cell analysis in samples derived from six CLL patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% similarity of paired samples (start vs end)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>16.6</td>
</tr>
<tr>
<td>Patient 2</td>
<td>56.2</td>
</tr>
<tr>
<td>Patient 3</td>
<td>39.2</td>
</tr>
<tr>
<td>Patient 4</td>
<td>42.7</td>
</tr>
<tr>
<td>Patient 5</td>
<td>44.8</td>
</tr>
<tr>
<td>Patient 6</td>
<td>24.7</td>
</tr>
</tbody>
</table>
It is of particular interest that analysis of V gene usage in the six paired samples revealed that the most frequently used V gene segments shared similarities with the previously reported TCR repertoires derived from CLL patients (Vardi et al., 2016, Vardi et al., 2017) (Figure 4.20a). This implies that T-cells utilizing these common V gene segments may be preferentially expanding in response to an auto-antigen or tumour antigen. When tracking the frequency of the 10 most abundant clones in individual patients, there was evidence of clonal expansion over time in 5/6 samples (Figure 4.19).

**Figure 4.20.** Frequency of V gene usage in the T-cells derived from CLL patients. (a) The heatmap shows the frequency of individual V gene segment usage in the samples and reveals commonality between all of the samples. Data analysed and graph produced by Adaptive Biotechnologies.
4.4 Discussion

CLL B-cells telomere lengths can vary from being in the normal range for non-malignant B-cells, to very short, and this shortening has been demonstrated to be highly prognostic. At the same time, CLL B-cells TL profiles have also been shown to be homogenous, consistent with clonal expansion. Although CLL is a disease that is frequently associated with profound telomere shortening, this chapter revealed that there is only slow TL erosion in CLL B-cells over long-term follow-up. Further, the erosion observed is comparable to that seen in non-leukaemic leukocyte and lymphocyte samples. Given the high level of proliferation that is apparent in CLL, up to 1% turn over of the entire tumour per day (Messmer et al., 2005), it is surprising that TL remains relatively stable over the course of the disease. It is interesting that the CLL cohort appears to manifest two different types of telomere dynamics; CLL tumour cells with mean TL above the fusogenic threshold show more marked telomere erosion than those tumour cells with short, dysfunctional telomeres. This suggests that telomerase may play a preferential role in maintaining TL in tumour cells with short telomeres (Ma et al., 2009, Palma et al., 2013). This may serve as a mechanism for sustained proliferative drive without the risk of catastrophic genomic instability.

The data in chapter 3.1 (Figure 3.2) demonstrated that whilst overall CLL B-cells mean TL was shorter than normal B-cells form CLL patients, the range of TL present in those populations overlapped significantly. Given the relative lack of telomere erosion seen in the CLL B-cells during long-term follow up, this data suggests that the telomere length of the malignant clone may be determined by the telomere length of the originating cell. In this case, a malignant transformation in a previously normal B-cell with long telomeres would result in a CLL clone with long telomeres, a good prognosis and some telomere length erosion would be expected to occur during disease follow up. Concurrently, a similar transformation to malignant disease from a normal B-cell with short
telomeres would result in a clone with short telomeres that in the absence of a functional DDR apparatus would avoid senescence and be prone to telomere dysfunction that would drive genomic instability. A similar model has previously been described by in colorectal adenomas and its association with chromosomal instability (Roger et al., 2013).

The precise temporal pattern of TL shortening seen in CLL remains unknown, with there being several different possibilities including step-wise, linear or episodic erosion. The limited data presented here in cells that start with telomeres longer than the fusogenic range (>3.81kbp) display both steady, linear decline in TL in as well as some that appear to favour of a parabolic curve in which the greatest rate of erosion occurs earliest during follow up when the telomeres are at their longest. However, it should be noted that the TL observed in CLL, particularly in patients with progressive disease, start short and remain so throughout the disease. Furthermore, the TL manifested by patients with high-risk CLL are not in any way analogous to age-related telomere shortening. Indeed, CLL demonstrates some of the shortest TL profiles ever recorded in human cells, analogous to patients with telomeropathies (Gutierrez-Rodrigues et al., 2014, Holohan et al., 2014, Lin et al., 2014, Stella et al., 2016).

Analysis of telomere dynamics as a function of stage of disease confirmed the findings from the total cohort – when telomeres were longer than the fusogenic range there was more marked telomere erosion than when the telomeres were short. Since the majority of stage C patients presented with short telomeres, they showed the reduced telomere erosion rates when compared to stage A patients; many of whom had longer telomeres. Treatment appeared to have little or no effect on TL profiles indicating that tumours cells with long (or short) telomeres were not preferentially targeted by treatment. However, it should be noted that the patient group who receive treatment predominantly have shorter telomere length profiles. It would appear that different treatments studied above in CLL reduces the numbers of CLL cells leading to improvement.
in symptoms whilst having no effect on the TL of the CLL B-cell clone. This would suggest there would be enough of the original clone remaining after treatment was completed that the same clonal TL would re-emerge. Treatment does not actively modulate telomere length and furthermore, three was no evidence that the amount of cyto-reduction led to a population bottleneck that was enough to change the TL distribution by random drift.

Although it was surprising to find that CLL B-cell TL remained relatively stable over long periods of time, it was perhaps more surprising to observe that T-cell telomere erosion was more pronounced over comparable timescales. The T-cells in the CLL patients are not malignant, but it would appear that they are prone to telomere shortening. The most likely explanation for this is that the T-cells are reacting to an auto-antigen or a tumour-specific antigen and are therefore chronically stimulated to divide. In keeping with this concept, the standard deviations of the T-cell TL profiles measured in this chapter were significantly reduced over time consistent with preferential clonal growth (and telomere shortening) in a subset of T-cells in response to antigenic drive. The erosion rate of the T-cells described here is greater than previously published reports (Rufer et al., 1999, Weng et al., 1995). It is possible therefore to deduce that T-cells in CLL are exhibiting a faster rate of biological ageing, and given that they are non-malignant and retain their DDR would lead to an increased proportion of senescent T-cells. This increasing proportion of senescent T-cells would likely lead to them being less effective in controlling CLL B-cells, therefore progression of CLL could accelerate due to the removal of T-cell surveillance (D’Arena et al., 2013).

It has long been established that T-cells derived from CLL patients show a skewing towards memory phenotypes and display a number of functional abnormalities (Taghiloo et al., 2017, McClanahan et al., 2015, Riches et al., 2013, Nunes et al., 2012, Christopoulos et al., 2011). It is possible that at least some of these abnormalities are caused by radical TL shortening. In this study,
no significant changes in the percentage of CD4 or CD8 subsets were apparent in the longitudinal samples and neither was there evidence of increased T-cell differentiation over time. However, there was a high percentage of memory T-cells in both the CD4\(^+\) and CD8\(^+\) compartments in all of the samples tested. Interestingly, there was a significant increase in the expression of the immunosuppressive marker PD1 on both CD4\(^+\) and CD8\(^+\) cells, perhaps suggesting a degree of T-cell exhaustion through the course of the disease (Rallon et al., 2018, Sponaas et al., 2018). Additional circumstantial evidence for the chronic antigen stimulation of the T-cell pool was derived from the analysis of the longitudinal samples. When plotting the time from diagnosis to sample acquisition against TL there was a remarkable inverse correlation \((r^2 = 0.93, P=0.0015)\). This suggests that T-cell TL is determined by how long the patient has had their disease, but this would need to be confirmed in a much larger cohort of patients. If proven, this would present a different scenario to that of tumour cell TL, which remain relatively stable and are not associated with the time since disease diagnosis.

In order to investigate the potential for preferential (oligoclonal) T-cell expansion as a mechanism for T-cell telomere shortening, the TCR repertoires of longitudinally collected samples, from six CLL patients, were evaluated. Consistent with previous reports, there appeared to be a skewed reliance on certain V gene segments in the TCR pool and some of these clones were expressed in multiple patients (Vardi et al., 2017, Vardi et al., 2016). Taken together, the results provide strong evidence for increased TCR clonality in CLL patients, which appears to become more pronounced over the course of the disease. When tracking the frequency of the 10 most abundant clones in individual patients, there was evidence of clonal expansion over time in 5/6 samples (Figure 4.19). These data provide a rationale for the marked reduction in the T-cell pool both in terms of TL and standard deviation. However, they should be regarded as preliminary at this stage and would need to be confirmed in a much larger cohort of CLL patients.
5.1 Abstract

In this chapter, a long-term in-vitro co-culture of peripheral blood from CLL patients was used to investigate temporal changes in cellular phenotype and function as well as telomere length dynamics. The long-term co-culture method was chosen as an effective method of promoting survival and inducing significant proliferation in the CLL B-cells. Analysis of CLL B-cells from these cultures revealed significantly increased Ki-67\(^+\) at day 14 when compared to day 0 (P<0.001) and this was evident for the duration of the cultures. Despite sustained tumour cell proliferation, CLL B-cell telomere length showed only minor erosion over the co-culture period. Further, the presence of T-cells was shown to be critical for the maintenance of the long-term cultures in two ways. Firstly, cultures that were treated with 4\(\mu\)M fludarabine showed a catastrophic reduction in T-cells (P=0.01), which was associated with a significantly shorter duration of survival of CLL B-cells when compared to untreated controls (median 17.5 days (range 7-70); P<0.001).

Secondly, it proved impossible to maintain T-cell depleted, purified CLL B-cells, in long-term culture. T-cells isolated from the long-term cultures showed evidence of proliferation with Ki-67\(^+\) again being increased at day 14 in comparison to baseline (P=0.003). Furthermore, T-cells derived from these cultures showed a significant alteration in subset composition over time with a decrease in the numbers of naive CD4\(^+\) (P=0.05) and CD8\(^+\) (P=0.02) T-cells and a corresponding increase in terminally differentiated effector memory (EMRA) subsets (P=0.07). The CLL B-cell TL findings with the in-vitro co-culture replicate those from the previous chapter where starting TL correlated with the degree of...
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TL erosion seen. The T-cell TL increase seen is likely to be related to the long-term culture conditions. Despite this, the further decrease of naïve T-cells is consistent with the in-vivo behaviour of these subsets.
5.2 Introduction

Telomere length is a prognostic factor in Chronic Lymphocytic Leukaemia (CLL) with short telomere length a powerful predictor of early time to first treatment and reduced overall survival. However, little is known about telomere dynamics through the course of an individual patient's disease.

There is growing evidence that interactions in the tumour microenvironment promote the survival, proliferation and drug resistance of primary chronic lymphocytic leukaemia (CLL) cells. Furthermore, progressive CLL is frequently associated with lymphadenopathy, pointing to a crucial role for signals emanating from the tissue microenvironment in the accumulation of malignant B-cells. Proliferation of CLL cells appears to be confined to specialized structures called pseudofollicles, which contain a number of cell types including CLL cells, T-cells, vascular endothelial cells and stromal cells. Chronic lymphocytic leukaemia (CLL) is a highly heterogenous disease with a very variable clinical outcome. It is now appreciated that it is a highly proliferative disorder with significant tumour cell turnover every day (Messmer et al., 2005) yet despite this, primary CLL cells are notoriously difficult to culture in-vitro and drug testing models are hindered by the poor survival of these cells. This raises the question as to what signals are provided in-vivo that enables these cells to survive and proliferate. Recent work has highlighted the role of accessory cells within the tumour microenvironment in the survival and induction of proliferation in these malignant cells (Patten et al., 2005, Buggins et al., 2010, Ferretti et al., 2011, Herishanu et al., 2011). As a result of this, a number of in-vitro co-culture systems have been developed to mimic the tumour microenvironment (Plander et al., 2009, Buggins et al., 2010, Coscia et al., 2011, Ferretti et al., 2011, Pepper et al., 2011). These studies indicate that it is interactions with accessory cells, such as activated T-lymphocytes, that play a role in sustaining CLL cells in-vivo.
5.2.1 Aims of the chapter

In the previous chapter, longitudinal analysis of CLL B-cell telomere length revealed very little dynamic change within individual patients with a mean erosion rate of -74.2bp/year (P=0.002). In marked contrast, T-cells derived from the same patients showed a significantly higher mean erosion rate of -119bp/year (P=0.02). Here, an in-vitro co-culture of peripheral blood from CLL patients was used to investigate temporal changes in cellular phenotype and function as well as telomere length dynamics.
5.3 Results

5.3.1 Co-culture CD40L density is critical for maintaining long-term survival

The CD40L co-culture system had been previously employed within the research group (Hamilton et al., 2012, Pepper et al., 2011) but there were two CD40L-transfected mouse fibroblast cell lines in use within the laboratory. As a first step, the level of CD40L expressed on each cell line was measured (Figure 5.1) with one line showing a log lower expression (cell line 1) of the human ligand than the other (cell line 2). As a result of these findings, cell line 2, showing the highest expression of CD40L, was initially employed in the long-term co-culture experiments. However, all of the cultures (n = 8) became non-viable within 14 days of seeding the primary PBMC. Consequently, co-cultures were switched to cell line 1, expressing the lower levels of CD40L, and this cell line was used in all subsequent experiments as it was shown to be able to sustain the survival and growth of primary PBMC derived from CLL patients. Although not proven experimentally, it was assumed that the higher level of CD40L shown by cell line 2 led to over stimulation of CLL cells in the culture and this was toxic following prolonged exposure. Interestingly, short-term stimulations using this cell line (24-48h), carried out by other members of the laboratory, were shown to result in NF-κB activation and CLL proliferation. So, it would appear that exposure of CLL cells to higher density of CD40L only results in apoptosis in the context of chronic exposure.
Figure 5.1. Human CD40L expression transfected into two different mouse fibroblast cell lines. CD40L FITC antibody was used to stain two different mouse fibroblast cell lines, both of which had been transfected with human CD40L, (a) shows the gating strategy used to identify viable fibroblasts, (b) shows an overlaid histogram of CD40L expression in the two mouse fibroblast cell lines.

5.3.2 CLL cells increase in size when maintained in co-culture

As part of the tracking of the long-term cultures, size and granularity (complexity) of the tumour cells were measured on a weekly basis. Figure 5.2 demonstrates that the average size of the CLL B-cells increased over time as did the side scatter (SSC-A), which denotes the granularity of the cells. An increase in size and complexity of the cells is consistent with activation and proliferation, so-called ‘blasting’ (Teague et al., 1993). It is worthy of note that in cultures treated with fludarabine, the CLL B-cells showed a significant reduced amount of increase in forward scatter (P=0.04; Wilcoxon signed-rank test), confirming the previously-described inhibitory effects of fludarabine on CLL cell growth and survival (Silber et al., 1994).
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CLL size during long term culture

Figure 5.2. CLL B-cell size increases over the course of the culture. Flow cytometry was performed on a BD Accuri C6 (a) Forward scatter and (b) side scatter from long-term cultures with and without the addition of 4μM fludarabine (n=17).

5.3.3 CD40L co-culture increased Ki-67 expression in CLL B-cells and T-cells in a temporal fashion

In order to investigate whether the observed increase in size was associated with specific markers of proliferation, patient-derived PBMCs, cultured in the presence or absence of fludarabine, were harvested and labelled with Ki-67. Cultures treated with fludarabine all showed a marked depletion in T-cell numbers, as evidenced by the reduction/absence of CD3+ cells (Figure 5.3b). Cultures without the addition of fludarabine showed a time-dependent increase in the percentage of both CLL B-cells and T-cells expressing Ki-67 (Figure 5.3c-f). This was shown to be the case in all of the long-term cultures evaluated (n = 17); Figure 5.4. It is interesting to note that although fludarabine was cytotoxic to both CLL B-cells and T-cells, the CLL B-cells that remained viable in these cultures were capable of being activated by co-culture with CD40L-expressing fibroblasts albeit to a lesser extent.
Figure 5.3. Gating strategy for CD19 CD3 and Ki-67 staining. Flow cytometry was performed on BD Accuri C6 with CD3 (PE) CD19 (APC) and Ki-67 (FITC). (a) shows the gating based on lymphocyte size selection. (b) shows the cultures containing fludarabine were depleted of all of their T-cells. (c) shows an example from day 14 of a long-term culture with CLL B-cells and T-cells present. At this time point the percentage of Ki-67+ CLL B-cells was less than 5%. Ki-67 expression increased over time in (e) T-cells and (f) CLL B-cells taken from the same culture.
Figure 5.4. CD19+ CLL B-cells and CD3+ T-cells showed a significant increase in Ki-67 expression in long-term cultures. Flow cytometry was performed on BD Accuri C6 flow cytometer using the gating strategy shown in Figure 5.3. (a) percentage of CD19+/Ki67+ CLL B-cell in the presence and absence of 4μM fludarabine (b) percentage of CD3+/Ki67+ T cells in the absence of fludarabine; too few T-cells remained viable following the addition of fludarabine to make evaluation of Ki-67 expression possible (n = 17).

5.3.4 Long-term survival in co-culture

5.3.4.1 T-cells play an important role in maintaining long-term cultures

Consistent with the increased Ki-67 expression in cultures without fludarabine, CLL cells were maintained in culture for many months. In contrast, cultures treated with fludarabine were usually non-viable within 3-4 weeks of initiation (Figure 5.5a-b). This reduced capacity to maintain the survival of CLL cells, even in the presence of CD40L-expressing fibroblasts, was shown to be caused, at least in part, by the cytotoxic effects of fludarabine on the T-cells within the PBMCs. Figure 5.5c shows an example of the effect on CLL cell survival in co-culture when the T-cells were depleted from the PBMCs using CD3 beads. T-cell depletion using CD3 beads was significantly more detrimental to the long-term survival of CLL B-cells than the presence of 4μM fludarabine in the culture (P=0.003; Wilcoxon signed-rank test, n = 7 pairs).
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Figure 5.5. Lymphocyte count during long-term co-culture. Cultures were sampled every 7 days for and cell numbers were determined using a V-Cell XR cell counter. (a) example of long-term survival of lymphocytes in one example culture (b) example of shorter survival (c) overall survival numbers including 8 samples depleted of CD3⁺ T-cells (n=20 in total).

5.3.4.2 T-cell derived cytokines are associated with B-CLL cell survival

Subsequent analysis of the supernatants derived from the co-cultures, using the MSD V-PLEX pro-inflammatory panel 1 immunoassay kit, confirmed that the T-cells in the long-term culture model secreted a number of pro-inflammatory cytokines which may confer a survival and/or growth advantage onto CLL B-cells (Figure 5.6a). The cellular origin of the cytokines was confirmed in cultures depleted of T-cells, using CD3 beads. In supernatants derived from these cultures, there was no detectable IL-2 and significantly reduced levels of INFγ, IL-6 and IL-10 (Figure 5.6b).
Figure 5.6. **T-cell-derived cytokine production in long-term cultures.** (a) The pro-inflammatory cytokines INFγ, IL-2, IL-6 and IL-10 were measured in supernatants derived from the long-term cultures. All of the supernatants showed significant increases in cytokine levels during long-term culture when compared to the Day 0 samples. (b) T-cell depletion of matched samples resulted in a significant reduction in INFγ, IL-6 and IL-10 and no detectable IL-2 after 28 days in co-culture suggesting that the origin of these cytokines was the T-cell pool.
5.3.5 Variation in the percentage of the CD19+ and CD3+ cells during the long-term cultures

Consistent with a diagnosis of a B-cell leukaemia, the PBMCs derived from CLL patients were dominated by CLL B-cells. In all 17 long-term cultures, CLL B-cells contributed at least 80% of the absolute lymphocyte count at the initiation of the cultures. Figure 5.7 shows examples of how the proportion of CD19+ CLL B-cells and CD3+ T-cells changed in individual samples over the course of the long-term cultures. Figure 5.7a shows an example where the proportion of CLL B-cells and T-cells remained unchanged. In contrast, Figure 5.7b shows an example of how the proportion T-cells increased over time in some cases. Figure 5.7c shows the sample-to-sample variation in proportions of CLL B-cells and T-cells (n=17) and reiterates the T-cell toxic impact of the addition of fludarabine to the co-cultures. Given the cytokine data shown above, it is worth noting that the mean percentage of CD3+ T-cells at the start of these long-term cultures was just 2.6%±3.4%. Despite the small numbers, their presence appears to be crucial for the long-term viability of the cultures, even in a co-culture system which provides immobilized CD40L presented on mouse fibroblasts. This highlights the likely role of T-cell-derived cytokines in promoting the long-term in-vitro survival of CLL B-cells.
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**Figure 5.7. CD19+ CLL B-cell and CD3+ T cell percentages in long-term cultures.**
Flow cytometry was performed on BD Accuri C6 using the gating strategy shown in Figure 5.3. (a) data from example culture that continued for over 140 days with steady CD3+ and CD19+ presence (b) data from a culture with markedly increased percentages of CD3+ cells that survived for 70 days (c) overall percentages for all the long-term cultures including both control (n=17) and 4μM fludarabine conditions (n=9).
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5.3.6 Longitudinal tracking of CLL B-cell telomere length profiles in long-term cultures

Given that the model used in this chapter allowed for the maintenance of PBMCs from primary CLL B-cells for a significant length of time, I next investigated whether telomere length was altered during the course of the co-cultures. Overall, the TL of the CLL B-cells was not significantly altered between the start and end of the co-culture period (Figure 5.8a). The addition of fludarabine to the cultures had no significant impact on CLL B-cell telomere length. However, given the cytotoxic and anti-proliferative effects mediated by fludarabine, this was not surprising. Figures 5.9 and 5.10 show examples of longitudinal tracking of CLL B-cell telomere length with and without the addition of fludarabine to the co-cultures respectively.

CD5+ CD19+ CLL B-cells

![Graph](image)

**Figure 5.8. CD19+ CD5+ CLL B-cell telomere length at the start and end of the long-term cultures.** Telomere length analysis was performed on purified CLL B-cells at time intervals throughout the course of the cultures. (a) shows that there was no significant change in CLL B-cell telomere length between the start and end of the cultures (n=17). (b) shows the impact of fludarabine on CLL B-cell telomere (n=9).
Figure 5.9. Longitudinal telomere length tracking in purified CLL B-cells (CD19+CD5+) sampled from long-term cultures. CLL B-cell telomere lengths were maintained in long-term culture despite evidence of on-going tumour cell proliferation in the cultures.
**Figure 5.10 Longitudinal tracking of CLL B-cell telomere length in long-term culture in the presence of 4μM fludarabine.** Samples were harvested at two weekly intervals and purified CD19+ cell pellets were obtained using CD19+ microbeads. DNA was then extracted, and STELA analysis performed.
5.3.7 Longitudinal tracking of CLL T-cell telomere length profiles in long-term cultures

The stable telomere length profiles of CLL B-cells observed in the long-term co-culture system raised the question of what was happening to T-cell telomere length. This was particularly relevant given the high, and sustained, levels of pro-inflammatory cytokines found in the supernatants, including IL-2. Cells were harvested at regular intervals throughout the course of the co-cultures and T-cells were isolated using positive selection (CD3 beads). Overall, the T-cell telomere length profiles showed a small, but significant, increase in length over the course of the co-cultures (Figure 5.11a). This was an unexpected finding given the level of Ki-67 expression consistently found in the T-cell pool. However, closer inspection of the telomere length profiles suggested that T-cells at the lower end of the length distribution were being selectively lost over time (Figure 5.11b and 5.11c).
Figure 5.11. Longitudinal telomere length tracking in purified T-cells sampled from long-term cultures. (a) T-cell telomere lengths showed apparent lengthening in long-term culture, (b) but this appeared to result from the preferential loss of shorter telomere T-cells from the T-cell pool as seen in this example patient, (c) shows minimal change in the telomere lengths present in this example patient.
5.3.8 Comparison of in-vitro and ex-vivo CLL B-cell telomere dynamics

In order to compare the telomere dynamics of the CLL B-cells in the long-term culture model and serial samples taken ex-vivo from patients, the change in telomere length from start to end of co-cultures were plotted. Figure 5.12a shows that the largest change in telomere length was observed in samples with the longest telomere length at the start of the culture. This was exactly the same as the finding from the ex-vivo samples (Figure 5.12b).
Figure 5.12 Correlation between the starting telomere length and telomere length change in CLL B-cells derived from CLL patients. (a) CLL B-cells were isolated at the start and end of the in-vitro culture period (n=17) and (b) serially over the course of the patients’ disease (n=41). In both cases, DNA was extracted and XpYp STELA was performed. TL was determined using Phoretrix 1D software and analysed in Graphpad Prism 6 software. Correlation and linear regression analyses were performed to assess the relationship between telomere length at the start of the observation period and extent of telomere length erosion expressed as the loss of telomere length.
5.3.9 Immunophenotypic changes from the beginning to the end of the in-vitro co-cultures

5.3.9.1 Relative changes in B- and T-lymphocytes

For 8/17 of the long-term co-cultures sufficient material was available to allow for the cryopreservation of cells at the beginning and end of the co-cultures. Subsequently, these cells were analysed using immunophenotyping to determine whether there were any changes in T-cell subsets. Figure 5.13 shows the changes in CD19, CD3, CD4 and CD8 subsets from the start to the end of each long-term culture. Overall, there was a significant reduction in the percentage of CLL B-cells in the culture, which was caused by a relative expansion of CD3+ T-cells. However, there was no change in the relative numbers of CD4 and CD8 T-cells.

![Figure 5.13](image.png)

**Figure 5.13. Relative changes in the immunophenotypic composition of the long-term cultures.** The overall change in the proportions of CD19+, CD3+, CD4+ and CD8+ subsets from start to end of the cultures. There was a significant reduction in the proportion of CD19+ B-cells, which was caused by the expansion of the CD3+ compartment (n = 8).
5.3.9.2 Relative changes in CD4$^+$ and CD8$^+$ subsets

In keeping with the increase in T-cell Ki-67 expression, and the elevated levels of pro-inflammatory cytokines in the long-term cultures, the percentage of naïve CD4$^+$ cells decreased over the course of the culture (Figure 5.14a). In addition, there was a reduction in central memory (CM) cells and terminally differentiated effector memory (EMRA) cells but neither of these changes reached significance (P=0.07 and P=0.07 respectively). In terms of CD8$^+$ T-cells (Figure 5.14b), there was a significant reduction in naïve cells with a trend towards fewer CM cells (P=0.07). In contrast, there was no apparent change in the proportions of EM or EMRA in the CD8 compartment. It is worthy of note that CLL is characterised by the skewing of the T-cell pool towards memory subsets (Hanna et al., 2016, Nunes et al., 2012)(Figure 5.15) so although there were no significant increases in CD4$^+$ or CD8$^+$ memory subsets over the course of the long-term cultures, both compartments were heavily skewed towards EM and EMRA subsets.
Figure 5.14. Relative changes in CD4$^+$ and CD8$^+$ subsets in the long-term cultures. (a) The relative proportion of naïve CD4$^+$ T-cells reduced over the course of the co-cultures. There was also a reduction in CM and EMRA cells but neither of these reached statistical significance. (b) In the CD8$^+$ compartment, there was reduction in the percentage of naïve CD8$^+$ T-cells and a trend towards reduced numbers of CM cells. There was no significant change in the proportions of EM or EMRA subsets. Naïve T-cells were CCR7$^+$CD45RO$^-$, Central memory (CM) T-cells were CCR7$^+$CD45RO$^+$, Effector memory (EM) T-cells were CCR7$^-$CD45RO$^+$ and terminally differentiated effector memory T-cells (EMRA) were CCR7$^-$CD45RO$^-$ (see figure 5.15 for further details).
Figure 5.15 T-cell subsets were skewed toward memory phenotypes in long-term cultures. In keeping with what is known about CLL, both (a) the CD4 and (b) the CD8 compartments showed a marked skewing of the T-cell pool towards memory phenotypes. Long-term co-culture led to a further reduction in the percentage of naïve cells in both the (c) CD4+ and (d) CD8+ compartments (n = 8). Naïve T-cells were CCR7-CD45RO-, Central memory (CM) T-cells were CCR7+CD45RO+, Effector memory (EM) T-cells were CCR7-CD45RO+ and terminally differentiated effector memory T-cells (EMRA) were CCR7-CD45RO-.
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5.4 Discussion

The culture model described in this chapter provides a tool for the long-term maintenance of primary CLL cells in-vitro. It is of course limited in that it is a static environment that supplies continual proliferation signals to the CLL cells, which as we know now, does not reflect the real physiology of the CLL B-cells. In-vivo, CLL cells traffic between lymph node, bone marrow and peripheral blood (Davids and Burger, 2012, Lafouresse et al., 2015, Vaisitti et al., 2015) and only divide when they enter the pro-proliferative microenvironment of the lymphoid tissues (Herndon et al., 2017). The constant stimulus, via CD40L, provided by this model may explain why the when a higher density of CD40L was employed it was not compatible with long-term CLL cell survival. Despite this limitation, the model proved useful in gaining further understanding the inter-dependency of CLL B-cells and autologous T-cells and in tracking the effects on cell proliferation on the telomere lengths of both CLL B-cells and T-cells.

Firstly, the addition of fludarabine to the long-term culture model prevented the sustained survival of CLL cells. Although a proportion CLL cells resisted the direct cytotoxic effects of fludarabine and showed evidence of an increase in Ki-67 expression, the cultures never survived beyond 42 days. This was caused, at least in part, by fludarabine-mediated eradication of autologous T-cells. The importance of T-cells in sustaining these long-term cultures was confirmed by depleting CD3+ T-cells with magnetic beads. These cultures too proved to be unsustainable beyond 21 days pointing to the vital role of autologous T-cells for long-term CLL cell survival. It also highlighted the fact that CD40L stimulation alone was not sufficient to promote the long-term growth and survival of CLL cells. These findings are in keeping with previous xenograft studies in which it was shown that without activated T-cells, CLL B-cells failed to engraft (Bagnara et al., 2011). The reasons for the vital role of autologous T-cells in the model are likely to be multiple. However, there was clear evidence that the presence of
even a small percentage of T-cells (2.6%±3.4%) resulted in relatively high levels of pro-inflammatory cytokines in the supernatants of the cultures. Furthermore, depletion of CD3+ T-cells significantly reduced the levels of IL-6, IL-10 and INFγ and in the absence of T-cells there was no evidence of IL-2. These cytokines have all be implicated in the growth and survival of CLL cells (Ghamlouch et al., 2017, Lai et al., 2002, Zhu et al., 2018) so it seems likely that a key role of T-cells in these cultures is the production of these pro-survival, pro-proliferative cytokines.

Serial samples were taken from the long-term cultures every 2 weeks. Given the high levels of Ki-67 expression induced in the model, it was hoped that it could be used to track the telomere dynamics of both CLL cells and autologous T-cells. In keeping with the ex-vivo data presented in the previous chapter, CLL cell telomere length was maintained over the course of the cultures. Furthermore, the pattern of telomere erosion was identical to that seen in longitudinal ex-vivo samples; CLL cells with long telomeres at the start of the culture showed a higher rate of telomere erosion when compared to CLL cells with shorter telomeres. It is possible that CLL cells with short telomeres show differentially increased levels of telomerase activity. That would explain the modest erosion rates observed in these CLL cells, but this hypothesis was not explored here.

In contrast to the ex-vivo analysis of T-cell telomere length, the in-vitro model showed a trend towards an increase in mean telomere length. However, this was shown to be due to the selective deletion of T-cells with short telomeres over the course of the cultures. This may be a consequence of the high levels of proliferation induced by the model, which would not be typical of the in-vivo situation. The loss of T-cells with short telomere may have been due to the co-culture conditions may not have been able to provide the environment needed for survival of T-cells that would have otherwise usually entered senescence to survive. Also, T-cells with short telomeres that were unable to proliferate due to reaching a senescence threshold would have made up progressively proportionally progressively less and less of the T-cell subset present.
In 50% (8/17) of the long-term cultures, it was possible to cryopreserve cells at regular timepoints in order to retrospectively analyse the phenotypic changes that may accrue during the course of the cultures. In all cases, the majority of cells at the start of the culture were CD19+ CLL B-cells. However, there was a trend towards an increase in the proportion of CD3+ T-cells in many of the long-term cultures over time (P=0.06) that was accompanied by a significant reduction in the proportion of CLL B-cells (P=0.04). In terms of the T-cell pool, the CD4 and CD8 compartments were dominated by memory subsets. This is consistent with the characteristic skewing towards memory commonly seen in CLL (Pourghesari et al., 2010, Gothert et al., 2013, Monserrat et al., 2014). During the long-term cultures, there was a further reduction in naïve CD4 and CD8 T-cells (P=0.05 and P=0.02 respectively).

Taken together, the data generated in this chapter point to the utility of this long-term co-culture model as a way of tracking both CLL cell proliferation and telomere dynamics in a more detailed fashion, albeit in the context of constant B-cell and T-cell stimulation rather than the episodic proliferative drive encountered in-vivo. The model confirms the central role that T-cells play in the maintenance and growth of CLL B-cells and reaffirms the concept that the telomere length of the tumour cell is determined at a very early timepoint in the disease and is not significantly modulated by proliferation.
Chapter 6

General discussion and future directions

6.1 General discussion

Telomeres are specialised protective structures at the end of eukaryotic chromosomes. They are essential to maintain chromosomal integrity and genome stability. Because of the end-replication problem, telomeres progressively shorten with repeated cell division, leading to telomere dysfunction and, ultimately, contributing to tumorigenesis. In tumour cells, short telomeres were associated with genetic instability and particularly with the origin of bridge-breakage events leading to genome reorganization (Gisselsson et al., 2001). Telomere dysfunction has been proposed as a prognostic factor in CLL as short telomere length (TL) was associated with unmutated status of the immunoglobulin heavy-chain variable region gene, high-risk genomic aberrations, and poor outcome (Rampazzo et al., 2012, Ricca et al., 2007, Roos et al., 2008, Rossi et al., 2009). Besides, an association between short TL and genetic complexity measured by fluorescence in situ hybridization (FISH) analysis and/or the large of copy number aberrations detected by high resolution SNP-arrays was described (Mansouri et al., 2013, Rossi et al., 2008). This study aimed to assess telomere dynamics in CLL patients with particular focus on CLL B-cells and T-cells from peripheral blood. In addition to the serial analysis of ex-vivo patient samples, a long-term in-vitro culture system was employed in an attempt to explore the effect of sustained proliferation signalling on the telomere length profiles of patient-derived PBMCs.
The aim of Chapter 3 was to describe in detail the telomere length profiles of unsorted PBMCs, as well as purified populations of normal B-cells, T-cells and CLL B-cells isolated by using high-speed cell sorting. The XpYp telomere length (TL) and standard deviation (SD) of 83 CLL B-cell samples, 86 T-cell samples from CLL patients, as well as 16 normal B-cell samples derived from CLL patients were measured and compared. The CLL B-cells’ mean TL was significantly shorter than all of the other populations with normal B-cells having significantly longer TL than any of the other cell populations. These findings serve as a reminder of the importance of obtaining purified cell populations before performing TL analysis and may explain why studies failing to do this have yielded only relatively weak associations between TL and disease outcomes (Graham and Meeker, 2017, Wang et al., 2017, Adam et al., 2017, Tahara et al., 2017). In keeping with previous studies of non-malignant leukocytes (Alder et al., 2018) in the background population, TL of normal B-cells was shown to significantly correlate with age. In contrast, the mean TLs of CLL B-cells showed no correlation with age pointing to the important contribution of malignant transformation in determining TL. More surprisingly, the mean T-cells TLs were not associated with age either but both CLL B-cell and T-cell TL were significantly shorter in advanced stage disease patients (Binet Stage B/C vs Stage A; P=0.01). This implies that although there was only a weak correlation between CLL B-cell TL and T-cell TL ($r^2=0.076; P=0.045$), telomere shortening, in both cell types, was more marked in the patients with progressive disease (Roth et al., 2008). In keeping with extensive telomere erosion in the T-cell compartment in CLL, the mean TL was shorter than healthy donor comparators. The SDs of the TL profiles were also lower than normal age-match controls suggesting an increase in clonality in the T-cell pool in CLL patients. This concept was dealt with in more detail in Chapter 5 of this thesis. Taken together these data reinforce the close inter-relationship between CLL B-cells and autologous T-cells and imply that the propagation of the leukaemia a) is in some way dependent on T-cell “help” and b) drives the proliferation, and hence telomere shortening, observed in CLL T-cells.
Chapter 4 aimed to investigate the effect of treatment on the telomere length of these different cell populations in a cohort of CLL patients. In keeping with previously published data in this field (Guieze et al., 2016, Thomay et al., 2017, Britt-Compton et al., 2012, Lin et al., 2010, Sellmann et al., 2016), CLL B-cell TL was significantly shorter in patients with advanced stage disease. Furthermore, significantly shorter CLL B-cells were seen in patients who were treated in comparison to untreated patients, though this was not replicated in the T-cell or normal B-cells. Given the clear association between telomere length and disease stage, it remains unclear whether CLL patients with short telomeres more often require treatment or whether treatment induces telomere shortening. From the data presented in this study, it would seem more likely that the former is more likely than the latter, but this would need to be proven in a larger cohort of patients. In addition to the treated group of patients showing an over-representation of short telomeres in the CLL B-cells, the standard deviation of the T-cell telomere length profiles was significantly decreased when compared with untreated samples. This suggests that the T-cell pool manifests reduced diversity following exposure to chemotherapy.

To further investigate the telomere dynamics of CLL B-cells and autologous T-cells, serial TL analysis was performed on a cohort of 41 patients at least twice over a median follow-up time of over 6 years. Overall, TL erosion was shown to be comparable between CLL B-cells and non-leukaemic lymphocyte samples. This was surprising given the clonal expansion observed in CLL. However, a more detailed analysis revealed that there was a significant difference in TL dynamics in CLL B-cells based on the starting mean TL of the population. Patients with short telomeres with the mean of the length distribution within the fusogenic range (Lin et al., 2010, Lin et al., 2014); the previously defined length at which chromosome fusion events can occur (≤3.81kbp), showed little evidence of telomere shortening. In contrast, patients with telomere lengths outside of the fusogenic range showed significant telomere length shortening over a comparable timescale. Indeed, there was a correlation between mean
starting TL and TL erosion rates with the longest starting telomeres demonstrating the most significant TL erosion rates.

In contrast to the CLL B-cells, the T-cell TL erosion was more pronounced in the 9 patients in whom repeat measurements were performed over the same timescale. In keeping with this erosion, immunophenotyping revealed that there was a significant increase in expression of PD1 consistent with increasing T-cell exhaustion through the course of the disease (Rallon et al., 2018, Sponaas et al., 2018). However, this conclusion may not supported by other data, which showed that there was no significant increase in CD57. Generally, CD57+ T-cells are defined as highly expanded oligoclonal, terminally differentiated, senescent, memory/effector T-cells. These T-cells are characterised by their limited proliferative capacity or even their inability to proliferate under stimulation (Arosa, 2002, Brenchley et al., 2003, Focosi et al., 2010). It might be expected that an increase in PD1 expression would be accompanied by an increase in CD57 expression, but there is evidence of uncoupling of the expression of these antigens in other pathologies (Cura et al., 2018, Grome et al., 2016).

Regardless of immunophenotypes, this chapter revealed a significant association between the T-cell TL and the time since diagnosis i.e. the longer the patient had been diagnosed with their disease, the shorter was their T-cell TL. This adds weight to the suggestion that the T-cell pool is being chronically stimulated in patients with CLL and that this chronic stimulation leads to inexorable shortening of the T-cell TL. In accord with this idea, T-cell receptor sequencing of serially collected T-cells from six patients with CLL showed clear evidence of clonality in the T-cell pool. Furthermore, when tracking the most frequently occurring clones over time, 5/6 patients showed a further increase in the size of these clones over time and an increased skewing of the TCR repertoire over the course of the disease. Interestingly, the TCRVβ sequences over-represented in the T-cells analysed from these patients showed a number of similarities with previously published TCRVβ sequences from other CLL cohorts (Vardi et al., 2016, Vardi et al., 2017). This implies that the oligoclonal
expansion may be in response to an as yet unidentified common (auto-)antigen (Cui et al., 2016, Zaleska et al., 2016, Kostareli et al., 2010).

In the final results chapter, an in-vitro co-culture model was used to maintain and propagate PBMCs from patients with CLL. The primary aim of this was to build a more detailed picture of CLL B-cell and T-cell telomere dynamics in a model that induced sustained CLL cell stimulation and growth. The use of CD40L-expressing fibroblasts as a feeder layer, coupled with the addition of exogenous IL-4, induced significant cellular activation and cell division although it also caused cell death, presumably due to the constant stimulation of the CLL cells. Indeed, this was previously shown to be the case in short-term co-cultures using the same CD40L system (Hamilton et al., 2012).

The addition of the purine nucleoside analogue, fludarabine, to the long-term culture model significantly increased the level of cell death observed and this led to early termination of the cultures. Although a proportion CLL cells resisted the direct cytotoxic effects of fludarabine the loss of autologous T-cells from the culture was shown to be critically important. This was confirmed by depleting CD3+ T-cells with magnetic beads. Without the addition of fludarabine, these cultures were not viable beyond 21 days highlighting the fact that CD40L stimulation without autologous T-cells was not sufficient to promote the long-term growth and survival of CLL cells. The explanation for this was found in the supernatants of cultures containing T-cells. There were high levels of the pro-inflammatory cytokines IL-6, IL-10 and INFγ as well as IL-2. In the absence of T-cells these cytokines were all absent or found at significantly lower levels. These cytokines have all be implicated in the growth and survival of CLL cells (Ghamlouch et al., 2017, Lai et al., 2002, Zhu et al., 2018) so it seems likely that a key role of T-cells in these cultures is the production of these pro-survival, pro-proliferative cytokines.

The pattern of telomere erosion observed in the long-term cultures was identical to that seen in longitudinal ex-vivo samples; CLL cells with long
telomeres at the start of the culture showed a higher rate of telomere erosion when compared to CLL cells with shorter telomeres. It is possible that CLL cells with short telomeres show differentially increased levels of telomerase activity, which allows them to maintain their telomeres, but this hypothesis was not explored here.

In contrast to the ex-vivo analysis of T-cell telomere length, the in-vitro model showed a trend towards an increase in mean telomere length. However, this was shown to be due to the selective deletion of T-cells with short telomeres over the course of the cultures. This may be a consequence of the high levels of proliferation induced by the model, which would not be typical of the in-vivo situation. The loss of T-cells with short telomeres may have been due differential senescence and/or apoptosis in the cultures but there is no easy way to test for this.

Immunophenotypic changes were assessed in 8/17 of the long-term cultures. In all cases, the cultures were dominated by CD19+ CLL B-cells at the start. However, there was a trend towards an increase in the proportion of CD3+ T-cells in many of the long-term cultures over time that was accompanied by a significant reduction in the proportion of CLL B-cells. In terms of the T-cell pool, the CD4 and CD8 compartments were dominated by memory subsets and over the course of the long-term cultures, there was a further reduction in naïve CD4 and CD8 T-cells. This mirrors what is seen in-vivo (Monserrat et al., 2014, Nunes et al., 2012), reiterating the physiological relevance of the model.
6.2 Conclusions and future directions

This study confirmed that the telomere length profiles found in CLL are often radically different to those seen in age-matched normal controls. This was reinforced by cell sorting of normal B-cells from CLL patients; these B-cells showed much longer telomere length profiles when compared to CLL B-cells. Although CLL B-cells often constitute the bulk of the lymphocytes in patient-derived PBMC, the significantly different telomere length profiles found in CLL B-cells, normal B-cells from CLL patients and autologous T-cells provides a strong rationale for the isolation of the leukaemic B-cells prior to telomere length assessment. This is particularly important in the context of providing accurate prognostic information to patients with early stage disease and those with low leukaemic burden. Recently published work from the Cardiff CLL group highlights the power of telomere length analysis as a prognostic tool (Lin et al., 2010) and as a predictive biomarker of response to therapy in CLL (Strefford et al., 2015). It seems likely that these studies were significantly enhanced by the purification of leukaemic B-cells prior to analysis.

The association between CLL B-cell telomere length and Binet Stage found in this study confirmed the previously published findings (Lin et al., 2010). A novel, and less expected, finding was that autologous T-cell telomere length was also associated with Stage of disease and was also linked to the longevity of the diagnosis. This implies that the T-cell pool is, in some way, “reacting” to the presence of the tumour B-cells and this “reaction” is modulated by the aggressiveness of the disease. This was further reinforced when the TCR repertoire of CLL patients was serially analysed. The samples evaluated all showed some degree of clonality and this was accentuated when repeat analysis was performed later in the disease course. The significant telomere erosion observed in CLL, coupled with the skewing of the TCR repertoire provides a “smoking gun” for some form of chronic antigen-driven response. The challenges in this regard is to determine whether the response of the T-cell
pool to the CLL B-cells can be blocked in some way and if so, will this prevent
the skewing in the TCR repertoire, and thereby promote better immune
function. Furthermore, given the crucial role of autologous T-cells
demonstrated in the long-term co-culture system, it would be tempting to
speculate that interference of the CLL B-cell/T-cell interaction (both cellular and
humoral) may limit tumour cell growth. Indeed, previous studies have shown
that blocking T-cell activation have some positive clinical effects in CLL so this
could be explored in more detail in the future (Cabrelle et al., 2009, Cortes et
al., 2001).

The remarkably stable CLL B-cell telomere lengths observed over the course of
the disease explain why telomere length is such a powerful predictor of clinical
outcome, even in early stage patients. It also implies that the telomere length is,
to some extent, fixed at an early timepoint in the pathogenesis of the disease
and may simply reflect a stochastic event i.e. if a normal B-cell with long
telomeres undergoes malignant transformation then the disease will have a
more indolent course that if the normal B-cell had short telomeres. This
inference is supported by the association between short telomeres and
genomic complexity. Intriguingly, the samples derived from CLL patients with
short telomeres showed much less telomere erosion over time than those with
long telomeres. This points to the possibility that these tumours have
differentially upregulated telomerase as a way of avoiding catastrophic genome
dysregulation. Unfortunately, this was not explored as part of this thesis but
would be a very important line of investigation for the future.
Supplemental Figure number 1. Long term co-culture.
(a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA.
(b) The same sample was cultured with the addition of 4uM fludarabine. Telomere length was quantified using Phoretix software.
Supplemental Figure number 2. Long term co-culture. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. Telomere length was quantified using Phoretrix software.
Supplemental Figure number 3. Long term co-culture. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) The same sample was cultured with the addition of 4uM fludarabine. Telomere length was quantified using Phoretrix software.
Supplemental Figure number 4. Long term co-culture 14. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length was determined. Telomere length was quantified using Phoretrix software.
Supplemental Figure number 5. Long term co-culture 15. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length were determined. Telomere length was quantified using Phoretix software.
Supplemental Figure number 6. Long term co-culture (a). The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length were determined and telomere length was quantified using Phoretix software.
Supplemental Figure number 7. Long term co-culture number 17. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length were determined. Telomere length was quantified using Phoretix software.
Supplemental Figure number 8. Long term co-culture. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length were determined. Telomere length was quantified using Phoretrix software.
Supplemental Figure number 9. Long term co-culture 19. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) CLL B-cells and (c) T-cells were obtained using FACS from cryopreserved samples from the start and end of the long-term co-culture. The XpYp telomere length were determined. Telomere length was quantified using Phoretix software.
Supplemental Figure number 10. Long term co-culture. (a) The XpYp telomere length distribution of CLL B-cells at two weekly intervals in long term culture were determined by STELA. (b) 4μM fludarabine was added to a paired culture. The XpYp telomere length were determined. Telomere length was quantified using Phoretix software.


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