Epidemiological Study of Chronic Lymphocytic Leukemia (CLL) in the Province of Manitoba, Canada

By

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Abstract

A previous population-based study of survival in Chronic Lymphocytic Leukemia (CLL) patients in the province of Manitoba demonstrated a lower five-year relative survival among CLL patients compared with the age- and gender-adjusted general population. This decreased relative survival was most pronounced among elderly male CLL patients.

In this study, we have demonstrated that the reduced five-year relative survival observed in CLL patients compared to the general population of Manitoba may partially be attributed to increased risk of second cancers and non-referral to specialized CLL clinics.

The increased risk of second cancers in CLL patients compared to Follicular Lymphoma (FL), a similar indolent B cell malignancy, was only observed after CLL diagnosis indicating that a CLL-specific factor may be responsible for the increased risk of second cancers in these patients. The risk of second cancers is independent of treatment and surveillance bias but is further increased with chemotherapy.

A superior outcome in CLL patients who have been referred to the CancerCare Manitoba (CCMB) specialized CLL clinic was observed that was independent of age, gender, treatment and history of previous cancers. This superior outcome was most pronounced in the elderly CLL patients.

We propose that CLL patients should be referred to CLL-specific hematologists and, where not possible, that guidelines created by such experts be followed. Appropriate screening for second cancers should be performed during routine follow up of CLL patients.

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List of Abbreviations

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AIHA	Autoimmune Hemolytic Anemia
AML	Acute Myeloid Leukemia
APAF-1	Apoptosis-Activating Factor 1
ATM	Ataxia Telangiectasia Mutated
BAG-1	Bcl-2 Associated Athanogene
BCL-2	B Cell Lymphoma 2
BCR	B Cell Receptor
BH	Bcl-2 Homology
BTK	Bruton Tyrosine Kinase
CCMB	CancerCare Manitoba
CD38	Cluster of Differentiation 38
CDC	Complement-Dependent Cytotoxicity
CDK	Cyclin-Dependent Kinase
CDR	Common Deleted Region
CHOP	Vincristine, Prednisone and Doxorubicin
CLL	Chronic Lymphocytic Leukemia
COP	Cyclophosphamide, Vincristine and Prednisone
CR	Complete Remission
CT	Computed Tomography
dATP	Deoxyadenosine Triphosphate
DED	Death Effector Domain
DISC	Death-Inducing Signaling Complex
DLEU	Deleted in Leukemia
DNA	Deoxyribonucleic Acid
DR	Death Receptor
Dx	Diagnosis
FasR	Fas Receptor

FC	Fludarabine and Cyclophosphamide
FCR	Fludarabine, Cyclophosphamide and Rituximab
FISH	Fluorescence *in situ* Hybridization
FL	Follicular Lymphoma
FLIP	FLICE-Like Inhibitory Protein
FR	Fludarabine and Rituximab
GP	General Population
GVHD	Graft Versus Host Disease
GVL	Graft Versus Leukemia
HCDR3	Heavy Complementarity-Determining Region 3
HER2	Human Epidermal Growth Factor 2
HR	Hazard Ratio
HSCT	Hematopoietic Stem Cell Transplantation
IAP	Inhibitors of Apoptosis
ICD	International Classification of Diseases
IGHV	Immunoglobulin Heavy Chain Variable Region
ITP	Immune Thrombocytopenic Purpura
iwCLL	International Workshop on Chronic Lymphocytic Leukemia
LDH	Lactate Dehydrogenase
LDT	Lymphocyte Doubling Time
MBI	Mass Body Index
MBL	Monoclonal B Cell Lymphoma
MCL-1	Myeloid Cell Leukemia 1
MDM2	Mouse Double Minute 2
MDR	Minimal Deleted Region
MDS	Myelodysplastic Syndrome
MHC	Major Histocompatibility Complex
miR	MicroRNA
MRI	Magnetic Resonance Imaging
Mth	Month
MYD88	Myeloid Differentiation Primary Response Gene 88

NCI-WG	National Cancer Institute – Working Group
NFκB	Nuclear Factor-Kappa B
NGS	Next Generation Sequencing
NMB	Non-Myeloablative
NMSC	Non-Melanoma Skin Cancer
OR	Odds Ratio
OS	Overall Survival
PFS	Progression-Free Survival
PI3K	phosphatidylinositol 3-kinase
PNA	Purine Nucleoside Analogue
POLH	Polymerase H
PR	Partial Remission
R-CHOP	Rituximab, Cyclophosphamide, Vincristine, Doxorubicin and Prednisone
RB	Retinoblastoma
RDX	Radixin
RIC	Reduced-Intensity Conditioning
RNA	Ribonucleic Acid
RNASEH2B	Ribonuclease H2 subunit B
SCC	Squamous Cell Carcinoma
SCT	Stem Cell Transplantation
SEER	Surveillance, Epidemiology and End Results
SF3B1	Splicing Factor 3B subunit 1
SHR	Sub-Hazard Ratio
SIR	Standardized Incidence Ratio
SLL	Small Lymphocytic Lymphoma
STAT3	Signal Transducer and Activator of Transcription 3
StDev	Standard Deviation
SYK	Spleen Tyrosine Kinase
TFS	Treatment-Free Survival
TNFR	Tumour Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand

TTFT	Time To First Treatment
Tx	Treatment
UV	Ultraviolet
VH3–21	Variable Heavy genes 3–21
XIAP	X-linked Inhibitor of Apoptosis
Y	Year
ZAP70	Zeta-Associated Protein 70

Chapter One

Introduction

1.1. Epidemiology of Chronic Lymphocytic Leukemia

1.1.1. Incidence Rate

Chronic Lymphocytic Leukemia/Small Lymphocytic Leukemia (CLL/SLL, subsequently referred to as CLL) is the most common type of leukemia in the Western world, accounting for approximately 20 – 30% of all diagnosed leukemias. The worldwide incidence of CLL is <1 – 5.5/100,000 persons with Australia having the highest incidence rate followed closely by the United States; Latin American and the East Asian countries have the lowest incidence rates. In general, the incidence of CLL is higher in males than in females (1).

A population-based study of CLL in the Canadian province of Manitoba has reported the age-adjusted incidence rate of CLL to be 7.99/100,000 persons (2). This study compared the incidence of CLL based on traditional techniques commonly used by cancer registries, e.g. bone marrow or lymph node histology and cytology, with the incidence rate based on flow cytometry results in the province of Manitoba and demonstrated that the incidence of CLL was 26% higher than previously reported. Interestingly, 13.5% of cases diagnosed by traditional techniques did not have CLL

while 27% of patients with CLL were diagnosed by flow cytometry and had not been on the registry. As a result of this study, all flow cytometry reports in Manitoba showing the presence of CLL cells are now reported to cancer registry, who, in consultation with clinicians, determine whether the patient has CLL, SLL or monoclonal B cell lymphocytosis (MBL).

The median age at the time of CLL diagnosis in Manitoba was 71.5 years old (range 36 – 101). As expected, male patients were predominant, with a male to female ratio of 1.3:1. Male patients also had a lower median age at CLL diagnosis compared to women (70 vs. 73 years, respectively) (2).

1.1.2. Five-Year Relative Survival

The five-year relative survival for men with CLL in Manitoba was 85% (95% CI 78 – 92) and 80% for females (95% CI 73 – 86). When patients were divided by age, a downward gradient in five-year relative survival was observed with advancing age. Patients in the youngest age group (<55 years) had the highest five-year relative survival of 92% (95% CI 82 – 97), while the oldest age group (≥75 year) had the poorest five-year relative survival of 68% (95% CI 58 – 78). The decline in relative survival was most prominent in the oldest category (≥75 year) (2).

When patients were subdivided by both age and gender, similar age-associated decline in five-year relative survival was observed in both genders. However, men in the oldest category (≥75 year) showed a particularly low five-year relative survival (62%, CI 47 – 76) as compared to females in the oldest category (≥75 year) (76%, 95% CI 62 – 88) (2). However, reasons for the poor relative survival of elderly male CLL patients are not known.

1.2. Etiology and Inheritance of CLL

1.2.1. Environmental Factors

The etiology of CLL is largely unknown. Physical agents such as ionizing and nonionizing radiations, as well as chemical exposures in manufacturing and agricultural industries, have been thought to play a role in the etiology of CLL. However, numerous studies investigating occupational exposures to benzene, styrene, butadiene and ethylene oxide in farmers and workers in different industries have led to controversial evidence for such a linkage. Smoking, diet and application of hair dyes were also investigated with no conclusive verdict (3). Despite compelling evidence for ionizing radiation as a cause of most forms of leukemia, CLL was not found to be radiogenic in early studies. A later study investigating US workers at four nuclear weapon facilities and a nuclear naval shipyard exposed to radiation from external sources and plutonium, as well as in uranium miners, did not find a consistent association between radiation and CLL (4).

The incidence of infections prior to the diagnosis of CLL has been shown to be higher than in normal individuals (5). As a result, it has been proposed that one of the consequences of chronic infections, allergic disorders, and autoimmune diseases is prolonged stimulation of the B cell lymphocytes with an increased risk of developing CLL. In support of this concept, it has been reported that approximately 20% of CLL patients, mostly with unmutated immunoglobulin heavy chain variable region (*IGHV*) gene status, have stereotypical or quasi-identical heavy complementarity-determining region 3 (HCDR3) sequences in their B cell receptors (BCRs), suggesting that stimulation by a common antigen might result in CLL (6–11). However, the increased

rate of infections prior to the diagnosis of CLL could also reflect the immune suppression that precedes the clinical diagnosis of CLL.

1.2.2. Familial CLL

Based on current evidence, a family history of CLL or other hematological malignancies is the strongest risk factor linked to the development of CLL (12–15). Thus, first-degree relatives of CLL patients are have a 30-fold increased risk of developing CLL. In addition, 13 – 18% of unaffected first-degree relatives have MBL, the precursor of CLL, and this is considerably higher than expected in the general population (3.5%) (16–18). Furthermore, the fact that CLL is less common in Asians than in North Americans, together with the fact that rates of CLL are similar between Asian populations in North America and in Asia indicates a stronger role for genetic factors as compared to environmental factors in the etiology of CLL (19).

Patients with at least one first-degree relative with CLL are considered to have familial CLL while other cases are considered sporadic CLL. Approximately 10% of CLL cases are estimated to be familial (13,20). Familial cases show an earlier age of onset, being approximately 10 years younger at the time of diagnosis compared with sporadic cases (14,20). Also anticipation, where an inherited disease is diagnosed at an earlier age in each successive generation, has been reported in multigenerational studies, showing the affected successive generation to be 15 – 20 years younger than the preceding generation at the time of onset (21–24). Shorter lengths of telomeres, high expression of cluster of differentiation 38 (CD38) and higher levels of B lymphocyte stimulator were reported in patients with familial CLL (25–27). Overall, however there

are no striking or consistent differences between familial and sporadic CLL in terms of prognostic markers or clinical outcome.

1.3. Diagnosis of Chronic Lymphocytic Leukemia

1.3.1. National Cancer Institute – Working Group Guidelines (1996)

When the National Cancer Institute – Working Group (NCI-WG) guidelines were developed in 1996, the diagnosis of CLL required a lymphocyte count of $\geq 5\times10^9$/L while patients with SLL had a lower count accompanied by lymphadenopathy and/or splenomegaly (28). In addition, the malignant lymphocytes had to have specific immunophenotypic markers: (i) coexpression of the B cell markers CD19, CD20 and CD23, together with T cell marker CD5 in the absence of other T cell markers; (ii) B cell monoclonality, with expression of either surface κ or λ light chains, and (iii) low density surface immunoglobulin.

A bone marrow examination was not required for the diagnosis of CLL, although it could provide prognostic information by determining whether marrow involvement was diffuse or non-diffuse (28,29).

1.3.2. International Workshop on CLL Update of the 1996 Guidelines (2008)

The 1996 NCI-WG guidelines for diagnosis and treatment of CLL were updated at the international workshop on CLL (iwCLL) in 2008. Based on these updated guidelines, the diagnosis of CLL now requires $\geq 5\times10^9$ B lymphocytes/L in peripheral blood. Individuals with $<5\times10^9$/L CLL cells in peripheral blood together with lymphadenopathy or splenomegaly are diagnosed as small lymphocytic lymphoma (SLL) whereas those with no organomegaly or other features of CLL are diagnosed as

monoclonal B cell lymphocytosis (MBL) (30). Patients with MBL are considered to have a pre-malignant condition and 1% to 2% will progress to CLL and require treatment each year (31).

CLL cells coexpress the T cell antigen, CD5, and the B cell antigens, CD19, CD20 and CD23 (30). However expression levels of CD20, CD79b and surface immunoglobulins are low in CLL cells compared with normal B cells (32,33) and each leukemia B cell clone expresses either κ or λ immunoglobulin light chains (32).

Molecular cytogenetic analysis, mutational status of *IGHV* gene and expression of CD38 and Zeta chain-associated protein 70 (ZAP70) are not required to establish the diagnosis of CLL but may provide useful information regarding the patient's prognosis and the most appropriate treatment options.

1.3.3. Presentation

The majority of newly diagnosed CLL patients are elderly male individuals. Patients usually present with an incidental finding of lymphocytosis during routine blood testing or lymphadenopathy or splenomegaly during a regular physical examination. However, some patients present with infections, the most common in CLL patients being bacterial pneumonias. Symptomatic patients usually present with lymphadenopathy, splenomegaly, fever, night sweats and weight loss, and require treatment shortly after diagnosis. Sometimes patients present with anemia that is usually due to marrow replacement by tumor, but it could also be related to an autoimmune hemolytic anemia (34).

The median lymphocyte count at diagnosis is $20 – 30 \times 10^9$/L and the leukemia cells are characteristically small and mature-appearing, with a dense nucleus and

inconspicuous nucleolus surrounded by a narrow ring of cytoplasm. These cells may be admixed with larger atypical cells, cleaved cells or prolymphocytes (28). As the disease progresses the number of prolymphocytes can increase and when more than 55% of the cells are prolymphocytes, the patient is considered to have prolymphocytic leukemia (34). Another characteristic feature of CLL is the presence of "smudge cells", which are fragile CLL cells that have become broken during the preparation of the smear. Interestingly, patients with >30% smudge cells have a good prognosis and are more likely to have the "good prognostic markers", such as a mutated *IGHV* gene and low expressions of CD38 and ZAP70 (35).

1.3.4. Monoclonal B Cell Lymphocytosis

Monoclonal B Cell Lymphocytosis (MBL) can be detected in 3.5% of normal individuals over the age of 40 years, with the incidence increasing with advancing age. The incidence is even higher in healthy relatives of individuals with CLL (13 – 18%) (16–18). A recent study indicated that the incidence of MBL in the general population could be as high as 12%, depending on the sensitivity of the assay (36).

Individuals with MBL can be divided into two groups; "population" MBL that is established by incidental finding or screening of healthy non-symptomatic individuals with sensitive tests, and "clinical" MBL that is detected when individuals with slightly elevated lymphocyte counts are tested. The chance of progression to a hematological malignancy is very low in individuals with population MBL (37). It has been shown that survival of individuals with clinical MBL who also have CD38-positive clones was significantly shorter than that of an age- and gender-matched control population,

whereas survival of individuals with clinical MBL who also have CD38-negative clones is similar to that of an age- and gender-matched control population (38).

The development of the iwCLL updated diagnostic criteria for CLL in 2008 has changed the number of patients with MBL or CLL. Approximately 40% of patients with Rai stage 0 CLL, based on the 1996 diagnostic criteria, would nowadays be considered to have MBL (39). Thus, today the number of patients with Rai stage 0 (see section 1.4) is approximately reduced to half, and the remaining Rai 0 patients have more aggressive disease than patients diagnosed using the older criteria.

1.4. Staging of Chronic Lymphocytic Leukemia

There are currently two staging systems for CLL, the Rai staging system (40) which is more commonly used in North America, and the Binet staging system (41) which is more popular in Europe. Both staging systems are based solely on physical examination and standard blood tests and do not require ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). Therefore they are both inexpensive and available worldwide. Both staging systems correlate clinical features with patient outcome and describe three patterns of survival, namely, low-, intermediate- and high-risk categories.

1.4.1. Rai Staging

Rai classification defines low-risk patients as patients with CLL cells in blood and marrow (Rai 0); intermediate-risk patients as those with lymphocytosis and lymphadenopathy (Rai I), or splenomegaly (Rai II); and high-risk patients as those with lymphocytosis and anemia (hemoglobin level <110 g/L) (Rai III), or thrombocytopenia

(platelet count <100 × 109/L) (Rai IV) related to marrow replacement by tumor (Table 1.1) (40).

Approximately 25% of all CLL patients eventually develop anemia or thrombocytopenia. Cytopenia may be due to marrow replacement by leukemia cells (54% of cases), presence of antibodies against red cells or platelets (18%) or other causes, such as chemotherapy (28%) (42). A bone marrow biopsy is useful to sort out the cause of the cytopenia for patients with Rai stages III and IV disease (34).

1.4.2. Binet Staging

The Binet staging system has three stages, namely, A, B and C and is based on the number of sites of disease involvement (nodes, spleen and liver) and the presence or absence of anemia or thrombocytopenia (41).

1.5. Prognosis

Traditionally, prognosis of patients with CLL was solely based on the clinical stage of the disease and this is still an excellent tool to assess survival. However, because of routine blood testing, 85% of patients nowadays present with Rai stages 0 and I disease and prognostic markers are thus required to determine in this group of patients who will progress, and who will have more stable, indolent disease. These cytological and hematological prognostic markers are summarized in Table 1.2.

1.5.1. Stage of the Disease

As mentioned earlier, stage of the disease has been traditionally used to assess the outcome of CLL patients. The median survival of low-risk patients, with Rai stage 0 is

Table 1.1. Patient characteristics based on Rai stage

Rai Stage	Risk	Symptoms	% at Diagnosis	Median Survival (y)*
0	Low	Lymphocytosis	~ 60	>10
I	Intermediate	Lymphocytosis + Lymphadenopathy	~ 30	6 – 9
II	Intermediate	Lymphocytosis + Splenomegaly	~ 10	
III	High	Lymphocytosis + Anemia (Hemoglobin <110g/L)	~ <2	1.5 – 6
IV	High	Lymphocytosis + Thrombocytopenia (Platelets $<100\times10^9$/L)	~ <2	

g: gram; L: litre; y: year

*(40,43,44)

Table 1.2. Summary of prognostic features

Prognostic Feature	Superior prognosis	Inferior Prognosis
Rai Stage	Stages 0, I and II	Stages III and IV
Age	≤75 years	>75 years
Gender	Female	Male
LDT	>12 months	≤12 months
Cytogenetic Abnormalities	Deletion 13q Normal Karyotype	Deletion 17p Deletion 11q
IGHV gene Status	Mutated	Unmutated
CD38 Expression	<20% B cells	≥20% B cells
ZAP70 Expression	<20% B cells	≥20% B cells
ß2-Microglobulin Level	Normal and Low	High
Creatinine	Normal and Low	High
Creatinine Clearance	≥60	<60

IGHV: immunoglobulin heavy chain variable region; LDT: lymphocyte doubling time

>10 years, intermediate-risk patients with Rai stages I and II have a median survival of 6 – 9 years, and high-risk patients with Rai stages III and IV have a median survival of 1.5 – 6 years (Table 1.1) (43–45).

1.5.2. Age and Gender

As reviewed earlier (section 1.1.2.), male CLL patients have a poorer five-year relative survival compared to female CLL patients (80%, 95% CI 73 – 86 vs. 85%, 95% CI 78 – 92 for male and female patients, respectively). Furthermore, an association was established between advancing age and declining relative survival in CLL patients of both genders, with elderly male patients having the poorest relative survival (2).

1.5.3. Lymphocyte Doubling Time

Lymphocyte Doubling Time (LDT) is a simple parameter that represents the speed of evolution and progression of CLL. A high LDT (>12 months) predicts longer treatment-free survival and is associated with good outcome, whereas a low LDT (≤12 months) predicts an aggressive disease in early-stage patients and indicates a poorer survival with a median of 58 months (46,47). A LDT <6 months is indication of active disease and need for treatment (30) (see section 1.7.1). LDT has been shown to be a strong and independent predictor of both time to first treatment (TTFT) and overall survival (OS) in early-stage CLL patients (48).

1.5.4. Cytogenetic Abnormalities

Chromosomal aberrations can be detected in approximately 82% of CLL cases using interphase fluorescence *in situ* hybridization (FISH) techniques, with the most frequent aberrations being deletion 13q14 (55%), followed by deletion 11q22-q23

(18%), trisomy 12q (16%), deletion 17p13 (7%), and deletion 6q (6%) (49). Based on these cytogenetic abnormalities, five prognostic categories were defined to reflect patients' response to treatment and length of survival. The median TTFT for patients with deletion 17p, deletion 11q, trisomy 12, normal karyotype, and deletion 13q was reported to be 9, 13, 33, 49 and 92 months, respectively. The median survival times for patients in these groups were 32, 79, 114, 111, and 133 months, respectively. The incidence of cytogenetic abnormalities can vary during the disease course as they may be acquired over time or in response to chemotherapy. It has been shown that 25% of patients will develop new chromosomal abnormalities within the first five years after diagnosis, with the risk being higher in patients with high expression of CD38 and ZAP70 and those with an unmutated *IGHV* gene (50).

1.5.4.1. Deletion 17p13

CLL patients with deletion 17p13 (hereafter referred to as deletion 17p) have the worst prognosis among all CLL patients, with a median survival of 2 – 3 years (49). Interestingly, it has been shown that the outcome of patients with *de novo* deletion 17p (5 – 7% of all CLL patients) is superior to those who acquired deletion 17p after treatment. A retrospective multicentre study from the Mayo Clinic and the MD Anderson Cancer Center on chemotherapy-naïve CLL patients revealed that approximately 50% of patients with *de novo* deletion 17p progressed within 12 to 18 month of diagnosis and required treatment, whereas the remaining patients had a relatively stable disease that extended to 70 months of follow up (51). Deletion 17p is associated with high expression of CD38 and ZAP70 (52,53) as well as with short telomeres (6,54,55).

The deletion 17p is usually accompanied by a *p53* mutation on the other allele and resistance to conventional chemotherapy. Fludarabine treatment has been shown to increase the expression of p53-dependant genes and consequently the probability of selection of 17p-deleted cells (56). Patients who have acquired a17p deletion by the time they require treatment (10% of all CLL cases) rarely achieve complete remission (CR) with fludarabine-containing combination therapies and have a median survival of less than three years (57). It is noteworthy that 33% of fludarabine resistant patients have deletion 17p and an additional 9% have a mutation in their *p53* gene (58). These patients have an inferior prognosis and a shorter survival time.

1.5.4.2. Deletion 11q22-q23

Deletion 11q22-q23 (hereafter referred to as deletion 11q) is the second most common chromosomal aberration seen in CLL patients and is associated with bulky lymphadenopathy, rapid progression and poor survival in younger patients (49,59–61). In these patients, certain adhesion proteins were expressed in reduced quantity, potentially explaining the observed marked lymphadenopathy (60). Deletion 11q has been shown to be associated with short telomeres and poor survival (6,54,55).

Two genes were proposed to be affected by this aberration, namely ataxia telangiectasia mutated (*ATM*) and radixin (*RDX*) genes (60). ATM is a protein kinase that phosphorylates and subsequently activates p53 in response to breaks in double stranded deoxyribonucleic acid (DNA), while *RDX* is a tumour suppressor gene. It has been shown that the second *ATM* allele is mutated in 36% of 11q-deleted CLL cases and these patients are therefore refractory to DNA damaging chemotherapeutic agents and respond poorly to radiation therapy. These patients have an inferior TFS and OS

compared to patients with deletion 11q and a functional *ATM* gene. It has been suggested that a deletion 11q might promote selection of clones with *ATM* mutations. Although *ATM* mutations are usually associated with an unmutated *IGHV* gene, they can still provide additional prognostic information over the *IGHV* mutational status (62–64).

Patients with deletion 11q can strongly benefit from addition of monoclonal antibodies to their treatment regimen. Addition of rituximab to fludarabine and cyclophosphamide (FCR) significantly improved CR and OS in these patients (CR 15% to 51%, p <0.001; and OS 83% to 94%, p 0.036) (57).

1.5.4.3. Trisomy 12q

Trisomy 12q (hereafter referred to as trisomy 12) is the third most common chromosomal abnormality in CLL patients and is caused by duplication of one of the homologues of chromosome 12. It can be seen in 16% of CLL cases (49). 12q13-15 contains the mouse double minute 2 (*MDM2*) homologue gene in humans, and the MDM2 protein can bind with and inactivate p53; thus, overexpression of the *MDM2* gene can simulate a *p53* mutation (65). Trisomy 12 is also associated with the presence of a *NOTCH1* mutation (66,67).

Trisomy 12 is frequently detected in atypical CLL or CLL/PLL and in CLL patients with more advanced disease and higher proliferative activity, which, in turn, predicts an inferior outcome (68). Approximately 50% of patients with trisomy 12 also have a mutation in *p53* gene, which could explain their poor prognosis (69). Interestingly, the significance of trisomy 12 in predicting survival appears to depend on the means of its detection. When trisomy 12 is detected by classic cytogenetics, it represents a subgroup of CLL patients with the poorest prognosis among CLL patients

with abnormal karyotypes; in contrast, when detected by FISH, it predicts a moderately inferior prognosis as compared with patients with a normal karyotype (49,70). In general, patients with trisomy 12 respond well to treatment (57) and CLL patients with trisomy 12 (detected by FISH) and normal karyotype were reported to have a similar OS and TTFT (71).

1.5.4.4. Deletion 13q14

Deletion 13q14 (hereafter referred to as deletion 13q) is the most common cytogenetic abnormality (55% of cases) and patients with this deletion are known to have the best prognosis compared to patients with other chromosomal abnormalities and to those with a normal karyotype (49).

Deletions 13q are usually large with anatomical heterogeneity that affects multiple genes. The heterogeneity of this aberration is reflected in the size of the deleted region, monoalleleic or bialleleic deletions, and whether or not they extend into centromeric or telomeric regions of 13q. The region defined as the minimal deleted region (MDR) includes the deleted in leukemia 2 (*DLEU2*) gene and microRNA (miR)-15A/16-1 cluster genes. The region telomeric to the MDR is often deleted as well and is referred to as the common deleted region (CDR). This region includes three genes, namely *DLEU1*, *DLEU7*, and ribonuclease H2 subunit B (*RNASEH2B*). A third region, located centromeric to MDR is also deleted in some cases and includes the retinoblastoma (*RB*) gene (72–75).

DLEU2 encodes a noncoding ribonucleic acid (RNA) with unknown function, while miR-15A/16-1 is a tumour suppressor that inhibits cell proliferation and promotes apoptosis by targeting myeloid cell leukemia 1 (MCL-1) and B cell leukemia 2 (BCL-2)

proteins (75,76). It has been shown that deletion of miR-15A/16-1 cluster genes leads to CLL and MBL in transgenic mice due to uncontrolled cell proliferation (74,77). *DLEU1* gene is not evolutionarily conserved and the function of its transcript is unknown, while *DLEU7* inhibits cell proliferation (74,78).

It has been shown that the percentage of nuclei with a deleted 13q and the size of the deletions provide useful prognostic information in CLL. Thus, patients with a 13q deletion in <70% of their nuclei and a deletion that only includes the MDR have a longer TTFT than patients whose cells carry the 13q deletion in <70% of nuclei and have a larger deletion that includes the *RB* gene, or patients with a 13q deletion in >70% of nuclei, regardless of involvement of *RB* (72,73). Furthermore, loss of CDR, in addition to MDR, leads to more aggressive disease (74). These reports suggest that the size and subtype of deletion 13q are important prognostic features.

1.5.5. *IGHV* gene mutational status

During normal B cell development, antigen-exposed B cells enter the lymphoid follicles in the secondary lymphoid organs to form germinal centres. In germinal centres, follicular dendritic cells present antigens and B cells undergo affinity maturation under the influence of T cells. During affinity maturation, random somatic mutations occur in the variable region of the immunoglobulin genes in B cells, leading to B cells with variable degrees of affinity for the presented antigen. Thereafter, B cells with the highest affinity to the presented antigen will be selected for further differentiation, while B cells with lower affinity are eliminated (79,80).

CLL cells originally were thought to be antigen-inexperienced or pre-follicular B cells. However Hamblin *et al.* (81) and Damle *et al.* (82) simultaneously and

independently identified CLL cases with mutated *IGHV* genes that showed less than 98% homology to the germline sequence. The 98% cutoff was chosen to account for polymorphisms that are quite common in *IGHV* genes (83). This cutoff has been reevaluated and was shown to be the most appropriate value for predicting the outcome of CLL patients (84–86).

Approximately 50% of all CLL cases have undergone somatic hypermutation in their *IGHV* gene. Patients with mutated *IGHV* genes have normal cell morphology and a favorable prognosis while patients with unmutated *IGHV* status have an inferior outcome (6,43,87–89). Mutated CLL cells are more likely to have deletion 13q, whereas unmutated cells have an increased risk of developing deletions in chromosomes 11q and 17p, mutations in *NOTCH1* gene and shorter telomeres (6,50,90,91). Ultimately, unmutated cases are more likely to develop drug resistance, progress or transform. Unmutated *IGHV* is also associated with shorter telomeres, increased telomerase activity and inferior outcome (54,55,92,93). Patients with unmutated *IGHV* have been shown to have a shorter TTFT and OS compared with mutated patients (48).

Interestingly, CLL cases that express the variable heavy genes 3–21 (*VH3–21)* usually have mutated *IGHV* while their survival has been shown to be comparable to that of patients with unmutated *IGHV* (94–96). These cases are also more likely to have a dysfunctional *p53* gene (97).

Although *IGHV* mutational status is one of the most important and accurate prognostic markers in CLL, it is not routinely determined in Canada as the assay is laborious and expensive (48). Comparative microarray experiments have thus been performed on mutated and unmutated cases to identify surrogate markers to replace

IGHV mutational status. Increased CD38 expression was first found to correlate with unmutated *IGHV* (82). Genome-wide gene expression assays subsequently identified increased ZAP70 expression in patients with unmutated *IGHV* as a possible surrogate marker (87,98).

1.5.6. CD38 Expression

CD38, also known as cluster of differentiation 38, is a 45 kDa transmembrane glycoprotein that acts as both an enzyme and a receptor (99). CD38 is expressed on the surface of more than 20% of leukemia cells in approximately 50% of CLL cases (100). CD38 activates ZAP70 and its downstream survival pathways (101) and is involved in cell proliferation, differentiation and survival (43,102). Increased CD38 expression is thus associated with progressive disease, atypical morphology, lymphadenopathy, increased ß2-microglobulin levels, short LDT and poor prognosis (100,103,104). CD38 expression has also been shown to be associated with ZAP70 expression and deletion 17p (52,53) as well as a diffuse bone marrow histology (105–107).

It has been shown that CD38 expression in lymph nodes reflects the proliferation status, suggesting that the number of CD38-positive cells in lymph nodes may have more clinical relevance than the number in the peripheral blood or bone marrow (108). The number of CD38-positive cells may change during the course of the disease and increases with disease progression, especially in cases with 17p deletions and a *p53* mutation (43,89,109,110). Hamblin *et al.* (111) observed an increase in CD38 expression in 24% of their cases. An increase in CD38 expression may occur with treatment, suggesting selective elimination of CD38-negative cells by chemotherapy and repopulation with CD38 expressing cells. They observed complete replacement of CLL

cells with CD38 expressing CLL cells after multiple rounds of chemotherapy in one patient and recovery of CD38-negative cells in another patient after discontinuing treatment. They also reported an increase in the proportion of CD38-expressing cells with progression of disease in untreated patients.

The original cutoff of 30% CD38-staining cells was chosen to classify CD38 positive and negative patients (82) and validity of this cutoff was later verified (111). However, lower cutoff levels have been proposed (7% and 20%) by other groups and therefore the optimal cutoff value for classification of CLL patients based on CD38 expression remains controversial (100,106,107,112–115).

Although CD38 expression cannot be considered a surrogate marker for the *IGHV* mutation status, it provides additional independent prognostic information for CLL patients (111,113,116,117).

1.5.7. ZAP70 Expression

Expression of ZAP70, an intracellular protein tyrosine kinase, was traditionally thought to be unique to T cells and natural killer cells. However, it has been shown that some CLL cells and even normal B cells also express ZAP70. ZAP70 has been shown to enhance BCR signaling in leukemia cells and in turn promote cell survival in CLL. Therefore, ZAP70-positive cells are associated with an aggressive disease and poor prognosis. Expression of ZAP70 in CLL cells is also associated with unmutated *IGHV* gene status (118–120), CD38 expression and deletion 17p (52,53).

It has been shown that CLL cells with unmutated *IGHV* gene have a higher expression of ZAP70 (87). This association was particularly strong in CLL cells that lack additional negative prognostic markers such as deletion 11q, deletion 17p or V3 –

21 usage (concordance was observed in 84% of cases). Alternatively, in the presence of other negative prognostic markers, the proportion of discordant cases increases up to 40%. Also, it was shown that discordant cases with cytogenetic abnormalities were almost exclusively ZAP70-negative and had unmutated *IGHV* (92%); while discordant cases with *V3 – 21* usage were exclusively ZAP70-positive and had mutated *IGHV* (89%) (53). The median survival of ZAP70-negative patients was reported to be notably longer than that of ZAP70-positive patients (24.4 years, 95% CI 15.1 – 33.8 and 9.3 years, 7.0 – 11.5, respectively; hazard ratio 5.5, 95% CI 2.8 – 10.8) (52). Accordingly, CLL patients with mutated *IGHV* status, which by itself is considered a good prognostic marker, do poorly if expressing high levels of ZAP70.

Expression of ZAP70 is strongly associated with the need for treatment in CLL patients. Patients with positive ZAP70 expression have a shorter TTFT compared with ZAP70-negative patients, regardless of their *IGHV* mutational status. ZAP70-positive patients have been reported to have a median TTFT of 2.8 and 4.2 years with unmutated and mutated *IGHV* status, respectively (p: 0.07); however, the median TTFT in ZAP70-negative patients was 11.0 years and 7.1 years with mutated and unmutated *IGHV* status, respectively ($p < 0.001$) (118).

In contrast to CD38, initial studies suggested that ZAP70 expression remained constant over time, but more recent studies have shown that ZAP70 expression levels change in 5% to 10% of patients (52,121–123).

1.5.8. Identification of Prognostic Factors Using Next Generation Sequencing

The development of next generation sequencing (NGS) has offered us the ability to obtain millions of sequences in a single experiment and enabled the sequencing of

complete genomes, exomes (all annotated exons), transcriptomes (all RNA transcripts) and methylomes (epigenetics) of different cancer types. Whole genome sequencing (WGS) allows detection of all DNA abnormalities, while whole exome sequencing (WES) or targeted re-sequencing focuses on coding DNA regions. WGS studies are more expensive and have a rather limited sample size compared to WES studies (124).

The first whole cancer genome sequence was completed in 2008 in a patient with acute myeloid leukemia (AML), demonstrating the value of NGS in detecting novel somatic mutations (125). NGS studies have subsequently been carried out in CLL and have shown that the CLL genome has a lower mutation burden compared to solid tumors (126,127).

The clinical outcome of CLL has been associated with somatic hypermutations and chromosomal aberrations. In addition to genomic alterations, cancer cells also undergo epigenetic changes such as DNA methylations, histone modifications and gene regulations through noncoding RNA (128). However, the clinical relevance of epigenetic changes in CLL is unclear.

It has been shown that genes involved in pathways associated with immune response, such as B cell signaling, T cell co-stimulation, and cytokine-cytokine interactions are more frequently hypomethylated (129). A number of epigenetic alterations such as hypermethylation and silencing of promoter regions of tumor suppressor genes have been shown to occur in CLL (130). Methylation levels of a single CpG in the *ZAP70* gene has been shown to influence ZAP70 expression and thus clinical behavior in CLL (131).

In recent years, WGS and WES in CLL revealed striking molecular heterogeneity between patients (132). These studies have found a number of genes that were mutated in 10% to 15% of patients while the majority of mutated genes were found only in a small fraction of cases (2% to 5%). Moreover, approximately 30% of patients did not show mutations.

Interestingly, the majority of mutated genes were involved in a few pathways that were differentially expressed in mutated and unmutated CLL patients. Genes involved in NOTCH1 signaling (*NOTCH1*), mRNA splicing, processing and transport (*SF3B1*), and DNA damage response pathways (*ATM* and *p53*), were more frequently mutated in cases with unmutated *IGHV*, while mutated patients showed a clustering of mutated genes involved in innate inflammatory pathways (*MYD88*) (133–135).

NOTCH1 encodes a transmembrane protein that serves as a ligand-activated transcription factor involved in cell differentiation, proliferation, and apoptosis. Upon binding to its ligand, NOTCH1 is cleaved and the intracellular domain of the protein is released and translocated to the nucleus to initiate transcriptional activation of several genes. Mutations in the *NOTCH1* gene generate a premature stop codon that results in a protein that lacks the C-terminal PEST domain (rich in proline, glutamic acid, serine and threonine) (127,134,136). The truncated protein is constitutively active and accumulates in CLL cells, leading to an increased cell survival and resistance to apoptosis (137).

The *SF3B1* gene encodes the splicing factor 3B subunit 1 protein, which is included in the SF3B splicing factor complex and plays a role in spliceosome assembly and mRNA splicing (138). *SF3B1* mutations lead to a faulty spliceosome complex and patients with *SF3B1* mutations showed a defective splicing activity (134). Myeloid

differentiation primary response gene 88 (*MYD88*) mutations lead to an elevated activation of signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NFκB). Identical mutations in the *MYD88* gene were reported across several lymphoid malignancies suggesting its potential proto-oncogenic activity in the pathogenesis of these lymphomas (139).

In one exome study, *NOTCH1* (12%) and *SF3B1* (10%) were recognized as the most common mutations in CLL (133), while in a second exome study (134) *p53* (15%), *SF3B1* (15%), *MYD88* (10%), and *ATM* (9%) were found to be the most commonly mutated genes in CLL. This difference may be, at least in part, explained by the diverse clinical characteristics of patients enrolled in the two studies. The majority of patients in the former study were early stage untreated patients, whereas the majority of patients in the latter study had adverse prognostic features such as advanced stage, chromosomal aberrations and high ZAP70 expression with approximately 30% having relapsed following chemotherapy. Also, patients in the former study were older than in the latter study (median ages of 54 and 62 years, respectively). This observation suggests that the mutation profile of patients may change during the progression of disease and certain mutations may be acquired over time. Alternatively, it may also implicate that aggressiveness of CLL may be pre-determined and that the mutation profiles of newly diagnosed patients may determine the aggressiveness of their disease at the time of diagnosis. More likely, however, a combination of pre-determined and acquired mutations may dictate the rate of progression in CLL. Interestingly, one study reported mutational activation of the NOTCH1 pathway in 8% of CLL patients at the time of

diagnosis and in 31% of patients at the time of disease progression and Richter transformation (see Section 1.9) and in 21% of refractory patients (126).

NOTCH1 mutations are also associated with advanced disease at the time of diagnosis and are more commonly seen in patients with trisomy 12 (66,140,141). Interestingly, CLL patients with *NOTCH1* mutations have an increased risk of developing a Richter's transformation and the transformation occurs more rapidly after the CLL diagnosis than is seen in patients without a mutation (91,127,142,143). In one study, it was demonstrated that all patients with a Richter's transformation had evidence of a *NOTCH1* mutation in their CLL cells at the time of CLL diagnosis (126). This suggests that *NOTCH1* mutations are present many years prior to transformation and that clonal selection of mutated CLL cells may lead to the clinical transformation. *SF3B1* mutations appear to be associated with deletion 11q, *ATM* mutations, fludarabine resistance, and advanced disease at the time of diagnosis. Patients with these mutations are reported to have a poorer prognosis with significantly shorter PFS and OS, suggesting that the mutations contribute to the poor prognosis (133,134,144,145). However, whether the poor prognosis in patients with mutated *NOTCH1* or *SF3B1* is associated with *IGHV* mutational status is unclear (91,142,145–147).

A higher frequency of A>C and T>G substitutions has been observed in CLL cases with mutated *IGHV* compared to those with unmutated *IGHV*. Interestingly, DNA polymerase h (*POLH*) also introduces similar mutations. *POLH* is abundantly expressed in follicular germinal centre cells and is responsible for repairing DNA breaks and contributes to the immunoglobulin gene diversity (127,129,133). This asymmetrical distribution of A>C and T>G substitutions may be indicative of different

microenvironments that each cell type is exposed to during development and that the different clinical behaviour of each subtype may be related to the diverse gene mutation profiles and subsequently deregulation of different molecular mechanisms in mutated and unmutated CLL patients.

Genomic and epigenomic studies using NGS emphasized the molecular complexity and heterogeneity of CLL and provided the capability to further explore different clinical and molecular subgroups of CLL. However, the clinical implications of these findings are not well understood. These newly found mutated genes may in future find their ways onto CLL prognostic panels or become the target of new therapies (148,149). This will provide CLL patients with truly individualized diagnosis, risk assessment and treatment.

1.5.9. Other Prognostic Factors

ß2-microglobulin, a component of the major histocompatibility complex (MHC) class I molecules which are present on all nucleated cells, is constantly shed by CLL cells and its level is thus directly related to tumour mass, LDT and CD38 expression (150). Plasma levels of ß2-microglobulin have been shown to be a predictor of TTFT and OS, with increased levels of ß2-microglobulin indicating a poor outcome (57,151–154). ß2-microglobulin is strongly associated with age and levels of plasma inflammatory cytokines that could also be important prognostic factors, especially in the elderly CLL patients (151). ß2-microglobulin is cleared by the kidneys, and the levels should be adjusted with glomerular filtration rate (154).

CLL cells characteristically express membrane CD23 that could increase plasma levels of CD23 due to spontaneous proteolysis. CD23 is a target gene for the NOTCH2

signaling pathway and deregulation of NOTCH2 signaling could lead to overexpression of CD23 in CLL cells. Therefore, an elevated level of CD23 is indicative of faulty apoptosis, cell proliferation and disease progression (155,156).

Creatinine, creatinine clearance and albumin levels may reflect internal organs function, comorbidities and the microenvironment conditions in CLL patients. It has been shown that an increased level of creatinine is an independent predictor of outcome and is associated with shorter OS in CLL patients (157).

There are three different patterns of bone marrow involvement in CLL patients: diffuse (interstitial or packed), non-diffuse and nodular. These patterns of involvement have prognostic value at all clinical stages. Diffuse involvement, in which fat tissue in bone marrow is replaced by leukemia cells, indicated poor prognosis (29,158).

In summary, these prognostic factors can predict the rate of disease progression and thus the need for more or less monitoring. The presence of a short LDT and advanced Rai stage may indicate the need for treatment, while the presence of a deletion 17p or 11q will provide guidance as to the type of treatment required.

1.6. Pathophysiology of the CLL Cell

Apoptotic defect is known to be the major mechanism for disease progression and drug resistance in CLL cells. Apoptosis is initiated through two major pathways, namely intrinsic and extrinsic apoptotic pathways. Both pathways are dependent upon activation of caspases. There is also a cross talk between the two pathways and they can activate the same downstream caspases (34).

1.6.1. Intrinsic Apoptotic Pathway

BAX and BCL-2 regulate the intrinsic apoptotic pathway by controlling the permeability of the mitochondrial membrane, and expression of these proteins is regulated by the tumor suppressor p53. DNA damage, or other types of severe stress, leads to upregulation of *p53* and subsequently an increase in the pro-apoptotic protein BAX and a decrease in the pro-survival protein BCL-2. This increases the permeability of the mitochondrial membrane and releases pro-apoptotic proteins, such as cytochrome *c* and Smac/DIABLO, into the cytosol. Cytochrome *c* binds to and activates apoptosis-activating factor-1 (APAF-1). APAF-1, in turn, binds to and activates pro-caspase 9 and subsequently caspase 3 (159). The triphosphate derivatives of fludarabine can substitute for deoxyadenosine triphosphate (dATP), which is required in the cytosol for the activation of APAF-1 by cytochrome *c*. Thus, apart from inducing DNA breaks and activating p53, fludarabine enhances apoptosis by activating APAF-1 and subsequently caspases 9 and 3 (160,161). Another protein released from mitochondria with cytochrome c is Smac/DIABLO, which binds to the inhibitor of apoptosis (IAP) family of proteins and induces apoptosis. IAPs prevent apoptosis by inactivation of a number of caspases, including caspase 3 (162).

1.6.2. Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway begins outside the cell through activation of the death receptors (DRs); these pro-apoptotic receptors include tumour necrosis factor receptor (TNFR), Fas receptor (FasR) and DR4/DR5 receptors. The ligand for DR4/5 is TNF-related apoptosis-inducing ligand (TRAIL). Death receptors contain a cytosolic death domain that, subsequent to ligand binding, recruits adaptor proteins to the receptor

complex to form a death-inducing signaling complex (DISC). Once the adaptor protein is recruited to the TNF receptor complex, its death effector domain (DED) binds to and activates caspases 8 and 10 (159). The extrinsic apoptotic pathway is regulated by inhibitor proteins that bind to the DED domain and prevent its binding to caspases. One of these inhibitor proteins is FLICE-like inhibitory protein (FLIP), which is expressed abundantly in CLL patients, causing resistance to TRAIL-induced apoptosis (163,164).

CLL cells secrete TRAIL and Fas ligand (165,166), which may suppress normal T cells and further immune deficiency in CLL patients (34).

1.7. Treatment

Traditionally, alkylating agents, such as chlorambucil and cyclophosphamide, were used to treat CLL. However, the treatment for CLL has changed dramatically in the past three decades, beginning with the introduction of purine nucleoside analogs (PNAs) and later with the development of monoclonal antibodies and combination therapies.

DNA damaging agents, such as alkylating agents and PNAs, induce apoptosis in CLL cells through both the intrinsic and extrinsic apoptotic pathways; while, type I antibodies (rituximab and ofatumumab) induce complement-dependent cytotoxicity (CDC) and type II antibodies (obinutuzumab also known as GA101) can directly induce caspase-independent programmed cell death upon binding to CD20. Both type I and type II antibodies are capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) (167). CD52 antibodies (alemtuzumab) similar to type I CD20 antibodies, can induce both CDC and ADCC (168).

The presently available CLL treatments are summarized in Table 1.3.

Table 1.3: Treatments for Chronic Lymphocytic Leukemia

Class	Example	Mechanism	Note
Alkylating Agents	Chlorambucil Cyclophosphamide Bendamustine	DNA Crosslinking, induction of apoptosis	Target: older patients/patients with high comorbidities Resistant: *p53* mutated patients
Purine Nucleoside Analogues (PNAs)	Pentostatin Cladaribine Fludarabine	Compromising the structural integrity of DNA	Resistant: patients with deletion 17p and *p53* gene mutation
Combination of alkylating agents and PNAs	Fludarabine and Cyclophosphamide (FC)	Synergistic antitumor activity	Better response rate compared to each agent alone
Monoclonal antibodies	Rituximab Ofatumumab Obinutuzumab (~CD20) Alemtuzumab (~CD52)	Induction of CDC, ADCC and apoptosis	Alemtuzumab target: patients with deletions 17p that are resistant to fludarabine
Chemoimmunotherapy	Fludarabine and rituximab (FR) Fludarabine, cyclophosphamide and rituximab (FCR) Alemtuzumab and fludarabine (FluCam)	Synergistic antitumor activity	FCR target: younger/fit patients who can tolerate this aggressive regimen, patients with deletion 11q FluCam target: resistant patients and those with deletion 17p
Stem Cell Transplantation (SCT)	AutoSCT	Marrow stem cells from peripheral blood, purged of leukemia cells	Target: younger patients with resistant disease Rarely performed nowadays as no survival advantage compared to FCR
	AlloSCT	Graft vs. leukemia	Target: younger patients with refractory disease and deletion 17p or *p53* gene mutation Induces long term remission and possible cure in 40% of patients

Table 1.3 (continued)

Class	Example	Mechanism	Note
Cyclin-dependent kinase (CDK) inhibitor	Flavopiridol	Transcriptional suppressor	Target: patients with deletions 17p and 11q, complex karyotypes or relapsed patients
Small molecule BCL-2 inhibitors	Navitoclax (ABT-263)	Inhibitor of pro-survival BCL-2 family of proteins	Target: Relapsed and refractory patients and patients with deletions 17p and 11q
Immunoregulators	Lenalidomide	Modulation of microenvironment and enhancement of immune effector cells	Target: previously untreated or heavily treated patients, elderly patients and patients with deletions 17p and 11q
Small molecule inhibitors of kinase	Fostamatinib Dasatinib Ibrutinib Idelalisib (GS-1101)	Inhibition of B cell receptor (BCR) signaling pathway	Target: relapsed and refractory patients with deletions 17p and 11q and heavily pre-treated patients Ibrutinib target: elderly untreated patients

ADCC: antibody-dependent cell-mediated cytotoxicity; CDC: complement-dependent cytotoxicity

1.7.1. Indication for Treatment

Chronic lymphocytic leukemia is currently considered an incurable, yet treatable disease. The majority of CLL patients present with asymptomatic, early stage CLL that requires monitoring without therapy (169). This "active observation" approach is based on historical studies demonstrating failure of early treatment to improve survival of early stage patients (170–172). Furthermore, it has been shown that the incidence of fatal second cancers was higher in early stage CLL patients who received treatment compared to those who did not (171). In practice, CLL patients in low- and medium-risk categories (Rai stages 0 – II) do not require therapy unless there is evidence of active disease such as progression or significant symptoms. Patients in high-risk categories (Rai stages III and IV) usually require treatment unless the patient is asymptomatic with a stable disease (30). Based on the 2008 iwCLL guidelines (30), indications for treatment are the presence of at least one of the following:

- Marrow failure, as evidenced by the development or worsening of anemia, thrombocytopenia or both,
- Massive splenomegaly (at least 6 cm below the left costal margin), or progressive or symptomatic splenomegaly,
- Massive lymphadenopathy (at least 10 cm in longest diameter), or progressive or symptomatic lymphadenopathy
- Progressive lymphocytosis with a >50% increase in lymphocyte count over two months, or a LDT of <6 months
- Autoimmune anemia or thrombocytopenia or both that is not responsive to corticosteroids or other standard therapy

- Constitutional symptoms defined as presence of at least one of the following: unintentional weight loss, significant fatigue, consistent fever or night sweats without evidence of infection

In CLL patients with cytopenias, the cause of cytopenia should be established prior to initiation of treatment. Cytopenias due to bone marrow replacement should be distinguished from immune-mediated cytopenias and each be treated accordingly (with chemotherapy or corticosteroids, respectively). Lymphocyte counts should be evaluated based on a trend rather than a single result, as a transient increase in lymphocyte count is expected after infections, inflammation or steroid use (30).

The standard therapy for CLL patients is FCR, as long as they have adequate performance status, a low number of comorbidities and normal renal function. This treatment is especially recommended for 11q-deleted patients. FR chemotherapy is used for the less fit and chlorambucil is reserved for the elderly and frail. Patients with deletion 17p are usually resistant to these treatments but may benefit from alemtuzumab, which does not require p53 for activity. Allogeneic hematopoietic stem cell transplantation (SCT) is reserved for patients less than 65 – 70 years who have relapsed within 2 years of FR or FCR.

Steroids are given to patients with immune cytopenias and should be avoided for patients receiving fludarabine-containing regimens as they increase the risk of infections. Radiotherapy is not commonly used in CLL patients, but it may be utilized to shrink bulky nodes in patients who otherwise do not require treatment or to shrink the spleen in patients that require splenectomy but are not surgical candidates. Radiation

therapy is most effective in untreated patients. Splenectomy is reserved for patients with painful spleens or patients with non-responsive immune cytopenias (34).

1.7.2. Definition of Terms Used in Assessment of CLL

The 2008 guidelines for diagnosis and treatment of CLL (30) define response to therapy in CLL as follows:

1.7.2.1. Complete Remission

Complete remission (CR) requires meeting all of the following conditions: a peripheral blood lymphocyte count of $<4\times10^9$/L; absence of significant lymphadenopathy, hepatomegaly or splenomegaly by physical examination; absence of symptoms; a blood count showing neutrophil count of $>1.5\times10^9$/L, platelet count of $>100\times10^9$/L and hemoglobin >110 g/L or 50% improvement over the baseline count without requiring red blood cell transfusions or exogenous erythropoietin (30).

1.7.2.2. Partial Remission

Partial remission (PR) is defined as one of the following: a decrease of greater than 50% in lymphocyte count; a reduction in lymphadenopathy by physical examination; a reduction of greater than 50% in size of spleen or liver by physical examination. In addition, a blood count showing at least one of the following results: neutrophil count of $>1.5\times10^9$/L, platelet counts of $>100\times10^9$/L or hemoglobin >110 g/L or 50% improvement over the baseline count without requiring red blood cell transfusions or exogenous erythropoietin (30).

1.7.2.3. Progression

Progression of disease after treatment requires at least one of the following: appearance of new lymphadenopathy or an increase of greater than 50% in size of any previous nodes; a greater than 50% increase in size of previous organomegaly or *de novo* appearance of hepatomegaly or splenomegaly; a greater than 50% increase in lymphocyte count; transformation to a more aggressive condition such as Richter's syndrome; post-treatment cytopenias that are due to marrow infiltration and not autoimmune mediated (30).

1.7.2.4. Stable Disease

Stable disease or non-responsive disease is seen in patients who neither achieved a CR or a PR, nor exhibited progressive disease (30).

1.7.2.5. Response and Failure

A clinically beneficial outcome (CR or PR) following treatment is considered a response to treatment. Everything else is considered treatment failure (30).

1.7.2.6. Time to First Treatment, Progression-Free Survival, and Overall Survival

TTFT is defined as the time from CLL diagnosis until administration of the first treatment. Progression-free survival (PFS) is defined as the time from beginning of observation or beginning of treatment until disease progression or death. Overall survival (OS) is defined as the time from beginning of the cancer diagnosis until death from any cause (30).

1.7.2.7. Relapse

Relapse is seen in patients who previously achieved CR or PR, but show signs of progression after six months (30).

1.7.2.8. Refractory disease

Refractory disease is used for patients who do not achieve a CR or PR following treatment or have disease progression in less than six months following treatment (30).

1.7.3. Alkylating Agents

Until three decades ago, the alkylating agent chlorambucil was the only treatment option for CLL patients. Chlorambucil still remains the preferred palliative treatment for progressive CLL, especially in older patients and in patients with comorbidities. Chlorambucil is a nitrogen mustard agent with alkylating properties; although its exact mechanism of action is unknown, it appears to bind to a number of cellular structures such as membranes, RNA, DNA and proteins. However, DNA crosslinking and induction of apoptosis appear to be the most important mechanism for the antitumor activities of this drug. Alterations in genes involved in apoptosis such as *p53*, *MDM2*, *BCL-2* and *BAX* may cause resistance to this agent. Mutations or deletions of *p53* occur in 10 – 15% of CLL patients at diagnosis and cause resistance to chlorambucil. However, it has been shown that chlorambucil in high concentrations can induce apoptosis through a p53-independent pathway (173).

Although chlorambucil has been used to treat CLL patients for the past 60 years, its optimum duration of treatment and schedule is still under debate, but in general it results in a response rate of 31 – 90% with 40 – 60% of patients achieving CR (174–182). To improve response rates, chlorambucil is combined with steroids or other

chemotherapeutic agents. Steroids lead to redistribution of leukemic cells and an increase in lymphocyte count. Prednisone is the most commonly used steroid to be administrated with chlorambucil, in order to rapidly reduce lymphadenopathy, splenomegaly or cytopenias due to bone marrow involvement (180).

Cyclophosphamide is another alkylating agent for treatment of CLL, but it is less marrow toxic than chlorambucil and is most usually used in combination regimens, such as FCR. Cyclophosphamide is usually combined with vincristine and prednisone (CVP) or vincristine, prednisone and doxorubicin (CHOP). The result of clinical trials appear to be controversial with no apparent advantage over chlorambucil with or without prednisone (172,183–185).

Like chlorambucil, bendamustine is an alkylating agent with a nitrogen mustard core but differs in having a benzimidazole ring similar to that of purine analogs. This latter feature is believed to be responsible for the unique action of this agent. Bendamustine causes more extensive DNA damage compared to other alkylating agents and is effective in cell lines that are resistance to the nitrogen mustards (186–188). Bendamustine produces a higher CR rate and a longer PFS compared with chlorambucil. At the same time, the risk of adverse events is two-fold higher with bendamustine compared to chlorambucil (179).

1.7.4. Purine Nucleoside Analogues

Pentostatin, cladaribine and fludarabine are among the purine nucleoside analogues (PNAs) that, since their arrival in the 1980s, played an important role in treating patients with lymphoproliferative disorders (189–192). This class of antitumour agents compromises the structural integrity of DNA by their incorporation into the DNA

structure during replication or repair, resulting in chain termination or breaks in the DNA (190). The alteration in deoxynucleotide pools leads to the accumulation of DNA breaks as the halogenated nucleotides are incorporated into DNA and RNA (34). All of these changes can lead to apoptosis.

Fludarabine is the most widely used PNA in CLL, and untreated CLL patients have a response rate of 70% and a CR rate of up to 40% (176,193–195). Fludarabine also produces a longer response in patients older than 65 years compared to chlorambucil, although survival is not influenced by the initial use of fludarabine or chlorambucil (178).

The most important adverse effect of fludarabine are myelo- and immune-suppression, which can lead to an increased incidence of infections, and may last several years following the treatment. The myelosuppressive effect is particularly pronounced in patients who were previously treated with alkylating agents, although altering the schedule and dosage of fludarabine may reduce the toxicity (196). Initial studies reported an association between PNAs and autoimmune anemia in CLL patients; however, the high rates were due to advanced and resistant disease in those patients and subsequent studies show no difference between fludarabine and chlorambucil in causing this adverse effect (180,181). Furthermore, an increased incidence of acute myeloid leukemia (AML) has been reported in fludarabine-treated CLL patients that is further increased in patients who received fludarabine in combination with chlorambucil (197,198).

Deletion 17p and *p53* mutations have been associated with resistance to PNAs (56,199). In addition, fludarabine resistance is strongly associated with poor survival,

and patients not responding to fludarabine or relapsing within 6 months of treatment have a median survival of 10 months (200,201). In general, resistance to fludarabine is associated with resistance to cladaribine suggesting a common mechanism of action for the two agents (202–204).

Combining the DNA damaging effects of alkylating agents with the inhibitory effects of PNAs on DNA repair can produce a synergistic antitumor activity (205–207). Thus, the combination of fludarabine and cyclophosphamide (FC) produces a higher response in untreated CLL patients than seen with fludarabine alone (overall response 80% and CR 35%) with a longer duration of response (180,208–212).

1.7.5. Monoclonal Antibodies

Monoclonal antibodies have revolutionized the treatment of CLL. Over the past few years, several monoclonal antibodies have been investigated in clinical trials for patients with CLL. Today, rituximab, an anti CD20 monoclonal antibody, and alemtuzumab, an anti CD52 monoclonal antibody, are commonly used in CLL.

Rituximab is a chimeric monoclonal antibody against CD20 that has both human and murine components. CD20 is a surface antigen that is expressed on normal B cells and most B cell lymphomas, and is involved in activation, proliferation and differentiation of B cells (213,214). The intensity of antigen expression or the number of receptors on the cell surface appears to correlate with the response to rituximab. In early clinical trials, rituximab showed less activity in CLL patients as compared to patients with follicular lymphoma (FL) (response, 13% vs. 50%, respectively), which was attributed to the low CD20 levels on CLL cells and the high levels of soluble CD20 in plasma (215,216). However, by increasing the dosage of rituximab in CLL, a response

rate of 40% was reached with tolerable toxicity (217,218). Rituximab can transiently reduce lymphocytosis, but has little effect on bone marrow and lymphadenopathy, perhaps due to a lower CD20 expression on leukemia cells at these sites (219–221). In patients with high lymphocyte counts, the initial rituximab treatments may cause adverse reactions such as fever, nausea, dyspnea and hypotension due to the sudden release of cytokines with rapid leukemia cell death. For unclear reasons, immune neutropenia may also occur in patients treated with rituximab. The severity of these reactions may be decreased by the administration of steroids and they usually stop after the tumor load decreases (217–220,222).

Ofatumumab is a fully humanized anti-CD20 antibody that binds to a distinct epitope than that of rituximab and demonstrates greater CDC and ADCC compared to rituximab (213,223). This antibody was initially developed to prevent graft vs. host disease (GVHD) and graft rejection in patients receiving stem cell transplants (224). Subsequently, it was demonstrated that there was no cross-resistance between ofatumumab and rituximab, and with minimal toxicity, approximately 50% of fludarabine-resistant patients will respond to ofatumumab (225).

Alemtuzumab (Campath-1H) is a humanized form of murine antibody that is directed against CD52, an epitope expressed on B and T lymphocytes as well as natural killer cells. As p53 defects are not associated with resistance to alemtuzumab, it is usually used to treat patients with deletions 17p and those that have developed resistance to standard therapy (224,226). Administration of alemtuzumab with high-dose steroids is highly effective in previously treated patients with deletion 17p and is now commonly used for patients with refractory disease (227). Increased rates of infections and

infusion-related toxicities such as fever, nausea and rash have been reported as adverse effects of alemtuzumab. Alemtuzumab effectively clears lymphocytosis of the peripheral blood as well as bone marrow and spleen; however, it is less effective in treating bulky lymphadenopathy (228,229).

While standard doses of rituximab have little effect in CLL when used alone, significant synergy is seen when rituximab is combined with chemotherapy, suggesting an overlap in their mechanism of actions. While it is not entirely clear why this synergy occurs, it is thought to be related to the ability of rituximab to reduce the levels of anti-apoptotic proteins, such as BCL-2, in CLL cells (34). The most common chemoimmunotherapy regimens are FR (fludarabine and rituximab) and FCR (fludarabine, cyclophosphamide and rituximab).

When FR was retrospectively compared to single agent fludarabine, the multivariate analyses controlling for pretreatment characteristics showed significantly improved PSF and OS for the FR group (67% and 93%, respectively, for FR vs. 45% and 81%, respectively, for fludarabine). In this comparative study, the CR and PR rates were 20% and 43%, respectively, for fludarabine and 38% and 46%, respectively, for FR (230). In addition, fludarabine and rituximab are more effective when given together rather than sequentially (47% CR and 43% PR vs. 28% CR and 49% PR, respectively) (231).

The MD Anderson Cancer Center developed FCR combination regimen that subsequently became the standard firs-line therapy for CLL patients (34). FCR is considered one of the most effective treatment regimens for CLL, but it does cause significant immune- and myelo-suppression and patients must meet a certain

performance status in order to qualify for this treatment (57). FCR produced 72% CR and 13% PR in chemotherapy-naïve CLL patients and showed superiority over both FR and single agent fludarabine (232). Despite its effectiveness, FCR has significant adverse effects that include cytopenias (in 20% of patients), serious infections (in 10% of patients) and therapy-related AML due to prolonged myelosuppression (in 5% of patients) (232,233). When FCR was compared with FC, its CR and PR were 44% and 46%, respectively for FCR and 22% and 58%, respectively for FC (CR p <0.0001); while, the median PSF was 52 and 33 months (p <0.0001) and OS was 87% and 83% (p 0.01) for FCR and FC, respectively. Patients with deletion 17p responded poorly to both FCR and FC; however PFS, but not OS, was slightly improved with FCR. On the other hand, patients with deletion 11q receiving FCR showed an improved outcome compared to those receiving FC, with CR of 51% and 15% (p <0.0001), PSF of longer than three years in 64% and 32% (p <0.0001) and OS of longer than three years in 94% and 83%, respectively (34,231,232).

It has also been shown that alemtuzumab can enhance the activity of fludarabine and that the combination of the two agents produces response in CLL patients who have become resistant to both agents when used alone (234). This combination has been shown to be an effective treatment for patients with relapsed or refractory CLL. It produced a CR and a PR of 30% and 50%, respectively, in previously heavily treated patients as well as a 66% response rate in fludarabine-refractory patients (235). When it was compared to single agent fludarabine in fludarabine-responsive pre-treated patients, it produced a PFS of 24 months vs. 17 months with single agent fludarabine (p: 0.0003) (236). Median OS was not reached for the combination, while it was 53 months for

single agent fludarabine (p: 0.021). In one small study, the combination of fludarabine and alemtuzumab (FluCam) produced a CR in almost all patients with non-functioning p53 or deletion 17p with tolerable toxicity (237).

1.7.6. Hematopoietic Stem Cell Transplantation

Autologous hematopoietic stem cell transplantation (HSCT) has been used in CLL, although nowadays it has generally been abandoned because of ineffectiveness and high risk of myelodysplasia/AML. In contrast, allogeneic HSCT has become a standard treatment for younger patients with fludarabine-refractory disease and patients with high-risk prognostic features such as deletion 17p or a *p53* mutation. While CLL patients cannot be cured with standard chemotherapy, long-term remissions and possibly cures can be obtained with allogeneic HSCT.

1.7.6.1. Autologous Stem Cell Transplantation

Autologous stem cell transplantation (autoSCT) uses stem cells from patients' peripheral blood as the source of hematopoietic stem cells. Therefore, the patient should be in a remission to ensure that the collected stem cells are not contaminated with CLL cells and purging of the collected cells has been carried out to try and eliminate any residual leukemia cells prior to reinfusion (238). Initial studies indicated that younger patients benefitted from autoSCT, if it is performed when patients are still sensitive to chemotherapy (239,240). Later studies showed that autoSCT produces no survival benefit when given as consolidation (therapy given after the initial therapy to further reduce tumour burden) when compared with observation (241). When first-line autoSCT was compared with first-line FCR, autoSCT produced an improvement in PFS but not in OS (242).

Unfortunately, autoSCT can result in myelodysplastic syndrome (MDS) or AML, with an incidence of 12% within five years (240,243). Thus, autoSCT is no longer used in CLL (34).

1.7.6.2. Allogeneic Stem Cell Transplantation

In allogeneic stem cell transplantation (alloSCT), stem cells from a donor are given to a recipient with CLL. One of the advantages of alloSCT is that there is no chance of CLL contamination when the donor is unrelated. However, when the donor is related, there is a chance of familial CLL or MBL that could lead to reinfusion of CLL-phenotyped cells from the undiagnosed donor to the recipient. As mentioned previously, it has been shown that 13% of first-degree relatives of CLL patients have MBL (16). Furthermore, the rate of MBL in the population is 3.5% in adults over 40 years of age and this rate is increasing with advancing age. Therefore, it has been suggested that donors, particularly related donors should be screened for MBL (16–18,31,244–247).

AlloSCT also provides the advantage of graft vs. leukemia (GVL) effect that may explain why alloSCT is more efficacious than autoSCT (248–251). GVL eliminates leukemia cells by immune mechanisms due to genetic differences between the donor and the recipient. A variety of immune cells have been implicated in the GVL effect, including T cells, natural killer cells and B cells (252,253). The downside to this phenomenon is graft vs. host disease (GVHD) that occurs when the same immune cells that recognize leukemia-associated antigens, also recognize similar antigens expressed on normal host cells. The incidence and severity of GVHD can be reduced by T cell depletion prior to infusion of the stem cells, but this increases risks of opportunistic

infections or disease relapse, and therefore, reducing the curative potential of alloSCT (250).

In contrast to autoSCT and T cell depleted SCT, there are fewer relapses after alloSCT. Additionally, the incidence of relapse decreases over time, indicating a pivotal role for GVL in the long-term remission of CLL (254–256). Unfortunately, although the risk of relapses is lower after alloSCT, its benefits are dampened by high rates of non-relapse mortality (50%) (256,257).

In an attempt to improve the tolerability of alloSCT, non-myeloablative (NMB) or reduced-intensity conditioning (RIC) was introduced. In this approach, myeloblative preparation is eliminated to reduce the risk of non-hematopoietic toxicity, opportunistic infections and severe cytopenias while retaining the benefits of GVL effect (256,258).

AlloSCT can prolong survival in patients who are fludarabine resistant, or have a deletion 17p or a *p53* mutation (259–261). Moreover, outcome is better for those who are in remission at the time of transplant, so care must be taken to identify appropriate patients and perform the transplantation while still sensitive to treatment (262).

1.7.7. Novel Treatments

A number of novel targeted therapies have been developed in the past 5 – 10 years and their use has the potential to dramatically improve the outcome of CLL patients.

1.7.7.1. Flavopiridol

Flavopiridol is a cyclin-dependent kinase (CDK) inhibitor that induces p53-independent apoptosis in CLL cells by decreasing proteins like MCL-1, X-linked inhibitor of apoptosis (XIAP) and BCL-2 associated athanogene (BAG-1) (263–265).

As a result it has been suggested that flavopiridol may sensitize cells to other antitumor agents. Flavopiridol is also a potent transcriptional suppressor and this is likely its mode of activity in CLL. This agent can induce rapid responses in CLL patients who are resistant to standard chemotherapy and have high-risk cytogenetic aberrations such as deletion 17p (50%), deletion 11q (59%) and complex karyotype (43%). The major side effect of flavopiridol is the very rapid tumor lysis that occurs and patients require careful monitoring and aggressive management. Because of this, flavopiridol is normally only administered in academic centers.

1.7.7.2. Small Molecule BCL-2 Inhibitors

All BCL-2 family members share BCL-2 homology (BH) domains namely BH1, BH2, BH3 and BH4. Multi-domain BCL-2 members function as inhibitors of apoptosis by binding to and inhibiting BAX and BAK, while BCL-2 members with BH3-only proteins have pro-apoptotic properties and either directly activate BAX and BAK or bind to anti-apoptotic proteins and prevent them from binding to BAX and BAK. BH3 mimetics have pro-apoptotic properties and function like BH3-only BCL-2 members. Navitoclax (ABT-263) is a potent BH3-mimetic inhibitor of pro-survival BCL-2 family members such as BCL-xL, BCL-2 and BCL-w, but has no activity against MCL-1. Navitoclax showed efficacy as a second line therapy in relapsed and fludarabine-refractory CLL patients and patients with deletion 17p and 11q. Navitoclax rapidly induces apoptosis in megakaryocytes, and thrombocytopenia is the major dose-limiting toxicity associated with this drug (264,266).

1.7.7.3. Lenalidomide

Lenalidomide is an immunomodulatory drug that has demonstrated efficacy in both untreated and heavily pre-treated patients with unfavourable cytogenetic abnormalities (deletion 17p and 11q) and in elderly CLL patients. This drug has shown considerable activity as single-agent first-line or salvage therapy. Lenalidomide is generally well tolerated with major toxicities being tumour flare initially, followed by myelosuppression and fatigue with prolonged use. These latter toxicities are dose limiting. Tumour flare manifests by painful swelling of lymph nodes, hepatosplenomegaly, skin rash, low-grade fever, and sometimes an increase in peripheral white blood cell counts. Tumour flare correlates with clinical outcome, suggesting it may account for the antitumour effect of this agent. Although the exact mechanism of action of lenalidomide is unknown, modulation of the microenvironment and enhancement of immune effector cells have been speculated to be responsible for its effects (263–265,267).

1.7.7.4. B Cell Receptor Targeted Agents

CLL is characterized by the accumulation of mature B cells, which undergo apoptosis unless exposed to a microenvironment that will support survival and induce proliferation. CLL cells in the peripheral blood thus home to the microenvironment of lymph nodes, marrow and spleen where they come in contact with stromal and T cells. Bothe the BCR and NFκB pathways that promote normal B cell development, expansion and survival, also provide survival support for CLL cells and are triggered by the microenvironment. Therefore, targeting these pathways may help disrupt the effect of

the microenvironment and allowing cells to undergo apoptosis and preventing proliferation (268).

Fostamatinib is a spleen tyrosine kinase (SYK) inhibitor that prevents BCR signaling, antagonizes the protective effect of stromal cells by reducing adhesion to stromal components, and induces a moderate degree of apoptosis in CLL cells *in vitro*. Fostamatinib has been shown to induce response in relapsed and refractory CLL patients with moderate side effects. The dose-limiting toxicities include diarrhea, neutropenia, and thrombocytopenia (269).

Dasatinib is a tyrosine kinase inhibitor that blocks the activity of several protein kinases and inhibits BCR signaling and stromal cell contact. Relapsed and refractory CLL patients have an OR of 20% to dasatinib. Dasatinib can also sensitize CLL cells to fludarabine with durable responses in heavily pretreated CLL patients. The primary toxicity with the combination is myelosuppression (266,269).

Ibrutinib is an irreversible Bruton's tyrosine kinase (BTK) inhibitor. Ibrutinib not only reduces BCR signaling, but also interferes with the crosstalk between CLL and stromal cells. It disrupts the protective effects of stromal cells and subsequently inhibits survival, proliferation, and migration of CLL cells. Ibrutinib is particularly active in refractory and relapsed CLL patients, and induces durable remissions even in high-risk patients. An overall response rate of 60% was reported in fludarabine-refractory patients and patients with deletion 17p. When high-risk patients were treated with a combination of ibrutinib and rituximab, an overall response of 85% was reached. Interestingly, ibrutinib does not affect normal B cells and may partially restore the humoral immune deficiency in advanced CLL patients and producing an increase in IgA levels. Ibrutinib

is well tolerated as a single agent, with diarrhea, fatigue and rash being the most common adverse effects. This agent appears to be suitable for treatment of the elderly treatment-naïve patients with CLL (263,266,268,269).

Idelalisib (GS-1101 formerly known as CAL-101) is a highly selective inhibitor of the phosphatidylinositol 3-kinase (PI3K) delta isoform and inhibits the supportive effect of microenvironment, such as BCR signaling. GS-1101 activates AKT and ERK and downregulates MCL-1, and subsequently induces apoptosis in CLL cells. Combining GS-1101 with rituximab or bendamustine improves OS in relapsed and refractory patients. Even patients with high-risk features such as deletion 17p respond to GS-1101 (266,268,269).

The advent of monoclonal antibodies and subsequently introduction of immunochemotherapy revolutionized CLL treatments. However, since combination therapies are usually aggressive, only younger, relatively healthier patients can benefit from them. Alkylating agents are still palliative treatment of choice for the elderly patients and patients with comorbidities. Furthermore, patients with defective p53 genes and chromosomal abnormalities, in particular deletion 17p, seem to be resistant to the majority of treatments that are available today. However, novel treatments such as CDK and small molecule BCL-2 inhibitors and agents such as Lenalidomide are shown to be promising treatments for resistant and refractory CLL patients.

1.8. Immune Deregulation

1.8.1. Immunosuppression in CLL

It is known that CLL patients are immunosuppressed. This immunosuppression is usually due to hypogammaglobulinemia or abnormal T cell function (270,271) and is

further increased by chemotherapy in particular PNAs, monoclonal antibodies or steroids. The pathogenesis of hypogammaglobulinemia in CLL patients is unknown.

In healthy individuals, T cells stimulate B cells to proliferate, induce B cell antibody class switching, and promote plasma cell differentiation. However in individuals with CLL, T cells appear to provide support for malignant B cells (244). Furthermore, CLL cells have been shown to lack immune function (272) and interfere with the function and interaction of activated T cells and normal B lymphocytes (273). CLL-derived natural killer cells have also been shown to suppress immunoglobulin secretion by normal B cells (274).

Although an increase in the number of circulating T cells was observed in CLL patients, these T cells have abnormal phenotype and function. T helper cells may be converted to regulatory T cells (Tregs), formerly known as T suppressors (272,275), leading to abnormal T cell function and consequently hypogammaglobulinemia (276). Increased numbers of Tregs have been associated with decreased T cell response to viral and malignant antigens, with this increase being more prominent in more advanced stage patients (277–279). Furthermore, it has been shown that Tregs secrete soluble IL-2 receptors that inhibit T helper cell differentiation (280).

The immune deficiency in CLL leads to an increased susceptibility to bacterial, viral, and fungal infections and failure to initiate an effective antitumor immune response. Recurrent infections can be seen in approximately 50% of CLL patients, and infections and second malignancies are two major causes of death in CLL (the third being drug-resistance and progression of the leukemia). Patients receiving PNAs, monoclonal antibodies and steroids are at higher risk of infections, in particular

opportunistic infections, while, patients receiving alkylating agents are at risk of recurrent bacterial infections. PNAs are known to deplete T cells, while monoclonal antibodies are toxic to B cells (rituximab) or both B and T cells (alemtuzumab). Prophylactic antibiotics are generally given to patients receiving PNAs, monoclonal antibodies or steroids (34).

The risk of infections has been associated with the duration and stage of the disease and the degree of hypogammaglobulinemia (270,271,281). It has been shown that low levels of IgG and IgA, but not IgM, at diagnosis are associated with shorter survival (282). Accordingly, an association between low serum IgG and IgA levels and recurrent infections has been observed. However, only IgA levels could predict recurrent infections and survival of patients independent of the stage of disease (282–284). However, in some cases patients with normal immunoglobulins have recurrent infections while some patients with low immunoglobulins have no infections (284).

Patients with hypogammaglobulinemia and recurrent infections may benefit from intermittent infusions of gammaglobulin to boost their IgG levels. This can reduce the risk of bacterial infections by 50%, but does not affect the incidence of opportunistic and non-bacterial infections. However, although gammaglobulin may reduce the risk of infections, there is no evidence that it improves survival (285,286).

While all CLL patients have reduced primary and secondary responses to vaccination, this response is even poorer in patients with low immunoglobulin levels (271). However, vaccination of CLL patients for pneumococcus and influenza is still recommended, while live vaccines should be avoided (270,287).

1.8.2. Autoimmune Cytopenias

Despite the commonness of immune deficiency in CLL, 4 – 10% of patients actually develop autoimmune cytopenias caused by antibodies generated by normal B cells (287–289). Autoimmune cytopenias may occur prior to, or at the time of CLL diagnosis, but the likelihood of developing this complication usually increases with the duration of the disease (290). In addition, they may be triggered by treatment, which presumably worsen the immune imbalance in CLL. The immune cytopenias are usually treated with immunosuppressive drugs, such as prednisone (34). Rituximab may also be used either as a single agent or in combination with cyclophosphamide when treatment of CLL is also required (291–293). It is noteworthy that the survival of patients with autoimmune cytopenias is similar to those without immune-mediated cytopenias (42,290).

Autoimmune hemolytic anemia (AIHA) is the most common autoimmune cytopenia in CLL patients, with approximately 10% of patients developing this complication (288–290,294). Interestingly, CLL is the most common cause of AIHA and patients with AIHA have an increased risk of MBL that could later progress to CLL (289,295). The risk of AIHA is higher in elderly male patients with advanced disease and patients with high lymphocyte count and poor prognostic markers such as unmutated *IGHV* gene status, high ZAP70 expression and ß2-microglobulin levels (42,289,290). Initial studies with fludarabine also showed a high incidence of AIHA, suggesting that this was a unique feature of this drug. However, in retrospect the high frequency was related to the fact that these patients had advanced disease (34). Later studies showed that the rate of AIHA was similar in patients who received either

fludarabine or chlorambucil, but it was lower in patients who received fludarabine in combination with cyclophosphamide, leading to the conclusion that cyclophosphamide may prevent AIHA (296). Patients with AIHA are treated with blood transfusions, immunosuppression or splenectomy/splenic irradiation (34,297,298).

Immune Thrombocytopenic Purpura (ITP) occurs in 1 – 2% of CLL patients and can be predicted by a reduced platelets count where there is no splenomegaly and the hemoglobin is normal (thrombocytopenia due to bone marrow infiltration is usually associated with anemia) (34). Treatment is as for AIHA.

1.9. Transformation

It has been reported that CLL can transform to diffuse large B cell lymphoma, Hodgkin's disease, prolymphocytic leukemia, hairy cell leukemia or acute B and T cell leukemias (34). In 1928, Richter first described transformation of CLL to a high-grade non-Hodgkin's lymphoma and, initially, all CLL transformations were known as Richter's transformation or Richter's syndrome (299). Today, the term Richter's transformation is reserved only for transformation of CLL or SLL to diffuse large B cell lymphoma (300). CLL patients have a 3 – 5 times increased risk of developing a second lymphoid malignancy, with diffuse large B cell lymphoma being the most common. The chance of developing a second lymphoid malignancy has been strongly associated with a prior PNA-containing treatment (301).

Richter's transformation has been shown to occur with an incidence of ~5% in the four years following diagnosis. The risk increases over time with the incidence being approximately 16% in the first10 years (302). The majority of cases of Richter's syndrome are clonally related to CLL (75% of all cases), while the rest are unrelated.

Risk of this transformation is higher in CLL patients with deletion 11q, trisomy 12, high expression of CD38 and *P53* (seen in 70% of transformations) and *NOTCH1* (seen in 40% of clonal transformations) mutations (143). Patients with non-functional p53, either due to mutation or deletion 17p, had a decreased median survival of 9 months as compared to 47 months in patients with functional p53 (303).

Clonally unrelated cases of Richter's syndrome tend to behave in a similar fashion to *de novo* diffuse large B cell lymphomas, are less aggressive and have the same median survival of 62 months. They are usually due to genetic predisposition to lymphoproliferative disorders or immunodeficiency due to treatment (304,305). Patients with clonally related cases of Richter's transformation have more aggressive disease and a median survival of only 14 months. Their symptoms include fever, weight loss, rapidly increasing lymphadenopathy, splenomegaly and involvement of lung, kidneys and digestive system (305,306). Patients with Richter's transformation are usually treated with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) (307).

In some cases, CLL may be transformed to Hodgkin's disease. Similar to Richter's transformation, the median time of onset is four years after CLL diagnosis. These patients may present with fever, weight loss and lymphadenopathy. The outcome is worse in patients who were previously treated with fludarabine, as compared to untreated patients (303).

CLL cases may gradually transform to prolymphocytic leukemia with the continued accumulation of prolymphocytes in the blood. This transformation has been associated with trisomy 12 and unmutated *IGHV* gene status (308). Similar to Richter's

transformation, prolymphocytic leukemia and multiple myeloma can also develop in CLL patients, either from CLL clones or independently (309–311).

1.10. Other Malignancies

1.10.1. Cancer Diagnoses Preceding CLL

A recent study at MD Anderson Cancer Center showed that 16% of CLL patients referred to their centre had a history of a previous malignancy at the time of presentation (312). In this study, high levels of ß2-microglobulin and creatinine and low levels of albumin were associated with a history of previous cancer.

A survival of 7.6 years (95% CI 6.4 – 10.0) was reported for CLL patients who had a history of a previous cancer as compared with 10.9 years (95% CI 10.2 – 11.9) for patients without a previous malignancy. The five-year survival was 70% (95% CI 63-76%) and 82% (95% CI 80 – 85%) for patients with and without a preexisting malignancy, respectively. Interestingly, a previous cancers was only a significant predictor of survival when chromosomal abnormalities were excluded from the model, indicating that there may be an association between chromosomal abnormalities and the development of previous cancers in CLL (312).

1.10.2. Cancer Diagnoses Following CLL

A retrospective population-based study in Manitoba 40 years ago showed that the rate of second cancers was higher in CLL patients compared the population of Manitoba residents of the same age and gender. In that study, the risk of all cancers in CLL patients was increased three-fold, the risk of skin cancers eight-fold and the risk of all cancers (excluding skin cancers) two-fold. The increased risk of developing a second

malignancy was significantly higher in both genders (3.25-fold and 2.99-fold in male and female patients, respectively). When skin cancers were excluded, the risk was significantly higher only in male CLL patients (313).

A more recent study at the MD Anderson Cancer Center (312) showed that 11% of CLL patients referred to that centre developed a neoplasm subsequent to their CLL diagnosis (at a median of 2.9 years after the initial visit). Approximately 15% of treated patients developed a second malignancy. As seen in the Manitoba CLL population, they observed a 2.2-fold increased risk of second cancers in their CLL patients. Cancers of skin, prostate, breast, gastrointestinal tract and lung were the most commonly diagnosed second cancers (312). An Australian population-based study also reported that 13% of CLL patients developed a second cancer at a median of 4 years after CLL diagnosis, and that patients had a 2.2-fold increased risk of developing a second cancer compared to the general population. This elevated risk was observed across both genders and all age groups and remained constant over the years. Significant excess risks for cancers of skin, head and neck, digestive system, lung, breast and female reproductive system, prostate, kidney and bladder were also observed (314). These findings were in agreement with previous studies (315–318).

The risk of second cancers was highest in male and in the elderly. Also the presence of chromosomal abnormalities was associated with a higher risk of second cancers, as 12% of patients with deletion 17p/deletion 6q, 14% of patients with deletion 11q/trisomy 12 and 10% of patients with other abnormalities developed a second cancer within 4 years of the diagnosis of CLL. Furthermore, elevated levels of ß2-microglobulin, creatinine and lactate dehydrogenase (LDH) were significantly

associated with the risk of a second cancer (312). Based on the fact that CLL patients appear to have an increased risk of smoking-related cancers such as pharynx, larynx, lung, kidney and bladder, it was suggested that the carcinogenic effects of smoking maybe amplified by immunosuppression in CLL patients (314).

In the same study, the overall mortality of CLL patients was 2.5-times higher than that of the general population, with a 72% increased risk of cancer-specific death (excluding death from lymphoproliferative disorders). Cancers of lung and skin (melanoma and non-melanoma skin cancers) accounted for the greatest cancer mortality. The increased risk of death due to all cancers was reported to be similar across both genders (314). It is noteworthy that 10% of people who died of squamous cell carcinoma (SCC) of the skin also had CLL, indicating that the disease is more aggressive and fatal in CLL patients, probably due to their deficient immune response (319).

The increased risk of second cancers in CLL patients may be attributed to the shared risk factors (e.g. genetic factors), immunosuppression due to CLL or treatment-induced immune deficiency. An increased incidence of non-Hodgkin's lymphoma, melanoma, lung and breast cancers was reported following a diagnosis of non-melanoma skin cancer (NMSC), suggesting a common factor in the etiology of these malignancies (320). An increased incidence of CLL has also been reported in relatives of colon cancer patients (321), while an increased incidence of breast cancer has been noted in first-degree relatives of CLL patients (322). Interestingly, some biochemical and genetic similarities may be seen between these different tumors. Firstly, estrogen receptors have been described in the leukemia cells of CLL patients who responded to

tamoxifen treatment (323,324). Secondly, CD5-positive breast cancer cells were reported in a CLL patient (325). Thirdly, deletion 13q, which is seen commonly in CLL, has been observed in breast and prostate cancer cells as well and contains the *BRCA2* gene (326–328). Finally, it has also been shown that human epidermal growth factor 2 (HER2/neu) is frequently overexpressed in lung cancer tissue of non-smokers with concurrent CLL, leading to the speculation of its role in the development and progression of lung cancer in CLL patients (329). This evidence suggests that there are certain genetic defects that may render individuals susceptible to CLL and other malignancies.

Patients with CLL have impaired humoral and cell-mediated immune responses leading to a weak surveillance system that may explain the increased rate of second cancers in these patients. It has been observed that CLL cells suppress normal T cell function by interfering with T cell synapse formation (330–333). The abnormal synapse formations lead to decreased binding of T cells to CLL cells and presumably to other malignant cells. Furthermore, the type of second cancers observed in CLL patients is similar to those observed in renal transplant recipients who receive immune suppressive drugs (334,335). In addition, an increased risk of melanomas was reported in patients with Hodgkin's disease, non-Hodgkin's lymphoma, renal transplant recipients and other individuals with immune deficiency (336). Ultraviolet (UV) exposure could suppress immune function and therefore may explain some of the association between skin cancers and lymphoid neoplasms (337,338). It has been suggested that shared risk factors such as immunosuppression together with UV exposure may lead to the increased rate of skin cancers in CLL patients (314).

Cytotoxic and immunosuppressive effects of chemotherapy may further increase immune deficiency in CLL patients. Common CLL treatments such as PNAs and monoclonal antibodies are potent immunosuppressants. The combination of cladaribine and cyclophosphamide has been shown to cause second malignancies in 13% of patients with advanced lymphoproliferative malignancy at a median of 16 months (339). However, another study showed that the rate of second cancers was higher in CLL patients who received fludarabine when compared to healthy individuals, but it was similar to CLL patients who did not receive fludarabine (340). As with the risk of infections, the risk of the development of a second cancer in CLL increases with the duration of disease (312).

Although the risk of second cancers has been shown to be higher in CLL patients compared to the general population, it is not known whether this risk is specific to CLL or would be seen in any of the indolent lymphoproliferative disorders, which are also treated with immunosuppressive drugs.

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Chapter Two

Objectives, Hypotheses and Aims

2.1. Objectives

It was previously shown that the five-year relative survival of Chronic Lymphocytic Leukemia (CLL) patients in the province of Manitoba was significantly shorter than the general population of Manitoba, when adjusted for age and gender. A downward trend in relative survival was observed with advancing age and this was most prominent in male patients. Thus, elderly male patients had the poorest relative survival (1). The objective of the present thesis is to evaluate the possible cause for the poor outcome of elderly male CLL patients. Specifically, 1) To compare the rates of pre- and post-CLL malignancies in CLL patients in Manitoba with an age- and sex-matched general population and with Follicular Lymphoma (FL) patients; 2) Evaluate the clinical features and outcome of patients referred to the CCMB CLL clinic with the overall CLL population and non-referred patients; and, 3) To investigate the role of prognostic factors in CLL according to age and gender, and determine whether older patients are more likely to have markers of poor prognosis.

2.2. Hypotheses

- The poorer relative survival of elderly male CLL patients in the population is related to an increased incidence of second malignancies in this group
- The poorer relative survival of elderly CLL patients in the population is because they are less likely to receive treatment due to referral bias
- The utility of prognostic markers in CLL depends on the age and sex of the patient so that older patients (and particularly men) are more likely to have markers indicating a worse prognosis

2.3. Aims

Specific Aim 1: Determine whether second cancers in the Manitoba CLL population explain the poor relative survival of elderly male CLL patients.

Rationale: Previous studies have shown that CLL patients have an increased risk of second cancers with the risk being highest in male patients and in those older than 60 years, suggesting that this could be a factor explaining the poor relative survival of elderly male CLL patients (2). Chronic lymphocytic leukemia patients have been shown to have a higher incidence of cancer-specific deaths (excluding deaths from CLL), as compared to the general population (3). This high cancer-specific mortality has been attributed to the increased rate of second cancers in CLL in conjunction with the increased aggressiveness of second cancers in CLL patients. Both the increased incidence and the more rapid growth rate of second malignancies have been attributed to the immune deficiency seen in CLL. It is shown that even benign cancers such as NMSCs tend to be more aggressive and even fatal in CLL patients due to the immune deficiency of these patients (4). Interestingly, patients with a cancer prior to the

diagnosis of CLL also have a shorter survival compared to patients without a preexisting malignancy (2). Based on the above, an increased rate of second cancers, either before or after the CLL diagnosis, could explain the poor relative survival of the elderly, and particularly males, with CLL.

Furthermore, while the risk of other cancers may be higher in CLL patients than in the general population, it is unclear whether this is specific for CLL or may also occur with other indolent B cell malignancies.

Approach: In this thesis I will compare the rates of second cancers in the Manitoba CLL population cohort (both prior and after CLL diagnosis), with rates of cancers in the general population of Manitoba to assess if elderly male patients were at most risk of second cancers. I will also compare the rates of second cancers in CLL and FL patients in Manitoba, both before and following the diagnosis of CLL or FL. Both CLL and FL are similar in that they are indolent B cell malignancies that are monitored and treated similarly.

Specific Aim 2) Determine whether elderly patients referred to a specialized CLL clinic have different outcome compared to non-referred elderly patients, and if the CLL clinic reflects the CLL population.

Rationale: It is possible that elderly patients in Manitoba, and particularly elderly men, are not being referred for therapy, which may explain the poor relative survival of these patients in the population. As Manitoba is a large province, it is also possible that patients outside Winnipeg are less likely to be referred for treatment than those within the city.

Approach: To address these concerns, I will compare the clinical features and outcome of referred and non-referred patients to the CLL clinic at CancerCare Manitoba (CCMB), a clinic that primarily sees patients referred in by family doctors across the province. A small percent of patients with aggressive disease are referred in by private practice hematologists for consideration of clinical trials or for more intensive treatment regimens.

Studies in CLL have generally been population or clinic based, but no studies have to date specifically compared the composition and outcome of the two groups. Many clinic studies come from highly specialized CLL Clinics where the age of diagnosis is about ten years younger than the age of diagnosis in the population, suggesting significant referral bias. Thus, comparing the clinical features and outcome of patients seen in the CLL clinic and in the population would be unique and provide information on referral bias and whether this influences patients' survival.

Specific Aim 3: Identify prognostic factors that are most useful in predicting the outcome of CLL patients.

Rationale: Negative prognostic factors such as advanced clinical stage (5), short LDT (5), high expression levels of CD38 (6–8) and ZAP70 (9–11), elevated levels of ß2-microglobulin (12–16) and creatinine (17), and unmutated *IGHV* (18–22) have been associated with poor prognosis. However, apart from clinical stage, there is considerable controversy regarding the utility of these prognostic factors for general practice.

As a result, most hematologists in Canada are unsure as to which prognostic markers they should use on a routine basis. The controversy is partly because the

markers have typically been evaluated in patients entered on clinical trials. Thus, markers are assessed at the time of treatment rather than at diagnosis and these patients are much younger than the average CLL patient. In addition, the markers have been assessed in highly specialized centres, where patients are also generally younger than in the average clinic and have been referred by other hematologists for specialized treatments. While the CLL clinic at CCMB specializes in CLL, it sees patients referred in by family doctors from across the province (referral population, 1.2 million) and thus sees a patient population that is more representative of patients seen in hematology clinics across the country.

Approach: I will analyze clinical features and prognostic markers for patients seen at diagnosis at CCMB. This would provide important information as to the utility of these markers. Moreover, it would demonstrate whether the utility of these markers depends on age and gender, and whether the elderly are more likely to have markers of aggressive disease, which would explain their poor relative survival.

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Chapter Three

Increased Risk of Second Malignancies in Chronic Lymphocytic Leukemia (CLL) Patients as Compared to Follicular Lymphoma (FL) Patients: A Canadian Population-Based Study

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Running Title: Second cancers in CLL

Condensed Abstract: Background: Chronic Lymphocytic Leukemia (CLL) patients have an increased risk of other malignancies. This may be due to surveillance bias, treatment or immunosuppression. Methods: Cohort of 612 consecutively diagnosed CLL patients in a Canadian province, with comparisons to Follicular Lymphoma (FL) patients. Results: Treated CLL patients had a 1.7-fold increased risk of second cancers compared to untreated CLL patients. As compared to untreated FL patients, untreated CLL patients had a 2-fold increased incidence of second malignancies. Conclusions: CLL patients have an inherent predisposition to second cancers and the incidence is further increased by treatment.

Key Words: Chronic Lymphocytic Leukemia (CLL), Follicular Lymphoma (FL), epidemiology, population-based study, second malignancies, non-melanoma skin cancers (NMSCs), treatment

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Contributions: Sara Beiggi designed the study, analyzed the data and wrote the first draft of the manuscript. James Johnston assisted in study design and critically reviewed the manuscript. Matthew Seftel critically reviewed the manuscript. Marshall Pitz conceived the study idea and critically reviewed the manuscript. Rajat Kumar critically reviewed the manuscript. Versha Banerji critically reviewed the manuscript. Jane Griffith coordinated data collection from the cancer registry and critically reviewed the manuscript. Spencer Gibson assisted in study design and critically reviewed the manuscript.

3.1. Abstract

Background: Chronic Lymphocytic Leukemia (CLL) patients are reported to have an increased risk of other malignancies. However, it is unclear whether this is due to surveillance bias, genetic or environmental factors that predispose patients to second cancers, immunosuppression associated with CLL, or a consequence of therapy. Methods: We conducted a retrospective cohort study, whereby records of all CLL (n: 612) and FL (n: 372) patients diagnosed between 1998 and 2003 in an unselected Canadian population were collected and followed to end of 2009. The relative risk of second cancers and its association with treatment was estimated. Results: The incidence of second malignancies was higher in CLL patients than in FL patients, another indolent B-cell malignancy (SIR 1.79 95% CI 1.30 – 2.45), with non-melanoma skin cancer (NMSC) being the most common type (SIR 2.27 95% CI 1.38 – 3.74) comprising 37% of all second cancers. Neither CLL nor FL patients had an increased probability of preceding cancers. Untreated CLL patients had a higher incidence of second

malignancies compared to untreated FL patients (SIR 2.05 95% CI 1.38 – 3.05). Treated CLL patients had a further increased risk of second cancers compared to untreated CLL patients (SHR 1.7 95% CI 1.10 – 2.62, p 0.016). Conclusions: CLL patients have an increased risk of second cancer diagnoses compared to FL patients independent of treatment or surveillance bias. It is likely that a CLL specific factor contributes to the elevated number of second malignancies observed in this population.

3.2. Introduction

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL) is the most common leukemia in adults (1). Primary causes of death consist of second cancers, progressive disease and infections (2,3). Patients with CLL/SLL have an impaired immune system, and this may partly explain the increased incidence of second malignancies (4). However, the increased incidence of second cancers may also be related to an inherent predisposition to malignancy, to the effects of systemic therapy, or to surveillance bias due to close lifetime monitoring.

A retrospective population study in Manitoba, Canada, forty years ago showed that the risk of all cancers was increased three-fold in CLL, while the increase in skin cancers was eight-fold (5). Subsequent studies have shown the same pattern (6–8). In these studies immunophenotyping for CLL/SLL diagnosis was not utilized and at least 10% of patients may have been misclassified (9). Secondly, most Cancer Registries do not capture non-melanoma skin cancers (NMSCs), the major contributor to the incidence of second cancers in CLL patients. Furthermore, a cancer-free control

population is commonly utilized that may lead to inaccuracy, as one cancer diagnosis increases the risk of development and detection of subsequent malignancies (10–12).

In the current study, we used Cancer Registry and immunophenotypic data in order to create a population-based cohort of confirmed CLL patients. Importantly, this cancer registry routinely captures NMSCs, as NMSCs are reportable in Manitoba. We compared CLL patients to the general population of Manitoba as well as to Follicular Lymphoma (FL) patients, another indolent B-cell malignancy with prognosis and long-term clinical surveillance similar to CLL. Furthermore, in Manitoba, FL patients received similar chemotherapy regimens to CLL patients (Chlorambucil and Fludarabine) for the majority of this study period. Comparison with a similar cancer cohort significantly reduces biases (e.g. surveillance and treatment bias) that could be introduced by comparing a cancer cohort to the cancer-free population. Furthermore, we examined the impact of systemic therapy on the incidence of second cancers. Finally, we examined malignancies in CLL patients both before and after the diagnosis of CLL.

3.3. Methods

3.3.1. Patients Selection

The CLL/SLL cohort (subsequently referred to as CLL) has been previously published (9). All immunophenotypically confirmed CLL/SLL and FL diagnoses in Manitoba between January 1998 and December 2003 were obtained from the Manitoba Cancer Registry. In accordance with the previous study (9), NCI-WG 1996 diagnosis criteria (13) were used. Ethics approval was obtained from the University of Manitoba Health Research Ethics Board.

3.3.2. Statistical Analysis

We used a retrospective cohort design. Standardized Incidence Ratios (SIR) were calculated as the ratio of observed number of second cancers in CLL patients to an expected number derived from age- and gender-standardized incidence of first cancers in the general population or second malignancies in FL patients. Person-years at risk was calculated for each person in the CLL or FL cohort from the date of diagnosis of CLL or FL, up to the date of diagnosis of the second cancer, date of death or 31st December 2009, whichever came first. Person-years at risk for the Manitoba population were estimated by the sum of the Manitoba population for each year of the study (1998 – 2009). Three patients (0.6%) were diagnosed with a second cancer within 30 days of their CLL diagnosis and they remained in the study. Patients with a history of previous cancer and patients diagnosed with CLL at time of death (n: 11) were excluded from SIR calculations. Sub-distribution hazard ratios (SHR) were calculated using a competing risk regression model considering age and gender as covariates and death before a second cancer a competing risk (14). For purposes of the analysis, if patients were initiated on systemic treatment less than 6 months prior to the diagnosis of a second cancer they were considered untreated. Odds ratios (OR) for history of previous cancers were estimated using a logistic regression model, adjusting for age, gender and year of diagnosis. Prevalence of previous cancers in the CLL cohort was compared with the prevalence of first cancers in the Manitoba population for the same age, gender and calendar year groups.

Data management and analysis were performed using SAS 9.2 (SAS Institute Inc., Cary, NC) and Stata 11.2 (StataCorp., College Station, TX).

3.4. Results

3.4.1. Patient Population

Between 1998 and 2003, 612 CLL and 372 FL patients were diagnosed in Manitoba. For CLL and FL cohorts, median age at diagnosis was 71 (31 – 101) years and 63 (24 – 92) years, male to female ratios were 1.3:1 and 1.2:1, median follow up was 6.4 (0 – 12) years and 6.9 (0 – 12) years, and median time to development of a second cancer was 3.3 (0 – 11.4) years and 4.0 (0 – 11.3) years, respectively (Table 3.1).

3.4.2. Second Cancers

CLL patients had a 1.8-fold increase in the relative risk of a second cancer (SIR 1.79, 95% CI 1.30 – 2.45) compared to age- and gender-standardized FL patients. This increased risk was evident for all ages and both genders. The risk of NMSCs alone was more than two-fold higher in CLL patients compared to FL patients (SIR 2.27, 95% CI 1.38 – 3.74) and when stratified by gender this was only significant in males (SIR 3.12, 95% CI 1.60 – 6.10 for males. CLL patients also showed an increased risk of second cancers when compared to the general population of Manitoba (SIR 1.93, 95% CI 1.40 – 2.68) (Table 3.2, Table 3.3, Table 3.4).

The most common second cancer among CLL patients was NMSC (37%), followed by cancers of digestive organs (16%), prostate (12%), breast (10%) and lung (9%). Second cancers in FL and the general population followed the same general pattern (Figure 3.1, Table 3.5, Table 3.6, Table 3.7).

Table 3.1: Baseline characteristics and follow up time for Chronic Lymphocytic Leukemia (CLL) and Follicular Lymphoma (FL) cohorts

		All Cancers			Invasive Cancers (Excluding NMSCs)		
CLL	Group	n	Median age at CLL Dx (Range) (yr)	M:F	n	Median age at CLL Dx (Range) (yr)	M:F
	Total	612	71 (31 – 101)	1.3:1	612	71 (31 – 101)	1.3:1
	A	455	69 (31 – 101)	1.3:1	499	69 (31 – 101)	1.4:1
	B	351	68 (31 – 101)	1.3:1	423	69 (31 – 101)	1.4:1
	C	104	70 (49 – 93)	1.7:1	76	70 (49 – 86)	1.5:1
	D	146	77 (38 – 95)	1.3:1	102	77 (38 – 93)	1.2:1
CLL	Group	n	Median Follow up (yr)	Range (yr)	n	Median Follow up (yr)	Range (yr)
	A	455	6.4	0.0 – 12.0	499	6.5	0.0 – 12.0
	B	351	7.0	0.0 – 12.0	423	6.9	0.0 – 12.0
	C	104	3.3	0.0 – 11.4	76	3.3	0.0 – 11.4
FL	Group	n	Median age at FL Dx (Range) (yr)	M:F	n	Median age at FL Dx (Range) (yr)	M:F
	Total	372	63 (24 – 92)	1.2:1	372	63 (24– 92)	1.2:1
	A	306	62 (24 – 92)	1.2:1	330	62 (24 – 92)	1.2:1
	B	270	62 (24 – 92)	1.2:1	300	62 (24 – 92)	1.1:1
	C	36	64 (33 – 87)	1.8:1	30	63 (33 – 87)	2.8:1
	D	66	71 (35 – 92)	0.9:1	42	70 (35 – 92)	0.9:1
FL	Group	n	Median Follow up (yr)	Range (yr)	n	Median Follow up (yr)	Range (yr)
	A	306	6.9	0.0 – 12.0	330	6.9	0.0 – 12.0
	B	270	7.0	0.0 – 12.0	300	7.0	0.0 – 12.0
	C	36	4.0	0.0 – 11.3	30	3.6	0.1 – 8.6

Table 3.1 (continued)

		NMSCs		
CLL	Group	n	Median age at CLL Dx (Range) (yr)	M:F
	Total	612	71 (31 – 101)	1.3:1
	A	538	70 (31 – 101)	1.3:1
	B	488	69 (31 – 101)	1.2:1
	C	50	71 (51 – 93)	2.3:1
	D	63	79 (55 – 93)	2.3:1
CLL	Group	n	Median Follow up (yr)	Range (yr)
	A	538	6.6	0.0 – 12.0
	B	488	6.7	0.0 – 12.0
	C	50	3.9	0.2 – 11.3
FL	Group	n	Median age at FL Dx (Range) (yr)	M:F
	Total	372	63 (24– 92)	1.2:1
	A	344	62 (24 – 92)	1.2:1
	B	333	62 (24 – 92)	1.2:1
	C	11	73 (55– 80)	1.2:1
	D	28	71 (38 – 88)	0.9:1
FL	Group	n	Median Follow up (yr)	Range (yr)
	A	344	6.9	0.0 – 12.0
	B	333	6.9	0.0 – 12.0
	C	11	4.3	0.0 – 11.3

Group A: Patients with a CLL or FL as the first cancer diagnosis

Group B: Patients with CLL or FL who never developed a second malignancy

Group C: Patients with CLL or FL who developed a 2nd malignancy during the follow up period

Group D: Patients diagnosed with another cancer prior to CLL or FL diagnoses

Patients were followed from time of CLL or FL diagnosis until a second cancer, death or end of 2009 (hence the follow up for group C equals the time to development of a second cancer)

Dx: diagnosis; F: female; M: male; NMSC: non-melanoma skin cancer; yr: year

Table 3.2: Increased rates of second malignancies in patients with Chronic Lymphocytic Leukemia (CLL) as compared with Follicular Lymphoma (FL) patients and the general population (GP) of Manitoba

	CLL Population			Compared with FL		Compared with GP	
Groups	**n**	**Time at risk (PY)**	**Second Cancers**	**SIR**	**95% CI**	**SIR**	**95% CI**
All Cancers							
All	455	2707.18	104	1.79*	1.30 – 2.45	1.93*	1.40 – 2.68
Male	260	1456.39	65	1.84*	1.23 – 2.75	2.05*	1.35 – 3.12
Female	195	1250.70	39	1.71*	1.02 – 2.84	1.76*	1.05 – 2.96
Invasive Cancers (excluding NMSCs)							
All	499	3033.02	76	1.51*	1.06 – 2.14	1.62*	1.13 – 2.32
Male	288	1662.09	45	1.19	0.77 – 1.82	1.59*	1.00 – 2.53
Female	211	1370.93	31	2.49*	1.29 – 4.78	1.66	0.94 – 2.93
NMSCs							
All	538	3268.53	50	2.27*	1.38 – 3.74	40.42*	5.28 – 309.32
Male	299	1712.79	35	3.12*	1.60 – 6.10	38.57*	3.65 – 408.04
Female	239	1555.73	15	1.39	0.64 – 3.03	44.00	0.84 – 2304.23

* Indicates statistical significance

CI: confidence interval; GP: general population; n: number of CLL cases; NMSCs: non-melanoma skin cancers; PY: person-years; SIR: standardized incidence ratio

Table 3.3: Risk of second cancers in Chronic Lymphocytic Leukemia (CLL) patients compared with Follicular Lymphoma (FL) patients calculated by competing risk regression model, adjusted for age and gender and considering death as competing risk

	All Cancers		Invasive Cancers (Excluding NMSCs)		NMSCs	
Variable	SHR	95% CI	SHR	95% CI	SHR	95% CI
Both Genders						
CLL	2.04*	1.39 – 2.97	1.70*	1.11 – 2.59	2.98*	1.55 – 5.71
CLL	1.72*	1.18 – 2.52	1.50	0.98 – 2.29	2.32*	1.20 – 4.49
Age	2.92*	1.61 – 5.28	2.55*	1.08 – 3.59	12.95*	1.76 – 95.18
CLL	1.70*	1.16 – 2.48	1.47	0.96 – 2.26	2.27*	1.17 – 4.39
Age	2.94*	1.63 – 5.33	1.99*	1.09 – 3.63	13.16*	1.79 – 96.52
Male	1.37	0.97 – 1.93	1.36	0.91 – 2.02	1.74*	1.02 – 2.97
Male						
CLL	1.91*	1.19 – 3.08	1.91*	1.19 – 3.08	3.77*	1.59 – 8.93
CLL	1.57	0.97 – 2.55	1.57	0.97 – 2.55	2.81*	1.16 – 6.82
Age	2.55*	1.26 – 5.20	2.55*	1.26 – 5.20	8.54*	1.13 – 64.08
Female						
CLL	2.22*	1.18 – 4.16	2.87*	1.32 – 6.25	2.00*	0.72 – 5.52
CLL	1.94*	1.04 – 3.62	2.59*	1.21 – 5.55	1.63	0.59 – 4.52
Age	4.19*	1.32 – 13.22	2.64	0.84 – 8.32		

* Indicates statistical significance

CI: confidence interval; NMSC: non-melanoma skin cancer; SHR: sub-hazard ratio

Table 3.4: Excess risk of second cancers in Chronic Lymphocytic Leukemia (CLL) patients compared with Follicular Lymphoma (FL) patients. Excess risk (calculated as observed rate – expected rate) is adjusted for age and gender and was estimated per 100,000 persons

Group	All Cancers	Invasive Cancers (Excluding NMSCs)	NMSCs
Male	29.59	7.02	23.78
Female	16.12	18.52	4.20
All	46.13	27.70	27.90

NMSC: non-melanoma skin cancer

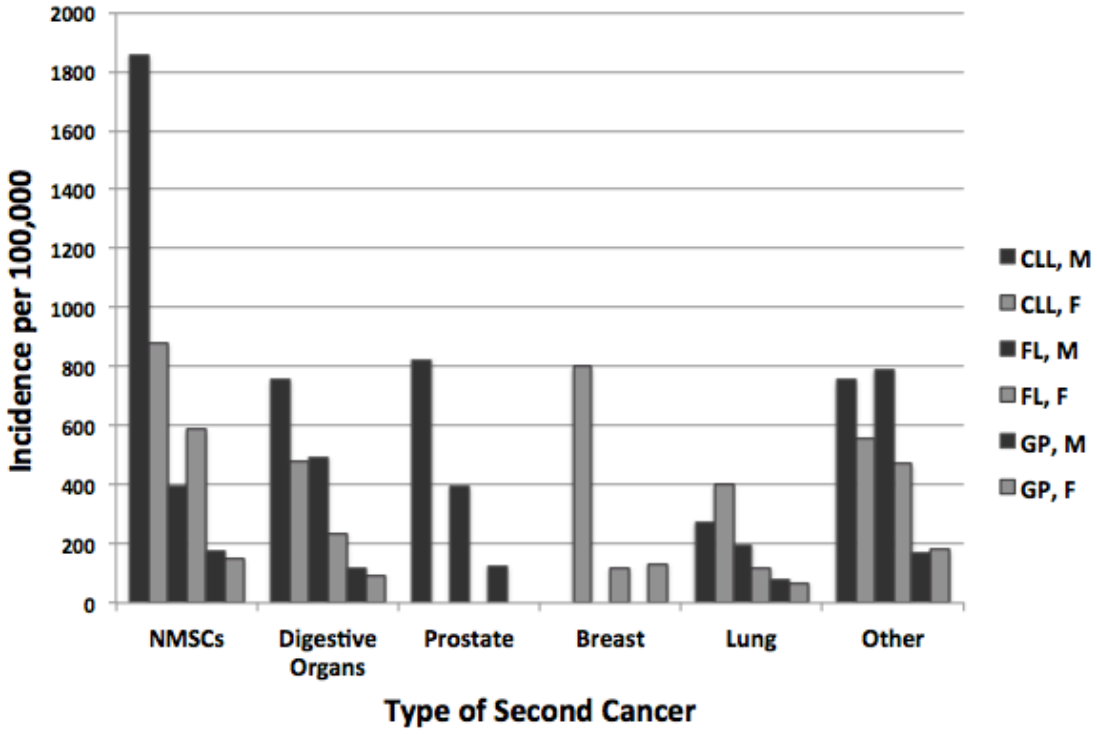

Figure 3.1: Site-specific incidence rates of second malignancies in Manitoba's Chronic Lymphocytic Leukemia (CLL) population as compared to Follicular Lymphoma (FL) and the general population (GP) of Manitoba by gender. Unadjusted incidence rates are calculated from 1998 to 2009 and are expressed per 100,000 persons

Table 3.5: Type of second cancers in Chronic Lymphocytic Leukemia (CLL) patients

Type of 2nd Cancer	n (M, F) [%][1]		
	<70 years	≥70 years	Total
NMSCs	17 (14, 3) [16]	21 (13, 8) [20]	38 (27, 11) [37]
Digestive Organs	7 (5, 2) [7]	10 (6, 4) [10]	17 (11, 6) [16]
Prostate	9 (9, 0) [9]	3 (3, 0) [3]	12 (12, 0) [12]
Breast	1 (0, 1) [1]	9 (0, 9) [9]	10 (0, 10) [10]
Lung	6 (3, 3) [6]	3 (1, 2) [3]	9 (4, 5) [9]
Thyroid	2 (1, 1) [2]	1 (-, 1) [1]	3 (1, 2) [3]
Melanoma	2 (1, 1) [2]	0	2 (1, 1) [2]
Follicular Lymphoma	2 (2, 0) [2]	0	2 (2, 0) [2]
Brain	1 (1, 0) [1]	1 (1, 0) [1]	2 (2, 0) [2]
Mouth	0	1 (0, 1) [1]	1 (0, 1) [1]
Nasal Cavity	0	1 (1, 0) [1]	1 (1, 0) [1]
Ovary	0	1 (0, 1) [1]	1 (0, 1) [1]
Renal Pelvis	0	1 (0, 1) [1]	1 (0, 1) [1]
Peritoneum	1 (1, 0) [1]	0	1 (1, 0) [1]
Testes	1 (1, 0) [1]	0	1 (1, 0) [1]
Multiple Myeloma	0	1 (1, 0) [1]	1 (1, 0) [1]
T-Cell Lymphoma	1 (1, 0) [1]	0	1 (1, 0) [1]
Non-Specific (blood)	0	1 (0, 1) [1]	1 (0, 1) [1]
Total	**50 (39, 11) [48]**	**54 (26, 28) [52]**	**104 (65, 39) [100]**

[1]Percentage may not sum to 100 due to rounding

F: female; M: male; NMSC: non-melanoma skin cancer

Table 3.6: Type of second cancers in Follicular Lymphoma (FL) patients

Type of 2nd Cancer	n (M, F) [%][1]		
	<70	≥70	Total
NMSCs	5 (3, 2) [14]	4 (1, 3) [11]	9 (4, 5) [25]
Digestive Organs	4 (3, 1) [11]	3 (2, 1) [8]	7 (5, 2) [19]
Prostate	4 (4, 0) [11]	0	4 (4, 0) [11]
Lung	2 (1, 1) [6]	1 (1, 0) [3]	3 (2, 1) [8]
Melanoma	3 (3, 0) [8]	0	3 (3, 0) [8]
Bladder	1 (1, 0) [3]	1 (1, 0) [3]	2 (2, 0) [6]
Breast	0	1 (0, 1) [3]	1 (0, 1) [3]
Kidney	1 (1, 0) [3]	1 (1, 0) [3]	2 (2, 0) [6]
Lip	1 (0, 1) [3]	0	1 (0, 1) [3]
Monocytic Leukemia	1(0, 1) [3]	0	1 (0, 1) [3]
Multiple Myeloma	0	1 (0, 1) [3]	1 (0, 1) [3]
Follicular Lymphoma	1 (1, 0) [3]	0	1 (1, 0) [3]
Non-Specific Site	1 (0, 1) [3]	0	1 (0, 1) [3]
Total	**24 (17, 7) [66]**	**12 (6, 6) [33]**	**36 (23, 13) [100]**

[1]Percentage may not sum to 100 due to rounding

F: female; M: male; NMSC: non-melanoma skin cancer

Table 3.7: Incidence of cancers in the province of Manitoba by gender and age groups from 1998 – 2009 (per 100,000 persons)

Type	Gender	00-29	30-39	40-49	50-59	60-69	70-79	80+	Total
All	F	26.19	155.73	396.46	867.58	1566.69	2291.75	2854.56	8158.96
	M	23.03	92.12	259.66	801.06	2140.92	3753.57	4684.32	11754.68
Invasive Cancers[1]	F	23.49	118.56	297.98	682.13	1238.83	1719.67	2035.41	6116.07
	M	21.46	64.04	168.73	585.06	1652.2	2776.29	3305.16	8572.94
NMSCs	F	2.7	37.17	98.58	185.69	328.38	573.79	821.04	2047.35
	M	1.57	28.28	90.93	216.7	489.09	979.68	1385.79	3192.04
Digestive System	F	0.93	8.17	31.64	92.26	213.57	415.46	610.84	1372.87
	M	0.85	11.82	40.05	150.38	372.61	673.66	884.37	2133.74
Breast	F	1.42	38.09	123.2	251.89	371.82	383.03	411.74	1581.19
Prostate	M	0	0.4	13.88	130.07	535.09	771.58	740.89	2191.91
Lung	F	0.14	2.66	17.79	81.56	221.42	324.99	267.9	916.46
	M	0.17	1.31	13.97	80.91	267.27	505.85	568.31	1437.79

[1]Excluding NMSCs

NMSC: Non-Melanoma Skin Cancer

3.4.3. Effect of Treatment on Second Cancers

Both treated CLL and FL patients had an increased risk of second cancers compared to untreated CLL and FL patients, respectively. Untreated CLL patients had a two-fold increased incidence of second malignancies compared to untreated FL patients (Table 3.8, Figure 3.2, Figure 3.3).

3.4.4. Cancers Diagnosed Prior to CLL Diagnosis

When previous cancers were investigated, patients with previous cancers showed a higher chance of developing CLL (OR 1.43, 95% CI 1.03 – 1.97, p 0.0322). However when adjusted for age, the association disappeared (OR 0.99, 95% CI 0.70 – 1.41, p 0.9712; OR age 1.58, 95% CI 1.42 – 1.76, p <0.0001). Gender and year of diagnosis did not significantly improve the model. In addition, no significant difference was seen in prevalence of previous cancers between the CLL patients at the time of CLL diagnosis and the general Manitoba population (data not shown).

3.5. Discussion

In this population-based cohort study, patients with CLL had an almost two-fold increased incidence of second malignancies compared to patients with FL. NMSCs were the most common second malignancy (37%), with a 2.3-fold increased risk in CLL compared to FL. When stratified by gender, this risk was only significant in male patients and may be explained by the fact that men are more likely to work outdoors and receive greater exposure to UV radiation (15). The elevated incidence of NMSCs is consistent with previous reports where skin cancers constituted 29.9% of all second

Table 3.8: Increased rates of second cancers in treated Chronic Lymphocytic Leukemia (CLL) and Follicular Lymphoma (FL) patients, and in untreated CLL patients compared with untreated FL patients

Variable	CLL	FL
Treated (%)	141 (30)	202 (66)
Median TTFT (Range)	5.3 mth (0 – 9.6 yr)	1.4 mth (0 – 9.0 yr)
2nd Cancer after Treatment (%)	32 (23)	25 (12)
Median time from treatment to second cancer Diagnosis (Range)	3.1 yr (6.4 mth – 11.2 yr)	4.0 yr (6.3 mth – 11.2 yr)
Treated vs. Untreated SHR (95% CI)	Tx 1.81 (1.18 – 2.78) Age 3.80 (1.57 – 9.18)	Tx 2.47 (1.05 – 5.80) Age 2.52 (1.08 – 5.85)
Untreated CLL vs. Untreated FL SIR (95% CI)	2.05 (1.38 – 3.05)	

Dx: diagnosis; mth: month; SHR: sub-hazard ratio; SIR: standardized incidence ratio; TTFT: time to first treatment; yr: year

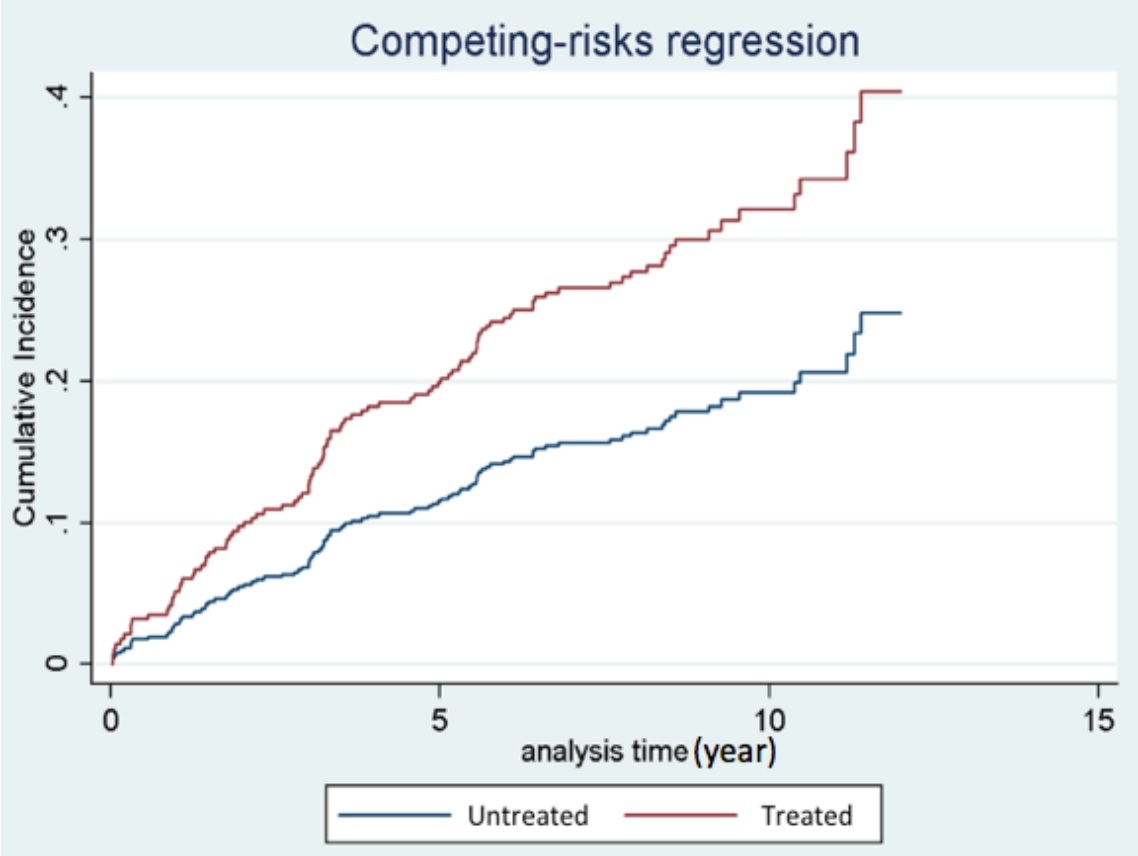

Figure 3.2: Cumulative Incidence Rate of second cancers in treated and untreated Chronic Lymphocytic Leukemia (CLL) patients (Table 3.8)

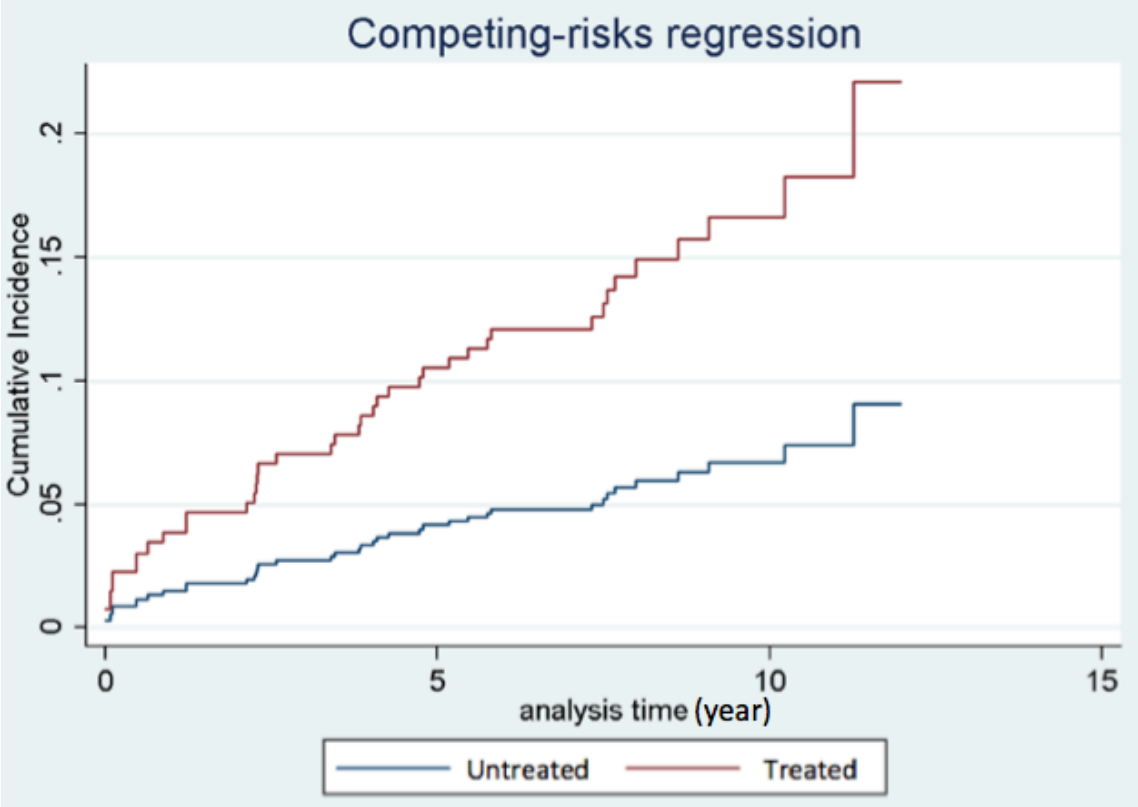

Figure 3.3: Fig. S2: Cumulative Incidence Rate of second cancers in treated and untreated Follicular Lymphoma (FL) patients (Table 2)

cancers (6) and patients with CLL had a 3.66-fold increased risk of NMSC when compared to the general population (7).

The increased incidence of second malignancies in CLL has been partly attributed to the profound immune deficiency seen in this disease (16). An increased incidence of NMSC has been closely related to immunosuppression after renal transplantation and other immunosuppressed individuals (17,18). Other studies have documented a high incidence of skin cancers in CLL patients and have noted a more aggressive disease course and increased mortality when compared with patients without CLL (8). In our CLL cohort, deeper and longer degrees of immunosuppression might explain the high incidence of skin cancers in these patients as compared to the FL patients. Furthermore, it has been shown that patients with breast and colorectal cancers and a pre-existent CLL have an inferior overall and cancer-specific survival. This is suggested to be due to a less effective immune response in CLL patients that consequently provides a more permissive environment for metastases (19).

Chemotherapy can increase the incidence of second malignancies in CLL patients (4). In our study, treated CLL and FL patients had substantially increased rates of second malignancies compared to untreated CLL and FL patients, respectively. However, it is important to highlight the 2-fold higher incidence in untreated CLL patients compared with untreated FL patients, suggesting treatment is not the sole contributor to the elevated risk of second cancers observed in CLL patients.

We have previously observed an increased incidence of non-Hodgkin lymphoma, melanoma, lung, prostate and breast cancers following a diagnosis of NMSC (10). This suggests a common factor in the etiology of these malignancies. In addition, an

increased incidence of CLL has been observed in families of patients with colon cancer (20) and an increased incidence of breast cancer has been noted in first-degree relatives of CLL patients (21). However, the role of genetic factors for secondary cancers in CLL patients is mitigated by our observation that the incidence of other malignancies is increased only in patients after the diagnosis of CLL, rather than beforehand. Finally, the increased rates of second cancers in CLL patients cannot be attributed to treatment or surveillance bias since in Manitoba FL patients undergo routine follow up in a similar fashion to CLL patients.

It should be noted that CLL patients were diagnosed using the diagnostic criteria from 1996, which required patients to have a peripheral blood lymphocyte count of $>5\times10^9$/L (13). The updated definition from 2008 requires a peripheral blood B cell count of $>5\times10^9$/L (13,22). Thus, a small proportion of our patients may have had monoclonal B cell lymphocytosis. Secondly, patients diagnosed between 1998 and 2003 would have received treatment with single agent chemotherapy. It remains to be seen if the second cancer rate will increase in patients receiving chemoimmunotherapy. Finally, our relatively small cohort and short follow up yielded few second malignancies.

In conclusion, we have observed that the risk of second cancers in CLL patients is not only greater than the general population, but is also greater than a closely related lymphoproliferative disorder, FL. This increased risk is independent of treatment or surveillance bias. Of the second malignancies, skin cancers were the most common type. Heightened awareness about risks of second cancers should be communicated to improve the care and outcome of CLL patients.

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Chapter Four

Comparison of Outcome between Referred and Non-Referred Chronic Lymphocytic Leukemia Patients to a Specialized CLL Clinic: A Canadian Population-based Study

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Running Title: Referred vs. non-referred CLL outcome

Condensed Abstract: Chronic Lymphocytic Leukemia (CLL) patients in Manitoba are either referred to the CancerCare Manitoba (CCMB) CLL clinic or are followed by hematologists/general practitioners. Of 555 CLL cases between 2007 – 2011, 51% were referred to the CLL clinic. Patients seen in the clinic had a >2-fold greater overall survival (OS) compared to the remaining patients (HR 2.39, 95% CI 1.5 – 3.82) when adjusted for age, gender, post-CLL cancer, pre-CLL cancer, requiring treatment/no treatment and urban/rural living.

Key Words: Chronic Lymphocytic Leukemia (CLL), Small Lymphocytic Lymphoma (SLL), outcome, overall survival, referral, CLL clinic

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Contributions: Sara Beiggi designed the study, analyzed the data and wrote the first draft of the manuscript. Versha Banerji critically reviewed the manuscript. Angela Deneka managed the data. Jane Griffith coordinated data collection from the cancer registry and critically reviewed the manuscript. Spencer Gibson critically reviewed the

manuscript. James Johnston conceived the study idea and critically reviewed the manuscript.

4.1. Abstract

Introduction: Chronic lymphocytic leukemia and small lymphocytic lymphoma (CLL/SLL) patients in Manitoba are either referred to the CLL Clinic at CancerCare Manitoba (CCMB) or are followed by other hematologists and general practitioners. However, it has been unclear whether referral to the CLL clinic influenced patient outcome. Patients: Overall survival (OS) was assessed for all CLL/SLL patients diagnosed in Manitoba between 2007 and 2011. Results: Of 555 patients, 281 (51%) were referred to the CLL clinic. Patients seen in this clinic had a two-fold increased OS compared to patients who were managed by other hematologists and general practitioners (HR 2.39, 95% CI 1.5 – 3.82) when adjusted for age, gender, presence of pre- or post-CLL cancer, treatment and urban/rural location. In the non-referred population there was a striking correlation between advancing age and decreasing OS. However, this correlation was almost eliminated in the referred population who were more likely to receive chemotherapy. Conclusion: Patients seen in the CLL clinic have an improved OS compared to non-referred patients and this appears to be primarily related to improved OS in the elderly. Whether this is related to referral bias is being evaluated.

4.2. Introduction

Chronic lymphocytic leukemia/small lymphocytic lymphoma (subsequently referred to as CLL) is the most common leukemia in the western world with an incidence rate of 7.9/100,000 persons in Manitoba (1). Over the past twenty years, the relative survival of CLL patients (calculated by correcting for the survival of an age- and sex-matched control population) both in the epidemiological studies and in CLL clinics has significantly improved, except for those aged greater than 70 to 80 years (1,2). The increased relative survival in younger patients may be related to improved therapies and supportive care, but it is unclear why the relative survival in the elderly has not improved (2–4).

In Manitoba, CLL patients are either referred to a specialized CLL Clinic at CancerCare Manitoba (CCMB) or are taken care of by other hematologists or by their family practitioners. To date, it has been unknown whether referral to the CLL clinic affected patient outcome. The concept that clinical outcome could be improved by treatment in a high volume centre was first introduced more than three decades ago (5). Since then, it has been shown in the literature that a physician's sub-specialty and caseload as well as centre volume are directly associated with more favourable outcome for non-surgical patients (6–9). Furthermore, it has been demonstrated that newly diagnosed CLL patients who are followed by CLL specialists at the Mayo Clinic have a longer time to first treatment (TTFT) and better overall survival (OS) compared with patients seen by other hematologists within the same centre (10).

In this study, we used Cancer Registry and immunophenotypic data to create a population-based cohort of CLL patients in the province of Manitoba, Canada, identified

over a five-year period (2007 – 2011). We evaluated the OS of these patients to determine whether there was a difference between patients referred to a specialized CLL clinic at CCMB to patients who were managed by other hematologists and family doctors.

4.3. Methods

4.3.1. Patients Selection

All cases diagnosed with CLL and SLL between January 1, 2007 and December 31, 2011 were obtained from the Manitoba Cancer Registry. In Manitoba, flow cytometry is carried out at the two teaching centres, Health Science Centre and St. Boniface General Hospital, and all reports showing the presence of CLL cells (monoclonal B cells that are CD19+, CD5+ and CD23+) are mandated to be reported to the Provincial Cancer Registry. Patients who received chemotherapy, or had other cancers were identified using the Provincial Cancer Registry database; however, data pertaining to the type of treatment and Rai stages were not available through the Cancer Registry. Other cancer diagnoses within 30 days prior or subsequent to the CLL diagnosis were considered concurrent malignancies. Data pertaining to causes of death was provided from death certificates through the Provincial Cancer Registry. Rural patients were defined as patients residing outside the city of Winnipeg as determined by postal codes. Patients referred to the CLL clinic at the CCMB were identified from the CCMB records.

In the CCMB clinic, the 2008 updated guideline for diagnosis and treatment of CLL were used (11), and accordingly patients with monoclonal B cell lymphocytosis

(MBL) were eliminated from the referred patient cohort; however, it is likely that the non-referred cohort included some MBL patients. Patients who had been initially followed by general hematologists and family physicians but later referred to the CLL clinic were included in the referred cohort.

Ethics approval was obtained from the University of Manitoba Health Research Ethics Board.

4.3.2. Statistical analysis

Time to first treatment (TTFT) was calculated from the date of CLL diagnosis to the date of administration of the first treatment. Overall Survival (OS) was calculated as the time between date of diagnosis and date of death or end of study (December 31, 2011). Association between age, referral status and treatment was measured by chi-squared test. OS plots were created using Kaplan-Meier methods and compared between groups by log-rank test. Hazard ratios (HR) were estimated using univariable and multivariable Cox proportional hazard regression models. A p-value <0.05 was considered to be statistically significant. Data management and analysis was performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

4.4. Results

4.4.1. Patient Population

There were 555 CLL diagnoses made in Manitoba between 2007 and 2011. Of the 555 patients, 281 (51%) were referred to the CCMB specialized CLL clinic (referred

patients) and 274 (49%) were seen by other hematologists (most usually in private practice) or followed by their family practitioners (non-referred patients). The clinical features of these two groups of patients are shown in Table 4.1. Patients referred to the clinic were younger than non-referred patients (p: 0.0033) and the median age of referred patients was 68 years compared to 72 years for non-referred patients (Figure 4.1). In addition, while the age for referred patients was in a normal distribution with a peak at 60 years, age for non-referred patients had a bimodal distribution with peaks at approximately 60 and 80 years. The primary causes of death in both referred and non-referred cohorts were CLL, the complications of CLL or second malignancies (Table 4.2). Comorbidities such as cardiovascular diseases had equal contributions to mortality in both populations (7% and 9.5% in referred and non-referred patients, respectively) (Table 4.2). When patients older than 70 years were investigated, elderly patients who were referred to the clinic were more likely to receive treatment (p: 0.0002).

4.4.2. Univariable analysis

Patients referred to the specialized CLL clinic had a superior OS compared with non-referred patients (HR 2.74, 95%CI 1.75 – 4.28) (Figure 4.2, Table 4.3). In referred patients, age ≥62 years, requiring treatment for CLL and having a previous cancer were associated with poor OS. In contrast, in non-referred patients, OS continued to decline with advancing age (Figure 4.3, Table 4.3). In this group, presence of a previous cancer and requiring treatment for other malignancies were also associated with poor survival. As opposed to referred patients, treatment for CLL was not associated with outcome in non-referred patients (Table 4.3).

Table 4.1: Characteristics of Manitoba CLL population

	Referred Patients	Non-Referred Patients
N (%)	281 (51)	274 (49)
Age Median (Range)	68 yr (39 – 99)	72 yr (45 – 96)
Male to Female Ratio	1.8:1	1.8:1
SLL (%)	46 (16)	34 (12)
Treated-CLL (%)	77 (27)	31 (11)
Median TTFT-CLL (Range)	4.7 mth (0 – 67.2)	2.7 mth (0 – 28.8)
Treated-Other Cancers (%)	10 (3.6)	11 (4.0)
Other Cancers (%)	Pre-CLL: 33 (12) Concurrent: 7 (2.5) Post-CLL: 15 (5)	Pre-CLL: 40 (15) Concurrent: 11 (4) Post-CLL: 13 (5)
Median Time to 2nd Cancer (Range)	24 mth (2 – 48)	15.6 mth (2.5 – 52.8)
Urban to Rural Ratio	1.1:1	1.4:1
Mortality (%)	28 (10)	63 (23)

CLL: Chronic Lymphocytic Leukemia; mth: month; SLL: Small Lymphocytic Lymphoma; TTFT: time to first treatment; yr: year

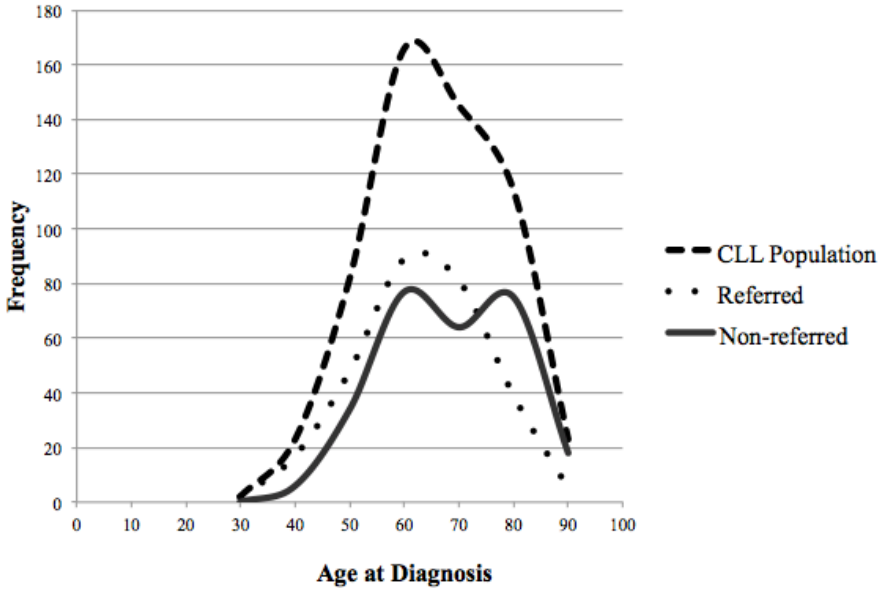


Figure 4.1: Age distribution of Manitoba CLL population

Table 4.2: Causes of death in referred and non-referred patients

Causes of Death	Referred Patients (%)	Non-Referred Patients (%)
CLL/SLL	16 (57)	22 (35)
CLL Complications[1]	1 (4)	2 (3)
Other Hematopoietic Malignancies[2]	2 (7)	6 (9.5)
Solid Tumours[3]	4 (14)	10 (16)
Non-Malignant[4]	2 (7)	6 (9.5)
Unknown	3 (11)	17 (27)
Total	28 (100)	63 (100)

[1]large B cell lymphoma (1) in referred patients and infection (1) and immune thrombocytopenic purpura (1) in non-referred patients

[2]multiple myeloma (1) in referred patients and acute myeloid leukemia (2) and multiple myeloma (4) in non-referred patients

[3]lung (1), non-melanoma skin cancer (1), endometrium (1) and prostate (1) in referred and digestive organs (2), lung (3), brain (2) and unspecified site (3) in non-referred patients

[4]heart disease (2) in referred and diabetes (2), heart disease (2), stroke (2), respiratory disease (1) and muscular disorder (1) in non-referred patients

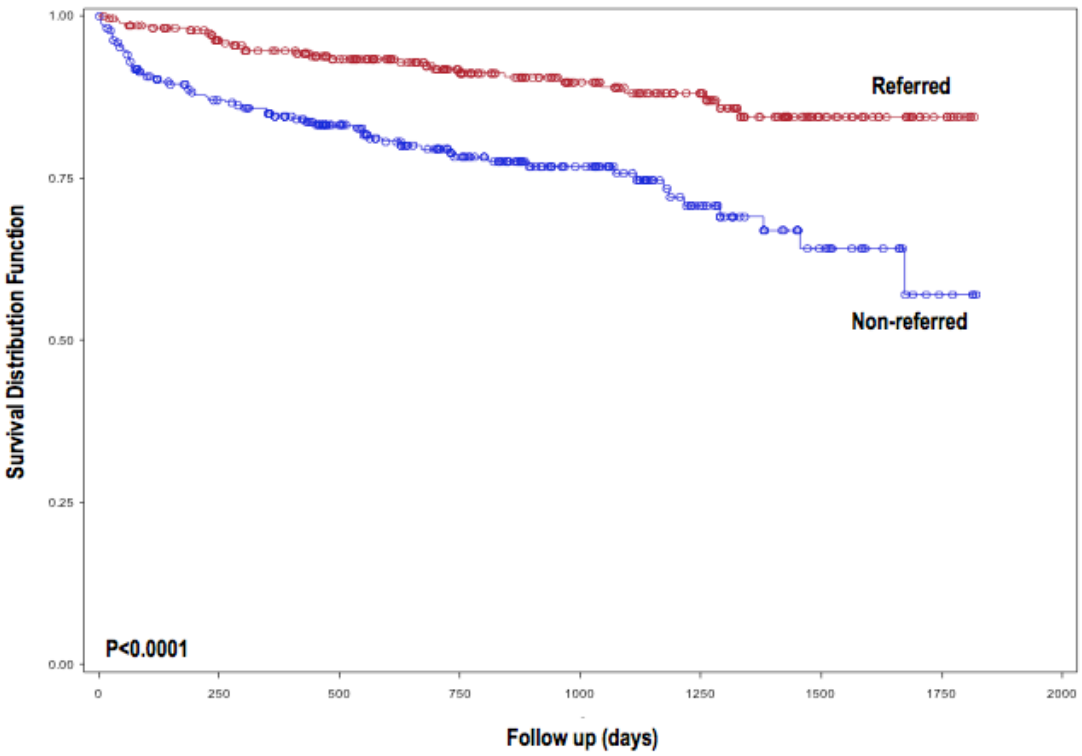


Figure 4.2: Kaplan-Meier plot of overall survival based on referral status

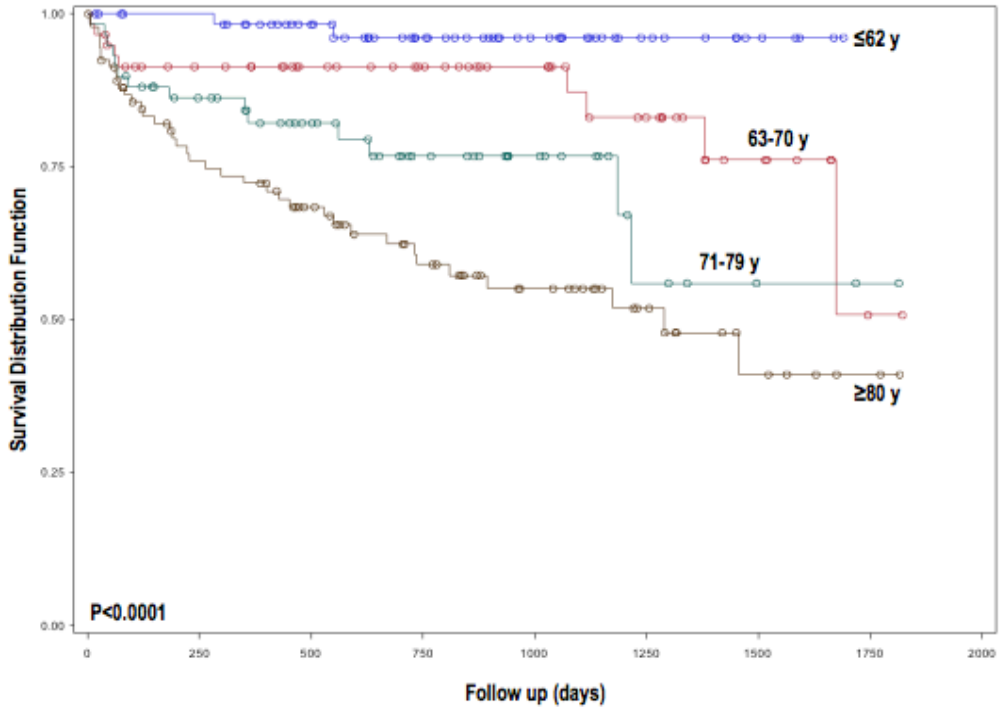
Table 4.3: Univariable analysis of overall survival

Univariable Analyses (Overall Survival)			
Variable	All CLL Patients HR (95% CI)	Referred Patients HR (95% CI)	Non-Referred Patients HR (95% CI)
Referral Status			
Referred	1.00	-	-
Non-Referred	2.74 (1.75 – 4.28)*		
Age quartile			
<62 yr	1.00	1.00	1.00
62 – 70 yr	4.34 (1.63 – 11.55)*	4.04 (1.13 – 14.49)*	4.73 (1.02 – 21.88)*
71– 79 yr	5.49 (2.08 – 14.50)*	3.20 (0.85 – 12.06)	8.94 (2.03 – 39.33)*
≥80	11.83 (4.70 – 29.85)*	4.56 (1.14 – 18.26)*	15.72 (3.79 – 65.14)*
Gender			
Female	1.00	1.00	1.00
Male	1.37 (0.88 – 2.14)	1.50 (0.66 – 3.41)	1.32 (0.78 – 2.25)
Tx-CLL			
Untreated	1.00	1.00	1.00
Treated	1.18 (0.73 – 1.90)	2.19 (1.04 – 4.60)*	1.12 (0.55 – 2.28)
Tx-Other			
Untreated	1.00	1.00	1.00
Treated	2.27 (1.05 – 4.91)*	1.94 (0.46 – 8.17)	2.59 (1.04 – 6.50)*
Pre-CLL Cancer			
Without	1.00	1.00	1.00
With	2.34 (1.42 – 3.87)*	2.80 (1.13 – 6.96)*	2.03 (1.11 – 3.70)*
Post-CLL Cancer			
Without	1.00	1.00	1.00
With	1.72 (0.86 – 3.42)	2.30 (0.80 – 6.64)	1.56 (0.63 – 3.89)
Residence			
Urban	1.00	1.00	1.00
Rural	1.03 (0.68 – 1.56)	1.21 (0.58 – 2.55)	1.02 (0.62 – 1.70)

CI: Confidence Interval; CLL: Chronic Lymphocytic Leukemia; HR: Hazard Ratio; Tx: Treatment; yr: year

* $p < 0.05$

Non-referred population



Referred population

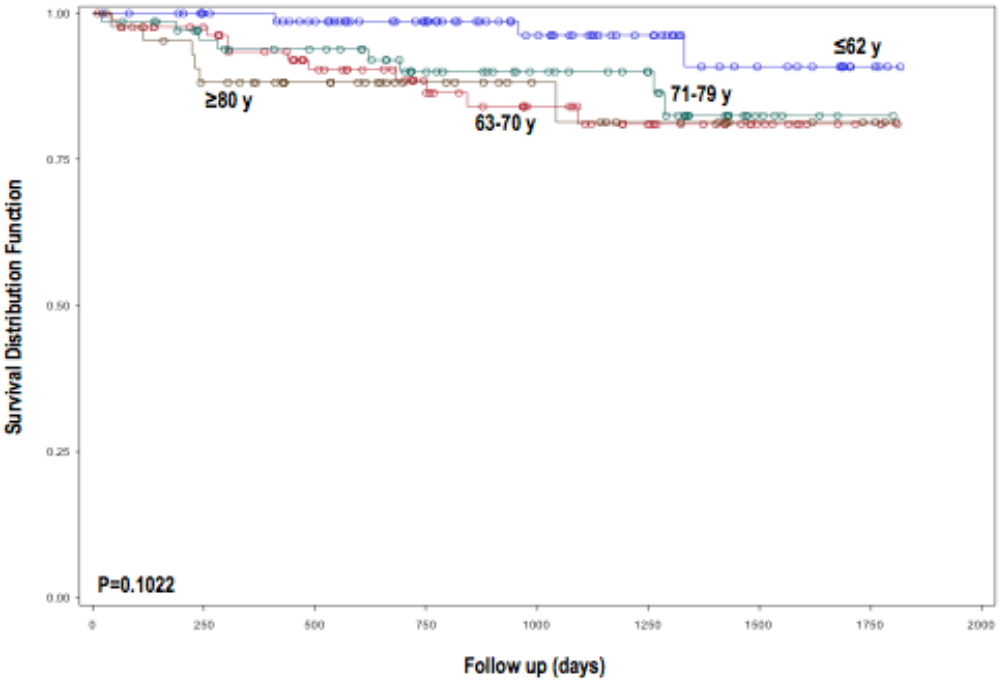


Figure 4.3: Kaplan-Meier plot of overall survival based on age in non-referred (top) and referred (bottom) patients; y: year

4.4.3. Multivariable analysis

When adjusted for age, gender, treatment, pre- and post-CLL cancers and residency in a multivariable model, referral to the CLL clinic was strongly associated with superior survival (HR 2.39, 95%CI 1.5 – 3.82). In referred patients, being treated for CLL, pre-CLL cancer and advancing age were associated with decreased OS; while, in non-referred patients, only age was significantly associated with OS (Table 4.4).

4.5. Discussion

Previous studies have suggested that the outcome of cancer patients is influenced by where they receive their care and treatment. Thus, outcome appears to be improved where caregivers have a specific interest in the disease in question and where large numbers of patients with the disease are managed. It has been suggested that this is related to cumulative expertise of support staff, better access to new agents through clinical trials and the better monitoring and management of comorbidities, toxicities and disease-specific complications (6–8,10,12). Moreover, these centres are more likely to develop treatment guidelines and to practice evidence-based medicine, factors that are associated with improved patient outcome (13,14). In the case of CLL, patients seen in a CLL clinic are more likely to be evaluated using molecular prognostic markers, to participate in clinical trials and to receive purine nucleoside-based therapy, rather than single alkylating agents or monoclonal antibodies (10).

Population studies show that the median age at diagnosis for CLL patients is 72 years (1), while in clinics the median age is much younger (64 years at the Mayo Clinic and 58 years at MD Anderson Cancer Center) (15,16). In the present study, the median age

Table 4.4: Multivariable analysis of overall survival

Multivariable Analysis (Overall Survival)			
Variable	All CLL Patients HR (95% CI)	Referred Patients HR (95% CI)	Non-Referred Patients HR (95% CI)
Referral Status			
Referred	1.00	-	-
Non-Referred	2.39 (1.5 – 3.82)*		
Age quartile			
<62 yr	1.00	1.00	1.00
62-70 yr	4.07 (1.52 – 10.86)*	4.33 (1.19 – 15.70)*	4.21 (0.91 – 19.54)
71-79 yr	5.44 (2.04 – 14.49)*	3.12 (0.80 – 12.28)	8.57 (1.94 – 37.87)*
≥80	10.76 (4.19 – 27.58)*	5.01 (1.16 – 21.68)*	16.38 (3.93 – 68.26)*
Gender			
Female	1.00	1.00	1.00
Male	1.61 (1.02 – 2.55)*	1.57 (0.67 – 3.65)	1.64 (0.95 – 2.84)
Tx-CLL			
Untreated	1.00	1.00	1.00
Treated	1.58 (0.96 – 2.60)	2.32 (1.06 – 5.08)*	1.16 (0.56 – 2.39)
Pre-CLL Cancer			
Without	1.00	1.00	1.00
With	1.85 (1.11 – 3.10)*	2.88 (1.07 – 7.74)*	1.78 (0.96 – 3.29)
Post-CLL Cancer			
Without	1.00	1.00	1.00
With	2.31 (1.14 – 4.70)*	2.66 (0.86 – 8.26)	1.98 (0.77 – 5.07)
Residence			
Rural	1.00	1.00	1.00
Urban	1.16 (0.76 – 1.77)	1.21 (0.56 – 2.59)	1.18 (0.50 – 3.18)

CI: Confidence Interval; CLL: Chronic Lymphocytic Leukemia; HR: Hazard Ratio; Tx: Treatment; yr: year

* $p < 0.05$

at diagnosis for referred patients was 68 years with a relatively normal age distribution, while non-referred patients had a bimodal age distribution with two equal age peaks at 60 and 80 years. In addition, while the median age of the CLL clinic is relatively close to the population median there are still a substantial number of elderly patients in the non-referred group indicating referral bias. This suggests that younger patients are being referred to the clinic for therapy; while elderly patients may not be referred as they are not considered fit enough to receive treatment. This would explain why the relative survival of the elderly patients has not changed over the past 20 years (4). While it might be expected that distance from the clinic would also influence referral practice, this did not appear to be a factor in our study.

Our findings would suggest that elderly patients are the primary beneficiaries of attending the CLL clinic. Thus, while patients, aged ≥62 years, had a poorer prognosis than younger patients, survival did not decrease further with advancing age. In contrast, there was a continued decrease in survival with age in non-referred patients. This difference may be related to the fact that older patients in the clinic were more likely to receive chemotherapy than non-referred patients. Alternatively, this may reflect referral bias, as elderly patients with comorbidities may not have been referred to the CLL clinic. However, the improved survival in the CLL clinic could also have been related to improved therapy and supportive care. Patient survival has improved in the past 15 years with the development of fludarabine and rituximab (17). Since rituximab must be infused in a supervise setting, it is possible that non-referred patients received less chemoimmunotherapy.

Shanafelt *et al.* (10) have shown that the survival of CLL patients seen at the Mayo Clinic was longer than patients in the Surveillance, Epidemiology and End Results (SEER) registry. Although this observation could also be attributed to referral bias, they also demonstrated a longer TTFT and OS for CLL patients seen by a hematologist specializing in CLL at the Mayo Clinic as compared to patients seen by other hematologists at the same centre. Thus, physician disease-specific expertise was an independent and important prognostic factor for CLL patients and referral bias did not explain the improved survival for patients treated by CLL-specialists.

Contrary to our previous report which evaluated patients diagnosed between 1998 and 2003 (1), we observed that gender did not influence survival in the present cohort, evaluating patients from 2007 to 2011. The improved survival of male patients may be attributed to the more effective treatments available today. In the previous study, most patients received single-agent chlorambucil or fludarabine, whereas in the latter cohort, most patients received rituximab with a fludarabine-containing regimen. Other investigators have also observed improved survival of male patients, as compared to females, over the past decade (3,4). As a result gender differences in survival has decreased over time.

For our patients in the clinic, a history of a previous cancer and requiring therapy for CLL were associated with a decreased OS. This is consistent with data from the MD Anderson Cancer Center, where having had a prior malignancy was associated with decreased survival (18). A postulated mechanism for this observation is that patients who had received prior chemotherapy or radiotherapy would be less able to tolerate

chemotherapy for their CLL. This was less of an issue for non-referred patients as they were less likely to receive treatment for their CLL.

It has previously been shown that geographic location is as an independent risk factor for survival in patients with lymphoma, with rural patients having an inferior outcome regardless of prognostic indicators (14). However, in our study, place of residence did not influence survival in CLL, either for referred or non-referred patients. This is likely because cancer patients in rural Manitoba are managed through local cancer clinics with treatment being overseen by the patient's hematologist from CCMB.

Despite our findings, in the present study we were expecting survival to be higher in the non-referred cohort as patients with more benign disease would not be referred. Moreover, it was thought likely that a number of the non-referred patients had MBL, whereas patients in the CLL clinic with MBL (15% of the population) were excluded from the analysis. Considering the benign nature of MBL, inclusion of these patients in the non-referred population should have improved survival in this group as compared to the referred patients. Moreover, some patients had been initially followed by general hematologists and family physicians but referred to the CLL clinic with disease progression. These patients were included in the referred cohort and therefore there are some crossovers between the two groups. Unfortunately data pertaining Rai staging, comorbidities and type of treatment was not available for the non-referred patients and therefore could not be used in this study.

In summary, this is the first study exploring the outcome of CLL patients in the population who were either referred or not referred to a CLL specific clinic in Canada. Patients seen in the specialized clinic tended to be younger than non-referred patients,

but correcting for age and gender, the OS was substantially higher for patients seen in the specialized clinic and this was primarily related to the improved survival in the elderly. Whether this is related to referral bias or to differences in practice is unclear.

Ongoing studies are determining whether differences in the survival of elderly CLL patients in the referred and non-referred cohorts can be attributable to differences in therapy, and why the elderly are not being referred to specialized clinics. These studies are particularly relevant at this time, as recently developed novel, non-toxic and highly effective antitumor agents could be tolerated by elderly patients with CLL.

This study exemplifies the difficulties in assessing the changes in disease outcome for the population as new therapies emerge. Similar studies should assess the impact of specialized clinics in other diseases and whether the development of guidelines can ensure that all patients receive appropriate therapy.

4.6. References

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Chapter Five

Characteristics of Chronic Lymphocytic Leukemia Patients at CancerCare Manitoba Referral-Based Clinic

5.1. Abstract

Two hundred and ninety three patients attended the CancerCare Manitoba (CCMB) referral-based clinic, between 2007 and 2011. Of the 293 patients, 15% had Monoclonal B Cell Lymphocytosis (MBL), 16% Small Lymphocytic Lymphoma (SLL) and 69% Chronic Lymphocytic Leukemia (CLL). The median age at the time of diagnosis was 68 years, and the male to female ratio was 1.7:1. Twenty seven percent of patients received treatment in a median time of 3.3 months after CLL diagnosis. Chlorambucil and a combination of fludarabine, cyclophosphamide and rituximab (FCR) were the most common regimens used. Twenty two percent of patients had a previous cancer diagnosis and 10% had a second malignancy diagnosis subsequent to CLL, with non-melanoma skin cancers (NMSCs) being the most common other malignancy.

5.2. Introduction

A previous population-based study of CLL in the province of Manitoba reported an age-adjusted incidence rate of 7.99/100,000 persons. This study investigated all CLL patients who were diagnosed in Manitoba between the years of 1998 and 2003. The median age at the time of CLL diagnosis in Manitoba was 71.5 years old and the male to female ratio was 1.3:1. A decreased five-year relative survival was observed in CLL patients and an association was established between advancing age and declining relative survival in CLL patients of both genders, with elderly male patients having the poorest relative survival (1).

This study was followed by a comparison of overall survival (OS) between referred and non-referred CLL patients to the CCMB CLL clinic in Manitoba (see Chapter 4). This study reported a greater than two-fold increased mortality in non-referred CLL patients seen in Manitoba between 2007 and 2011. This difference was related to a markedly improved survival of elderly referred patients who were more likely to receive chemotherapy than if they were not referred.

The CCMB CLL clinic is the only CLL clinic in the province of Manitoba. The purpose of this study is to describe the characteristics of patients seen at this clinic to determine if there were age- and gender-based differences that may explain the poor relative survival of elderly men in the population.

5.3. Methods

5.3.1. Patient Collection

All CLL, SLL and MBL patients diagnosed between January 1, 2007 and December 31, 2011 in the province of Manitoba who attended the CCMB referral-based

CLL clinic were included in this study (n: 293). CLL patients who were referred to the CCMB clinic upon diagnosis, as well as CLL patients who had been followed by other physicians initially and later referred to the CCMB clinic were included in this cohort. CLL diagnoses were based on upgraded 2008 iwCLL criteria (2). The Manitoba Cancer Registry provided information pertaining to date and cause of death (collected from death certificates), as well as International Classification of Diseases (ICD) codes for other malignancies. Gender, date of birth, date of diagnosis, Rai stage, laboratory tests, comorbidities, treatment data and cause of death were collected from the CCMB patient charts. Cause of death based on death certificates was only used if it was not available in patients' charts. Rai stage, and comorbidity data used in this study were all recorded at the time of CLL diagnosis. All data pertaining to MBL patients were collected from CCMB patient charts. *Immunoglobulin heavy chain variable region* (*IGHV*) gene mutational status was provided by the Manitoba Tumour Bank.

Ethics approval was obtained from the University of Manitoba Health Research Ethics Board and the CancerCare Manitoba Research Resources Impact Committee.

5.3.2. Laboratory Tests

Only the first laboratory tests performed after CLL diagnosis were considered in this study. If the first test was performed more than 120 days after the CLL diagnosis, it was considered as missing.

ß2-microglobulin was z-transformed due to utilization of two different measuring techniques in medical laboratories and the impracticality of conversion between the two units. To calculate z-scores, the mean value of ß2-microglobulin in our CLL cohort was measured, and multiples of standard deviation from the mean was

calculated for all patients. Patients were divided into five groups based on their z-score and creatinine value: (i) those with a ß2-microglobulin value below the mean, (ii) those with a ß2-microglobulin value that stood between the mean and +1 standard deviation away from the mean, (iii) those with a ß2-microglobulin value that stood between +1 and +2 standard deviations away from the mean, (iv) those with a ß2-microglobulin value that stood more than +2 standard deviations away from the mean, and finally (v) those with high creatinine regardless of their ß2-microglobulin level. This category was created because ß2-microglobulin is eliminated by the kidneys and individuals with poor kidney function and high creatinine usually have high ß2-microglobulin. In these patients, a high ß2-microglobulin does not necessarily reflect their CLL outcome.

CD38 and ZAP70 expressions were measured by flow cytometry at the Health Sciences Centre or St. Boniface Hospital medical laboratories. Patients were considered CD38-positive or ZAP70-positive if ≥20% of cells expressed CD38 or ZAP70. *IGHV* mutational status was determined by the Manitoba Tumour Bank. *IGHV* genes that acquired mutation in their sequence with ≥2% deviation from the germ line sequence were considered mutated (3,4). Laboratory test values are summarized in Table 5.1.

5.3.3. Statistical Analysis

The degree of correlation between continuous prognostic factors was estimated by Spearman's Rank correlation coefficient (ρ) due to a skewed distribution and presence of outliers in the data. Association between discrete prognostic factors was measured by chi-squared test. Direction of association between prognostic markers was assessed using Kandall Tau Rank test.

Table 5.1: Range of laboratory test values

Laboratory Test	Value	Unit
Creatinine	44 – 106 (Normal)	µmol/L
Creatinine Clearance	>60 (Normal)	Ml/min/1.7m^2
IgG	6.9 – 16.2 (Normal)	g/L
IgA	0.7 – 3.8 (Normal)	g/L
IgM	0.6 – 2.6 (Normal)	g/L
ß2-microglobulin	<3.5 (population mean) 3.5 – 6.9 (0 – 1 StDev) 7 – 12 (1 – 2 StDev) >12 (>2 StDev) <2,368 (population mean) 2,368 – 3,799 (0 – 1 StDev) 3,800 – 5,000 (1– 2 StDev) >5,000 (>2 StDev)	mg/L µg/L
CD38	Negative <20% Positive ≥20%	N/A
ZAP70	Negative <20% Positive ≥20%	N/A
IGHV Status	Unmutated <2% Mutated ≥2%	N/A

IGHV: *immunoglobulin heavy chain variable region*; StDev: standard deviation

A p-value <0.05 was considered to be statistically significant. Data management and descriptive analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

5.4. Results

5.4.1. Patient Population

From January 1, 2007 to December 31, 2011, 293 patients with CLL, SLL or MBL diagnoses were managed at the CCMB referral-based CLL clinic in Winnipeg. The median age of patients seen at CCMB clinic was 68 years (range 39 – 99 years). Patients were predominantly male, with a male to female ratio of 1.7:1. Of the 293 patients, 78 (27%) received treatment at a median time of 3.3 months after CLL diagnosis (range 0 – 53 months) (Table 5.2). The majority of treated patients received chlorambucil (32%) as the first line of therapy, followed by prednisone (23%), a fludarabine, cyclophosphamide, rituximab (FCR) regimen (17%), and a fludarabine, rituximab (FR) combination (14%) (Table 5.3). Eighteen patients (6%) received chemotherapy for other malignancies.

Of 293 patients, 65 (22%) had a pre-CLL cancer diagnosis with a male to female ratio of 2.6:1, 11 (4%) had a concurrent cancer diagnosis with a male to female ratio of 1.2:1 and 28 (10%) had a post-CLL cancer diagnosis with a male to female ratio of 2.1:1 (Table 5.2). The most common other malignancy among CLL patients was non-melanoma skin cancers (NMSC) (40% of other cancers), followed by cancers of prostate (13% of other cancers), breast (10% of other cancers) and digestive system (8% of other cancers) (Table 5.4).

Table 5.2: Characteristic features of patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at the CancerCare Manitoba referral-based clinic

Variable	Subset	All Clinic Patients
n		293
Follow up	Median	2.2 years
	Range	0 – 5.0
Age	Median	68 years
	Range	39 – 99
Gender	Male to Female Ratio	1.7:1
CLL-Treatment (%)	Treated	78 (27)
Age at First Treatment	Median	71 years
	Range	39 – 93
Time to First Treatment	Median	3.3 months
	Range	0 – 53
Other malignancies (%)	Pre-CLL	65 (22)
	Concurrent	11 (4)
	Post-CLL	28 (10)
Deceased (%)		29 (10)
Age at Death	Median	72 years
	Range	51 – 94
Survival	Median	10 months
(of Deceased Patients)	Range	0.6 – 44

Table 5.3: First line treatment in patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at the CancerCare Manitoba referral-based clinic

Agent	n (%)	Median Age at Tx Range
Prednisone	18 (23)	78 yr 49 – 93
Chlorambucil	25 (32)	78 yr 62 – 91
Cyclophosphamide	1 (1)	65 yr
Fludarabine	6 (8)	67 yr 63 – 70
FR	11 (14)	65 yr 39 – 80
FCR	13 (17)	59 yr 53 – 72
RCD	2 (3)	71.5 yr 65 – 78
FC	1 (1)	49 yr
CVPR	1 (1)	88 yr

FR: fludarabine, rituximab; FCR: fludarabine, cyclophosphamide, rituximab; FC: fludarabine, cyclophosphamide; CVPR: cyclophosphamide, vincristine, rituximab, prednisone; RCD: cyclophosphamide, rituximab, dexamethasone; mth: months; TTFT: time to first treatment; Tx: treatment; yr: years

Table 5.4: Types of other cancers in patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at the CancerCare Manitoba referral-based clinic

Type of Cancer	n
Pre-CLL Diagnoses	
NMSC	31
Prostate	11
Breast	7
Digestive Organs	5
Connective and Soft Tissue	3
Uterus	2
Bladder	2
Lung	1
Melanoma	1
Kidney	1
Thyroid	1
Total	**65**
Concurrent Diagnoses	
Larynx	2
Lung	2
Multiple Myeloma	2
Breast	2
Melanoma	1
Bladder	1
Diffuse B-Cell Lymphoma	1
Total	**11**
Post-CLL Diagnoses	
NMSC	10
Digestive Organs	3
Diffused B-Cell Lymphoma	3
Endometrium	2
Prostate	2
Chronic Myeloid Leukemia	2
Lung	1
Breast	1
Brain	1
Thyroid	1
Non-Hodgkin's Lymphoma	1
Unknown	1
Total	**28**

Of the 293 patients 238 (81%) had at least one comorbidity at the time of CLL diagnosis, while, 55 (19%) reported none. Twenty four percent of patients reported one, 20% reported two, 19% reported three and 18% reported four or more comorbidities at the time of diagnosis. The most common comorbidities among CLL patients were hypertension, hypercholesterolemia, arthritis and cardiovascular diseases (Table 5.5). Autoimmune diseases were reported in 38 (13%) patients, of which 24 (8%) were present at the time of CLL diagnosis. The most common autoimmune complications were immune thrombocytopenic purpura (ITP) (7%) and autoimmune hemolytic anemia (AIHA) (4%) (Table 5.6).

Of the 293 patients, 29 (10%) died during the follow up period (Table 5.2) with a male to female ratio of 2.6:1. The most common causes of death were CLL/SLL (55% of deceased patients), lung and skin cancers (melanoma and NMSCs) as well as myocardial infarctions, each contributing to 7% of causes of death (Table 5.7).

5.4.2. Primary Diagnoses

Of 293 patients who visited the CCMB CLL clinic, 44 (15%) had MBL, 48 (16%) had SLL and 201 (69%) had CLL. For MBL, SLL and CLL, the median age at diagnosis was 70, 68 and 67 years, the male to female ratios 1.3:1, 2:1 and 1.8:1, percentage treated 7%, 35% and 26%, median time to first treatment 36, 3.5 and 6.5 months and the percentage of deceased patients 2%, 13% and 11%, respectively (Table 5.8). In terms of laboratory tests, the majority of CLL, SLL and MBL patients exhibited negative expression of CD38 and ZAP70 (except SLL patients with 50% being CD38-positive), mutated *IGHV* status and normal serum creatinine, ß2-microglobulin, immunoglobulin G (IgG) and immunoglobulin A (IgA). However, approximately half of

Table 5.5: Comorbidities in patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at diagnosis at the CancerCare Manitoba referral-based clinic

Comorbidity	n (%)
Hypertension	132 (45)
Hypercholesterolemia	77 (26)
Arthritis	67 (23)
Cardiovascular Disease	66 (23)
Gastrointestinal Disease	54 (18)
Diabetes	52 (18)
Other endocrine Disorders	25 (9)
Anxiety/Depression	22 (7.5)
Benign Prostatic Hyperplasia	21 (11 male patients)
Kidney Disease	12 (4)
Asthma	11 (4)
Other pulmonary Disease	10 (3.5)
Others	46 (16)
Total	**595 (100)**

Table 5.6: Autoimmune disorders in patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at the CancerCare Manitoba referral-based clinic

Autoimmune disease subset	n (%)
AIHA/ITP	33 (11)
AI Neutropenia	2 (1)
Red Cell aplasia	1 (0.5)
Lupus inhibitor (with thrombosis)	1 (0.5)
Time of Diagnosis	**n (%)**
At CLL diagnosis	24 (8)
Before treatment	8 (3)
After treatment	5[1] (2)
Total	**38[2] (13)**

[1] After administration of chlorambucil (2), fludarabine (2) and FR (1)

[2] 12 patients received prednisone as first line of treatment due to AI diseases

AIHA: autoimmune hemolytic anemia; ITP: immune thrombocytopenic purpura

Table 5.7: Causes of death in patients with Chronic Lymphocytic Leukemia, Small Lymphocytic Leukemia and Monoclonal B Cell Lymphocytosis at the CancerCare Manitoba referral-based clinic

Cause of Death	n
CLL/SLL	16
Diffuse Large B-Cell Lymphoma (Richter's Transformation)	1
Lung Cancer	2
Skin Cancer	2
Prostate	1
Endometrium	1
Heart Attack	2
Stroke	1
Unknown Causes	3
Total	**29**

Table 5.8: Summary of characteristic features and prognostic markers of patients at CancerCare Manitoba referral-based clinic by diagnosis

Factor	Subset	MBL	SLL	CLL
n (%)		44 (15)	48 (16)	201 (69)
Age	Median	70 yr	68 yr	67 yr
	Range	49 – 93	39 – 89	39 – 99
Gender	M:F	1.3:1	2:1	1.8:1
Treated (%)	CLL	3 (7)	17 (35)	53 (26)
	Other Cancers	1 (2)	5 (10)	12 (6)
TTFT	Median	36 mth	3.5 mth	6.5 mth
	Range	9 – 41	0.5 – 46	0 – 53
Deceased (%)		1 (2)	6 (13)	22 (11)
LDT	<6 mth	7 (16)		16 (8)
	6 – 12 mth	2 (5)	N/A	36 (18)
	>12 mth	34 (77)		132 (66)
	Not determined	1 (2)		17 (8)
CD38	Positive	11 (25)	22 (46)	50 (25)
	Negative	25 (57)	22 (46)	133 (66)
	Not determined	8 (18)	4 (8)	18 (9)
ZAP70	Positive	9 (20)	9 (19)	48 (24)
	Negative	31 (70)	26 (54)	136 (68)
	Not determined	4 (10)	13 (27)	8 (17)
IGHV	Mutated	26 (59)	16 (33)	87 (43)
	Unmutated	6 (14)	14 (29)	62 (31)
	Not determined	12 (27)	18 (38)	52 (26)
Creatinine	Normal	35 (80)	40 (83)	171 (85)
	High	9 (20)	8 (17)	29 (14.5)
	Nor determined	0 (0)	0 (0)	1 (0.5)
β2-Microglobulin[1]	<Mean	30 (68)	26 (54)	135 (67)
	0 – 1 StDev	5 (11)	12 (25)	23 (11.5)
	1– 2 StDev	0 (0)	0 (0)	5 (2.5)
	>2 StDev	0 (0)	0 (0)	5 (2.5)
	High Creatinine	9 (21)	8 (17)	27 (13.5)
	Not determined	0 (0)	0 (0)	6 (3)

Table 5.8 (continued)

Factor	Subset	MBL	SLL	CLL
Immunoglobulin G	Low	9 (20)	10 (21)	49 (24)
	Normal	32 (73)	32 (67)	140 (70)
	High	2 (5)	5 (10)	11 (5.5)
	Not determined	1 (2)	1 (2)	1 (0.5)
Immunoglobulin A	Low	7 (16)	8 (17)	35 (17.5)
	Normal	35 (80)	36 (75)	157 (78)
	High	1 (2)	3 (6)	8 (4)
	Not determined	1 (2)	1 (2)	1 (0.5)
Immunoglobulin M	Low	21 (48)	21 (44)	111 (55)
	Normal	19 (43)	24 (50)	82 (41)
	High	3 (7)	2 (4)	7 (3.5)
	Not determined	1 (2)	1 (2)	1 (0.5)

[1]ß2-Microglobulin was z-transformed

CLL: Chronic Lymphocytic Leukemia; F: female; HR: hazard ratio; *IGHV*: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time; M: male; MBL: Monoclonal B Cell Lymphocytosis; mth: month; N/A: not applicable; SLL: Small Lymphocytic Lymphoma; StDev: standard deviation (away from the mean); TTFT: time to first treatment; yr: year

all patients had low immunoglobulin M (IgM) levels (Table 5.8).

Of the 201 CLL patients in the clinic, 161 (80%) were diagnosed with Rai stages 0 and I, and 40 (20%) were diagnosed with Rai stages II, III or IV. The median age at diagnosis was 66 and 70 years, the male to female ratios 1.4:1 and 5.7: 1, percentage of patients treated 15% and 73%, median time to first treatment 19.5 and 2.5 months and percentage of deceased patients 4% and 73% for the Rai stages 0/I and Rai stages II/III/IV, respectively. As expected, the majority of patients with Rai stages I and II were negative for CD38 and ZAP70 expressions, had mutated *IGHV* status and normal serum creatinine, ß2-microglobulin, IgG and IgA; while, the majority of patients with Rai stages II, III and IV had the negative prognostic markers such as positive CD38 expression, unmutated *IGHV* status and high serum ß2-microglobulin (Table 5.9.

5.4.3. Association between Prognostic Factors

Correlations between age and serum levels of creatinine, IgG, IgA and IgM were investigated (as continuous variables). Statistically significant yet clinically irrelevant correlations between age and creatinine levels as well as between age and IgG levels were detected (ρ: 0.19, p: 0.0006 and ρ: 0.14, p: 0.0437, respectively). When prognostic factors were investigated as discrete variables, associations were found between age and ß2-microglobulin, creatinine, creatinine clearance, immunoglobulin levels, comorbidities and history of other malignancies. Gender was associated with serum levels of IgA, ß2-microglobulin, creatinine and creatinine clearance. CD38 and *IGHV* mutational status were also shown to be associated with LDT and ZAP70 (Table 5.10).

Table 5.9: Summary of characteristic features and prognostic markers of Chronic Lymphocytic Leukemia patients by Rai stage

Factor	Subset	Rai 0 and I	Rai II, III and IV
n		161 (80)	40 (20)
Age	Median	66 yr	69.5 yr
	Range	42 – 99	39 – 87
Gender	M:F	1.4:1	5.7:1
Treated (%)	CLL	24 (15)	29 (73)
	Other Cancers	9 (6)	3 (8)
TTFT	Median	19.5 mth	2.5 mth
	Range	0 – 53	0 – 31
Deceased (%)		7 (4)	15 (38)
LDT	<6 mth	9 (5)	7 (17.5)
	6 – 12 mth	32 (20)	4 (10)
	>12 mth	112 (70)	20 (50)
	Not determined	8 (5)	9 (22.5)
CD38	Positive	30 (19)	20 (50)
	Negative	121 (75)	12 (30)
	Not determined	10 (6)	8 (20)
ZAP70	Positive	34 (21)	14 (35)
	Negative	115 (71.5)	21 (52.5)
	Not determined	12 (7.5)	5 (12.5)
IGHV	Mutated	80 (50)	7 (17.5)
	Unmutated	45 (28)	17 (42.5)
	Not determined	36 (22)	16 (40)
Creatinine	Normal	146 (90.5)	25 (62.5)
	High	14 (9)	15 (37.5)
	Not determined	1 (0.5)	0 (0)
β2-Microglobulin[1]	<Mean	126 (78)	9 (22.5)
	0 – 1 StDev	17 (11)	6 (15)
	1 – 2 StDev	1 (0.5)	4 (10)
	>2 StDev	1 (0.5)	4 (10)
	High Creatinine	14 (9)	13 (32.5)
	Not determined	2 (1)	4 (10)

all patients had low immunoglobulin M (IgM) levels (Table 5.8).

Of the 201 CLL patients in the clinic, 161 (80%) were diagnosed with Rai stages 0 and I, and 40 (20%) were diagnosed with Rai stages II, III or IV. The median age at diagnosis was 66 and 70 years, the male to female ratios 1.4:1 and 5.7: 1, percentage of patients treated 15% and 73%, median time to first treatment 19.5 and 2.5 months and percentage of deceased patients 4% and 73% for the Rai stages 0/I and Rai stages II/III/IV, respectively. As expected, the majority of patients with Rai stages I and II were negative for CD38 and ZAP70 expressions, had mutated *IGHV* status and normal serum creatinine, ß2-microglobulin, IgG and IgA; while, the majority of patients with Rai stages II, III and IV had the negative prognostic markers such as positive CD38 expression, unmutated *IGHV* status and high serum ß2-microglobulin (Table 5.9.

5.4.4. Association between Prognostic Factors

Correlations between age and serum levels of creatinine, IgG, IgA and IgM were investigated (as continuous variables). Statistically significant yet clinically irrelevant correlations between age and creatinine levels as well as between age and IgG levels were detected (ρ: 0.19, p: 0.0006 and ρ: 0.14, p: 0.0437, respectively). When prognostic factors were investigated as discrete variables, associations were found between age and ß2-microglobulin, creatinine, creatinine clearance, immunoglobulin levels, comorbidities and history of other malignancies. Gender was associated with serum levels of IgA, ß2-microglobulin, creatinine and creatinine clearance. CD38 and *IGHV* mutational status were also shown to be associated with LDT and ZAP70 (Table 5.10).

Table 5.9 **(continued)**

Factor	Subset	Rai 0 and I	Rai II, III and IV
Immunoglobulin G	Low	32 (20)	17 (42.5)
	Normal	123 (76.5)	17 (42.5)
	High	5 (3)	6 (15)
	Not determined	1 (0.5)	0 (0)
Immunoglobulin A	Low	22 (13.5)	13 (32.5)
	Normal	132 (82)	25 (62.5)
	High	6 (4)	2 (5)
	Not determined	1 (0.5)	0 (0)
Immunoglobulin M	Low	88 (54.5)	23 (57.5)
	Normal	69 (43)	13 (32.5)
	High	3 (2)	4 (10)
	Not determined	1 (0.5)	0 (0)

[1]ß2-Microglobulin was z-transformed

CLL: Chronic Lymphocytic Leukemia; F: female; HR: hazard ratio; *IGHV*: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time; M: male; mth: month; StDev: standard deviation (away from the mean); TTFT: time to first treatment; yr: year

Table 5.10: Association between prognostic factors as determined by χ^2 and Kendall Tau Rank tests

Variable 1	Variable 2	Direction	p-Value
Older Age	IgA	Normal	0.0006
	ß2-microglobulin	Normal	0.0019
	Creatinine	Normal	0.0035
	Creatinine Clearance	Normal	<0.0001
	Comorbidities	Low	0.0009
	History of Malignancies	Negative	<0.0001
Male Gender	IgA	Normal	0.0006
	ß2-microglobulin	High	0.0122
	Creatinine	High	0.0003
High ß2-microglobulin	Age	Younger	0.0019
	Gender	Male	0.0122
	IgA	Low	0.0220
	CD38	Positive	0.0005
	Creatinine Clearance	Low	<0.0001
	Comorbidities	High	0.0066
Positive CD38	IgA	Low/High	0.0333
	LDT	Short	0.0004
	ZAP70	Positive	<0.0001
	ß2-microglobulin	High	0.0005
	Creatinine	High	0.0209
High Creatinine	Age	Younger	0.0035
	Gender	Male	0.0003
	IgG	High	0.0129
	CD38	Expression	0.0209
Low Creatinine Clearance	Age	Younger	<0.0001
	Gender	Female	0.0030
	IgA	High/Low	<0.0001
	ß2-microglobulin	High	<0.0001
	Comorbidities	High	0.0404
	History of Malignancies	High	0.0228

Table 5.10 (continued)

Variable 1	Variable 2	Direction	p-Value
Low/High IgA	Age	Younger	0.0006
	ß2-microglobulin	High	0.0220
	CD38	Positive	0.0333
	Creatinine Clearance	High	<0.0001
	History of Malignancies	Positive	0.0030
Low/High IgG	LDT	Short	0.0494
	Creatinine	High	0.0129
Short LDT	IgG	Low	0.0494
	CD38	Positive	0.0004
	IGHV Status	Unmutated	0.0001
Positive ZAP70	CD38	Positive	<0.0001
	IGHV Status	Unmutated	<0.0001
Unmutated *IGHV* Status	LDT	Shorter	0.0001
	ZAP70	Positive	<0.0001
Comorbidities	Age	Younger	0.0009
	ß2-microglobulin	High	0.0066
	Creatinine Clearance	Low	0.0404
History of Malignancies	Age	Younger	<0.0001
	Creatinine Clearance	Low	0.0228
	IgA	High	0.0030

IGHV: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time

5.5. Discussion

We observed a median age at diagnosis of 68 years and a male to female ratio of 1.7:1 in the CCMB referral-based CLL clinic population (Table 5.2). As had been observed in other referral-based centres, the median age at our CLL clinic was also slightly younger than the median age of 71 years that was previously reported for the CLL population of Manitoba (1,5). The median age of CLL patients at the time of diagnosis was reported to be 64 years at the Mayo Clinic and 58 years at MD Anderson Cancer Center (6,7). Also it appeared that male patients were more likely to be referred to specialized clinics as the male to female ratio for the CLL population of Manitoba as a whole was 1.3:1 (1,5).

The most common comorbidities in our cohort were hypertension (45%), high cholesterol (26%), arthritis (23%) and cardiovascular diseases (23%). Eighty one percent of patients had at least one comorbidity at the time of CLL diagnosis (not including other malignancies) (Table 5.5). Similar to our findings, the Mayo clinic reported at least one comorbidity in 89% of their CLL patients, with 40% having a major comorbidity, including cardiopulmonary or cardiovascular disease, diabetes or other cancers). Similarly, they reported hypertension, joint diseases and hyperlipidemia as the most common comorbidities in their CLL patients (8). It is noteworthy that only the fittest patients are qualified for treatments and the degree of fitness is determined by Cumulative Index Rating Scale (CIRS) that takes into account the number and type of comorbidities to assign a fitness score to each patient (9,10). Therefore comorbidities are important determining factors both for prognosis and management in CLL patients.

In our clinic, 22% of patients had a prior cancer diagnosis at the time of the CLL diagnosis, while the MD Anderson Cancer Center and the Mayo clinic reported 16% and 15%, respectively. The higher rates of previous cancers observed in our clinic is likely because our report included NMSC (10% of the total cohort and 48% of all previous cancers), while this malignancy is usually not reported by other centres (8,11). In addition, the age of our patient population is older than in these other centres. The most common other malignancies in our CLL patients (diagnosed pre-CLL, concurrent to CLL or post-CLL) after NMSC were cancers of breast, prostate and digestive systems (Table 5.4). This is similar to what was previously observed in the CLL population of Manitoba as a whole (5). Autoimmune cytopenias were reported in 13% of our CLL patients, with ITP and AIHA diagnosed for 7% and 4% of our CLL cohort, respectively (Table 5.6). This is in agreement with the general observation of 5 – 10% ITP and AIHA in CLL patients reported by other studies (12–14). Speculatively, patients with CLL and autoimmune disorders may have a reduced incidence of second malignancies as they have a hyperactive immune system. However, since patients with ITP and AIHA are treated with immune suppressants, it is likely that CLL patients with autoimmune disorders will also have an increased risk of second malignancies.

Associations were observed between age, ß2-microglobulin, creatinine, creatinine clearance, immunoglobulins as well as number of comorbidities and a history of other malignancies (Table 5.10). Serum creatinine and creatinine clearance reflect kidney function, and the ß2-microglobulin level is increased with reduced renal function; higher levels of creatinine and ß2-microglobulin are observed with a lower creatinine clearance (15,16). Furthermore, high levels of ß2-microglobulin and

creatinine have been shown to be associated with a history of previous cancers (11). This observation may be attributed to the fact that elderly patients are more likely to have a preceding cancer. ß2-microglobulin has been shown to increase with age and the plasma levels of inflammatory cytokines, both of which are associated with poor survival in CLL (17). Based on these facts, we expected to see an increased number of comorbidities, higher ß2-microglobulin and creatinine levels, lower creatinine clearance and a greater number of previous cancers in older patients. To the contrary, in this cohort, older patients had better general health compared to the younger patients. This likely explains the improved survival of elderly patients in the referred population, as discussed in the previous chapter (see Chapter 4), as these patients could tolerate chemoimmunotherapy (Table 5.3). The better general health in the older patients may be attributed to a referral bias where healthier elderly individuals are more likely to be referred to the clinic. However, we did demonstrate that male patients were more likely than females to have higher plasma levels of creatinine and ß2-microglobulin. These features suggest that men have more aggressive CLL than women, but further patients need to be studied to confirm this point.

There were also associations observed between increased expressions of CD38 and ZAP70 and shorter LDT, high ß2-microglobulin and high/low IgA (Table 5.10). The fact that CD38 measures cell proliferation explains the association of CD38 with LDT, which is also a measure of cell proliferation. Increased proliferation may consequently lead to low levels of IgA due to tumour burden in CLL patients (18–22). The association between CD38 or ZAP70 with ß2-microglobulin has been previously described in the literature (23,24). CD38 and ZAP70 are known to be functionally

linked (25) and ß2-microglobulin reflects tumour burden and the aggressiveness of CLL (26,27).

IGHV gene status was also associated with ZAP70 and LDT. It was found that unmutated *IGHV* status was associated with ZAP70 positivity and a short LDT. The association of ZAP70 and CD38 expressions with *IGHV* mutational status has been previously described (28–31). Association of *IGHV* and CD38 (both measures of cell proliferation) may manifest as shorter LDT in *IGHV* mutated patients.

The limitations of this study include missing laboratory tests, particularly *IGHV* mutational status which is only measured for patients that donated blood to the Manitoba Tumour Bank. In addition, fluorescent *in situ* hybridization assay (FISH) studies were not carried out in these patients as FISH is only performed at our centre for patients initiating treatment.

In summary, this study is unique as it is the first epidemiological study to investigate CLL patients referred to a specialized clinic in Canada using the updated iwCLL 2008 diagnostic criteria to distinguish MBL from CLL and SLL (2). The study showed that approximately 15% of referred patients have MBL, while 16% have SLL. Moreover, with a median follow up time of two years, one-third of patients required therapy, with the median time to treatment being three months. In addition, 10% of patients died within a median time of 10 months after the CLL diagnosis with CLL being the main cause of death. Surprisingly, elderly patients in the clinic were relatively fit with fewer comorbidities and lower levels of creatinine and β2-microglobulin than seen in younger patients. Thus, survival for older individuals in the clinic was only slightly poorer than for younger patients.

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Chapter Six

Prediction of Overall and Treatment-Free Survival in Chronic Lymphocytic Leukemia Patients at CancerCare Manitoba Referral-Based Clinic

6.1. Abstract

Two hundred and ninety three new patients were seen at the chronic lymphocytic leukemia (CLL) Clinic at CancerCare Manitoba (CCMB) over the five-year period, from 2007 to 2011. Of the 293 patients 69% had Chronic Lymphocytic Leukemia (CLL) and were the subject of this study (n: 201).

Overall survival (OS) was investigated using Kaplan-Meier and Cox proportional hazard regression models. Treatment-free survival (TFS) was estimated using a competing risk regression model and considering death before treatment as a competing risk.

Rai stage, lymphocyte doubling time (LDT), ß2-microglobulin and IgA levels and another cancer diagnosis were independent predictors of OS. Rai stage was the only independent predictor of TFS when all patients were examined. However, in patients with low Rai stage, high ß2-microglobulin, unmutated *IGHV* gene and low IgA were independent predictors of shorter TFS.

6.2. Introduction

Traditionally, the prognosis for Chronic Lymphocytic Leukemia (CLL) patients has been solely based on the clinical stage of the disease (1,2). A population-based study of CLL patients in Manitoba more than 30 years ago showed a significant difference between patients with Rai stage 0, Rai stages I and II, and Rai stages III and IV in terms of survival (3). However, the investigators failed to find any significant difference between Rai stages I and II and between Rai stages III and IV. These findings were confirmed by later studies (4–6).

The median survival in CLL is 10 years, but the disease is highly heterogeneous with some patients having an indolent course while others having a rapid downhill course over several years (7). It has been shown that treatment of early asymptomatic CLL patients with chlorambucil offers no benefit in survival (8). However, whether stratifying patients according to risk factors and using immunochemotherapy only for the high-risk patients can improve survival is still unknown. Answering this question requires the identification of robust prognostic markers to predict disease aggressiveness and survival, which will allow only those patients with poor prognosis to be treated at diagnosis.

Staging systems reflect overall tumour burden and allow an accurate prognosis. However, most patients today are diagnosed with early stage disease and staging systems do not distinguish between early stage patients who are likely to progress and may benefit from earlier more aggressive treatment, and those with stable indolent disease who do not require treatment for many years. A need for an accurate prognostic marker to distinguish different risk categories within each Rai stage has long been

recognized by clinicians. Major advances were achieved by identification of cytogenetic aberrations (9) and *immunoglobulin heavy chain variable region* (*IGHV*) gene mutational status (10,11) as useful markers to predict outcome and response to therapy. These breakthrough markers were complemented by other prognostic factors such as age, gender, lymphocyte doubling time (LDT) (12,13), serum levels of ß2-microglobulin and creatinine (14,15) and cellular expressions of CD38 (16) and ZAP70 (17,18).

In this study, the utility of prognostic factors in terms of treatment-free survival (TFS) and overall survival (OS) were evaluated in CLL patients at the CancerCare Manitoba (CCMB) referral-based CLL clinic.

6.3. Methods

6.3.1. Patient Collection

All CLL patients diagnosed between January 1, 2007 and December 31, 2011 in the province of Manitoba who attended the CCMB referral-based CLL clinic were included in this study (n: 201). OS analysis was performed on all CLL patients with Rai stages I to IV (n: 201), while TFS analysis was performed on CLL patients with Rai stages I and II (n: 161). Patient selection and data collection criteria were described in detail in the previous chapter (see Chapter 5).

Ethics approval was obtained from the University of Manitoba Health Research Ethics Board and the CancerCare Manitoba Research Resources Impact Committee.

6.3.2. Laboratory Tests

Laboratory tests are described in detail in Chapter 5.

LDT was divided into three categories, <6 months, 6 – 12 months and >12 months, for OS analysis, and into two groups, <12 months and ≥12 months, for TFS analysis due to a smaller cohort.

6.3.3. Statistical Analysis

OS was calculated as the time between date of diagnosis and date of death or end of study (December 31, 2011). OS plots were created using Kaplan-Meier methods and compared between groups by log-rank test. Hazard ratios (HR) were estimated using univariable and multivariable Cox proportional hazard regression models.

TFS was calculated as the time between date of diagnosis and date of first chemotherapy administrated to patients, date of death, or end of study (December 31, 2011). Sub-distribution hazard ratios (SHR) were calculated using a competing risk regression model, considering death before treatment a competing risk.

Kolmogorov-Smirnov test was performed to detect clustering in the data. For this, the expected distributions of negative markers (in both deceased and treated cohorts) were determined using Monte Carlo methods and were compared to the observed distributions.

A p-value of <0.05 was considered to be statistically significant. Data management and analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Competing risk regression models and plots were created using Stata 11.2 (Stata Corp., College Station, TX, USA).

6.4. Results

6.4.1. Overall Survival

6.4.1.1. Univariable Analyses

In a univariable model age was a predictor of survival as a continuous variable (HR 1.05, 95% CI 1.01 – 1.10). When CLL patients were divided by median age, mortality was 2.4-times higher in patients ≥68 years (95% CI 0.99 – 5.35) (Table 6.1, Figure 6.1).

Rai stage was the strongest predictor of OS in CLL patients. Patients with Rai stages II, III and IV had a significantly shorter OS compared with patients with Rai stages 0 and I (Table 6.1, Figure 6.2).

Patients with a LDT of <6 months had a 7-times increased risk of mortality compared to patients with LDT of 6–12 and >12 months (95% CI 2.19 – 21.85). However, patients with LDT of 6–12 and >12 months had a similar outcome in terms of OS (Table 6.1, Figure 6.3).

CD38 expression was associated with a decrease in overall survival and 2.6-fold increased risk of mortality (95% CI 1.03 – 6.58) (Table 6.1, Figure 6.4).

Creatinine and creatinine clearance also showed a statistically significant association with OS. CLL patients with high creatinine and low creatinine clearance had a shorter OS (HR 3.45, 95% CI 1.39– 8.35 and HR 3.08, 95% CI 1.21 – 7.82, for patients with high creatinine and low creatinine clearance, respectively) (Table 6.1, Figure 6.5, Figure 6.6).

Table 6.1: Univariable analysis of overall survival

Univariable Analysis (Overall Survival)			
Variable	**Subset**	**HR**	**95% CI**
Gender	Female	1.00	-
	Male	1.66	0.65 – 4.26
Age	<68 yr	1.00	-
	≥68 yr	2.43	0.99 – 5.95
Age	Continuous	1.05*	1.01 – 1.10
LDT	>12 mth	1.00	-
	6 – 12 mth	1.08	0.22 – 5.19
	<6 mth	6.92*	2.19 – 21.85
Stage	Rai 0	1.00	-
	Rai I	0.41	0.05 – 3.43
	Rai II	6.67*	2.02 – 22.17
	Rai IIII	12.54*	4.03 – 39.02
	Rai IV	11.02*	3.10 – 39.15
Chemotherapy	Untreated	1.00	-
(CLL)	Treated	1.37	0.57 – 3.27
Chemotherapy	Untreated	1.00	-
(Other Cancers)	Treated	2.16	0.64 – 7.31
Previous Cancers	Without history	1.00	-
	With history	3.92*	1.50 – 10.23
Other cancers	No other cancer	1.00	-
(Pre-/Post-CLL)	With other cancer	2.55*	1.07 – 6.08
CD 38	Negative	1.00	-
	Positive	2.60*	1.03 – 6.58
ZAP70	Negative	1.00	-
	Positive	1.27	0.44 – 3.66
IGHV	Mutated	1.00	-
	Unmutated	1.30	0.38 – 4.53
Creatinine	Normal	1.00	-
	High	3.45*	1.39 – 8.55
Creatinine Clearance	≥60	1.00	-
	<60	3.08*	1.21 – 7.82

Table 6.1 (continued)

Univariable Analysis (Overall Survival)			
Variable	**Subset**	**HR**	**95% CI**
β2-Microglobulin[1]	<Mean	1.00	-
	High Creatinine	6.84	2.06 – 22.43
	0 – 1 StDev	5.15*	1.38 – 19.21
	1– 2 StDev	11.61*	2.24 – 60.13
	>2 StDev	9.43*	1.82 – 48.79
Immunoglobulin G	Normal	1.00	-
	Low	0.81	0.27 – 2.47
	High	4.47*	1.47 – 13.62
Immunoglobulin A	Normal	1.00	-
	Low	1.73	0.62 – 4.80
	High	4.84*	1.39 – 16.86
Immunoglobulin M	Normal	1.00	-
	Low	1.71	0.65 – 4.50
	High	8.44*	2.09 – 34.16
Comorbidity	<3	1.00	-
	≥3	3.13*	1.31 – 7.46
BMI	Normal	1.00	-
	Overweight/Obese	1.91	0.80 – 4.55

* Statistical significance with $p < 0.05$

[1]ß2-Microglobulin was z-transformed

BMI: body mass index; CI: confidence interval; CLL: Chronic Lymphocytic Leukemia; F: female; HR: hazard ratio; *IGHV*: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time; M: male; mth: month; StDev: standard deviation (away from the mean); TTFT: time to first treatment; yr: year

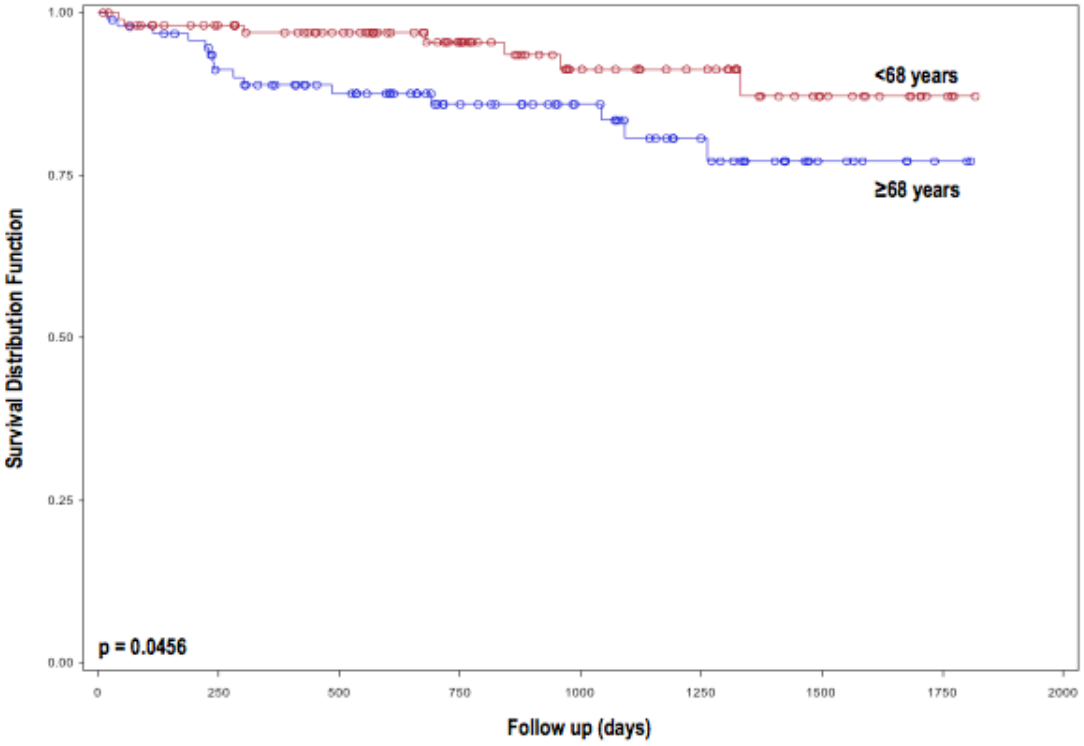

Figure 6.1: Kaplan-Meier plot of overall survival based on age

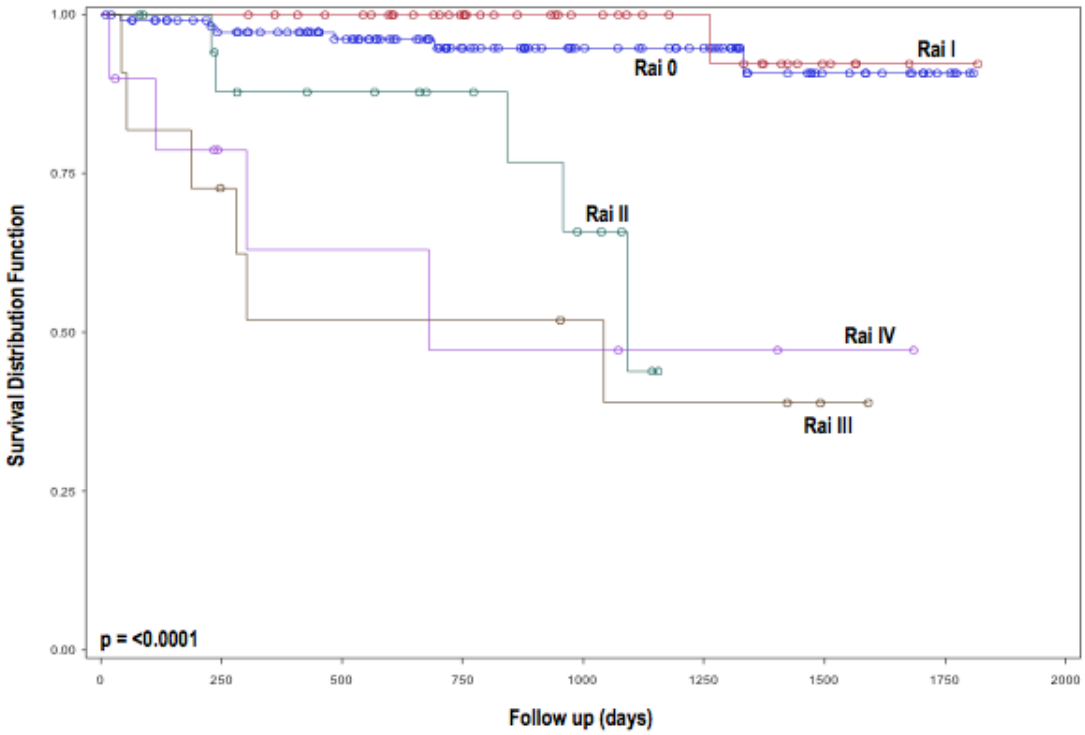

Figure 6.2: Kaplan-Meier plot of overall survival based on Rai stage

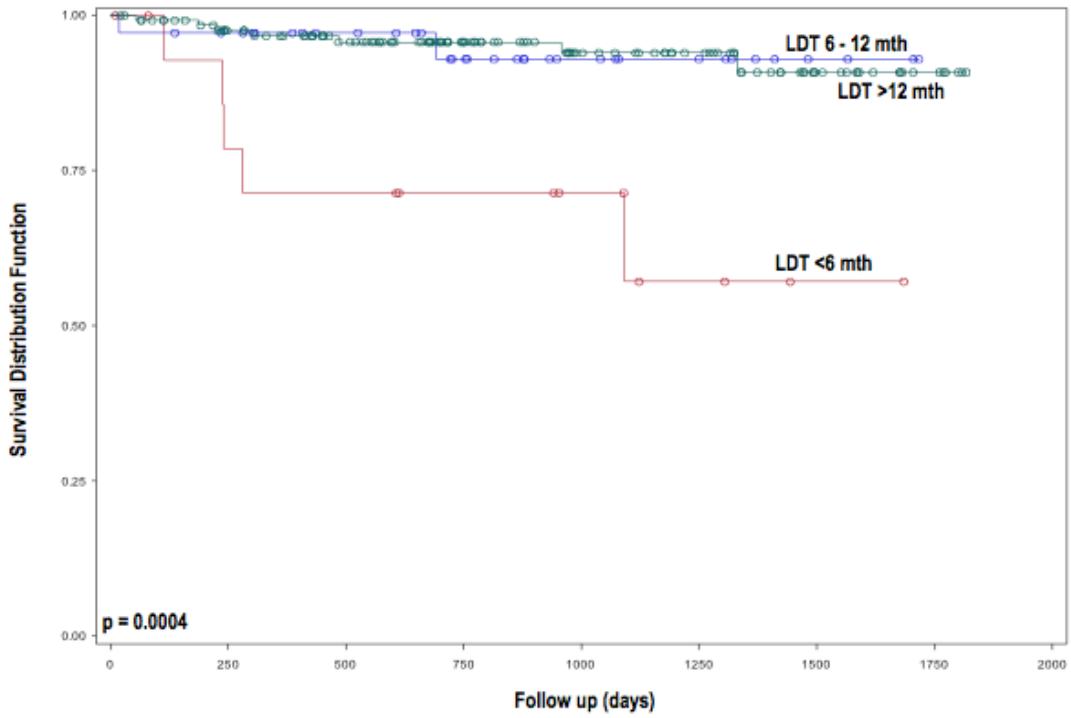

Figure 6.3: Kaplan-Meier plot of overall survival based on lymphocyte doubling time (LDT)

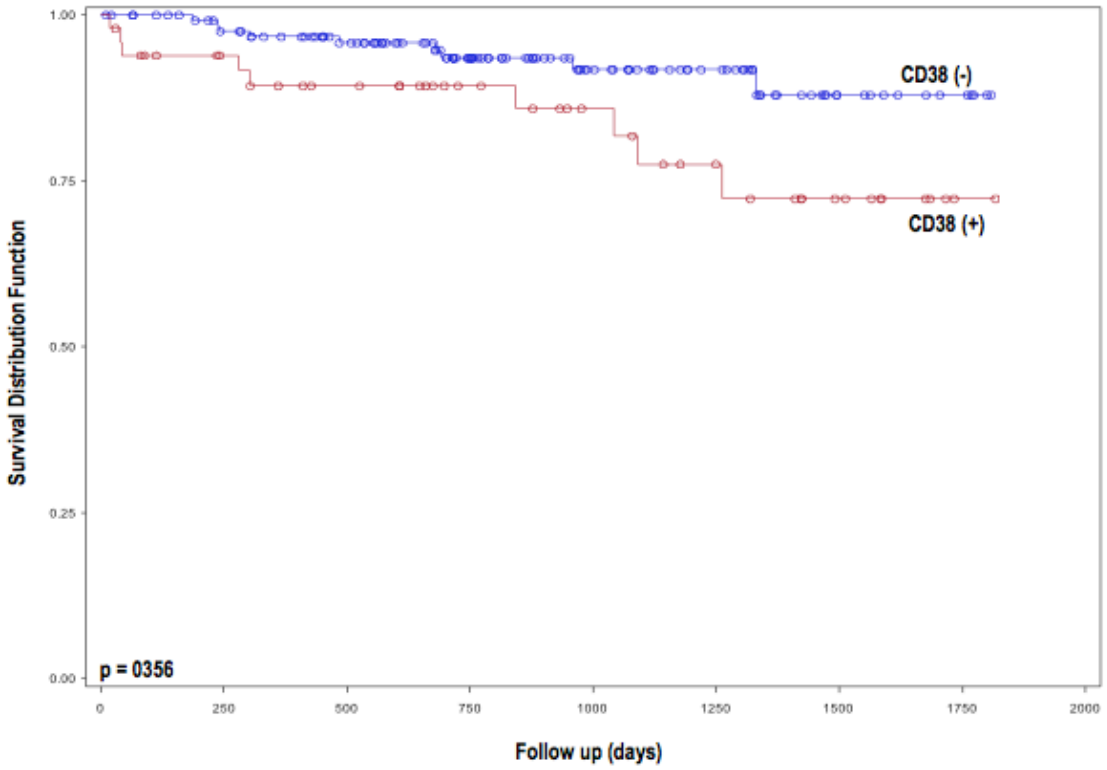

Figure 6.4: Kaplan-Meier plot of overall survival based on CD38 expression

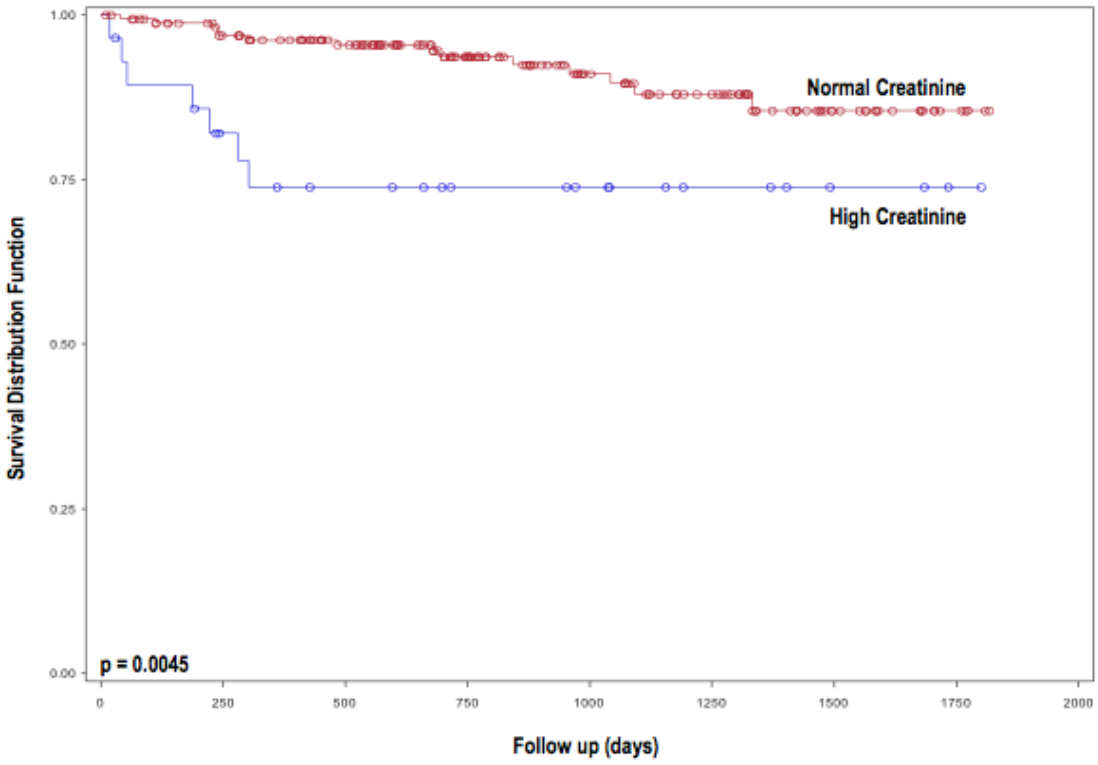

Figure 6.5: Kaplan-Meier plot of overall survival based on plasma level of creatinine

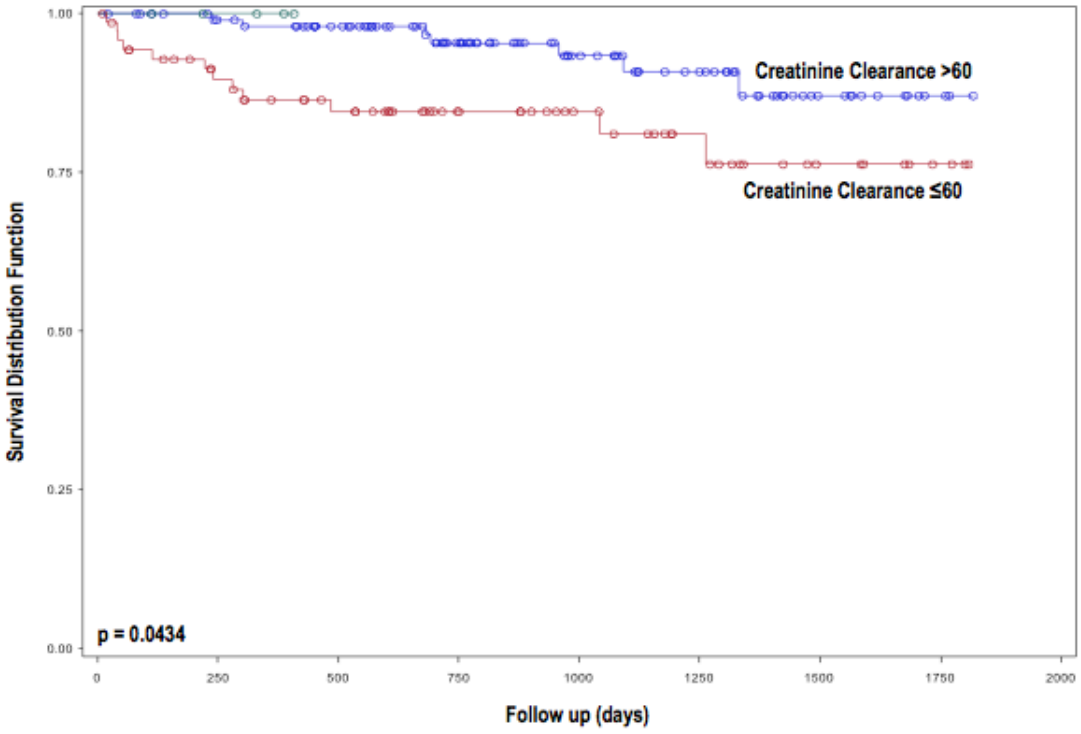

Figure 6.6: Kaplan-Meier plot of overall survival based on creatinine clearance

ß2-microglobulin was one of the strongest predictor of OS in CLL patients. Patients with ß2-microglobulin levels that were higher than the mean, as well as patients with both high creatinine and high ß2-microglobulin, had a significantly shorter OS as compared with patients with ß2-microglobulin lower than the mean (Table 6.1, Figure 6.7).

High IgG, IgA and IgM were also associated with a decreased OS, while patients with low and normal immunoglobulin levels had a similar outcome in terms of OS (Table 6.1, Figure 6.8, Figure 6.9, Figure 6.10).

Interestingly, patients who had a history of previous cancers at the time of CLL diagnosis had a high mortality, almost 4-fold greater than those without a previous malignancy (95% CI 1.50 – 10.23) (Table 6.1, Figure 6.11). Patients with another cancer diagnosed before CLL, concurrent with CLL or post-CLL, had a 2.5-fold increased mortality compared to patients without another cancer (95% CI 1.07 – 6.08) (Table 6.1, Figure 6.12).

Patients with three or more comorbidities had 3-fold increased risk of death compared with patients with less than three or no comorbidity (95% CI 1.31 – 7.46) (Table 6.1, Figure 6.13).

Gender, requiring chemotherapy (either for CLL or other cancers), ZAP70 expression, *IGHV* gene mutational status and mass body index (MBI) did not show any statistically significant predictive value for OS in CLL patients as single variables (Table 6.1).

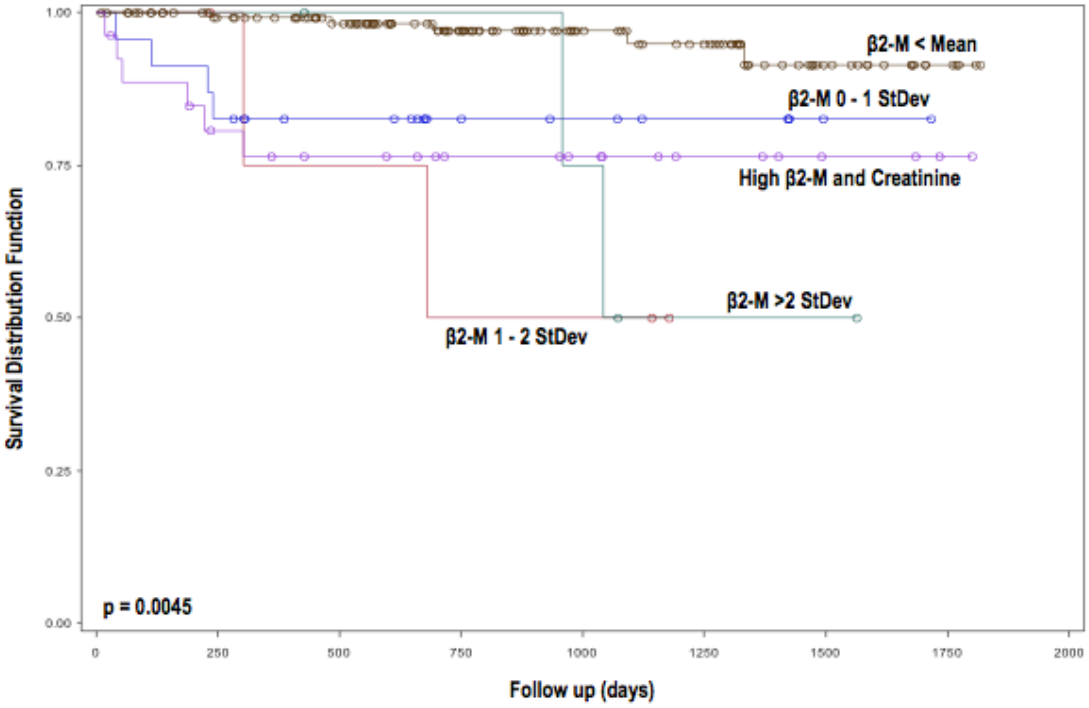

Figure 6.7: Kaplan-Meier plot of overall survival based on plasma level of ß2-microglobulin (ß2-microglobulin is z-transformed)

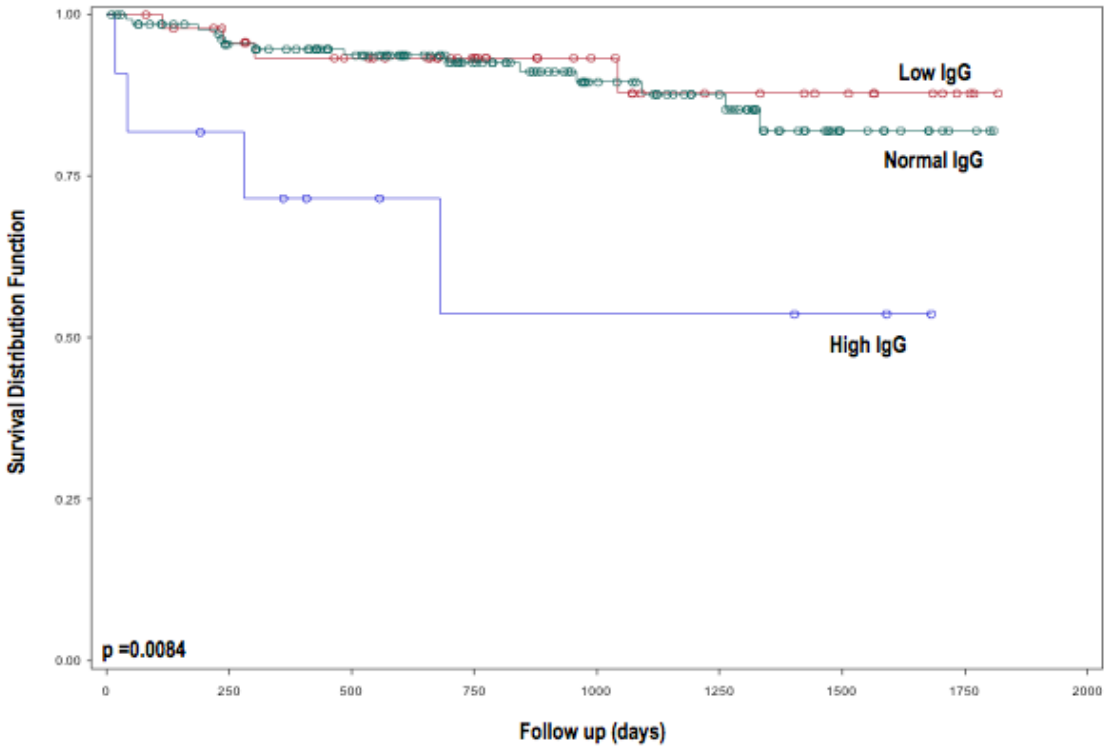

Figure 6.8: Kaplan-Meier plot of overall survival based on plasma IgG level

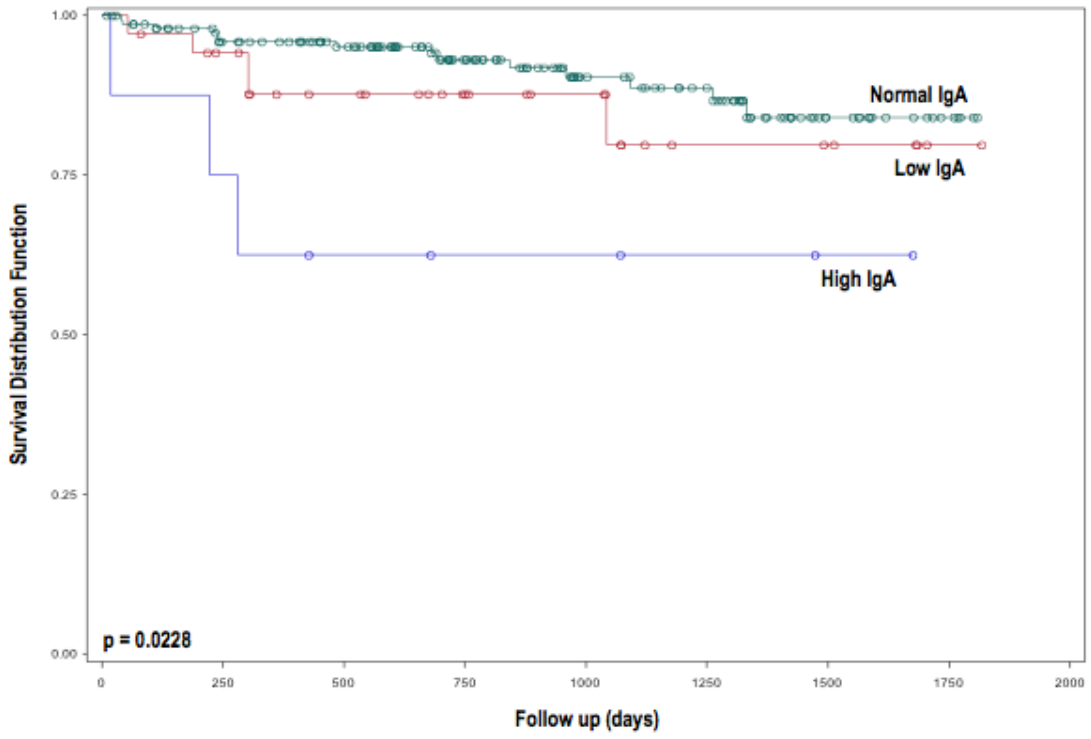

Figure 6.9: Kaplan-Meier plot of overall survival based on plasma IgA level

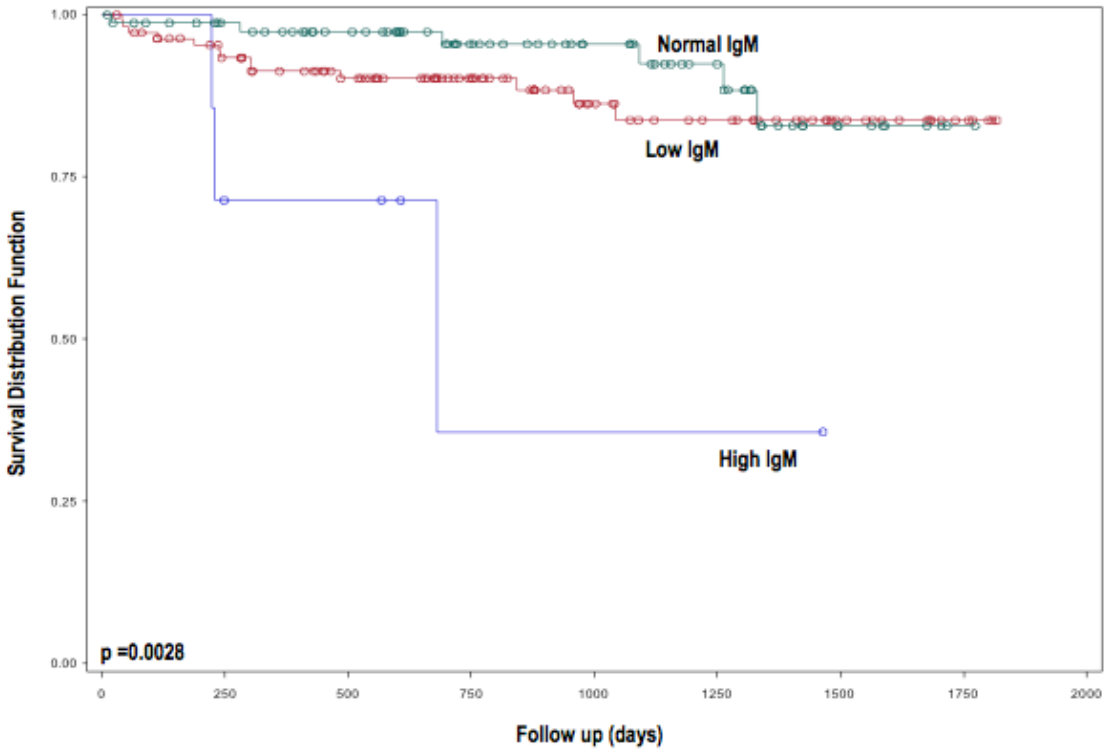

Figure 6.10: Kaplan-Meier plot of overall survival based on plasma IgM level

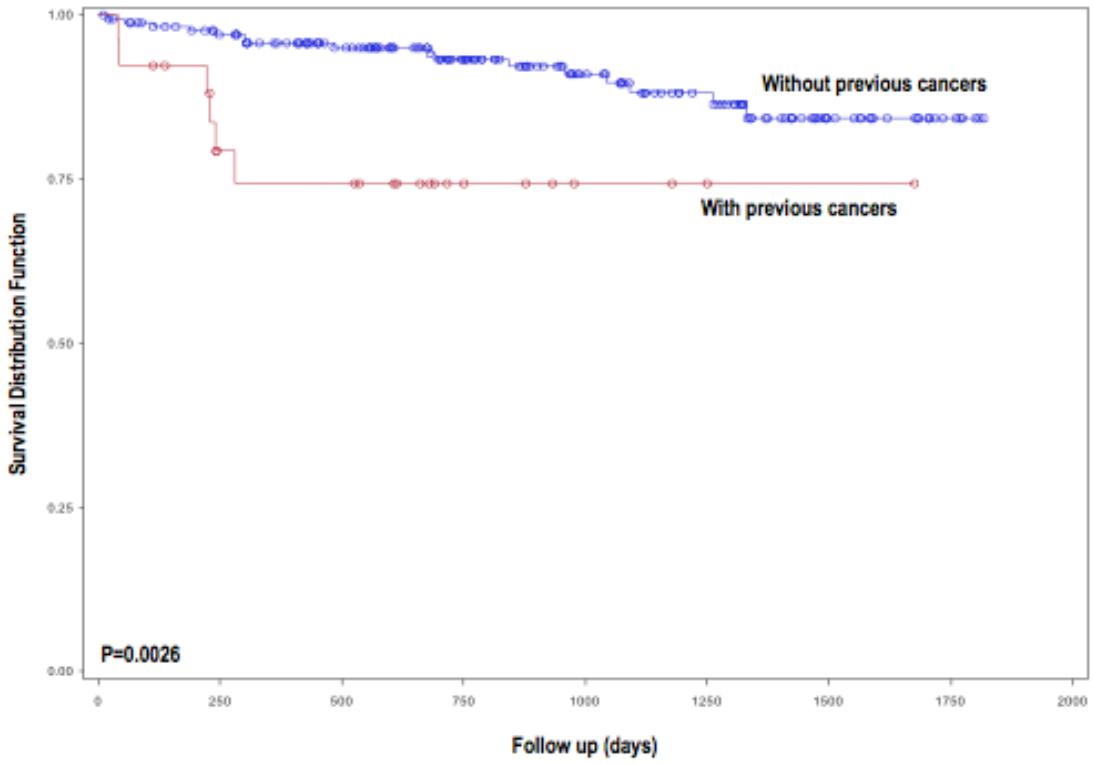

Figure 6.11: Kaplan-Meier plot of overall survival based on history of previous malignancies

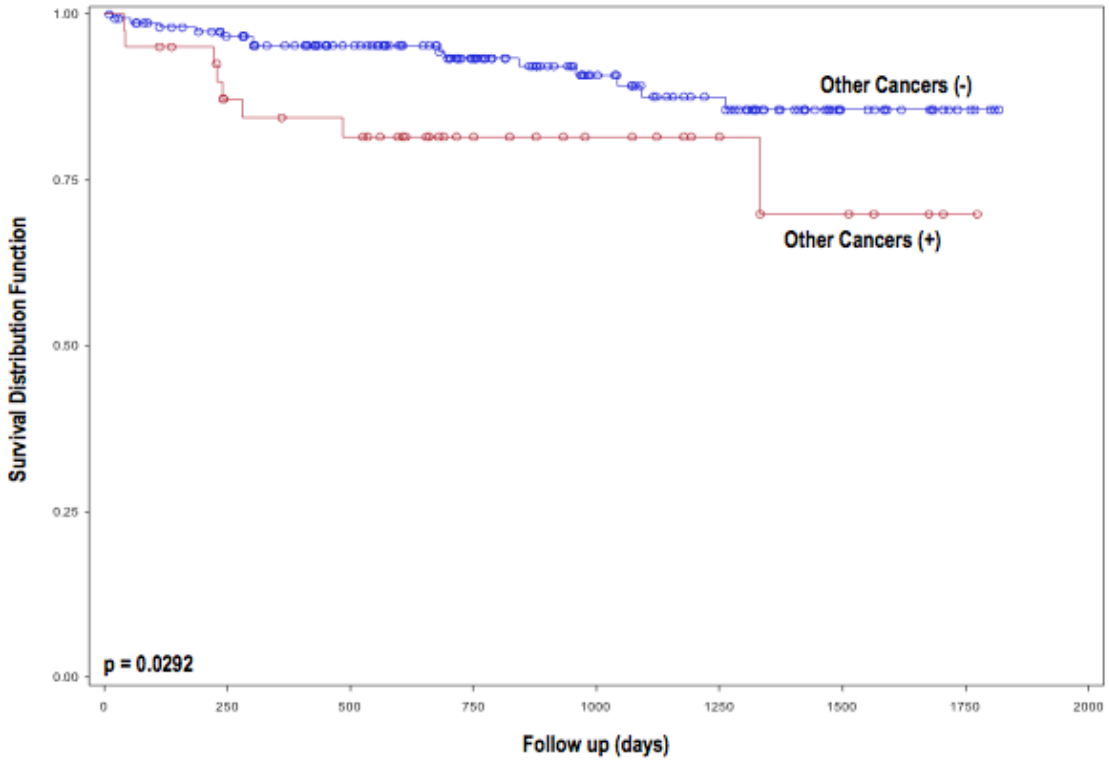

Figure 6.12: Kaplan-Meier plot of overall survival based on other cancer diagnoses

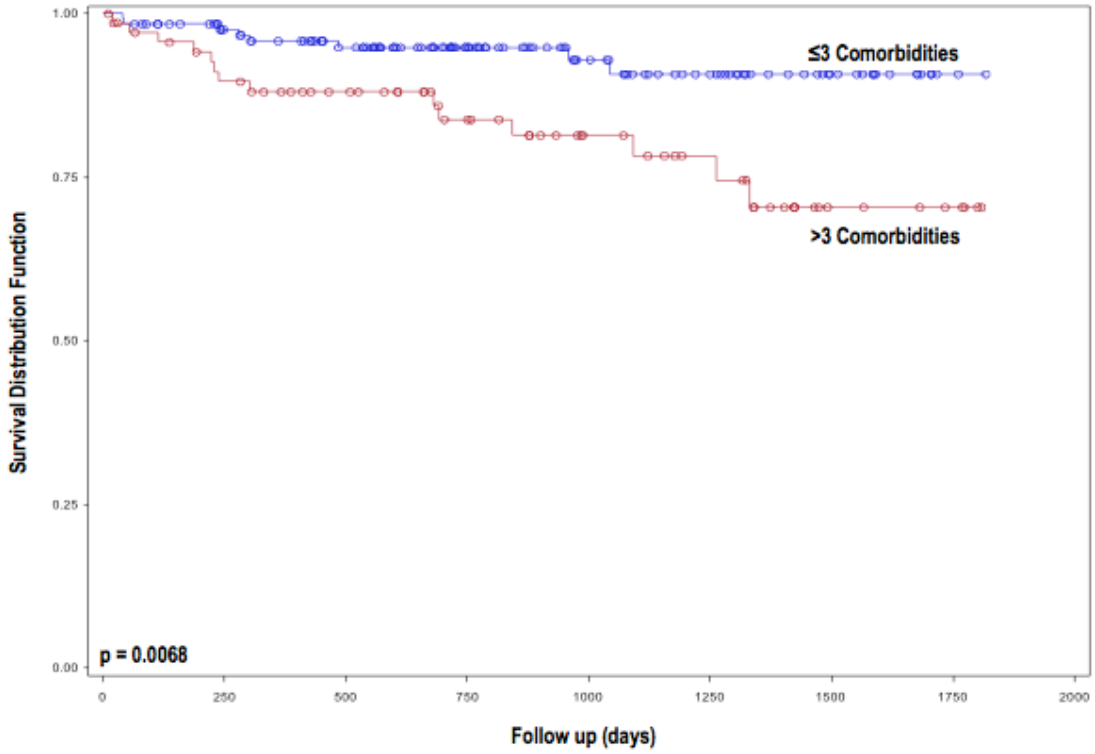

Figure 6.13: Kaplan-Meier plot of overall survival based on number of comorbidities

6.4.1.2. Multivariable Analyses

Initially, age, LDT and Rai stage, as the easiest to obtain and commonly used prognostic markers in clinics, were analyzed in a multivariable model. Both LDT and Rai stage were independent predictors of OS, when adjusted for age. Mortality was 4.5 times higher in patients with LDT <6 months (95% CI 1.28 – 16.05) and 6.8-times (95% CI 1.58 – 29.19) and 7.7-times (95% CI 1.32 – 44.55) higher for CLL patients with Rai stages III and IV, respectively (Table 6.2).

As a second step, a multivariable model was constructed with all the variables with a statistically significant HR in univariable models. However, none of the variables showed any statistical significance (data not shown). Therefore, as the next step, variables that predicted OS in univariable models were added to the initial multivariable model to adjust for age, LDT and Rai stage.

When ß2-microglobulin was added to the model, adjusting for age, LDT and Rai stage, patients with serum ß2-microglobulin levels greater than +2 standard deviations away from the mean had 28.9-times higher mortality compared with other patients (95% CI 1.31 – 639.58) (Table 6.3).

The only immunoglobulin that independently predicted OS after adjusting for age, LDT and Rai stage, was IgA. Mortality was 5.2 times higher in patients with high IgA compared with patients with normal or low IgA (95% CI 1.06 – 25.02) (Table 6.4).

Other prognostic factors that were significant predictors of OS in univariable models such as expression of CD38, creatinine clearance, comorbidities and other cancers were no longer associated with OS when adjusted for age, LDT and Rai stage in a multivariable model (data not shown).

Table 6.2: Multivariable analysis of overall survival

Multivariable Analysis (Overall Survival)			
Variable	**Subset**	**HR**	**95% CI**
Age	Continuous	1.04	0.99 – 1.1
LDT	>12 mth	1.00	-
	6 –12 mth	2.05	0.40 – 10.35
	<6 mth	4.54*	1.28 – 16.05
Stage	Rai 0	1.00	-
	Rai I	0.00	0.00 – .
	Rai II	4.21	0.95 – 18.57
	Rai III	6.79*	1.58 – 29.19
	Rai IV	7.67*	1.32 – 44.55

* Statistical significance with $p < 0.05$

CI: confidence interval; HR: hazard ratio; LDT: lymphocyte doubling time; mth: month

Table 6.3: Multivariable analysis of overall survival

Multivariable Analysis (Overall Survival)			
Variable	**Subset**	**HR**	**p-Value**
Age	Continuous	1.07*	1.00 – 1.13
LDT	>12 mth	1.00	-
	6-12 mth	3.17	0.57 – 17.67
	<6 mth	4.95*	1.16 – 21.19
Stage	Rai 0	1.00	-
	Rai I	0.00	0.00 – .
	Rai II	2.67	0.48 – 14.72
	Rai III	5.80	0.69 – 48.53
	Rai IV	10.69*	1.04 – 1.10.39
β2-Microglobulin[1]	<Mean	1.00	-
	High Creatinine	1.29	0.20 – 8.57
	0 – 1 StDev	0.77	0.12 – 5.13
	1 – 2 StDev	0.00	0.00 – .
	>2 StDev	28.90*	1.31 – 639.58

* Statistical significance with $p < 0.05$

[1]ß2-Microglobulin was z-transformed

HR: hazard ratio; LDT: lymphocyte doubling time; mth: month; StDev: standard deviation (away from the mean)

Table 6.4: Multivariable analysis of overall survival

Multivariable Analysis (Overall Survival)			
Variable	**Subset**	**HR**	**p-Value**
Age	Continuous	1.04	0.99 – 1.11
LDT	>12 mth	1.00	-
	6 – 12 mth	2.06	0.41 – 10.36
	<6 mth	3.81	0.92 – 15.76
Stage	Rai 0	1.00	-
	Rai I	0.00	0.00 – .
	Rai II	5.26*	1.13 – 24.43
	Rai III	4.84	0.97 – 24.02
	Rai IV	8.12*	1.24 – 53.07
Immunoglobulin A	Normal	1.00	-
	Low	1.95	0.42 – 8.99
	High	5.15*	1.06 – 25.02

* Statistical significance with $p < 0.05$

HR: hazard ratio; LDT: lymphocyte doubling time; mth: month

6.4.2. Treatment-Free Survival

When TFS was investigated in all CLL patients with Rai stages 0 – IV, Rai stage was a very strong predictor of treatment in the univariable model (Figure 6.14) and the only independent predictor of treatment in the multivariable model (data not shown). Therefore, TFS was investigated in CLL patients with Rai stages 0 and I to investigate the utility of prognostic markers in predicting TFS in patients with less advanced disease.

6.4.2.1. Univariable Analyses

In univariable models, LDT, Rai stage, ß2-microglobulin, CD38 expression, *IGHV* mutational status and plasma IgA level predicted TFS.

Rai stage was a predictor of TFS in CLL patients with Rai stages 0 and I. Patients with Rai stage I had 6.6-times increased risk of requiring treatment compared with patients with Rai 0 (95% CI 2.87 – 15.40) (Table 6.5).

LDT was a strong predictor of TFS and patients with LDT <12 months had more than 3-times higher risk of requiring treatment compared to patients with LDT ≥12 months (95% CI 1.36 – 7.12) (Table 6.5, Figure 6.15).

ß2-microglobulin was the strongest predictor for requiring treatment in CLL patients after taking kidney function into account. Patients with ß2-microglobulin levels between +1 and +2 standard deviation away from the mean, and patients with ß2-microglobulin levels more than +2 standard deviation away from the mean had a respectively 17-fold and 96-fold increased risk of requiring treatment, compared to patients with ß2-microglobulin levels lower than the mean (95% CI 8.07 – 34.60 and 21.28 – 434.06, respectively) (Table 6.5, Figure 6.16).

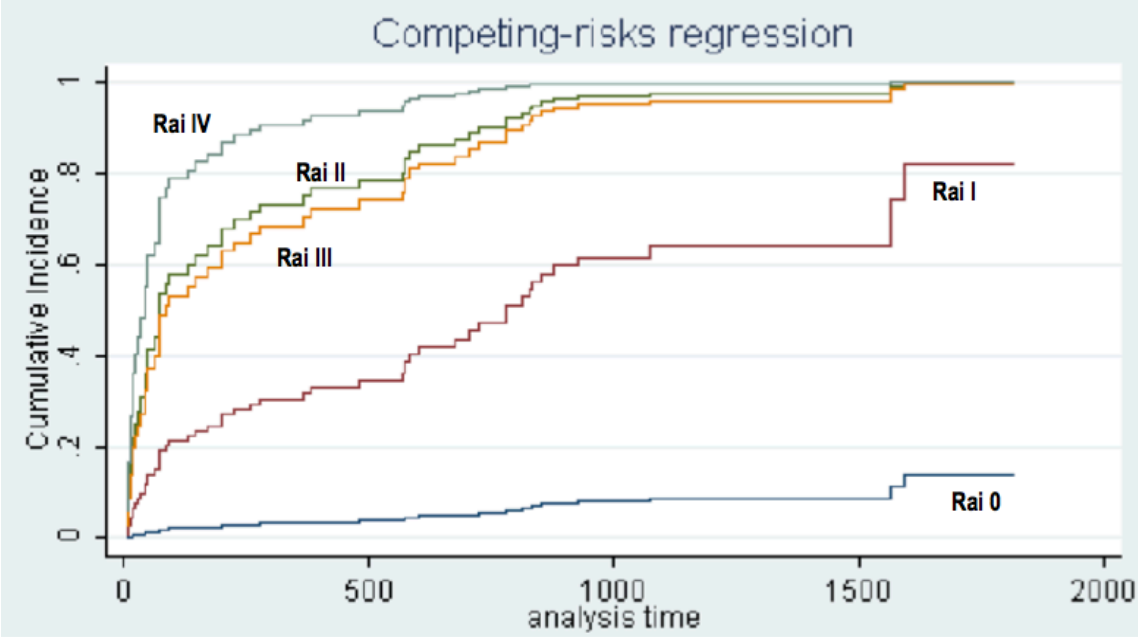

Figure 6.14: Competing risk regression plot for treatment-free survival (TFS) (days) based on Rai stage

Table 6.5: Univariable competing risk regression analysis of treatment-free survival

Univariable Analysis (Treatment-Free Survival)			
Variable	**Subset**	**HR**	**95% CI**
Gender	Female	1.00	-
	Male	1.30	0.55 – 3.06
Age	≥68 yr	1.00	-
	<68 yr	1.04	0.46 – 2.38
LDT	≥12 mth	1.00	-
	<12 mth	3.12*	1.36 – 7.12
Stage	Rai 0	1.00	-
	Rai I	6.64 *	2.87 – 15.40
Creatinine	Normal	1.00	-
	High	2.43	0.83 – 7.12
B2-Microglobulin[1]	> Mean	1.00	-
	0 – 1 StDev	0.90	0.22 – 3.78
	1 – 2 StDev	16.71*	8.07 – 34.60
	2+ StDev	96.11*	21.28 – 434.06
	Creatinine-High	2.68	0.87 – 8.21
CD38 Expression	Negative	1.00	-
	Positive	2.38*	0.99 – 5.75
ZAP70 Expression	Negative	1.00	-
	Positive	1.02	0.38 – 2.78
IGHV Status	Mutated	1.00	-
	Unmutated	3.73*	1.52 – 9.17
IgG Level	Normal	1.00	-
	Low	1.89	0.76 – 4.69
IgA Level	Normal	1.00	-
	Low	4.05*	1.72 – 9.52
IgM Level	Normal	1.00	-
	Low	1.47	0.63 – 3.47

Table 6.5 (continued)

Univariable Analysis (Treatment-Free Survival)			
Variable	**Subset**	**HR**	**95% CI**
Tx (Other Cancers)	Untreated	1.00	-
	Treated	2.92	0.83 – 10.25
Comorbidity	<3	1.00	-
	≥3	1.65	0.75 – 3.61
BMI	Normal	1.00	-
	Overweight/Obese	0.93	0.35 – 2.43

* Statistically significant

[1]ß2-Microglobulin was z-transformed

CI: confidence interval; HR: hazard ratio; *IGHV*: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time; mth: month; StDev: standard deviation (away from the mean); yr: year

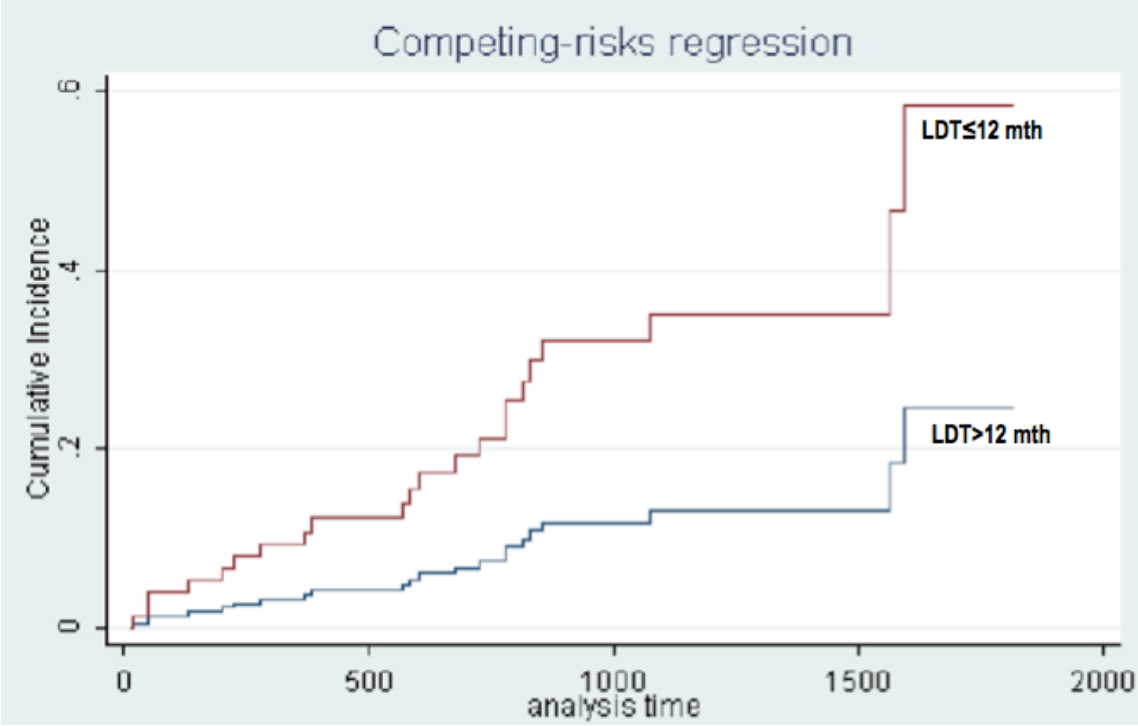

Figure 6.15: Competing risk regression plot for treatment-free survival (TFS) (days) based on lymphocyte doubling time (LDT)

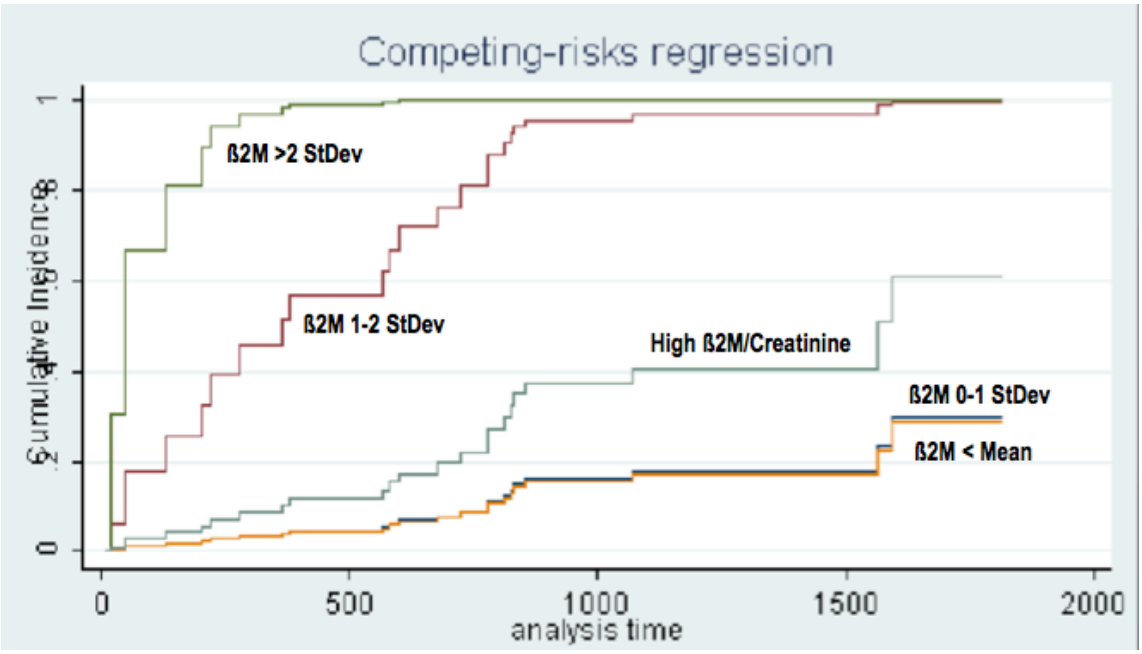

Figure 6.16: Competing risk regression plot for treatment-free survival (TFS) (days) based on ß2-microglobulin level (ß2-microglobulin levels were z-transformed)

Patients with CD38 expression had an almost 2.5-times increased risk of requiring treatment compared to those who did not express CD38 (95% CI 0.99 – 5.75) (Table 6.5, Figure 6.17).

CLL patients with unmutated *IGHV* gene status were at higher risk of requiring treatment compared with patients with mutated *IGHV* status (HR 3.73, 95% CI 1.52 – 9.17) (Table 6.5, Figure 6.18).

Patients with low plasma IgA levels had 4-times increased risk of requiring treatment compared to patients with normal IgA levels (95% CI 1.72 – 9.52) (Table 6.5, Figure 6.19).

6.4.2.2. Multivariable Analyses

When Rai stage, LDT, ß2-microglobulin, IgA and *IGHV* mutational status were investigated in a multivariable model, after adjusting for age and gender, LDT, age and gender did not show independency in predicting TFS (Table 6.6).

6.4.3. Cluster Analysis

When the expected distribution of negative markers was compared with the observed distribution, significant clustering of data was not detected in deceased patients or in treated patients ($p > 0.1$ for both cohorts).

However, upon manual examination of prognostic marker distribution in deceased patients, nine patients (41%) had 3 – 4 negative prognostic markers and another nine patients (41%) had five or more negative prognostic markers. Of the 24 treated patients with stages 0 and I, 12 patients (50%) had three or more negative prognostic markers.

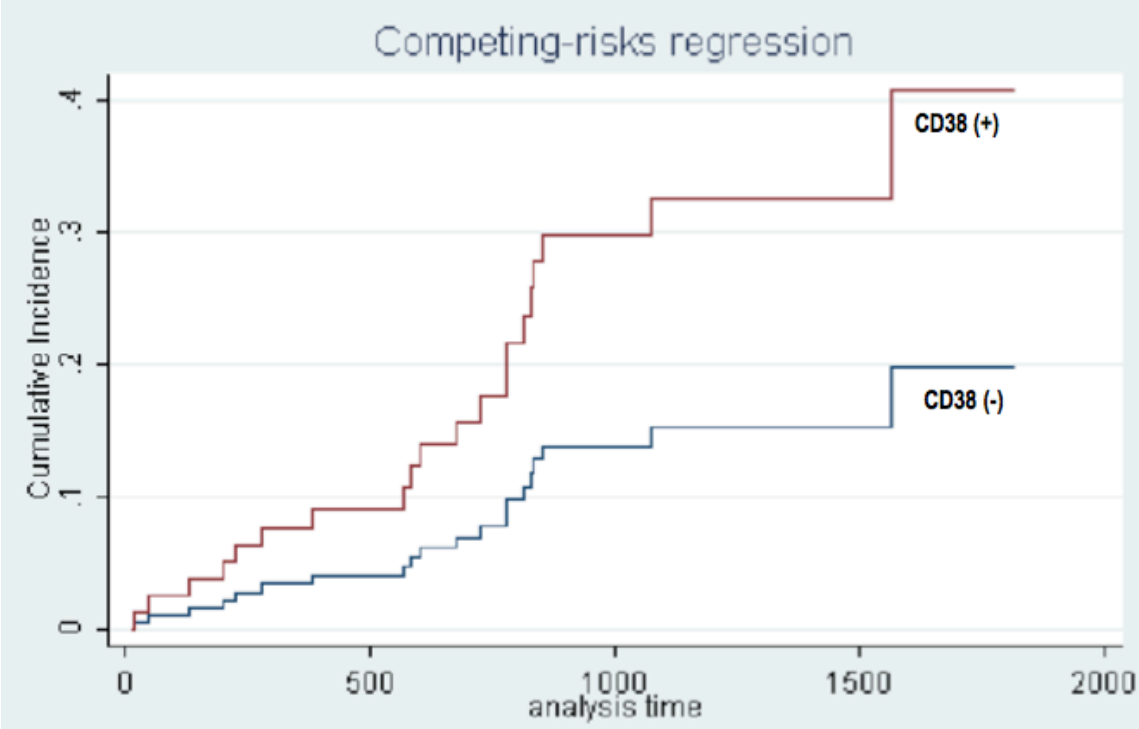

Figure 6.17: Competing risk regression plot for treatment-free survival (TFS) (days) based onCD38 expression

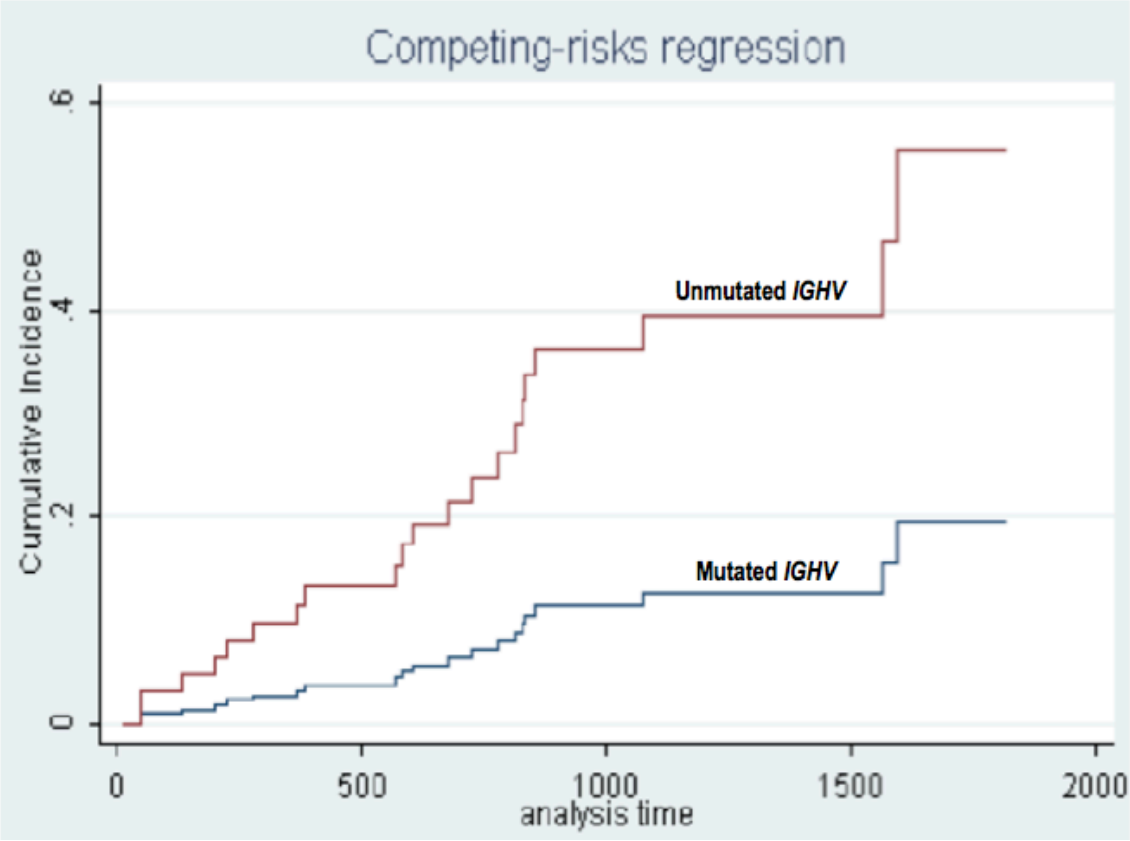

Figure 6.18: Competing risk regression plot for treatment-free survival (TFS) (days) based on *IGHV* gene mutational status

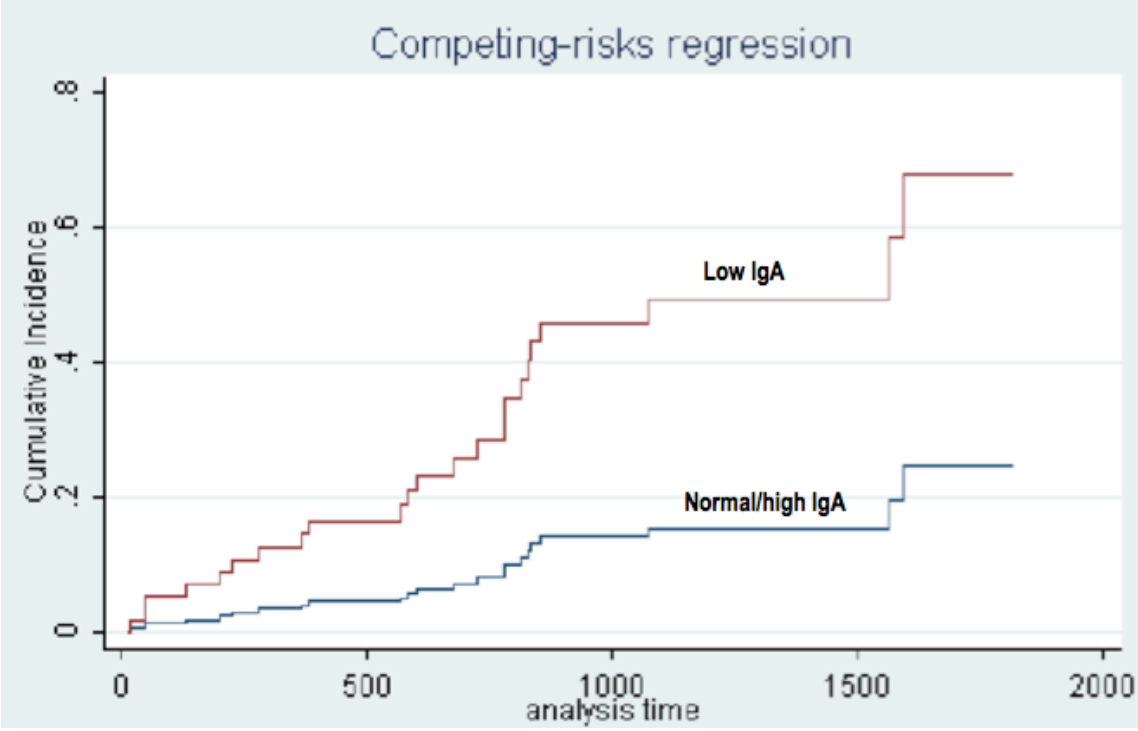

Figure 6.19: Competing risk regression plot for treatment-free survival (TFS) (days) based on serum IgA level

Table 6.6: Multivariable competing risk regression analysis of treatment-free survival

Multivariable Analysis (Treatment-Free Survival)		
Variable	**HR**	**95% CI**
Gender		
Male	0.52	0.16 – 1.66
Female	1.00	-
Age		
<68 yr	1.36	0.44 – 4.26
≥68 yr	1.00	-
LDT		
<12 mth	1.92	0.55 – 6.71
≥12 mth	1.00	-
Stage		
Rai I	4.80 *	1.45 – 15.88
Rai 0	1.00	-
B2-Microglobulin[1]		
0 – 1 StDev	1.20	0.32 – 4.53
1 – 2 StDev	1.36	0.27 – 6.78
2+ StDev	105.77*	14.92 – 750.10
Creatinine-High	4.59*	1.29 – 16.40
>Mean	1.00	-
IGHV status		
Unmutated	3.60*	1.13 – 11.49
Mutated	1.00	-
IgA Level		
Low	4.60*	1.63 – 12.98
Normal	1.00	-

* Statistically significant

[1]B2-Microglobulin was z-transformed

CI: confidence interval; HR: hazard ratio; *IGHV*: *immunoglobulin heavy chain variable region*; LDT: lymphocyte doubling time; mth: month; StDev: standard deviation (away from the mean); yr: year

6.5. Discussion

The Rai staging system has been used for the last 40 years to predict survival in CLL, but has limited use as the majority of patients are diagnosed with either Rai stage 0 or I disease, as a result of routine blood work. While many of the patients with early-stage CLL do not require chemotherapy for many years and have a prolonged survival, others will require treatment within months to years. Thus, there is considerable interest in developing accurate prognostic markers to counsel and monitor patients. Moreover, if patients were known to have a poor prognosis, they might be considered for earlier and more aggressive therapy.

While innumerable prognostic markers have been described, there is no consensus as to which should be used when patients are initially diagnosed with CLL. One reason for this is because not all prognostic markers will measure the same clinical outcome. Thus, a predictor for progression-free survival (PFS), which predicts the durability of response to therapy, may not predict OS. Secondly, the value of a prognostic marker may depend on the age of a patient, at which point during the disease course it is measured, and whether a patient has received prior therapy. Thus, the utility of prognostic markers depends on the outcome in question and the stage at which they are measured.

In the present study we elected to investigate a variety of common prognostic markers in a patient cohort that would be similar to that seen by general hematologists in Canada. All newly diagnosed CLL patients (201 patients) seen at the CLL Clinic over a five-years period (2007 – 2011) were assessed. These patients were referred to the CLL clinic by family doctors from across the province of Manitoba. When TFS was

evaluated, Rai stage was the only independent predictor of how quickly treatment was going to be required. Therefore, TFS was investigated in the 161 CLL patients with Rai stages 0 and I. Factors that were associated with a short TFS when evaluated as a single variable included Rai stage I disease, short LDT, CD38 positivity, high ß2-microglobulin, low IgA, and unmutated *IGHV* gene (Table 6.5). Using a multivariable model Rai stage I, high ß2-microglobulin, low IgA levels, and unmutated *IGHV* remained independent predictors of TFS (Table 6.6). These results were in agreement with previous reports (19–22). Association of high ß2-microglobulin with short TFS was expected, as ß2-microglobulin is a measure of tumour burden (23). Although high ß2-microglobulin is not an independent indication to treat, it may bias a physician's decision to treat in borderline scenarios. Interestingly, although a short LDT is one of the indicators to initiate treatment, LDT did not show any independent predictive value in this cohort. This is likely because increased CD38 expression and unmutated *IGHV* status reflect increased cell proliferation, which in turn results in a short LDT (5,24).

The utility of the prognostic markers to measure OS was then examined for all 201 CLL patients. In these patients, advanced Rai stage, short LDT, CD38 positivity, low creatinine clearance and increased plasma ß2-microglobulin, creatinine and immunoglobulin levels all correlated with reduced survival as single variables. In addition, simple clinical features, such as age, history of a previous cancer, and the presence of >3 comorbidities were associated with poor survival. Thus, many different and varying factors influenced survival (Table 6.1). In previous studies, Rai stage (4–6), LDT (19) and age (25) have been shown to be independent predictors of OS in CLL. Serum creatinine levels reflect kidney function and typically increase with age and

comorbidities (15) while ß2-microglobulin levels reflect tumor burden (Rai stage) (21,26) and renal function (creatinine) (20). The high levels of immunoglobulins at diagnosis could have represented the presence of infection at the time of diagnosis.

In contrast to previous reports (5,27), increased levels of ZAP70 and unmutated *IGHV* did not correlate with a short survival. It is possible that the study was underpowered to detect association of ZAP70 expression and IGHV status as not all patients had ZAP70 and *IGHV* measurements. The association between these prognostic markers and OS should later be re-evaluated using a larger cohort with a longer follow-up period. In addition, requiring treatment for either CLL or other malignancies did not influence OS. In contrast, when we previously combined CLL and SLL patients (281 patients) there was a correlation between the need for treatment and short survival, suggesting that the number of patients in the present study was too small to see a statistical correlation between treatment and survival. Again, whether this is a reflection of the smaller number of patients in the present study or differences between SLL and CLL is not clear. Finally, although high BMI and male gender have been associated with poor survival in the hematological malignancies, this was not seen in the present study (25,28). Interestingly, an improved outcome for male patients over the last 20 years has been observed in other studies suggesting that the development of the nucleoside analogs and monoclonal antibodies has particularly benefited male patients (7,29).

By multivariable analysis, both Rai stage and LDT showed independent value for predicting OS (Table 6.2). Being the most important prognostic markers, other markers were adjusted for age, Rai stage and LDT to test their utility in predicting OS. High levels of ß2-microglobulin and IgA were strong independent predictors of OS, whereas

CD38 expression, IgG and IgM levels, and number of comorbidities were not independent predictors of OS (Table 6.3, Table 6.4). This could be attributed to the strong association between these factors and age, LDT and Rai stage that was described earlier (see Chapter 5), in addition to the low mortality observed in this cohort.

Cluster analysis showed no significant clustering in our data. However, due to a small number of events and a high number of variables, these results may be underestimated. Clustering in the data may explain some of the unexpected results that were observed in this study such as association of high IgA with decreased OS.

Limitations of this study included the small cohort, few events and the short follow up that made it difficult to confirm the correlation between prognostic markers and TFS and OS. Furthermore, *IGHV* mutational status was only available in 75% of patients as the assay is only carried out on patients who consented and donated blood to the Manitoba Tumour Bank. Similarly, data pertaining to chromosomal abnormalities were not available for this study.

In conclusion, this study demonstrated that Rai stage is the only independent predictor of TFS when all CLL patients are assessed. When patients with Rai stages 0 and I disease are assessed, Rai stage I disease, high ß2-microglobulin, unmutated *IGHV* gene and low IgA are independent predictors of a short TFS. For OS, Rai stage, LDT, high ß2-microglobulin and low IgA levels independently predict a short survival. The results of this study suggest that simply measuring Rai stage, LDT and ß2-microglobulin levels provide sufficient information to predict time to treatment and survival. Ongoing studies with a larger number of patients with a longer follow-up is being undertaken to confirm these results.

6.6. Chapter References

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Chapter Seven

Discussion

7.1. Factors Affecting the Outcome of CLL Patients

CLL is an important disease accounting for 48% of all leukemias and 1% of all cancer diagnoses in Manitoba in 2010. CLL deaths accounted for 34% of all leukemia and 1% of all cancer deaths in Manitoba (1). A population-based study in the province of Manitoba (1998 – 2003) demonstrated a lower five-year relative survival among CLL patients compared with the age- and gender-adjusted general population (2). Moreover, the five-year relative survival in male patients was inferior to that of female patients for all age groups. A downward gradient in five-year relative survival was observed with advancing age, with elderly male patients having the poorest five-year relative survival. In this thesis, I have explored these findings by assessing the effects of other cancers, referral status, age and gender on outcome of CLL patients, both in the population and in the clinic settings.

7.1.1. Risk of Secondary Malignancies in CLL patients

An initial hypothesis to explain the reduced relative survival in elderly CLL patients was increased risk of second malignancies. The MD Anderson Cancer Center reported increased rates of second malignancies in CLL patients older than 60 years (3). It is known that the cellular immune surveillance declines with advancing age and this

contributes to the increase in incidence and aggressiveness of malignancies in the elderly CLL patients (4–7). Functional and phenotypic changes observed in T cells include a decrease in the number of naive T cells, an increase in memory T cells, decreased response to T cell receptor stimulations and impaired proliferation. A decline of 20% to 90% in IL-2 production occurs with aging. Since naive T cells produce higher levels of IL-2 compared to the memory T cells, it is suggested that the decline in the number of naive T cells in the elderly may contribute to the decrease in IL-2 production and subsequently a decrease in immune function (6,8–11). More recently, it has been shown that the physical interaction between CLL and T cells results in paralysis of T cell function; while this helps CLL cells evade immune surveillance, it increases the susceptibility of patients to recurrent infections and second malignancies (12–14). Accordingly, CLL patients with a second malignancy may die from the second malignancy or may die from progressive CLL, if treatment for the CLL is prevented by the second malignancy.

An Australian population-based study reported a 72% increased risk of death due to cancers (excluding deaths from lymphoproliferative malignancies) in CLL patients compared with the general population; lung, skin and colorectal cancers were the major causes of cancer mortality in that study (15). The MD Anderson Cancer Center reported that second cancers (16%) and infections (16%) were major causes of death in CLL patients, following active CLL (37%) (16). The same centre had shown that a history of malignancy prior to the diagnosis of CLL also reduced patient survival. The median durations of survival for patients with and without a history of a previous cancer were 7.6 and 10.9 years, respectively (95% CI 6.4 – 10.0 and 10.2 – 11.9, respectively) (3).

The five-year survival times for these two groups were 70% and 82%, respectively (95% CI 63% – 76% and 80% - 85%, respectively) (3). Similarly, in our population-based study we found that CLL patients are at increased risk of second malignancies.

Patients at the CLL clinic with a history of a previous cancer had a 4-fold increased risk of death compared to patient without another malignancy (Table 6.1). Breast, colorectal and NMSCs are known to be more aggressive in CLL patients than in a control population, and this has been ascribed to the decreased immunity in these patients (15,17). Alternatively, these carcinomas may share genetic features of their tumours with the CLL cells or may receive less aggressive treatment because of their underlying CLL (17). As discussed above, the immunity of CLL patients is known to be diminished prior to the diagnosis of CLL, it is likely that cancers developing at that time are also more aggressive and likely to recur, when compared to the non-CLL patients. Thus, the increased risk of second cancers may partly explain the reduced five-year relative survival of CLL patients compared to the general population, although it is unlikely to explain the worse prognosis for elderly male patients as both sexes and all age groups appear to be equally susceptible to invasive cancers.

We also showed that CLL patients had higher rates of second cancers compared to age- and gender-matched patients with Follicular Lymphoma (FL), another indolent B cell lymphoma, which is monitored and treated in a similar way to CLL (Table 3.2), and that this rate is higher only after the CLL diagnosis when compared with the general population as well as FL patients. This finding has three implications. First, the observed increased rate of second cancers is not likely due to detection bias, as both CLL and FL patients require long-term follow up after diagnosis. Second, both CLL and

FL patients are treated with similar rituximab-containing regimens, making chemotherapy an unlikely explanation for the increased incidence of other cancers in CLL patients. Finally, this increased rate of second cancers is likely due to a CLL-specific factor such as immune deficiency inherent in CLL.

7.1.2. Role of Age in Outcome of CLL Patients

A second factor that was thought to explain the poor relative survival of elderly CLL patients was referral bias, with elderly patients not being referred for evaluation and therapy. If this was a general phenomenon, it might explain why the only age group to not have shown an improvement in relative survival in Surveillance, Epidemiology and End Results (SEER) CLL population data over the past 20 years has been patients aged >80 years (18). We did demonstrate a significant referral bias for the clinic, in that there was a sizable group of patients, with an average age of approximately 85 years who were not being referred. Non-referred patients would have been followed by their family doctors or by a hematologist in the community. Indeed, overall mortality was more than 2-fold higher in non-referred patients than in referred patients, after adjusting for age, gender, treatment and other malignancies (Table 4.4). A number of factors may explain these findings. Firstly, frail elderly patients may not have been referred, as their other comorbidities may have been a greater concern than their CLL, while the elderly "fit" patients were referred. Secondly, low stage patients in the clinic were treated less often than non-referred patients, while elderly patients were more likely to receive chemotherapy if they were referred to the clinic. Also, clinic patients were more likely to be treated with fludarabine and rituximab containing combinations, which are more effective than single agent chlorambucil, which was commonly used in non-referred

patients. Finally, patients generally do better in high volume specialized clinics as staff may have more experience in monitoring and treating a disease, are more likely to follow treatment guidelines and to use more effective treatment regimens, are more familiar with managing drug toxicities and disease-specific complications. Patients at these clinics may have better access to advanced technologies and clinical trials (19–23). Thus, elderly patients who are not referred to the CLL clinic are less likely to receive treatment and have a markedly poorer survival than elderly patients who have been referred to the clinic.

As individuals age, they have an increased likelihood of developing other diseases, such as diabetes and hypertension, with associated risk of cardiovascular disease. The presence of comorbidities would be predicted to influence a patient's ability to tolerate the complications of CLL and chemotherapy and to influence survival. Indeed, we did demonstrate that having more than two comorbidities was associated with 3-fold increased mortality (Table 4.12). In addition, in a previous study we showed that the plasma levels of ß2-microglobulin increased with age, and were associated with increased plasma levels of inflammatory cytokines, increased creatinine and the presence of cardiovascular disease (24). Surprisingly, in the CLL clinic, we found that elderly patients had fewer comorbidities than the younger patients, and this was associated with lower ß2-microglobulin, normal IgA, normal creatinine and normal creatinine clearance (Table 5.10). These data demonstrate that the worsened outcome for elderly patients appears to be related to their comorbidities and only the healthiest elderly CLL patients are being referred for evaluation and treatment.

At the present time, it is unknown why some elderly patients are not being referred. It does not appear to be related to geographic location, but could be attributed to having other more significant comorbidities or being too frail to tolerate therapy. However, it is possible that the quality of these patients' lives might actually be improved with treatment of their CLL. Over the last 5-10 years there has been the rapid development of well tolerated new therapies, including monoclonal antibodies, eg, ofatumumab and GA101, and agents that influence the microenvironment, eg, ibrutinib and lenalidomide, which will likely benefit the older patients with CLL (13,25).

7.1.3. Role of gender in Outcome of CLL Patients

As we demonstrated in our CLL population study that male patients, and particularly elderly male patients, had a significantly worse prognosis than females, we elected to study gender differences in CLL. MD Anderson Cancer Center has demonstrated that male CLL patients have slightly higher rates of second malignancies compared to SEER data (3). In addition, a study of male CLL patients at a Veteran's hospital reported second malignancies to be the primary cause of death in CLL patients, accounting for 34% of CLL-related deaths (26). In our CLL population cohort, both male and female CLL patients had increased rate of all cancers. When cancers were subdivided by type, NMSCs were significantly more common in male CLL patients compared to the male general population, while female patients did not show any increased risk. A population-based National Cancer Institute (NCI) study reported a gender-specific risk for certain cancer sites in CLL patients (27). The mechanism underlying the increased incidence of second malignancies for males in CLL is

unknown, but this predilection likely contributes to the worse relative survival of male patients in the population.

In the CLL clinic, males were more likely to have high ß2-microglobulin and creatinine levels (Table 4.11). High plasma levels of ß2-microglobulin may reflect tumor burden, although increased levels are also associated with renal dysfunction and increased plasma inflammatory cytokines, which occur with the comorbidities, hypertension and diabetes (24,28–30). High levels of ß2-microglobulin and creatinine predict short OS in CLL patients (24,29–32). This may contribute to higher mortality in male CLL patients.

Despite these findings suggesting that males should do more poorly than females, they actually had a similar prognosis as females (Table 6.1). Two other recent studies have also found that the prognosis for males has improved in the past 10-20 years, suggesting that males have particularly benefited from the new therapies that have emerged in the past 15 years (18,33). For example, in our population cohort (1998 – 2003), most patients received single-agent chlorambucil or fludarabine, whereas in the clinic cohort (2007 – 2011), most patients received rituximab and a fludarabine-containing regimen.

7.1.4. Role of Prognostic Markers in Outcome of CLL Patients

There is tremendous clinical heterogeneity in the natural history, prognosis and treatment outcome of CLL patients. The list of promising prognostic markers for CLL is ever increasing and it is important to find out how to best utilize these markers. These prognostic markers are primarily useful for predicting the outcome of early stage CLL patients (Rai stages 0 and I) and should be evaluated at the time of diagnosis as they

may change in the course of the disease. In addition, although the majority of CLL patients are elderly, most studies have focused on evaluation of molecular prognostic markers in clinical trial patients who are relatively younger than the CLL population as a whole. Shanafelt *et al*, (34) suggested that molecular markers were less valuable in predicting survival of the elderly CLL patients. Age-related cytokines have been suggested to be better predictors of survival in the elderly CLL patients (24). Ongoing studies should determine the utility of these markers according to patients' age and timing during the disease course.

It is also important to recognize that prognostic markers interact with each other, and that they should be interpreted as such and not individually. Also each patient may have a combination of positive and negative markers and focusing on only one or two markers may be misleading. Yet, many studies have focused on one or a few markers, thereby limiting the utility of these markers. MD Anderson Cancer Center has developed a prognostic nomogram that incorporates a range of prognostic markers with clinical application (16); this nomogram is further validated by the Mayo Clinic group (34). It takes age, gender, ß2-microglobulin, absolute lymphocyte count, Rai stage and number of involved lymph node groups into account to predict outcome of CLL patients. The median survival for the low risk group was not reached, while the median survival for intermediate risk group was 10 years and for high risk group was 7.2 years. More importantly, it remained valid in predicting the outcome of low stage CLL patients. One disadvantage of this index was that it did not include modern prognostic markers. However, one may argue that, in fact, this may be considered an advantage as

it could be utilized worldwide with results of a basic blood test and a physical examination.

Reproducibility and evolution of prognostic markers also should be taken into account. For example, while *IGHV* mutational status is a static marker and remains the same during the course of disease, the same cannot be said about Fluorescent *in Situ* Hybridization (FISH) data, CD38 or ZAP70 expression, as they are more dynamic and may change during the course of the disease. Cytogenetic abnormalities may be acquired with disease progression (35) and CD38 and ZAP70 expression levels have been shown to change over time (36–39) and are indicative of level of aggressiveness at the time of sampling. Furthermore, a lack of a standard threshold for expression levels of CD38 and ZAP70 should be addressed.

It should be noted that all prognostic markers could be shown to influence survival if the sample size is large enough. However, those markers that influence decision-making process in the clinic should be considered the most important markers. For example, although *IGHV* mutational status is a very strong marker in predicting TFS and OS, it has no effect in choice of therapy, while deletion 17q indicates resistance to standard therapy regimens and potential benefits of investigational agents offered in clinical trials.

Future screening for genomic and proteomic markers at diagnosis will provide insight into deregulated pathways that make each patient either susceptible or resistant to certain treatment combinations. The goal of personalized therapy is to choose the best available regimen based on each patient's biological and clinical characteristics. Accordingly, the ability to distinguish early stage patients with an aggressive or

treatment-resistant disease and offer them treatment at an earlier stage may prevent clonal evolution and improve patient outcome.

The majority of treated CLL patients relapses after first line therapy, and eventually become refractory to therapy due to acquisition of new genetic aberrations that render them resistant to current treatments. Novel agents such as the cyclin-dependent kinase inhibitor flavopiridol (40–42), the immunomodulatory agent lenalidomide (13,40–42), small molecule Bcl2 inhibitors (41,43) and microenvironment modulating agents (43–45) are currently under investigation in clinical trials and will hopefully improve outcomes of high-risk CLL patients.

7.2. Conclusions

This thesis has demonstrated that the continued reduction in five-year relative survival of CLL patients with advancing age may be partly related to the increased risk of second cancers in CLL and to the fact that many of these patients are not referred and treated for their cancer. Interestingly, those elderly patients who are referred to the CLL clinic have fewer comorbidities than younger patients, can tolerate treatment and have a survival that is similar to young patients. In addition, males in the clinic, who notoriously had a poor prognosis, did as well as females suggesting that they are particularly benefiting from new therapies.

The increased risk of second cancers appears to be unique for CLL, only occurs after the diagnosis of CLL and is likely related to immune deficiency. The increased risk of second cancers is further increased by chemotherapy, which presumably also initially worsens the immune state. Based on these results, we are recommending the routine screening of CLL patients for second cancers and that all CLL patients should be

assessed by a CLL-specific hematologist. While some elderly patients may not tolerate treatment, many could receive therapy with the newer agents, which may improve the quality and duration of their lives.

7.3. Future Directions

7.3.1. Role of Immune System in Second Malignancies

Although this research has provided some explanations for the declining five-year relative survival that has been reported in CLL patients compared to the general population, especially in male and elderly patients, there are still questions that remain unanswered. We showed that the risk of second cancers is higher in CLL patients compared to FL, a similar B cell malignancy, and that this risk is only increased after CLL diagnosis and not prior to it. As second malignancies are thought to be related to immunosuppression, which may be worsened or improved by therapy, further studies are required to assess the risk of this complication with new therapies. Thus, the impact of new treatments on immune status and the incidence of second malignancies should be monitored. Assessment of immune status has become an area of active research, and can be monitored by measuring immunoglobulin levels along with B and T cell number and function (12,14).

Finally, as skin cancers appear to be so common in CLL patients and are a clinical manifestation of immunosuppression, it would be interesting to determine whether the development of a NMSC in CLL increases the likelihood of developing an invasive malignancy.

7.3.2. Type of Cancers and Survival of CLL Patients

The increased risk of cancer in CLL should continue to be evaluated, particularly as new agents are being used which can either increase (chemotherapy and monoclonal antibodies) or decrease (e.g. lenalidomide) immunosuppression. Collaborations with other clinics will be required to ensure adequate patient numbers.

It has been shown that CLL patients with other cancers have a higher mortality rates. However, to prove that the reduced survival in male elderly CLL patients is related to second cancers, the incidence of second cancers and the cancer-specific mortality in elderly male CLL patients should be compared with other CLL patients. In our study, we were not able to divide our cohort based on cancer-site or age due to the limited events in each category. Thus, a larger cohort with longer follow up is required to provide enough power to answer these questions.

7.3.3. Defining Referral Bias in the Specialized CLL Clinics

Our research showed that CLL patients, especially elderly CLL patients who were referred to the CLL clinic have a longer survival compared to non-referred patients. A comparison of comorbidities and accurate causes of death (based on chart review) between referred and non-referred patients could assist us to confirm the existence of a referral bias. Higher rates of comorbidities and non-CLL mortality in non-referred patients would indicate the existence of referral bias, whereas similar rates of comorbidities and CLL-related mortality in both groups would indicate the absence of such bias.

In our non-referred cohort we were not able to separate patients who were managed by their family physician from those that were referred to a general

hematologist. This information should be obtained and the outcome of patients seen by general practitioners, general hematologists and CLL-specific hematologists should be investigated separately in order to properly demonstrate the effects of physicians' disease expertise on survival of CLL patients in Manitoba.

Importantly, the phenomenon we described may be unique for Manitoba and this study should be repeated at other centres to determine whether referral bias is similar in other parts of the country.

7.3.4. Prognostic Factors and Overall Survival

To fully investigate the association between prognostic markers and TFS or OS, the number of events (e.g. treatment and death) should be increased. This will allow us to create a nomogram to predict outcome of patients with multivariable models that takes into account several prognostic markers. This may be achieved by increasing the number of patients and the length of follow up. The number of patients might be increased by incorporating data from other provincial cancer centres and the length of follow up could be increased by including CLL patients diagnosed prior to 2007 to our cohort or re-visiting this cohort in future.

Association between rates of second cancers in CLL patients and well-established prognostic markers such as levels of ß2-microglobulin and creatinine, CD38 and ZAP70 expression and *IGHV* mutational status, as well as more recent prognostic markers such as the mutational status of NOTCH1 should be investigated. Furthermore, addition of FISH may provide valuable information about risk of second cancers as well as treatment-free and overall survival of CLL patients in Manitoba.

These future studies will help us understand the effects of new treatments on immune status and subsequently their effect on rates of second malignancy and mortality in CLL patients. Understanding reasons behind the non-referral of the elderly patients may help eliminate referral bias and improve the outcome of elderly CLL patients.

7.4. Chapter References

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