The Effects of the Retinoid Drug Acitretin on CLL Cells with Emphasis on CD38 Expression and Cell Homing

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CITATION

Mohammed, Sally (2021): The Effects of the Retinoid Drug Acitretin on CLL Cells with Emphasis on CD38 Expression and Cell Homing. Royal College of Surgeons in Ireland. Thesis.
https://doi.org/10.25419/rcsi.15036228.v1

DOI

10.25419/rcsi.15036228.v1

URL

https://repository.rcsi.com/articles/thesis/The_Effects_of_the_Retinoid_Drug_Acitretin_on_CLL_Cells_with_Emphasis_on_CD38_Expression_and_Cell_Homing/15036228/1

The effects of the retinoid drug acitretin on CLL cells with emphasis on CD38 expression and cell homing

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Medicine

Under the academic supervision of:
Dr Philip Murphy
Dr John Quinn
Professor Patrick Thornton

September 2019

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Medicine, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Table of Contents

List of abbreviations 8

List of Figures: 11

List of Tables: 12

Summary 13

Acknowledgements 15

1. Introduction 17

Chronic Lymphocytic Leukaemia 17

1.1. Diagnosis 17

Peripheral blood smear 17

Immunophenotype 17

Monoclonal B-cell Lymphocytosis (MBL): 18

1.2. Evaluating the CLL patient 19

Symptoms and signs 19

Laboratory tests 19

Clinical Staging 19

1.3. Prognosis 21

Stage 21

Clinical and laboratory parameters 22

The molecular markers 22

Genetic abnormalities 27

Prognostic scoring 28

2. CLL pathophysiology 28

2.1. Normal B-lymphocyte biology 28

2.2. CLL cell origin 29

2.4. B-cell receptor (BCR) 30

BCR signalling: 30

BCR in CLL: 30

2.5. The microenvironment 31

Accessory cells 31

Cell migration 32

Chemokines and chemokine receptors 32

Integrins 33

BAFF and APRIL 33

3. Treating CLL 35

3.1. Indications for treatment 35

3.2. Standard Chemotherapy 36

3.3. Anti-CD20 monoclonal antibody drugs 36

Rituximab 36

Obinutuzumab 37
3.4. Alternative chemo-immunotherapy 37
3.5. Treatment of CLL with *TP53* mutation / 17p deletion 37
Alemtuzumab 37
Lenalidomide 38
B-cell receptor pathway inhibition 38
BCL-2 Inhibition 40
3.6. Allogeneic haematopoietic stem cell transplantation (alloSCT) 41
3.7. Chimeric Antigen Receptor T (CAR-T) cell therapy 41
3.8. Anti-CD38 monoclonal antibodies 42
3.9. Other agents in study 42
4. Retinoids 43
4.1. Retinoic Acid Receptor pathway 43
Co-activator 44
4.2. Gene modulation: 44
4.3. Retinoids in Cancer prevention and treatment: 45
Retinoids in haematological diseases 46
Retinoids in Cutaneous T Cell Lymphoma (CTCL) and other cancers: 47
Retinoids in CLL 47
5. Study rationale and Objectives 49
Objectives: 49
6. MATERIALS AND METHODS 50
6.1.Ethics 50
6.2. Patients 50
6.3. Samples 50
6.4. Cell line 51
6.5. Culture media 51
6.6. Stock cell-line development 52
Cell Growing 52
Cell Passaging or 'Splitting' 52
Cell Freezing 52
6.7. Patient derived CLL cell isolation 53
6.8. Cell counting 54
6.9. Drugs and treatments 54
Acitretin 54
Ibrutinib 54
6.10. Cell Viability assays 55
Setting up the assay 55
6.11. Migration Assay: cell homing in response to CXCL12 chemokine 56
6.12. Apoptosis measurement 59
Antibodies 59
Flowcytometric analysis 59
6.13. CD38 Expression 60

Treatement: 60
6.14. Data analysis and statistics 60
7. RESULTS 62
7.1. Patient Characteristics 62
7.2. CD38 status: 64
7.3. Cell purity 65
..... 65
7.4. Growth Assays 66
7.5. Cell viability assays 67
MEC-1 viability 67
Primary Patient-derived cell viability 68
7.6. Cell Migration 72
7.7. Apoptosis 74
7.9. CD38 Expression 76
8. Discussion 81
Acitretin and modulation of CD38 expression 81
Acitretin's effect on cell migration 82
Acitretin and cell viability and growth 83
Acitretin and Apoptosis 84
The role of the MEC-1 cell line 85
9. Conclusions: 86
10. Recommendations and future perspectives 87
References 88
Appendices 112
Patient Information Leaflet 113
Consent form 116

List of abbreviations

ADCC	antibody-dependent cell cytotoxicity
ADCP	antibody-dependent cell phagocytosis
ADP	adenosine diphosphate
alloSCT	allogeneic haematopoietic stem cell transplantation
APML	acute promyelocytic leukaemia
APRIL	A proliferation inducing ligand
ATM	ataxia telangiectasia mutation
ATRA	all-*trans* retinoic acid
BAFF	B-cell activating factor
BAFF-R	BAFF receptor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BMSC	bone marrow stroma cell
BTK	Bruton's tyrosine kinase
CAR-T	chimeric antigen receptor T
CLL	chronic lymphocytic leukaemia
CR	complete remission
CTCL	cutaneous T-cell lymphoma
CXCL12	C-X-C motif chemokine 12
CXCL13	C-X-C motif ligand 13
CXCR4	C-X-C chemokine receptor type 4
DAT	direct agglutination test
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
FCR	fludarabine + cyclophosphamide + rituximab
FDA	Food and Drug Administration
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GvHD	graft-versus-host disease
GWAS	genome-wide association study
IC50	half-maximal inhibitory concentration
IgD	immunoglobulin D
IgM	immunoglobulin M
IgVH	immunoglobulin heavy-chain variable region
Igα	immunoglobulin alpha
Igβ	immunoglobulin beta
IMDM	Iscove's modified Dulbecco's media

IPI	International Prognostic Indicator
IWCLL	International Workshop on CLL
LDH	lactate dehydrogenase
LDT	lymphocyte doubling time
MAPK	mitogen-associated phosphokinase
MBL	monoclonal B-cell lymphocytosis
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NAD^+	oxidised nicotinamide adenine dinucleotide
NCRI	National Cancer Registry of Ireland
NFκB	nuclear factor kappa B
NLC	nurse-like cell
NSCLC	non-small-cell lung carcinoma
OS	overall survival
PARP	poly-ADP-ribose polymerase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFS	progression-free survival
PI	propidium iodide
PI3K	phosphoinositol-3-kinase
PML	promyelocytic leukaemia
PPAR	peroxisome proliferator-reactivated receptor
PS	phosphatidylserine
RA	retinoid acid
RAR	retinoid acid receptor
RARA	retinoid acid receptor alpha
RARE	retinoic acid response element
RCF	relative centrifugal force
RXR	retinoid X receptor
SDF-1	stroma cell derived factor 1
SEER	Surveillance Epidemiology and End Results
SEM	Standard Error of the Mean
SFM	serum-free media
smIg	surface immunoglobulin
SNP	single-nucleotide polymorphism
SYK	spleen tyrosine kinase
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TGF-β	tissue growth factor beta
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor

VLA-4	Very late antigen 4
WHO	World Health Organisation
ZAP-70	zeta-associated protein 70
β2M	beta-2 microglobulin

List of Figures:

Figure 1: The CLL micro-environment. 34

Figure 2: Retinoid drug interaction with the nuclear retinoic acid receptor family.44

Figure 3: Schema of lower chamber duplicates. 58

Figure 4: B-cell purity in cells separated from patient whole blood. 65

Figure 5. Growth curves for MEC-1 cell line and primary CLL cells..... 66

Figure 6: Effect of acitretin on MEC-1 cell viability. 68

Figure 7. Primary CLL cell viability. 69

Figure 8: Primary CLL cell viability following treatment with Ibrutinib. 71

Figure 10: **Primary CLL cell homing in response to CXCL12**. 73

Figure 11: Effect of Acitretin on CLL cell apoptosis. 75

Figure 13: CD38 modulation on primary CLL cells from CD38 negative patients... 78

Figure 15. Modulation of CD38 expression in MEC-1. 79

Figure 16: CD38 modulation as fold difference in CD38 expression. 80

List of Tables:

Table 1: Immunophenotype scoring for the diagnosis of CLL. 18

Table 2*:* Clinical staging of CLL using the Rai and Binet staging systems. 20

Table 3: Indications for treatment in CLL as outlined by IWCLL. 35

Table 4: Patient characteristics. 63

Table 5 :Patient break down according to CD38 expression status. 64

Summary

Chronic lymphocytic leukaemia is a B- cell malignancy that has been the subject of a great deal of progress both in understanding disease pathophysiology and in the development of new therapies. The recognition of a subgroup of patients that responds poorly to standard therapy has inspired a plethora of research into improving treatment outcomes.

Retinoids have well-reported effects as anti-proliferation agents and have an established role in promoting cell differentiation. These properties have led them to be extensively studied in cancers in the contexts of prophylaxis and treatment.

In this study we explore the effects of the retinoid derivative acitretin on CLL cell viability, migration and CD38 expression *in vitro*.

In our clinical practice, a patient with stage A CLL as well as a diagnosis of squamous cell carcinoma of the skin was started on acitretin for the purpose of controlling his skin cancer. During follow-up it was noted that his lymphocyte count normalised within a month of starting acitretin. This observation led to preliminary *in vitro* studies investigating acitretin on primary patient cells.

Acitretin significantly increased CD38 expression in MEC-1 cells as well as in CLL cells from CD38-positive patient samples but did so to a far lesser degree on samples from CD38-negative patients. We also show that acitretin reduced the migration of CLL cells toward CXCL12 in the majority of samples tested. Acitretin however had a very modest effect on CLL viability and apoptosis.

Our results provide evidence that retinoids may have a role to play as an adjunct to drug treatment in CLL. We suggest that they should be considered for further studies *in vivo* and in perhaps in clinical trials, and that particular consideration should be given to studying them in combination with anti-CD38 monoclonal antibody drugs.

Acknowledgements

My sincere gratitude goes to my mentors; Dr Philip Murphy, Dr John Quinn and Professor Patrick Thornton. Thank you for taking me on, introducing me to the world of scientific discourse and thought. You were all very generous with your time and support at every turn. Thank you, Dr Murphy, for your wisdom and insight. Special thanks to Dr Quinn for accepting our invitation to become a part of the project and then becoming one of my main sources of inspiration and support. Professor Thornton, thank you for creating the whole opportunity out of pure goodwill and confidence in my ability to see it through. I am indeed very privileged to have worked with the three of you.

I would like to thank Professor Bryan Hennessey for granting us use of his distinguished laboratory and all the guidance and encouragement that you gave me. Your input at our project progress meetings was invaluable.

I am indebted to Dr Olive McCabe for making sure my journey in the cell culture lab was an enjoyable and seamless one. To Ozlem Aslan, Alex Eustace, Sinead Toomey, Aoife Carr, Naomi Elster, Angela Farrelly and all my other laboratory colleagues, a massive thank you for all your help.

My heartfelt thanks to the Beaumont Hospital haematology day unit staff who pulled endless charts, printed consent forms and made space and hot drinks.

Finally, I am forever grateful to my husband and family for bearing with me in this journey while I stopped and started. You make it all worthwhile.

1.Introduction

Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is the accumulation of mature clonal B-lymphocytes in blood, bone marrow and secondary lymphoid organs. It is the most commonly occurring leukaemia in the Western world, accounting for more than 40% of leukaemia diagnosed in individuals above 65 years of age (1,2). CLL is more prevalent in males and is uncommon in the less than 55 age group. According to the National Cancer Registry of Ireland (NCRI), an average of 200 new cases of CLL were diagnosed each year between 2005 and 2014 in the Republic of Ireland.

1.1. Diagnosis

CLL is increasingly diagnosed following an incidental finding of persistent lymphocytosis in an asymptomatic individual. The World Health Organisation (WHO) guidelines require the presence of an absolute lymphocytosis of >5x109 cells/L along with characteristic lymphocyte morphology and immunophenotype in order to make a diagnosis of CLL (3).

Peripheral blood smear

Peripheral blood film examination will typically show the accumulation of small mature lymphocytes with clumped chromatin, indistinct nucleoli and scanty cytoplasm as well as numerous smear cells. In 15% of patients the morphology is atypical owing to the presence of >10% prolymphocytes or lymphoplasmacytoid cells and/or cleaved nuclei(4).

Immunophenotype

CLL cells express CD19, CD20, CD5, and CD23, and weakly express CD79b and surface IgM and IgD (5,6). The CLL immunophenotype score was designed to aid the accurate diagnosis of CLL as other B-cell neoplasms may masquerade as CLL, such as marginal zone lymphoma, mantle zone lymphoma, follicular lymphoma or splenic marginal zone lymphoma with villous lymphocytes (5–7). Table(1) outlines five immunophenotypic parameters and a score of 1 allocated to each as described (8). A score of >3 is indicative

of CLL, whereas a score of <3 is more typical of other lymphoid neoplasms (5,6). A diagnostic panel will typically also include CD3, CD10 and CD38.

Table 1: Immunophenotype scoring for the diagnosis of CLL. (DiGiuseppe JA, Borowitz MJ, 1998).

The score is based on five immunophenotypic parameters. Strong expression of CD23 and CD5, weak expression of CD79b and surface immunoglobulin (smIg) and negative expression of FMC7 each get one point, while the opposite finding scores zero. CLL is

	Score points	
Marker	1	0
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
CD22 or CD79b	Weak	Strong
Surface Immunoglobulin (smIg)	Weak	Strong

diagnosed if the immunophenotype score is >3. Another lymphoid neoplasm is more likely if the score is <3.

Monoclonal B-cell Lymphocytosis (MBL):

The presence of monoclonal lymphocytes of $<5x10^9$/L with a characteristic CLL phenotype in the absence of lymphadenopathy or splenomegaly is known as monoclonal B-cell lymphocytosis. The prevalence of MBL in the population is higher than that of CLL, with estimates of 3-5% (9). On follow-up, it has been noted that most

of these individuals never achieve the B-cell count threshold required for a formal diagnosis of CLL, and the majority of those that do develop CLL do not require subsequent treatment. MBL does however progress to CLL at a rate of 1-2% per year and virtually all cases of CLL are preceded by MBL (9–11).

1.2. Evaluating the CLL patient

Symptoms and signs

The majority of CLL patients are asymptomatic at diagnosis. Every patient, however, should be evaluated for a history of constitutional disease related symptoms such as significant weight loss, night sweats, fever >38°C (B-symptoms), or profound fatigue. Patients can also present with symptoms of anaemia, bleeding or recurrent infection and a family history of lymphoid malignancies should be established at the initial assessment.

Examination of the patient should note involved lymph node areas and their size as well as presence or absence of spleen and the size in centimetres below the diaphragm (12).

Laboratory tests

Lab tests are of diagnostic and prognostic importance; baseline full blood and reticulocyte count, liver and kidney biochemistry, including serum LDH and direct agglutination test, should be performed on every patient. Serial full blood counts will determine lymphocyte doubling time or a rapid rise in lymphocytosis (13). Renal and liver biochemistry are vital in defining the patients' fitness for treatment, particularly for chemotherapy. Lactate dehydrogenase (LDH) is raised in high cell turnover states and a rising LDH points to progressive disease. Direct agglutination test (DAT) and reticulocyte count are essential to exclude or diagnose haemolysis. In addition to the above haematological and biochemical tests, CLL guidelines recommend the measurement of immunoglobulin levels and viral screening on all CLL patients (12). It is also important to determine whether disease-related symptoms and signs are a result of marrow infiltration, hypersplenism, an immune process or an alternative cause. Initial evaluation of the patient should culminate in disease staging.

Clinical Staging

The Rai and Binet clinical staging systems were developed over four decades ago and are considered to be the first prognostic assessment tools used in CLL (14,15). These two well-validated systems were devised from collating patient data in the 1970s and employ patient blood count and examination for lymphadenopathy and spleen or liver enlargement to predict patient outcomes. The Rai staging system uses five stages (stage 0, I, II, III, and IV), which are further simplified to three groups: low risk (stage 0), intermediate risk (stage I and II), and high risk (stage III and IV) (14). Assessment is made according to the presence of lymphocytosis, anaemia, and thrombocytopenia, as well as the presence or absence of lymphadenopathy or splenomegaly. Patients with lymphocytosis in the peripheral blood or bone marrow (>30% lymphoid cells) but no other clinical signs are considered to have low-risk disease (stage 0). Patients with lymphocytosis, enlarged lymph nodes, and/or splenomegaly or hepatomegaly are classified as having intermediate-risk disease (stage I-II). Patients with disease-related anaemia (haemoglobin level <11g/dl) are classified as stage III and those with thrombocytopenia (platelet count $<100x10^9$/L) as stage IV disease, regardless of the presence or absence of lymphadenopathy and splenomegaly; these two later stages constitute the high-risk disease group (14).

The Binet staging system uses three stages: A, B, and C; staging is based on the number of sites involved with CLL, as defined by lymph nodes >1 cm in diameter or organomegaly, in addition to presence of anaemia and/or thrombocytopenia (15). Similar to the Rai staging system, the three Binet stages reflect low (A), intermediate (B), and high-risk disease (C), with high-risk patients defined as having anaemia and/or thrombocytopenia, independently of the number of lymph node sites involved (15,16). Both the Rai and Binet staging systems remain the mainstay of patient staging and are widely used in clinical practice (Table 2) (12,17,18).

Table 2: Clinical staging of CLL using the Rai and Binet staging systems. (Oscier D, 2012)

The Rai staging system categorises patients into low-, intermediate- or high-risk groups based on the presence or absence of enlarged lymph nodes, spleen or liver as well as red cell or platelet cytopenias. Binet et al divide patients into three groups: A, B, and C, corresponding to risk categories of low, intermediate and high. Treatment is indicated for Rai stages III/IV or Binet C. Early-stage management is the 'watchful waiting' approach.

Rai Stage		Clinical characteristics	Median survival (years)
0	Low-risk	Lymphocytosis in blood and bone marrow only	>10
I	intermediate	Lymphocytosis and enlarged nodes	
II		Lymphocytosis and enlarged spleen and/or liver	6
III	high	Lymphocytosis and anaemia (Hb <11g/dl)	2
IV		Lymphocytosis and thrombocytopenia (Platelet count <100x10^9/L)	
Binet stage			
A		Haemoglobin ≥10g/dl, platelet count ≥100x10^9/L AND <3 involved areas	>7
B		Haemoglobin ≥10g/dl, platelet count ≥100x10^9/L, ≥3 areas involved	<5
C		Haemoglobin <10g/dl, platelet count <100x10^9/L, or both, independently of number of areas involved	<2

1.3. Prognosis

A number of patient- and disease-specific factors determine disease prognosis in CLL.

Stage

The Rai and Binet staging systems described above can predict survival in patients with bulky lymphadenopathy, splenomegaly or marrow failure. However, both have inherent limitations in predicting which of the early-stage patients will progress (13).

Clinical and laboratory parameters

In addition to advanced Rai and Binet stages, numerous clinical and biochemical parameters have been proposed as predictors of poor prognosis. According to analysis of data from the 1st Medical Research Council trial in CLL, males did not do as well as females, regardless of age and stage of disease (19).

The lymphocyte doubling time (LDT) and beta 2 microglobulin are among the factors shown to predict disease outcome. LDT is by definition the period of time during which the absolute lymphocyte count is doubled and is an indicator of disease activity. The prognostic significance of the LDT has been shown in a number of studies; an LDT <12 months is associated with poor prognosis and survival, while an LDT >12 months correlates with a favourable disease course (13,20).

Beta 2 microglobulin (β2M) is one of the two polypeptide chains that make up the MHC Class I complex and is necessary for the cell surface expression of MHC class I and the stability of the peptide binding groove (21). β2M is known to be released by CLL cells on a constitutive basis and is elevated in the serum of CLL patients (22,23). The β2M level correlates with bulky disease and was shown to predict for poor prognosis in several studies (23,24).

The morphology of CLL cells in a blood smear or in the bone marrow is an important prognostic factor for CLL; atypical, prolymphocytic morphology with a mainly large cell size and cleaved nucleus is associated with poor prognosis, whereas granular and small-sized lymphocytes predict good disease outcomes (25). In an analysis of CLL cells from 270 patients at Binet stage A, an atypical morphology of CLL cells was shown to predict adverse disease. A diffuse pattern of involvement of the bone marrow by CLL was also linked to worse outcomes (26).

The molecular markers

In addition to clinical and pathological parameters, several molecular markers have been shown to add substantial prognostic information to a diagnosis of CLL.

TP53

TP53 is a tumour suppressor gene which plays a central role in DNA damage repair. The product of the *TP53* gene, the p53 protein, acts as a transcription factor that can trigger cell cycle arrest, DNA repair, and apoptosis or cause the cell to lose its power to divide – an effect known as cell senescence (27).

TP53 is mutated in up to 50% of all cancers (28). The gene is highly polymorphic, meaning that different alleles can occupy its coding and non-coding portions. This has interesting implications; mutations of the gene are so diverse, it is often possible to distinguish cancer type and aetiology by the pattern of *TP53* mutation (29). Mutations are mostly single-base substitutions that either result in a change in one amino acid (missense) or lead to truncation of protein product (nonsense or frameshift). The consequence is most commonly disruption of the DNA binding domain of the p53 protein but can also be conformational change in the protein, severely impairing its function (29).

17p deletion

Deletion of the short arm of the 17th chromosome (17p deletion) leads to disruption of the *TP53* gene. Fluorescence *in situ* hybridization (FISH) of metaphase chromosomes is used to demonstrate 17p deletion and in practice is the more accessible modality for assessing a *TP53* gene abnormality. However, a PCR to look for mutations of the *TP53* gene should be performed in all these cases to fully exclude a *TP53* mutation. *TP53* mutations have a high concordance with 17p deletion, where one locus is mutated and the other is deleted in about 80-90% of cases of *TP53* disruption. Few patients who harbour an abnormal *TP53* have one or the other lesion in isolation; 17p deletion without *TP53* mutation is found in around 20% while mutations without deletions in around 3-4% (30).

***TP53* mutation and 17p deletion incidence and impact in CLL**

The incidence of *TP53* lesions in CLL varies across the course of the disease. Whilst they

are detected in around 3–6% of CLL patients at diagnosis (31), the incidence rises to 8-12% of patients at initial treatment (32) and to 30-40% in patients refractory to fludarabine (33,34). *TP53* mutations are associated with a particularly inferior outcome and response to conventional therapies in CLL; a multivariate analysis by Oscier et al described the impact of the various prognostic markers on median survival, highlighting that disruptions in *TP53* are independent indicators of unfavourable prognosis. Patients harbouring *TP53* mutations or deletions were found to have a median survival of 47 months in stark contrast to 209 months in patients with a normal karyotype and 292 months in patients with isolated 13q deletion (31). Later, in a patient series, *TP53*-mutated CLL predicted an overall survival of 27% at 5 years, compared to 83% in CLL with normal *TP53* (30).

Interestingly, although *TP53* lesions on the whole invoke poor risk disease, a small subset of patients with *TP53* lesions detected at diagnosis exhibit stable disease that behaves like indolent CLL (30).

IgVH genes' mutation status

Immunoglobulin forms part of the B-cell receptor for antigens. Somatic hypermutation is a crucial step in the normal humoral immune response. In CLL, one of the most significant discoveries in disease behaviour is the impact of the mutational status of the genes coding for the immunoglobulin heavy-chain variable region (IgVH). When more than 2% of the IgVH gene sequence differs from the germline, this is identified as 'mutated' and when there is a less than 2% difference or in other words >98% homology with the germline, this denotes 'unmutated' immunoglobulin genes. Unmutated IgVH genes correlate strongly with very poor prognosis CLL (35–38). The reverse is true of CLL with mutated IgVH genes, which carries a good prognosis and responds favourably to chemo-immunotherapy (39). Hamblin and colleagues in their landmark study reported the median survival in patients with mutated IgVH genes to be 25 years compared to 8 years in those with unmutated genes (35). Despite the strong prognostic value of IgVH mutation status, most clinical diagnostic laboratories are not equipped to routinely perform IgVH mutation analysis, which is currently time-consuming and

expensive. IgVH mutation analysis has therefore not yet been incorporated into routine diagnostic testing.

CD38 expression

CD38 is a type 2, single-chain transmembrane protein expressed in most haematopoietic cells at some stage in their maturation. It is expressed in immature B-lymphocytes and expression levels fall as the cells mature (40). The CD38 protein functions as an ectoenzyme, a receptor and an adhesion molecule (41–43). As an enzyme, the CD38 glycoprotein hydrolyses NAD^+ to cyclic ADP ribose, which in turn helps in intracellular Ca^{++} regulation. As a receptor it binds the CD31 non-substrate ligand (41).

CD38 expression is a marker of poor prognosis. Damle and colleagues first described that CD38 expression is linked to poor outcomes in CLL in 1999 (37). They reported an association between unmutated IgVH gene status and high CD38 expression, showing that patients with unmutated immunoglobulin genes had CD38 expression levels of >30% whereas those with mutated immunoglobulin genes expressed CD38 at levels of <30%. High CD38 expression was later shown to confer poor prognosis independently of other risk factors (40,43–46). The level of expression that best correlates with adverse prognosis is not certain but has been reported to be between 20% and 30% (47) and as low as 7% (48). It has also been suggested that any level of CD38 expression denotes poor risk (40). Furthermore, expression may vary during the course of illness in as many as 25% of patients and tends to increase with time (43). CD38-negative patients in general have been found to stay negative for the duration of their illness (40).

CD38 in CLL pathophysiology

Numerous studies have looked at the functional link between CD38 signalling and poor-prognosis disease. The CD38 molecule was shown to participate directly in the delivery of growth and survival signals (49–51). Studies have also shown that CD38-positive CLL

cells have high migratory potential (52) and are found with more abundance in the lymph node proliferation centres (53). CD38-expressing cells are thus said to be primed for proliferation (54).

Vaisatti et al investigated the role of CD38 in cell migration. They demonstrated that CD38 co-operates with CXCR4 to enhance CLL cell homing induced by CXCL12 and has been linked to the close physical proximity of the two receptors. $CD38^{-}$positive cells responded to CXCL12 chemotaxis and CD38-negative cells had less of a response (55). The group later showed that blocking the enzymatic activity of CD38 in CLL blocks cell chemotaxis, adhesion and *in vivo* homing – trapping CLL cells in the blood (56).

CD38 signalling promotes CLL cell survival and induces proliferation. CD38-CD31 interaction promotes proliferation and survival of the cell both directly and indirectly, through the regulation of other survival signals (56). Furthermore, CD38-negative cells have genetic signatures distinctly different from those of CD38-positive cells (57). Later, the genetic signature that results from CD38-CD31 interaction was shown to favour the activation of pathways leading to proliferation and migration (58).

Zeta-associated protein 70 (ZAP-70)

ZAP-70 is an intracellular tyrosine kinase involved in the normal signalling of T-cell and natural killer cells, and is normally absent in mature B-lymphocytes (59). ZAP-70 expression is a feature of poor prognosis in CLL; ZAP-70 positive CLL has an average survival of 8 years while ZAP-70 negative CLL patients can expect an average of 25-year survival (49).

On a functional level CD38 and ZAP-70 work together to enhance cell chemotaxis toward CXCL12 and were shown to be functionally linked as CD38 ligation results in phosphorylation of ZAP-70 (49). ZAP-70 expression therefore enhances the ability of malignant B-cells to respond to migratory and survival signals and renders them more sensitive to BCR signalling.

ZAP-70 expression is assessed by flow cytometry; however, variability in the results reported from different laboratories (60) has reduced the reliability of the protein as a clinically applicable prognostic marker.

Genetic abnormalities

The triggering events in CLL oncogenesis are not entirely known but the monoclonal nature of B lymphocytes implies that genetic aberrations occur in an early progenitor clone (61). Cytogenetic lesions are rare in the early clone; however, around 80% of CLL will acquire a genetic insult over time (2). There is no one disease defining mutation in CLL, but despite that, a large number of gene mutations re-occur if having relatively low frequency (62). The most common is a deletion of the short arm of chromosome 13 (del 13q14.3) and is present in 50% of CLL over time (63). Other more ominous chromosomal lesions such as deletion of 17p13, which involves the *TP53* gene, and deletion of 11q, which involves the ataxia telangiectasia mutation (*ATM*) gene, carry a poor prognostic signal and are associated with resistance to chemotherapy (63–65). Of note, the poor prognostic impact of 11q deletion was shown to be largely overcome by the addition of the monoclonal antibody drug rituximab (32).

Mutations in five particular genes have been found to be relatively more frequent in CLL and are of clinical interest owing to their association with poor prognosis. In a large series of 406 untreated patients, Nadeu et al used ultra-deep next-generation sequencing to detect clonal and sub-clonal mutations in CLL. The group reported mutations of *TP53* in 10.6%*, NOTCH1* in 21.8%*, SF3B1* in 12.6%*, ATM* in 11.1% and *BIRC3* in 4.3%. They also made important associations between the mutations and time to first treatment and overall survival (64).

Two studies published in *Nature* recently delve deeper into defining the genetic signature of CLL using whole-genome and whole-exome sequencing to uncover recurrently mutated genes (66,67). What is clear is that even in these large-scale sequencing studies there are differences in the mutated genes ascribed to CLL, which reflect the profound genetic heterogeneity of CLL.

One of the newer methods employed in studying the molecular heterogeneity of CLL is genome-wide association studies (GWAS). GWAS is a relatively new method of utilising high-throughput sequencing techniques, to gather information about (

single-nucleotide polymorphisms (SNPs) on large cohorts of patient samples. As the name implies, an SNP is a single-nucleotide variation at a specific position in the genome that is present to an appreciable frequency in a population—for example, >1%.

Whole-genome sequencing and subsequent genome-wide association studies have examined SNPs at locus sites that have been shown to be associated with CLL. Berndt et al put together masses of SNP information in a meta–analysis of four genome-wide association studies and expanded the work with further discovery of pertinent SNPs. They reported 14 SNPs in 11 novel loci associated with CLL risk. Importantly they performed pathway analyses and concluded that apoptosis plays an important role in CLL pathogenesis as consistently shown by the genomic data (68).

Prognostic scoring

The use of prognostic scores helps to inform clinical decisions on treatment and follow-up through the collation of information on the relative impact of prognostic factors into one tool. There have been several suggested prognostication tools for CLL. Most recently a large meta-analysis was undertaken by a consortium of CLL practitioners: the CLL International Prognostic Indicator (IPI) working group. The group proposed a tool that analysed the impact of 27 prognostic factors on overall survival in a large data set of patients from 13 CLL trials. The outcome of this impressive work is the CLL International Prognostic Indicator (CLL-IPI), which classifies patients into four prognostic categories with significantly differing overall survival at five years. The tool employs a weighted grading of five prognostic factors: *TP53* (deletion or mutation or both), IgVH (mutated vs unmutated), serum ß2M (≤3.5mg/L vs ≥3.5mg/L), clinical stage (early Rai 0/Binet A vs late-stage Rai I–III/IV or Binet C) and age (≤65 years vs >65 years). It divides patients into the following five risk groups and gives the values for percentage of patients expected to be alive at 5 years: low risk (93.2% overall survival at 5 years), intermediate (79.3%), high (63.3%) and very high risk (23.3% OS at 5 years) (69).

2. CLL pathophysiology

To understand the evolution of CLL, we must first understand the normal B-lymphocyte: its proliferation, migration, mutation and differentiation.

2.1. Normal B-lymphocyte biology

A normal B-lymphocyte goes through an elaborate set of maturation steps before becoming a functioning immune cell. A naïve B lymphocyte is activated by exposure to an antigen, following which it begins to differentiate. It acquires cell features such as

the B-cell specific CD19 antigen and then either forms foci of antibody-producing cells locally or migrates to nearby lymphoid follicles to form germinal centres (70). These migrated antigen-specific B-cells are presented with antigens by follicular dendritic cells and undergo further rounds of proliferation, leading to the formation of the germinal centre (71).

In the germinal centre, somatic hypermutation of the variable region of the heavy-chain (VH) genes occurs. This is in order to produce an immunoglobulin that is highly specific for a particular inciting antigen. The resulting germinal centre B-cells then mature into plasma cells or memory cells capable of producing antibodies. They also continue to divide in the presence of antigens as they acquire these additional mutations. Cells that do not encounter antigens are eliminated (70).

2.2. CLL cell origin

Two models for the cellular origin of CLL have been proposed. The first model is based on the observation that CLL cells carry either mutated or unmutated IgVH. It suggests that mutated CLL and unmutated CLL cells are derived from two distinct populations: mutated CLL cells are derived from antigen-experienced B cells expanded through the germinal centre, where somatic hypermutation occurs, while unmutated CLL cells are derived from marginal-zone B cells by T-cell independent processes. The second model proposes a common origin for both mutated and unmutated CLL (72). Evidence from gene expression profiling supports the second model, as unmutated and mutated CLL cells were shown to have a homogeneous phenotype bearing markers of memory B cells (73).

2.3. Apoptosis in CLL

CLL is considered the quintessential example of a cancer that primarily involves defects in apoptosis. CLL cells undergo rapid apoptosis *in vitro*, suggesting that apoptosis resistance is to a large extent influenced by the CLL micro-environment as will be discussed later. There is, however, a certain amount of inter-patient heterogeneity in the rate of spontaneous apoptosis suggesting an additional intrinsic resistance to apoptosis in CLL cells. This is supported by the fact that all CLL cells express high levels of the anti-apoptotic protein BCL-2 (74), where the promotor region in the BCL-2 gene

is hypomethylated leading to increased transcription and therefore expression of the BCL-2 protein (75). Moreover, CLL cells have an increase in the ratio of BCL-2 to the pro-apoptotic protein Bax, compared to controls. In a study by Saxena et al, sequence alterations in the promotor region of the BAX gene was noted in some patients resulting in reduced expression of the Bax protein (76).

2.4. B-cell receptor (BCR)

'The B cell receptor (BCR) stands sentry on the front lines of the body's defences against infection' (Robinson 2006).

The B-cell receptor is composed of a transmembrane immunoglobulin linked with heterodimer co-receptors CD79a and CD79b (Igα and Igβ respectively). The BCR function is twofold: the first is antigen binding and internalisation, the second is signalling. When a BCR-bound antigen is internalised, it is processed into fragments and displayed on the cell surface to T-cells. A proportion of BCRs remain on the cell surface to partake in signalling (77).

BCR signalling: One of the key signals that CLL cells likely receive in proliferation centres is antigenic stimulation. Microarray analyses of CLL cells from lymph nodes showed the upregulation of genes implicated in ongoing BCR signalling and activation of the NFκB pathway when compared to CLL cells from peripheral blood. Once the BCR is activated through encountering an antigen, the receptor recruits kinases, namely spleen tyrosine kinase (SYK) and tyrosine kinase LYN, which phosphorylate the Ig co-receptors and set off a cascade of phosphorylation leading to downstream signalling events, including the activation of Bruton's tyrosine kinase (BTK) and phosphoinositol 3 kinase (PI3K) (78). BCR signalling leads to the proliferation and survival of mature B-lymphocytes and signalling in normal B-cells is constitutive (tonic), probably as a way of maintaining the memory function of the cell (79).

BCR in CLL: The importance of the BCR in CLL pathogenesis is deduced from the fact that the mutational status of the immunoglobulin chain of the BCR is one of the strongest predictors of survival in CLL patients (37,46,80). Furthermore, BCRs from different patients are often structurally similar and sometimes even identical, providing further evidence of a probable role of the BCR in CLL pathogenesis (78). Even though

BCR signalling is thought to drive CLL proliferation and survival, BCR expression is curiously low in CLL cells in stark contrast to its high expression in normal B-lymphocytes and in other B-cell malignancies (7,78). In addition to mediating pro-survival signals, sustained BCR signalling mediated through SYK also appears to upregulate a number of adhesion molecules and to increase the ability of CLL cells to migrate toward the chemokines C-X-C motif chemokine 12 (CXCL12) and C-X-C motif ligand 13 (CXCL13), both important for homing of CLL cells to the tissue microenvironment. Following entry into the tissue microenvironment, BCR triggering plays a role in retaining CLL cells in the microenvironment by downregulating C-X-C chemokine receptor type 4 (CXCR4), and CD62L, preventing re-entry of CLL cells into the bloodstream. Interestingly, unmutated CLL cells or cells from patients with aggressive disease were shown to be more responsive to BCR-mediated retention in the tissue microenvironment. Sustained BCR signalling is particularly important in the pro-survival effects of B- cell activating factor (BAFF) and A proliferation inducing ligand (APRIL), both of which are elevated in CLL (78).

2.5. The microenvironment

"Cancer as a disease must simultaneously subvert the microenvironmental controls as well as the genetic programme." —Mina Bissell, biologist

Malignant cells in general rely heavily on a permissive 'oncogenic' microenvironment for survival and expansion. Far from being an innocent, if genetically unsound, bystander, the cancer cell produces many outgoing signals recruiting the microenvironment to its cause. CLL is a quintessential example of this co-operation between malignant cell and host tissue constituents. The critical role of the microenvironment in promoting CLL cell survival was in part deduced by the behaviour of these cells *in vitro*. One of the hallmarks of CLL cells, whether from aggressive or indolent disease, is their resistance to apoptosis *in vivo*. However, when cultured *in vitro*, CLL cells undergo apoptosis spontaneously and generally fail to proliferate without external stimuli (81).

Accessory cells

Lymphoid tissue and bone marrow proliferation centres or pseudo-follicles are an important hub in the CLL microenvironment. Here, bone marrow stroma cells (BMSCs),

macrophage-derived cells called nurse-like cells (NLCs) and T-cells are in close physical proximity to the CLL lymphocyte. CLL cells are driven by chemokines to migrate under and beneath bone marrow stroma cells (82–85). Furthermore, CLL cells seem to recruit accessory cells and thereby create 'pseudo-follicles' that support their own survival. There, CD3-positive T-cells, which are also CD40 ligand expressing and CD4-positive, form clusters in and around pseudo-follicles. These cells can stimulate CLL cells through the interaction of CD40 and CD40L, and this stimulus is synergistic with BCR signalling (86). The apoptosis of CLL cells *in vitro* can therefore be rescued by co-culturing with stromal cells or addition of a number of soluble factors mimicking those found in the tissue microenvironments *in vivo* which co-operate to sustain CLL cells.

Cell migration

CLL cells move between blood and lymphoid organ compartments in a to-and-fro fashion, referred to as CLL cell trafficking (78,82). Fluorescence labelling of cells in *in vivo* experiments showed cells moving from the blood to lymph nodes and back to blood (87). This is presumed to be in line with the cell's need to receive signals, before continuing its pre-ordained journey of circulating in the peripheral blood. This movement is orchestrated by factors both on and from the CLL cell as well as factors linked to the accessory cells in the microenvironment. The net result of this migration is increased proliferation and often drug resistance of the CLL cell (85).

Chemokines and chemokine receptors

CXCL12 and CXCL13 are chemokines secreted by NLCs and BMSCs and work to summon the CLL cell through cognate receptor interaction with resultant movement of the CLL cell down a chemokine gradient. This gradient also succeeds in retaining migrated cells in the vicinity of 'nourishing' NLCs. CCL3 and CCL4 on the other hand are chemokines secreted by the CLL cell (88).

CXCR4- CXCL12 axis

CXCR4 is expressed on CLL cells and is the receptor for CXCL12. CXCL12 is the most

prominent of the chemokines. CXCR4 is highly expressed on circulating CLL cells – a characteristic that distinguishes them from tissue CLL cells, which exhibit low CXCL4 expression (89). Functionally it aids the cell in migration across the endothelium with the help of integrins, toward a higher concentration of CXCL12 and beneath and underneath BMSCs. In addition to attracting CLL cells towards BMSCs, CXCL12 also induces the upregulation of CXCR4 and conveys survival signals (82,88). CXCR4 can be blocked by specific CXCR4 antagonists, thereby allowing the CLL cell to become 'vulnerable' to apoptosis. CXCR4 signally can also be blocked by SYK, BTK and PI3K inhibitors (90).

Integrins

Integrins are glycoproteins that mediate cell-cell adhesion. VLA-4, also known as CD49d, is expressed on lymphocytes, monocytes, and most other haematopoietic cells and plays a role in lymphocyte trafficking and homing as part of immune surveillance (7,82).

BAFF and APRIL

The B-cell-activating factor of the TNF family (BAFF) and A proliferation inducing ligand (APRIL) are important molecules for the survival, proliferation, and differentiation of B-cells (78,90). CLL cells are activated in the tissues by NLCs through BAFF and APRIL, which promote the survival and proliferation of CLL cells. BAFF activates B-cells by binding to the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFF-R), whereas APRIL binds to TACI and BCMA, but not to BAFF-R (Figure 1) (91).

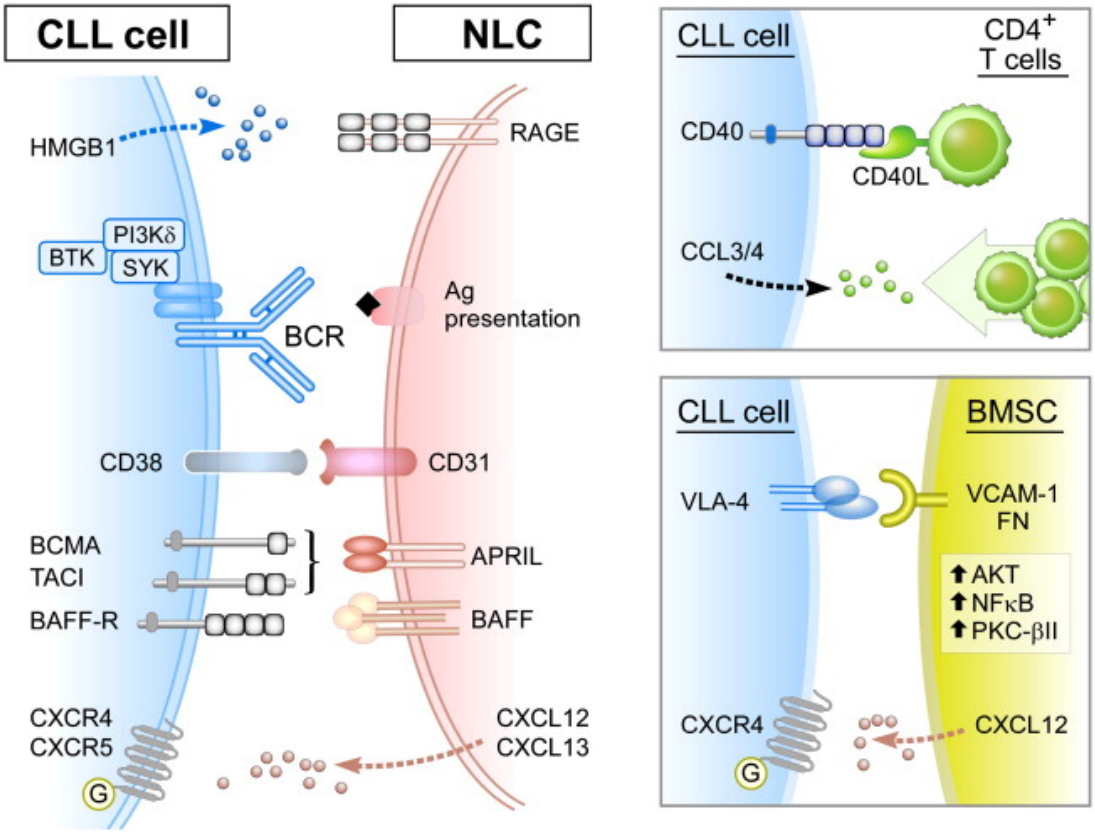

Figure 1: The CLL microenvironment.

A pseudo-follicle is formed in secondary lymphoid organs where accessory cells gather around CLL cells, allowing to-and-fro messages through soluble agents as well as direct cell-cell interactions. Microenvironmental stimulation of CLL cells leads to changes in gene signatures, resulting in improved survival and treatment resistance. Adapted from Ten Hacken et al, *Pharmacology and Therapeutics*, 2014 (92).

3. Treating CLL

3.1. Indications for treatment

A fundamental issue for clinicians treating CLL patients is the question of when to initiate treatment. Asymptomatic patients are unanimously monitored through 'watchful waiting' (17,93). The indications for treatment have been set forth by the International Workshop on CLL (94). Treatment is indicated when a patient presents with or develops disease-related systemic symptoms (fever, sweats, weight loss) or progressive lymphadenopathy (>10cm) and/or splenomegaly (>6 cm below the coastal margin) and/or bone marrow failure and its symptoms. Two thirds of all CLL patients will require CLL-specific therapy; of these one half will warrant treatment at the outset and the other half will have early-stage disease but progress to requiring treatment. Treatment is aimed at achieving deep and durable remission while minimising therapy-related toxicity and the patient's wishes are an important determining factor in treatment choice (94,95).

Table 3: Indications for treatment in CLL as outlined by IWCLL.

Indications for treatment	
Symptoms	Weight loss >10% over last 6 months Fever >38°C for >2 weeks Night sweats Profound fatigue
Progressive lymphocytosis	>50% increase over 2 months Lymphocyte doubling time <6 months
Lymphadenopathy **Splenomegaly**	>10 cm or progressive Massive >6 cm or progressive
Progressive marrow failure	Development or worsening of anaemia Development or worsening of thrombocytopenia

Adapted from Oscier et al, *Guidelines for the Diagnosis and Management of CLL*, 2012

3.2. Standard Chemotherapy

Another cardinal question for the treating physician is which drugs to offer which patient.

Chlorambucil is a nitrogen mustard drug that was used as a single agent in the treatment of CLL for several decades (96,97). Chlorambucil has good efficacy and a tolerable safety profile in the mostly elderly CLL population (98). It continues to be a benchmark against which many new therapies are tested in CLL clinical trials, and is used in combination with monoclonal antibodies as will be described below.

In the 1990s, the purine analogue fludarabine was used as a second-line agent in patients who did not respond to chlorambucil. Rai and colleagues evaluated the efficacy of fludarabine as a first-line agent and compared it in a randomised control trial to chlorambucil. Fludarabine was found to improve the quality and duration of response specifically in younger, fit patients (99).

Cyclophosphamide was combined with fludarabine and the drug duo was shown in studies to achieve a better response than fludarabine alone (32). Catovsky et al conducted a large randomised control study with the aim of determining whether the combination impacted survival. The addition of cyclophosphamide to fludarabine was shown to extend progression-free survival (PFS) but not overall survival (100).

3.3. Anti-CD20 monoclonal antibody drugs

Rituximab

CD20 is an important target in all B-cell malignancies, including CLL. The addition of the anti-CD20 monoclonal antibody rituximab to fludarabine and cyclophosphamide (FCR) created the first CLL treatment regimen that was shown to prolong overall survival as well as induce more complete remissions (32). The survival benefit imparted by FCR was found, through multivariate analysis, to be largely limited to the group of patients who were less than 70 years of age, had a good performance status and had no major

co-morbid condition (32,101). FCR therefore became the standard of care for younger fit patients who were deemed able to tolerate the chemo-immunotherapy regime. Notably, the greater proportion of CLL patients are above 65 years of age: the Surveillance Epidemiology and End Results (SEER) study reports that 75% of all CLL patients at diagnosis are in the above 65 age group (1). Furthermore, the majority of patients present with early-stage disease and in those with progressive disease, treatment is more likely to be needed nearer the age of 70. It thus became necessary to devise alternative therapy regimes better suited to the elderly patient with equally good efficacy as that of FCR.

Obinutuzumab

Obinutuzumab is a humanized, glycol-engineered type 2 antibody that also targets CD20. In preclinical studies, obinutuzumab showed superior efficacy, as compared with rituximab, by inducing direct cell death and enhanced antibody-dependent cellular cytotoxicity with less complement-dependent cytotoxicity (102). The combination of obinutuzumab with chlorambucil resulted in an improved response and survival in older patients with co-morbidities (103).

3.4. Alternative chemo-immunotherapy

Bendamustine has structural similarities to both alkylating agents and purine analogues and has shown considerable efficacy as a single agent for lymphoid malignancies, including CLL. Combined with rituximab, it was shown to be safe and active in previously untreated CLL (104). The CLL10 trial then showed significant survival benefit for patients treated with bendamustine who were otherwise not suitable for treatment with fludarabine (104,105).

3.5. Treatment of CLL with *TP53* mutation / 17p deletion

CLL clinical trials have consistently highlighted that patients harbouring *TP53* gene mutations or deletions are strikingly resistant to conventional chemo-immunotherapy (30,33,65,106). Treatment modalities targeting this group of patients have varied in the last two decades. BCR pathway targeted therapies have revolutionized the outcome of relapsed/refractory patients with or without high-risk molecular markers. Below we describe these and other treatment modalities previously used to treat high-risk CLL.

Alemtuzumab

Alemtuzumab is an anti-CD52 monoclonal antibody that functions through a *TP53*-independent mechanism and combined with methylprednisolone gave previously untreated patients with a *TP53* aberration a mean overall survival rate of just above 30 months (107). However, this favourable response in high-risk CLL came with a serious side effect profile of 50% infection rate in the over 60 age group. Alemtuzumab was used in several trials in relapsed patients of all descriptions but infectious complications were a prominent feature throughout (108).

Lenalidomide

The immunomodulatory drug lenalidomide was found to be effective in inducing remission in CLL (109). Lenalidomide improves T-cell function by downregulating expression of T-cell inhibitory molecules (such as PD-1) and enhancing T-cell motility (110). Its mechanism of action appears to be disease-specific and since it is not directly cytotoxic *in vitro*, its clinical activity in CLL has been presumed to be secondary to its immune-modulating activity.

Combined with monoclonal anti-CD20 antibodies in patients with relapsed/refractory disease, it was efficacious with no major toxicity concerns (109,111,112).

B-cell receptor pathway inhibition

BTK Inhibition

Ibrutinib is a first-in-class Bruton's tyrosine kinase (BTK) inhibitor that proved an exceptional new addition to the CLL drug stockpile. Ibrutinib blocks BCR signalling *in vitro* (113) and *in vivo* (114). Both *in vitro* and in patients, ibrutinib impairs microenvironment-induced survival and proliferation as well as release of and migration towards tissue-homing chemokines (113–115).

The inhibition of adhesion/migration explains the CLL cell redistribution seen with ibrutinib, which is characterized by a rapid and sustained decrease in lymphadenopathy accompanied by transient lymphocytosis. Furthermore, ibrutinib might have an immune-modulating potential by affecting the repertoire of CD4+ T-cells. However, this has to be explored further in future studies.

In patients, the RESONATE trial compared ibrutinib with ofatumumab in relapsed/refractory CLL patients with poor risk markers. Ibrutinib did remarkably better in the group as a whole and notably stood out as the superior drug in patients carrying poor risk cytogenetic markers, including del17p, del11q, *TP53*, *NOTCH1*, *SF3B* and *BIRC3* (116).

RESONATE-2 pitched the BTK inhibitor against chlorambucil as a first-line treatment. The results were a resoundingly better response rate and progression-free and overall survival in patients who received ibrutinib (117).

Another permutation of the ibrutinib trials was RESONATE-17, which specifically selected relapsed/refractory patients with del17p. The results re-affirmed the drug's efficacy in this CLL subtype, making it the unequivocal drug of choice in these particularly difficult-to-treat patients at the time of first treatment (118).

The HELIOS trial teamed ibrutinib up with bendamustine/rituximab, comparing it to bendamustine/rituximab plus placebo. The ibrutinib-containing arm gave superior results. Ibrutinib received breakthrough drug approval from the FDA for use in CLL in early 2014 (119).

Resistance to ibrutinib generally occurs in the context of Richter's transformation and less commonly, in CLL progression. Resistance was shown in some cases to involve mutation of the cysteine residue where binding occurs (120). This mutation prevents irreversible drug binding, leading to increased BCR signalling (121). Another resistance mechanism is mutation in PLCγ2, immediately downstream of BTK, which potentially allows BTK-independent BCR activation (120).

Ibrutinib carries an increased bleeding risk that is thought to be due to platelet pathway blocking effects (118,122,123). Ibrutinib is thus not the drug of choice in clinical scenarios where patients are on anticoagulation or dual anti-platelet therapy or for patients with bleeding diathesis.

In summary, ibrutinib represents a significant addition to the drug repertoire in CLL. However, it is not a curative option and does come with potential adverse effects as well as risk of resistance (116,117,124).

Phosphoinositol-3 (PI3) Kinase Inhibition

Signalling from several major pathways converges on PI3K, which has a key role in regulating B-cell function and survival (125). Idelalisib is an orally available, highly specific and reversible inhibitor of PI3Kδ. Idelalisib inhibits chemotaxis toward chemokines, pro-survival cytokines and secretion of chemokines (CCL3/4) from CLL cells *in vitro* as well as in treated patients (126). Idelalisib also decreases CLL adhesion by interfering with CD49d/VCAM-1 binding (127). Finally, idelalisib might have immune-modulating capacity as inhibition of regulatory T-cells has been shown *in vitro* (128).

In the relapsed setting, idelalisib plus rituximab was compared to rituximab/placebo in a group of patients with baseline characteristics precluding the safe use of chemotherapy agents. The results revealed significantly better response and survival rates in the group treated with idelalasib/rituximab (129). Idelalisib was also studied in the frontline setting in older patients. Combined with rituximab, treated patients had highly favourable response rates, where 83% of patients had no progression of disease at 36 months (130). Side effects associated with idelalasib therapy include diarrhoea and colitis but the trial investigators felt that these were manageable and the benefit/risk balance was still favourable. Idelalisib is thus another drug to consider in the upfront setting for patients harbouring unfavourable risk cytogenetic parameters and a particularly attractive choice in relapsed patients with co-morbid conditions (131).

BCL-2 Inhibition

Impaired apoptosis in cells is a hallmark of most cancers and in CLL is a well-studied phenomenon. The BCL-2 protein is an anti-apoptotic molecule that when targeted can lead to cell death or render a cell more susceptible to chemotherapy. Venetoclax (ABT-199) is a very specific BCL-2 inhibitor which suppresses the growth of BCL-2 dependent tumours *in vivo* (132).

A phase 2 trial reported responses in relapsed and refractory patients with 17p deletion and indicated that patients receiving venetoclax achieve deeper, better-quality remissions – an objective that may preclude the need for 'lifelong' medication. One major cause for concern initially was a high rate of tumour lysis, which was

subsequently abrogated by gradually escalating the venetoclax dose (133). Venetoclax received FDA approval in 2016 for use in patients who progress on or are unsuitable for BCR small-molecule kinase inhibitors (131,133). Ongoing phase 3 studies are looking at combinations with monoclonal antibodies and other targeted therapies. The MURANO study compared the combination of venecolax and rituximab to bendamustine and rituximab; preliminary results suggest improved progression-free survival in the venetoclax arm (134).

3.6. Allogeneic haematopoietic stem cell transplantation (alloSCT)

Allogeneic stem cell transplantation is a much-debated treatment modality in this era of targeted therapies. It remains however a treatment option in eligible patients with CLL who have failed chemoimmunotherapy and BCR inhibitor therapy irrespective of *TP53* status and in those patients with *TP53* disruption who have not responded to or have lost response to BCR inhibitor therapy. AlloSCT should also be considered for all eligible patients with Richter's transformation (94,95).

3.7. Chimeric Antigen Receptor T (CAR-T) cell therapy

Chimeric antigen receptor T (CAR-T) cell therapy is a novel immune-based method that utilises autologous T-cells in which the receptor has been chimerised to an antibody targeting a specific cancer cell molecule. In the case of CLL the chimerisation is with an anti-CD19 antibody. CAR-T cell therapy provokes a type of graft-versus-leukaemia effect with the added benefit of highly specific targeting of malignant B cells, avoiding the GVHD that comes with allografted stem cells (135). A number of phase I/II clinical trials were undertaken using anti-CD19 CAR-T cells with impressive results observed in CLL (136). A number of trials showed responses to be independent of age, number of prior therapies and TP53 abnormalities. More recently, a larger trial treated 24 patients who had received ibrutinib and were either refractory or intolerant of it. A proportion of the patients in the trial were also refractory to venetoclax. The trial concluded that CD19 CAR-T cells are highly effective in high-risk patients with CLL after they experience treatment failure with BTK inhibitor therapy (136).

3.8. Anti-CD38 monoclonal antibodies

Monoclonal antibody agents targeting the CD38 molecule have been extensively studied in multiple myeloma and are being investigated in other haematological malignancies, including CLL. Daratumumab, a human IgG1 monoclonal antibody, is the most advanced in the development of anti-CD38 antibodies and has been shown to have a wide spectrum of anti-tumour activity, including complement and antibody-dependent cell cytotoxicity and phagocytosis as well as enzyme modulation and apoptosis induction (11,137).

Matas-C et al reported pre-clinical evidence of the effectiveness of daratumumab in CLL. The group demonstrated efficient lysis of patient-derived CLL cells and cell lines by antibody-dependent cell cytotoxicity (ADCC) *in vitro* and antibody-dependent cell phagocytosis (ADCP) both *in vitro* and *in vivo* (11). A phase I clinical trial is currently recruiting previously untreated CLL patients to test the combination of ibrutinib and daratumumab (ClinicalTrials.gov, 2018).

3.9. Other agents in study

Targeting the CXCR4-CXCL-12 axis

Plerixafor is a selective inhibitor of the chemokine receptor CXCR4. It inhibits CXCL12-mediated chemotaxis and binding. It is approved by the FDA in lymphoma and multiple myeloma for mobilization of haematopoietic stem cells (90). Early phase II data from relapsed CLL patients treated with plerixafor in combination with rituximab demonstrated a dose-dependent mobilization of CLL cells from tissues into the blood as well as some responses (138).

NOX-A12 is an RNA oligonucleotide that binds and neutralizes CXCL12. It effectively inhibits CXCL12-induced chemotaxis of CLL cells and enhances their sensitivity to fludarabine and bendamustine in preclinical models (139).

4. Retinoids

Retinoid is a term used to encompass both naturally occurring and synthetic compounds that are chemically related to vitamin A. The naturally occurring compounds are found ubiquitously in all tissues and regulate a range of cellular functions, including cell growth, differentiation and apoptosis (140).

Retinoid drugs in cancer therapeutics is an immense field of study that has attracted oncologists for numerous decades.

4.1. Retinoic Acid Receptor pathway

For the most part, retinoids deploy their message through interaction with the retinoid receptor family of nuclear receptors that act as transcription factors. There are two main subtypes of receptors: retinoid acid receptors (RARs) and retinoid X receptors (RXRs). Each has three isoforms—alpha, beta and gamma—which bind to specific ligands. Our drug of interest, acitretin, binds RARs (140,141).

The classical RA pathway of activation involves four main steps: ligand involvement, receptor dimerization, DNA binding (at the retinoic acid response element, RARE), and the resulting transcriptional modulation of the target gene. RARs and RXRs are not active as monomers. Briefly, ligation of a retinoid induces receptor dimerization. The RAR forms a heterodimer with RXR complexed with corepressor proteins which occupy a region in the ligand binding site. RXR can form both heterodimers and homodimers. The receptor then binds to a retinoic acid response element (RARE) – a unique DNA sequence mostly found in the promotor region of target genes. When a ligand binds to the heterodimer, the corepressor protein dissociates, and an activator protein is bound in its stead. This is followed by chromatin remodelling allowing transcription machinery proteins access to the intended gene promoter site (142).

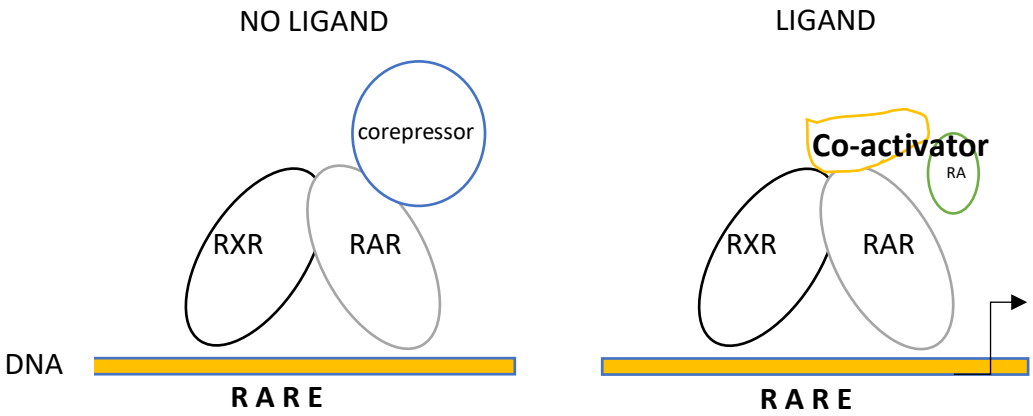

Figure 2 : Retinoid drug interaction with the nuclear retinoic acid receptor family.

Retinoic Acid Receptor and retinoic X receptor form a heterodimer. When no ligand is present in the binding groove of the receptor, the grove is occupied by a 'co-pressor' compound. When drug is bound to the groove , a co-activator is needed. The receptor – drug-activator complex then binds the Retinoid acid response element (RARE) sequence of DNA usually located in the promotor region of target genes. This acts as a transcription factor for those genes and results in a variety of cellular effects including promotion of cell maturation and apoptosis and inhibition of proliferation.

The retinoid receptors also form heterodimers with other nuclear receptor groups namely; Oestrogen receptor alpha, AP-1, Peroxisome Proliferator reactivated receptor(PPAR), Liver X receptors and Vitamin D receptors (VDR)(143). By joining forces with these various receptors, retinoids partake in regulating the partner receptors pathway.

4.2. Gene modulation:

Retinoids exert pleiotropic effects on cellular mechanisms. In some instances, affecting opposing effects on the same gene (transcriptionally activated versus repressed) subject to the context – for example activated during differentiation and repressed during growth inhibition (142). Balmer and Blomhoff (2002) elegantly condensed down 1191 studies pertaining to retinoic acid regulated genes, suggesting that as many as 532

genes are targets of these compounds. Through their analysis of the literature they grouped the genes according to strength of evidence linking them to retinoid regulation. They conclusively deduced that 27 genes were identified as definite targets of the classical retinoic acid pathway – 26 of them were upregulated, whilst in one gene, the effect was variable. The direction of transcriptional modulation in 311 genes was upregulation, regardless of pathway and in 212 genes it was variable (142).

To add to the complexity of retinoid function, indirect gene modulation has been described and is evident from data in 124 genes. Methods of indirect gene regulation can occur through the expression of intermediary proteins and the modulation of the expression of other transcription factors therefore affecting downstream genes independently of RAR/RXR binding. Intriguingly it was shown that activation through partnered receptors such as Vitamin D3 receptors, often results in opposing effects to those of the classical pathway activation.

4.3. Retinoids in Cancer prevention and treatment:

In cancer therapy, retinoids represent an interesting departure from the concept of cytotoxicity to a principle of diverting a malignant cell to a more 'physiologic' phenotype through differentiation (141,144). A link between vitamin A and cancer was first noticed in the 1920's when animal experiments demonstrated carcinogenesis in response to induced Vitamin A deficiency (see Bollag & Holdener, 1992 and references therein). Following that, numerous studies have demonstrated the involvement of retinoids in cellular processes key to carcinogenesis, such as inhibition of cell proliferation, promotion of differentiation and apoptosis. Functioning through the classical RA pathway, retinoids were shown to effect; Nuclear Factor Kappa B (NFKB), Interferon gamma, Tissue growth factor beta (TGF ß), vascular endothelial growth factor (VEGF), mitogen Associated PhophoKinase (MAPK) and chromatin remodelling (146).

Prevention of carcinogenesis, both primary and secondary has been a subject of a substantial number of clinical trials; 9-cis-retinoid in primary prevention of lung cancer in one study showed reduction in metaplasia when compared to placebo (146). Disappointingly, several other studies failed to prove a benefit for retinoids in the

setting of either primary, secondary or tertiary prevention (147). ATRA was combined with cisplatin and paclitaxel in a phase 2 trial in advanced NSCLC and favoured addition of ATRA but in contrast to this Bexarotene in combination with chemotherapy made no difference (146).

In breast cancer, fenretinide was quite comprehensively studied as a preventative agent as it selectively accumulates in breast tissue. In one large scale (2867 subjects) randomized study of fentretinide in secondary prevention of recurrent tumours, no differences in the incidence of 2nd tumours were observed between the retinoid arm and placebo after eight years of follow up. But when the results were analysed using menopause as a stratification, an interesting contrast in the response of pre-menopausal and post-menopausal women responses was seen. Premenopausal women had a mean 35% reduction in recurrent tumours a fact that was confirmed at 15 years of follow up. Meanwhile in postmenopausal women, fenretinide was thought to show a detrimental effect (148). An ongoing phase 3 trial is looking specifically at fenretinide in pre-menopausal women at high risk of breast cancer (149).

Our drug of interest, acitretin, is a 2nd generation synthetic metabolite of etretinate, with a shorter half-life and therefore a more favourable side effect profile. Common side effects include increased triglyceride levels, hair loss and skin dryness. Clinically it is used in the treatment of severe Psoriasis and has been used in control of squamous skin cancer (150).

Retinoids in haematological diseases

Retinoids brought about a fundamental change in the treatment of Acute Promyelocytic Leukemia (APML). Huang et all first published a case series of 24 patients treated with ATRA who attained complete remission(CR) of their APML in 1988 and also described morphological evidence of maturation of the blast cell (151). In this, retinoids represent an interesting departure from other anti-cancer drugs by inducing differentiation rather than causing cytotoxicity. Later international joint efforts in optimizing ATRA-based regimens in the 90's resulted in CR rates up to 90%-95%, and 5-year disease free survival of up to 74% (152). On account of retinoids, APML went from being a highly malignant leukaemia to a singularly curable one.

On a molecular level, this success was found to be linked to a fusion gene product resulting from a balanced translocation between chromosomes 15 and 17 (153). The PML/RARA onco-protein is the product of this translocation and is essentially a faulty receptor, unable to transmit functional transcription signals ordinarily induced by retinoids. ATRA promotes degradation of this fusion protein by a mechanism involving the induction of a proteinase, allowing the normal functioning of the receptor and subsequent differentiation of malignant pro-myelocytes to mature granulocytes (154).

Retinoids in Cutaneous T Cell Lymphoma (CTCL) and other cancers:

Retinoids have an established role in CTCL namely Mycosis Fungoidis and Sezary Syndrome in combination with immune modulators and light therapy (155). They are mostly well tolerated but do cause unique side effects such as hyperlipidaemia and hypothyroidism that require monitoring and sometimes treatment. The 3rd generation rexinoid Bexarotene – so called as it selectively binds the RXR receptor subtype- has proven efficacy in both persistent early stage and refractory CTCL (156).

Retinoids in CLL

A few pre-clinical studies looked at aspects of treating CLL cells with various retinoid compounds. One study indicated that Retinoid-induced apoptosis in B-cell chronic lymphocytic leukaemia cells is mediated through caspase-3 activation and is independent of p53 and the retinoic acid receptor and differentiation (157). One group showed synergistic induction of apoptosis in B-cell chronic lymphocytic leukemia cells after treatment with all-trans retinoic acid in combination with interleukin-21 and rituximab (158). Another group demonstrated enhanced *ex-vivo* fludarabine sensitivity in cells pre-treated with ATRA (159). Yet another study looked into the effect of ATRA on the proliferation of CLL cells versus normal B-lymphocytes. The study reports inhibition of proliferation in the malignant cells in response to ATRA but markedly enhanced proliferation of normal B-lymphocytes (160). This last study illustrates the oft times contradictory effects of retinoids on a particular cell function which entirely depends on the circumstance of the cell.

Of particular relevance to our study, CD38 is modulated by retinoids on various cell types. CD38 expression was shown to be increased by ATRA in myeloid cell lines (161),

on bone marrow stem cells (162) and on myeloma cells (163). The CD38 gene contains a RARE sequence in the 1st intron (164), substantiating the finding that retinoids upregulate CD38 expression in the different cell lines.

5. Study rationale and Objectives

In the preceding section, we reviewed literature that supports a role for retinoic acid derivatives in cancer treatment in general and the roles in haematology both established and experimental. Our interest in the retinoid drug acitretin was sparked by a case of a Stage A CLL patient, who was under surveillance at our haematology out patients' clinic. The patient in question was a male patient who receiving acitretin for the control of squamous cell carcinoma of the skin. His lymphocyte count was noted to have normalised at one of his visits and remained normal on a 1 year follow up visit. Inspired by this observation, our group set out to address the question of whether acitretin reduced CLL cell viability and/or reduced CLL cell migration.

In light of previous evidence and promising initial results from our group, the work described in this thesis was undertaken to examine the effects of CLL cells in more detail, with the aim of outlining a potential role in the treatment of CLL patients.

Objectives:

The objective of this thesis was to explore the role of the retinoid drug, acitretin in CLL through the performance of in vitro assays. More specifically we set out the following objectives:

1. To determine whether acitretin is anti-proliferative to CLL cells and a CLL like cell line MEC-1 though cell viability assays.
2. To assess apoptosis induction in CLL cells treated with acitretin versus untreated controls by using flow cytometry to measure Annexin V labelled apoptotic cells and Propidium iodide labelled dead cells.
3. To analyse the effect of acitretin treatment on CLL cell homing in response to the CLL specific chemokine CXCL12.
4. To establish whether acitretin modulates CD38 expression in CLL and describe the pattern of modulation in the different patient groups.
5. To present a hypothesis and plan for future experiments involving retinoids in CLL both pre-clinical and clinical.
6. To suggest how acitretin might fit into the clinical treatment algorithm of CLL.

6. MATERIALS AND METHODS

6.1.Ethics

Ethical approval for the study was granted by the ethics committee in Beaumont University Hospital in accordance with the declaration of Helsiniki. Patient study information leaflet and consent form were also approved by the committee.

6.2. Patients

We recruited patients from the haematology day ward initially and later from both the day ward and outpatient clinic of Beaumont Hospital. Patient accrual was completed between January 2014 and October 2015. Our study subjects were patients diagnosed with CLL according to standard WHO immunophenotypic and morphological criteria. We approached patients in a sequential fashion from those not currently on active treatment and were either in remission or relapsed but pre-treatment. A telephone call was made to each patient prior to the date of attendance to clinic in order to allow for time to consider participation in the study. We provided patients with further details with respect to the study aims, objectives in clinic, both verbally and in the form of a pre-designed information leaflet (see appendix). Participating patients gave written informed consent (see appendix for consent form).

6.3. Samples

We collected peripheral blood samples from 32 patients. Whole blood (15 ml) was collected in EDTA and transported to the lab at room temperature.

Three patients were recalled to give repeat samples for further experiments. In the second half of the study, we collected samples specifically from patients with CD38 positive CLL. For this purpose patient records were accessed and patients contacted and invited to volunteer blood samples.

We accessed patient records to take note of demographic and clinical information including age, year of diagnosis, baseline laboratory parameters, IgV_H gene mutation status, *TP53* mutation status, CD38 expression.

6.4. Cell line

Leukemic Cell lines are an important part of laboratory research as they provide a readily expandable in vitro cell lineage that is representative of the disease being studied. Continuous CLL cell lines however have been difficult to establish owing to the fact that CLL cells undergo rapid spontaneous apoptosis in standard culture conditions. Attempts to establish cell line in CLL cells include methods using EBV to immortalise the cells. With longer time in culture however the cells tend to lose the distinguishing pan T-cell antigen CD5.

For this study we used the MEC-1, CLL- like cell line which we obtained from DSMZ Germany. This cell line was established in 1993 from the peripheral blood of a 61-year-old Caucasian man with B-CLL in prolymphocytoid transformation to B-Prolymphocytic Leukemia. The cells bear 94.3% homology of the Ig genes to germ line and are *TP53* mutated (165). MEC-1 cells have been used in many CLL studies both in vitro and in vivo. The cells grow in suspension and morphologically are larger and more polymorphic that primary CLL cells. MEC-1 cells are CD19, CD20, CD22 and CD38 positive. However, the lack of CD5 expression on these cells is not typical of the CLL phenotype, and are thus given the name 'CLL-like' cell line in light of the phenotypic differences. Data from experiments involving MEC-1 therefor need to be interpreted with caution given the differences between the two cell types. In the literature new CLL cell lines include the OSU-CLL and MDA-BM5 that both retains CD5 expression. The OSU-CLL was developed through EBV induced transformations (166). The MDA-BM5 cell line was isolated from the bone marrow of a del17p*, TP53* mutated CLL patient and developed in continuous co-culture with bone marrow stroma cell(167)

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6.5. Culture media

Cell culture media contain a standard set of essential amino acids, inorganic salts, buffer solution, glucose, sodium pyruvate as an additional source of energy and phenol red sodium salt as a PH indicator**.** When choosing from the large variety of commercially

available culture media we considered the particularly delicate nature of primary CLL cells. We chose Iscove's modified Dulbecco's Media (IMDM) with L-Glutamine 25mM Hepes (hepes is superior to bicarbonate buffers in maintaining PH of the culture medium) which we obtained from Biosera®. IMDM is a modification of the widely used DMEM with a higher amino acid content and the addition of the non-essential amino acid L-Glutamine. For the culturing procedure we supplemented the cell media with Foetal Bovine Serum (FBS) to a concentration of 20% FBS for the initial stages of cell line establishment and later 10% FBS supplementation was used. Cells were cultured in T25 ($25cm^3$) culture flasks (Corning) which allowed for a larger density of cells, in turn providing the cells with a greater chance for survival. MEC-1 cells were cultured in T75 flasks also in large density.

6.6. Stock cell-line development

Cell Growing

When received, MEC-1 cells are preserved in cryovials of $1x10^7$cells/ml. Cells were thawed in 37° Celsius water bath and quickly transferred to a 15ml falcon tube, washed twice with 15ml of complete medium and centrifuged. We made stocks of cells by growing the cell for 2 to 3 days until 75% to 80% confluent.

Cell Passaging or 'Splitting'

Following 48 to 72 hours we harvested confluent cells (transferred from flasks to falcon tubes) washed and re-suspended them in fresh culture media. From this cell suspension, we transferred 1 ml into new culture flasks and the growing procedure repeated. Once several culture flasks of cell suspensions were established, we prepared the cells for freezing as back up stocks as follows:

Glutamine, 25mM Hepes (Biosera) supplemented with 10% FBS, 1% Penicillin/Streptokinase at 37^o celcius and 5% CO_2, humidified incubator.

Cell Freezing

We made stocks of cells for back up by cryopreserving cells in liquid nitrogen. Cells were harvested as described. Cells were suspended in 70% medium, 20% FBS, 10% DMSO to a cell concentration of $1x10^7$cells/ml and preserved in sterile cryovials in a liquid nitrogen storage tank until further use.

6.7. Patient derived CLL cell isolation

We isolated CLL cells from whole patient blood using a density centrifugation method utilising a negative selection technique and immunodensity reagent. The rosettesep™ B-cell enrichment media crosslinks unwanted cells with red cells by way of an antibody antigen reaction, that does not include CD19 expressing cells, forming 'immune-rosettes' . These rosettes pellet down with red cells during centrifugation leaving a pure layer of CD19 positive B-cells between plasma and density medium (168).

We performed the CLL isolation procedure in a type 2 laminar flow hood with U.V. light facility under strict aseptic conditions. To each 1 ml of whole blood, we added 50 µL of Rosettesep Human B-cell Enrichment Cocktail, (Stemcell Technologies), and mixed by gentle pipetting. We then incubated the mixture at room temperature for 20 minutes after which an equal volume (1:1) of sterile Phosphate Buffer Saline (PBS), supplemented with 2% FBS solution was added to dilute the blood. This mixture was then carefully layered using a soft transfer (Beral) pipette onto 15 ml of Ficoll Paque density medium (GE Healthcare life sciences) in a 50ml Falcon tube. The layered blood and ficoll were then centrifuged at 1200g force in a Beckman Coulter centrifuge set to room temperature for 20 minutes with the break set to zero. This prevents the separated layers from mixing by sudden stop jolting of the centrifuge. The cell layer appears as a cloudy buffy coat like layer between the plasma and Ficoll. We used a transfer pipette to remove the cell layer onto a sterile Falcon tube and re-suspended the isolated cells in PBS supplemented with 2% FBS. We repeated the centrifugation as a way of washing the cells. This step is repeated again before the cells are finally suspended in a volume of complete media – usually 35ml in a 50ml falcon tube. At the final step we inspect the cells and count them as described below, in readiness for plating.

6.8. Cell counting

A sample of the isolated cells are counted in a way that is both quantitative and qualitative. We used the Trypan blue exclusion method in cell counting in order to differentiate between viable cells which do not take up dye, versus dead cells which take up the Trypan blue. A volume of 10 µL of cell suspension was placed in a 0.5 ml Eppendorf tube and 10 µL of Trypan Blue exclusion dye 0.4% (Sigma Aldrich) added to it and gently mixed by pipetting. Following that, 10µL of the cell/dye mixture was pipetted onto a haematocytometer chamber under a glass slide. Using a 10X objective lens on a light microscope, we counted cells in 4 separate fields, calculated the average and multiplied by the dilution factor of 2. The cell count was expressed as number x 10^4 cells/ml. Following isolation, we used fresh primary cells in Iscoves Modified Dulbecco's Media (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin.

6.9. Drugs and treatments

Acitretin

Acitretin is a metabolite of etretinate with a shorter half-life. We obtained acitretin from sigma Aldrich in powder form and dissolved in DMSO according to the manufacturer's instructions. We made 10 mM stock solutions aliquots and stored these in a -20^{o}C freezer until use. The solution was protected from direct light at all times.

Ibrutinib

We obtained the Bruton Tyrosine Kinase (BTK) inhibitor, Ibrutinib from Selleckchem, Houston, Tx, USA. The 10 mM solution obtained was further aliquoted into 1 mM aliquots, stored in a -20^{o}C freezer until further use. The solution was kept on ice when out of the freezer and thawed at room temperature.

6.10. Cell Viability assays

In vitro assays to test cells for sensitivity to drugs can be divided into three main categories; clonogenic assays, proliferation assays and total cell kill or cell viability assays. Here we use the MTS assay- a form of cell viability assay. The MTS assay kit we used is comprised of the following**:** CellTiter 96® AQueous One Solution Cell Proliferation assay reagent, PROMEGA (Madison, Wisconsin, USA) which contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. The MTS compound is reduced to a purple compound – formazan - by mitochondrial enzymes, indicating metabolically active, viable cells. Thus, the MTS assay is a form of colorimetric viability assay. The amount of formazan present in each well is directly proportional to the number of viable cells – darker shades of purple denote more viable cells. The intensity of the colour change is detected using a spectrophotometer which measures light absorption of the coloured solution at 570 nm wavelength. The MTS assay is not a cell proliferation assay as it does distinguish the metabolic activity of dividing cells, rather it is purely a measure of viable cells.

Setting up the assay

To determine primary CLL and MEC-1 cell sensitivity to acitretin, we counted and seeded cells at concentrations of $1x10^5$ cells in 100 µL of 10% FBS media in 96 well plates. We treated cells with different concentrations of acitretin with the aim of determining the drugs IC50 in these cells. IC50 is the concentration of drug in molar that achieves 50% cell kill. We used a top concentration of 100 µM and serially diluted this down by 50%, 10 times. All assays were set up in triplicate wells. Following 24,72 and 96 hours of incubation, we added 20 µL of CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay reagent (PROMEGA) to each 100 µL cell suspension and mixed with gentle pipetting. We then incubated the plates for a further 1-2 hours to allow for the MTS reaction, which is time dependant. Given that excess incubation time can be detrimental to cells and can result in cell cytotoxicity we limited this time to 1.5 hours. We analysed the plates on a spectrophotometer with a 96 well plate reader and recorded the absorbance of the solution at a wavelength of 520 nM in one second.

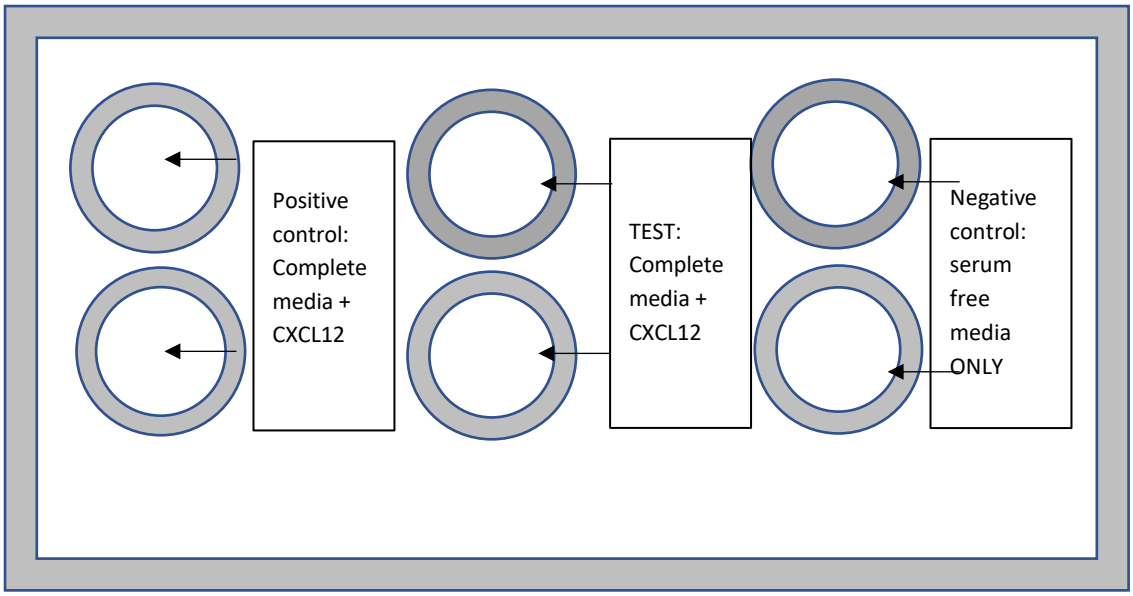
Viabilities were normalised to the relative viabilities of vehicle (DMSO) treated control samples as a negative control for spontaneous apoptosis in different samples. We used the BTK inhibitor, Ibrutinib as the positive control. We set up serial dilutions from a top concentration of 10 µM of Ibrutinib simultaneously alongside the acitretin experiments and incubated again for 24, 72 and 96 hours. The same MTS procedure describes above was applied to the Ibrutinib treated cells.
We constructed dose response curves in order to calculate IC50.

6.11. Migration Assay: cell homing in response to CXCL12 chemokine

The aim of the migration experiments was to investigate the effect of treating cells with acitretin on the cells ability to migrate (home) toward the higher concentration of chemokine.

We selected the recombinant chemokine CXCL12 (R&D SYSTEMS), also known as Stroma Cell Derived Factor 1(SDF-1), as a CLL specific chemoattractant. We seeded $2X10^6$ primary cells and $1X10^6$ MEC-1 cells in 24 well plates in duplicates and treated these with either 10 µM acitretin and kept cells untreated in complete media as controls. Plates were incubated overnight at 37^oC, 5% CO2, humidified incubator.
The following day, we set up treated and untreated cell suspensions on Corning transwell polycarbonate membrane cell culture inserts with 5 µm size pores (Sigma Aldrich, St. Louis). We placed 400 µL of media in the lower chambers of each of the wells; for the test wells (those containing cells treated with acitretin) complete media was placed and 200 ng/ml of CXCL12 chemokine added. We placed the same media and CXCL12 underneath a duplicate of untreated cells to act as positive control. Serum free media (SFM) without CXCL12 was placed in the negative control wells.
We placed the upper chamber inserts on top and into them, 250 µL of cell suspension was transferred from the treatment plates. We replaced all cells into the incubator and allowed to migrate for 4 hours.
We counted cells in the upper and lower chambers using a haematocytometer and trypan blue exclusion method as described above and calculated the mean cell count

of migrated and un-migrated cells. The proportion of migrated cells was expressed as the percentage of cells in the lower chamber to cells in the upper chamber.



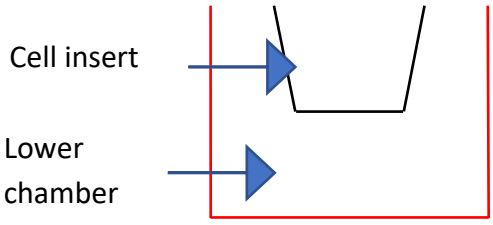


Figure 3: Schema of lower chamber duplicates.

Treated and untreated cells where placed in cell inserts (lower schema) and each placed over lower chambers containing either complete media and CXCL12 (two duplicates on the right as shown) or serum free media to determine background cell movement

6.12. Apoptosis measurement

Antibodies

For the detection of apoptosis, we stained cells with Annexin V /FITC (5µl per 100µl of cell suspension) and Propidium Iodide (PI) (5 µl per 100 µl of cell suspension) as per manufacturer's instructions (BD Pharmingen, Annexin V: FITC apoptosis detection kit 1). All Antibodies were stored in -4^{o}C refrigerator and protected from light at all times.

The appearance of phosphotidylserine (PS) residues on the cell surface is a marker of early apoptosis and detection of these residues can be used as a measure of the presence of early apoptotic cells (169). Annexin V has a strong affinity for PS residues and can therefore be used as a probe for detection of early apoptotic cells. Propidium Iodide (PI) binds fragmented DNA in the nucleus of necrotic cells and therefor is used coupled with annexin V to differentiate apoptotic from necrotic cells.

In experiments to quantify apoptosis, we used flow cytometry to measure phosphatidyl serine (PS) externalisation through labelling cells with annexin V conjugated to FITC (fluorescein Isothyocyanate). PI was added to label and thus exclude necrotic cells. We seeded 2x10^{6} primary cells and 1x10^{5} MEC-1 cells suspended in 10%FBS media in 24 well plates in duplicates and treated all cells with 10 µM or 20 µM of acitretin. Control cells were treated with 1 µM of Ibrutinib for positive controls and 0.01% DMSO for negative controls. Plates were incubated for 24, 48 and 72 hours.

We retrieved cell suspensions from individual wells and placed them in 1.5 ml Eppendorf tubes and centrifuged each at 4500 RCF (relative centrifugal force) and discarded the supernatant. The cell pellet was washed once in sterile PBS and re-suspended in 500 µl of Annexin binding buffer. We added 5 µL of Annexin5/FITC (Annexin V:FITC apoptosis detection kit 1, BD Pharmingen, BD biosciences) and 5 µL of PI to each tube of cells and incubated them at room temperature in the dark for 10 to 15 minutes.

Flowcytometric analysis

We analysed cells for apoptosis detection on a Beckman Coulter Cytomics FC 500 flowcytometer. Cell acquisition data was analysed using CXP software. Cell scatter was displayed in dot and density plots where Annexin V –FITC labelling was plotted on the X-axis and PI labelling on the Y-axis. Percentage of Annexin V/FITC staining in acitretin treated cells was compared to DMSO treated controls.

6.13. CD38 Expression

CD38 is a transmembrane glycoprotein expressed in a subgroup of CLL. CD38 expression is part of the routine immunophenotype panel in CLL diagnosis. In the CD38 modulation experiments we sought to measure CD38 expression following treatment with acitretin.

Treatement: 10^5 primary cells and MEC-1 cells suspended in complete media (IMDM + 10% FBS), were seeded in 24 well plates in duplicates as described above. In one set of experiments we treated cells with 10 µM acitretin and 0.01% DMSO for control. Cells were incubated following the different treatments for 24 and 72 hours. We also treated a group of cells for shorter durations of time and measured CD38 expression at the following time points: 30 minutes, 4 hours, 24 hours.

At the specified time point, we retrieved cells from the wells, transferred them into Eppendorf tubes, washed each with PBS in readiness for labelling with antibody-dye conjugates.

Treated and untreated cells were then labelled with CD19 PE-Cy7 mouse anti-human antibody (1:20 dilution), BD Pharmingen and CD38 PE-Cy5.5 mouse anti human antibody (1:10 dilution), BD Biosciences. In another set of experiments we treated cells with the following concentrations of acitretin; (100 µM, 50 µM, 10 µM, 1 µM, 100 nM). Cells were incubated with antibody for 20 minutes at room temperature in the dark before being transported to the flowcytometer. We measured CD38 expression levels using flowcytometry. Results were expressed as Mean Fluorescence Intensity (MFI) mean +/- SEM.

6.14. Data analysis and statistics

We utilised Microsoft Excel and GraphPad Prism software to analyse and graph our data. All results that were deemed to be Guassian or approximately Guassian in distribution were compared using Students paired *t* test or 1-way ANOVA.

7. RESULTS

7.1. Patient Characteristics

Samples were taken from a total of 32 patients. The mean age of patients was 71.8 years and the median age was 73 years. Nineteen (59%) of the patients were male and 13 (41%) female. Twelve (38%) patients had received previous treatment for CLL and had relapsed at the time of sampling. Twenty (63%) patients had stable disease not requiring treatment and had not received any CLL specific therapy prior to participating in the study. FISH analysis was available in 10 patients; two patients had 17p deletion and one patient was *TP53* mutated. Chromosome 13q deletion was detected in three patients and in another four patients, a cytogenetic report stating absence of deletion 17p and 11q was available (Table 4).

Table 4: Patient characteristics.

A total of 32 patients provided samples for the study of which 19 (59%) were males and 13 (41%) females. Twelve samples were from patients who had been previously treated for CLL and had relapsed at the time of sampling while 20 samples were from treatment naïve patients with stable disease. Twelve (38%) patients had received previous treatment for CLL and had relapsed at the time of sampling. Twenty (63%) patients had stable disease not requiring treatment and had not received any CLL specific therapy prior to participating in the study. FISH analysis was available in 10 patients; two patients had 17p deletion and one patient was TP53 mutated. Chromosome 13q deletion was detected in three patients and in another four patients, a cytogenetic report stating absence of deletion 17p and 11q was available.

No.	GENDER	AGE	CD38	Tx	FISH
CLL1	F	72	<1%	N	ND
CCL2	M	76	1%	N	ND
CLL3	M	77	<1%	Y	ND
CLL4	M	52	13%	Y	ND
CLL5	M	78	<1%	N	ND
CLL6	F	65	6%	N	13q del
CLL7	F	66	6%	N	ND
CLL8	M	54	1%	Y	13q del
CLL9	M	78	88%	Y	ND
CLL10	F	54	1%	N	ND
CLL11	F	72	1%	N	No 17p/11q
CLL12	M	82	43%	Y	ND
CLL13	M	76	19%	Y	17p del
CLL14	F	89	2%	N	ND
CLL15	F	58	0.62	N	ND
CLL16	F	71	1%	N	ND
CLL17	M	68	NA	N	No 17p/11q
CLL18	M	78	43%	Y	ND
CLL19	M	67	<1%	N	ND
CLL20	F	66	1%	Y	13q,11q del
CLL21	F	57	4%	N	ND
CLL22	F	73	<1%	Y	ND
CLL23	M	75	<1%	Y	17p del
CLL24	M	90	1%	N	ND
CLL25	M	72	2%	Y	ND
CLL26	M	81	Neg	N	ND
CLL27	M	64	NA	Y	p53 mut
CLL28	F	74	Neg	N	ND
CLL29	M	83	9%	N	ND
CLL30	M	83	1%	N	no 17p/11q
CLL31	F	73	NA	N	No 17p/11q
CLL32	M	73	<1%	N	ND

7.2. CD38 status:

In our centre, CD38 expression is measured using routine immunohistochemistry at diagnosis. In patients diagnosed with CLL prior to 2009 (two patients) positive or negative was given as the CD38 status. After 2009, CD38 expression levels as percentage positive cells was measured for each CLL patient (27 patients). In our sample, four (13%) patients were $CD38^{+}$ by clinical definition (CD38 expression level ≥ 20%). 25 patients had CD38 expression level ≤19%. Of these, three patients had 'negative' as the stated CD38 expression status. Three patients had no CD38 expression levels recorded (**Table 5**).

Table 5 :Patient break down according to CD38 expression status.

Four (13%) of the patient sample were CD38 positive (CD38 expression ≥ 20%). Six patients had CD38 expression level between 6% and 19%, while 17 patients had ≤ 5% $CD38^{+}$ cells at the time of testing. Two patients were stated as 'negative' CD38 and a further three patients had no documented CD38 expression levels.

CD38 status/%	Unknown	NEG	≤5	6 to 19	≥20	TOTAL
%/ No. of patients	9% (n=3)	6% (n=2)	53% (n=17)	19% (n=6)	13% (n=4)	100% (n=32)

7.3. Cell purity

Percentage of CD19 expressing B- lymphocytes was quantified using flow-cytometry of the cell sample. A mean cell purity of 95% CD19 positive cells (+/- 2.48%) was achieved using the negative selection method (
Figure ***4***).

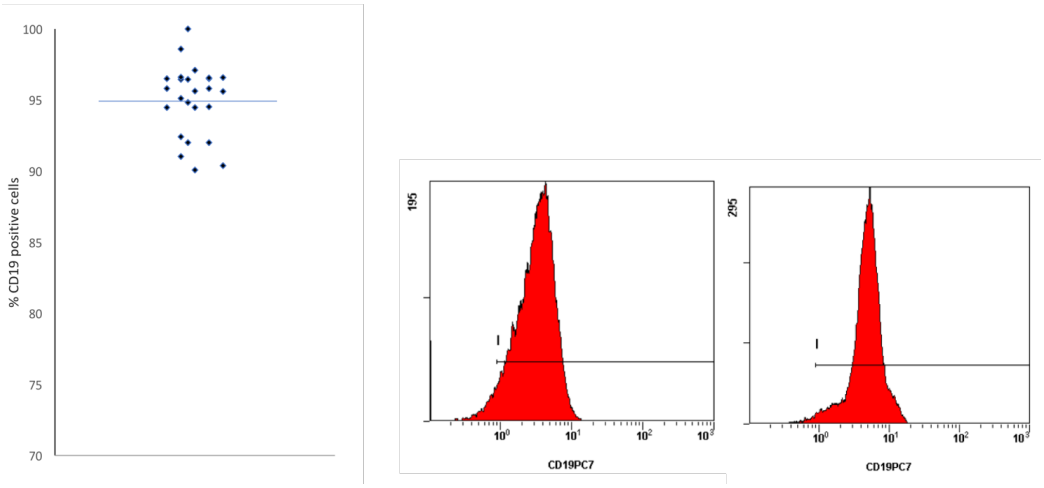


Figure 4: B-cell purity in cells separated from patient whole blood.

A mean of 95% of cells separated from whole blood were CD19 positive lymphocytes (+/- 2.48%). Left graph shows the spread of CD19 positive expression values obtained in our experiments. On the right, representative flow plots showing CD19 positive expression in cells.

7.4. Growth Assays

Primary CLL cells and the MEC-1 cell line are non-adherent in culture. When cultured cells/ml at baseline to 29.3x10^5cells/ml at 24 hours of incubation. At 72 hours, the mean MEC-1 cell count was 59.3x10^5 cells/ml, thus MEC-1 cells had a doubling time of between 24 to 48 hours (n=4). In contrast to the rapid proliferation rate of MEC-1 cells, mean primary CLL cell count declined from 16.75x10^5 cells/ml at baseline to 5.4x10^5 cells/ml at 48 hours and to 3.15x10^5 cells/ml at 72 hours of incubation (n=4)(Figure 5).

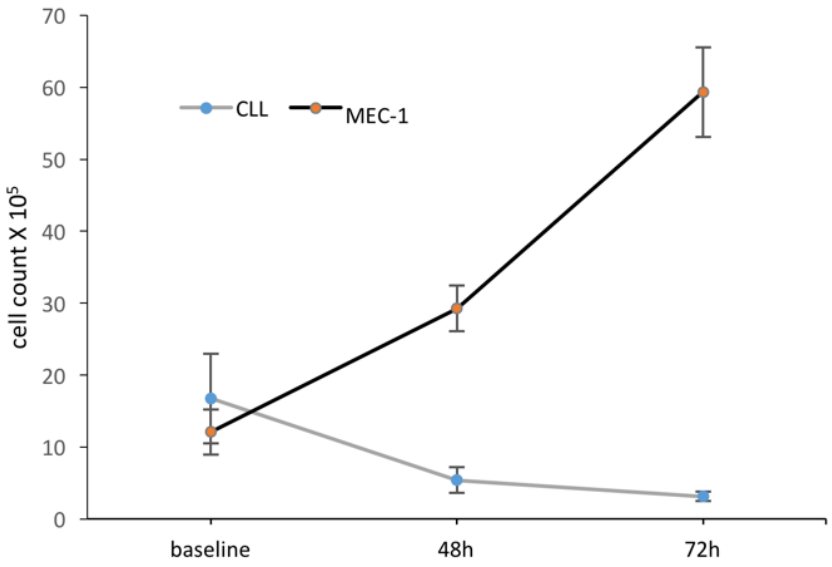


Figure 5. Growth curves for MEC-1 cell line and primary CLL cells.
At 48 hours the MEC-1 count was X 2.4 of baseline count (n=4). Primary CLL cell count declined in culture. Primary cell count fell to 32% of baseline at 48 hours and 18.7% at 72 hours. Displayed are mean counts +/- S.E.M.

7.5. Cell viability assays

MEC-1 viability

The MTS assay was used to measure cell viability following drug treatments as previously described. The assay provided a convenient platform for testing primary CLL and MEC-1 cell sensitivity to drugs as the need for minimal assay steps ensured preservation of cell numbers in wells. This was of particular importance given the non-adherent nature and the small size of these cells. In order to determine the effects of acitretin on cell viability, cells were treated for 24, 48, 72 and 120 hours, with different concentrations of acitretin and control wells were treated with the drug vehicle in this case DMSO. Figure 6)shows the effect of acitretin on MEC-1 cell viability using serial dilutions of acitretin, at 24, 48, 72 and 120 hours of treatment. Acitretin lead to a significant reduction in MEC-1 cell viability after 120 hours of treatment. The lowest mean cell viability was 43.1% (56.9% mean reduction) when cells were treated with 6.25µM acitretin. A variable response to acitretin was seen with shorter durations of incubation. For example, at 48 hours of incubation, the lowest mean percentage cell viability was 76.8% (23.2% mean reduction) in cells treated with 12.5µM acitretin and at 96 hours the lowest relative cell viability achieved was 89% (11% mean reduction) in cells treated with 25µM acitretin (n=2 for each time point).

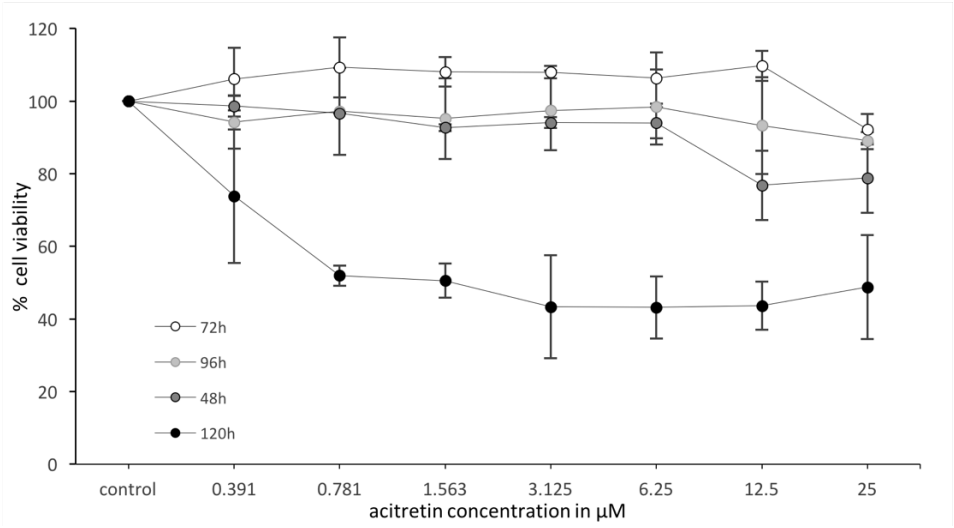


Figure 6: Effect of acitretin on MEC-1 cell viability.
Treatment with two-fold serial dilutions of acitretin for 48, 72, 96 and 120 hours of acitretin with a maximum concentration of 25µM. Maximum reduction in mean cell viability was achieved when cells were treated with acitretin for 120 hours (minimum mean cell viability 43.1%, 76.8% reduction). A variable dose response was seen at all other time points. Viabilities are normalised to the relative viability of control samples treated with the acitretin drug vehicle DMSO (100%). Displayed are means +/- SEM.

Primary Patient-derived cell viability

Acitretin had a modest effect on the viability of primary CLL cells derived from treatment naïve patients *in vitro.* Incubation of primary cells for 72 hours with 20µM acitretin resulted in a significant reduction in mean cell viability in four patient samples to 81.7% (18.3% reduction) (figure 2.2(B), n=4, mean +/- SEM, *$p=0.042$). However, the effect of acitretin on viability was less apparent at 48 and 96 hours of treatment (Figure 7). Incubation for longer durations produced similar relative cell viability results as those seen at 96 hours of incubation. Primary cells derived from previously treated patients showed little to no response to acitretin in cell viability assays.

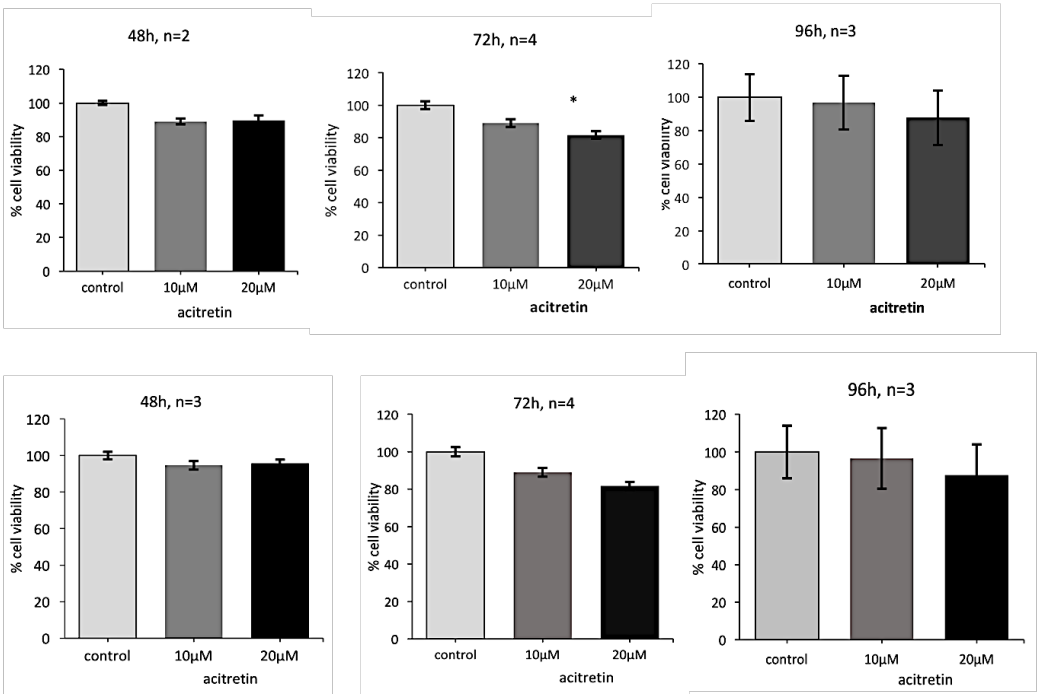


Figure 7. Primary CLL cell viability.

Following 48, 72 and 96 hours incubation with either DMSO in the control cells or 10μM or 20μM of acitretin. **Top Row** : graphs show dose response for samples from treatment naïve patients. **Bottom row**: shows graphs for cells from relapsed patients. Acitretin reduced the viability of primary CLL cells derived from previously untreated patients but did not affect cells from relapsed patients. Significant reduction was achieved only in cells derived from therapy naïve patients incubated with 20μM acitretin for 72 hours (n=4) *($p<.05$).

The BTK inhibitor Ibrutinib (PCI -32765) was utilised in cell viability experiments as a positive control. Ibrutinib was cytotoxic to MEC-1 cells in a dose and time dependent manner. At 72 hours incubation, a maximum reduction in viability was achieved in cells treated with 10µM ibrutinib (31.3% relative viability, 68.7% reduction). Ibrutinib treatment of primary CLL cells also resulted in a dose and time dependent reduction in cell viability. Shown in (Figure 8;B, C and D) are representative patient samples of cells that have been treated with either 1 or 10µM ibrutinib or incubated with DMSO. Ibrutinib (10µM) reduced CLL cell viability after 48 hours to 60% relative to control (40% reduction), whilst at 96 hours of treatment, maximum reduction was to 38.2% of control cells (62% reduction) (mean +/- SEM, *$**p \leq .01.$).*

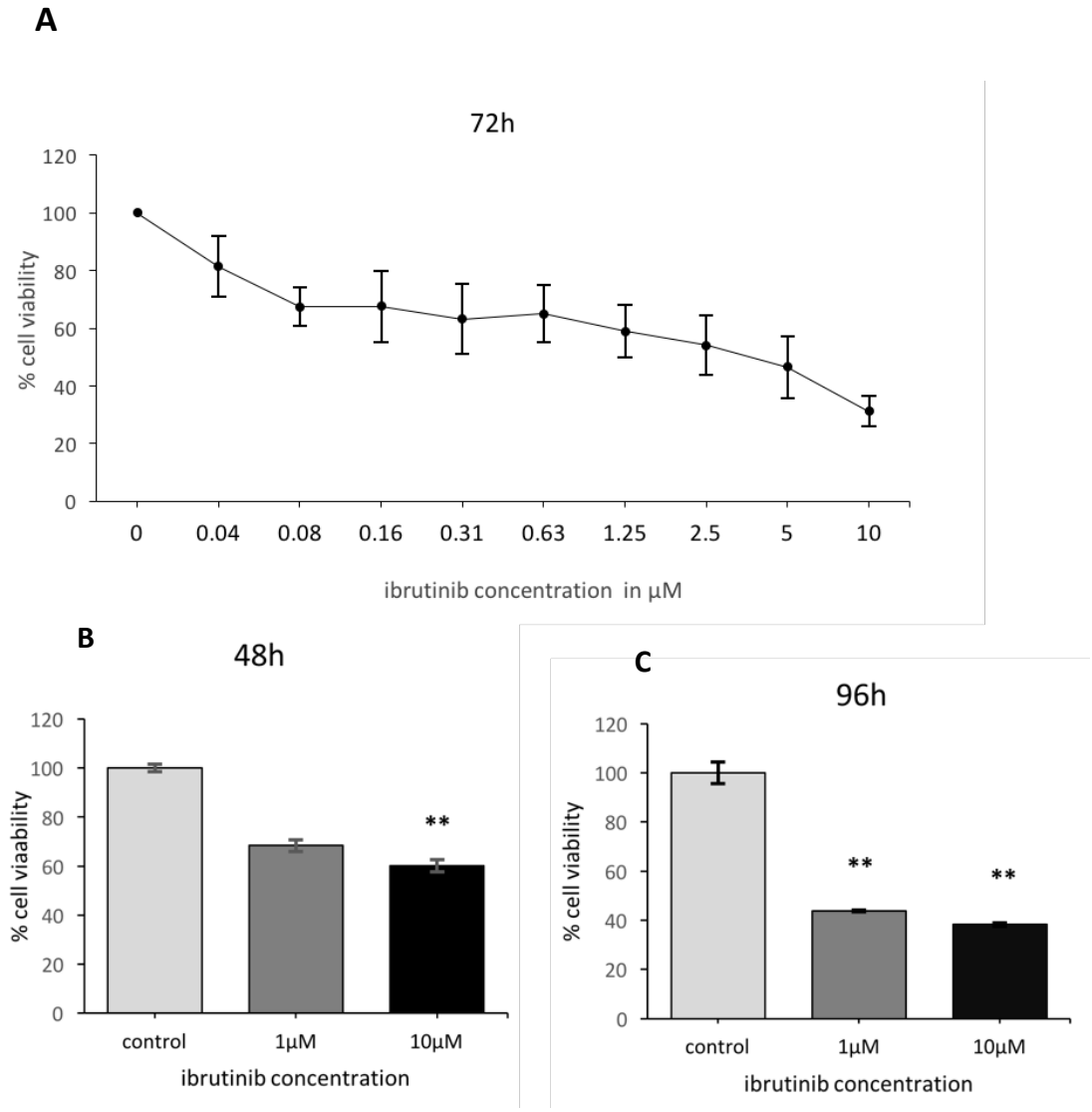


Figure 8: Primary CLL cell viability following treatment with Ibrutinib.

(A) graph showing MEC-1 cells treated with two-fold serial dilutions of acitretin. Maximum reduction in cell viability was in cells treated with 10µM ibrutinib (68.7% reduction). Primary cells were incubated with either 1µM, 10µM ibrutinib or DMSO. Graphs **(B)** and **(C)** depict cell viabilities in ibrutinib treated cells following 48 and 96 hours of treatment respectively. Ibrutinib was cytotoxic to MEC-1 and primary CLL cells in a time and dose dependant manner. Viabilities are normalised relative to controls. Displayed are mean +/- SEM. **p<0.01.

7.6. Cell Migration

Cell migration or “homing” in CLL is influenced by several CLL specific chemokines.
In order to determine whether acitretin influences CLL cell homing in response to micro environmental stimuli *in vitro*, we used CXCL12 as a CLL cell specific chemo-attractant. Acitretin treatment resulted in a significant reduction in CLL cell homing toward CXCL12 in 14 out of 17 (82%) patient samples. Mean relative migration was 23.5% (+/- 16.5) in untreated cells compared to 11.9% (+/- 12.5) in cells treated with acitretin (11.6% less cell migration, ***p=.00018*, mean +/- SEM). Samples from eight of the 14 patients were from previously treated, relapsed patients while the other six were from treatment naïve patient samples. CD38 expression in three of these 14 samples was ≥ 13% while nine were negative and two had no documented CD38 levels, therefor no link could be made between CD38 expression status and change in cell homing ability in this case.
In the remaining three patient samples tested, mean cell migration followed the opposite pattern with a mean 6.5% (+/- 1.5) of cells moving towards CXCL12 in the control wells, while 20.7% (+/-4.2) of acitretin treated cells moved to the CXCL12 containing chamber (14.2% higher cell migration**p=.023,* n=3, mean +/- SEM). Two of the three samples were from stable untreated patients and the third was from a heavily pre-treated relapsed patient. One of the three patients had CD38 expression of 88% while the other 2 were CD38 negative (Figure 9).

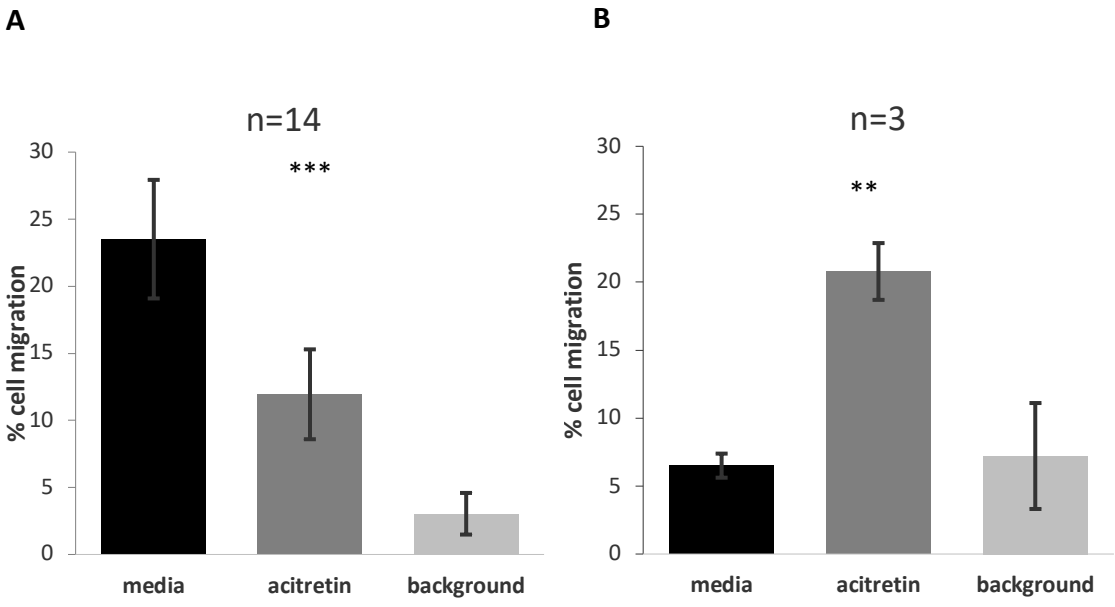


Figure 9: **Primary CLL cell homing in response to CXCL12**.
(A) Patient derived CLL cells from 14 of 17 patient samples showed significantly reduced homing in response to the chemokine CXCL12 when pre-treated overnight with 10µM acitretin as compared to untreated cells (23.5% migration in untreated cells versus 11.9% in acitretin treated cells, 11.6% reduction, **p=.00018). **(B)** In 3 out of 17 patients, acitretin appeared to increase cell homing toward CXCL12 (20.7% migration in acitretin treated cells versus 6.5% in untreated control cells (*p= .023).

7.7. Apoptosis

Apoptotic cells exhibit characteristic morphological and molecular features which are exploited in *in vitro* to study drug effects on cell apoptosis.

In our study we aimed to ascertain whether acitretin induces apoptosis in CLL cells. We measured apoptosis in acitretin treated primary CLL cells from 12 patients through flow cytometric analysis of Annexin V labelling of early apoptotic cells as described in the methods section.

Acitretin 10μM did not affect the rate of apoptosis in primary CLL cells at the time points tested (24 and 72 hours). Following 24 hours of incubation, the mean percentage of apoptotic cells in the acitretin treated group versus DMSO treated controls was very similar 28.4% versus 31.1% (n=8) and after 72 hours of treatment, the a mean of 15.6% apoptotic cells was seen in acitretin treated cells and 7.9% in DMSO treated controls (n=4, p=.11).

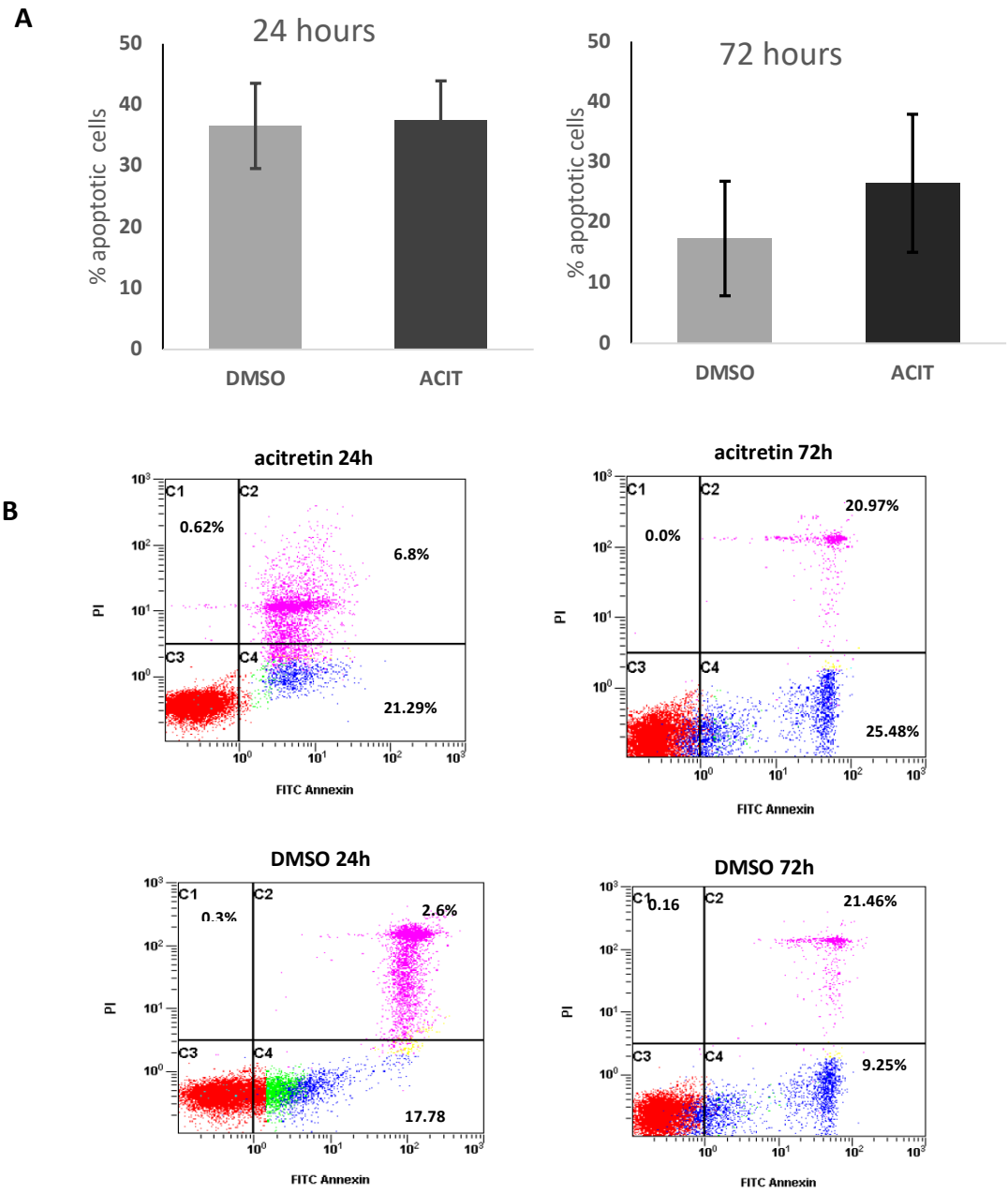


Figure 10 : Effect of Acitretin on CLL cell apoptosis.

(A) Bar diagram showing proportion of apoptotic CLL cells following 24 and 72h of treatment with 10µM acitretin Acitretin did not significantly increase the proportion of apoptotic cells as compared to control. **(B)**Flow cytometry plots depicting one representative patient sample. The proportion of early apoptotic cells is shown in segment C4 of the plot. Acitretin treatment did not increase the rate of early apoptosis following 24 and 72 hours of incubation as evidenced by the similar proportion of apoptotic cells in the DMSO and acitretin plots.

7.9. CD38 Expression

Flow cytometric analysis of CD38 expression on primary CLL cells and the MEC-1 cell line was performed in order to determine the effect of acitretin on CD38 expression in these cells. Cells were either treated with 0.01% DMSO or 10µM acitretin for 24 and 72 hours. MEC-1 cells are CD38 positive with mean expression of 69.5% at baseline (Mean Fluorescence Intensity of 5.09).

In primary cells, acitretin treatment resulted in different responses in cells from CD38 positive patients versus cells from CD38 negative patient samples. The increase in CD38 expression was more pronounced in cells from patient samples with CD38 expression ≥ 9% at baseline (Figure 12). While cells with baseline CD38 expression of < 9% had only a marginal rise in CD38 levels following treatment with acitretin (Figure 13).

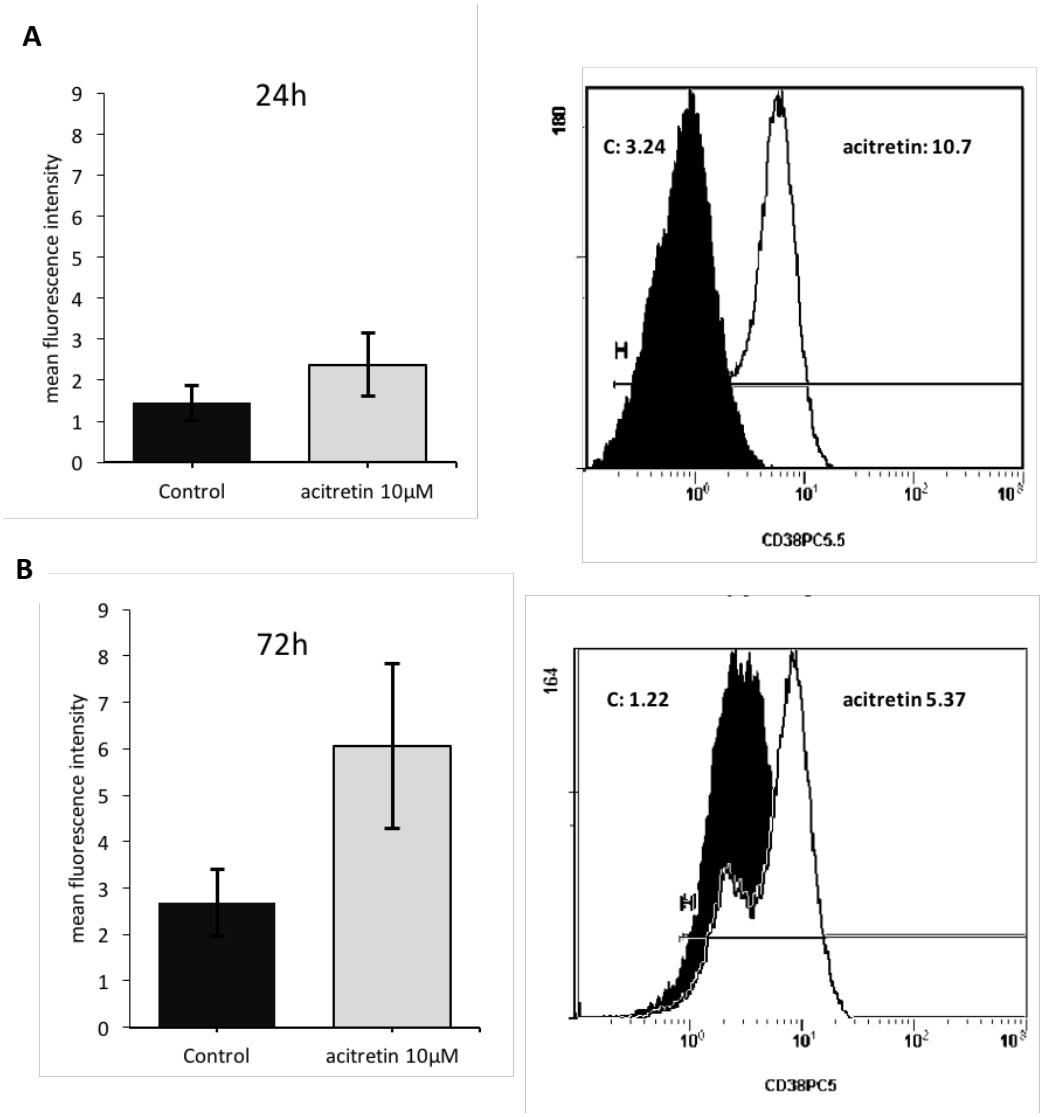


Figure 12: CD38 expression modulation in CD38 positive CLL cells.

(A) At 24 hours of treatment a small increase in CD38 expression was seen in acitrerin treated cells. **(B)** Acitretin treatment significantly increases the expression of the CD38 protein following 72 hours of treatment (n=5, * p=.02). Displayed are mean +/-SEM.

CD38 negative cells

Acitretin treatment of cells that were negative for CD38 at baseline (CD38 expression < 9%), only resulted in an increase of CD38 expression at significantly higher concentrations of acitretin and this increase was only significant in cells treated with 50µM acitretin for 72h (figure 5.2, Graph B, n=2, *p=.022).

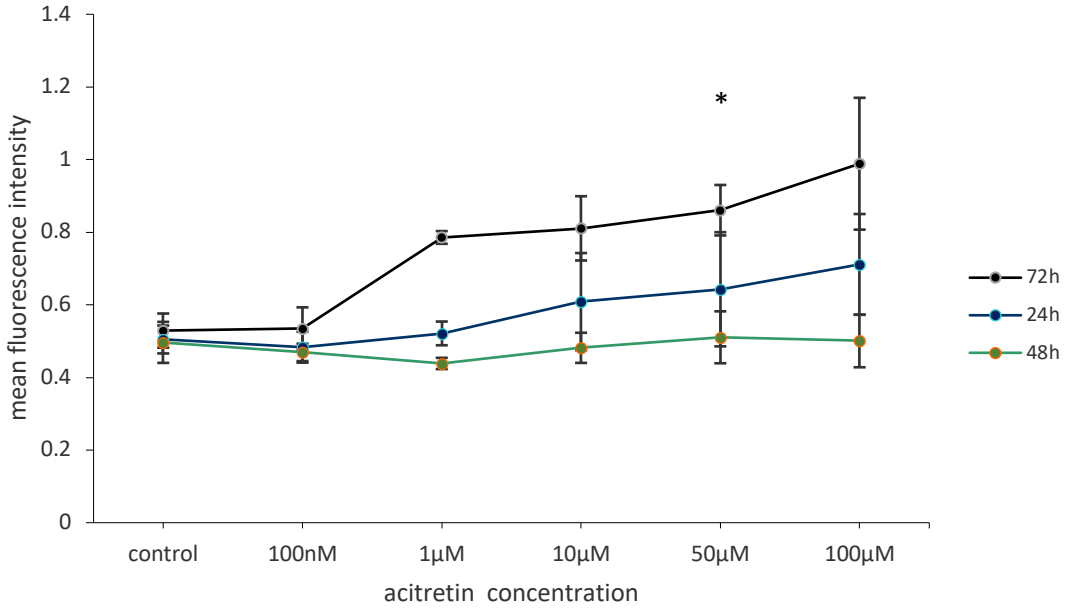


Figure 11: CD38 modulation on primary CLL cells from CD38 negative patients.

Following 24, 48, 72 hours of incubation with 100nM, 1µM, 10µM, 50µM and 100µM acitretin. Acitretin upregulates CD38 expression in cells from CD38 negative patients following 72h of incubation, although this was only significant with 50µM of acitretin at 72h (B), n=2 for each time point shown in series legend. Displayed are mean values +/- SEM, (*P=.022).

CD38 expression in MEC-1 cells

In MEC-1 cells, 24 hours of treatment resulted in a mean of Mean Fluorescence Intensity (MFI) of 11.96(+/-8.56) in control cells and 23.26(+/-12.87) in acitretin treated cells (1.94-fold difference). After 72 hours, acitretin treatment produced a pronounced rise in CD38 expression on MEC-1 cells; mean MFI was 9.4(+/-3.96) in control cells and 64.3(+/-13.92) in acitretin treated cells (fold difference 6.89, n=3, **p=.00075) (Figure 15). Modulation of CD38 expression in all cells was time dependant.

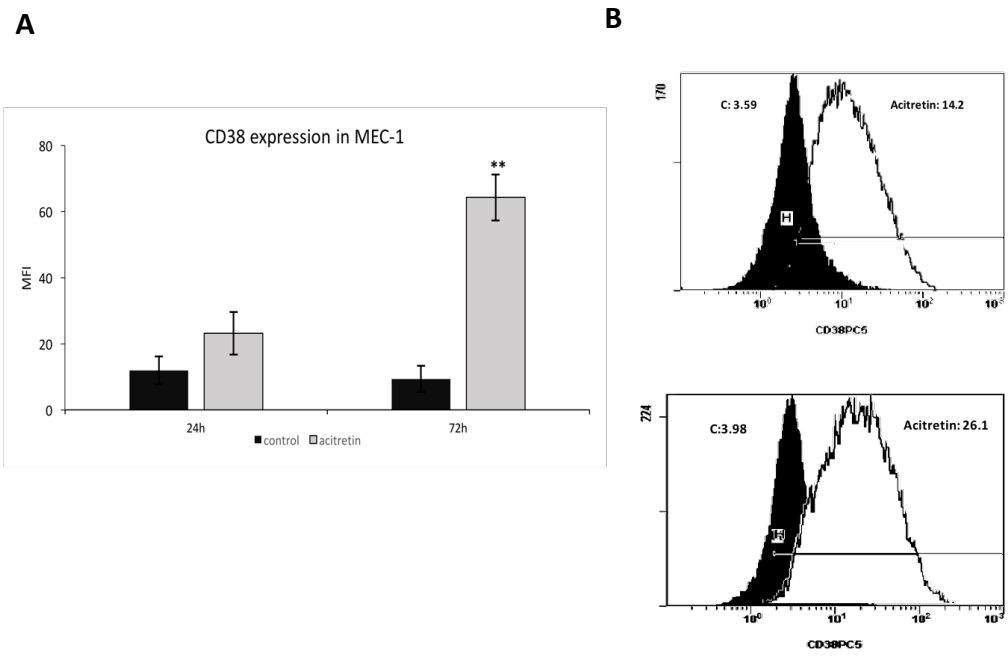


Figure 12. Modulation of CD38 expression in MEC-1.

(A) Following 24 hours of treatment, CD38 expression was 1.94 fold higher in treated versus untreated cells. CD38 expression following 72 hours of incubation was significantly higher in treated cells (6.89 fold higher, n=3, **p=.00075) compared to control. **(B)**Flow cytometry plots depicting a representative sample of MEC-1 cells following 24 hours of treatment (top plot), and 72 hours treatment (bottom plot). Shown are Mean Fluorescence Intensity values for control cells (closed black plot) and acitretin treated cells (open plot) at 24 and 72 hours respectively

Modulation of CD38 expression in all cells was time dependant. Significant upregulation of CD38 expression was seen in MEC-1 cells treated with 10µM acitretin for 72 hours with a relative fold increase in expression of 3.55. CD38 expression ≥9% at baseline the relative difference in expression was 2.55-fold higher at 72 hours compared to that at 24 hours. In cells with <9% at baseline, relative expression in acitretin treated cells at 72 hours of treatment was 1.27 higher than at 24 hours.

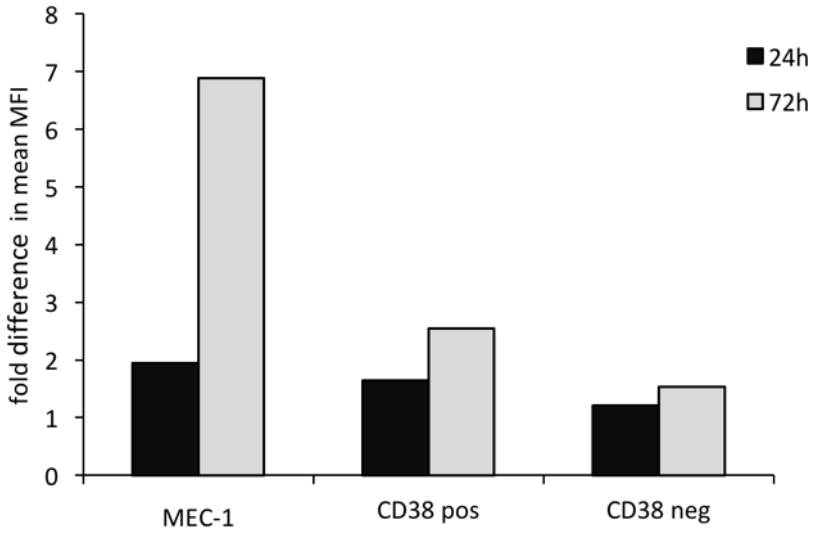


Figure 13: CD38 modulation as fold difference in CD38 expression.

Bar diagram depicting time dependant fold difference in CD38 expression between treated and untreated cells in the different subsets of cells as shown on the x-axis. CD38 expression on MEC-1 cells was 1.94-fold higher in acitretin treated cells as compared to controls at 24 hours of treatment and 6.89-fold higher at 72 hours of treatment. In CD38 positive cells, acitretin resulted in 1.64-fold higher CD38 expression as compared to DMSO treated controls at 24 hours and 2.55-fold higher expression at 72 hours, whilst in CD38 negative cells it was a much smaller difference of 1.21 higher and 1.53 at 24 and 72 hours respectively.

8. Discussion

CLL has a distinctive heterogeneous disease course. Adverse outcome is determined by the presence of a combination of patient and disease related factors. Knowledge of the role of molecular indicators has signalled a risk adapted approach to treatment and novel approaches to therapy are in development.

Acitretin, the drug of interest in our study, is a safe and inexpensive drug that is used successfully in the dermatology setting to treat aggressive psoriasis and in control of squamous cell carcinoma. The anti-proliferative and differentiating properties of retinoid drugs in general, have been well described and interest in this group of drugs as potential anti-cancer drugs has a long history.

Anecdotal evidence for a possible role for the retinoid derivative drug acitretin in CLL was brought to light in our haematology service in Beaumont hospital by the observation of a haematology patient in clinic with a diagnosis of stage A CLL. The patient in question was being managed for squamous cell skin cancer and as part of maintenance therapy was started on 25mg of acitretin. In his haematology follow up it was noted that his lymphocyte count normalised and continued to be normal one year into follow up. Preliminary lab investigations inspired by this observation showed that acitretin reduced cell viability and cell migration in the MEC-1 cell line.

Acitretin and modulation of CD38 expression

In this thesis we set out to outline a role for acitretin in CLL. Our data show that acitretin is a potent inducer of CD38 expression, particularly in CD38 positive cells. What we have demonstrated in CLL cells corroborates what others have shown to be true of CD38 expression induction by retinoids in other cell types. Acitretin induced CD38 expression in CD38 negative cells but only at much greater drug concentrations and even then, did so to a much lesser degree. The reason for this disparity in response was not clear. One possibility is a link between acitretin activation of the retinoid receptor and the transcriptional process by which the CD38 gene is regulated. We hypothesize that the mechanism might involve the RARE element as we believe acitretin functions through

the classical RA pathway to modulate CD38 expression. The CD38 gene may become in some way 'sensitised' to receiving regulatory messages in those cells which express CD38 at baseline. The reverse may be true of CD38 negative cells, in which the CD38 gene is possibly unable to receive transcriptional messages.

CD38 upregulation may constitute part of a novel treatment approach. Nijhoff et al have demonstrated that upregulating CD38 expression leads to greater cell kill with the anti CD38 monoclonal antibody drug daratumumab. We propose that in the setting of CLL, acitretin in combination with anti CD38 monoclonal antibody drugs deserves further study.

Acitretin's effect on cell migration

CLL cell homing to the micro-environmental proliferation centres is a topic of great interest in CLL in light of the potential for targetable pathways. CXCL12 is one of the well characterised chemokines involved in CLL cell tissue homing.

We studied the effect of treating CLL cells with acitretin and then exposing them to CXCL12 on a transwell migration assay platform. Our results show variable effects on each patient sample in terms of numbers of migrated cells. However, in 14/17 patient samples analysed, acitretin treatment had an overall effect of reducing cell homing in response to CXCL12. Taken together we found significantly reduced cell migration in the acitretin treated cells in these patients. In the remaining three patient samples, acitretin treated cells curiously exhibited the reverse behaviour by homing towards CXCL12 enriched media in greater numbers compared to untreated cells. One of these samples was from a CD38 negative patient, previously treated with multiple lines of chemo and was on his 3rd relapse. The other patient had early stage, untreated, CD38 negative CLL. The third patient was another relapsed sample but was CD38 positive. We can therefore draw no unifying conclusion about these three patients. We hypothesize that the three patients may possess a common variable that is of particular relevance to migration, perhaps CXCR4 receptor expression levels or MMP9 or L-selectin expression. Therefor we suggest that further functional studies to deduce the mechanism by which acitretin achieves this effect on migration, would be an attractive line of enquiry. Unexpectedly, in the CLL samples in which acitretin increased cell

migration, the migration into the media was much less that in those CLL patient samples where acitretin reduced cell migration. Whether this discrepancy in migratory response to CXCL-12 is due to a common factor or characteristic in these cells resulting in the treated cells showing increased migration is not evident.

One explanation for the varied response of cells in culture to acitretin treatment could be the diverse function of acitretin at the gene transcription level. Acitretin can induce as well as downregulate transcription of the same set of genes, depending on a variety of cellular factors as reported by Balmer et al(142).

Acitretin and cell viability and growth

Primary CLL cells are challenging to work with in the in vitro setting. Under traditional culture conditions of media supplemented with foetal bovine serum, primary CLL cells undergo apoptosis. Studying the effect of acitretin on Primary CLL cells on the standard platform used for other primary cells was fraught with obstacles.

The 96 well plate platform on which viability assays are ordinarily performed was never-the-less our chosen platform. We undertook a series of optimisation steps in order to demonstrate the effect of acitretin on CLL cell viability. In the first set of experiments, the number of cells seeded per well was 1×10^3 per well, but it quickly became apparent that this number was not sufficient with cell numbers of cells declining rapidly in both treated and untreated wells. We therefor increased the proportion of cells per well as this was shown to dramatically improve survival (83). We increased the number of cells per well first to 1×10^4 cells/well and later to 1×10^5 cells per well in the 96 well plate setting and to 2×10^6 in the 24 well plate settings for other assays.

It was also evident from the start that the MTS assay we were conducting was to demonstrate cytotoxicity rather than anti-proliferative effects of acitretin. This was in view of the fact that the primary cells isolated from peripheral blood represent the non-proliferating circulating pool of CLL cells. Thereby demonstrating anti-proliferative action was not entirely possible on a group of cells that were largely in the resting phase of the cell cycle.

Indeed, our cell expansion or “growth assays” prove the lack of proliferation in primary CLL cells in standard culture when compared to the immortalised MEC-1 cell line.

In order to improve on the proportion of cells surviving the culture process, we adopted several steps; after several trials of using frozen primary CLL cells, we opted to use cells freshly isolated from whole blood. We also eliminated the intermediary step of incubating cells in culture flasks for several days before plating for the different assays -a step that is ordinarily recommended in all immortalised cell line experiments. We ultimately resorted to setting up the desired assays on the day of sample collection and cell isolation.

We were able to show a modest effect on cell viability in primary cells from treatment naïve patients at 72 hours of incubation with concentrations of 20µM and in MEC-1 cells after 96 to 120 hours but with smaller concentrations. The long incubation time needed to achieve a significant response in MEC-1 cell viability may be explained by the fact that acitretin exerts it’s effects at a molecular level through gene transcription modulation, which would by virtue of the process likely necessitate a longer exposer of the cells to the drug.

We did not show a consistent time and dose dependant effect on CLL viability on primary cells. We cannot however conclude that acitretin did not have a cytotoxic effect on these cells, as primary CLL cells underwent spontaneous apoptosis most probably before it was possible for acitretin to exert a significant effect on gene transcription.

Acitretin and Apoptosis

We chose to investigate the effect of acitretin on apoptosis using the Annexin V/PI method which utilises flow cytometry as previously discussed. When we compared proportions of apoptotic cells in each of the treatment and control cell groups, there was a higher proportion of apoptotic cells in the acitretin treated cells when treated for 72 hours.

It is important to note that we obtained varying levels of cell apoptosis in the primary cells from individual patients. This, we believe reflects the heterogeneity of CLL in

different patients. It may also reflect the widely varying actions of acitretin on cellular functions.

Future experiments could look specifically at mediators of the apoptotic pathway, for example caspase 3 levels or Poly -ADP-ribose polymerase (PARP) to further characterise the mechanism by which acitretin may increase cell apoptosis.

The role of the MEC-1 cell line

MEC-1 cells were valuable as a proliferating cell line as we sought to show an anti-proliferative effect of acitretin on CLL. That being said, they are not entirely representative of CLL and are duly characterised as CLL cell 'like' cells. For that reason, we have considered that results obtained from the MEC-1 cell experiments were suggestive of acitretin function but need to be interpreted with caution. *In vivo* studies using a mouse model CLL could further enhance the reliability of apoptosis and viability experiments using acitretin in CLL.

9. Conclusions:

In this study we found the following:

1. Acitretin up-regulates CD38 expression in CLL cells in vitro
2. CD38 expression modulation in cells from patients with positive CD38 at clinical measurement was more evident than in cells from CD38 negative patients.
3. Upregulation of CD38 expression by acitretin was dose and time dependant.
4. Acitretin reduced migration of CLL cells in response to the chemokine CXCL12, suggesting an effect on the CLL micro-environment.
5. Cell viability was reduced by acitretin but to a minor degree *in vitro* when CLL cells are cultured in isolation of a supportive micro-environment.
6. Acitretin does not affect primary CLL cell viability or apoptosis to an appreciable degree in our experiments.

10. Recommendations and future perspectives

In light of our findings we recommend that further studies could include:

- Examining the effect of adding an anti-CD38 monoclonal antibody to cells treated with acitretin, with the aim of describing whether cell kill is improved in cells pre- treated with acitretin.
- Expanding migration assays to include other microenvironmental chemokines such as CXCL13.
- Future experiments designed to include a pro-survival environment such as co-culture with bone marrow stroma cells or the addition of cytokines such as CD40L.
- Describing the mechanism by which acitretin inhibits cell migration. This could be through the measurement of changes in cell markers that are differentially over-expressed in the migrating pool of CLL cells, such as L-selectin and CD49d (VLA-4).
- Determining the effect of retinoids on CLL cell apoptosis and cell viability *in vivo.*
- In addition to further research into the effects of retinoids on CLL cell biology, potential future work might include a phase 1 clinical trial to describe the safety and effects of adding acitretin to standard CLL treatment.
- A clinical trial designed to examine the safety and efficacy of combining anti-CD38 monoclonal antibody therapy with retinoids in the treatment of CLL.

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Appendices

1.Patient information leaflet.

2.Patient consent form.

Website: www.beaumont.ie

Ospidéal Beaumont

BEAUMONT HOSPITAL
P. O. Box 1297 Beaumont Road Dublin 9
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Patient Information Leaflet

Study title: The effect of acitretin on B-Chronic Lymphocytic Leukemia (CLL) cell migration, proliferation and apoptosis.

Principal investigator's name:	**Dr. Philip Murphy**
Principal investigator's title:	**Consultant Haematologist**
Telephone number of principal investigator:	**01 809 2622**

You are being invited to take part in a clinical research study to be carried out at Beaumont Hospital.

Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision.

You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.

You don't have to take part in this study. If you decide not to take part it won't affect your future medical care.

You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don't have to give us a reason. If you do opt out, rest assured it won't affect the quality of treatment you get in the future.

Why is this study being done?

This study is being carried out to test a drug called Acitretin on Chronic Lymphocytic Leukemia cells, in the laboratory, in order to find out whether it effectively kills the leukemia cells, whether it makes them less likely to migrate through tissues and also whether it induces them to undergo natural cell death. **Please note that this drug will be tested on Chronic Lymphocytic Leukemia cells from blood samples and not directly on the patients, therefor involvement in this study will not change patients' normal treatment.**

Who is organising and funding this study?

This study is being organised by the haematology department here in Beaumont Hospital and is being funded by the Haemtology department Research Fund. This is a private fund that is being managed by the haematology department for use in haematology research in the hospital.

How will the study be carried out?

This study will take place in the RCSI laboratory in Beaumont Hospital. It will be carried out over a 2 year period, 2014-2016. We anticipate that around 30 patients and 30 volunteers will participate in the study. Participants will be asked to donate a sample of blood to the study when they are attending their routine clinic appointment.

What will happen to me if I agree to take part?

To take part in this study you will be required to donate the equivalent of 3 tablespoonful of blood, when you attend the haematology out patient's clinic**. Please note that the blood sample will be taken as an additional sample at the routine clinic as part of routine care, therefore you will not be asked to attend especially for the purpose of giving a blood sample for this study.** Your blood sample will then be sent to the Royal College of Surgeons laboratory for analysis as per the study. The samples may be retained for use in future research projects.

Participation in this study will also require some blood test results to be reviewed so your consent will allow the principal investigator to have access to your results. Only Dr. Philip Murphy, Dr. Patrick Thornton and Dr. Sally Mohammed, will have access to

this information. It is possible that this information may be discussed with the other Consultant Haematologists in Beaumont Hospital. **If you take part in this study and then decide to withdraw from it, all data collected previously during your consent (electronic or hard copies) will be destroyed.**

What are the benefits?

The benefit of this research is to hopefully improve the future treatment of Chronic Lymphocytic Leukemia. This project aims to find alternative therapies for the treatment of Chronic Lymphocytic Leukemia.

What are the risks?

The only risk associated with taking part in this study is that of having a blood sample taken. Giving a blood sample may be associated with some pain at the needle prick site, occasionally bruising as well as a small possibility of oozing from the insertion site.

Is the study confidential?

Yes. If you choose to take part in this study, you will be given a unique identification number so your name will not be known throughout the duration of the study. The principal and co-investigators will be aware of this system. Your personal information will be treated as confidential at all times and never be disclosed.
The blood sample collected will be transported to the Royal College of Surgeons laboratory here in Beaumont Hospital by Dr. Sally Mohammed who is a co-investigator in this project and will be carrying out the laboratory experiments for this study.

Where can I get further information?

If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you get in the future.
If you need any further information now or at any time in the future, please contact:

Name: Dr. Philip Murphy
Address: Haematology Department, Beaumont Hospital, Dublin 9

Website: www.beaumont.ie

Ospidéal Beaumont

BEAUMONT HOSPITAL
P. O. Box 1297 Beaumont Road Dublin 9
Telephone: 809 3000 / 837 7755 Facsimile: 837 6982

Consent form

Study title: The effect of the retinoid drug, Acitretin on Chronic Lymphocytic Leukemia cell proliferation, migration and apoptosis.

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study and that I can opt out at any time. I understand that I don't have to give a reason for opting out and I understand that opting out won't affect my future medical care.	**Yes** ☐	**No** ☐
I am aware of the potential risks of this research study.	**Yes** ☐	**No** ☐
I give permission for researchers to look at my medical records to get information. I have been assured that information about me will be kept private and confidential.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
Storage and future use of information: I give my permission for information collected about me to be stored or electronically processed for the purpose of scientific research and to be used in <u>related studies or other studies in the future</u> but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐
I agree to give a blood sample or samples for this research project. I understand that giving a blood sample or samples for this research is my own decision.	**Yes** ☐	**No** ☐
Storage and future use of biological material: I give permission for my samples and information collected about me to be stored for <u>possible future research studies</u> but only if the research is approved by a Research Ethics Committee.	**Yes** ☐	**No** ☐

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

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----------Name (Block Capitals) | Qualifications | Signature | Date

3 copies to be made: 1 for patient, 1 for PI and 1 for hospital records.