

CD200 EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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DECLARATION

I, Karissa Thamanna Mannaru, declare that this thesis is my own work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Day of 2016

DEDICATION

For my grandparents Sunny, Pooby and Kitty, my parents Mogie and Devan and sister, Alicia.

Finally for Shahan, “...not be afraid of confrontation... Even the planets collide and from chaos the stars born”.

PUBLICATIONS AND PRESENTATIONS

Part of this work was presented as a poster at Unipath 2014.

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ABSTRACT

Introduction:

Chronic Lymphocytic Leukemia (CLL) is a small cell B cell neoplasm, demonstrating CD19/CD5 co-expression with a heterogeneous prognosis. Its distinction from Mantle Cell Leukemia/lymphoma (MCL) is necessary for accurate treatment strategy and prognostication. CD200 is a transmembrane antigen found on a range of haemopoietic cells, with a multifactorial immune-suppressive role. It is postulated to contribute to tumour biology by evading immune surveillance and enhancing immune tolerance. The differential expression of CD200 in CLL and MCL may be used as an immunophenotypic tool in separating these diagnoses. Its role as a prognostic and diagnostic marker is not established in the context of CLL in an African setting.

Aims:

To verify the value of CD200 in differentiating CLL from MCL during immunophenotypic assessment and to ascertain if its expression may be utilized as a prognostic marker in the context of CLL.

Methods:

Flow cytometric analysis of CD200 expression on normal lymphocytes and CD19/CD5 co-expressing B cell neoplasms was performed. A cut off value for identification of CD200 positivity was established. Statistical analysis of tumour expression of CD200 was then undertaken to determine its sensitivity and specificity as a diagnostic tool in CLL assessment.

Established prognostic markers were collected for each case, for comparison to CD200 expression. Statistical analysis was used to establish the utility of CD200 expression as a surrogate prognostic marker.

Results:

CD200 is weakly positive on normal B-lymphocytes and negative on normal T lymphocytes. A cut-off mean fluorescence intensity of 20 was determined to constitute positivity for CD200 expression. Within the CD19/CD5 cohort obtained (n=57), 46/57 were identified as CLL and a further 7 as MCL. The sensitivity of CD200 was 97.8% with a specificity of 85.7%, with statistically significant positive and negative predictive value during immunophenotypic diagnosis of CLL. Requisition of laboratory prognostic markers was suboptimal in this cohort. There was no reliable association of CD200 expression with other markers of prognostic significance in this study.

Conclusion:

Within this small cohort, CD200 proved to be a sensitive marker for the discrimination of CLL from MCL during immunophenotypic analysis of CD19/CD5 co-expressing tumours. The role of CD200 as a prognostic tool in CLL could not be confirmed.

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LIST OF ABBREVIATIONS

ATM: Ataxia Telangectasia Mutated gene

BCL2: B cell Lymphoma-2

BCR: B cell immunoglobulin receptor

BLPD: B-cell lymphoproliferative disorder

B-PLL: B-Prolymphocytic Leukaemia

CCND1: Cyclin D1

CCND3: Cydlin D3

CDK: Cyclin dependent kinase

CLL: Chronic Lymphocytic Leukaemia

EBV: Epstein Barr Virus

FISH: Fluorescence *in-situ* Hybridization

FL: Follicular Lymphoma

HIV: Human Immunodeficiency virus

IgVH: Immunoglobulin Heavy Chain

LPL: Lymphoplasmacytic Lymphoma

LIS: Laboratory Information System

MBL: Monoclonal B cell Lymphocytosis

MAP3K: MAP kinase kinase kinase

MCL: Mantle Cell Lymphoma/ Leukaemia

M-CLL: mutated CLL

MDM1: Mouse double minute 2 homologue (E3 ubiquitin ligase)

MFI: Mean Fluorescence Intensity

NFKB: nuclear factor kappa-light-chain-enhancer of activated B cells

PBS: Phosphate Buffered Solution

PCR: Polymerase Chain Reaction

RT-PCR: Reverse Transcription PCR

SF3B1: Splicing factor 3B subunit 1

TP53: Tumour protein53

UM-CLL: Unmutated CLL

WCC: White cell count

ZAP-70: Zeta Associated Protein-70

CHAPTER 1: INTRODUCTION

1. Chronic Lymphocytic Leukaemia (CLL)

1.1 Definition

CLL is the most common lymphoid malignancy of adulthood arising from a clone of small mature B-lymphocytes, typically affecting middle-aged to elderly patients [1].

1.2 Epidemiology

CLL accounts for 6-7% of Non-Hodgkin Lymphoma in Western countries [2]. In the South African context, CLL constitutes $\pm$ 14% of Non-Hodgkin Lymphomas [3]. Age and gender distributions are comparable to that seen worldwide, however, it has been observed that black patients present at younger ages with more aggressive disease [4]. The mean age at diagnosis is 65 years with a male: female ratio of 1.5-2:1 [2].

1.3 Pathogenesis

1.3.1 The cell of origin

The CLL clonal cell is postulated to be an antigen-experienced B cell. Various factors have been identified in CLL pathogenesis, including genetic abnormalities, epigenetic dysregulation, and aberrant cell signaling through chronic antigenic stimulation as well as microenvironment subversion.

Links to viral aetiologies, such as hepatitis B and C as well as Epstein-Barr Virus (EBV) have largely been disproven [5, 6]. In addition, CLL is neither a Human Immunodeficiency

Virus (HIV) -defining or associated B cell tumour. CLL shows an inherited genetic predisposition [2].

1.3.2 Precursor Lesion

Monoclonal B cell lymphocytosis (MBL) often precedes CLL [7, 8]. MBL is an expansion of small mature clonal B-lymphocytes comprising less than $5 \times 10^9/l$ in the peripheral blood [1,7,9]. The incidence of MBL in patients older than 60 years is approximately 5-14%, with an estimated risk of progression to CLL of 1% per annum [7,8]. Cytogenetic abnormalities in MBL do not necessarily predict progression [10].

1.3.3 Molecular pathophysiology

Cytogenetic abnormalities associated with CLL include mutations, chromosomal deletions and gene dosage alteration that can sustain and transform the cell of origin or precursor lesion. These factors ultimately converge to deregulate tumour suppressor function, DNA repair and apoptosis- resulting in abnormal cell survival and progressive genetic instability.

1.3.3.1 Mutations in tumour suppressor genes

Tumour suppressor malfunction in CLL is most commonly attributed to TP53 and Ataxia Telangectasia Mutated gene (ATM) instability through complex interconnected mechanisms.

Loss of TP53 as a chromosomal deletion of the short arm of chromosome 17 occurs in 10% of CLL cases [2]. Considered the guardian of the genome, P53 protein is a master regulator that oversees tumour suppression through a myriad of pathways [11]. Mutations of the remaining/ homologous P53 allele occurs in up to 80% of patients with 17p deletions [11]. Loss or attenuation of P53 as a protector of genomic integrity is a well characterized

pathogenic factor in CLL genesis [11]. Abnormalities of the tumour suppressor ATM gene are also implicated in CLL pathogenesis. ATM function is important for pro-apoptotic activity mediated by P53. Deletional loss or mutations of the long arm of chromosome 11 can be biallelic as both deletion and mutation can occur simultaneously [12]. The phenotypic consequences are impaired DNA damage control, which contributes to tumour genetic instability [12].

1.3.3.2 Epigenetic insults

Epigenetic disease mechanisms include disruption of methylation patterns, spliceosome modifications and changes in micro-RNA profiles [13,14]. Micro-RNA 15/16a reduction, caused by chromosome deletion of the long arm of 13, is responsible for the negative regulation of genes BCL2, cyclins CCND1 and CCND3, as well as cyclin dependent kinase 6 (CDK6). Loss of these regulatory micro-RNAs favours constitutive activation of cell cycling and resistance to apoptosis [1,7,9,15,16]. Methylation pattern changes are demonstrated in CLL to derange cell programming further in favour of tumourigenicity [13].

Mutations of SF3B1, a spliceosome subunit that affects RNA processing, have been described more recently in CLL. While the frequency of the latter in CLL is significant, the direct consequences of these mutations are still under investigation [12].

1.3.3.3 Proto-oncogene dose effect

Another common genetic insult in CLL is trisomy of chromosome 12. Trisomy of chromosome 12 is demonstrable in $\pm 15\%$ of CLL cases [8]. Whilst the precise mechanism of action is not fully understood, it is thought that this abnormality alters proto-oncogene dose [1,7,9,15,16]. Since the whole chromosome is involved, several candidate proto-oncogenes have been suggested [17]. Of these a gene dosage increase in MDM2 an E3 ubiquitin ligase,

is thought to contribute to CLL pathogenesis. MDM2 is involved in P53 degradation and cyclin-dependent-kinase-4 essential for cell cycle [17]. Augmented MDM2 cell dosage is therefore an important negative regulator of P53 tumour suppressor effect and of cell cycle progression.

1.3.3.4 Signaling pathway insults

Chronic abnormal antigenic stimulation of surface B-Cell Immunoglobulin Receptor (BCR) is an established driver of CLL behaviour [18]. The origin of the antigens are postulated to be autologous in nature [19]. Two clinical subsets of CLL have been recognized based on the mutational status of CLL cells' immunoglobulin heavy chain genes (IgVH) [7]. A variation of >2% from germ-line IgVH configuration is considered mutated [19].

Mutated heavy chain genes (M-CLL) are associated with a more favourable phenotypic behavior than unmutated cases (UM-CLL) [19]. BCR signaling influences anergy or proliferation and is ultimately a determinant of clonal progression [18].

Molecular signaling aberrations recently identified include NOTCH1 and BIRC3 disruption[14]. NOTCH1 protein is transmembrane; upon ligand-binding it acts as a transcription factor regulating a number of downstream target genes that involve P53, NFkB and MYC pathways. Sustained signaling through mutated NOTCH protein, ultimately results in evasion of apoptosis with subsequent cell immortalization [14].

Other signaling cascades such as MAP3K and NFkB are under BIRC3 negative regulation. Mutations affecting BIRC3 act as an activator of constitutive NFkB oncogenic signaling, compounding tumour survival [14].

1.3.3.5 Tumour co-optation of the reticuloendothelial microenvironment

Understanding the nexus between CLL and its environment is in its infancy. Elements of the microenvironment include tumour-associated macrophages, nurse cells, mesenchymal stromal cells, growth factors, non-tumour lymphocytes, chemokines and extracellular matrix components and cell ligands. CLL subverts the microenvironment in a complex signaling exchange that assists survival, proliferation and escape from chemotherapy and immune surveillance [20]. The rate of progression and course is variable, which makes CLL a clinically heterogeneous entity. Overall survival rates range from 1-15 years [14].

The rate of progression is dependent on the overall molecular profile of CLL (as previously described), which determines tumour phenotypic behaviour. The culmination of cumulative CLL genomic instability is progression to a high-grade B cell neoplasm such as a Prolymphocytic Leukaemia or Diffuse Large B cell Lymphoma [2, 7], a process known as Richter's Transformation, which occurs in up to 10% of CLL cases [2, 21].

1.4 The Diagnosis of CLL

The process of diagnosis is directed by several guidelines including those of the International Workshop on Chronic Lymphocytic Leukaemia [22] and the World Health Organization (WHO) [2]. The minimal requirements include:-

1. The full blood count (FBC) and differential count will demonstrate an absolute lymphocytosis of $> 5 \times 10^9/l$ consisting of small monomorphic lymphoid cells (Refer to Figure 1.)

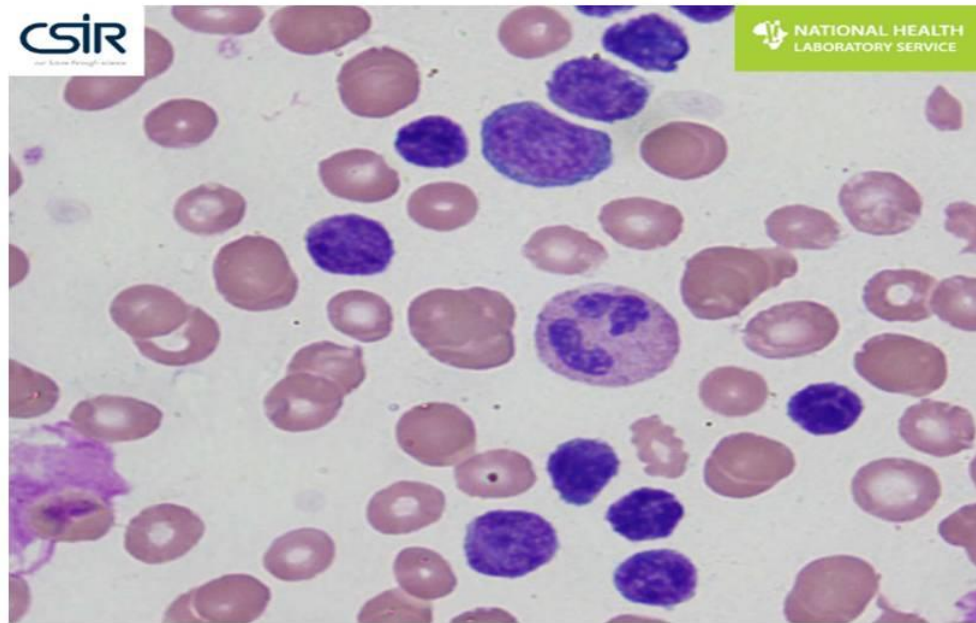


Figure 1: Peripheral blood morphology of a typical CLL.
Image reproduced with permission.

2. Cytological or histological analysis of peripheral blood, bone marrow, spleen or lymph node reveal small cells, clumped nuclear chromatin without nucleoli and high nuclear:cytoplasmic ratio. Smudge cells are frequently seen. Prolymphocytes may be present but should constitute less than 55% of the tumour population.

3. The immunophenotype is required to establish clonality and remains the gold standard for diagnosis (Refer to Figure 2.). Flow cytometry or immunohistochemical staining will in typical cases demonstrate a CD19/ CD5 co-expressing tumour population with CD23 and generally low (dim) surface immunoglobulin expression, while FMC-7 (an epitope of CD20) is not usually expressed. B cell antigens CD20 and CD79a are weakly expressed [2]. The relative expression of these antigens can be used in a predictive scoring matrix for CLL known as Matutes score [23].

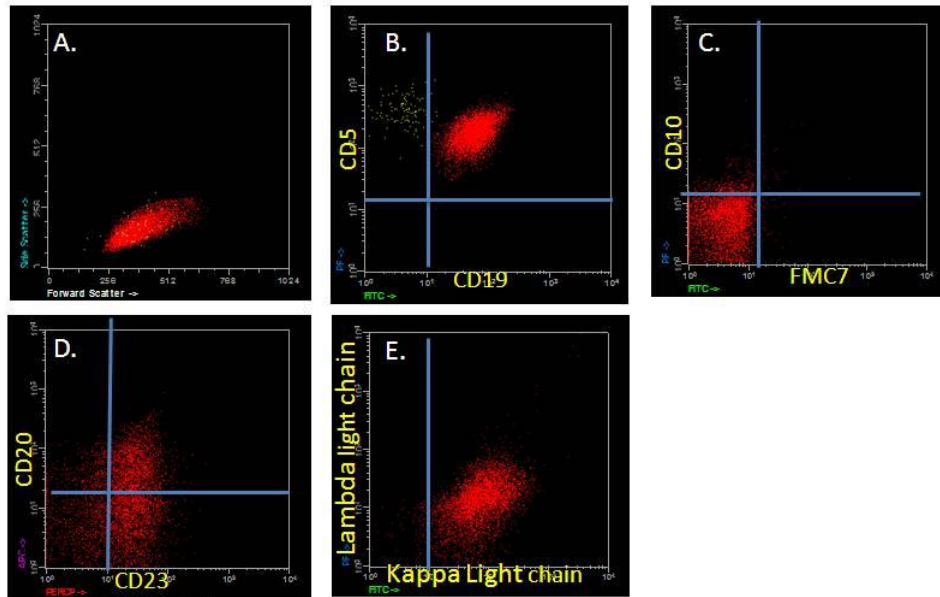


Figure 2. Immunophenotypic scatter plots for a typical CLL case
 A Tumour isolated by orthogonal light scatter (red population).
 B. Tumour positive for CD19/CD5 co-expression.
 C. Tumour negative for FMC7 and CD10.
 D. Tumour positive for CD23 and CD20 (dimmer intensity).
 E. Tumour positive for kappa light chain restriction (dimmer intensity).

Other tests performed at diagnosis include tumour chromosomal analysis, Fluorescent *in-situ* Hybridization (FISH) and other biochemical markers. These investigations may provide prognostic information and assist in the design of a therapeutic strategy. They are, however, not necessary for diagnosis and, in some centres, the performance of a bone marrow investigation is not mandatory [22].

1.4.1 Variants of CLL

Small Lymphocytic Lymphoma (SLL) is a localized form of CLL, where peripheral blood clonal lymphocytosis does not exceed $5 \times 10^9/l$ and bone marrow clonal lymphocytes comprise $<30\%$ [2]. The absence of a leukemic phase in CLL is however, infrequent [2].

A diagnosis of SLL is based on histological examination of involved tissues including lymph nodes, spleen, liver and rarely extranodal sites. B-lineage infiltrate of small cells with an

immunohistochemical profile showing dim CD20, CD5, and CD23 in the absence of cyclin D1 would best fit SLL.

Atypical CLL was first defined in 1997 [24] and is described in the current WHO [2]. This variant is based on morphological variations of CLL as well immunophenotypic aberrancy [2, 24]. The former has been defined as cases having plasmacytoid morphology, >10% cleaved forms, >15% (but less than 55%) prolymphocytes, and a lymphocyte doubling time of less than a year [25]. Subsequent studies verify that atypical CLL cases present with higher mean white cell count, more frequent association with trisomy 12 and a higher probability of progression than typical cases [25].

(Refer to Figure 3. for an example of an atypical CLL immunophenotype).

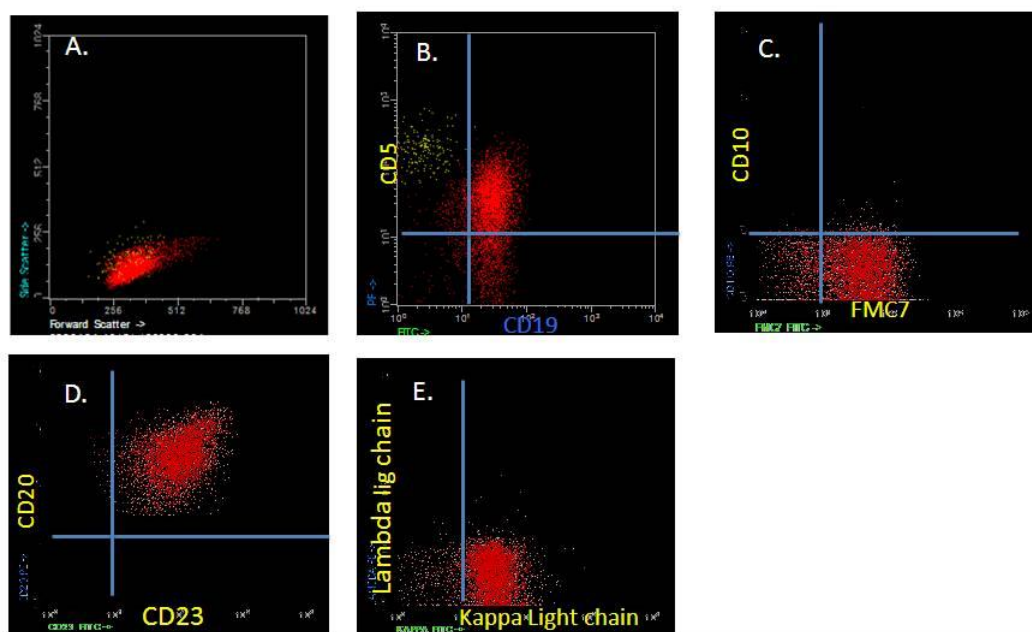


Figure 3. Immunophenotypic scatter plots for an atypical CLL
A. Tumour isolated by orthogonal light scatter (red population).
B. Tumour positive for CD19/CD5 coexpression.
C. Tumour positive for FMC7 and negative for CD10.
D. Tumour positive for CD23 and CD20 (stronger intensity).
E. Tumour positive for kappa light chain restriction (dim intensity).

1.5 The Prognosis of CLL

Classified as indolent, CLL is heterogeneous in course, more so than other mature B cell leukaemia/ lymphoma [9]. To date several clinical and laboratory factors have been identified for prognostication and risk stratification in order to individualize treatment options [1,9,15,26].

Prognosis may be guided by clinical stage, serum biochemical and haematological markers, and tumour characteristics such CD38 expression, genetics, ZAP70 expression and IgVH status (M-CLL vs UM-CLL).

1.5.1 Clinical Prognostication

The Rai and Binet classifications are used for clinical staging (summarized in Tables 1 and 2). These staging systems are dependent on clinical findings of organomegaly and lymphadenopathy and the presence of a low platelet count (thrombocytopenia) or a low haemoglobin concentration (anaemia) on the FBC. The cytopenias are exclusive of CLL-related autoimmune effect [2].

Table 1. RAI CLASSIFICATION [27]

Stage	Lymphadenopathy	Hepatosplenomegaly	Haemoglobin (g/dl)	Platelets (10 ⁹ /l)
0	Not present	Not present	>= 11	>= 100
I	Present	Not present	>= 11	>= 100
II	Present	Present	>= 11	>= 100
III	Present	Present	<11	>=100
IV	Present	Present		<100

Table 2. BINET STAGING CLASSIFICATION [28]

Stage	Lymph node areas involved	Haemoglobin (g/dl)	Platelets ($10^9/l$)
A	<3	≥ 11	≥ 100
B	≥ 3	≥ 11	≥ 100
C	Not applicable	<10	<100

The Rai classification is risk-stratified into low (Stage 0), intermediate (Stages I-II) and high (Stages III-IV) [1]. The median overall survival of these groups are >10, 7-9 and 1.5-5 years respectively [1]. These staging systems are easy to use and may assist with treatment choices. The disadvantage of these staging systems is that it may not predict prognosis accurately in the early stages of disease [29]. Re-staging is not clinically relevant. For these reasons, recently proposed scoring systems exclude the clinical staging models in favour of molecular and biochemical markers [30].

1.5.2 Laboratory Prognostication

Several laboratory-based markers are well characterized for their prognostic value [1,9,15,22]. In addition, some markers are surrogate measures, while others remain independent adverse prognostic markers. Prognostication in CLL is currently limited by the absence of a comprehensive validated prognostic model [19, 31].

Commonly used markers for prognosis: (prognostic marker significance is summarized in Table 3 [1,19,31]).

1.5.2.1 Tumour phenotypic markers

- a) Lymphocyte doubling time (LDT). This is an indirect assessment of proliferation, measured as the length of time taken to double the absolute lymphocyte count [19].
- b) CD38 expression. CD38 is a transmembrane glycoprotein involved in cell adhesion, signaling and increased intracellular calcium. CD38 expression is a correlate of lymphocyte proliferation.
- c) The pattern of tumour infiltration on histological bone marrow examination.

1.5.2.2 Tumour Genotypic markers

- a) PCR sequencing for Immunoglobulin Heavy Chain (IgVH) mutational status (M-CLL or UM-CLL) is one of the principle prognostic markers.
- b) Zeta Associated Protein (ZAP)-70 expression. ZAP-70 is a tyrosine kinase involved in T-cell signaling. This parameter may be assessed by flow cytometry or reverse transcriptase polymerase chain reaction (RT-PCR). ZAP-70 is not expressed in normal B lymphocytes and is a surrogate marker for IgVH mutational status [9]. When used in combination with CD38 expression, ZAP-70 defines three separate prognostic groups. The latter prognostic model is restricted to settings where both markers are routinely performed.
- c) Fluorescent *in-situ* hybridization (FISH): deletions of 17p, 13q, 11q and trisomy 12 as well as complex karyotype.

1.5.2.3 Serum biochemistry

- a) Beta 2 microglobulin, a component of HLA class 1 molecule is a marker of disease activity. It is not specific for CLL.

- b) Thymidine kinase, which is involved in DNA synthesis salvage pathway, is an additional tumour proliferation measure.

Table 3. Summary of commonly used prognostic markers in CLL [1.9].

Prognostic marker	Prognostic value	Bearing on clinical outcome	Limitations of testing	Advantages as a prognostic tool
1. LDT	Adverse if <12 months.	-MS of 36 months.	Requires serial measurement.	Ease of use.
2. Pattern of BM involvement	Adverse if diffuse.	-Decreased OS.	Invasive.	Relatively easy to perform.
3. CD30%	Adverse if expression >30%.	-Faster progression. -Decreased OS. -Decrease MS	Expression is variable over disease course.	Relatively easy to perform.
4. IgVH status	Adverse if unmutated or mutated at VH3-21.	-Mutated cases have a 50% longer survival, with an MS of 293 months. -Unmutated cases MS of 95 months	Not routinely available.	Remains prognostic after autologous stem cell transplant
5. ZAP70 expression	Adverse if >20% expression by intracellular flow cytometry analysis.	-Predicts shorter time to treatment.	-Analysis may be obscured by T cells (which normally express ZAP70). -Not routinely available.	Surrogate marker of IgVH status is the majority of cases.
6. Cytogenetics	Adverse if complex. Intermediate if normal karyotype.		Culture success often limited.	Availability is restricted.
7. 17 p abnormalities	Deletion of 17p or mutation alone is adverse.	-Aggressive course -Reduced PFS -Predicts purine analogue resistance.	Mutational analysis is not routinely available.	First line treatment predictive.
8. 11q abnormalities	Deletion 11q or mutation is adverse.	-Aggressive course -Reduced TFS -Predicts use of cyclophosphamide based regimens.	Mutational analysis is not routinely available.	Treatment predictive.
9. Trisomy 12	Intermediate risk.	-Higher response rate with Rituximab based regimen.		
10. 13q deletion	Good prognosis if occurring in isolation.			
11. Serum thymidine kinase	>15 U/l	Indicator of disease progression. If >10 U/l there is lower rate of treatment response.	Not routinely available.	
12. B2 microglobulin	>3.5 mg/l	-Decreased OS -Lower CR -Correlate of PFS.	Must be adjusted for creatinine clearance of patient.	Routinely available.
Abbreviations: CR=complete remission, MS= mean survival, PFS=Progression free survival, OS= Overall survival, TFS= treatment free survival.				

1.6 Limitations in diagnosing CLL

CD19 and CD5 co-expression may occur in a range of small cell B neoplasms [32]. The primary and most common differential diagnoses considered in CD19/CD5 co-expressing tumours are Mantle Cell Lymphoma (MCL) or CLL in Richter transformation [2].

MCL constitutes 3-10% of B-cell Non-Hodgkin Lymphoma. It may behave as both a high and low grade Non-Hodgkin Lymphoma, however, its overall course is generally aggressive and relapsing [33]. Furthermore, CLL and MCL share morphological and immunophenotypic overlap, which may obscure definitive diagnosis.

Other B cell neoplasms that may demonstrate CD19/CD5 expression include Marginal Zone Lymphoma [34], Lymphoplasmacytic Lymphoma / Waldenstrom's Macroglobulinaemia [35] and very rarely, Follicular Lymphoma [36]. Rare cases of Diffuse Large B-Cell Lymphoma (DLBCL) are also CD5 positive [2] although these generally have larger cells than MCL or CLL.

It is paramount to accurately differentiate small B-cell neoplasms with CD19/CD5 co-expression, particularly CLL from MCL, as prognosis and treatment differ significantly [31].

1.6.1 Similarities between MCL and CLL

1.6.1.1 Presentation

CLL and MCL generally present in the 6th decade and show a male preponderance [33,37, 38].

Bone marrow and peripheral blood involvement is common to both entities at presentation. Lymph nodes and spleen may also be involved at presentation [2].

1.6.1.2 Morphological and Histological similarities

MCL presents as a histo- and cytological spectrum including a small mature tumour cell phenotype [39]. The distinction of CLL from MCL on morphological grounds can be uninformative especially with atypical CLL morphology [40]. Refer to Figure. 4 for comparative MCL morphology.

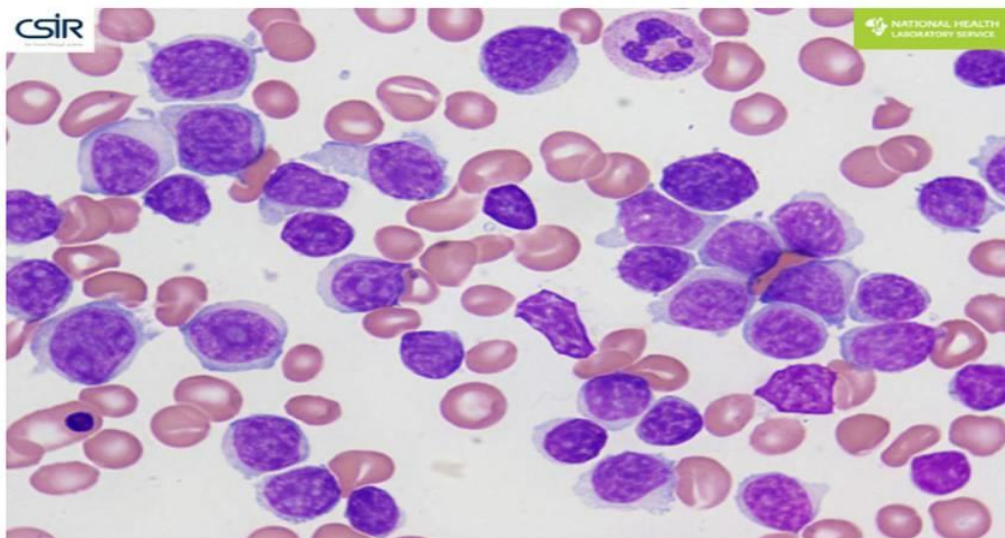


Figure 4 : Peripheral blood morphology of a typical leukemic phase MCL.
Image reproduced with permission.

1.6.1.3 Immunophenotypic similarities

MCL classically expresses CD19, CD5 and FMC7 with brighter light chain restriction, but does not *typically* express CD23 [37] (See Figure 5). Immunophenotypes of both CLL and MCL may, nonetheless, overlap [41, 42]. 11% of MCL cases lack FMC-7 expression and up to 20% express CD23 [41, 43]. In addition, atypical or transforming CLL cases can demonstrate Mantle Cell like phenotypes [42] (Figure 5.).

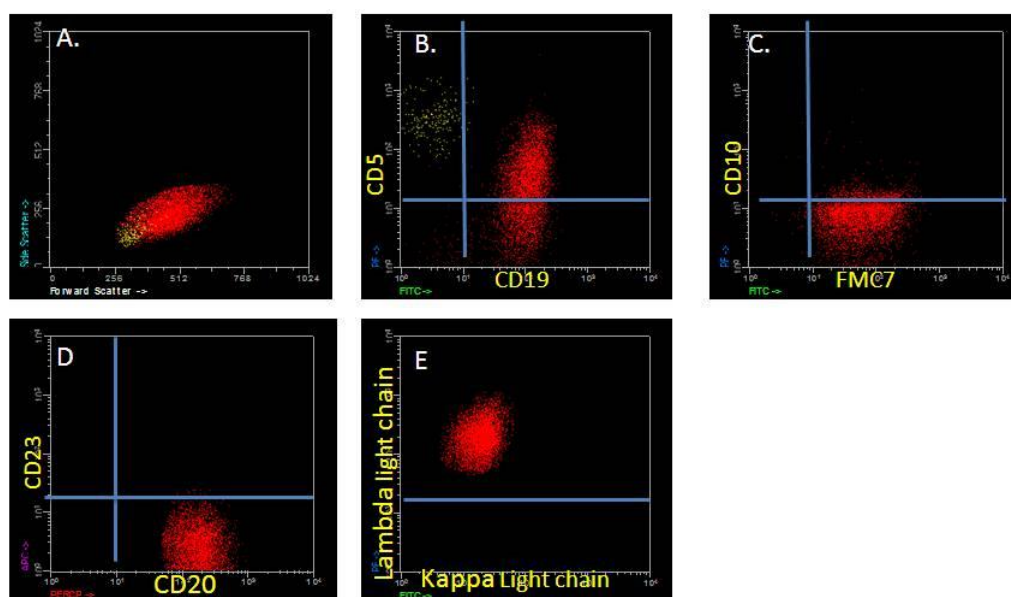


Figure 5. Flow cytometry scatter plots for typical MCL
A. Tumour isolated by orthogonal light scatter (red population).
B. Tumour positive for CD19/CD5 co-expression.
C. Tumour positive for FMC7 and negative for CD10.
D. Tumour negative for CD23 and CD20 (stronger intensity).
E. Tumour positive for lambda light chain restriction (normal intensity).

The Matutes score was established in 1994 [23] to aid the differentiation of CLL and MCL. The score was subsequently revised in 1997 [44]. This scoring system assesses the probability of CLL. Scores of 4 or more are predictive of CLL in 87% of cases [23]. A score of 2 or less suggests an alternative diagnosis, as this low score is associated with other small B neoplasms including MCL, in the overwhelming majority of cases (Table 4).

Table 4. Revised Matutes score [23, 44]

<u>Antigen</u>	<u>0 points</u>	<u>1 point</u>
CD5	Negative	Positive
CD23	Negative	Positive
FMC7	Positive	Negative
Surface Immunoglobulin expression	Strong	Dim
CD22/ CD79a	Strong	Weak

Other scores proposed [45] have yet to be validated for diagnostic use. Matutes score remains the most useful adjunct for CLL vs. MCL diagnosis during immunophenotypic assessment. A proposed new classification will include CD200 [46].

1.6.1.4 Genetic similarities

Trisomy 12 can be found in up to a quarter of MCL [2]. Furthermore ATM and P53 mutations are also implicated in MCL pathogenesis [2]. Such abnormalities, therefore, lack diagnostic specificity.

1.6.2 The pathognomonic difference between MCL and CLL: Cyclin D1 overexpression in MCL

At a molecular level, MCL is typified by translocation between breakpoints 11q13 (cyclin D1 gene) and 14q32 (immunoglobulin heavy chain gene). The resulting translocation causes constitutive over-expression of the cyclin D1 gene, a cell cycle regulator, leading to unchecked and uncontrolled tumour proliferation [38]. Immunohistochemistry for cyclin D1 is also used as an equivalent diagnostic modality, and is positive in the majority of MCL [37, 47]. Demonstration of t(11;14) or cyclin D1 histologically is therefore considered highly suggestive of MCL. Cyclin D1 (translocation and /or immunohistochemical positivity) has also been demonstrated recently in B-Prolymphocytic Leukaemia (B-PLL) [48], reducing diagnostic specificity.

1.6.3 Limitations in demonstrating cyclin over expression in a MCL diagnosis.

Sensitivity of FISH analysis for diagnostic confirmation of MCL is reportedly 70-75% [49]. Confirmation of t(11;14) by FISH is labour-intensive and FISH expertise and availability is limited to central laboratories in the South African setting. Poor sample quality may reduce

test sensitivity. Furthermore, it is suggested that both FISH for t(11;14) and cyclin D1 immunohistochemistry be performed concurrently, as discordant positivity has been reported [50].

MCL variants exist including CD5-negative and cyclin D1-negative MCL subtypes [2, 51-53]. The latter, in particular, give rise to diagnostic uncertainty. Cyclin D1 immunostain or t(11;14) negative cases, may involve alternative cyclin abnormalities. These include overexpression of cyclin D2 (more commonly) and cyclin D3 or rearrangements with immunoglobulin light chain genes (chromosomes 2 and 22) [53, 54]. Gene expression profiling has been used to authenticate such cases [53]. Demonstration of increased cyclin RNA levels by Polymerase Chain Reaction (PCR) is currently not routinely available.

In a case of small B cell neoplasm with CD19/CD5 co-expression in the absence of t(11;14) or cyclin-D1 immunohistochemistry, both a MCL-subtype or CLL need to be considered in the differential diagnosis.

1.7 Implications of an accurate diagnosis in CLL

CLL is largely incurable. Treatment is directed by age, presence of co-morbidity, clinical stage at presentation, performance status as well as the prognostic variables earlier described [31]. The course of CLL is generally indolent, when compared with MCL, which is aggressive. An accurate immunophenotypic and molecular diagnosis during the workup of CD19/CD5 small cell B tumours is therefore essential for treatment initiation and the design of individual therapeutic strategy.

2. CD200

Investigation of tumour immunophenotypes has implications for diagnosis, prognosis and therapy in the personalized medicine era [55]. CD200 is an emerging immunophenotypic biomarker, identified in a number of B cell neoplasms including CLL [56-59].

2.1 Definition and structure

CD200 is a 41-47 kDa glycoprotein, comprised of two immunoglobulin-like extracellular domains extending into the membrane [60]. This molecule is found on B lymphocytes, activated T lymphocytes and dendritic cells (as well as other non-haematological cells) [61]. Its cognate receptor (i.e. CD200-R) is found on myeloid cells, including neutrophils, basophils, mast cells, monocytes, macrophages and dendritic cells [61]. CD200-R is structured as two immunoglobulin-like extracellular domains, a trans-membrane portion and small cytoplasmic extension for signaling. In addition, crystallographic resolution shows that CD200 and CD200-R has the capacity to function within the immunologic synapse [62]. The mechanism of signaling is not fully understood [63].

2.2 Functional mechanisms of CD200-CD200R within the immune system

Normal CD200- CD200 receptor engagement is involved in suppression of the immune response, through modulation of adaptive and innate immunity [61, 64, 65]. Monocyte and macrophage function is inhibited by CD200-CD200 receptor engagement (for example by the induction of Indoleamine 2,3 dioxygenase)[61, 66]. Similarly, cytokine production and degranulation of mast cells is suppressed by the interaction of CD200 and its receptor[61, 63, 67].

T-cell subsets include cytotoxic and helper cells. Helper cells are further divided into Th1, Th2, T-follicular helpers, Th17 and T-regulatory (T-reg) subsets. The latter is involved in immune tolerance. The normal function of the Th1 response is cellular immunity particularly targeting intracellular parasites [68]. The normal Th2 response is associated with humoral immunity, aimed at extracellular-parasitic, bacterial infections and allergic responses [68]. CD200-CD200R binding biases the T-helper response toward a T-suppressor or T-regulatory phenotype [69].

Binding of CD200 to CD200R is thought to induce tolerance of tumour antigens and the expansion of T-regulatory cells, which facilitate tumour escape from immune regulation [70]. Reduction of cytotoxic T-cell function and dendritic cell tumour antigen presentation may also play a role. Several malignancies demonstrate CD200 upregulation including B cells neoplasms, Acute Leukaemia [71] and Myeloma [59].

2.3 CD200 expression and B cell neoplasms

CD200 is variably expressed by chronic B-cell neoplasms [57]. It is significantly expressed in CLL/SLL, Hairy cell leukemia (HCL), up to 80% of Lymphoplasmacytic Lymphoma/leukaemia (LPL) [57] and shows variable expression in Marginal Zone Lymphoma (MZL) [72]. It is reportedly negative in MCL [72] and Follicular Lymphoma [57].

2.4 CD200 and CLL

2.4.1 Pathological role of CD200 in CLL

CD200 in soluble form is significantly elevated in CLL patients and correlates with more advanced clinical stage [73]. In murine models, increased CD200 expression was permissive for the establishment of CLL. CLL engraftment in mice could be abrogated by the infusion of anti-CD200 antibodies [73].

T-cell proliferation in response to CLL cells was enhanced with anti-CD200 antibodies [73] and T-reg frequencies were reduced [74]. CD200 blockade is therefore a potential immunotherapeutic target [75, 76].

2.4.2. Utility of CD200 expression in CLL

2.4.2.1 CD200 expression as a diagnostic marker

The inclusion of CD200 in multiparameter flow cytometry of CD19/CD5 co-expressing tumours may help to differentiate CLL and Mantle cell Lymphoma [77, 78] and a revision of the Matutes score proposes to use CD200 as a component [46].

Validation of a revised Matutes score using CD200 expression as a marker of CLL suggested superior specificity in the diagnosis of CLL in comparison with the traditional score [46].

Overall, the sensitivity of the *modified* Matutes score improved to 97.1% (from 87%) with a specificity of 87.2%, demonstrating an improvement in score utility during immunophenotypic assessment [46].

2.4.2.2 CD200 as a prognostic marker in CLL

CD200 has also been identified as prognostic marker in haematological malignancies [79, 80].

The role of CD200 as a prognostic marker in CLL has been tested to a limited extent [58, 73, 74]. Validation of CD200 as a prognostic marker in CLL may allow its use as a cost-effective alternative to traditional prognostic markers like FISH.

2.4.2.3 CD200 as a predictive marker in CLL

The treatment predictive value of CD200 measurement is an area of active research. Targeted and immunological based therapies are increasingly important in cancer therapy. The clinical efficacy of therapies such as Rituximab (human anti-CD20 monoclonal antibody) and Alemtuzumab (human anti-CD52 monoclonal antibody) is now well substantiated in lymphoid malignancies [31].

CD200 blockade has been demonstrated to be an effective anti-tumour strategy [81]. Anti-CD200 antibodies and other methods for CD200-bearing cell depletion have been very recently patented for investigation into therapeutic uses in cancer and autoimmune disease [21]. Provisional results for a CD200 monoclonal antibody therapy (Samalizumab, Alexion Pharmaceuticals) have demonstrated efficacy in CLL [82].

2.5 Study Aim

The use of CD200 in the immunophenotypic assessment of CD19/CD5 tumours in a South African context requires validation, as does evaluation of its potential role as a prognostic tool. In addition, documentation of tumour CD200 positivity may have therapeutic implications in the future.

This study aimed to evaluate the diagnostic and prognostic utility of CD200 in CLL in the South African population.

CHAPTER 2: AIMS AND OBJECTIVES

Aim 1:

To verify the diagnostic benefit of measuring CD200 expression in CD19/CD5 co-expressing B cell tumours.

Objectives:

- Optimize CD200 monoclonal antibody for use during immunophenotypic assessment within the setting of this study.
- Establish baseline levels of CD200 expression on normal lymphocytes for comparison to clonal B-lymphocytes.
- Identification of tumours with CD19/CD5 co-expression for evaluation of CD200 expression.
- Verify whether CD200 expression is beneficial as a discriminatory marker of CLL from non-CLL cases.
- To determine if the traditional Matutes score and CD200 are synergistic during immunophenotypic assessment of CLL and MCL.

Aim 2:

To ascertain if CD200 expression can be used as surrogate prognostic marker in comparison with markers of known prognostic value in CLL.

Objectives:

- To describe the profile of the commonly used prognostic markers found in this cohort of CLL patients including:
 - a) LDT
 - b) CD38 tumour expression
 - c) Genetic markers identified by cytogenetic and FISH analysis

d) B2 microglobulin

- To determine if CD200 expression correlates with known prognostic markers in CLL.
- To describe the epidemiological profile of CLL in this local cohort.

CHAPTER 3: MATERIALS AND METHODS

Ethical permission was obtained from The Human Research Ethics Committee- number: MI30279 (see appendix). Patient confidentiality was maintained throughout and the data derived was not linked to any unique patient identifier. Control and Patient samples were allocated consecutive numbers. Analysis of data was performed retrospectively-results were not communicated with the attending physician and therefore had no bearing on diagnosis. Research funding was obtained through the National Research Fund Thuthuka grant (numberTTK20110801000022866) and University of the Witwatersrand Faculty of Research Committee fund (number0125484681051211050000000000000000004990)

3.1 Acquisition and optimization of CD200 monoclonal antibody for use in this study

CD200 monoclonal antibody was obtained from BD Biosciences in conjunction with its respective isotypic control as allophycocyanin (APC) stain.

In order to maximize staining with the CD200 antibody whilst minimizing background auto-fluorescence and antibody wastage, a minimal optimal antibody volume was determined by a titration assay. Aliquots of a single cell suspension in were incubated with series of antibody concentrations, maintained under the same conditions. Five biological replicates were used. Mean fluorescence intensity (MFI) of each monoclonal antibody (MFI as a log value) was calculated for each tube using Flow Jo analysis software (Ashland, Oregon). MFI was plotted as a function of volume (refer to Figure 6). The point of curve plateaux was considered optimal for staining, extrapolated to be 10 microlitres of antibody.

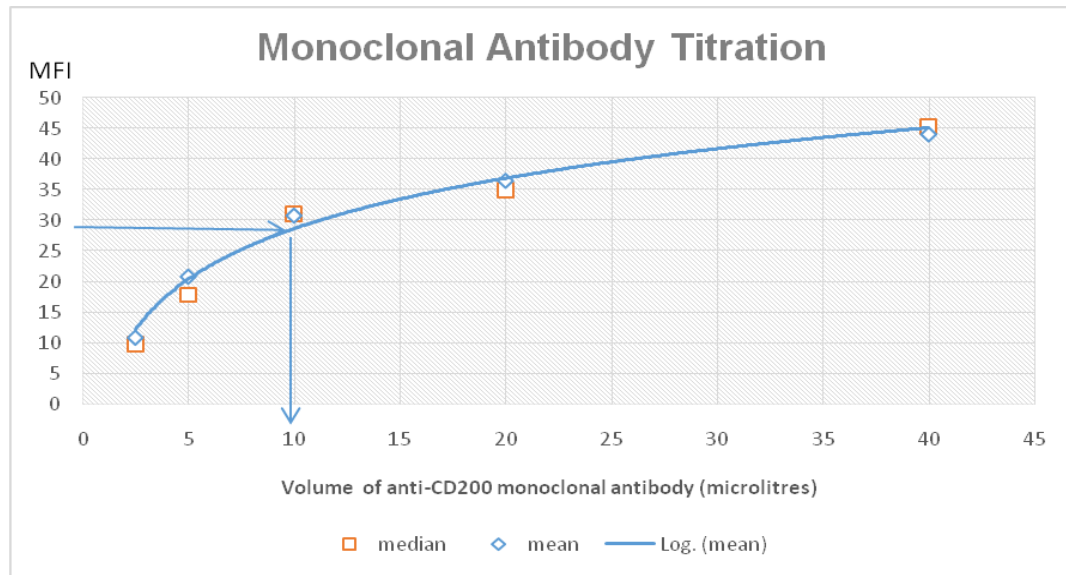


Figure 16. Median MFI, Mean MFI and Log (mean) or geometric mean (to account for log-normal behaviour of the data) of CD200 expression. The point at which the curve began to plateau was taken as the minimal antibody concentration required for

3.2. Selection of samples for study inclusion

3.2.1 Controls

20 controls were selected from adult samples received for routine immunophenotyping referred for exclusion of a mature B lymphoproliferative neoplasm, by a minimum limited screening of CD19, CD5, Kappa and Lambda light chain expression. Once a clonal B cell population was excluded then residual material containing a total mature B cell population of >1% was used for the control experiments.

3.2.2 Pathological samples

All cases of a clonal B cell neoplasm demonstrating the major inclusion criteria of CD19 and CD5 co-expression were selected from routine samples referred to the Charlotte Maxeke

Johannesburg Academic Hospital between May 2013 and November 2014 (an 18 month study period). Inclusion prerequisites were adequate sample viability and volume. Samples included blood, bone marrow or fluid specimens and previously diagnosed follow-up samples were also accepted. All samples that fulfilled the above criteria were utilized regardless of the referral centre (inter or intra-provincial). Repeat samples from the same patient were excluded. Study cohort demographic details were captured in order to gather complementary results from laboratory databases (DISA and TRAKCARE).

3.3 Methods for Preparation and Staining of samples for immunophenotypic analysis

All samples referred to the flow cytometry routine immunophenotyping bench were prepared in accordance with laboratory standard operating procedures (SOPs). Both the control and patient samples were prepared and analyzed similarly.

Briefly, a mononuclear cell layer was derived by a density gradient technique. 4.5 ml Ficoll/Histopaque(Sigma Aldrich, St Louis, Missouri) was aliquoted into a standard conical tube, to which sample (blood, bone marrow or fluid) was added. The tubes were then centrifuged at 2000 g/ min for 15 minutes, to concentrate the mononuclear cell layer containing the population of interest. The enriched mononuclear layer was removed and placed into new glass tubes. Phosphate buffered solution (PBS) was added, followed by centrifugation and discarding of the supernatant. The resulting cell button was dislodged by vortex agitation. Red cell lysis was achieved by the addition of isotonic ammonium chloride followed by incubation of 2-4 minutes. Contaminating red cells were lysed and removed by repeating washing in PBS. The resulting cell button was re-suspended in 5ml PBS, from which aliquots

of 100 microlitres were used for testing. Monoclonal antibodies were subsequently added to the prepared cells for incubation period of 10 minutes in the dark.

All controls had a minimum screen of Kappa/Lambda/CD19/CD5 to enumerate T and B-cells and to confirm the absence of a clonal B-cell population. Additional antibodies for CLL diagnosis were selected at the discretion of the reporting pathologist and were dependent on the quality and quantity of the sample received for testing, in conjunction with the estimated tumour burden. A minimum screen included a K/L/CD19/CD5/FMC7/CD10/CD20/CD23 $\pm$ CD38 (see Table 5).

Table 5. Antibodies used in this study.

<u>Antibodies</u>	<u>Manufacturer</u>
1. Mouse IgG1	Beckman-Coulter, Brea, California
2. Isotypic control, APC	BD Bioscience, New Jersey USA
4. Kappa FITC/ Lambda PE	Dako, Glostrup, Denmark
6. CD23 FITC/CD20 PE or CD20 PerCP/CD23 APC	Dako/ Beckman-Coulter
8. FMC7 FITC /CD10 PE	Beckman-Coulter
9. CD19 FITC/ CD5 PE or CD19 PerCP/CD5 APC	BD Bioscience
10.CD38 PE	BD Bioscience
11.CD200 APC/ Mouse1 IgG APC	BD Bioscience

3.4 Sample acquisition

Samples were acquired on the FacsCalibur flow cytometer (BD Bioscience, New Jersey) using CellQuest software (BD Bioscience). The flow cytometer was calibrated daily using Calibrite beads (BD Biosciences). Internal and external quality control processes were appropriately maintained during the study period.

5000 fluorescent events were mandatory for an adequate analysis. Data was analysed using Paint-a-Gate software (BD Bioscience, New Jersey) with routine gating-strategies to identify and quantify cell populations of interest.

3.4.1 Controls

20 controls were selected to establish cut off values for positivity or negativity for CD200 expression and to identify any internal control population. The choice of 20 control samples was influenced by the availability of normal residual samples subsequent to limited screening for clonal B cells. Control samples were stained using monoclonal antibodies against CD19 (to identify B cells), CD5 (to identify T cells) and CD200. An isotypic antibody control assisted in the exclusion of non-specific binding of antibodies. Polyclonality of B-cells was confirmed by a normal light chain (kappa to lambda) ratio of 2-3:1.

MFI values for CD200 expression were calculated for normal T lymphocytes, as the internal negative control. The gating strategy for control samples is demonstrated in Figures 7 and 8.

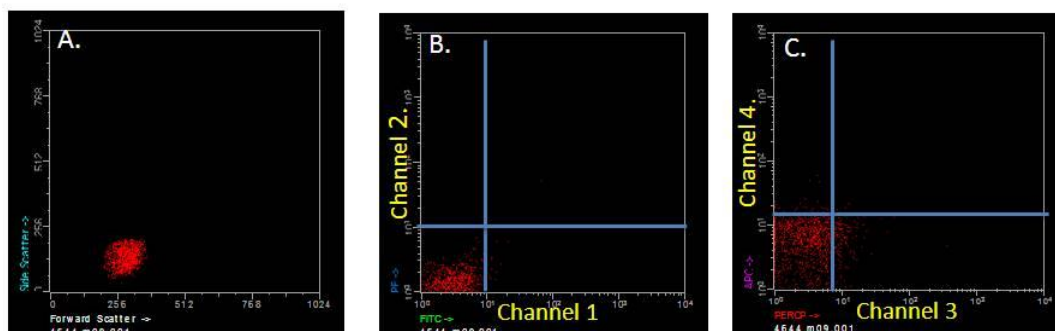


Figure 7. Gating strategy for Isotypic control

A: Lymphocytes isolated by orthogonal light scatter

B: Autofluorescence and non-specific binding excluded in channels 1 and 2.

C: Autofluorescence and non-specific binding excluded in channels 3 and 4.

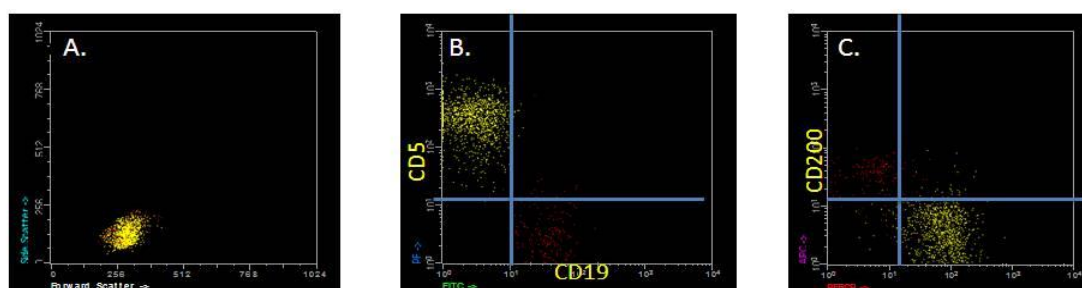


Figure 8 . Gating strategy for controls

A: Lymphocytes isolated by orthogonal light scatter.

B: Lymphocytes defined and separated by CD5 (T cells- yellow) and CD19 (B cells-red)

C: T cells are negative for CD200.

3.4.2 Pathological samples

Appropriate samples with CD19/CD5 co-expressing tumours were stained with 10ul of anti-CD200 monoclonal antibody and the sample was reacquired. The MFI of CD200 (in the APC channel) was then assessed on the tumour population. Based on values derived from the normal controls, a CD200 MFI value of >20 was considered positive. To exclude operator and analysis bias, a single operator calculated all MFIs on controls and on tumour samples.

3.5 Collection of complementary results for study samples

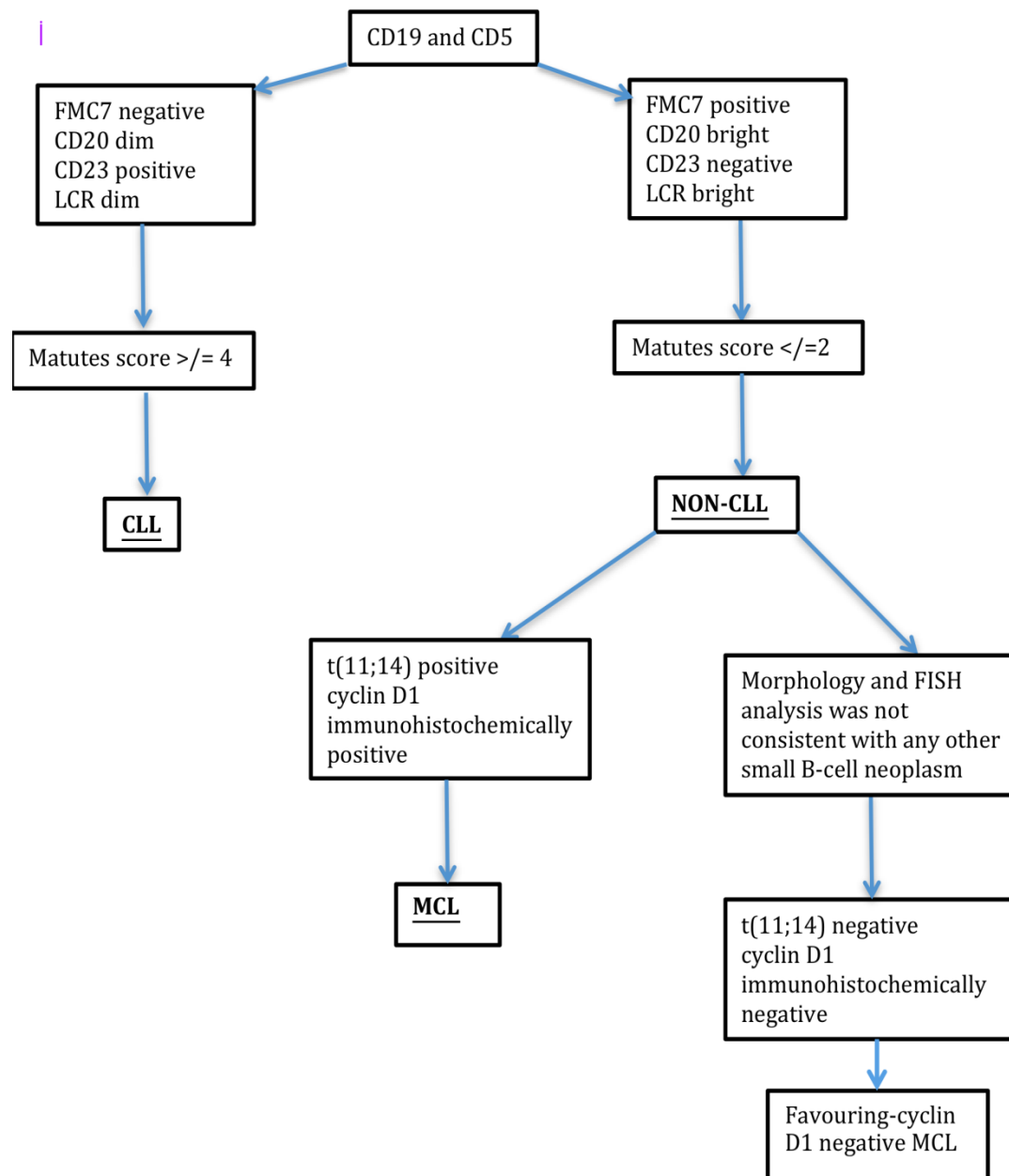
The DISA and Trakcarelaboratory information systems results database of the National

Health Laboratory were accessed for each patient. Available data including white cell counts, bone marrow or lymph node histology, immunohistochemistry, cytogenetics, FISH analysis and B2 microglobulin were collated. Clinical details provided on the histology reports were also collected where available. Please see appendix 1 for the data collection sheet.

3.6 Diagnostic assessment

A diagnosis for each case was made on a hierarchical basis using reported favoured diagnosis (flow cytometry remains the gold standard for CLL diagnosis [2]), followed by Matutes score and FISH analysis or cyclin D1 immunohistochemistry to resolve cases with differential diagnoses. A grey zone Matutes score of 3 was not used for diagnostic allocation in this study. Cases otherwise classical for MCL but lacking either translocation t(11;14) or cyclin D1 were assigned a presumptive diagnosis of cyclin-D1 negative MCL. Please refer to Figure 9 for the diagnostic strategy.

Figure 29. Algorithm of diagnostic strategy undertaken in this study.



3.7 Statistical analysis

Statistical analysis was performed with the assistance of a statistician, using statistical software package (Stata version 4, Statacorp) as well as Graph Pad Prism (version 6). Box and whisker plots illustrate and summarise numerical data. The raw data was normalized to proportion categorical and continuous variables for concordance analyses. Receiver operator curve was used to assess the accuracy of CD200 as a marker of CLL. Sensitivity and specificity for the use of CD200 expression to distinguish CLL from MCL was also calculated. A Mann Whitney test was used to compare clinical and other characteristics of CLL vs. MCL. Results are reported as a two-tailed P value (significance defined as a p value <0.05). Concordance analyses by logistic regression analyses were performed to assess the secondary objectives. These analyses were used to determine the strength of variable relatedness, where CD200 expression was the primary dependent variable. A ratio of >0.85 was defined as statistically significant. Bonferroni corrections were applied to adjust the P-values as several statistical tests (both dependent and independent) were performed.

Descriptive analyses were undertaken, where small sub-group variable numbers hindered statistical power. The profiles of CLL prognostic markers within this cohort are represented graphically.

CHAPTER 4: RESULTS

4.1 Establishment of baseline levels of CD200 expression on normal lymphocytes.

A total of 18 adult control samples were analyzed to establish the range of CD200 on normal lymphocytes. Two controls were excluded *post-hoc* as insufficient cells were collected on re-acquisition. A sample number of 20 was considered acceptable for convenient and timeous control selection.

The isotypic controls were successful in excluding non-specific binding. B cells CD200 MFI expression was evaluated for completeness.

MFI for CD200 expression was predictably low on T cells with a median MFI of 4.46 (2.41-7.48). The findings of the T cell CD200 MFIs were used to define a cut- off for assessing positive CD200 expression. An MFI value of >20 was established as a cut-off positive for CD200 expression.

4.2 Identification of tumours with CD19/CD5 co-expression for evaluation of CD200 expression.

Based on the hierarchical assignment of diagnosis (Figure 8.), CD200 expression was evaluated on a total of 56 patient samples obtained within the collection period of 18 months (see Figure 10. for cohort breakdown).

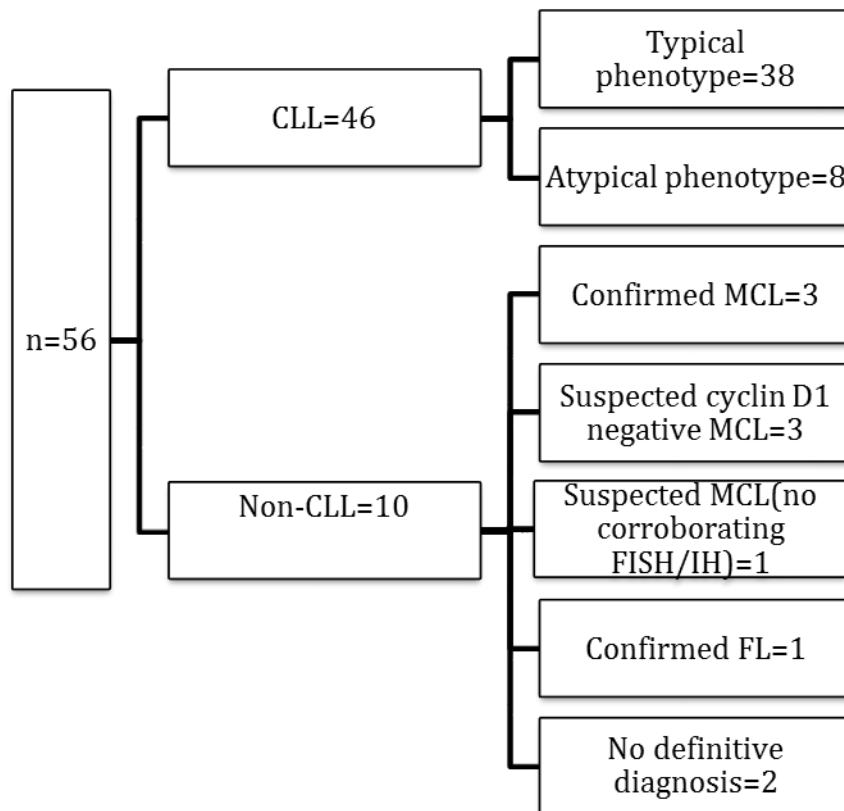


Figure 310. CD19/CD5 co-expressing tumours in this study.

Based on immunophenotypic analysis of CD19/CD5/CD23 and dimmer light chain restriction in combination with a Matutes score of 4 or greater, 46 out of 56 samples fulfilled the diagnosis of CLL.

3 out of 56 were molecularly confirmed MCL. A further 4 of the 56 were highly suspicious for MCL, demonstrating a typical immunophenotype of CD19/CD5/FMC7 and absent CD23. These cases could not be confirmed as MCL owing to a negative t(11;14) or negative cyclin D1 result on immunohistochemical analysis. These cases were postulated to represent cyclin D1-negative MCL variants as they did not fulfil WHO criteria for any other small B cell neoplasm.

The rest of the cohort consisted of a single case of molecularly confirmed FL and 2 cases that were not classifiable (Refer to appendix 3 for raw cohort data).

4.2.1 Epidemiology of CLL in this local cohort

Please refer to Table 6. for CD19/CD5 co-expressing case characteristics.

Table 6. Characteristics of CD19/CD5 tumours in this study including patient demographics

	<u>CLL=46</u>		<u>MCL=7</u>
	<u>Typical</u> <u>immunophenotype</u> <u>=38</u>	<u>Atypical</u> <u>immunophenotype</u> <u>=8*</u>	
<u>Male</u>	22	5	6
<u>Female</u>	16	3	1
<u>Age (years)</u>	65.6 (40-84)	70.4 (60-85)	62.4 (57-73)
<u>WCC (x10⁹/l)</u>	188.4 (3.3-524)	88.6 (3.3-303.9)	39.4 (3.92-94)
<u>Mean CD200 MFI</u>	115.7 (20.4-378)	82.2 (12.2-155.5)	12.5 (5.2-45.8)
<u>Mean CD38%</u>	38.3(0-100)	39.2 (0-98)	n/a
<u>Average Matutes</u>	4	3	2

4.2.1.1 CLL vs. atypical CLL

Within the cohort of 46 CLL patients, 20% demonstrated ambiguous immunophenotypes (with either FMC7 or brighter light chain expression, no case was negative for CD23, which would have been defined as atypical CLL).

The age and gender distribution in CLL vs. atypical CLL were not clinically significant with median ages of 68 (n=8) and 66.5 years (n=32) respectively (p=0.41). The median white cell counts (WCC) of CLL cases (n=36, median WCC $150 \times 10^9/l$) vs atypical CLL cases (n=8, median WCC $90.5 \times 10^9/l$) were not significantly different (p=0.41). CD200 expression was positive (>20 MFI) and comparable in both atypical (n=8, median MFI 81.8) and typical CLL (n=38, median MFI 81) (p=0.75). CD38 expression in atypical and typical cases did not reveal a statistical difference with medians of 23.5% (n=8) and 33.9% (n=26) (p=0.9). The small sample size may be affecting significance. In terms of Matutes score and CLL cases, all typical CLL scored at least 4 (n=38), while the atypical cases scored 3 (n=8).

4.2.1.2 CLL vs. MCL

A stronger male predilection was seen in MCL (n=7). Age differences were insignificant (n=7, median age 60 years) compared with CLL (n= 46, median age 67 years). Differential CD200 expression by tumour cells in MCL and CLL was statistically significant (median MFI of 6 vs. 82 respectively, p<0.001). White cell counts were also significantly different, with lower counts in MCL (median WCC $37 \times 10^9/l$ vs $130 \times 10^9/l$, p=0.0131). Matutes score was predictably 2 or less for MCL cases.

4.3 CD200 expression as a discriminatory marker of CLL from non-CLL cases

CD200 was therefore evaluable on 46 cases of CLL and 7 cases of MCL. CLL populations demonstrated a median CD200 MFI of 109.9 ± 84 (12.2-378) while MCL populations revealed a median of 12.6 ± 14.8 (5.2-45.8). (Refer to Figures 11. and 12. for gating strategies to determine CD200 expression).

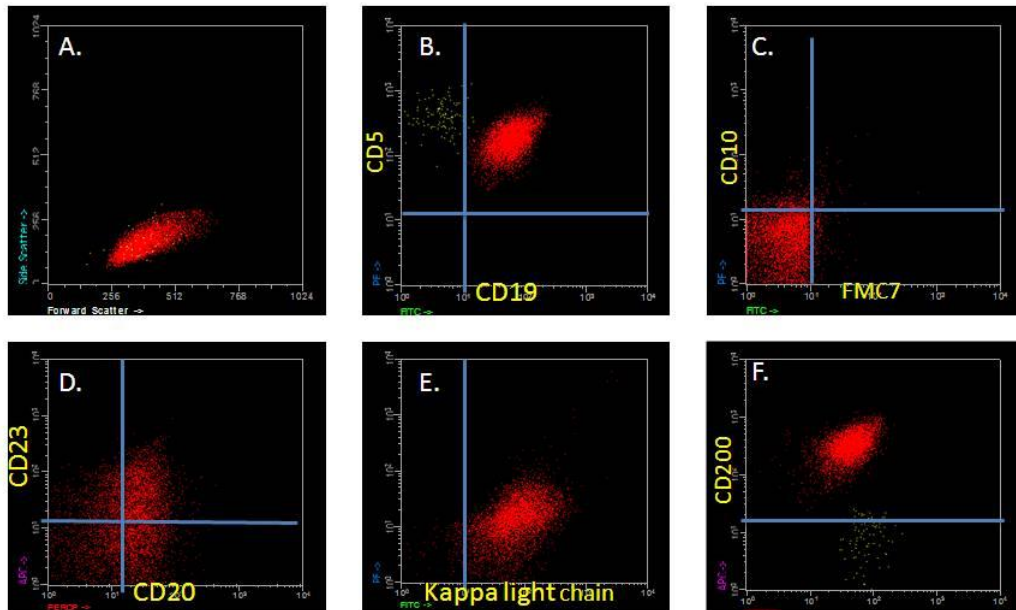


Figure 11. Gating strategy for a case of CLL

A: Lymphocytes isolated by orthogonal light scatter

B: Tumour identified by CD19/CD5 co-expression.

C, D and E: Tumour positive for CD23 and CD20 with light chain restriction, and negative for FMC7 and negative for CD10,

F: Tumour positive for CD200.

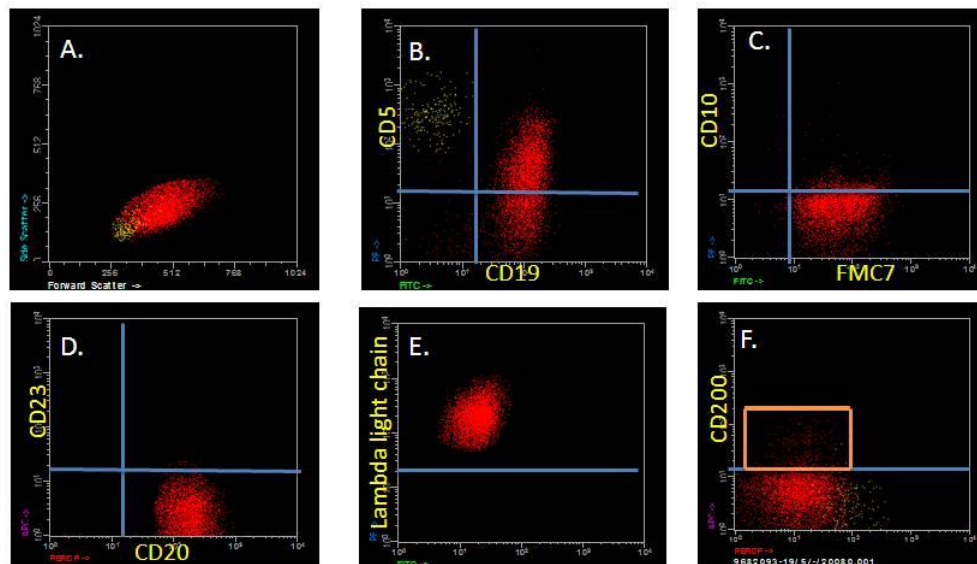


Figure 12. Gating strategy for a case of MCL

A: Lymphocytes isolated by orthogonal light scatter

B: Tumour identified by CD19/CD5 co-expression.

C: Tumour positive for FMC7 and negative for CD10.

D and E: Tumour positive for CD20 and light chain restriction, but not CD23.

F: Tumour negative for CD200. The inset square demonstrates that non-clonal background B cells may be dimly positive for CD200.

Figure 13. demonstrates the relative distribution of CD200 expression in CLL vs MCL (p<0.001).

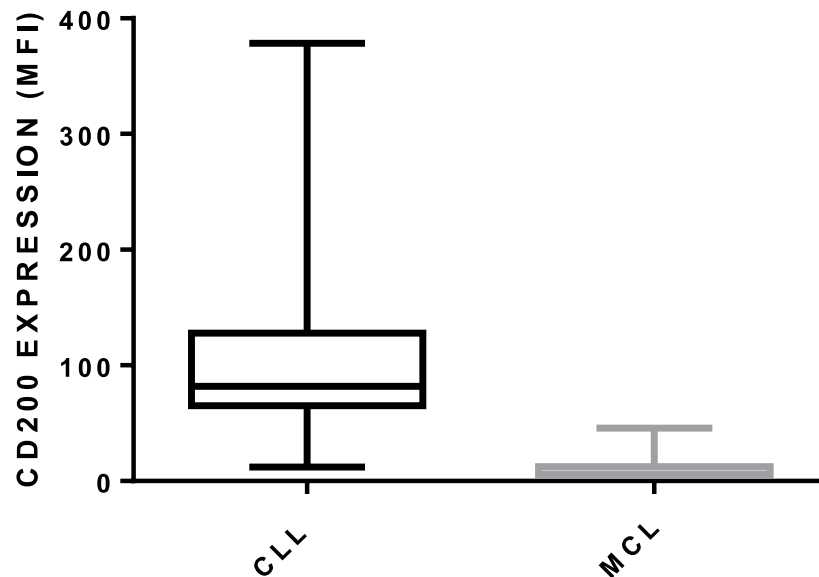


Figure 413. Box and whisker plot illustrating median CD200 expression in CLL vs MCL.

The sensitivity of CD200 expression to differentiate CLL from MCL by immunophenotypic analysis was 97.8% (88.71-99.95) with a 95% confidence index (CI), and with a specificity of 85.7% (42.13-99.64) 95% CI (refer to contingency Table 7). Likelihood ratio for the prediction of a true case of CLL using CD200 expression was calculated at 6.8 (1.12-42.08) 95% CI, while the negative likelihood ratio proved to be 0.02 (0-0.18) 95% CI.

Table 7. Calculation of Sensitivity and Specificity in a diagnosis of CLL

	<u>CLL (n=46)</u>	<u>MCL cases (n=7)</u>
CD200 positive (as defined by an CD200 MFI of >20)	45	1
CD200 negative(as defined by an CD200 MFI of <20)	1	6

4.4 Traditional Matutes score and CD200 expression are comparable and synergistic during immunophenotypic assessment of CLL and MCL.

Receiver operating characteristics (ROC) demonstrate that CD200 expression in CLL is specific and sensitive, and highly concordant with Matutes scoring (demonstrated in Figure 14).

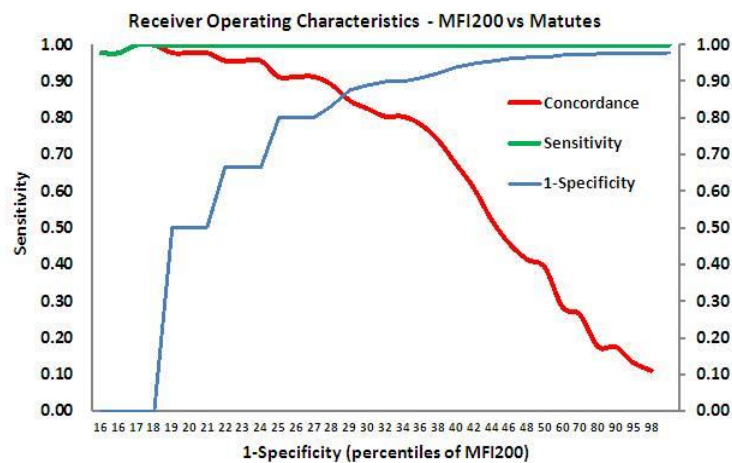


Figure 14. Receiver operating curve for CD200 expression and Matutes score. All curves represented are >45 degrees, indicating that both tests perform well and are concordant.

4.5 The profile of the commonly used prognostic markers found in this cohort of CLL patients.

This study aimed to evaluate CD200 expression as a prognostic marker in CLL compared with other traditional prognostic markers. In this study the small numbers of tests requested however, made analysis of B2 microglobulin, chromosomal studies, and LDT difficult. CD38% expression, FISH analysis and WCC remained evaluable.

The findings of the raw data analysis indicate that of the commonly available prognostic biomarkers, CD38 expression is the most widely used (46 out of 46). FISH analysis was available in 28 out of 46 (60% of cases). Unfortunately the use of B2 microglobulin was found to be unpredictable and clinician-dependent, available in only 6 cases. Similarly cytogenetic analysis was requested in only 11 out of 46 (24% of cases), where karyotypic culture failed in 5 out of 11 and normal karyotypes were yielded in the remaining 6 out of 11. Follow-up white cell counts within the study period were not available in the majority of cases for the calculation of LDT.

Owing to these unexpected findings during *post-hoc* data review, and for the purpose of maximizing statistical power, B2 microglobulin, chromosomal studies and LDT were eliminated from the analysis. CD38 expression, FISH findings and presentation white cell counts were therefore selected for concordance analyses.

4.5.1 CD200 expression as a prognostic marker vs. CD38%.

Percentage CD38 expression did not correlate statistically meaningfully with CD200 expression when compared as both a categorical value as evidenced by logistic regression analyses (concordance ratio 0.69). See figure 15.

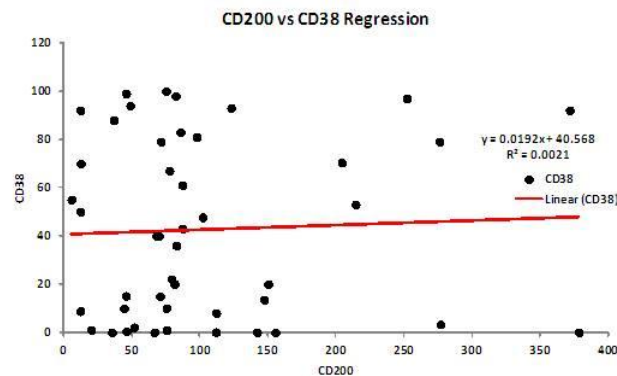


Figure 15: Simple linear regression

This analysis showed an insignificant relationship between absolute CD38 % and intensity of CD200 expression.

4.5.2 CD200 expression as a prognostic marker vs. FISH analysis.

A non-significant association was found between CD200 expression and individual FISH abnormalities, where concordance ratios ranged from 0.3- 0.56. The analysis was limited by the small sample sizes on subgroup analysis.

4.5.3 CD200 expression vs. absolute White cell counts.

In the majority of cases within the 18 months data collection period, a chronologically appropriate follow-up white cell count was not traceable. Lymphocyte doubling times were therefore excluded from analysis. Evaluation of the absolute presenting white cell count instead revealed that CD200 expression was highly concordant (dependent relationship) with the white cell count (ratio 0.97). A non-significant association was found between WCC and CD38 expression (ratio 0.5) See figure 16. White cell count and CD38 expression appear independent in this study ($p=0.54$).

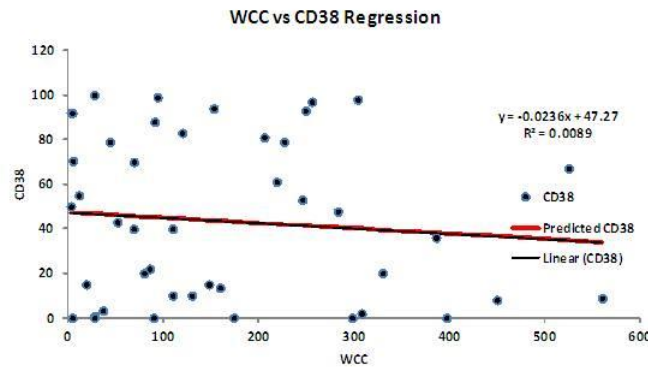


Figure 16: Simple linear regression
This analysis showed an insignificant relationship between absolute CD38 % and absolute white cell count.

4.6 FISH findings in this cohort

Of all CLL cases 29 out of 46 had successful FISH analysis. Abnormalities were found in 21 out of 29 (72%) cases. Two simultaneous FISH abnormalities were the second commonest finding (24.4%) after trisomy 12 (27.6%), isolated 13q deletion comprised $\pm 7\%$ of all findings. 8 out of 29 (27.5%) of these cases had no abnormality detected. 17p deletion was detected in 4 cases but always as part of a complex FISH abnormality (Refer to Figure 17: Graphical breakdown of FISH lesions in this study).

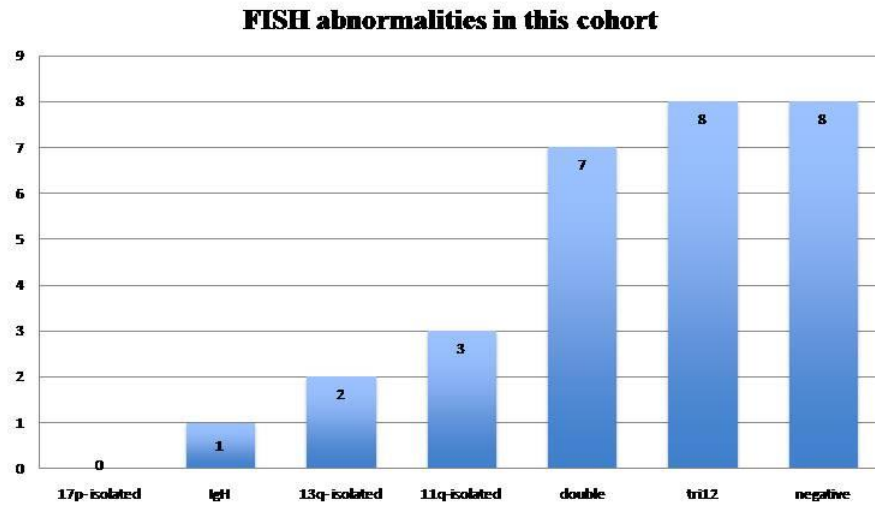


Figure 17. FISH findings in this CLL cohort. The most common abnormality was trisomy 12. compared to 13q deletion (most common as documented by the W.H.O²)

4.7 Poor prognostic markers in this cohort

While subgroup statistical analyses were not feasible in view of the limited numbers of cases referred for analysis, some observations were made. Poor prognostic FISH findings and tumour expression of CD38 of >30% (i.e. poorer prognosis [1,9]) did not aggregate in the majority of cases , refer to Table 8. Double abnormalities including 17p- were found most frequently in relation to CD38 expression of <30% (generally associated with a better prognosis [1,9]), refer to Table 9. Higher CD38 expression was associated most often with trisomy 12 followed by negative FISH analysis (refer to Table 8). The combined significance of conflicting independent prognostic markers is not clarified in the literature.

Table 8 : Descriptive Comparison of CD38% as poor prognostic marker and respective FISH findings.

<u>CD38>30%</u>	<u>FISH</u>	<u>Frequency (n=15)</u>	<u>% of cases</u>
	Isolated Trisomy 12	5	33
	All FISH negative	5	3
	Isolated 11 q-	2	13
	Double abnormality	2	13
	IgH@ rearrangement	1	6.7
	Isolated 13q-	0	0
	Isolated 17p-	0	0

Table 9. Descriptive Comparison of CD38% as a favourable prognostic marker and respective FISH findings.

<u>CD38<30%</u>	<u>FISH</u>	<u>Frequency (n=14 cases)</u>	<u>% of cases</u>
	Double abnormality	5	35.7
	Isolated Trisomy 12	3	21.4
	Negative	3	21.4
	Isolated 13q-	2	14.2
	Isolated 11q-	1	7
	Isolated 17p-	0	0

CHAPTER 5. DISCUSSION

5.1 The diagnostic benefit of measuring CD200 expression in CD19/CD5 co-expressing B cell tumours, particularly its expression in CLL.

This study concurred with current literature in showing that CD200 is useful in conventional immunophenotyping, to distinguish CLL from MCL. CD200 will be added routinely to all panels for the evaluation of mature B-cell lymphoproliferative disorders in our centre. An MFI value of 20 was established as a valid positive value for the flow cytometer in comparison with the normal controls sourced within our setting. It was established that T cells may act as an appropriate negative control for CD200 expression, while normal (non-clonal) B cells are negative to weakly positive, in keeping with the literature [72]. Sensitivity and specificity for CD200 expression in CLL cases was 97% and 85% respectively. CD200 was dimly to intensely positive in all cases of CLL except one in this cohort, with an MFI range of 20.4-378. CD200 expression was negative in all except one case with a presumptive diagnosis of MCL (5.1-12.2). All MCL cases were positive for FMC7, no case expressed CD23 in this cohort and average Matutes score was valid in distinguishing MCL from CLL during initial immunophenotypic assessment.

The single false negative case for CD200 expression was a histologically diagnosed SLL. The tissue submitted for immunophenotypic analysis was a blood sample with absolute WCC of $3.3 \times 10^9/l$ of which 7% was determined to be disease. Bone marrow investigation and FISH analysis were not submitted, however, immunohistochemistry confirmed a small cell B lymphoid infiltrate, showing immunoreactivity for CD20, CD5 and CD23 with absent cyclin D1. The clinical presentation of mediastinal disease and complicating Superior Vena Caval Syndrome, in the presence of lymphadenopathy was unusual in this case.

The false negative result may be attributable to the minimal disease burden within the sample referred. The detection of CD200 is dependent on the number of leucocytes present as well as the size of the CD200 expressing target population. An alternative hypothesis is that SLL is phenotypically different in its CD200 expression to CLL, possibly mechanistic in the lymphomatous versus leukaemic stages of the same disease.

The single false positive case for CD200 expression in a case of MCL had an otherwise typical MCL immunophenotype, a Matutes score of 1 and was negative for both t(11;14) or cyclin D1 immunohistochemistry. The sample submitted for immunophenotyping was peripheral blood with an absolute WCC of $94 \times 10^9/l$ of which 70-75% comprised disease. The latter expressed CD20 (normal intensity), FMC7, light chain restriction of normal intensity and absent CD23. FISH analysis was positive for trisomy 12. The clinical presentation was that of a hepatomegaly. This tumour is still a diagnostic challenge. It may be a B cell neoplasm, not yet classifiable. The possibility that this represents a variant MCL (for example with cyclin D2 or D3 abnormality) could not be excluded since these assays are not performed routinely at our centre. Attempts to trace the patient failed and it is unclear what the clinical course in this patient was.

5.2 Matutes score and CD200 expression

CD200 expression in conjunction with Matutes score is synergistic during immunophenotypic assessment. The modified Matutes score which includes evaluation of CD200 [46] will further refine the differential diagnostic evaluation of CD19/CD5 co-expressing tumours, supplementing immunophenotyping when validated for diagnostic use.

5.3 Limitations of CD200 measurement immunophenotypically

The limitations of this test must be considered during its performance, which rely on the number of cellular events examinable by flow cytometry. The total white cell count as well as the absolute number of clonal cells present in the sample determines the limits of detection. Specimens that are pauci-cellular (e.g. leucopenia, lymphopenia, fluids with minimal clonal infiltration) may not give a reliable representation of CD200 expression. It is recommended that CD200 expression be interpreted in the context of the following analytical prerequisites:

- 1) An adequate number of viable flow cytometry events.
- 2) Defined gating strategies where T cells (defined by CD5 expression), reactive B cells (defined by CD19 expression) and clonal B cell (defined by CD19/CD5 co-expression) are separable and enumerable.
- 3) The presence of background T cells used as the negative internal control.
- 4) Analysis of CD200 on small clonal populations (of <10%) should be interpreted with marked reserve and reported as such.

In addition, it should be noted that other small lymphoid B-cell neoplasms may exhibit CD200 (with or without CD19/CD5 co-expression), such as LPL and subsets of MZL as described in the literature [57, 72].

In such cases, a consensus diagnosis should be reached in conjunction with the morphological, molecular and clinical contexts. Owing to the small sample size and the rarity of these tumours, a comparison was not possible between CLL and non-mantle cell CD19/CD5 co-expressing B-cell neoplasms. The presence of CD200 expression is not validated for use in such cases in the literature.

5.4 Comparison of CD200 expression with laboratory markers of known prognostic significance.

A secondary aim of this study was to assess whether CD200 would add value to the established prognostic markers, which are offered at our centre. Unfortunately, not all prognostic markers were requested by the clinicians for all patients and many markers like the B2 microglobulin had to be excluded in this study. No significant relationships between the intensity of CD200 expression by the CLL tumour cells and other prognostic markers (CD38 expression, 13q-, trisomy 12 or 17p-) could be described. In some cases, the individual subgroups were too small to allow adequate comparison. Interestingly, CD38 expression did also not correlate with the traditional FISH markers of poor prognosis. The utility of CD38 expression as a prognostic marker should be compared with clinical outcomes to decide its utility in our setting.

Cytogenetic samples, referred in less than half the cases, yielded no growth or normal karyotypes in all other cases. Conventional karyotyping is the least sensitive and least cost effective prognostic marker for CLL in the setting of this study and a recommendation will be made to discontinue its use.

Based on this small sample size, the findings suggest that CD200 expression intensity may be a function of the WCC. There was no convincing relationship between CD38 expression and WCC. Examination of this relationship was particularly useful, as CD38 as a function of WCC has not been formally investigated in the literature. CD38 expression as a prognostic marker, independent of white cell count as a confounder (as in the case of CD200 expression), is therefore meaningful.

The findings of other studies [57,59,79], suggest that CD200 still has relevance as a prognostic marker in other haematological neoplasms such as Myeloma and Acute Myeloid Leukemia.

5.5 Epidemiology of CLL in South African Context

CLL is the commonest CD19/CD5 co-expressing small mature B cell neoplasm in this cohort, in line with world statistics [2]. The age and gender distributions were similar to that described in the literature [2]. Atypical immunophenotypes were not uncommon, comprising 17% of cases, unfortunately morphological atypia was not co-assessed during this study. CD38 expression of >30% on the tumour population (associated with poorer prognosis in the literature) was seen in half the cases studied.

FISH analysis was requested in two thirds of cases. The most common abnormality in this group of patients was trisomy 12, which is disparate from WHO statistics, which describes 13q deletion as the commonest finding[2]. Trisomy 12, the commonest documented finding in *atypical* CLL[83], was found to be the comparable in this cohort of atypical cases.

A compelling finding that requires further elucidation is that CD38 expression did not aggregate with poor and better prognostic FISH findings. 17p deletion and double abnormalities were more frequent in tumours with reduced CD38 expression. The bearing of CD38 expression as a prognostic tool in the context of a concomitant 17p or 11q deletion is therefore uncertain. Moreover, double FISH abnormalities were the second most common finding in this cohort. The clinical significance of the latter is not clarified in the literature. Follow-up and evaluation of this subset of cases in terms of clinical response is unquestionably needed.

5.6 Prognostication of CLL in South African Context

To date, there is no integrated prognostic calculator available for CLL that would be applicable in a resource-restricted setting. Local guidelines for diagnosis and treatment stratification are required to standardize and optimize care across centres. This is of particular relevance when prognostic markers are at variance (such as 17p deletion and low CD38 expression).

5.7 Observations from this study with potential clinical consequences

Chronic Lymphomas, by virtue of their “indolent nature”, appear sub-optimally investigated in the public health setting. Clinical prognostication by staging systems, patient fitness and comorbidities are only of use in the decision to initiate therapy and not in prediction of treatment-free or overall survival.

Laboratory markers of disease behavior are useful in treatment planning. This should ultimately outweigh the perceived cost-saving benefits of omitting certain tests during

workup. 17 out of 46 cases appear to have had no other laboratory-based prognostication other than CD38 expression. It would have been of value in examining the reasons for the lack of laboratory-based prognostication in these instances.

In addition, it is noted that not all cases were followed through with bone marrow biopsies. Current international literature states that bone marrow investigation is not necessary for diagnosis of CLL [31]. While HIV status was not investigated in this cohort, it is recommended that a bone marrow biopsy be performed especially where concomitant infections such as TB and HIV are prevalent. In addition the pattern of infiltration is prognostically valuable [19].

5.8 Observations from this study with potential biological consequences

CLL and MCL remain the foremost differentials in CD19/CD5 co-expressing tumours. Cases that were excluded from analysis in this cohort, however, included one case of molecularly confirmed Follicular Lymphoma and two other cases that were not classifiable. The wider differential diagnosis of small B neoplasms that should always mandate suspicion during immunophenotypic analysis includes Follicular lymphoma, Lymphoplasmacytic Lymphoma, Marginal Zone Lymphomas and Splenic Lymphomas. Although the latter B cell lymphoproliferative neoplasms are less common, our cohort confirms that such entities can sporadically complicate definitive diagnosis. A molecular characterization of disease should not be compromised, even in a resource-restricted environment.

Secondly, of the MCL cases in this study group, 3 out of 7 (43%) were neither t(11;14) *nor* cyclin D1 immunohistochemically reactive. Unfortunately both modalities could not be tested

side-by-side in these three cases, to exclude the possibility of discordance. In addition, the diagnosis of the rare sub-group of cyclinD1 negative MCL could not be confirmed. New molecular methods for the confirmation of cyclin variants would have been useful in these exceptional cases. In order to justify the cost: benefit ratio of more sophisticated Cyclin detection methods, further study of a larger South African cohort is needed to verify the true incidence of this subset of patients.

5.9 Recommendations for the routine diagnosis of CLL in a South African context.

The incorporation of CD200 in the diagnostic processing of CD19/CD5 co-expressing tumours has utility. Its inclusion is recommended as part of the chronic lymphoid panel during immunophenotypic assessment. A recommended algorithm for its use is illustrated by flow diagram (refer to figure 18).

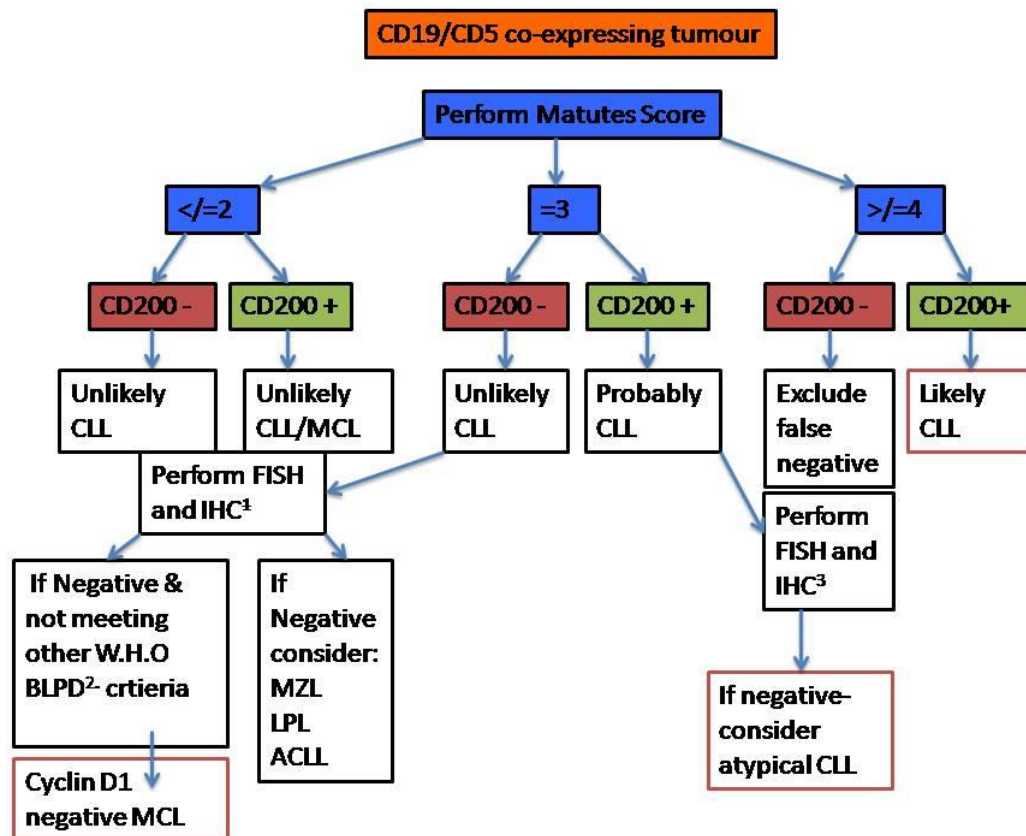


Figure 18: Schema for use of CD200 expression in the diagnosis of CLL.

¹FISH: translocation t(11;14), CyclinD1 Break apart, Immunohistochemistry (IHC)-cyclin D1, if negative then add translocation t(14;18).

²BLPD- B- lymphoproliferative neoplasm as per WHO classification of Haematolymphoid pathology.

³FISH: translocation t(11;14), CyclinD1 Break apart, IHC- cyclin D1.

It is highly suggested that bone marrow investigation be included as a mandatory diagnostic tool, to rule out concomitant infections, determine extent of infiltrate and to assess large cell transformation. The bone marrow tissue can also be used for cyclin D1 immunohistochemistry. A paucity of involved tissue for additional testing often complicates the course of definitive diagnosis (as noted during this study).

Cytogenetic analysis appears to be of little value in resource-constricted environment, unlike FISH analysis. From a cost: benefit perspective the poor prognostic FISH 17p and 11q should be prioritized- as these variables have bearing on treatment and clinical response prediction. The clinical significance of double cytogenetic abnormalities remains to be clarified. Previous local studies have demonstrated that double abnormalities are not infrequent in our setting. Further study with regard to the clinical implications of these findings is required on a larger scale.

In addition, an integrated laboratory prognostic scoring system for CLL is required, particularly in view of the number of prognostic variables. Under-utilization of prognostic markers in our setting may be attributable to the complexity and disagreement of independent markers. A simplified prognostic score would, therefore be of particular value when markers such as CD38 expression and 17p- deletion are discordant.

5.10 Other interesting observations

A number of unusual cases presented themselves within this cohort. One of these cases was included within the CLL study group that demonstrated an IgH translocation. Chromosome 14q translocations in the context of CLL have been described as an infrequent occurrence [84]. They may signify clonal evolution [84]. The translocation partner was not identified of the case in point (translocation t(11;14), and t(14;18) FISH analysis were negative). Since the case morphologically fulfilled the profile of a small cell tumour, translocation with MYC-t(8;14) was not performed. The immunophenotypic differential of CLL/ MCL (and borderline Matutes score) of 3 could therefore not be resolved molecularly. The patient presented non-specifically with hepatosplenomegaly and constitutional symptoms. It would have been of

value in assessing the clinical outcome of this patient in order to clarify the implications of the IgH translocation in this case, which did not fulfill WHO criteria for any other small B cell neoplasm.

The second case was peculiar in that the immunophenotype was atypical demonstrating small cells with CD19, CD5, dim CD20, FMC7, lambda light chain restriction, CD10 and no CD23. The tumour population was negative for CD200. The patient presented with a deep venous thrombosis as a result of intra-abdominal adenopathy. This case exhibited classical t(14;18) and was thus diagnosed as a Follicular Lymphoma.

Two other cases of B cell neoplasms with CD19/CD5 co-expression and unusual immunophenotype were excluded from analysis, as a definitive WHO diagnosis could not be made. This underscores fact that not all small cell “indolent” B neoplasms behave in a way that is currently classifiable. Laboratory (including molecular studies) and clinical consensus are indicated in these cases.

5.11. Limitations of this study

5.11.1 Logistical impediments

Over the time period of this study, the laboratory information system (LIS) used was changed. Owing to this, location of test sets and result histories were hindered. The inclusion of cases from across province also proved problematic, since provincial LISs were not linked prior to the transition. Access to complementary tests such as bone marrow investigations was therefore not always possible.

The number of cases obtained within the given period, limited this study. In addition to small total cohort number, the information required for secondary objectives assessment, was not available for every case. This further constrained statistical analyses of subgroups. Ethnic differences were not explored as demographic information was lacking in the majority of cases. Similarly clinical information was not available for a significant proportion of the cohort.

5.11.2 Technical impediments

As mentioned, data was insufficient for assessment of three of the secondary objectives, which had to be excluded ultimately. Two controls were omitted post hoc, owing to paucicellularity. Tissue for FISH analysis and immunohistochemistry was not available in some cases. Two of the cases were therefore excluded, in which diagnosis was not definitive.

Clinical follow up of this cohort would have also been highly useful to contextualize the findings of this study. Relative chronicity of these B cell entities prevented the feasibility of follow up. This was further hampered by the distribution of samples, which were obtained across provinces.

CHAPTER 6: CONCLUSION

Unlike acute leukaemias, indolent leukaemias/lymphomas are often underestimated in terms of overall severity and clinical outcome. CLL is in fact a heterogeneous entity with the potential to behave aggressively. MCL, an alternative CD19/CD5 diagnosis, is by its biological nature more aggressive, requiring different chemotherapy to that offered in CLL. Accurate laboratory differentiation of these B cell neoplasms is therefore critical to patient care. In addition, prognostic markers in CLL are imperative in informing therapeutic strategy and predicting disease course. The value of molecular diagnostics and prognostication cannot be undermined for health economic frugality. Inclusion and ongoing validation of novel biomarkers are essential to the advancement of any health care platform whether first or third-world, private or public. Conclusions made in this study follow:

CD200 expression assists in the diagnosis of CLL and is a qualitative measure.

Its use as a surrogate prognostic marker was not verified, however, the sample size was too small to confirm this.

Whether documentation of its expression will be useful for future targeted therapy remains to be seen.

There are limitations to the measurement of CD200 expression, which should take into consideration the technical requisites when performed. These include type of sample and expected cellularity, tumour burden within sample and the presence of reactive T cells for use as an internal control.

While some epidemiological similarities remain, such as sex and age distribution, the FISH profile of this cohort are sufficiently disparate from WHO based statistics[2] to warrant larger cohort studies of CLL in the South African setting. Double FISH abnormalities were

common, while 13q deletion did not occur as the most frequent lesion in the sample investigated. The clinical significance of such differences requires further study.

A worrying trend of inadequate workup of small cell B lymphoid neoplasms was noted in this cohort. Concessions should not be made during workup such as bone marrow investigation and molecular techniques. Indolence is relative to the subtype of CLL and should be treated with the same priority that is accorded to all malignancies.

Future research avenues in CLL for a South African/ African setting:

- Validation of an inclusive scoring matrix, aiming to simplify CLL prognostics in a resource-restricted background.
- Determination of the clinical and prognostic relevance of Double Fish abnormalities in CLL, which is not formally documented in the literature.
- A guideline on appropriate investigation for chronic lymphomas (quaternary to secondary level use) to ensure prompt recognition and appropriate referral of indolent lymphomas that may otherwise be neglected.

APPENDIX

Appendix 1. Data collection sheet

Patient Record Number	Age	Gender	Immunophenotype and % disease	Reported diagnosis	CD38% reported	CD200 MFI	Matures score	Cytogenetic analysis	FISH /IHC	1.WCC 2. follow up WCC	Serum B2 microglobulin

Appendix 2. Data CLL cohort

sex	age	diagnosis	MFI 200	CD38	wcc	matutes Score	FISH 17p	11q	trisomy 12	13q-	igH
m	68	CLL	27,86	0		4					
m	70	CLL	36,82	1	91	3	0	0	1	0	
m	75	CLL	44,29	0	130	3	0	1	1	0	
f	78	CLL	45,62	0	148	4					
f	74	CLL	46,12	0	28	4	0	0	0	1	
f	81	CLL	48,84	1	153	4	0	0	1	0	
f	67	CLL	61,02	0	nd	4					
m	68	CLL	69,85	1	68.93	4	0	0	0	0	
m	82	CLL	70,72	0	19.52	4					
f	67	CLL	71,31	1	227	4	0	1	0	0	
m	66	CLL	75,2	1	27.7	3					
m	54	CLL	75,66	0	28	4	0	1	0	0	
f	74	CLL	79,15	1		4					
f	50	CLL	79,24	0	86	4	0	0	1	0	
f	57	CLL	82,71	1	386	4			1		
f	46	CLL	85,82	1	120	3	0	0	0	0	
m	65	CLL	87,24	1	52	3					
f	78	CLL	97,69	1	206	4	0	0	0	0	
f	70	CLL	99,52	1		4					
m	47	CLL	112	0	450	4					
f	69	CLL	150,24	0	330	4					
m	76	CLL	252,12	1	256	4	0	0	0	0	
f	60	CLL	77,66	1	524,9	4					
m	69	CLL	51,68	0	308	4					
f	63	CLL	111,79	0	28	3	0	0	1	0	
m	40	CLL	378,57	0	4,8	4	0	0	0	0	
m	79	CLL	147,2	0	159,6	4	0	0	0	0	
m	66	CLL	66,6	0	397	4	1	0	0	1	
m	72	CLL	155,49	0	90	3	1	0	0	1	
m	64	CLL	82,17	1	303,9	3	1	0	0	1	
f	85	SLL	12,2	1	3,3	2					
m	82	CLL	142,05	0	174	4					
m	64	CLL	204,39	1	5,52	3	0	0	1	0	
m	62	CLL	276,1	1	44	4					
m	41	CLL	123	1	249	4	0	0	1	0	
m	60	CLL	102,2	1	283	3	0	0	0	0	1
m	52	CLL	214,45	1	246	4	0	0	0	0	
m	82	CLL	75,47	0	110	4					

m	65	CLL	67,97	1	110	4	0	0	1	1	
m	58	CLL	94	0		4					
sex	age	diagnosis	MFI 200	CD38	wcc	matutes Score	FISH 17p	11q	trisomy 12	13q-	igh
m	69	CLL	371,9	1	3,3	4	0	1	0	0	
f	75	CLL (t(11;14) negative	20,14	0	272	4	1	0	0	1	
f	50	CLL	35,39	0	298	4	0	0	0	1	
m	84	CLL	87,17	1	219	3					
f	67	CLL	81,4	0	80	3	0	0	1	1	
f	66	CLL	276,9	0	37	4	0	0	0	0	

Appendix 3. Data non-CLL CD19/CD5 co-expressing tumours.

Sex	Age	Diagnosis	CD200	WCC	Matutes score	FISH 17p	11q	tril2	13q	IgH	t(11;14)	Cyclin B/A	
m	59	MCL t(11;14) neg	5,18	3,92	2						0	0	t(11;14)/b/a unsuccessful
m	57	MCL cyclin d1 pos	5,74	11,5	3								cyclin d1 pos
m	71	MCL t(11;14) neg	6,04	45	1						0	0	t(11;14)neg/b/a unsuccessful
m	57	blastoid MCL	7,45	37	2			1	1		1		tri 12, 13q14, bcl6, igh, t(11;14), 14;18
f	73	MCL (t(11;14) negative/cyclin D1 negative	45,8	94	1	0	0	1	0		0		tri12 t(11;14) neg/cyclin D1 negative
m	60	MCL t(11;14) positive	5,22	15	1						1		t(11;14) positive

m	60	MCL (no FISH)	12,5	69,37	2								inadequate for FISH/IH
f	57	FL	12,2	4,6	1								t(14:18)
m	24	?other t(11;14) negative	114,16	394	2	0	0	0	0	1	0		Igh/ -t(11;14)neg, cyclin d1 neg
m	60	no definitive (fish not complete)	12,2	560	4	0	0	0	0				neg/ t(11;14) not done

Appendix 4. Raw control Data

CD200 MFI on B cells	CD200 MFI on T cells	CD200 MFI isotypic control
11,07	2,41	3,55
9,62	3,05	3,71
32,19	3,14	4,70
16,12	3,60	5,48
30,70	4,03	9,92
31,38	4,20	9,32
15,44	4,22	5,00
19,44	4,22	5,88
11,45	4,37	6,51
19,36	4,53	6,60
26,70	4,58	6,89
15,58	4,71	9,52
11,09	4,81	7,36
23,55	4,93	12,65
18,86	5,26	7,64
23,09	5,42	6,03
11,60	5,57	9,40
16,56	7,48	13,42

17,71	4,45	6,75
19,10	4,47	7,42

Appendix 5. Contingency tables

Contingency table

Matutes MF200 29 percentile

	MF200 29 percentile		Total	Sensitivity	Specificity
	T=1	F=0		1 - β	1 - α
Matutes T =1	38	7	45	1.0000	
Matutes F=0	0	1	1		0.1250
	38	8	46	1.0000	0.1250

Concordance 0.8478

In this contingency table a positive (true=T) Matutes score was defined as all scores of $\geq$ to 4, conversely scores of 3 or less were defined as negative (false=F). As previously stated, cases positive for CD200 expression demonstrated MFIs of >20 .

Matutes score v.s CD200 expression in CLL were concordant (concordance 0.85).

Contingency table

Prognosis: CD38

	CD38/ 1=none/0=yes		<i>Total</i>	Sensitivity 1 - β	Specificity 1 - α
	T=1	F=0			
Prognosis T=1 good	12	5	17	0.6667	0.7222
Prognosis F=0 bad	6	13	19		
	18	18	36	0.6667	0.7222
Concordance				0.6944	

In this contingency table Prognosis T=1 represents CD38 of <30% which is good in comparison to the converse, where CD38 of >30% (Prognosis F=0) is considered poorer prognosis. Intensity of CD200 expression was shown to have a weak clinically insignificant relationship with the prognosis conferred by by tumour expression of CD38 (concordance 0.69).

Contingency table

Prognosis: C13q

	C13q/ 1=none/0=yes		Total	Sensitivity	Specificity
	T=1	F=0		1 - β	1 - α
Prognosis T=1 good	6	11	17	0.5455	
Prognosis F=0 bad	5	14	19		0.5600
	11	25	36	0.5455	0.5600

Contingency table

Prognosis: FISH17p

Concordance 0.5556

	FISH17p/ 1=none/0=yes		Total	Sensitivity	Specificity
	T=1	F=0		1 - β	1 - α
Prognosis T=1 good	6	11	17	0.4615	
Prognosis F=0 bad	7	12	19		0.5217
	13	23	36	0.4615	0.5217

Concordance 0.5000

Contingency table

Prognosis: Trisomy12

	Trisomy12/ 1=none/0=yes		Total	Sensitivity	Specificity
	T=1	F=0		1 - β	1 - α
Prognosis T=1 good	0	17	17	0.0000	
Prognosis F=0 bad	8	11	19		0.3929
	8	28	36	0.0000	0.3929

Concordance 0.3056

Contingency table

Prognosis: C11q

	C11q/ 1=none/0=yes		Total	Sensitivity	Specificity
	T=1	F=0		1 - β	1 - α
Prognosis T=1 good	6	11	17	0.4615	
Prognosis F=0 bad	7	12	19		0.5217
	13	23	36	0.4615	0.5217

Concordance 0.5000

Contingency table

Prognosis: Trisomy 10

This set of contingency tables maintain CD200 expression as the dependent variable against each FISH prognostic value. For each Prognosis T=1 signifies good prognosis, converse for Prognosis F=0. Concordance analyses demonstrate that no FISH lesion is concordant with the intensity of CD200 expression.

Contingency table

MR200 WCC 17:14 percentiles

	MR200 17 percentile		Total	Sensitivity	Specificity
	T=1	F=0		1 - !	1 - "
WCC 14 percentile T=1	45	1	46	1.0000	
WCC 14 percentile F=0	0	0	0		0.0000
	45	1	46	1.0000	0.0000
Concordance				0.9783	

CD200 intensity as dependent variable against WCC= white cell count revealed high concordance (0.98).

Appendix 6. Ethics Approval



M130279

R14/49 Dr Karissa Mannaru

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130279

NAME:
(Principal Investigator)

Dr Karissa Mannaru

DEPARTMENT:

Molecular Medicine & Haematology
National Health Laboratory Services

PROJECT TITLE:

CD200 Expression in Chronic Lymphocytic
Leukemia (under blanket approval M090688)

DATE CONSIDERED:

Ad hoc

DECISION:

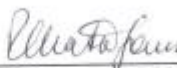
Approved unconditionally

CONDITIONS:

SUPERVISOR:

Professor Lesley Scott

APPROVED BY:


Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 10/06/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**

Principal Investigator Signature

Date

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