Extracellular vesicles in chronic lymphocytic leukaemia: exploration of their miRNA cargo as biomarkers

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Abstract

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The lymph node microenvironment provides essential signals for the proliferation and survival of chronic lymphocytic leukaemia (CLL) cells and contributes to resistance to chemotherapy. There is no readily available access to lymph node tissue and currently no markers of leukaemic cell activity specifically due to stimulation within lymph nodes. Extracellular vesicles (EVs) are produced by CLL cells and their cargo, which includes miRNA, mRNA and proteins, is important for intercellular signalling. Following CD40L/IL-4 stimulation EVs are enriched in miR-363-3p and miR-374b. These miRNA are only detectable at lower levels in plasma from normal subjects and in this thesis the idea that microRNAs might be predictive biomarkers reflecting leukaemic activity in the tumour microenvironment was investigated. To pursue the hypothesis that plasma levels of miR-363 might correlate with disease activity I established real-time PCR assays and showed that patients had higher miR-363 levels than normal subjects and confirmed this result in a repository study using samples from patients in the ARCTIC and CLEAR clinical trials. There were no associations between miR-363 levels and prognostic markers. Numbers of EVs, measured by dynamic light scattering are higher in CLL patients than normal subjects. To examine the source of circulating miRNA size exclusion chromatography was carried out followed by real-time PCR and showed that circulating miR-363 was derived from both plasma and particle bound fractions in healthy subjects but in patients a greater proportion was found in the particle fractions. Finally, I investigated the function of miR-363 in CLL. In contrast to T-cells miR-363 did not appear to have effects on the expression or function of CD69 in CLL B-cells. Overall, numbers of EVs and miR-363 levels associate with CLL but not with survival. An observation, which may have implications for identifying disease associated miRNA and can be followed up is that there appears to be a disease specific distribution of circulating miR-363 between plasma protein and particle bound fractions.

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With Love

Afaf

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List of abbreviations

APC	Antigen-presenting cells
AIHA	Autoimmune haemolytic anaemia
AGO2	Argonaute2
ABI	Applied Biosystems
BMSC	Bone marrow stromal cells
BTK	Bruton tyrosine kinase
BCR	B- Cell Receptor
BCL-2	B-cell lymphoma 2
CLL	Chronic lymphocytic leukaemia
CCL17	Chemokine (C-C motif) ligand 17
Ct	Cycle threshold
EVs	Extracellular vesicles
FISH	Fluorescence in-situ hybridisation
FA	Fludarabine and alemtuzumab
FC	Fludarabine and cyclophosphamide
FCR	Fludarabine and cyclophosphamide with rituximab
FACS	Fluorescence-activated cell sorter.
FSC	Forward scatter
SSC	Side scatter
HCL-v	Hairy cell eukaemia variants
HCL	hairy cell leukaemia
IL21	Interleukin 21

IGHV	Immunoglobulin heavy chain variable gene
lTP	Immune thrombocytopenic purpura
IFNy	Interferon gamma
LDT	Lymphocyte doubling time
LDH	Lactate dehydrogenase
LNA	Locked nucleic acid
MS	Multiple sclerosis
MRD	Minimal residual disease
MBL	Monoclonal B cell lymphocytosis
MCL	Mantle cell lymphoma
MiRNAs	Micro RNAs
mRNA	Messenger RNA
pri-miRNA	Primary miRNA
miRISC	Multiprotein RNA-induced silencing complex
mTOR	Mechanistic target of rapamycin
NLC	Nurse like cells
NF-ƙB	Nuclear factor ƙB
NCI	National Cancer Institute
OD	Optical density
PI3K	Phosphoinositide -3 kinase
PLL	Prolymphocytic leukaemia
PSA	Prostate specific antigen
PBMNC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

RA	Rheumatoid arthritis
RS	Richter's syndrome
RISC	RNA-induced silencing complex
RT-PC	Reverse transcription PCR
SLE	Systemic lupus and erythematosu
SCT	Stem cell transplantation
SYK	Spleen tyrosin kinase
SLL	Small lymphatic lymphoma
SLVL	Splenic lymphoma with villous lymphocytes
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
T-PLL	T-cell prolymphocytic leukaemia
Tfh	T follicular helper
TCL1	T-cell leukemia/lymphoma protein 1
tRNAs	Transfer RNAs
TME	Tumour microenvironment
3’-UTR	3’-untranslated region
WHO	World Health Organization
ZAP70	Zeta-chain-associated protein kinase 70

Chapter 1 Introduction

1.1 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is a lymphoproliferative disorder characterised by a clonal expansion of a B lymphocyte, displaying unique phenotypic and molecular features.

1.1.1 Characterization of chronic lymphocytic leukaemia

In 1891 with the emerging of histochemical staining, it became possible to differentiate the chronic leukaemias as either chronic lymphocytic leukaemia (CLL) or chronic myeloid leukaemia (CML) (Hamblin, 2000).

In the past, patients diagnosed with chronic lymphocytic leukaemia could have had any one of lymphoproliferative disorders characterised by the relatively slow rate of accumulation of lymphocytes of different origins (B cells, T cells and NK cells). Lymphocytic leukaemias were characterised by accumulation of agranular leucocytes with smooth nuclei, whereas splenic leukaemias had granular leucocytes with trefoil-like nuclei.

In the early 1970's, when B cells were identified as expressing surface immunoglobulin (Aisenberg and Bloch, 1972), it became obvious that most cases of "chronic lymphocytic leukaemia" (CLL) are tumours of B cells. With the recognition that lymphoid malignancies derive from distinct cell types and lineages "chronic lymphocytic leukaemia" was eventually subdivided into several diseases that separated true CLL from lymphoid tumours such as prolymphocytic leukaemia (PLL), hairy cell leukaemia (HCL), hairy cell variants (HCL-v), splenic lymphoma with villous lymphocytes (SLVL) and mantle cell lymphoma (MCL) (Hamblin, 2000).

The World Health Organization (WHO) classification of haematopoietic neoplasias describes CLL as leukaemic, lymphocytic lymphoma, being distinguishable from SLL (small lymphocytic lymphoma) by its leukaemic appearance.

In the WHO classification CLL is always a disease of neoplastic B cells, while the entity formerly described as T- CLL is now called T-cell prolymphocytic leukaemia (T-PLL) (Hallek, Cheson et al., 2008, Furman, Sharman et al., 2014).

1.1.2 Immunophenotyping

In diagnosing lymphoproliferative disorders, surface immunophenotyping is an important technique. However, typical chronic lymphocytic leukemia (CLL) can be confused with other conditions, particularly mantle cell lymphoma (MCL) because both express CD19 and CD5. The typical CLL phenotype is $CD5^+$, $CD19^+$, $CD23^+$ and *FMC7*$^-$with weak monotypic surface immunoglobulin (sIg) and weak or absent CD79b. Based on the markers CD5 (+), CD22 (weak), CD23 (+), FMC7 (-) and sIg (weak) as a recommended panel of monoclonal antibodies, a scoring system for the diagnosis of CLL was developed to distinguish typical (≥3/5 positive markers) and atypical <3/5 positive markers) for CLL (Matutes, Owusu-Ankomah et al., 1994). Later, CD79b was added (Table 1.1) (Moreau et al, 1997*).* Using this scoring system, 92% of CLL cases score 4 or 5, 6% score 3 and 2% score 1 or 2. Most other chronic B cell lymphomas and leukaemias score 1 or 2 (Oscier, Fegan et al., 2004). Mantle cell lymphoma, on this panel, expresses surface immunoglobulin more strongly than CLL and CD23 more weakly. Several markers such as CD11c, CD38, and CD43, which have been added in the lymphoproliferative disease panel, were more valuable than CD22, CD79b, and FMC7 in the diagnosis of CLL. Moreover, more recently it has been suggested that CD200 and CD43 may contribute to the diagnosis of CLL (Falay and Ozet, 2017).

Table 1.1 Scoring system based on immunophenotyping for the diagnosis of chronic lymphocytic leukaemia (CLL).

	Score Points	
Marker	**1**	**0**
sIg	Weak	Strong
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
CD22 OR CD79b	Weak	Strong

Scores in CLL are usually >3, in other B cell malignancies the scores are usually <3.

Adapted from (Oscier, Fegan et al., 2004).

1.1.3 Epidemiology

CLL accounts for 40% of all leukaemias in individuals over the age of 65 years (Oscier, Fegan et al., 2004). It is the most common form of leukaemia in Western countries, with an age-adjusted incidence of 4.7 per 100,000 per year (USA, 2011-2015) and a prevalence of 153,000 individuals in the European Union (Figure 1.1) (European Medicine agency, 2014). There is a 20-30 times higher incidence in European, Australasian and North American white and black populations than in Indian, Chinese and Japanese populations. CLL is extremely rare below the age of 30 years, but 20-30% of patients present under the age of 55 years (Oscier, Fegan et al., 2004), with a median age at diagnosis is 80 years (Noone, Cronin et al., 2017). Incidence rates in men are nearly twice as high as in women. Studies have consistently reported female patients as having a reduced incidence and a superior survival (Molica, 2006). Results of a population-based study on the 'Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001' demonstrated males had higher rates than females for most lymphoid neoplasm sub-types. The male-to-female ratio was 1.5-1.6 for all lymphoid neoplasms combined and 1.9 for CLL/SLL for white

patients (Morton, Wang et al., 2006). Data available for 3000 patients in a population-based registry of leukaemias and lymphomas from parts of England and Wales (1984 - 1993), revealed a 5-fold male CLL excess around the age of 40, a 2-fold excess around the age of 60 years and a similar excess around the age of 75 years (Cartwright, Gurney et al., 2002).

The better survival of women with malignant disease might just reflect their better general longevity and an earlier disease stage at diagnosis. However, some authors have hypothesised the hormonal status to have an impact on the course of neoplastic disease (Shaw, Milton et al., 1978), although recent evidence cannot confirm this (Lens and Bataille, 2008). Others have suggested genetic and environmental factors, such as X chromosomal genes, which have been shown to influence immune function (Waldron, 1983). A definitive answer will however, need further investigation.

Increased incidence is also observed in family members of CLL patients. In a study of familial CLL using the Danish Central Population Register, first-degree relatives of CLL cases were found to be at significantly increased risk for CLL [relative risk (RR) = 7.5,95% confidence interval (Cl): 3.63- 5.56] (Goldin and Caporaso, 2007). Families with multiple individuals affected with CLL and other related B cell tumours have been described (Houlston, Sellick et al., 2003). Large, population-based case-control and cohort studies have also shown a familial association of CLL and related conditions including non-Hodgkin and Hodgkin lymphoma. Monoclonal B cell lymphocytosis (MBL) is also associated with families with CLL (Marti, Abbasi et al., 2007, Fuller, Papaemmanuil et al., 2008).

Linkage studies have been conducted in high-risk CLL families to screen the whole genome for loci that contribute to susceptibility, but no gene mutations have yet been identified by this method. Houlston and colleagues (2003) concluded that 'at present there is no compelling evidence that any specific gene acts as a major susceptibility locus and part of the inherited susceptibility to the disease is mediated through low-risk alleles'. The same conclusions were reinforced by the same group (Sellick, Catovsky et al., 2006).

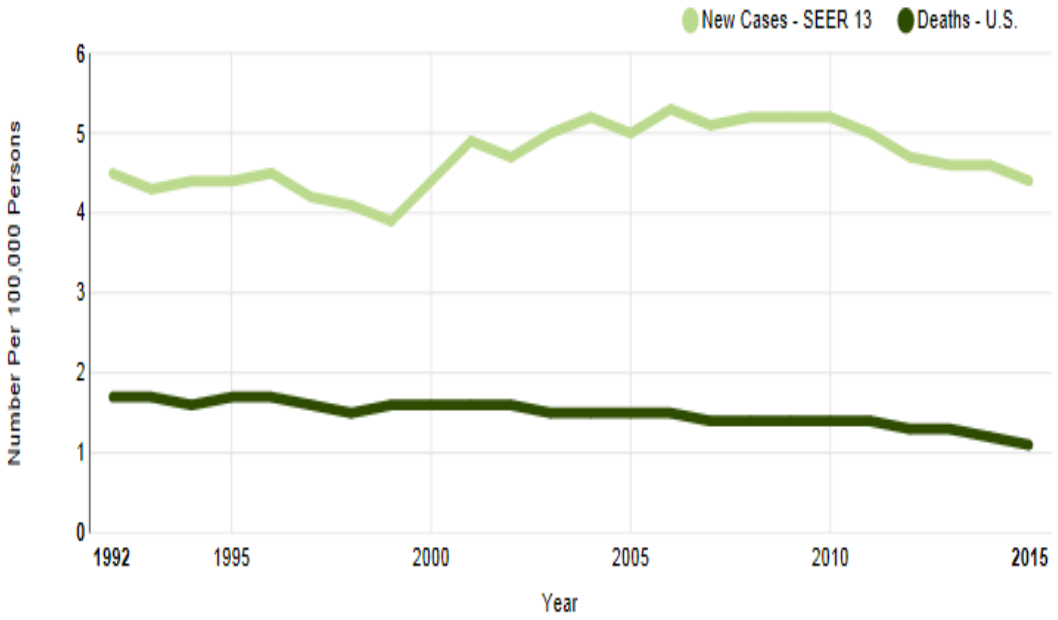

Figure 1.1 **Number of New Cases and Deaths per 100,000.**

The figure was adapted from National cancer institute and based on 2011-2015 cases and deaths (https://seer.cancer.gov/statfacts/html/clyl.html).

1.1.4 **Clinical Features**

70-80% of CLL patients are now diagnosed as an incidental finding on a routine full blood count. Others may present with lymphadenopathy, systemic symptoms such as tiredness, night sweats and weight loss or the symptoms of anaemia or infection (Oscier, Fegan et al., 2004). Approximately 40% of CLL patients will die from causes unrelated to their CLL. The most frequent causes of CLL-related death are; severe systemic functions such as, pneumonia and septicaemia, bleeding, and severe wasting and weakness. In a small number of cases, CLL will transform into Richter's syndrome (RS). Richter's syndrome (RS) is the transformation of CLL to a high grade non- Hodgkin's lymphoma. Clinical observations typically associated with RS transformation include rapid clinical deterioration with fever in the absence of infection, progressive lymph node enlargement, and an elevation in serum lactate dehydrogenase (LDH) (CLL FAQ, 2007; CLL Support Association). Diagnostic biopsy of affected sites usually reveals large cell lymphomas, however, Hodgkin variant cases have been described (Brecher and Banks, 1990). Richter's transformation occurs in approximately 5% of CLL patients. The large cells of RS arise through transformation of the original CLL clone in the majority of patients and as a separate and independent neoplasm in fewer cases.

Autoimmune haemolytic anaemia (AIHA) is commonly associated with CLL. It is estimated that 10-20% of CLL patients may develop AIHA during the course of their disease. Clinically significant AIHA shows fatigue, shortness of breath, rapid heart rate, jaundice and splenomegaly. CLL associated AlHA is treated with steroids and sometimes splenectomy or, if require, chemotherapy (Ding and Zent, 2007).

Idiopathic thrombocytopenic purpura or immune thrombocytopenic purpura (lTP) develops in approximately 2-3% of CLL patients. It can occur at any stage of CLL. The main symptom of ITP is bleeding which can include bruising and tiny red dots on the skin or mucous membranes. ITP is the diagnosis when platelets are abnormally low and other diseases that cause low platelets have been ruled out. Treatment is based on symptoms: steroids and intravenous immunoglobin are the first lines of treatment. Splenectomy is successful in about 75% of cases who have not responded to other therapy (Ding and Zent, 2007).

1.2 Clinical indications for the treatment of B-CLL

1.2.1 Clinical Staging Systems

Traditional clinical prognostic factors are disease staging and lymphocyte doubling time (LDT: the time required for doubling of the absolute lymphocyte count). The tests described below are not needed to establish the diagnosis of CLL, but may help to predict the prognosis or to assess the tumour burden. The median survival in CLL is approximately 10 years but to know this figure is of little value to an individual patient, due to the extraordinary heterogeneity in the natural history of this disorder. (Rai, Sawitsky et al., 1975) (Rai, Sawitsky et al., 1975) Rai et al (1975) and Binet et al (1981) developed clinical staging systems that would assess tumour burden and help predict prognosis. These two staging systems are simple, inexpensive, and can be applied by physicians worldwide (Tables 1.2 and 1.3).

1.2.1.1 Rai Classification

The original Rai classification was modified to reduce the number of prognostic groups from five to three (Table 1.2); both systems now describe three major subgroups with discrete clinical outcomes.

Table 1.2 Rai classification system for chronic lymphocytic leukaemia.

Adapted from (Oscier, Fegan et al., 2004).

Risk level (Modified classification)	**Stage** (Former classification)	**Clinical features at diagnosis**	**Median survival time (yrs)**
Low	0	Blood and marrow lymphocytosis	≥10
Intermediate	I	Lymphocytosis and lymphadenopathy	9
	II	Lymphocytosis and splenomegaly or hepatomegaly	7
High	III	Lymphocytosis and anaemia (Hb <11g/dL)	5
	IV	Lymphocytosis and thrombocytopenia (<100,000/μl)	5

1.2.1.2 Binet Staging System

Staging is based on the number of involved areas, as defined by the presence of enlarged lymph nodes of greater than 1 cm in diameter or organomegaly, and on whether there is anaemia or thrombocytopenia (Table 1.3).

Table 1.3 Binet clasification systems in chronic lymphocytic leukaemia.

Adapted from (Oscier, Fegan et al., 2004)

Binet stage	Features	Median survival time (yrs)
A	<3 lymphoid areas	>7-10
B	>3 lymphoid areas	5-7
C	Haemoglobin <10.0g/dL or Platelets <100x10^9/L	<2-5

1.3 Treatment

1.3.1 Conventional Treatment

The clinical course of CLL is heterogeneous with some patients having an indolent course and often dying of unrelated causes, while others progress very rapidly and die from their leukaemia. Currently, there is no cure for CLL, but it can be treated (Chiorazzi and Ferrarini, 2003) However, in many cases it is not necessary to begin treatment immediately, because CLL often progresses slowly and poses little threat to the patient's well-being when first diagnosed. Physicians refer to this "treatment" strategy as "watchful waiting" (Chiorazzi, Hatzi et al., 2005). Treatment of patients with stage A disease at presentation with chlorambucil has not been shown to prolong survival (Dighiero, Maloum et al., 1998), thus treatment is not usually initiated until symptoms start to interfere with quality of life.

New and more reliable prognostic factors (discussed below) will facilitate the design of randomised clinical trials to determine whether early intensive treatment of patients with a low tumour burden and poor risk factors can prolong survival. They may also influence the choice of initial treatment and subsequently the need for, and potential benefit of, additional treatments for patients with advanced or progressive disease.

Recently the range of therapeutic options for patients with progressive disease and those who present with advanced disease have increased. In the 1990s, chlorambucil was the standard treatment for patients with CLL. Phase II trials of purine analogues have shown promising results and the effect of fludarabine on DNA and RNA synthesis including DNA repair, opened up the possibility of potentiating its effect in combination with other drugs (Chiorazzi and Ferrarini, 2003). The MD Anderson group was the first to show, in a non-randomised phase II trial, that the combination of fludarabine with cyclophosphamide was effective in previously untreated patients with CLL (O'Brien, Kantarjian et al., 2001).

In 1999, the UK CLL 4 trial was initiated; a multicentre randomised trial in previously untreated patients comparing fludarabine, with or without cyclophosphamide, with chlorambucil, given at higher doses than in previous trials. The aim was to examine the results in terms of overall survival, response rates, progression-free survival, toxic effects, health-related quality of life and establish whether this treatment combination provided greater survival benefit than that of chlorambucil or fludarabine.

The trial showed that there was no significant difference in overall survival between patients given fludarabine plus cyclophosphamide, fludarabine, or chlorambucil.

Complete and overall response rates were better with fludarabine plus cyclophosphamide than with fludarabine alone, which were in turn better than with chlorambucil. Progression-free survival at 5 years was significantly better with Fludarabine plus cyclophosphamide than with fludarabine or chlorambucil alone ($p<0.00005$) and a meta-analysis of these data and those of two published phase III trials showed a consistent benefit for the fludarabine plus cyclophosphamide regimen in terms of progression-free survival. It was concluded that 'fludarabine plus cyclophosphamide should now become

the standard treatment for chronic lymphocytic leukaemia and be the basis for new protocols that incorporate monoclonal antibodies' (Catovsky, Richards et al., 2007).

Subsequently, the combination of fludarabine and cyclophosphamide (FC) with rituximab (FCR); an anti-CD20 monoclonal antibody, was investigated in a phase II trial on 300 patients with previously untreated CLL. FCR resulted in an overall response rate of 95%, with complete remission in 72% (Hallek, Cheson et al., 2008). Preliminary results of two large, phase III, prospective, randomised studies of FCR verses Fe confirmed the superior overall response and complete remission rates for FCR as a front-line treatment. However, FCR did not improve the progression free survival or overall survival of patients with a deleted chromosome 17p (Hallek, Cheson et al., 2008).

Monotherapy with alemtuzumab, a recombinant, fully humanized, monoclonal antibody against the CD52 antigen, has been shown to be effective in patients with high-risk genetic markers such as deletions of chromosome llq (del llq) or 17p (del 17p) and pS3 mutations (Stilgenbauer, Bullinger et al., 2002, Lozanski, Heerema et al., 2004). In one study, 48 previously untreated patients with high-risk CLL including del 17p were treated with FCR plus Alemtuzamab (Cf AR), this produced more minimal residual disease (MRD) eradication than FCR, but greater myelosuppression (Wierda, W.O. et al., 2008). Previously, the synergistic activity of fludarabine and alemtuzumab (FA) was investigated in a phase II trial, enrolling patients with relapsed CLL (Elter, Borchmann et al., 2005). This combination proved feasible, safe, very effective and well tolerated, with an overall response rate of 83% (30/36 patients) and a 30% complete remission rate.

With the increasing potential of newer chemoimmunotherapy combinations, treatment is becoming patient tailored. It has been suggested that patients in good physical condition should be offered an FCR combination therapy, whilst patients with relevant comorbidity may be offered either chlorambucil or a dose-reduced fludarabine containing regimen for symptom control.

If a patient presents with del 17p or p53 mutations it is now established that conventional FCR chemotherapy is not effective and treatment should be with ibrutinib (see following section).

1.3.2 Recent approaches to treatment

Risk-based individualized therapy is now potentially possible for CLL, ranging from chemo immunotherapy for low-risk (IGHV-mutated) CLL to novel molecularly targeted therapy for high-risk (del(17p), IGHV-unmutated) disease (Burger and O'Brien, 2018). Other methods of treatment include radiotherapy, in some cases splenectomy is beneficial and as mentioned above in some circumstances a bone marrow transplant may be considered.

1.3.2.1 Agents targeting B-cell receptor signalling

B-cell receptor signalling plays a significant role in the survival of CLL cells (Petlickovski, Laurenti et al., 2005, Stevenson, Krysov et al., 2011). Various components of the B-cell receptor signalling pathway have been identified as prognostic markers in CLL, such as immunoglobulin heavy chain variable gene (IGHV) mutational status and Zeta-chain-associated protein kinase 70 (ZAP70) level.

Constant BCR signalling supports CLL cell survival. This might explain why inhibition of BCR signalling is a new and potent strategy to treat CLL (Wiestner, 2012). The B-cell receptor signalling in CLL cells is mediated by different tyrosine kinases, such as Bruton tyrosine kinase (BTK), Spleen tyrosine kinase (Syk), ZAP70, Src family kinases (in particular Lyn), and PI3K (Wiestner, 2012). A diagram illustrating the location of these kinases (Figure1.2) shows how they control the major B-cell signalling pathways.

Several inhibitors of protein kinases involved in BCR signalling have produced good clinical outcomes of which the most important is the BTK inhibitor (Ibrutinib). However, a PI3Kδ inhibitor (Idelalisib) has also entered clinical practice and a LYN inhibitor (Bafetinib) and a SYK inhibitor (Fostamatinib) have been trialled (Robak and Robak, 2013).

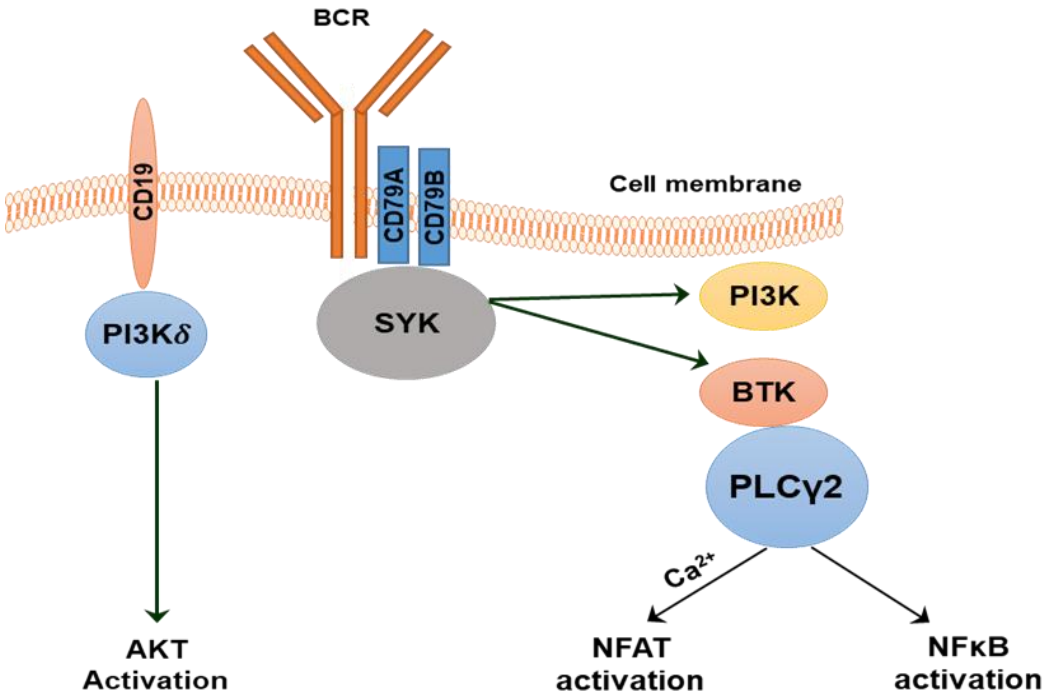

Figure 1.2 The B-cell receptor signaling in CLL cells.

The diagram shows the location of different tyrosine kinases mediated The B-cell receptor signalling in CLL cells, such as Bruton tyrosine kinase (BTK), Spleen tyrosine kinase (Syk), and PI3K and how they control the major B-cell signalling pathways. The signals generated on BCR ligation lead to the activation and phosphorylation of the ITAMs (immunoreceptor tyrosine-based activation motifs) of CD79a/b and further to the phosphorylation of the tyrosine kinase, Syk. Subsequently Syk is trans- autophosphorylated leading to full enzymatic activation. Several intermediate signalling molecules are then phosphorylated by Syk in turn activating the downstream signalling molecules AKT, PLCy2, NFAT and NFκB.

Ibrutinib's antileukemia actions, illustrating remarkable and immediate inhibition of CLL cell proliferation and promotion of high rates of CLL cell death in vitro (Burger, Li et al., 2017). Bruton tyrosine kinase (BTK) upregulates cell survival pathways such as NF-ƙB and MAP kinases through Src family kinases (Herman, Gordon et al., 2011). These pathways are important aspects in the B-cell receptor (BCR) signalling. Ibrutinib is an orally active, small molecule BTK inhibitor that promotes apoptosis in B-cell lymphomas and CLL cells (Herman, Gordon et al., 2011).

However, with continuing use it is clear that there are relapses during ibrutinib maintenance. With a median follow-up time of 3.4 years patients showed a cumulative prevalence of progression at 4 years of 19% (Woyach, Ruppert et al., 2017). Baseline karyotypic complexity, existence of del (17) (p13.1), and age <65 years were risk factors for progression. 85% of patients who developed relapse, gained mutations of BTK or PLCG2. These mutations were found at an approximate median of 9.3 months before relapse. Therefore, the BTK inhibitor ibrutinib has made a major impact on the treatment of CLL but it is apparent that resistance mechanisms can emerge.

Class I phosphatidylinositol 3-kinases (PI3Ks) control cellular functions important in oncogenesis (Plunkett, Gandhi et al., 1993). Expression of the PI3K p110 d isoform (PI3K- d) is bound to cells of hematopoietic origin where it has a notable role in B cell proliferation and survival. In CLL the PI3K pathway is stimulated by PI3K d (Rai, Peterson et al., 2000).

Idelalisib is an oral PI3Kd-isoform-selective inhibitor, which encourage apoptosis in primary CLL cells in a time- and dose-dependent manner without promoting apoptosis in normal T cells or natural killer cells. Idelalisib inhibits CLL cell chemotaxis toward CXCL12 and CXCL13 and migration below stromal cells (pseudoemperipolesis). Idelalisib also activates secretion of chemokines in stromal cultures and after BCR triggering (Rai, Peterson et al., 2000). Idelalisib decreases survival signals derived from the BCR or from nurse-like cells, and inhibits BCR- and chemokine-receptor- promoted AKT and MAP kinase (ERK) activation (Rai, Peterson et al., 2000).

Venetoclax is a BH3-mimetic drug designed to block the function of BCL-2 protein (Calin, Dumitru et al., 2002). Venetoclax inhibits the growth of BCL-2-dependent tumors

in vivo but spares human platelets. A single oral dose of venetoclax in 3 patients with refractory CLL led to tumor lysis within 24 hours (Calin, Dumitru et al., 2002). Subsequently, a dose increase strategy was identified to avoid these matters with a weekly dose build-up level through 4–5 weeks (Stilgenbauer, Eichhorst et al., 2016).

Overall, the outcomes of the two trials reveal that venetoclax monotherapy is potent and well tolerated in patients with relapsed or refractory del (17p) CLL, providing a new therapeutic option for this very poor prognosis population.

1.3.3 Stem cell Transplantation

Many potential treatments are available for CLL, but appropriate patient selection and the timing of stem cell transplantation (SCT) remain controversial and the focus of ongoing clinical trials. The use of SCT must always be weighed against the risk of the underlying disease, particularly in a setting where improvements in treatment are leading to improved outcome (Gribben, 2009). B-CLL patients were successfully transplanted with autologous stem cells subsequently becoming minimal residual disease (MRD) negative (Esteve, Villamor et al., 1998). Moreover, the clinical outcome can be highly successful and a recent study demonstrated that 8 out of 12 patients became MRD negative following transplantation (Schetelig, Schaich et al., 2015).

1.4 Prognostic markers

Currently, 70% to 80% of patients with CLL present with a low tumour burden and are frequently diagnosed from a blood count performed for an incidental reason. Traditional clinical prognostic factors such as disease staging and lymphocyte doubling time are still the cornerstones to discriminate those patients who need to start treatment during the clinical follow-up. However, they are both limited in their inability to predict the course of the disease when CLL is diagnosed at an early stage and patients have a low tumour burden. In recent years a number of prognostic markers have been identified which can better predict the likelihood of clinical progression in CLL and in some cases predict subsets of patients who will not respond to a given therapy, thus avoiding unnecessary treatment toxicity.

1.4.1 **Cytogenetic Abnormalities**

Using interphase fluorescence in-situ hybridisation (FISH), cytogenetic lesions can be identified in more than 80% of all CLL cases. The most common cytogenetic alterations are deletions in the long arm of chromosome 13q (del 13q; 55%), deletion in the long arm of chromosomes 11q (del 11q; 18%), trisomy of chromosome 12 (16%), deletion in the short arm of chromosome 17p (del 17p; 7%) and deletion in chromosome 6q (del 6q; 7%) (Dohner, Stilgenbauer et al., 2000). In addition certain translocations can help distinguish other lymphoproliferative diseases from CLL (e.g. t(11;14), generally found in mantle cell lymphoma (Hallek, Cheson et al., 2008).

Cytogenetic analysis has shown correlations between chromosome abnormalities and clinical features in CLL. Patients with leukaemic cells that have del 17p have an inferior prognosis and appear resistant to standard chemotherapy regimens employing alkylating drugs and/or purine analogues (Dohner, Stilgenbauer et al., 1995, Grever, Lucas et al., 2007). Deletion in chromosome 11q is associated with extensive lymph node involvement and poor survival. It has been shown that patients who have CLL cells with the chromosomal aberrations del 11q or del 17p have an inferior outcome compared to patients who have leukaemic cells with a normal karyotype or del 13q as the sole genetic abnormality (Dohner, Stilgenbauer et al., 2000). Genetic defects are also acquired during the course of the disease (Shanafelt, Jelinek et al., 2006) and clonal evolution can lead to more dangerous clonal variants (Keating, Chiorazzi et al., 2003).

In CLL clonal evolution has been shown to occur in nearly a third of CLL patients with single or complex abnormal genetic karyotypes developing over time (Berkova, Zemanova et al., 2009, Norin, Kimby et al., 2010).

1.4.2 **IGHV Mutational status**

In 1999, it was discovered that patients whose CLL cells express immunoglobulin heavy chain variable region (IGHV) genes that differ from the germline sequence by more than 2% i.e. mutated, have a significantly better clinical outcome than CLL patients with

'unmutated' IGHV genes (98% or more homology with the germline sequence) (Damle, Wasil et al., 1999, Hamblin, Davis et al., 1999).

IGHV gene mutational status has been defined as the 'gold standard' of CLL prognostic markers. Hamblin and colleagues (1999) found that in patients with mutated IGHV genes the median survival was nearly 25 years, compared to less than 8 years for patients with unmutated IGHV genes. Since then there has been some controversy as to the percentage of mutations which best correlates with clinical outcome, with some authors choosing 97% (Krober, Seiler et al., 2002) or even 95% (Lin, Sherrington et al., 2002) homology as a possible more appropriate cut-off, however, the general consensus now is to use the original cut-off of 98%, yet some caution is needed in borderline cases. In addition to this, Rosenquist and colleagues (2002) observed that the usage of the IGHV3-21 gene was an independent unfavourable prognostic marker irrespective of the IGHV gene mutational status. This was the first time that an exception to the mutational status rule had been identified and thus suggested the possibility that other exceptions existed.

1.4.3 **CD38 Expression**

Based on the fact that IGHV gene mutational analysis requires sequencing it was originally considered to be impractical in a routine haematology setting. This created the need for a surrogate marker. CD38 expression was reported to be able to predict IGHV gene mutational status with 92% accuracy (Damle, Wasil et al., 1999). Expression of CD38 has since been validated as an independent prognostic marker associated with a poor outcome, but the variation in concordance with mutational status, the ongoing controversy over the percentage cut-off (Hallek, 2005, Boonstra, van Lom et al., 2006, Seiler, Dohner et al., 2006) and the fact that CD38 expression has been shown to change over the course of the disease prevented CD38 being a surrogate for mutational status.(Hamblin, Orchard et al., 2002).

1.4.4 ZAP-70 Expression

Microarray analysis of the unmutated and mutated subsets in CLL identified another potential surrogate marker for IGHV gene mutational status (Rosenwald, Alizadeh et al., 2001). These studies identified ZAP-70 as being differentially expressed in the two subgroups. The correlation between ZAP-70 expression and IGHV gene mutational status was confirmed in a large cohort showing that ZAP-70 expression is associated with a more aggressive clinical course (Wiestner, Rosenwald et al., 2003). The development of a method to detect ZAP-70 by flow cytometry made ZAP-70 an attractive surrogate. Rassenti, Jain et al. (2008) demonstrated ZAP-70 as an independent prognostic marker and that it might even be a better predictor of prognosis than IGHV gene mutational status. However, the lack of a standardised method and difficulties in reproducibility between different laboratories have hindered ZAP-70 becoming a routine prognostic test. For both CD38 and ZAP-70 further clinical trials are needed to standardise the assessment of these parameters and to determine whether they should affect the management of patients with CLL.

1.5 Overview of the Biology of CLL

1.5.1 Tumour microenvironment

The secondary lymphatic tissues are the principal site for expansion of normal mature B cells, ultimately leading to the generation of antigen-specific B cells and maturation into antibody-producing plasma cells. Normal B cell growth in germinal centres is based on antigen selection and clonal expansion, which is reinforced by antigen specificity, along with co-stimulatory signals from T cells and antigen-presenting cells (APC) (Caligaris-Cappio and Ghia, 2008). Generally, the pathways responsible for the growth of antigen-specific normal B cells appear to be also functional in their malignant counterparts.

CLL proliferation occurs mainly in peripheral lymphoid organs and in the bone marrow (BM) where microenvironments are established and maintained through a dynamic, interactive development of tumour and normal observer cells (Caligaris-Cappio and Ghia, 2008).CLL cells interact with different types of stromal cells, such as mesenchymal

stromal cells (Calissano, Damle et al., 2009), monocyte-derived nurse like cells (NLC) (Burger, Tsukada et al., 2000) (Damle, Ghiotto et al., 2002, Rosen, Murray et al., 2010) as well as T cells (Catera, Silverman et al., 2008, Hoogeboom, van Kessel et al., 2013) all together referred to as the "microenvironment", and they proliferate in the context of micro-anatomical tissue sites called proliferation centres (pseudo-follicles), a hallmark histopathological finding in CLL (Duhren-von Minden, Ubelhart et al., 2012). CLL microenvironments share the general properties of cancer microenvironment: new vessels provide nutrients, growth factors are produced locally and leukemic cells are protected from immune elimination.

Early evidence of microenvironment-dependency came from the notion that CLL cells normally undergo spontaneous apoptosis in suspension culture unless they are co-cultured with bone marrow stromal cells (BMSC) or nurse-like cells (NLC) (Panayiotidis, Jones et al., 1996, Tinhofer, Marschitz et al., 1998, Burger, Tsukada et al., 2000).

Many molecules must be involved in the interaction between CLL cells and the microenvironment and these will include those that mediate direct cell-cell interactions e.g. CD40 (Ibrahim, Jilani et al. 2003), BAFF and April receptors, and growth factors. Recently, our group has demonstrated that interleukin 21 (IL21) is functional in CLL, and IL21 and interleukin 4 (IL4) act together to enhance leukaemic cell proliferation without apoptosis or differentiation. IL21 and IL4 are the major cytokines produced by CD4+ T follicular helper (Tfh) cell subset (Ahearne, Willimott et al., 2013). These findings suggest that the Tfh cytokines, IL4 and IL21, promote leukaemic cell proliferation in the lymph node microenvironment, and could contribute to the specific production of cells resistant to conventional chemotherapy.

Therefore, the tumour microenvironment includes several cell types and growth factors. There is cross talk between the leukaemic and non-leukaemic cells, which is important in controlling the growth of CLL and the responses of the normal cells of the microenvironment.

1.5.2 **Balance between apoptosis and proliferation**

There has been long-standing uncertainty about whether CLL is primarily a condition of reduced apoptosis or whether increased proliferation is important. There are aberrant signals conducted through CD40 receptors with down regulation of CD95 receptors on the B-CLL surface. All these factors play important role in prolongation of cell survival both by preventing apoptosis (Laytragoon-Lewin, Duhony et al., 1998, Younes, Snell et al., 1998, Chiorazzi, 2007, Willimott, Baou et al., 2007). D Kater, Evers et al. (2004) and Willimott, Baou et al. (2007) have further confirmed that anti-CD40 stimulated B-CLL cells express enhanced levels of the anti-apoptotic proteins Bcl-xL and Bfl-1 and downregulate BH3-only protein Harakiri. Additionally, there is a suggestion that B-CLL cells may even be promoting their own survival and growth by the production of CCL17 and CCL22, chemokines involved in the attraction of T cells to the anatomic zones (Ghia, Chiorazzi et al., 2008).

According to Bomstein, Yuklea et al. (2003), soluble factors, such as IL-4 or IL-6, are found in the serum of CLL patients, which are not present in a normal individual's serum. Both of these cytokines released either by malignant cells or by some non-malignant cells, can rescue the of B-CLL cells from apoptosis. Interestingly, prevention of apoptosis is not seen in purified B-CLL cells, which propose strongly that spontaneous apoptosis of B-CLL cells in cultures is dependent on the interaction of immune complexes with accessory molecules, such as IFNy and IL-4 (Levesque, Misukonis et al., 2003).

PI-3K is a protein kinase involved in the regulation of the growth of normal and neoplastic cells. It has been shown that this protein is constitutively activated in B-CLL cells leading to inhibition of apoptosis. In addition, Preclinical studies in CLL demonstrated that the crucial role for PI-3K with regard to the proliferation and survival of B-CLL cells. (Burger, Li et al., 2017).

However, experiments in which patient cells were labelled with heavy water (D_2O) in order to measure turnover have suggested that there is a proportion of CLL cells that are actively proliferating (Messmer, Albesiano et al., 2004). Therefore, CLL is likely to be a disease both of reduced apoptosis and increased proliferation.

1.5.3 Are Regulatory B cells the normal counterpart of CLL cells?

Immune response–associated inflammation is an important part of the host defense system including many biological structures and pathways that protects against foreign pathogens; however, if deregulated, it can contribute to permanent cellular injury (Mauri and Menon, 2017).

Although microbial infections or injury initiates the inflammatory responses, numerous autoimmune disorders and cancers can induce chronic inflammation (Medzhitov, 2010). If these continue, repeated presentation to pro inflammatory soluble factors, such as TNF-α, IL-17, IL-6, and IFN-α, leads to the generation of a variety of autoimmune and inflammatory diseases, including rheumatoid arthritis (RA), multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Moudgil and Choubey, 2011).

In order to prevent permanent cellular injury, prompt creation of an anti-inflammatory cellular response that reduces tissue damages and induces maintenance of homeostasis is needed (Chaudhry and Rudensky, 2013). Therefore, various regulatory process that inhibit further immune responses and supress current inflammatory pathways are available to maintain immune homeostasis.

Together with Tregs that plays an important role in the restoration of immune homeostasis, regulatory B cells (Bregs) are a recently discovered subset that regulate immune response associated inflammation, through the production of IL-10 and other anti-inflammatory factors (Blair, Norena et al., 2010, Carter, Vasconcellos et al., 2011, Flores-Borja, Bosma et al., 2013). The significance of human Bregs in the restoration of immune homeostasis comes from various immune-related pathologies, such as autoimmune diseases, cancers, and chronic infections that are linked to aberrant Breg numbers or function (Blair, Norena et al., 2010, Flores-Borja, Bosma et al., 2013, (Khoder, Sarvaria et al., 2014, Oka, Ishihara et al., 2014).

There is extensive evidence from mouse models studies emphasise the key role for Bregs in cancer immunology. In a mouse model of breast cancer, a subset of $CD25^{hi}$ $CD69^{hi}$ cancer-evoked Bregs was demonstrated to assist lung metastasis by promoting the

differentiation of FoxP3+ Tregs in a TGF-β–dependent pathway (Olkhanud, Damdinsuren et al., 2011). Interestingly, cancer-evoked Bregs produced high amounts of CD80 and CD86, proposing that CD80- and CD86-facilitated crosslinking between Bregs and their target cells is significant both in repression of effector T cell response and in the differentiation of Breg-induced Tregs.

Therefore, there are some similarities between normal Bregs and CLL cells. Both express CD5 (Sarvaria, Madrigal et al., 2017) and both are immunosuppressive through an action on T helper cells (Yanaba, Bouaziz et al., 2008) and finally both produce IL-10 (Drennan, D'Avola et al., 2017, Sarvaria, Madrigal et al., 2017). Previous work (Smallwood, Apollonio et al., 2016) has demonstrated that CLL extracellular vesicles (see section 1.7 for extracellular vesicles and their role) perturb T-cell function. Speculatively this might also be an adaptation from normal Breg responses.

1.5.4 **Mouse models of CLL**

There are difficulties to investigating communication between CLL and T cells in the human body. Access to tissue from lymph nodes or other lymphatic organs is limited, and the establishment ex vivo of an entire microenvironment for immune system interactions is complicated. Therefore, modelling cancer–host interactions has been a field of intensive research. The most widely used in vivo model for CLL is the transgenic TCL1 mouse, in which the human TCL1 gene is expressed under the regulation of the immunoglobulin heavy chain variable region promoter and enhancer (Bichi, Shinton et al., 2002) in addition, a knockout mouse model recapitulating the chromosomal deletion at 13q14 has been utilized (Klein, Lia et al., 2010). Although both of these mouse strains model CLL, they are based on either the overexpression of an oncogene or the deletion of a specific regulatory region and as such reflect a specific disease genotype. A complementary route has been to xenograft the Mec-1 cell line (Bertilaccio, Scielzo et al., 2010) or primary CLL cells into immune-compromised mice (Durig, Ebeling et al., 2007, Bagnara, Kaufman et al., 2011). Bagnara et al. (2011), using primary human CLL cells, showed that peripheral blood mononuclear cells (PBMCs) from CLL patients xenografted into NOD/ scid/gamma chain null (NSG) mice accumulated mostly in the spleen (SP). Moreover, these findings demonstrated that proliferation of CLL cells in vivo

was enhanced by human T cells. Likewise, utilizing a NOD/scid CLL xenograft model determined cancer localization in the spleen (Aydin, Grabellus et al., 2011). Therefore, these xenograft models may be an appropriate but complex methodology to investigate tumour microenvironment communication.

1.5.5 MicroRNA in Normal Subjects and CLL Patients

There are many types of RNA, and they have different distinct roles. Messenger RNAs (mRNAs) specify amino acid sequence of proteins and ribosomal RNAs (rRNAs) organise and catalyse the synthesis of proteins. Transfer RNAs (tRNAs) are essential for translation of mRNA to protein, and snRNAs and snoRNAs, small RNAs in the nucleus, are directing many of the RNA processing steps.

MiRNAs are a class of noncoding single-stranded RNAs of approximately 22 nucleotides in length, which are cleaved from 60- to 110-nt hairpin precursors and are encoded in the genome ((Calin, Liu et al., 2004). miRNAs are able to modulate messenger RNA (mRNA) translation (Nicoloso, Kipps et al., 2007). Recent discoveries have shown that miRNAs have key roles in both physiologic and pathologic processes and may be useful as biomarkers. For example, miR-155 controls the germinal centre response in normal immunity (Vigorito, Perks et al., 2007), but might also be a prognostic marker in CLL (Ferrajoli, Shanafelt et al., 2013).

MiRNAs have a central role in the pathogenesis of chronic lymphocytic leukaemia (CLL). Earlier studies have demonstrated a significant association between multiple miRNAs and CLL disease progression and prognosis. A minimum two marked miRNAs clusters of CLL cases were linked to the presence or absence of ZAP-70 expression, a biomarker of early disease progression (Calin, Liu et al., 2004). Moreover, two miRNA marks were correlated with the presence or absence of mutations in the expressed Ig variable-region genes or with deletions at 13q14, a region that is deleted in more than half of all patients with B-cell chronic lymphocytic leukaemia (B-CLL), respectively (Calin, Liu et al. (2004). Analysing the chromosome region 13q14, demonstrated that miR-15a and miR-16a were either absent or down regulated in approximately 68% of patients (Calin, Dumitru et al., 2002). Further studies have shown that miR-15 and miR-16 act as

tumour suppressors by targeting the oncogene BCL2, which encodes a protein involved in cell survival (Stahlhut and Slack, 2013).

There is other evidence suggesting that miRNA have a role in the pathogenesis of CLL. A new study has identified a specific exosomal microRNA signature from patients with CLL, encompassing highly up regulation of miR-150, miR-155, and miR-29 family members and down regulation of miR-223 (Yeh, Ozer et al., 2015). Moreover, it is found that miR-155 was upregulated in B cells from CLL patients, more than those from healthy volunteers (Ferrajoli, Shanafelt et al., 2013). These cellular microRNAs have been earlier accompanied with the CLL disease (Calin, Ferracin et al., 2005, Cui, Chen et al., 2014, Mraz, Chen et al., 2014). Interestingly, it has been demonstrated that miR-155 expression levels were significantly higher in patients who did not respond to treatment compared to those who achieved successful response. Suggesting that cellular and plasma levels of miR-155 could be used as biomarkers to recognize patients with CLL who may fail to response well to treatments (Ferrajoli, Shanafelt et al., 2013). Therefore, cellular microRNAs might be potential non-invasive prognostic biomarkers for CLL tumour cells.

1.6 RNAs and classification.

RNAs carrying the code for protein synthesis are called “coding RNAs” or “messenger RNAs (mRNAs)”. However, “non-coding RNAs (ncRNAs)” do not undergo translation to synthesize proteins. Many of them are reported to play an important role as various regulatory elements in the genome, whereas most are still of unknown role to gene regulation (St Laurent, Wahlestedt et al., 2015).

1.6.1 Coding-RNA

Messenger RNA; mRNA

Messenger RNA (mRNA) carries the genetic code from DNA in a form that can be recognized to make proteins. The coding sequence of the mRNA determines the amino acid sequence in the protein produced. Once transcribed from DNA, eukaryotic mRNA briefly exists in a form called “precursor mRNA (pre-mRNA)” before it is fully processed into mature mRNA. This processing step, which is called “RNA splicing”, removes the

introns—non-coding sections of the pre-mRNA. There are approximately 23,000 mRNAs encoded in human genome.

1.6.2 Non-coding RNA (ncRNA)

Ribosomal RNA (rRNA)

Ribosomal RNA is the catalytic component of the ribosomes. In the cytoplasm, rRNAs and protein components combine to form a nucleoprotein complex called the ribosome which binds mRNA and synthesizes proteins (also called translation).

Transfer RNA (tRNA)

Transfer RNA is a small RNA chain of about 80 nucleotides. During translation, tRNA transfers specific amino acids that correspond to the mRNA sequence into the growing polypeptide chain at the ribosome.

Small nuclear RNAs (snRNA; 150 nt)

Small nuclear RNAs are always associated with a group of specific proteins to form the complexes referred to as “small nuclear ribonucleoproteins (snRNP)” in the nucleus. Their primary function is to process the precursor mRNA (pre-mRNA).

Small nucleolar RNAs (snoRNA; 60-300 nt)

Small nucleolar RNAs are components of small nucleolar ribonucleoproteins (snoRNPs), which are complexes that are responsible for sequence-specific nucleotide modification.

Piwi-interacting RNAs (piRNA; 24-30 nt)

Piwi-interacting RNAs bind the PIWI subfamily proteins that are involved in maintaining genome stability in germline cells. Piwi-interacting RNAs also play a role in gametogenesis.

MicroRNAs (miRNA; 21-22 nt

MicroRNAs are small ncRNAs of ~22 nucleotides (nt) and the most widely studied class of ncRNAs. These RNA species mediate post-transcriptional gene silencing through RNA interference (RNAi), where an effector complex of miRNA and enzymes can target complementary mRNA by blocking the mRNA from being translated or accelerating its degradation. In human, miRNAs are estimated to regulate the translation of >60% of protein-coding genes.

Long noncoding RNAs (lncRNA)

Long noncoding RNAs are a heterogeneous group of non-coding transcripts larger than 200 nt in size and make up the largest portion of the mammalian non-coding transcriptome. It is estimated that more than 8,000 lncRNAs encoded in the human genome. lncRNAs are essential in many physiological processes. To date, various mechanisms of gene regulation by some lncRNAs have been reported, whereas most are still of unknown function (St Laurent, Wahlestedt et al., 2015).

Table 1.4 Different known classes of lncRNAs

Adapted from (St Laurent, Wahlestedt et al., 2015)

Classification	Examples
Classification based on transcript length	Long non-coding RNA, Long-intergenic non-coding RNA; large Intervening Non-Coding RNA, long-intervening non-coding RNA.
Classification based on association with annotated protein-coding genes	Intronic ncRNA; Stable intronic sequence RNA; totally intronic RNA, partially intronic RNA.
Classification based on association with other DNA elements of known function	Enhancer-associated RNA, Promoter-associated long RNA, Upstream antisense RNA
Classification based on association with repeats	C0T-1 repeat RNA, Long interspersed nuclear element, Transcribed endogenous retroviruses
Classification based on protein-coding RNA resemblance	mRNA-like noncoding RNAs, Long-intergenic non-coding RNA; large Intervening Non-Coding RNA, long-intervening non-coding RNA
Classification based on association with a biochemical pathway or stability	Nrd1-unterminated transcript, miRNA primary transcripts, piRNA primary transcripts
Classification based on sequence and structure conservation	Transcribed-Ultraconserved Regions, Hypoxia-induced noncoding ultraconserved transcript
Classification based on expression in different biological states	Long stress-induced non-coding transcript
Classification based on association with subcellular structures	Chromatin-associated RNA
Classification based on function	Long noncoding RNAs with enhancer- like function; ncRNA-activating

1.7 MiRNA Biology

MiRNAs have been reported to regulate important biological functions in the cell such as growth, differentiation, apoptosis, and adhesion, and for this reason they have provided importance into the mechanisms of human cancer as dysregulation of miRNAs is a crucial feature of cancer in general, including lymphomas (Lawrie, 2013).

Abnormal expression of miRNA is common in oncogenesis (McManus, 2003). MiRNAs play a central role in cancer biology through regulation of gene expression by targeting and binding to complementary sequences on messenger RNA (mRNA) for degradation or blocking translation, to enhance tumour growth, invasion, angiogenesis, and immune evasion (Lu, Getz et al., 2005, Hayes, Peruzzi et al., 2014).

miR-21 is a great example of an oncogenic miRNA (a so-called oncomir). It is highly expressed in most cancers e.g., breast cancer, colorectal cancer, lung cancer, pancreatic cancer, glioblastoma, neuroblastoma, leukaemia, and lymphoma (Iorio, Ferracin et al., 2005). Higher expression of this miRNA can result in tumour growth, maintenance, and survival in vivo. Interestingly, these tumours are completely dependent on expression of miR-21 (Medina, Nolde et al., 2010). Many tumour-suppressor genes are targeted by miR-21, including PTEN, to enhance proliferation and decrease apoptosis (Gabriely, Wurdinger et al., 2008). MiR-21 could also promote tumour migration by targeting pro-invasion genes (Sarkar, Gou et al., 2010).

Moreover, it has been suggested that the regulation of approximately 30% of protein-coding genes may be impacted by miRNAs (Moussay, Wang et al., 2011). Therefore, they may show promise as biomarkers for cancer.

1.7.1 Production and processing of miRNA

Production of mature miRNAs involves a complex maturation steps begins with the transcript of the primary miRNA (pri-miRNA) from the genomes, by RNA polymerase II to produce an approximately 70–100 nt hairpin precursor miRNA (pri-miRNA) and is followed by production of a precursor miRNA (pre-miRNA) in the nucleus by the RNase

III enzyme Drosha and the processing of the double-stranded DNA binding protein DGCR8 (Cortez, Bueso-Ramos et al., 2011).

The next step is the exportation of pre-miRNAs to cytoplasm by the nuclear export receptor exportin 5, and then they undergo an additional digestion step by the RNase III endonuclease Dicer (Figure 1.3): RNase III enzyme and Dicer cut the double- stranded RNA (the duplex of the miRNA sequence and the miRNA star-sequence) from the pre-miRNA hairpin.

The miRNA–miRNA star-sequence duplex is then carried on the miRNA-associated, multiprotein RNA-induced silencing complex (miRISC). The mature miRNA strand is preferentially retained in the miRISC, where it guides the RISC to target sequences. In most animals, miRNAs direct gene regulation at the level of translation. Mature miRNAs, carried by the RISC, attach through the seed regions (the 8 nt starting from the second position of the miRNA 5'-end), to mRNAs, mainly at their 3'-untranslated region (3'-UTR), through a significant but not complete sequence complementarity. By a mechanism that is not fully characterized, the bound mRNA remains un-translated, resulting in reduced levels of the corresponding protein or on alternative way, can be digested, leading decreased amount of both the mRNA transcript and the corresponding protein (Nicoloso, Kipps et al., 2007).

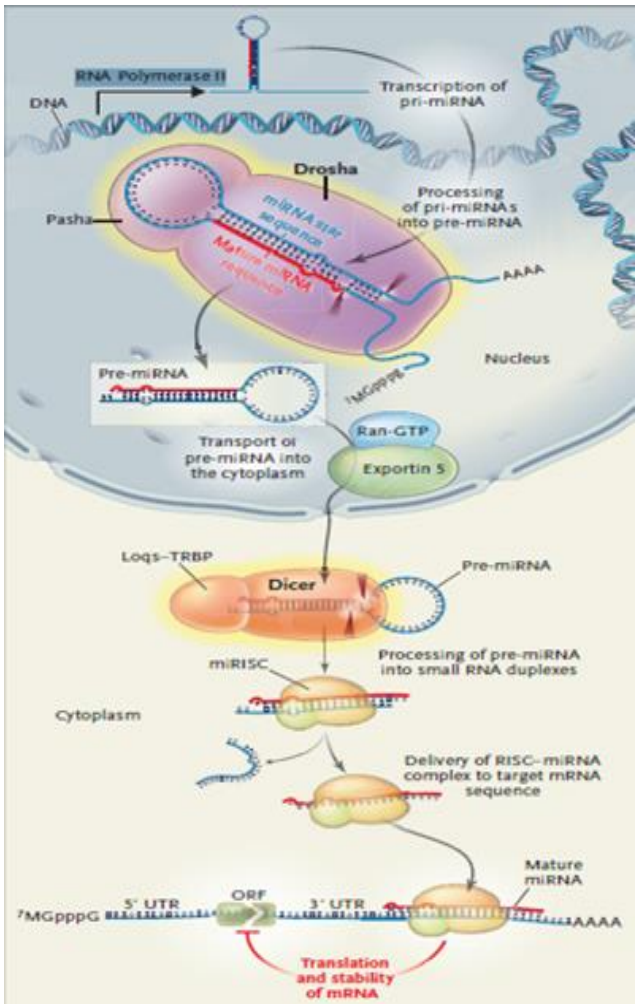

Figure 1.3 Drosha and Dicer and the Processing and Function of miRNA.

Pri-miRNAs, large miRNA precursors, are transcribed from the genome by RNA polymerase II and fold into a hairpin structure that is the substrate for RNA processing by Drosha (cleavage sites shown as dark red arrows) to a pre-miRNA. Pre-miRNAs enter the cytoplasm, where they are further processed by, resulting in an RNA duplex containing the mature miRNA and the miRNA star sequences. The mature miRNA preferentially enters the miRNA-associated, multiprotein RNA–induced silencing complex (miRISC) and guides it to partially complementary sequences in target messenger RNA (mRNA), leading to repression of their translation. AAAA represents the poly-A tail, and 7MGpppG denotes the 5′ cap. ORF denotes open reading frame, Ran-GTP Ran guanosine triphosphate, and UTR untranslated region Adapted from.(Slack and Weidhaas, 2008).

1.7.2 **Circulating miRNA and potential as biomarkers**

MiRNAs in body fluids are attractive non-invasive biomarkers in cancer. (See section 1.8 for principles of biomarkers). Tumour types show a unique profile of up regulated and down regulated miRNAs (Lu, Getz et al., 2005). Moreover, circulating miRNAs are highly stable and resistant to degradation on storage. They were found to be present in human serum in a remarkably stable and cell-independent form (Grasedieck, Scholer et al., 2012).

Cancer cells can release microRNAs into the circulation and it is believed that their stability is, preserved by being incorporated into microvesicles. Indeed plasma levels of miRNA are stable after several freeze–thaw cycles and long exposure to room temperature (Mitchell, Parkin et al., 2008). Another factor maintaining stable miRNA levels is binding to proteins, such as AGO2 (Argonaute2) (Arroyo, Chevillet et al., 2011).

MicroRNAs have also shown stability in other bodily fluids, such as urine and saliva (Schwarzenbach, Nishida et al., 2014). Isolation of exosomes from serum showed that a signature involving two microRNAs and one small non-coding RNA could be used for non-invasive diagnosis of glioblastoma (Manterola, Guruceaga et al., 2014).

Interestingly, one study has demonstrated the existence of tumour-derived exosomes (Taylor, Homesley et al., 1980) and a miRNA signature of circulating ovarian cancer exosomes (Taylor and Gercel-Taylor, 2008). This miRNA fingerprint was significantly correlated with primary tumour-miRNA expression in women with cancer compared to women with benign disease and was not identified in normal controls. A similarity between miRNA signatures in circulating exosomal miRNA and originating tumour cells was also found in lung adenocarcinoma with a significant difference in exosomal miRNA levels between cancer patients and controls (Rabinowits, Gercel-Taylor et al., 2009).

Therefore, expression of miRNAs in body fluids plays an important role in the field of biomarkers and could create a great opportunity for novel non-invasive biomarkers for prognosis, as well as for the improvement of new RNA based cancer therapies.

1.8 Extracellular vesicles and their role in cancer and CLL

Cells can secrete various types of extracellular vesicles (EVs), which have been difficult to classify in a specific way (Gould and Raposo, 2013). EVs include two principal vesicle types. Microvesicles, which are large (>200 nm diameter) and dense, emerge from plasma membrane. This phenomenon may have evolved to clean regions of damaged cell membrane from the cell in response to the injury. Therefore, is thought by many as a form of debris related to cellular damage (Pilzer, Gasser et al., 2005). Exosome vesicles, which have an endocytic origin, are generally smaller (30–150 nm diameter) (Johnstone, Adam et al., 1987), have a characteristic density of 1.1–1.2 g/ml (Figure 1.4) (Raposo, Nijman et al., 1996).

Both microvesicles and exosomes are a physiologic process that accompanies cell activation and growth (Ghosh, Secreto et al., 2010), and conserve cytoplasmic materials, but exosomes have distinct surface markers, such as CD63, CD9, CD81, and TSG101, not identified on other released particles (Wang, Liu et al., 2008). They are released by different type of primarily hematopoietic cells such as reticulocytes, platelets, dendritic cells, B and T lymphocytes, and macrophages (Denzer, Kleijmeer et al., 2000). However, exosomes are also released by numerous epithelial and tumor cells (Valadi, Ekstrom et al., 2007). Examples of epithelial cells that secrete exosomes encompass alevolar lung tissue (Denzer, Kleijmeer et al., 2000), tubule cells and podocytes from nephrons (Zhou, Cheruvanky et al., 2008), and intestinal cells (Buning, von Smolinski et al., 2008).

Categorizing vesicles based on their size or subcellular origin remains problematic (Lotvall, Hill et al., 2014) and whether or not one form of vesicle is biologically more effective than another is indistinct within the field at the present time.

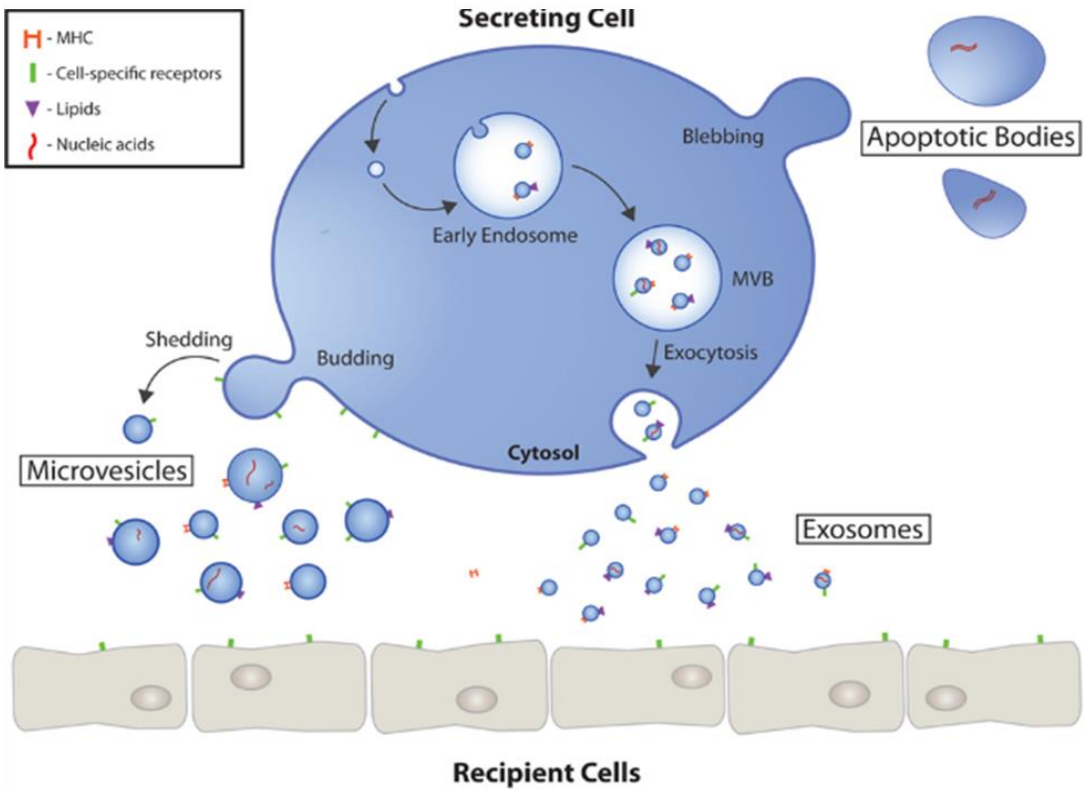

Figure 1.4 Types and origin of extracellular vesicles (EVs) secreted from cells.

EVs include Exosome vesicles, which have an endocytic origin, are generally smaller (30–150 nm diameter), microvesicles, which are large (>200 nm diameter) and dense, emerge from plasma membrane. The largest EVs, apoptotic bodies, are produced during programmed cell death by membrane blebbing. EVs have numerous markers ranging from proteins, to lipids, to nucleic acids. MVB, multivesicular body. Adapted from (Gustafson, Veitch et al., 2017).

1.8.1 Extracellular vesicles in cancer

Recently, researchers have shown an increased interest in the transportation of EVs from cancer cells to other cell types. It is a mode, which provides a significant form of cellular communication and interaction through the transfer of cargo, which includes proteins, lipids, messenger RNA (mRNA), and microRNAs (miRNAs) packaged within a vesicle beside classical receptor–ligand interactions, from cancer cells to other TME cell types (Smallwood, Apollonio et al., 2016). Recipient TME cells usually engulf EVs through endocytosis (Svensson, Christianson et al., 2013), and/or for microvesicles through membrane fusion (Del Conde, Shrimpton et al., 2005, Al-Nedawi, Meehan et al., 2008). Therefore, the biological function of EV transmission is likely to be very important and there is much to discover.

Although EV secretion can be demonstrated in various normal proliferating cell types, there are many well-described examples of EV effects in cancer, many of which may become characterized as a mediator way of mechanisms that stimulates disease (Webber, Yeung et al., 2015). EV production is increased in tumor cells, as evidenced by their increased existence in plasma, ascites, and pleural effusions of patients with cancer (Andre, Schartz et al., 2002, Valenti, Huber et al., 2006).

Recently, the increased presence of EVs and their transmission, from cancer cells to other TME cell types has been the subject of intensive study in the solid cancers. Microenvironmental studies have investigated the origin and function of EVs in multiple malignancies including breast, ovarian, prostate, lung and gastric cancer, glioblastoma multiforme, melanoma, and hepatocellular carcinoma (Zhang, Valencia et al., 2015). These studies revealed wide potential implications of EVs as a way of intercellular communication, thus promoting oncogenesis and tumor progression (Handunnetti, Polliack et al., 2017).

In the clinical setting, exosomes are detected in various types of bodily fluids, encompassing blood, plasma, urine, amniotic fluid, and tumor malignant effusions (Lakkaraju and Rodriguez-Boulan, 2008). Given the relative ease and non-invasive mode of extracting exosomes from patient samples, and their distinctive contents, many studies have suggested using exosomal biomarkers for disease diagnostic purposes (Skog,

Wurdinger et al., 2008, Taylor and Gercel-Taylor, 2008, Zhou, Cheruvanky et al., 2008). The majority of these studies investigated exosomes isolated from serum, although several papers have focused on exosomal biomarkers in urine for renal disease (Zhou, Cheruvanky et al., 2008, Gonzales, Pisitkun et al., 2009) prostate cancer (Mitchell, Welton et al., 2009) and saliva (Kapsogeorgou, Abu-Helu et al., 2005, Gonzales, Pisitkun et al., 2009). Beyond diagnostics, exosomes have also emerged as an exciting potential candidate for immunotherapy and vaccination modalities (De La Pena, Madrigal et al., 2009), as well as a novel vector for gene therapy (Seow and Wood, 2009, Michael, Bajracharya et al., 2010). And could be a potential biomarker for disease progression and response for treatment.

1.8.2 **Extracellular vesicles in CLL**

EVs have become an exciting subject in the field of lymphoid malignancies. Recent work has showed that B-cell receptor (BCR) stimulation, a central survival signaling within the CLL TME, promotes the release of CLL exosomes that encapsulate a notable miRNA profile compared to healthy donors (Koga, Matsumoto et al., 2005). Moreover, it is revealed the presence of exosomal RNA and showed the transmition of genetic materials between cells (Ratajczak, Miekus et al., 2006). Another study determined that secreted exosomes carry both cellular mRNA and miRNA, which might be received by target cells (Valadi, Ekstrom et al., 2007).

Other studies show that miRNA carried by tumor exosomes can repress the mRNA for signaling within T cells (Taylor, Lyons et al., 2002). Because secreted exosomes carry RNA populations, including miRNA, it might be that exosomal miRNA represents the miRNA profile of the parental tumor. In addition, CLL-EVs and their cargo are actively transmitted to stromal cells promoting intracellular pathway and the modifying of these cells into pro inflammatory cancer- associated fibroblasts (Ghosh, Secreto et al., 2010, Farahani, Rubbi et al., 2015, Paggetti, Haderk et al., 2015).

The secretion of EVs is promoted when primary murine B cells are activated by T-cell signals (Saunderson, Schuberth et al., 2008). The effect of CD40 and IL-4 activation, is one of the most critical immune-derived TME stimuli on the secretion and cargo of CLL-

Evs (Jacob, Pound et al., 1998, Herishanu, Katz et al., 2013). Our previous study has showed an enrichment of specific cellular miRNAs compassing miR-363 within EVs transferred from CD40/IL 4 activated CLL cells compared to parental cell miRNA cargo and control EVs from un activated CLL cells. It has revealed that autologous patient CD4+ T cells internalize CLL-EVs carrying miR-363 and that this miRNA controls the immunomodulatory molecule CD69. Furthermore, it is found that autologous CD4+ T cells that are exposed to EVs from CD40/IL 4 activated CLL cells show migration, immunological synapse signaling, and interactions with tumor cells. Knock down of miR-363 in CLL cells prior to CD40/IL 4 activation prevented CLL-EVs promoting increased synapse signaling or providing altered functional characteristics to CD41 T cells. Overall, these results demonstrate a novel role for CLL-EVs and their molecular cargo, which might have critical implications for commination between the TME and CLL cells (Smallwood, Apollonio et al., 2016).

In this context, EVs might be important in the establishment of a pro-survival microenvironment in CLL. Accordingly, increased numbers of larger secreted vesicles have been detected in blood plasma of CLL patients with advanced disease stage and a vesicle-mediated stimulation of bone marrow stromal cells has been shown (Ghosh, Secreto et al., 2010). Engulfment of CLL-derived EVs by stromal cells has been shown to induce activation of mechanistic target of rapamycin (mTOR)/AKT signaling (Ghosh, Secreto et al., 2010) and the induction of an inflammatory phenotype that is associated with enhanced tumor growth in mice (Paggetti, Haderk et al., 2015). It has previously been demonstrated that monocytes and macrophages are the major targets of CLL-derived exosomes (Paggetti, Haderk et al., 2015), but little is known about how exosomes influence the function of these immune cells (Haderk, Schulz et al., 2017).

1.8.3 **Methods of EVs isolation and characterisation**

Differential ultracentrifugation or ultrafiltration are the most important and commonly used methods. Both are capable of producing pure populations of EVs but are labour intensive and require specialised apparatus. Variants of these techniques, such as density gradients, precipitation, filtration, size exclusion chromatography, and immunoisolation have been added to the basic techniques. To achieve better specificity of EV or EV

subtype separation, most researchers use one or more additional techniques following the primary step, such as washing in EV-free buffer, ultrafiltration, application of density gradients (velocity or flotation), or chromatography.

A variety of additional techniques or combinations of techniques have been or are currently being developed, some of which may become more prominent in the coming years if they achieve better recovery or specificity than legacy methods. Such methods include variations on size exclusion chromatography (SEC), microfiltration, novel immune isolation or other affinity isolation technologies, including lipid affinity, novel precipitation/combination techniques, hydrostatic filtration dialysis, high through put/high-pressure methods such as fast protein/high performance liquid chromatography (FPLC/ HPLC) that involve some form of chromatography and a wide variety of microfluidics devices that harness one or more principles (Thery, Witwer et al., 2018). A summary of different EV isolation methods is shown in Table 1.5.

Table 1.5 Summary of different EV isolation methods

Adapted from (https://www.sigmaaldrich.com/life-science/protein-sample-preparation/extracellular-vesicle-preparation.html).

EV Preparation Method	Pros	Cons
Ultracentrifugation (sometimes with sucrose gradient)	• Reproducible • Relatively accurate • Generates functional vesicles	• Time-consuming • Requires use of large, specialized shared equipment (ultracentrifuge) • Prep may also include other soluble proteins and aggregates • EVs may get damaged
Affinity Purification	• High selectivity • Relatively fast and simple	• Lower yields • Markers may not be representative of the targeted EV population • Solution conditions may be incompatible with downstream assays
Ultrafiltration (i.e. size exclusion through low MWCO membranes)	• Generates functional vesicles, as reported for preparation of therapeutic EVs • Relatively fast and simple	• Depending on the membrane and force applied, EVs may be deformed or broken up
Polymeric Precipitation	• Relatively fast and simple	• Contamination by other soluble proteins

1.9 Principle of biomarkers

Biomarkers can be any molecular, biochemical, physiological, or anatomical property that can be quantified or measured in the body and is associated either with the presence of disease (a diagnostic biomarker) or the level of disease (predictive biomarker). The National Cancer Institute (NCI), in particular, defines biomarker as a biological molecule detected in body fluids, or tissues that is an indication of a physiological or a pathological condition. Biomarkers are helpful in a number of approaches, including evaluating the progress of disease, assisting the most effective treatment plans for a specific cancer type, and demonstrating long-term vulnerability to cancer or its recurrence. Ideally, such biomarkers can be detected in non-invasive ways (Mishra and Verma, 2010).

Biomarkers can be useful in non-malignant disease to help to make an accurate diagnosis and choose a therapeutic regime. For instance, the measurement of some autoantibodies in patients' blood sample is a strong biomarker for autoimmune disease and specifically the detection of rheumatoid factors has been a reliable diagnostic marker for rheumatoid arthritis (RA) for over 50 years (Waaler, 2007).

Currently, biomarkers are the interest of intensive research in oncology. There are many biomarkers already discovered and others in development for use in detecting and evaluating several types of cancer. These novel biomarkers have become essential for preventive medicine that identifies tumor or the risk of disease early and takes specific countermeasures to prevent the progress of disease. Many cancer patients are diagnosed at a late stage where the cancer has poorly progressed and too far to be managed but molecular biomarkers have the potential to improve cancer detection, such as increased prostate specific antigen (PSA) indicating the presence of prostate cancer and the need for further investigations (Li and Chan, 2014).

Additionally, biomarkers have the potential to enable physicians to develop individualized therapeutic regimes for their cancer patients. For instance, the role of KRAS in colorectal cancer and other EGFR-related cancers. In patients, whose tumors express the mutated KRAS downstream EGFR signalling is up regulated to cause continued cancer cell growth and proliferation. Assaying a cancer for its KRAS type (wild-type vs. mutant) assists in recognizing those patients who will benefit most from

treatment with cetuximab a monoclonal antibody directed against EGFR. Analysis of mutation status helps to choose treatments specific to their patient's tumor type and the mutant can, therefore, be considered a biomarker. This route to using a biomarker to tailor treatment ensures that drug response rate will improve, drug toxicity will be limited and costs associated with testing various therapies and the ensuing treatment for side effects will decrease (Ludwig and Weinstein, 2005).

Biomarkers can also be quantitative. For example, lactate dehydrogenase (LDH) has been shown to be prognostic in lymphomas such as Hodgkin's lymphoma and diffuse large B-cell lymphoma (Zhou et al., 2014). LDH is a blood marker and therefore, useful as it is the basis for non-invasive testing.

MicroRNAs are detectable in the circulation (Cortez and Calin, 2009) and there is interest in using miRNA as biomarkers in cancer (Tang, Shen et al., 2013). Moreover, many miRNAs have been shown to be down regulated in CLL. Decreased expression of the genes encoding miR-15a and miR-16-1 associate with a good prognosis while, low levels of miR-29 miRNAs associate with poor prognosis in CLL (Bartels and Tsongalis, 2009). In order to introduce a biomarker into clinical practice a candidate biomarker needs extensive testing in terms of repository studies followed by testing in prospective clinical trials. In this thesis a candidate miRNA biomarker, miR-363, which was discovered to be enriched in extracellular vesicles from stimulated CLL cells was tested in a repository study.

1.10 Preliminary work

The lymph node microenvironment provides essential signals for the proliferation and survival of chronic lymphocytic leukaemia (CLL) cells and contributes to resistance to conventional chemotherapy. There is no readily available access to lymph node tissue from patients and currently no markers of leukaemic cell activity specifically due to stimulation within lymph nodes.

CD154 is a T-cell surface molecule delivering essential signals for normal B-cell proliferation in the germinal centre. CD154, either as soluble protein or in a cell-membrane bound context with addition of IL-4 is capable of delivering signals to CLL

cells. CD154, which is likely be a physiological ligand encountered by leukemic B-cells in the lymph node, is sufficient to up regulate CD38 (Willimott, Baou et al., 2007). Extracellular vesicles play also an important role in cell communication. They transport signalling molecules including miRNA between cells by either directly fuse into the plasma membrane or be endocytosis delivering their cargo into the cytoplasm of the recipient cells (Masyuk, Masyuk et al., 2013). Exosomes which carry a cargo including miRNA are believed to be produced by stimulated but not resting B-cells and are recoverable from peripheral blood (Valadi, Ekstrom et al., 2007).

Our laboratory has used CD154/IL-4 stimulation to mimic the effects of encounter with T-cells in the tissue microenvironment and drive proliferation of CLL cells in vitro. When the miRNA content of exosomes from CD154/IL-4 stimulated CLL cells was investigated they were found to contain miRNA, which differed in amount from that within cells. Characterisation by low-density miRNA microarrays showed that 7 were most highly altered: miR-363, miR-374b, miR-323-3p, and miR-494 were upregulated and miR-150, miR-484, and miR-17.

Characterization and miRNA profiling of CLL-EVs derived from CD40/IL-4–stimulated CLL cells reveals enrichment of specific miRNA content including miR-363. miR-363 showed ∼270-fold increased expression in EVs compared with parental CD40/IL-4–stimulated CLL cells and we identified the T-cell immunomodulatory protein CD69 as having the highest context score of all miR-363 target genes (targetscan.org) (Smallwood, Apollonio et al., 2016).

To investigate the hypothesis that EVs from CLL cells can alter T-cell function we exposed autologous $CD4^+$ T cells to EVs from CD40/IL-4–stimulated CLL cells and demonstrated enhanced immunological synapse signaling and migration. In order to confirm this finding knockdown of miR-363 in CLL cells was carried out and shown to prevent the ability of released CLL-EVs to confer altered functional properties to $CD4^+$ T cells.

Therefore, this work suggests that CLL cells stimulated in the tissue microenvironment produce EVs, which have a role in intercellular communication and may perturb T-cell

function. The importance of these effects in the pathogenesis of CLL or maintenance of the disease requires further work.

In addition, through the preliminary work mentioned above we have suggested candidate miRNAs, miR-363 located on chromosome X (Xq26.2) and miR-374b located on chromosome X (Xq13.2) that are enriched in exosomes from chronic lymphocytic leukaemia B-cells and have demonstrated the functional importance of one of these in regulating T-cell function. MiRNAs are only produced by stimulated primary B-cells and we reason that CLL cells produce exosomes only when receiving stimuli from cells in the lymph node microenvironment and furthermore that miR-363 and miR-374b may be biomarkers if detectable in the blood of CLL patients. Although levels of cellular miRNA have been shown to be prognostic in CLL, circulating miRNA have not been analysed and the proposed work will, therefore, provide new data.

Data from others suggests that the candidate exosomal miRNA that we have identified are not present in significant amounts in plasma from normal subjects (Moussay, Wang et al., 2011). In this thesis, the potential of miR-363 and miR-374b as biomarkers for disease activity in CLL will be investigated.

1.11 Summary and Aims

A hypothesis of this study is that miRNA enriched in EVs produced in the TME might be biomarkers of disease activity or response to therapy if detectable in the circulation. The lymph node microenvironment provides essential signals for the proliferation and survival of chronic lymphocytic leukaemia (CLL) cells and contributes to resistance to conventional chemotherapy. There is no readily available access to lymph node tissue and currently no markers of leukaemic cell activity specifically due to stimulation within lymph nodes. Biomarkers derived from leukaemic cells within this microenvironment will be clinically useful in assessing disease progression and as predictive markers of response to treatment. Importantly peripheral blood biomarkers derived from the lymph node microenvironment will be clinically important in the low-grade lymphomas, which are common diseases and for which assessment of progression currently depends on clinical assessment and CT scans. EVs carry a cargo including miRNA and are only produced by stimulated B-cells. They are recoverable from peripheral blood. We will

assess candidate miRNA enriched in EVs measured in peripheral blood for their utility in low-grade lymphoproliferative disorders, focusing on CLL.

We have already established candidate miRNA, miR-363 and miR-374b that are enriched in exosomes from chronic lymphocytic leukaemia B-cells and have demonstrated the functional importance of one of these in regulating T-cell function. MiRNA are only produced by stimulated primary B-cells and we reason that CLL cells produce exosomes only when receiving stimuli from cells in the lymph node microenvironment. Data from others suggests that the candidate exosomal miRNA that we have identified are not present in significant amounts in plasma from normal subjects and we will, therefore, pursue the hypothesis that levels of miR-363 and miR-374b correlate with disease activity within lymph nodes

Aim 1: To determine numbers of circulating extracellular vesicle and size distribution in CLL patients and normal subjects.

Aim 2: To carry out a repository study to measure circulating miR-363 levels in CLL patients requiring treatment and asymptomatic patients and in a group of normal subjects.

Aim 3: To carry out functional characterisation of candidate biomarker miR-363 in CLL cells and to determine whether knockdown alters specific surface markers, proliferation or apoptosis.

Chapter 2 Materials and Methods

2.1 Clinical samples and processing

2.1.1 Ethical Approval

Human blood samples from healthy donors and CLL patients were obtained after written informed consent was obtained, and with ethical approval from the local institutional review boards.

2.1.2 Primary Material

Plasma samples from normal subjects (n = 11) and from Leicester patients (n = 23) were obtained after informed consent was obtained (Leicester Research Ethics Committee 06/Q2501/122). Characteristics of Leicester patients, age, gender, VH gene mutational status and FISH cytogenetic results are presented in Table 2.1. Total white cell count (WCC) and platelet count at the time samples were taken for EV isolation are given in columns to the right (Table 2.1).

Additional Plasma samples were obtained through the UK CLL Trials Biobank (University of Liverpool) (North-West England Research Ethics Committee 14/NW/1014) from CLL patients enrolled in two clinical trials: the ARCTIC trial which was funded by the NIHR Health Technology Assessment Programme (NIHR HTA project number 07/01/38; ISRCTN16544962) (University of Leeds) (Howard, Munir et al., 2017) (n = 100) and CLEAR [A trial looking at using antibiotics for chronic lymphocytic leukaemia (http://www.cancerresearchuk.org/about-cancer/find-a-clinical-trial/a-trial-looking-using-antibiotics-for-chronic-lymphocytic-leukaemia-the-clear-trial)] (n = 50). For ARCTIC, a trial investigating advanced disease requiring treatment, median age was 63 years, interquartile range 58–67 years and M:F was 69:31. 48 patients had unmutated immunoglobulin genes, 36 mutated and 16 not determined. 14 patients showed 11q23 deletion and 4 patients 17p deletion by FISH interphase cytogenetics. Clinical information was not available for patients enrolled in CLEAR, a trial enrolling asymptomatic patients with early stage disease. It was not possible to complete processing of 5 ARCTIC samples and 2 CLEAR samples, either because miRNA isolation failed or RT-PCR failed, and these cases were, therefore, excluded from the study.

Table 2.1 characteristics of Leicester patients.

Patient ID	Age	Gender	Mutational Status	VH Gene Segment	FISH Cytogenetics				WCC ($x10^9$ /l)	Platelets ($x10^9$ /l)
					11q del	13q del	17p del	Trisomy 12		
1	77	M	U	7-04	-	Y	-	-	11.8	41
2	68	M	M	3-48	-	Y	-		44.8	149
3	69	F	U	3-21	Y	-	-	-	93.5	86
4	81	F	M	3-15	-	-	-	-	13.8	183
5	80	F	M	3-21	-	Y	-	-	317.1	151
6	73	M	U	1 69	-	-	-	-	42.1	227
7	83	F	M	4 34	-	Y	-	-	31.9	243
8	66	M	M	3 23	-	-	-	-	5.5	193
9	77	F	M	3 74	-	-	-	-	206.6	167
10	69	M	U	1-02	-	-	Y	-	27.3	168
11	75	M	M	7 81	-	-	-	Y	5.9	150
12	69	M	M	2 05	-	-	-	-	93.5	192
13	61	F	U	3 23	-	Y	-	-	16.8	140
14	46	M	M	3 21	Y	-	-	-	57.2	221
15	86	M	M	4 34	-	-	-	-	19.7	245
16	91	M	M	3 15	-	Y	-	-	14.2	166
17	84	F	M	3 21	-	Y	-	-	6.1	205
18	60	M	U	1 69	Y	-	-	-	74.9	220
19	79	M	U	4 39	-	Y	-	-	64.5	228
20	58	F	M	1 03	-	-	-	-	20.3	174
21	82	M	ND	ND	-	Y	-	-	5.3	154
22	71	M	M	3 07	-	Y	-	-	3.38	221
23	68	F	ND	ND	-	-	-	-	5.77	130

Age, gender, VH gene mutational status and FISH cytogenetic results are presented. Total white cell count (WCC) and platelet count at the time samples were taken for EV isolation are given in columns to the right.

2.1.3 **Peripheral Blood Mononuclear Cell Isolation**

Ficoll histopaque density gradient separation was performed as the standard procedure to separate peripheral blood mononuclear cells (PBMNC) from fresh whole blood in a suitable cell culture facility. The following steps were undertaken:

1- Remove histopaque solution (17-1440-03, Ficoll-Paque PLUS) and RPMI 1640 (supplemented with 10% FCS, Glutamine and Pen/Strep) from fridge to warm to room temperature.

2- After labelling all tubes and lids with sample number, add 15 ml of histopaque into a 50 ml falcon tube.

3- Next, gently layer blood (up to 30 ml) onto histopaque and avoid mixing blood with histopaque layer.

4- The sample was centrifuged at 400 g and stopped without braking at 20 °C.

5- Collect WBCs into a Falcon tube using a proper pipette and add the re-warmed fresh culture media to make up to 30 ml.

6- After centrifuging at 200 g for 10 minutes, discard the supernatant and resuspend the cell pellet in about 10-50 ml of media (depending on the pellet size).

7- Count cells using hemocytometer and TC20 automated cell counter-based trypan blue staining (BIO-RAD).

2.1.4 Extracellular vesicle preparation from plasma

K_2EDTA plasma was isolated by centrifugation of whole blood at 4,200 rpm (250 g) for 5 minutes at room temperature. The top three quarters of the plasma supernatant was transferred to fresh tubes by using a plastic transfer pipette. Samples were aliquoted in to vials and then frozen at −80 °C and thawed on ice before use.

The general scheme of extracellular vesicle purification by ultracentrifugation is depicted in Figure 2.1.

For EV preparation from plasma, samples were centrifuged again twice for 15 minute at 2500 g 4°C to remove cells, cell debris and apoptotic bodies. This will still leave us with a mixed population of micro particles, exosomes and plasma protein and lipids. The large proportion of the micro particles was pelleted using a higher speed centrifuge, 30,000 g for 30 minutes. This yielded a suspension of extracellular vesicles and plasma protein/lipids. This suspension was used in the NanoSight machine to measure particle number and concentration. EVs were also isolated from *in vitro* culture supernatants (see Section 2.1.5 and Figure 2.1). Samples were put on ice before use.

2.1.5 Extracellular vesicles purification from CLL culture supernatants

The first steps are designed to eliminate large dead cells and large cell debris by successive centrifugations at increasing speeds (as for plasma) (see Figure 2.1).

At each of these steps, the pellet is discarded, and the supernatant is used for the following step. The final supernatant is ultra-centrifuged at 100,000 g to pellet the small vesicles that correspond to extracellular vesicles. The pellet is washed in a large volume of PBS, to eliminate contaminating proteins, and centrifuged one last time at the same high speed. Finally, the invisible pellets were re-suspended by leaving 20ul Aim V covering predicted pellet area with tube on its side for 30 minutes (Figure 2.1).

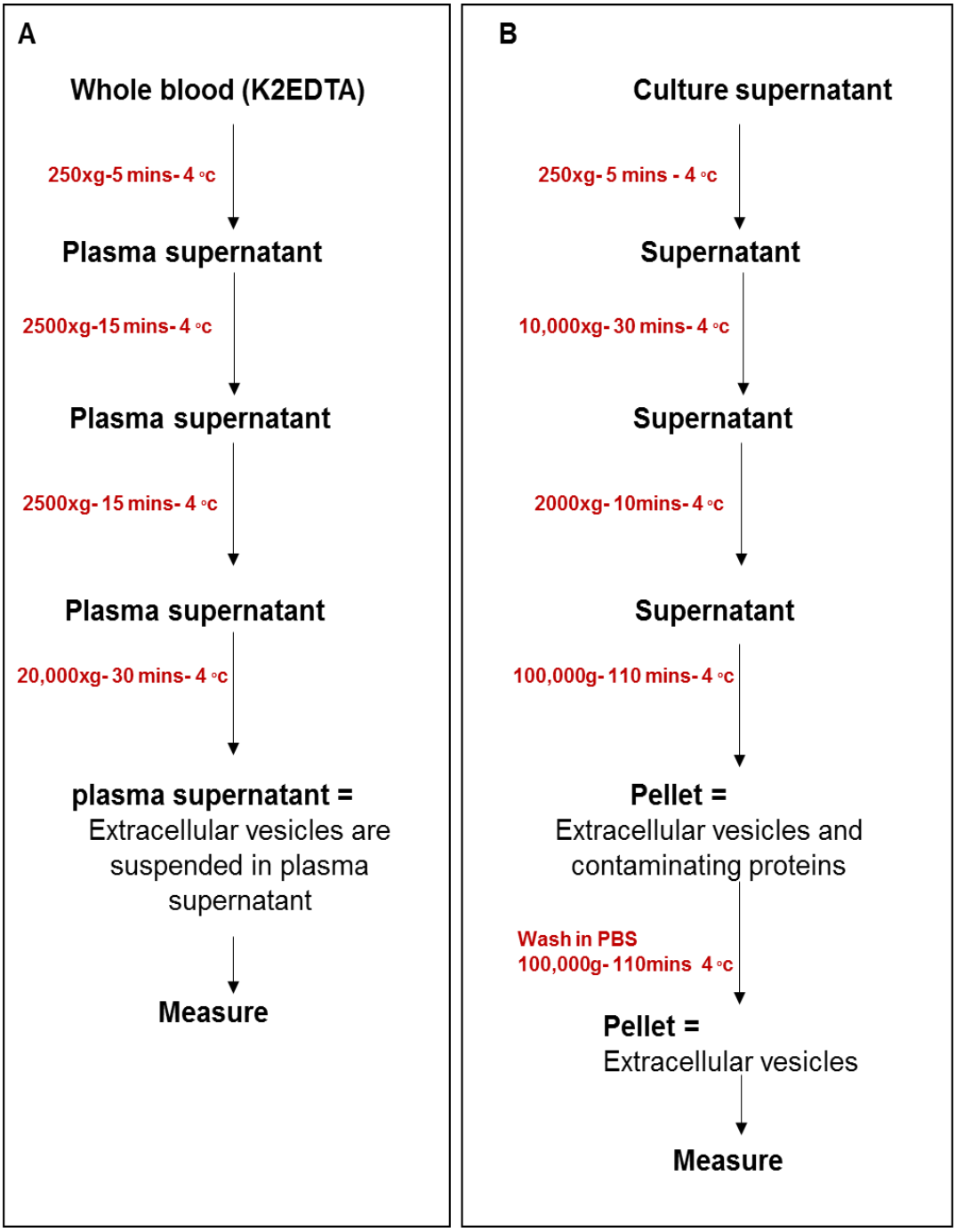

Figure 2.1 Steps of extracellular vesicles purification.

(A) from whole blood and (B) from CLL culture supernatant by differential ultracentrifugation.

2.2 Vesicle measurements (NanoSight)

Particle size distributions and counts were measured on a NanoSight NS500 (Malvern Instruments), which uses the properties of light scattering due to Brownian motion to obtain particle size distributions and concentration of samples in liquid suspension. A laser beam is passed through a prism edged glass flat within the sample chamber into the nanoparticle suspension. The beam refracts to an intense low profile resulting in a compressed beam with a reduced profile and a high power density. The particles in suspension in the path of this beam scatter light in such a manner that they can be easily visualized via a long working distance, x20 magnification microscope objective fitted high-sensitivity CMOS camera, operating at 30 frames per second (fps). The camera subsequently captures a video file of particles moving under Brownian motion within a field of view of approximately 100µm x 80µm x 10µm (Figure 2.2). Following manufacturer's protocol, EVs plasma samples were diluted 1000x in filtered PBS, inverted 2–3 times immediately prior the measurement and then analysed with the NanoSight. The number of particles per ml and the size distributions were calculated using NanoSight NS500 gating software.

2.2.1 Problems Encountered With NanoSight

Generating statistically significant results from plasma using Nanosight requires different settings for each sample due in part to biological materials variations. These settings are an iterative process between Camera Level, Sample Concentration, Beam Position, and Focus. Sample concentrations were the most common problem, as too high concentration prevented accurate particle tracking. On the other hand, lower concentrations required longer capture and analysis time to produce remarkable results. Image focus comes in the second place of the common problems, the particles are constantly moving, so it is difficult to achieve a uniform perfect spherical focus, and indistinct particles, gives inaccurate results. Therefore, a long time was spent to provide an optimal setting for each sample. See Table 2.2 and Figure 2.3.

Table 2.2 Nanosight Settings for vesicle measurements .

Setting	Protocol	Modification	Notes
Dilution	1:1000	Sample dependent	Used to achieve optimal particle concentration
Measurements	6 x 1min	3 x 1min	
Temperature	23	23	
Viscosity	0.89-0.92	0.89	
Camera shutter	25-32ms	11-15ms	Shorter avoids over exposure of larger particles but may miss some smaller particles
Frames/s	24.98-24.99	24.98-24.99	
Drift velocity	5011 – 6970 nm/s	~1000 nm/s	Drift is caused by the sample moving under flow, ordinarily you want this to be close to 0. Their values suggest they have an attachment which induces flow
Analysis			
Blur	Auto	Auto	
Detection threshold	5-6	10	Higher values mean more certainty that an event is actually a vesicle rather than an anomaly
Min track length	Auto	Auto	
Min expected size	Auto	Auto	

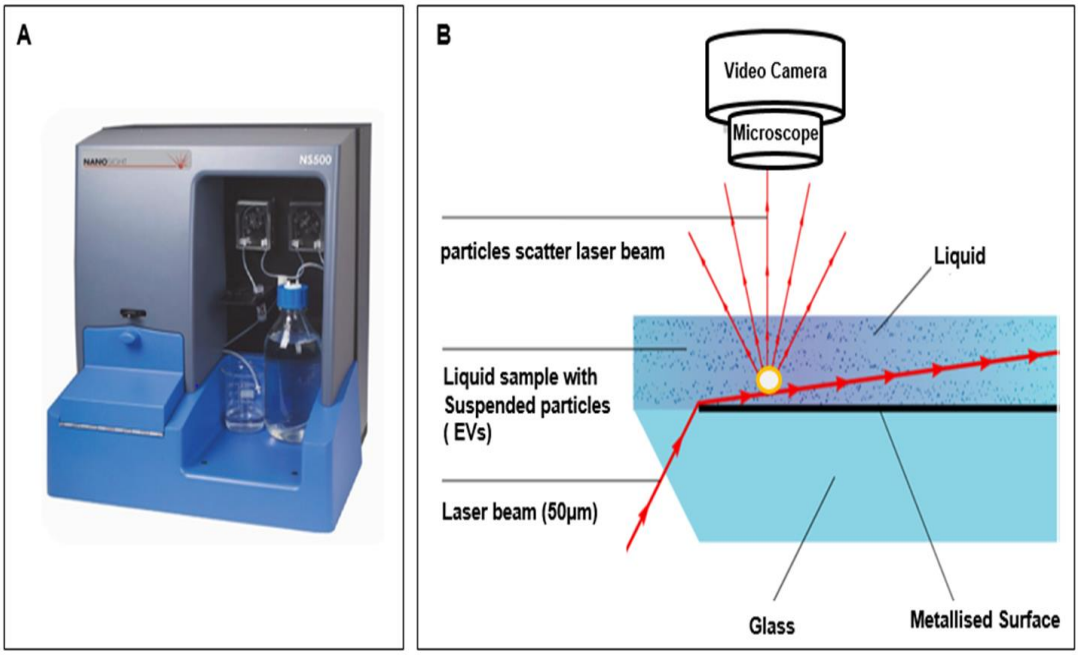

Figure 2.2 NanoSight NS500.

(A) (Malvern Instruments). (B) Principal configuration of NanoSight for NTA of EVs adapted from (Dragovic, Gardiner et al., 2011).

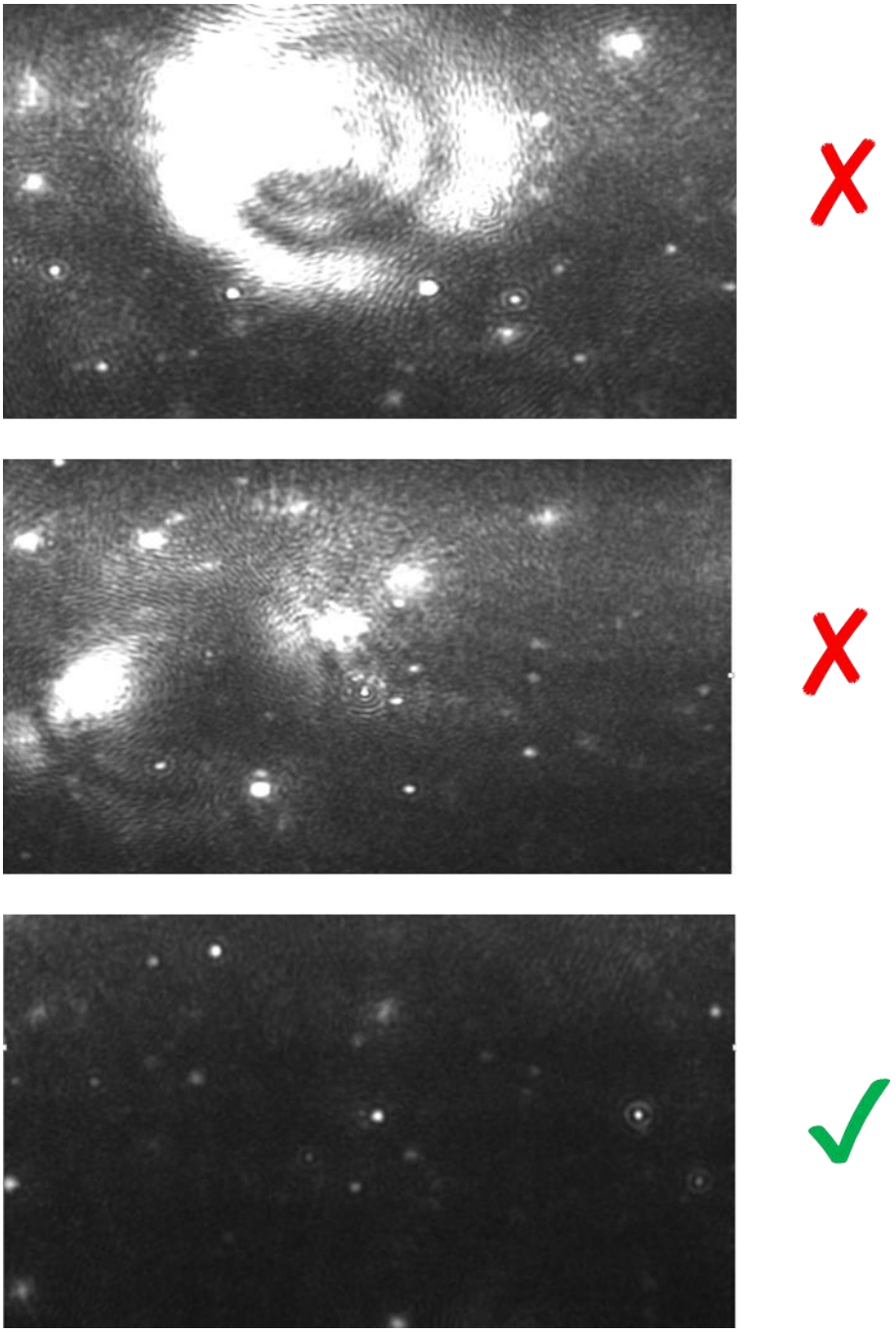

Figure 2.3 Problems Encountered With NanoSight.

Images show moving particles, and the difficulty of achieving a uniform perfect spherical focus. Unclear particles, give inaccurate results. Therefore, a long time was spent providing an optimal setting for each sample.

2.3 Size exclusion chromatography (SEC)

SEC separates molecules according to differences in size as they pass through a SEC medium packed in a column.

An ÄKTA prime (GE Healthcare, Little Chalfont, UK) with a sephacryl S-500 resin chromatography column (0.9 × 30 cm, 19.1 ml bed volume) was employed. Before injection, the column was equilibrated with 25 ml of phosphate buffered saline (PBS) (pH 7.4) solution at 0.5 ml/ minute at room temperature. Platelets were depleted from fresh plasma by two rounds of centrifugation. The column was then injected with 7 ml of undiluted plasma and eluted at room temperature for approximately 1 hour with PBS solution at a flow rate of 0.5 ml/minute. A total of 31 to 37 fractions (HV: 1,4,5,7,9,11,13,16,17,19,21,23,25,29,33,37) (Pt: 1,5,9,13,17,21,25,29, 33,37) of 4 ml each were collected . The column was flushed with 75 ml of PBS solution at 0.5 ml/min (3.75 column volumes) between plasma fractionation to eliminate carryover. We used blue dextran as a control and monitored the flow of this trace in the chromatography column (Figure 2.4. A). Protein molecular weight standard BSA (67 kDa; GE Healthcare) were also used, by measuring the absorbance (A280) in the collected fractions. Protein molecules started to elute at fraction 17 (Figure 2.4 B). Fractions were stored at 4 °C before use.

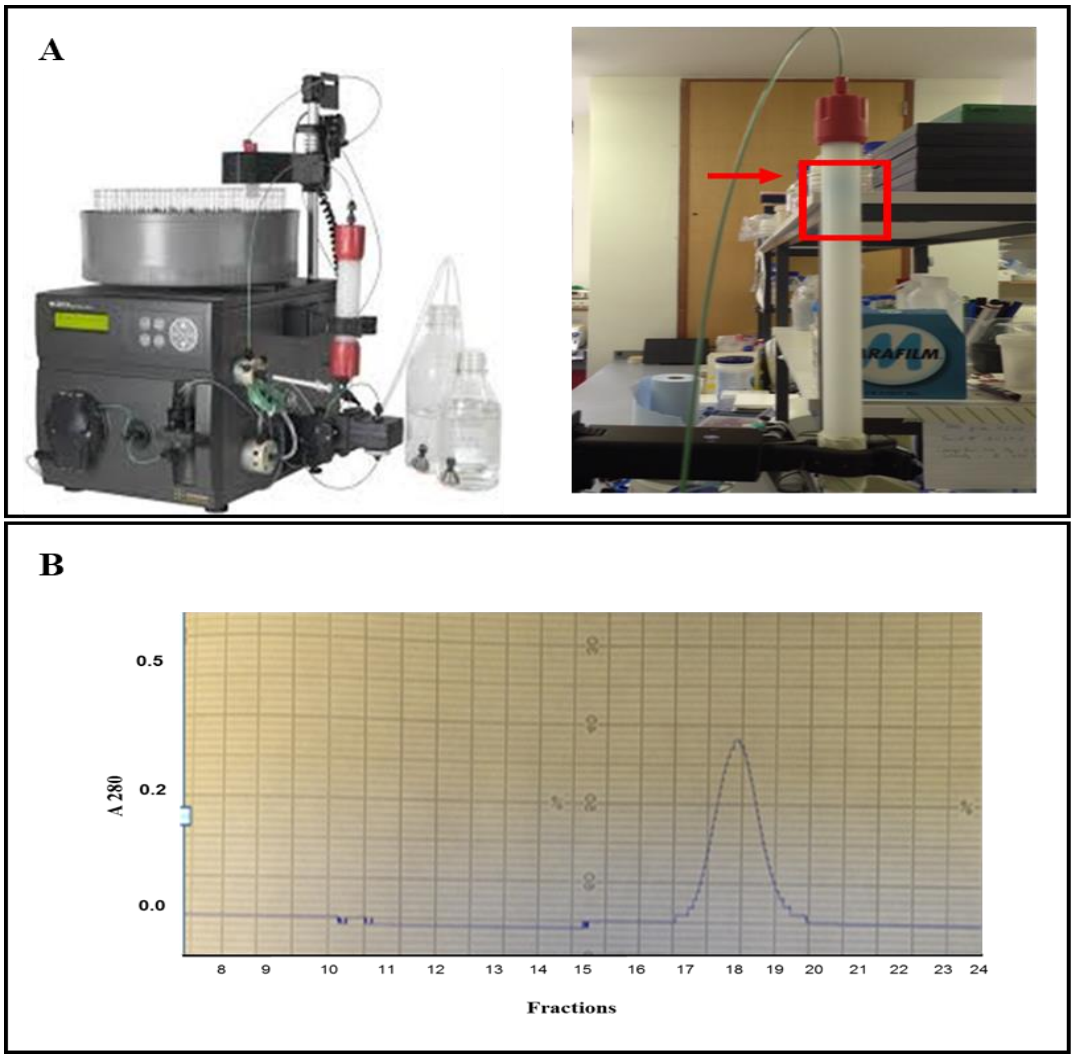

Figure 2.4 AKTA prime plus.

(A) An automated liquid chromatography system, panel to the right shows a size exclusion chromatography column with trypan blue moving down the column as indicated by the red rectangle and arrow. (B) Flowchart of protein standards (BSA) elusion: column resolves particles from ~1 to 120 nm in diameter, particles 120 nm and larger eluting near fraction 8. Particles 1 nm or smaller eluting near fraction 17**.**

2.4 Quantitative PCR for miRNAs

2.4.1 RNA extraction

RNA was isolated from all samples using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany, # 217184) according to the manufacturer's instructions.

QIAzol Lysis Reagent (Qiagen, Hilden, Germany, #79306) was added to ultra-centrifuged plasma samples, after chloroform was added, the lysate is separated into aqueous and organic phases by centrifugation. RNA separated to the upper, aqueous phase, while DNA separated to the interphase and proteins to the lower, organic phase or the interphase.

The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water.

To assess recovery and stability of RNA, each sample was spiked with an identical amount of synthetic UniSp2 RNA (Exiqon, Vedbaek, Denmark, #203203).

2.4.2 Heparinase treatment of RNA before quantitative real-time RT-PCR

Quantitative real-time reverse transcription PCR (RT-PCR) is a highly sensitive method for detecting changes in gene expression. Heparin was identified as an inhibitor of enzymatic reactions including DNA polymerase. Methods for removing heparin from DNA and RNA using heparinase have been developed (Johnson, Navanukraw et al., 2003).

Initial experiments did not use heparinase and miR-363 was found to be undetectable in plasma samples from ARCTIC patients (n=10), which had been taken into tubes with Li heparin as an anticoagulant. Because this miR-363 was readily detectable previously from

samples taken into K_2EDTA the hypothesis was made that heparin was inhibiting the DNA polymerase.

In order to test this hypothesis heparin was eliminated from RNA isolated from ARCTIC and CLEAR patient plasma samples using heparinase I (Sigma-Aldrich Corp., St. Louis, MO, # H2519). According to the manufacturer's protocol, Heparinase I (55 units) was dissolved at 1 mg/ml in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM $CaCl_2$, and 0.01% BSA. And added to a final concentration of 140 USP units per 1 ml of sample, which is expected to nearly completely remove heparin.

To test the effect of heparinase treatment on Ct variability, we compared the amplification of miR-363, miR-16, UniSp2 and UniSp6 from heparinase-treated with that of control miRNAs water solution (Figure 2.5).

Two controls were used: UniSp2, which was spiked into the plasma and UniSp6, which was added to the isolated RNA just before reverse transcription and was not, therefore, exposed to heparin in the plasma. While miR-363 was undetectable without heparinise treatment, miR-16 and UniSp2 were detectable but almost at the limits of the assay. UniSp6 as expected, because it was added after RNA isolation, is detectable at almost the same level in heparinise treated and untreated. Use of heparinise showed that miR-363 was present, but in lower amounts than miR-16 or UniSp2.

Subsequently all the ARCTIC samples were treated with heparinise before RT-PCR.

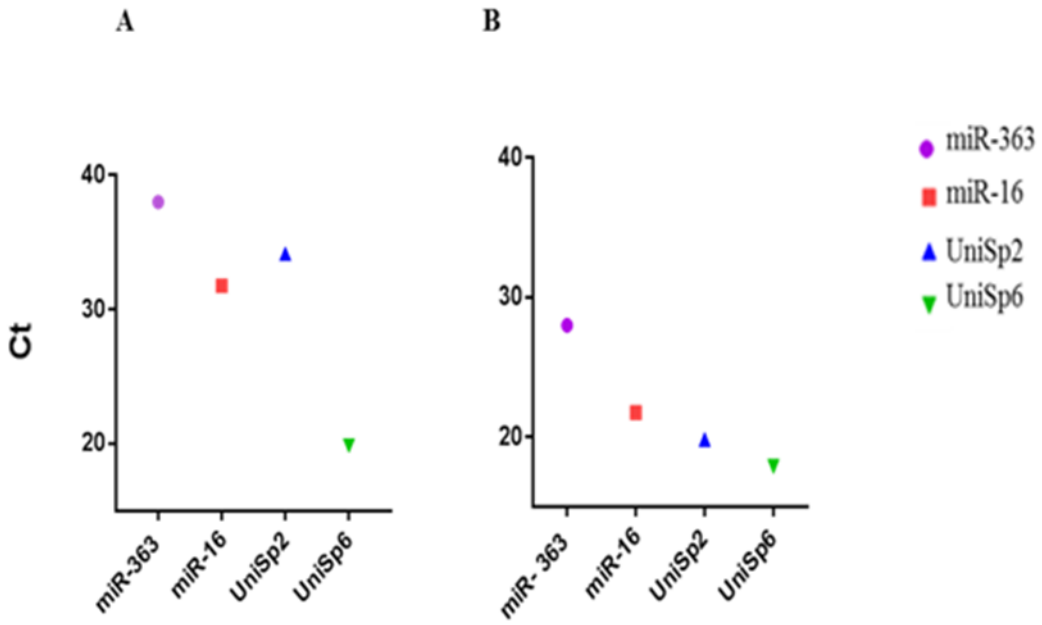

Figure 2.5 The effect of heparinase treatment on Ct variability.

Comparison of the amplification between miR-363. MiR-16, UniSp2 and UniSp6 from water selution (A) with that of heparinase-treated (B). Ct= 37 considered not determined. UniSp6 was spiked in to the mixture after miRNA isolation but before reverse transcription and therefore it is not affected by heparin in the patient sample. However, UniSp2 is spiked in to the plasma sample before miRNA isolation and is affected by heparin in the sample.

2.4.3 **Standardization and normalization**

To ensure standardized input, exactly 200 µl of the plasma sample was used. To assess recovery and stability of RNA, we spiked each sample with an identical amount of synthetic RNA spike-in (UniSp2 and UniSP6) during RNA extraction and cDNA synthesis respectively. The RNA abundance in each sample was normalized to synthetic RNA recovery. Quantitative PCR reactions were performed using SYBR® Green master and miRNA-specific primers (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Copy DNA produced in the RT reaction was amplified in Micro Amp ™ optical 96-well reaction plates in triplicate 10 µl reactions on an Applied Biosystems 7900HT Thermocycler (ABI). Moreover level of miR-363 were measured twice in 8 ARCTIC patients, in order to investigate reproducibility, and data demonstrated no significant differences between two measurements, (Wilcoxon- paired t-test). See chapter 4 Figure 4.2.

We normalized the data across samples using a median normalization procedure adapted from (Mitchell, Parkin et al., 2008). For each sample, the Ct values obtained for the two spiked-in UniSp2 and UniSp 6 were averaged to generate Spike-in Average Ct value. The median of the Spike-in Average Ct values obtained from all of the samples to be compared was next calculated (designated here as the Median Spike-in Ct value). A normalization factor was then calculated for each sample based on the following formula: Normalization factor =1/[2ˆ(Median Spike-in Ct value) - (Spike-in Average Ct value of the given sample). The number of copies of a given miRNA in each sample (calculated using the standard curves Figure 2.6) was multiplied by the normalization factor corresponding to the sample to obtain a normalized copy number value.

For some results Ct values are presented. These were calculated by the formula:

Normalized Ct value for the miRNA in the sample = Raw Ct value - [(Spike-in Average Ct value of the given sample) - (Median Spike-in Ct value)]

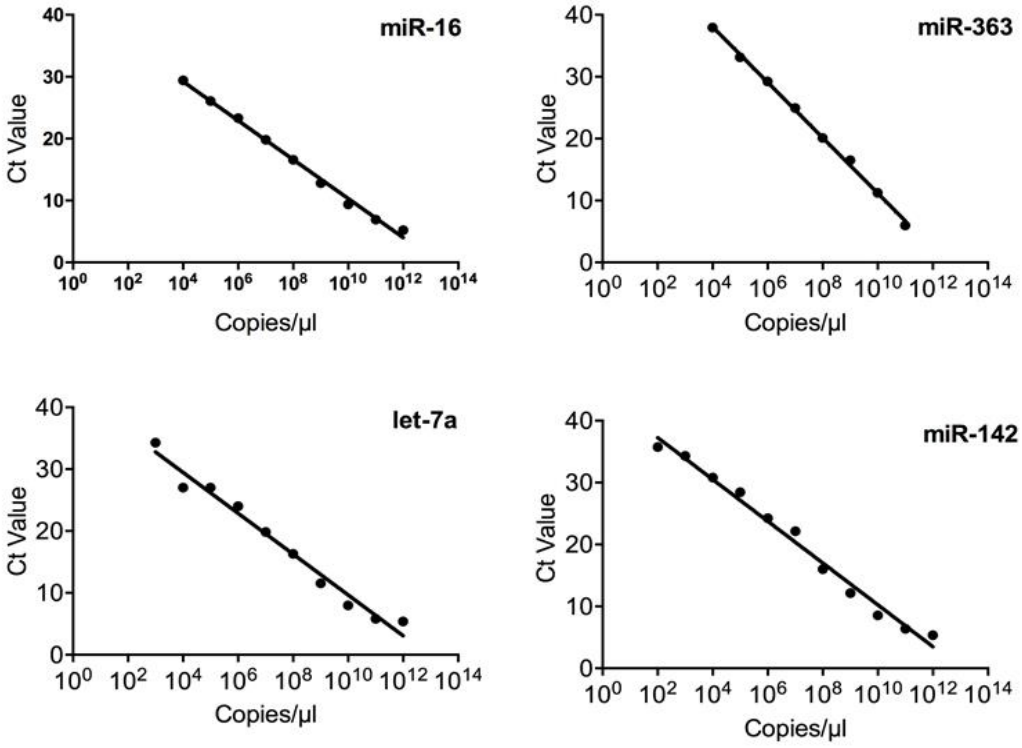

Figure 2.6 Standard curves for quantitative RT-PCR.

Standard curves were constructed for mir-363, miR-16, miR142 and let-7a. Using known numbers of copies of each oligonucleotide as template RT-PCR was carried out and Ct values obtained. Trend line was interpolated using GraphPad Prism v6.0.

2.4.4 **Quantification of nucleic acids**

Concentration and quality of nucleic acids were checked using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). DNA and RNA concentration were determined by measuring the optical density (OD) at 260 nm, where 1 OD unit equals 50 µg/ml of DNA and 40 µg/ml of RNA.

2.4.5 **First-strand cDNA synthesis**

Extracted micro RNAs were reverse transcribed using Universal cDNA synthesis kit II, (Exiqon- product number 203301). According to the manufacture's protocol with some modifications, the template RNA samples were diluted to a concentration of 40 ng/µl using nuclease free water. RT working solution of the 5x Reaction buffer, water, Enzyme mix and RNA spike-ins Reagents was performed (Table 2.3). Finally, the reaction was incubated for 60 min at 42˚C, and then heated-inactivate the reverse transcriptase for 5 min at 95˚C and immediately cooled to 4°C using thermo cycler.

Table 2.3 Reverse transcription reaction setup.

Reagent	**Volume (µL),RT reaction**
5x Reaction buffer	2
Nuclease-free water	4.5
Enzyme mix	1
Synthetic RNA spike ins, optional replace with H2O if omitted	0.5
Template total RNA (5 ng/ µl)	2
Total volume	10

2.4.6 **Quantitative reverse transcriptase PCR (qRT-PCR)**

Real-time RT-PCR is a power full tool to quantify gene expression. The quantitative endpoint for real-time PCR is the threshold cycle (CT). The CT is described as the PCR cycle at which the fluorescent signal of the reporter dye (amplification curve) crosses the threshold. It is a relative measure of the concentration of target in the PCR reaction.

The numerical value of the Ct is inversely related to the amount of amplicon in the reaction (i.e., the lower the CT, the greater the amount of amplicon).

Quantitative PCR reactions were performed using ExiLENT SYBR® Green master mix (Exiqon, #203402) and miRNA-specific primers (Exiqon, hsa-miR-363-3p LNA PCR primer set #204726, hsa-miR-142-3p LNA PCR primer set #204291, hsa-let-7a-5p LNA PCR primer set #206084, hsa-miR-16-5p LNA PCR primer set #205702 and hsa-miR-374b-5p LNA PCR primer set # 204608). A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide in which the ribose moiety is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form duplexes. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired and hybridize with DNA or RNA according to Watson-Crick base-pairing rules (Kaur, Arora et al., 2006).

According to the manufacturer’s instructions, cDNA produced in the RT reaction was amplified in MicroAmpTM optical 96-well reaction plates in triplicate 10 µl reactions on an Applied Biosystems 7900HT Thermocycler. Concentration and quality of nucleic acids were checked using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA). Results were normalised to control for differences in amount of RNA loading and efficiency of RT-PCR using Sp2 and Sp6 RNA.

For some experiments, levels of miR-363 were expressed as fold change relative to control (Sp2 and Sp6 RNA)

For each sample, the Ct values obtained for the two spiked-in UniSp2 and UniSp 6 were averaged to generate Spike-in Average Ct value. The median of the Spike-in Average Ct values obtained from all of the samples to be compared was next calculated (designated here as the Median Spike-in Ct value)

Fold change for each target gene is calculated in the following way:

Normalise each sample to its control (Sp2 and Sp6 RNA) = Ct (sample) – Ct (Spike-in Average Ct value).

Delta Ct = each normalised sample Ct value calculated above - the median of the Spike-in Average Ct values obtained from all of the samples.

Due to exponential nature of PCR, "fold change" is calculated as 2^-DeltaCt. The results are shown as "fold change +/- S.E.M".

Absolute expression provides the exact copy number following transformation of the data via a standard curve (Figure 2.6). For that, standard curves were constructed for mir-363, miR-16, miR142 and let-7a. Using known numbers of copies of each oligonucleotide as template. RT-PCR was carried out and Ct values obtained. Then we drew a standard curve (DNA concentration vs Ct values). Next, we run the unknown sample and then plotted the Ct value to the standard curve and found the matched concentration of DNA in our sample(s). Trend line was interpolated using GraphPad Prism v6.

Standard curves (Figure 2.6) were constructed using synthetic oligonucleotides (Sigma, St. Louis, MO, USA) for miR-16, miR-363, miR-142 and let-7a (ThermoFisher, Waltham, MA, USA) (see Table 2.5).

RT-PCR was carried out and Ct values obtained. Trend line was interpolated using GraphPad Prism v6.

For some experiments levels of *CD 69 mRNA* and *S1PR1 mRNA* were measured using TaqMan® Gene Expression Assays (Applied Biosystems, TaqMan® Fast Advanced Master Mix, # 4444557, *CD69* TaqMan® Gene Expression Assays # 2244506 and *S1PR1* TaqMan® Gene Expression Assays # 4224566) (Table 2.6). These differ from SYBR Green assays in that there is a probe labelled with two fluorophores as well as the forward and reverse PCR primers.

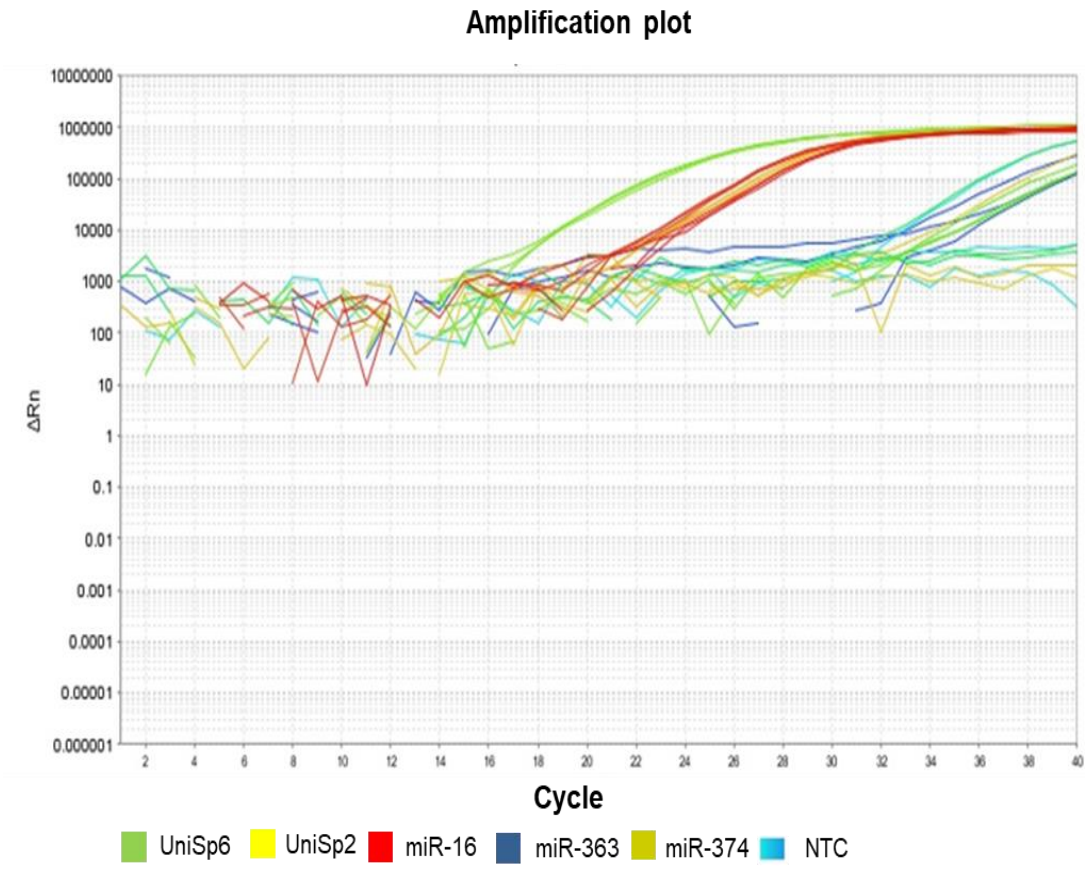

Figure 2.7 Illustrative exported files of PCR amplification plot.

SYBER GREEN Real Time PCR Assay (Applied Biosystems, #4398965).

Table 2.4 Real -time PCR using SYBR® Green, per. 10 µL reaction.

Reagent	Volume (µL),RT reaction
PCR Master mix	5
PCR primer mix	1
Diluted cDNA template	4
Total volume	10

Table 2.5 Primer sequences of synthetic oligonucleotides for miR-16 miR-16, miR-363, miR-142 and let-7a.

Primer	Sequence (5' to 3')
MiR-363	AAUUGCACGGUAUCCAUCUGUA
MiR-16	UAGCAGCACGUAAAUAUUGGCG
MiR-142	UGUAGUGUUUCCUACUUUAUGGA
Let-7	UGAGGUAGUAGGUUGUAUAGUU

Table 2.6 Real -time PCR using TaqMan® Gene Expression Assays.

Component	Volume(µl) 1 reaction	Final Concentration 96-well plates
TaqMan ® Fast Advanced Master Mix (2x)	10.0	1x
TaqMan ® Gene Expression Assay (20x)	1.0	1x
cDNA template	2	100 ng to pg
Nuclease- free water	7.0	-
Total volume per reaction	20	-

2.4.7 **Real-time PCR amplification**

Real-time PCR amplification was performed followed by melting curve analysis according to Table 2.7. Cycle threshold (Ct) values were calculated using manual constant thresholding and miRNA assays were normalized between the samples using the UniSp6 and UniSP2.

Table 2.7 Real-time PCR cycle conditions (Applied Biosystems).

Process step	Settings
Polymerase Activation/Denaturation	95˚C, 10 min
Amplification 60˚C, 1 min,	40 amplification cycles at 95˚C, 10 s ramp-rate 1.6˚C/s Optical read
Melting curve analysis7	Yes

2.5 Cell Culture

2.5.1 Cell Culture and CD40L/IL4 activation

CLL B-cells were cultured in Aim V with AlbuMAX serum-free media (Invitrogen) at a concentration of 5 x10^6/mL compared to 3 x10^6 in our previous study(Smallwood, Apollonio et al., 2016) . To stimulate the release of CLL B-cell EVs, CLL cells were incubated with recombinant human CD40L (sCD154) (1 mg/ml; R&D Systems) and IL-4 (20 ng/ml; R&D Systems) and anti-CD40 antibody (clone EA-5) at 1 mg/ml (sc65264; Santa Cruz Biotechnology) for 36 hours. CLL-EVs were harvested from 36 x 10^6 cultured leukemic cells at 4˚C by differential centrifugation of culture supernatant. Supernatants were centrifuged at 250 g for 5 minutes followed by twice centrifugation at 2000 g for 15 minutes at 4°C to pellet cells and debris, and then the supernatant was further centrifuged at 30,000 g for 30 minutes at 4˚C again to pellet debris and larger membrane-bound particles. Finally supernatants containing suspended EVs were collected and store at -80 °C to use later.

2.5.2 siRNA Knockdown of miR-363

To determine whether CLL-EV miR-363 is a significant regulator of CD41T-cell biology, we knocked down expression of this miR in CLL cells using LNA-based antisense oligonucleotides.

Antisense oligonucleotide is a nucleotide sequence that is complementary to a sequence of messenger RNA (mRNA). When antisense DNA or RNA is added to a cell, it binds to a specific mRNA molecule and inactivates it by physically blocking the ability of ribosomes to move along the messenger RNA or by simply accelerating its degradation. The Antisense LNA™ GapmeRs are stable and potent that they can be added directly to serum-containing culture medium without the need for transfection reagents, which is great advantage when working with primary cell cultures.

Prior to CD40/IL-4 stimulation and isolation of released EVs. Transfection methods, which can cause apoptosis and disrupt cells were not used but addition of miRNA to the

culture medium was sufficient to allow effective concentrations (as demonstrated by reduction in amount of miRNA by RT-PCR) to enter cells.

CLL cells were cultured in the presence or absence of CD40/IL-4 stimulation in 6-well plates at $5X10^6$/mL with a volume of 2 ml per well. Either miRCURY LNA Power microRNA inhibitor miR-363 (Exiqon) or miRCURY inhibitor negative control A (Exiqon) were added to the cultures at a final concentration of 50 nM for 36 hours before harvesting.

In order to achieve the optimal knock down, 5 $X10^6$/mL cells in the presence or absence of CD40/IL-4 stimulation were transfected with anti-miR-363 at 0, 5, 20 and 50 nM. For comparison a negative control scrambled (Scr) oligonucleotide was used. Then PCR Screening was carried out to identify the gene expression level (Figure 2.8).

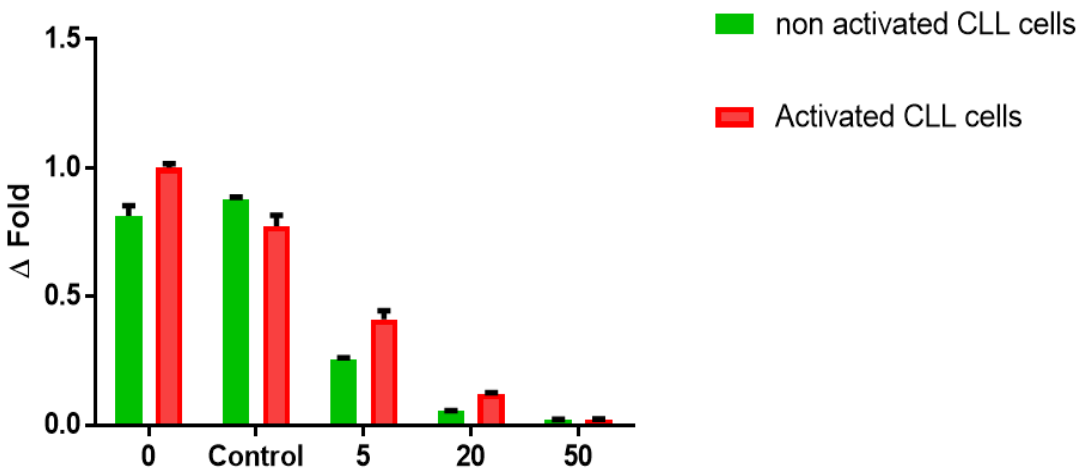

Figure 2.8 The effect of miR-363 knockdown on miR-363 level.

Different concentrations of anti-miR-363 were used (5, 20, 50 nM). For comparison a negative control scrambled (Scr) oligonucleotide was used.

2.5.3 **Viability and proliferation**

Viability of cells and growth was measured by using the CellTiter-Glo® (CTG) luminescent cell viability assay (CellTiter Glo, Promega, Madison, WI, USA). It is worth noting that the assay system is designed for utilise a multi-well plate format. It relies on the feature of a proprietary thermo stable luciferase and generates a stable luminescent signal relative to the amount of ATP. The CTG is a homogeneous approach to determine the number of viable cells present in culture depending on quantitation of the ATP, which signals the existence of metabolically active cells. Additionally, the amount of ATP present is directly proportional to the number of cells present in culture. Briefly, CTG reagent (100 µl) was added to 100 µl of medium containing cells, usually equal volume of the reagent is added to the volume of cell culture medium for a 96-well plate. After adding the reagent, mix contents on an orbital shaker to induce cell lysis for about 2 minutes. Then the plate was incubated to stabilize luminescent signal at room temperature

for 10 minutes. The effect of CD40L/IL4 on cells viability was determined using the Wallac VICTOR2 multilabel counter (PerkinElmer) for measuring luminescence at room temperature. Percentage of cell viability was calculated to dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) used a vehicle control.

2.5.4 **Confocal Microscopy**

The particles suspensions were labelled with PKH67 dye (Fluorescent Cell Linker Kits use proprietary membrane labeling technology to stably incorporate a green fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane) (Sigma – Aldrich, #PKH67GL) and incubated for 24 hours with two different culture conditions of CLL cells: activated with CD40L/IL-4 and not activated. Images were then acquired by A1R (Nikon) confocal microscope using a 60x/1.40 oil objective with NIS-elements imaging software (Nikon). Images were obtained at time =0 just after EVs were added to the cells and at 24 hours after adding EVs. Technical assistance for microscopy was obtained from Dr. David Read, MRC Toxicology Unit. In order to label EVs and subsequently carry out immunofluorescence staining the following steps were undertaken:

(a) EV Labelling:

1- Re-suspend EVs in 180 µL PBS
2- Mix 180 µl Diluent C (Sigma Linker kit) and 20 µl diluted PKH67 Dye in a clean Eppendorf.
3- Mix the 180 µl EVs With 180 µl of the dye solution and incubate 5 minutes at RT in the dark
4- Transfer Dye/EVs Solution to labelled UC Tubes and top up the tubes with 5 mL Sterile PBS.
5- Centrifuge the vessels at (Rotor SW41, 20000 x g for 1hr)
6- Collect the tubes with forceps, tip over the supernatants
7- Re-suspend EVs in 200 µl AIM V Media and add 100 µl of the solution to the different culture conditions (0h, 24h).

(b) Immuno-fluorescence staining:

1- Centrifuge the cells on to a poly-lysine slide using cytofuge or cytospin

2- Fix cells with fix buffer (3%formaldehyde In PBS) for 15 minutes

3- Remove fix buffer and wash once with PBS.

4- Permeabilize with perm buffer (0.1% Triton X-100 (Sigma-Aldrich), 4% BSA In PBS) for 20 minutes.

5- Remove perm buffer and wash once with PBS.

6- Add Rhodamine Phalloidin buffer (Life technologies) (1:40) diluted in PBS and incubate for 30 minutes.

7- Remove Rhodamine Phalloidin buffer and wash once PBS.

8- Add DAPI Solution and incubate for 5 minutes.

9- Remove DAPI and wash once with PBS.

10-Mount (add 1 drop of fluorescent mounting medium (Dako).

11-Let the slides dry overnight at 4°C

12-Acquire images to microscope.

2.5.5 **Reagents and Flow cytometry**

Flow cytometry was used to investigate the effects of CD40L stimulation and siRNA knockdown of miR-363 on apoptosis and surface expression of CD69 (shown to be a miR-363 target in T-cells (Smallwood, Apollonio et al., 2016) and S1PR1 a molecule involved in lymphocyte trafficking.

For flow cytometry, 5 µl of the antibodies (anti-CD69-PE-Cy7 antibody, #557745, BD PharMingen, Oxford, UK, anti S1PR1 -APC-A antibody, # LS-A1013-50, Biocompare, anti CD5-PE-A antibody, # MA5-17790, ThermoFisher and anti CD19-Qdot 605 antibody, # Q10306 ThermoFisher) was added to 50 µl (containing $1x10^6$ cells) and incubated for 30 minutes on ice. The stained cells were re-suspend in 400 µL of FC staining buffer and acquire data on a flow cytometry using BD FACS Canto TM II. The data were analysed with FlowJo software (vX.0.7). Gating and selection of viable B-cells prior to analysis shown in Figure 2.9 and Figure 2.10.

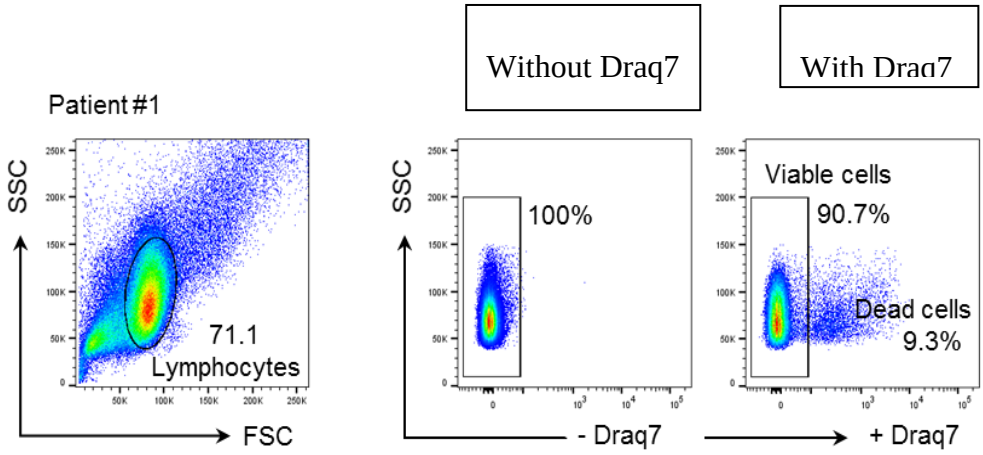

Figure 2.9 Example of selection of viable cells prior to analysis.

Selection of cells based on the forward scatter (FSC) and side scatter (SSC). To exclude dead cells, Draq7 was added to cell suspension before acquisition on a FACS Aria. Viable cells, which are negative for Draq7 dye, were selected for B-cells gating strategy in CLL.

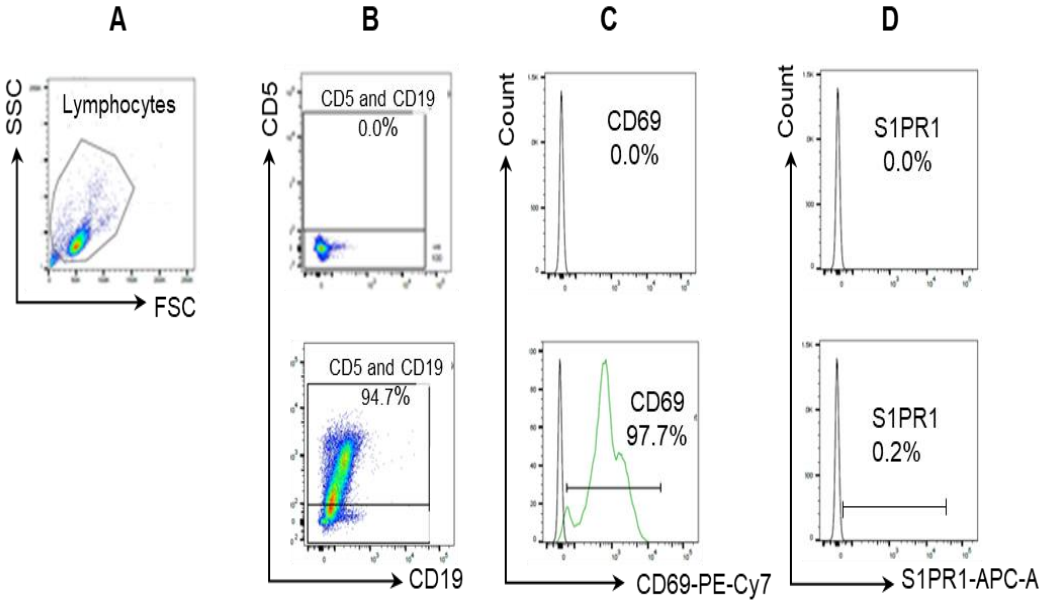

Figure 2.10 Histograms of gating steps for CD5+ and CD19+ B-Lymphocytes using Flow cytometric analysis.

(A) Gating viable lymphocyte population. (B) Gating of CD5+ and CD19+ B-Lymphocytes. Top histograms showed unstained sample for control and histograms in bottom illustrate stained samples. (C) Expression levels of CD69 within CD5+ and CD19 B-lymphocytes. (D) S1PR1 levels within CD5+ and CD19 B-lymphocytes. After isolation cell population was > 90% pure. The data was analysed by FlowJo.

2.5.5.1 Determination of apoptosis by flow cytometry

To measure the effects apoptosis of activated and not activated CLL cells following miR-363 knockdown, Cell pellets were harvested at 200 g for 5 minutes at room temperature, then the pellets were washed twice with cold PBS prior to analysis and re-suspended with 100 µl annexin binding buffer and 5 µl annexin-V. The cells were incubated in 20 minutes at room temperature, and then 400 µl of 1 X annexin binding buffer was added to the cells and kept on ice. Apoptotic cells were subsequently determined using a FITC Annexin V Apoptosis Detection kit I (Bioscience) and draq7 TM (BioLegend). The acquisition and analysis of laboratory data report was automatically generated using BD FACS Canto TM II.

2.6 Statistics

All experiments were performed in duplicate or triplicate and the data were expressed as mean ± SEM. Data were compared with the use of either the 2-tailed Student t test or the nonparametric Mann-Whitney test. Analysis was done using GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA). $P< 0.05$ was considered statistically significant.

Chapter 3 Characterization of circulating extracellular vesicles

3.1 Introduction

CLL cells activated *in vitro* have characteristic miRNA signatures (Paggetti, Haderk et al., 2015) and various functions have been suggested for extracellular vesicle derived miRNA. These currently include modifying the immune synapse and other T-cell functions (Smallwood, Apollonio et al., 2016, Haderk, Schulz et al., 2017) or modifying stromal cells differentiation or function (Farahani, Rubbi et al., 2015, Paggetti, Haderk et al., 2015).

CLL cells receive stimuli for activation, either through the B-cell receptor or CD40, within the tumour microenvironment. As described in Chapter 1 (pages 18 to 19) the tumour microenvironment is believed to have roles in driving CLL cell proliferation and be an important target for therapy.

We, therefore, made the hypothesis that because CLL extracellular vesicles appear to be released from activated leukaemic cells within the tumour microenvironment, they or their cargo might be a source of clinically useful biomarkers for disease activity.

It is an open question as to whether circulating extracellular vesicles are derived from the tumour microenvironment but it is reasonable to suppose that a proportion are produced in this tissue context. Previous work on circulating miRNA has suggested that they might have some clinical predictive power (Moussay, Wang et al., 2011).

In this chapter, I firstly compare numbers and size of extracellular vesicles between healthy volunteers and patients using dynamic light scattering. One prediction is that miRNA that are enriched in extracellular vesicles derived from the tumour microenvironment will also be enriched in vesicles within the blood. I, therefore, determined the distribution of specific miRNA between plasma and particles using size exclusion columns.

Overall patients have greater numbers of circulating extracellular vesicles than normal subjects and surprisingly a different distribution of specific miRNA between vesicles and plasma.

3.2 Results

3.2.1 Circulating particle size distributions in CLL patients and healthy volunteers

To determine the numbers and size distribution of extracellular vesicles in the plasma we isolated these particles by differential centrifugation from the plasma of 10 normal donors and 23 CLL patients and then used dynamic light scattering (NanoSight; Malvern Instruments) as described in Material and Methods (pages 42 to 45).

The results demonstrated two patterns of size distribution profiles in the plasma of both groups. The first profile showed a single major and broader peak ranged 30 to 400 nm in diameter. This distribution (which is called Pattern A) was the dominant pattern in both normal subjects and patients (Figure 3.1A and C). Pattern B was different from Pattern A in that although the major peaks were at approximately the same average particle diameter at 200 nm, there were more substantial minor peaks of larger diameter particles and size range extended approximately from 200 to 490 nm in diameters. Pattern B was observed in 10 % of patients and 20% of healthy volunteers (Figure 3.1B and C). Overall, this finding indicates that there was no appreciable difference in size distribution profiles among patients with CLL and healthy volunteers and the dominant vesicles showed in plasma samples are within the range of extracellular vesicles. The difference between patterns A and B is not patient dependent but seems to be sample dependent. The relatively small amount of larger particles in both groups could be due to some biological materials such as aggregated proteins or surface membrane blebs, which contaminated the samples despite the centrifugation steps in the isolation procedure.

Although, biological heterogeneity is likely to contribute to patterns A and B, but unavoidable differences in sample processing might also play a part. We made every effort to process samples from normal subjects and patients in exactly the same manner but it is known that sample handling can affect the results of assays (Pritchard, Cheng et al., 2012) and there is unavoidable variability in the collection of patient samples.

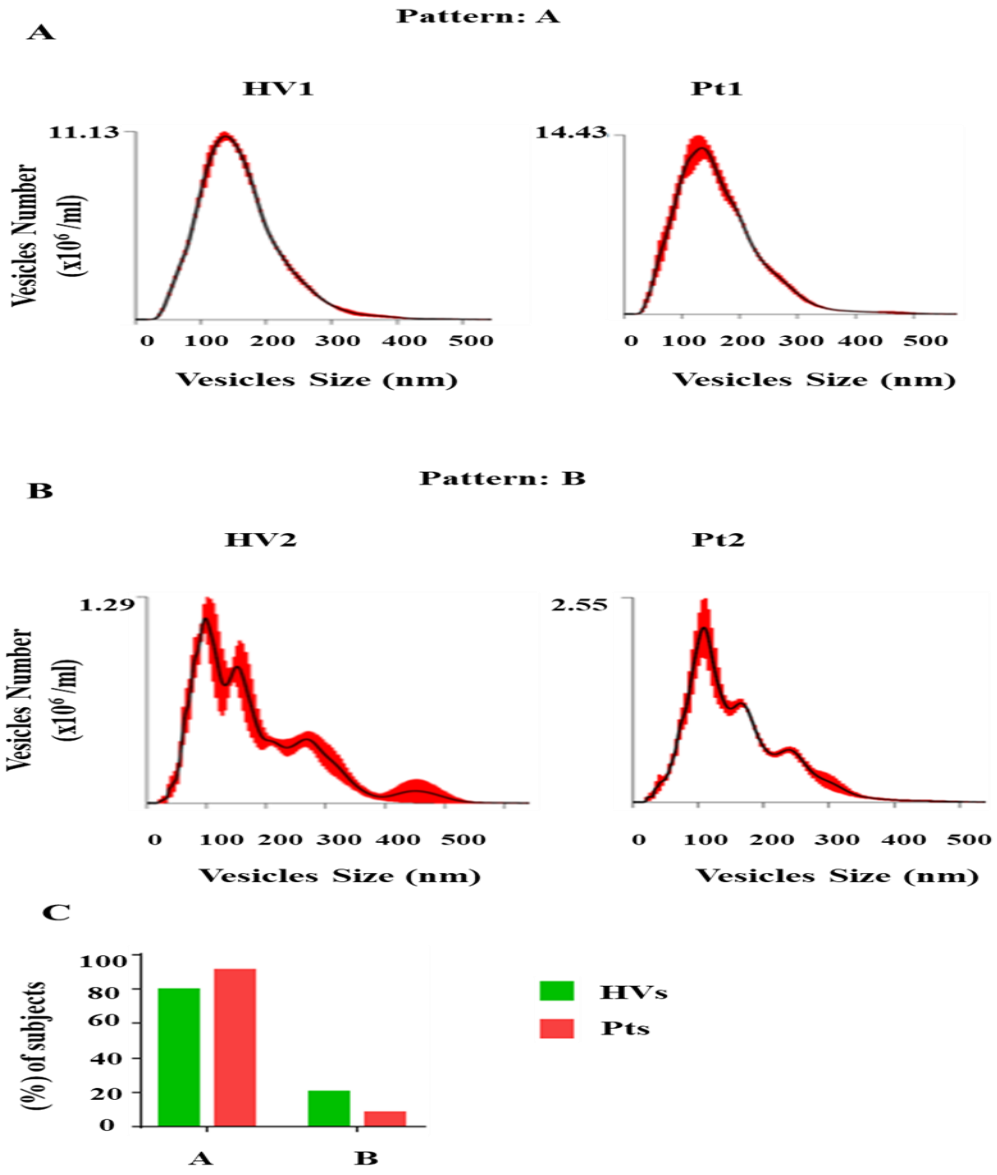

Figure 3.1 Data profiles obtained from the NanoSight.

Showed different EV Size distribution patterns in plasma of CLL patients (Pts) and healthy volunteers (HVs). (A) Pattern A it is likely that there is one main and broader peak. (B) pattern B shows many peaks, which decrease in peak-height as size increases. (C) Comparison of EV size distribution pattern in CLL patients (Pts) (n=23) and Healthy volunteers (HVs) (n=10). Red lines indicate mean -/+ SD.

3.2.2 Comparison of vesicle size and numbers between CLL patients and healthy volunteers

Numbers of vesicles from the plasma of patients with CLL (n=23) and healthy volunteers (n=10) were then measured and compared by dynamic light scattering (NanoSight; Malvern Instruments). The measurement of plasma extracellular vesicles (EVs) revealed significantly increased numbers in CLL patients compared to healthy volunteers (Mann-Whitney U-test; **P*=0.01) (Figure 3.2 A). However, mean particle size distribution between two groups was very similar (Figure 3.2 B).

Comparison of the size distribution profiles from individual patients showed these to be very similar irrespective of the numbers of circulating vesicles (Figure 3.3). This suggests that these two variables are independently regulated.

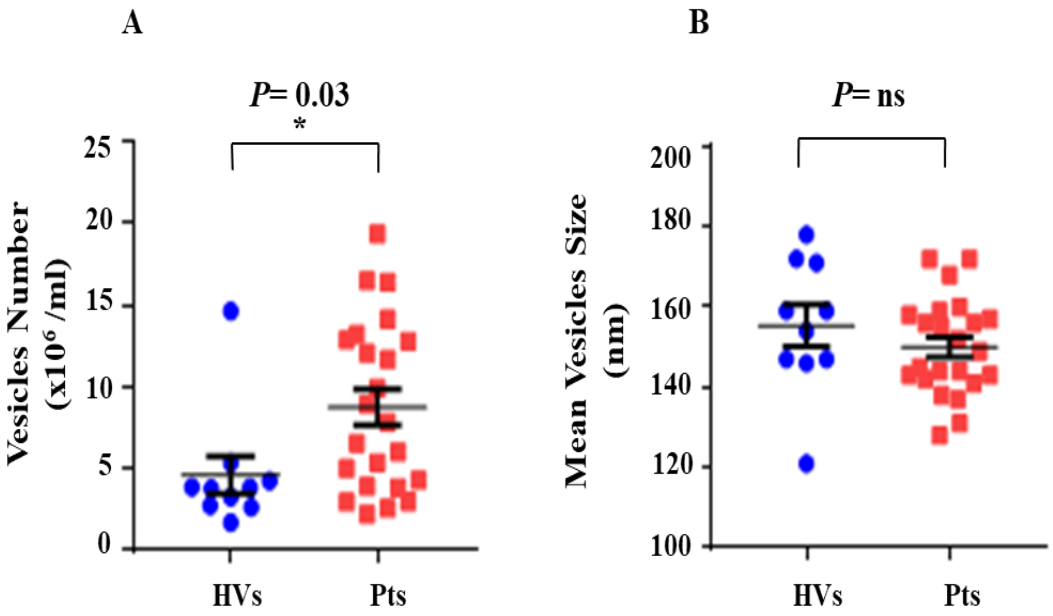

Figure 3.2 Characterisation of vesicles (EVs) isolated from plasma of CLL patients (Pts) and healthy volunteers (HVs).

(A) Histogram showing vesicles concentration from plasma samples from patients with CLL (Pts) (n=23), following isolation by two rounds of centrifugation, measured by NanoSight. For comparison EVs were isolated from normal subjects (HVs) (n=10). There was a significant difference in the number of vesicles (Mann-Whitney U-test; * *P*=0.01). (B) Comparison of EV sizes (mean vesicles diameter) between patients and normal subjects. Error bars indicate mean +/- SEM.

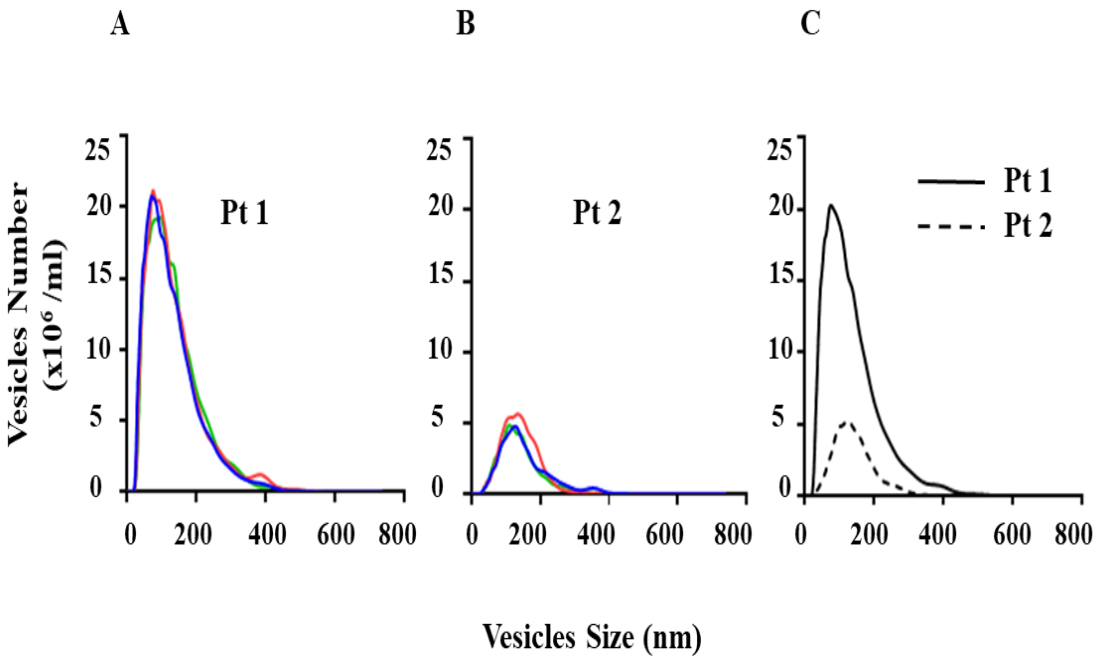

Figure 3.3 Triplicate measures of extracellular vesicle (EV) size distribution in two plasma samples of patients.

(A) CLL Patient with a high particle number. (B) With lower numbers. (C) Comparison of EV size distribution in the two patients; one with a high vesicle number (solid line) and the other with a lower numbers (dashed line).

Table 3.1 Particle concentrations and mean particle diameter in plasma from healthy volunteers (+/- SEM).

The pattern of size distribution (either A or B) (see section 3.2.1) is presented to the right.

Subject ID	Particle concentrations: E8 particles/ml	Size distribution: nm	Size distribution: Pattern
1	3.86 +/- 0.23	147 +/- 1.8	A
2	5.36 +/- 0.41	146 +/- 3.6	A
3	4.21 +/- 0.08	152 +/- 4.8	A
4	3.89 +/- 0.19	121 +/- 3.2	A
5	4.21 +/- 0.23	150 +/- 5.7	A
6	1.71 +/- 0.15	178 +/- 15.5	B
7	2.75 +/- 0.07	147+/-10.2	A
8	3.28 +/- 0.18	172 +/- 17.5	B
9	2.26 +/- 0.22	159 +/- 2.7	A
10	14.64 +/- 0.29	159 +/- 1.7	A

Table 3.2 Particle concentrations and mean particle diameter in plasma from patients with CLL (+/- SEM).

The pattern of size distribution (either A or B) (see section 3.2.1) is presented to the right.

Subject ID	Particle concentrations: E8 particles/ml	Size distribution: nm	Size distribution: Pattern
1	9.91 +/- 0.17	152 +/- 2.8	A
2	16.39 +/- 0.46	145 +/- 1.9	A
3	2.97 +/- 0.28	168 +/- 6.2	A
4	6.58 +/- 0.49	143 +/- 6.2	A
5	2.59 +/- 0.29	172 +/- 7.5	B
6	3.92 +/- 0.34	155 +/- 2.1	A
7	14.14 +/- 0.51	156 +/- 3.3	A
8	12.89 +/- 0.38	141 +/- 1.5	A
9	19.35 +/- 0.11	128 +/- 1.4	A
10	16.49 +/- 1.01	172 +/- 5.9	B
11	12.05 +/- 0.24	149 +/- 4.6	A
12	8.93 +/- 0.55	157 +/- 6.1	A
13	4.31 +/- 0.12	159 +/- 3.3	A
14	11.67 +/- 0.13	137 +/- 1.9	A
15	14.06 +/- 0.23	150 +/- 2.7	A
16	5.38 +/- 0.11	158 +/- 3.2	A
17	3.00 +/- 0.10	160 +/- 9.4	A
18	6.05 +/- 0.03	156 +/- 3.4	A
19	2.23 +/- 0.02	150 +/- 7.2	A
20	7.84 +/- 0.08	143 +/- 2.6	A
21	12.80 +/- 0.27	138 +/- 0.4	A
22	12.86 +/- 0.34	145 +/- 5.1	A
23	3.36 +/- 0.46	159 +/- 5.7	A

3.2.3 Extracellular vesicle numbers in the plasma of CLL patients are not associated with circulating whit cells count

There is a potential problem with patient samples that might influence circulating EV numbers: circulating EVs might be derived from circulating white cells or platelets and because CLL patients will have higher white cell counts than normal subjects this could explain the findings above (section 3.2.2).

In order to address this issue, absolute lymphocyte and also platelet counts were correlated with EV numbers. EV numbers in CLL patients were not significantly associated with absolute lymphocyte or platelet counts (Figure 3.4). Indicating that, EV numbers in CLL do not simply reflect circulating tumour load.

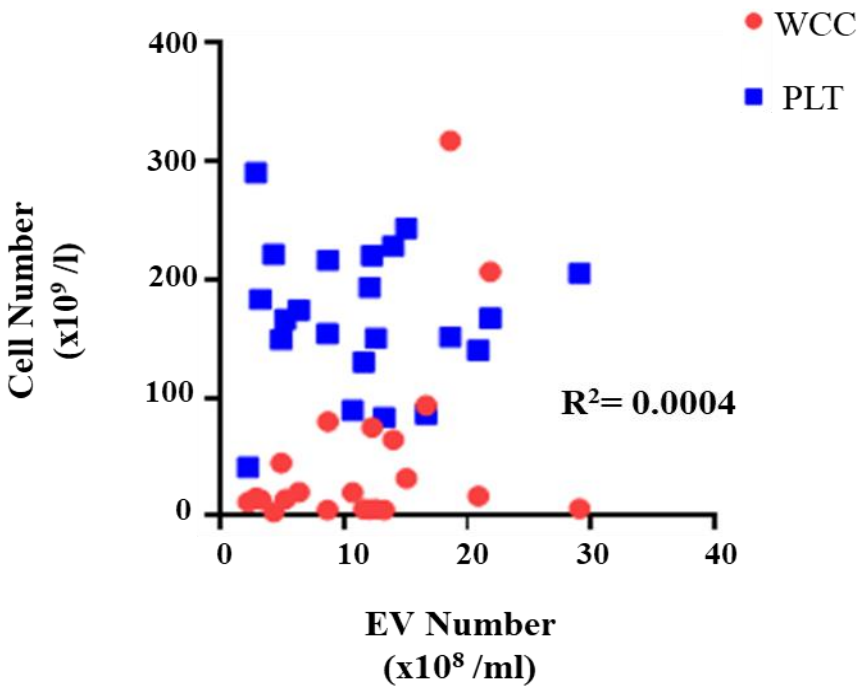

Figure 3.4 Correlation between total white cell count (WCC), platelet count (PLT) and EV numbers,

Demonstrating a lack of association.

3.2.4 Are miR-374b and miR363 detected in CLL Patients plasma

Based on our previous observations Willimott et al. (2012) and Smallwood et al. (2016) that specific miRNAs, including miR-363 and miR-374b, were detectable in EVs produced following stimulation of CLL cells by CD40L/IL4, we hypothesized that these miRNAs could be biomarkers present in the peripheral blood. miRs have stable molecular structure and therefore can be detected in body fluid and tissues (Mitchell, Parkin et al., 2008)

Therefore, assays were established to detect miR-363 and miR- 374b in peripheral blood (plasm). As a control miRNA, which is not believed to be produced by activated CLL cells we used miR-16. Cellular miR-16 has been shown to be prognostic (Calin et al., 2005). The results showed that CLL patients (n=24) had significantly higher levels of miR-363 than healthy volunteers (n=11) (Mann-Whitney U-test; * P=0.03), whereas miR-16 and miR-374b showed no significant differences between the two groups (Figure 3.5). Patient characteristic (Table 2.1) showed that 10 patients had 13q deletion thus could not have miR16. However**,** it is likely that the 13q deleted patients had monoallelic loss and miR-16 was produced normally from the other chromosome. Only if there was biallelic loss of miR-16 would it be expected that this miRNA is undetectable. Our finding confirms the results of others (Moussay, Wang et al., 2011) who showed that levels of miR-16 in CLL patients plasma were 6.45+/- 0.76 (mean +/- SEM) higher than normal subjects.

This finding led to the next investigation; whether miR-363 is present in particle or non-particle fractions in the circulation using size exclusion chromatography to fractionate the plasma based on particle size.

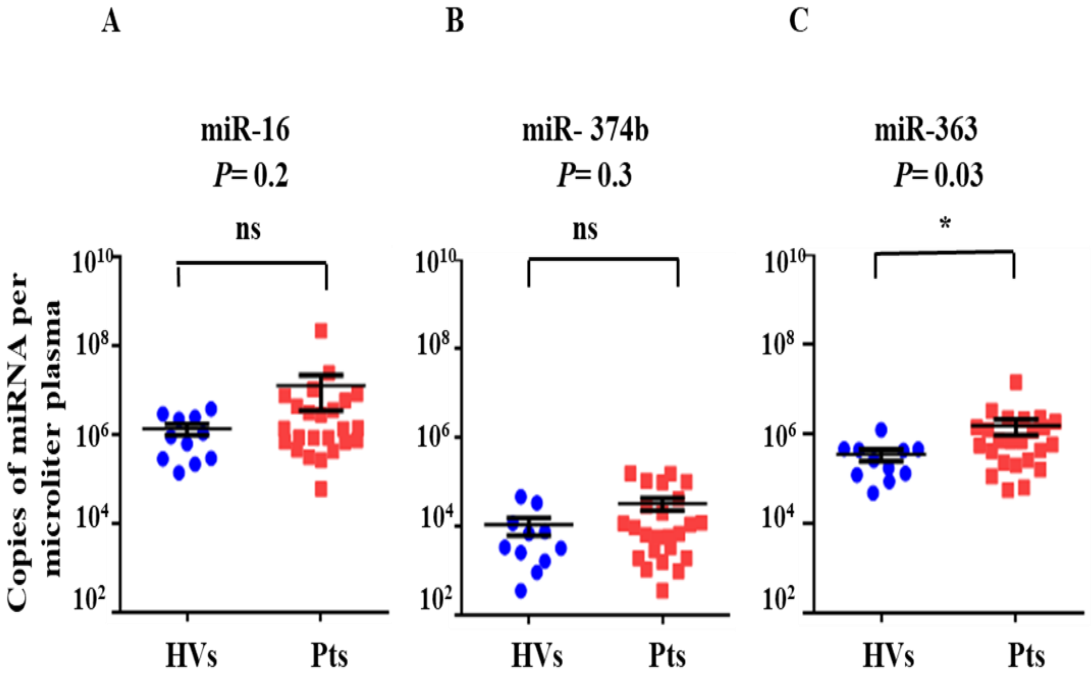

Figure 3.5 Plasma from patients with CLL is enriched for miR363 compared to healthy volunteers.

PCR was used to measure and compare 3 candidates miRNAs (miR-16, miR-374b and miR363) between plasma of patients (Pts) (n = 24) and age-matched healthy volunteers (HVs) (n=11). MiR-363 demonstrates significant increased expression in CLL patients (Mann-Whitney U-test; **P*=0.03), whereas miR-16 and miR-374 showed no significant differences between two groups. Error bars indicate mean +/- SEM.

3.2.5 Size exclusion chromatography confirms that some microRNAs are selectively found in EVs rather than plasma.

MiRNAs were detectable in plasma of CLL patients and healthy volunteers (see previous section 3.2.4) but this analysis did not show whether the microRNAs were in the particle or plasma protein fractions. Therefore, this was investigated by fractionating miRNA-containing particles and plasma protein fractions under gentle conditions with Sephacryl S-500 resin size exclusion chromatography.

Plasma from healthy donors (n=3) (Figure 3.6 panels to the left) and four pooled CLL samples (n = 9 individuals per pool in 4 pools) (Figure 3.7) were applied separately to the column. Based on a protein standard (blue dextran) (see Materials and Methods, pages 46 to 47), the column recovers particles from approximately 1 to 120 nm in diameter, with particles 120 nm and larger eluting near fraction 13, and particles 1 nm or smaller eluting near fraction 20. We determined late-eluting fractions that likely to represent a size range corresponding to protein by the presence of blue colure of dextran blue and the Protein levels of each fraction by absorbance at 280 nm.

Based on our previous work, we chose to examine the abundance of three circulating potential cancer biomarkers (miR-142, miR-363 and let7a). MiR-363 was our test but miR-142 and let7a were chosen as controls. Others Arroyo, Chevillet et al. (2011) have shown that miR 142 and let7a are predominantly particle bound in normal subjects and these miRNA were, therefore, controls for our experiments. MiR-16 is highly expressed in CLL (Calin, Ferracin et al., 2005) and also circulating levels are higher in patients than in normal subjects (Moussay, Wang et al., 2011). The locus of mir-16, together with miR-15, which is at chromosome 13q14, is deleted in about 50% of CLL cases. These miRNA are believed to normally have a role in reducing BCL2 levels and high cellular levels of miR-16 are associated with poor prognostic factors (unmutated immunoglobulin genes and ZAP70 expression) (Calin, Ferracin et al., 2005). However, the association has never been proved in a prospective trial and circulating levels do not appear to associate with clinical outcome (Moussay, Wang et al., 2011). Therefore, in this study of circulating

miRNA we chose to employ miR-16 as a highly expressed control miRNA and also Arroyo, Chevillet et al. (2011) showed that miR-16 predominantly associated with plasma protein fractions in normal subjects.

The copy numbers of miRNAs were quantified in selected plasma fractions by quantitative RT-PCR and normalized to spiked-in synthetic UniSp2 RNA to control for fraction-to-fraction technical differences in miRNA isolation efficiency and experimental variation in reverse transcriptase efficiency.

Using size exclusion chromatography early fractions (1 to 13) are associated with vesicles whereas the later fractions are associated with plasma proteins (see Materials and Methods, pages 46 to 47). This was established using BSA and blue dextran as described in Materials and Methods (page 47).

In healthy volunteers, approximately 95% of the circulating miR-16 and miR-363 separated late, in fractions 17 to 25 that are derived from the plasma protein fractions (Figure 3.6 panels to the left). However, distribution of circulating let-7a and miR-142 differed from this very skewed distribution with amounts of these miRNA being approximately equally distributed between early and late fractions (Figure 3.6 panels to the left).

By contrast, in CLL patients a higher proportion of miR-16 and miR-363 were detected in the early fractions i.e. particle containing, than was observed in normal subjects (Figure 3.7 panels to the right). Circulating let-7a and miR142 was distributed between particle and non-particle fractions similarly in patients and normal subjects (Figure 3.7 panels to the right).

Thus, the findings demonstrate that particle and plasma protein fractions of circulating miRNA can be readily distinguished and that not only do some miRNAs distribute preferentially to either one or the other but that this distribution is affected by CLL for miR-16 and miR-363.

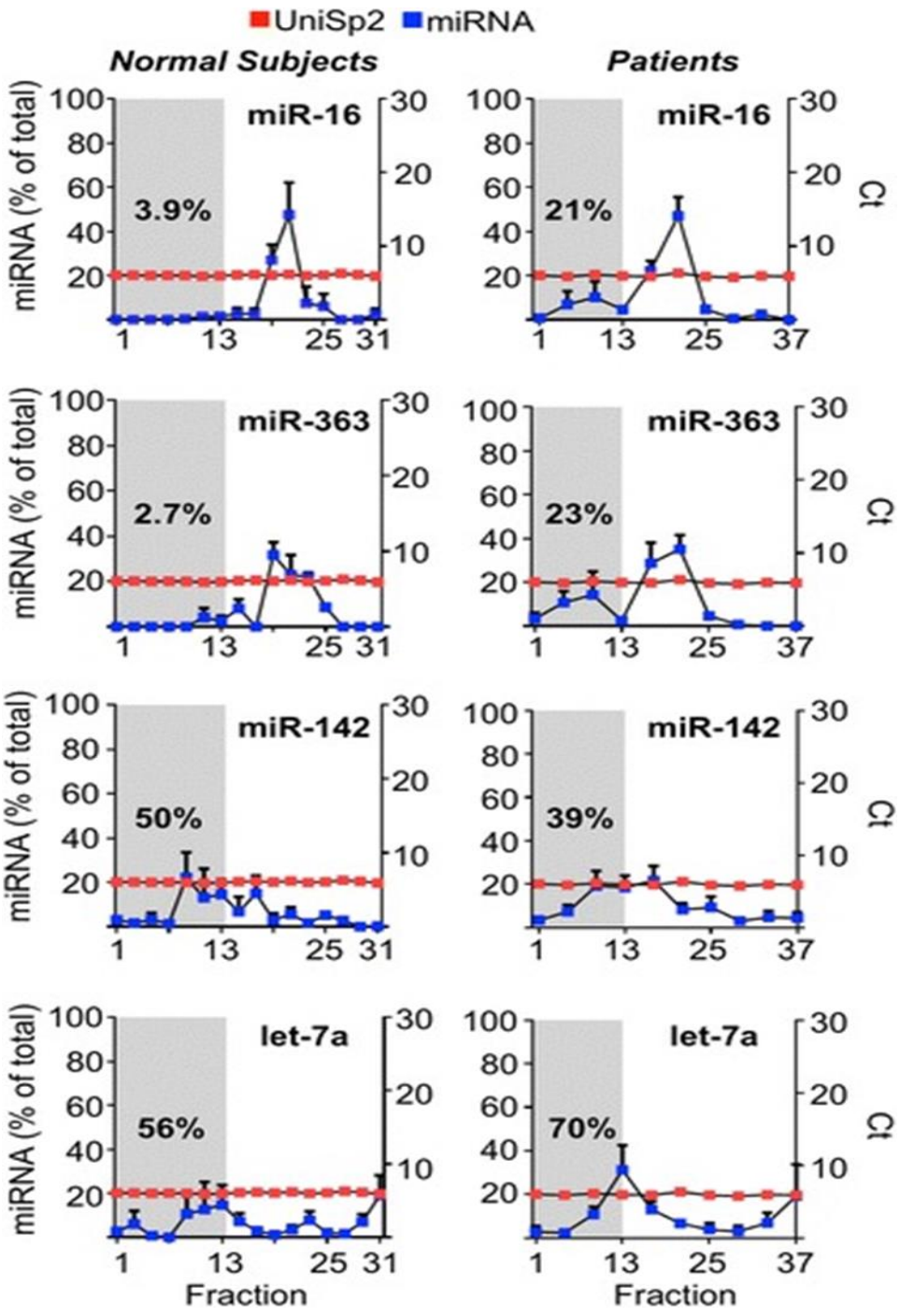

Figure 3.6 Two populations of circulating miRNAs are detected by size exclusion Chromatography.

Distribution of selected miRNA between plasma protein and vesicles bound fractions of plasma. Between 31 and 37 plasma fractions were obtained by size exclusion chromatography from either healthy volunteers (panels to the left) or patients (panels to the right) as indicated on the x-axis.

Fractions 1–13 are designated early vesicle containing fractions (grey shaded area). UniSp2 RNA spike-in was employed as a control and quantitative RT-PCR Ct values obtained are shown (red squares and right-y-axis). For each individual miRNA tested (mir-363, miR-16, miR142 and let-7a) the percentage of the total amount of miRNA in each fraction is plotted (blue squares and left-y-axis) of each individual graph. Percentage of total within fractions 1–13 is presented within the grey shaded area.

3.3 Discussion

Flow cytometry, which is used to detect cells has a resolution of ~10 µm, but extracellular vesicles have diameters of ~100 nm. The earliest characterisation of EVs employed transmission electron microscopy but this is a cumbersome and time-consuming technology. More recently instruments for the detection of EV sized particles have been developed, which include a number of technologies: nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing (Lotvall, Hill et al., 2014). NanoSight (Malvern Instruments), which employs dynamic light scattering, was used in this thesis. The technology takes images from a wide field and also close-shot images of single vesicles and the data is then analysed using the company's software (Sokolova, Ludwig et al., 2011).

3.3.1 Vesicle size distribution

Our data identified two patterns of size distribution profiles in different individuals. One profile, which is called Pattern A, had a single peak of particles with diameters between 30 and 200 nm, the characteristic size range of small extracellular vesicles (Figure 3.1 A). The other (Pattern B) also showed a predominant peak at this size range but there were also several other peaks at greater particle diameters up to ~400 nm. These larger particles might represent that might be attributed to biological compartments such as aggregated proteins or membrane blebs, and were only present in a minority of 10% of CLL patients (10 %) or normal subjects (20 %) (Figure 3.1 B).

Therefore, there do not appear to be major differences in vesicle size distribution between normal subjects and CLL patients.

3.3.2 Numbers of extracellular vesicles in plasma

EVs are released by activated CLL cells (Willimott and Wagner, 2012; Yeh et al., 2015) suggesting that this occurs in the tumour microenvironment (TME). The TME plays an essential role in the biology of CLL and is a target for therapy. In the current study, we made a hypothesis that EV and their miRNA cargo might be a source of clinically useful biomarkers for disease activity. One prediction from this is that patients should have higher numbers of circulating EV, "spilling" over from the TME, than normal subjects. Comparison of numbers of extracellular vesicles between healthy volunteers and patients with CLL demonstrates significantly increased numbers of the plasma EVs in CLL patients compared to normal subjects (Figure 3.2 A). This result is in agreement with other's showing that CLL patients demonstrated significant higher levels of EVs in plasma than normal subjects (Yeh, Ozer et al., 2015).

There is the possibility that high amount of circulating particles in patients with CLL is associated with numbers of leukemic-B cells in the circulation. However our data showed that EV levels were not significantly correlated with absolute lymphocyte or platelet counts. These results are also consistent with previous work (Ghosh, Secreto et al., 2010, Yeh, Ozer et al., 2015) and indicate that EV levels in CLL patients do not rise in parallel with numbers of circulating leukaemic cells.

3.3.3 MiR-363 detected in stimulated CLL cells by CD40L/IL4 is also detectable in plasma from CLL patients and normal subjects

MiRNAs are an important component of the molecular cargo of EVs and recent studies have demonstrated a disease-associated EV miRNA signature in CLL (Farahani, Rubbi et al., 2015, Yeh, Ozer et al., 2015). Functional work is just beginning but several studies have suggested effects of CLL EVs on stromal cells (Ghosh, Secreto et al., 2010, Farahani, Rubbi et al., 2015, Paggetti, Haderk et al., 2015) while our laboratory has shown that CLL derived EVs can modify CD4+ T-cell function (Smallwood, Apollonio et al.,

2016). Therefore, different lines of work converge on the notion that CLL cell-derived EVs are able to regulate and reprogramme the tumour microenvironment.

Moreover, our recent work has shown that specific miRNAs associate with EVs from stimulated primary CLL cells. In particular, there was an enrichment of specific cellular miRNAs including miR-363 within EVs produced from CD40L/IL-4 activated CLL cells compared to control EVs from non-activated CLL cells.

MiRNAs derived from the TME but detectable in the peripheral blood would potentially provide biomarkers correlating with leukaemic cell activity within tissues that could guide decisions on treatment and provide early and relatively non-invasive measures of response. Therefore, based on our previous work, we selected miR-363 and miR-374b. We first determined that miR-363 and miR-374b, which are detectable in activated CLL cells, are also detectable in plasma of CLL patients and healthy volunteers. Therefore, we utilised size exclusion chromatography to demonstrate the presence of miR-363 in the circulation.

3.3.4 Different proportion of miRNAs in plasma and vesicles between patients and healthy volunteers

Others Arroyo, Chevillet et al. (2011) have demonstrated that, in normal subjects, individual miRNA appear to have specific patterns of distribution between particle containing and plasma protein fractions. These authors found that let-7a was predominantly found in the early particle containing fractions whereas the vast majority of miR-16 was in the plasma protein fractions. The results described here (Figures 3.6) are very comparable to those of Arroyo, Chevillet et al. (2011) for miR-16 with most miRNA being present in the late eluting fractions. For let-7a the results show ~50% in early fractions (generally less than found by Arroyo, Chevillet et al. (2011), although one of their normal subjects appears to show a similar distribution) and similarly miR-142, which was found more evenly distributed across fractions than by Arroyo, Chevillet et al. (2011), although this could partly be due to the way in which fractions were collected e.g.differences in fraction volume or machine settings. Overall the control miRNA (miR-16, miR-142 and let-7a) had similar distributions to that previously described for normal

subjects. The distribution of miR-363 was also investigated by Arroyo et al. (their Figure 4A) and was found to be present predominantly in the plasma protein fractions. Results presented here for miR-363 in normal subjects is in agreement with this.

In this thesis the first analysis of miRNA distribution between particle and plasma protein fractions in CLL has been carried out. While the distribution of miR-142 and let-7a appeared to be similar in health and disease a greater proportion of the miR-16 and miR-363, found mainly in late eluting fractions, were present in the particle fractions in CLL patients.

Our results show that there are specific changes to the distribution of two miRNA (miR-16 and miR-363) in CLL patients. While in health these miRNA are found almost exclusively in plasma protein fractions in CLL there are increased amounts in the particle fractions. This is consistent with the hypothesis that a proportion of circulating miR-363 is derived from EVs produced by stimulated CLL cells in the TME.

It is interesting that miR-16 levels are associated with clinical outcome (Calin, Ferracin et al., 2005). For this miRNA higher levels are associated with poor prognostic factors (unmutated immunoglobulin gene status and ZAP70 expression). MiR-363 was found to be enriched in CD40L/IL4 activated CLL (Willimott and Wagner, 2012). It has also been suggested that miR-363 levels are higher in CLL than in either hairy cell leukaemia, another low grade B-cell lymphoproliferative disorder, or normal subjects (Moussay, Wang et al., 2011).

The same authors additionally suggested that miR-363 "showed progressive changes along with the severity of the disease" such that levels increased in association with Rai clinical stage.

In the next chapter this is further tested by carrying out a study on samples stored from CLL patients enrolled in clinical trials.

Chapter 4 Circulating miR-363 levels in normal subjects and patients

4.1 Introduction

A biomarker derived from the TME but present in the peripheral blood would potentially provide a measure of leukaemic activity within tissues that could guide decisions on treatment and provide early and relatively non-invasive measures of response.

We made the hypothesis that miR-363 is a potential biomarker of the microenvironment in chronic lymphocytic leukaemia because extracellular vesicles containing this miRNA appear to be released from activated leukaemic cells. Because leukaemic cells are activated and driven to proliferate within the TME, it is possible that their cargo, might be a source of clinically useful biomarkers for disease activity.

Various studies have demonstrated the clinical potential of circulating miRNAs, For instance, One of the early works measuring miRNA amounts in serum was described by Lawrie, Gal et al. (2008) who revealed that miR-21 was combined with disease-free survival in diffuse large B-cell lymphoma patients; therefore, miR-21 may have usefulness as a diagnostic biomarker for this disease. Another work has shown that the serum amounts of miR-141, could be a diagnostic measure that might identify patients with prostate cancer from control (Mitchell, Parkin et al., 2008).

In order to pursue this hypothesis size exclusion chromatography was utilized to demonstrate that miR-363 was present in both non-particle and particle fractions of plasma (Chapter 3). Our results are consistent with the hypothesis that a proportion of circulating miR-363 is derived from EVs produced by stimulated CLL cells in the TME.

In order to investigate the potential clinical usefulness of these observations I carried out a retrospective study using samples obtained from patients enrolled in two national clinical trials. Both trials are now completed. ARCTIC (Howard, Munir et al., 2017) was a trial of a conventional chemotherapy combination (fludarabine, mitxantrone and dexamethasone) in patients meeting the definitions of requiring treatment by recognised criteria (Hallek, Cheson et al., 2008). CLEAR, by contrast, enrolled asymptomatic patients in a trial to investigate the effects of antibiotics on the short term clinical course. I have investigated associations of miRNA levels in plasma (obtained from the UK

Haematological Malignancies Biobank, University of Liverpool) with survival, prognostic markers and response to treatment.

4.2 Results

4.2.1 Repository study of miR-363 expression in patients from the ARCTIC and CLEAR clinical trials

In order to investigate the potential of miR-363 as a prognostic biomarker in CLL, plasma samples were obtained from patients enrolled in two clinical trials, ARCTIC and CLEAR. Fully quantitative RT-PCR assays were established (as described in Materials and Methods, page 53 to 57), and the number of copies or miR-363 was calculated from the raw Ct values.

The expression miR-363 was significantly increased in ARCTIC compared to CLEAR patients (Mann-Whitney, P=0.0091) (Figure 4.1). Data of healthy volunteers are the same presented in chapter 3 (Figure 3.5, page 76). MiR-363 levels were also significantly higher in ARCTIC patients than in healthy volunteers (Mann-Whitney, P =0.031). However, miR-363 levels were not significantly different between CLEAR patients and health volunteers.

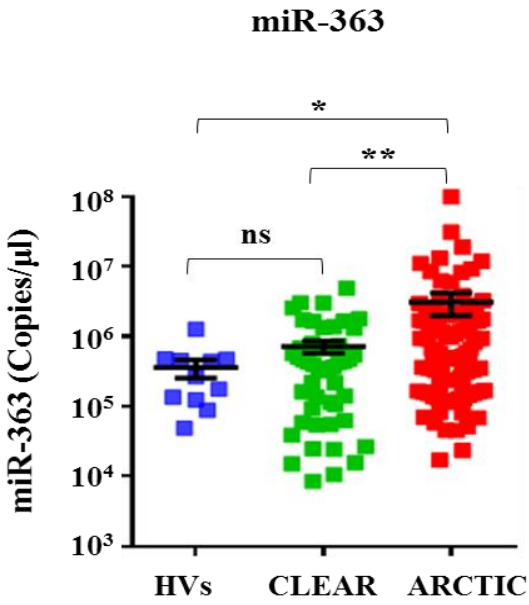

Figure 4.1 MiR-363 levels in plasma of ARCTIC and CLEAR patients and healthy volunteers.

MiR-363 showed significant differences (Mann-Whitney test, P value= 0.0091) in expression between ARCTIC patients and CLEAR patients. Data of healthy volunteers are the same presented in chapter 3 (figure 3.5, p 77) and is significantly different from those in ARCTIC patients (Mann-Whitney U-test, P value= 0.031). Error bars mean+/-SEM.

4.2.2 Replicate measurements of miR-363 in 8 ARCTIC patients

There are some possible sources of error in carrying out multiple PCR assays such as time difference, freezing time and pipetting errors. Therefore, in order to investigate the reliability of miR-363 results, levels of miR-363 were measured twice in 8 ARCTIC patients with high and low miR-363 expression but otherwise chosen at random. In patients 5 to 8 Assay 1 results are consistently below Assay 2. However, this was not the case for the patients 1 to 4. In order to determine if the differences (comparing assay 1 and 2) are important. A Wilcoxon-paired t-test was carried out because the sample data might be not normally distributed, and they cannot be transformed to a Normal distribution by means of a logarithmic transformation, and no significant differences were observed. Overall, therefore, there is no evidence of systematic errors in these experiments (Figure 4.2).

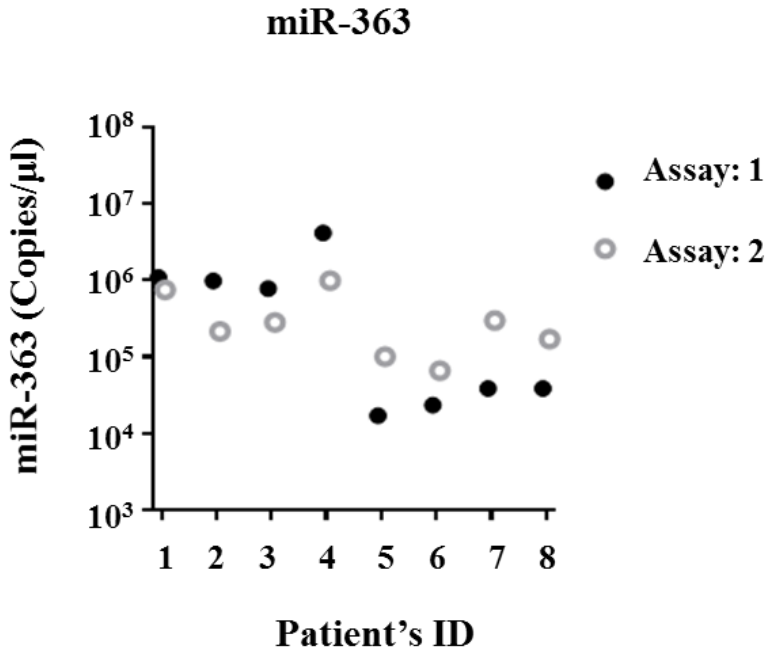

Figure 4.2 A mount of miR-363 measured twice in 8 ARCTIC patients.

The data demonstrated no significant differences between two measurements, (Wilcoxon- paired t-test).

4.2.3 **MiR-363 and miR-16 in ARCTIC CLL patients**

Calin, Ferracin et al. (2005) showed miR16 within CLL cells to be prognostic. Levels of plasma miR-16 in CLL patients are higher than those of healthy volunteers and also higher than in other lymphoproliferative disorders (Moussay, Wang et al., 2011). The overall amounts of miR-363 in comparison to miR-16 were determined. The data showed that the mean level of miR-16 was higher in ARCTIC patients than in healthy volunteers, although this did not reach statistical significance (Figure 4.3 B) and that the absolute levels of miR-363 was not significantly higher than that of miR-16 for a group of patients (n=30) (Mann-Whitney U-test , P=0.7) (Figure 4.3 A).

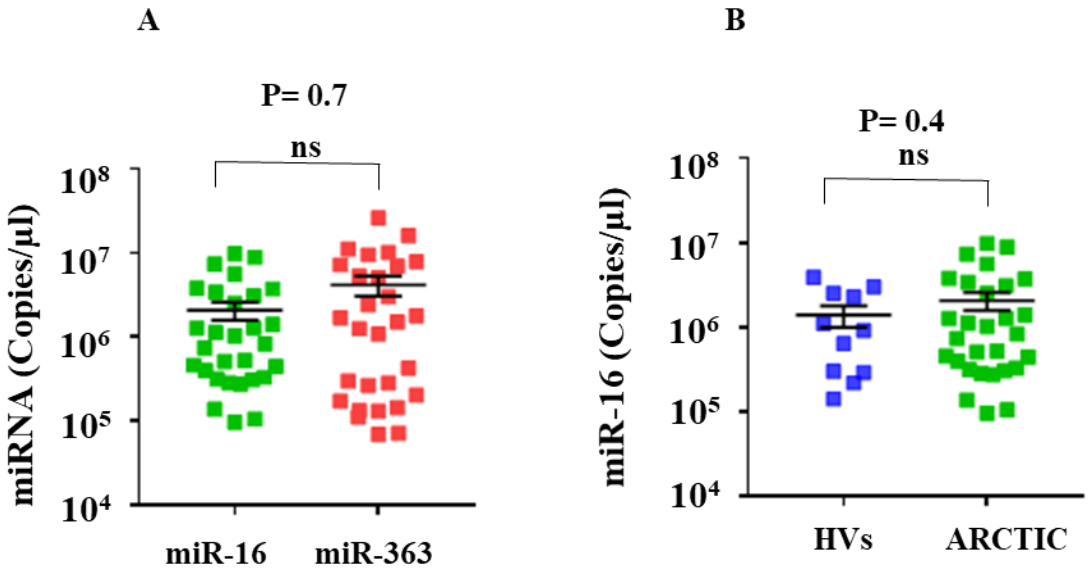

Figure 4.3 Comparison of miR-16 and miR-363 levels.

(A) MiR-363 revealed no significant increase compared to miR-16 in ARCTIC patients. (Mann-Whitney U-test , P=0.7) (B) Comparison of miR-16 from ARCTIC patients and healthy volunteers showed no significant differences. Error bars (Mean+/- SEM).

4.2.4 Association between miR-363 and miR-16

In order to investigate the correlation between miR-16 and miR-363 levels in plasma a linear regression was carried out. The result demonstrated no association between miR-16 and miR-363 (R^2= 0.18, P= 0.18) (Figure 4.4). When displayed using a linear scale (Figure 4.4 A) some data points are crowded into the bottom left corner of the graph. In order to show the points more clearly the data was displayed using a log10 scale (Figure 4.4 B). The regression line is curved for this reason and there was no correlation as the data is the same in both parts of the figure.

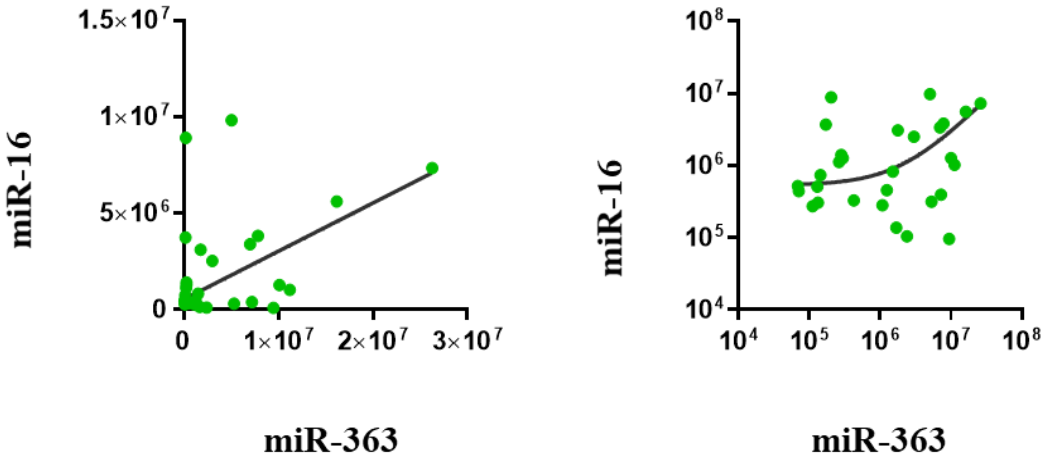

Figure 4.4 Linear regression analysis.

(A) Employing a linear scale and (B) Employing a log scale. Demonstrating no association between miR-16 and miR-363 levels. R^2= = 0.18, P= 0.18.

4.2.5 **Clinical correlation**

ARCTIC patients have good clinical annotations. In order to investigate the relationship between miR-16 and miR-363 and other prognostic markers in ARCTIC patients, Kaplan-Meier survival graphs were produced. Patients were ranked based on miR-363 expression from low to high into two equal groups, one with low miR-363 Expression and the other with high miR-363 Expression.

The data demonstrated identical survival with 75% overall survival by 60 months in the two groups with low and high expression of miR-363 (Figure 4.5). Similarly, analysis of progression free survival (PFS) was performed and was found to be not significantly different between the two groups, with 60% PFS by 60 months in the both cases (Figure 4.6).

Moreover, relationship between miR-363 gene mutational status were investigated, the result demonstrated no significant difference between miR-363 levels in un mutated and mutated groups (Figure 4.7).

Analysis of ARCTIC data showed there was no significant difference in miR-363 levels in patients with either 17p deletion (n = 4) or 11q23 (n = 14) deletion as compared to those with a normal karyotype (Figure 4.8). Similarly neither gender nor Binet clinical stage were associated with significant differences in amounts of miR-363 (Figure 4.9). We compared outcomes for patients with miR-363 levels greater than the median for the group with those whose miR-363 level was less than that of the median (Figure 4.5 and 4.6). There was no significant difference between these groups in either overall or progression free survival.

Kaplan-Meier survival curves were also produced for miR-16, and there was no evidence of a statistically significant difference between groups. 74% and 45% overall survival in 60 and 50 months for patients with low and high miR-16 levels respectively (Figure 4.11). The progression free survival probabilities for the two groups were also not significantly different. PFS was 45% and 60% in 60 and 50 months for patients with low and high expression of miR-16 respectively (Figure 4.11).

In addition, unmutated and mutated gene status showed no significant difference between both groups (Figure 4.12).

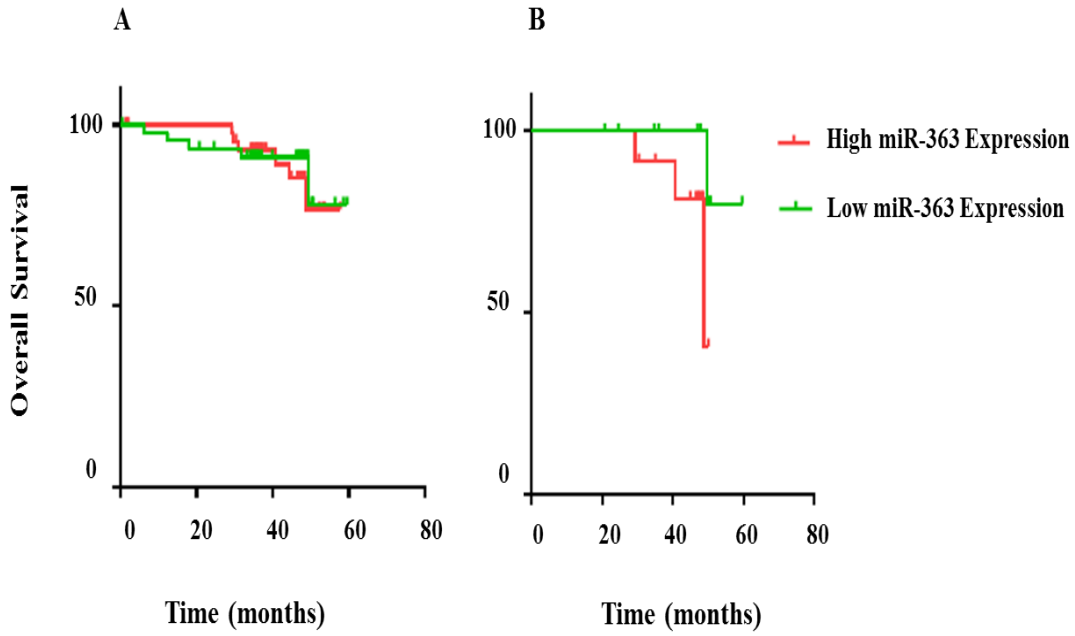

Figure 4.5 Kaplan Meier survival curves showing effects of plasma miR-363 levels on overall survival.

(A) The figure shows the overall survival (time to death) in two groups of patients from ARCTIC one with low miR-363 expression (below median, green line) and other with high expression (above median, red line). Notice that the survival curves do not show much separation, and there was no significant difference (Log-Rank (Mantel-Cox) test). B) The figure shows the overall survival (time to death) in two groups of patients from ARCTIC. The lowest patient quartile (green line) is compared to the highest patient quartile (red line). There was no-significant difference (Log-Rank (Mantel-Cox) test).

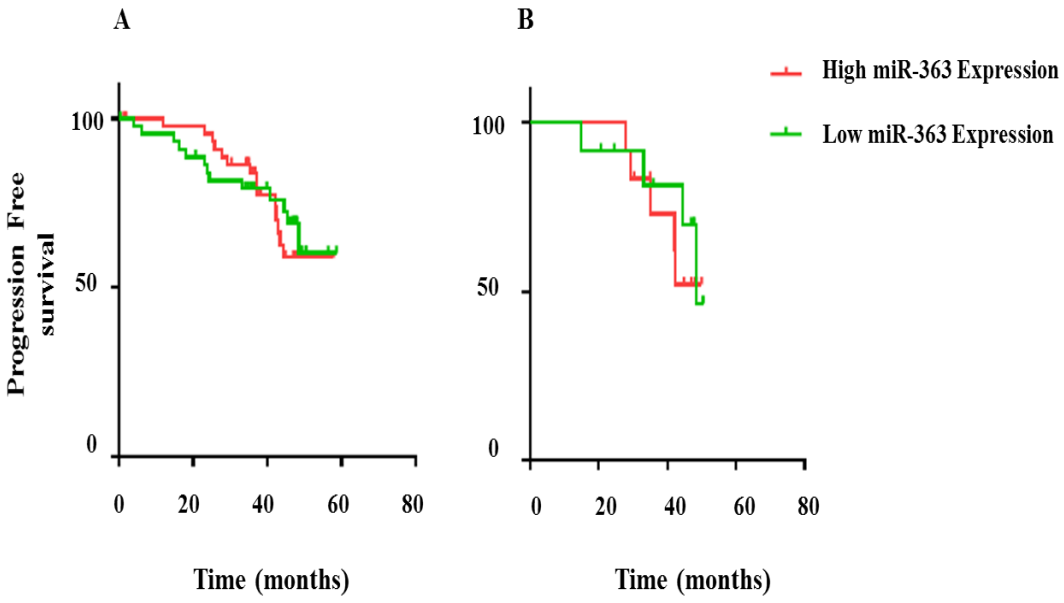

Figure 4.6 Kaplan Meier survival curves showing effects of plasma miR-363 levels on progression free survival.

Curves demonstrate the progression free survival (time to progress) in two ARCTIC patient's groups one with high miR-363 expression (red line) and other with low miR-363 expression (green line). Notice that the progression free survival curves do not show much separation, and there was non-significant difference (Log-Rank (Mantel-Cox) test).

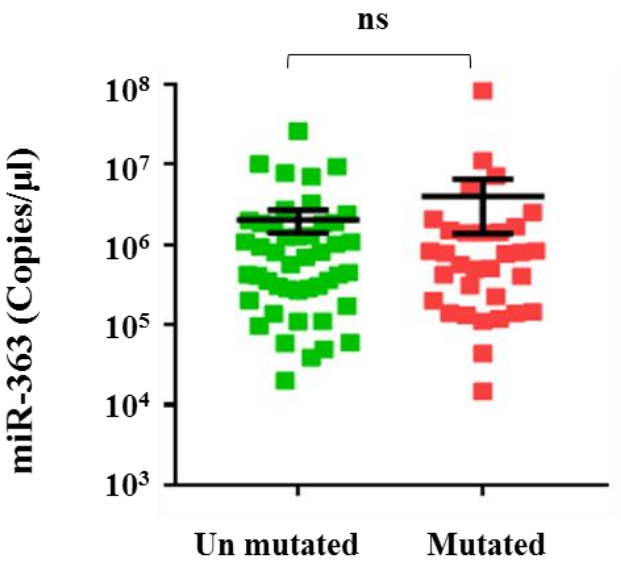

Figure 4.7 Effect of mutational status on miR-363 levels.

Comparison of miR-363 in 2 ARCTIC patient's groups, one showed un mutated gene and the other with mutated gene. The data demonstrated no significant differences between the two groups. (Mann-Whitney U- test). Error bars (Mean+/- SEM).

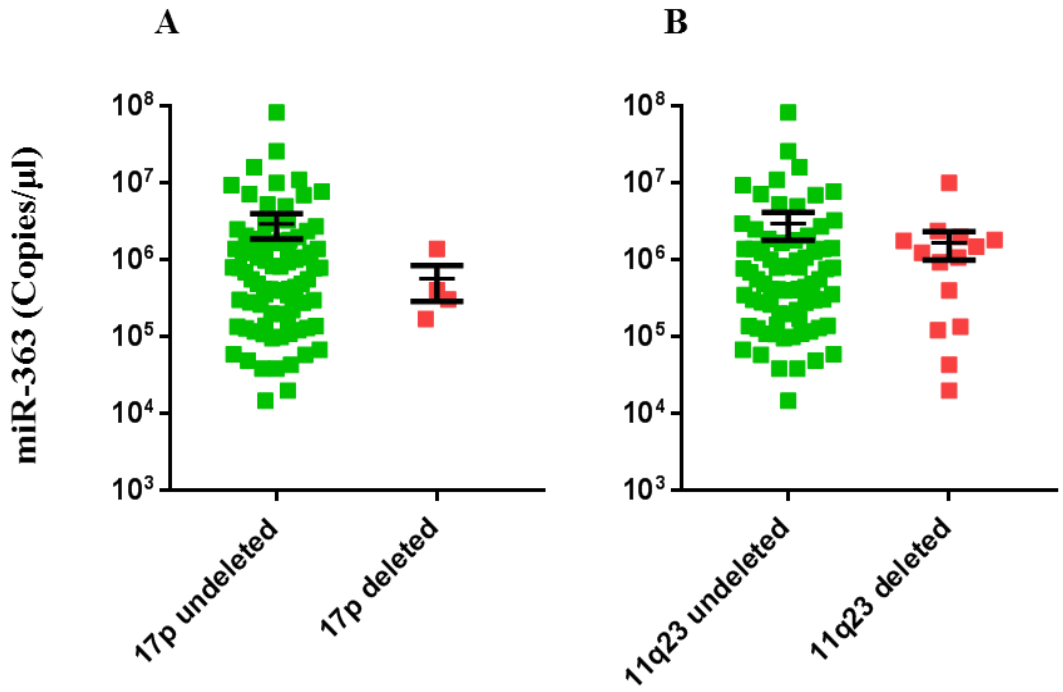

Figure 4.8 MiR-363 and cytogenetic aberrations.

Analysis of ARCTIC data showed there was no significant difference in miR-363 levels in patients with either (A) 17p deletion (n = 4) or (B) 11q23 (n = 14) deletion as compared to those with a normal karyotype. Error bars (Mean+/- SEM).

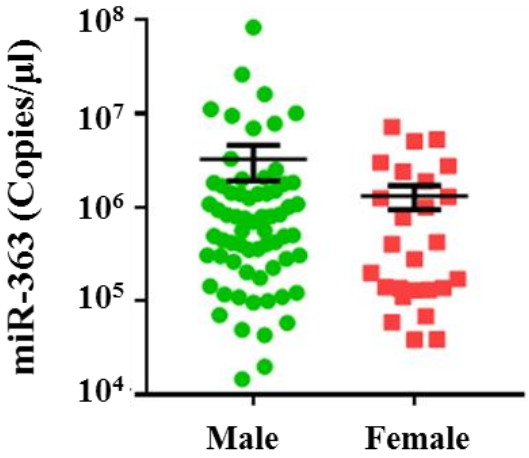

Figure 4.9 MiR-363 levels of ARCTIC patients are compared by gender.

Median and interquartile ranges are indicated. There was no significant difference (Mann–Whitney U-test) between groups. Error bars (Mean+/- SEM).

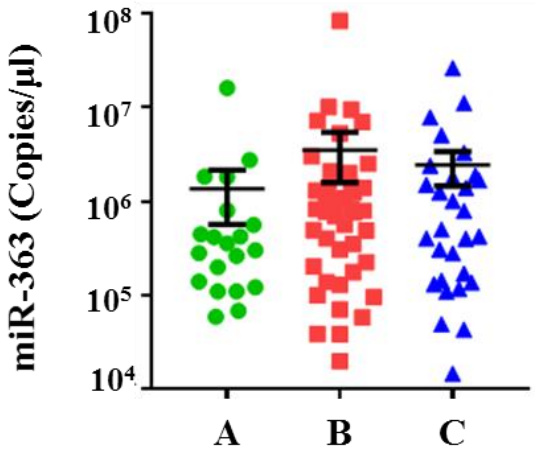

Figure 4.10 MiR-363 levels of ARCTIC patients are compared by Binet clinical stage.

Binet A indicates progressive stage A disease. Median and interquartile ranges are indicated. There was no significant difference (Mann–Whitney U-test) between groups Error bars (Mean+/-SEM).

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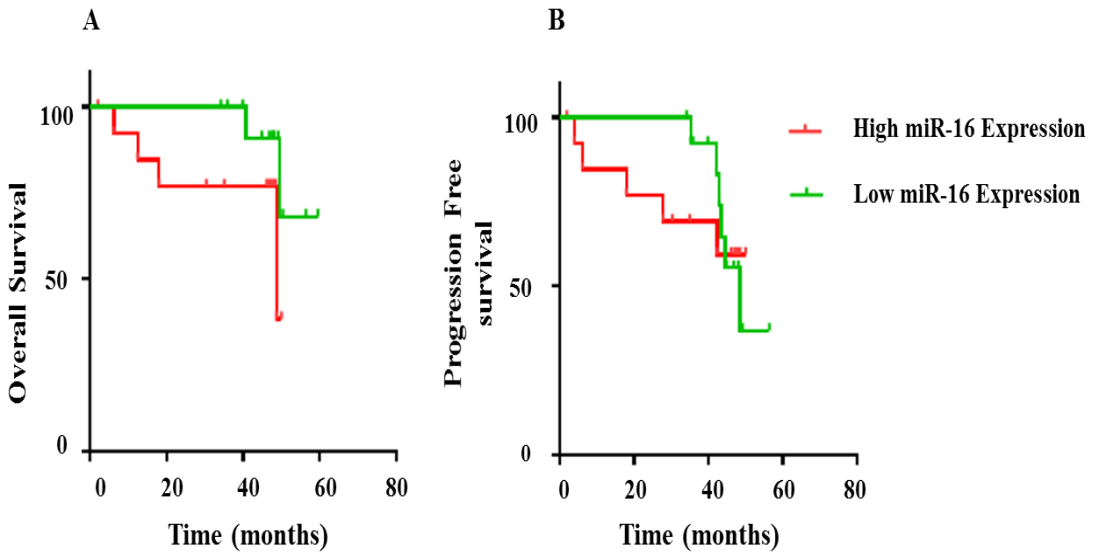

Figure 4.11 Kaplan–Meier survival curves of ARCTIC patients.

Patients were grouped into those with miR-16 levels above the median (red line) and those with levels below the median (green line). There was no significant difference (Log-Rank (Mantel-Cox) test) in (A) overall survival or (B) progression free survival.

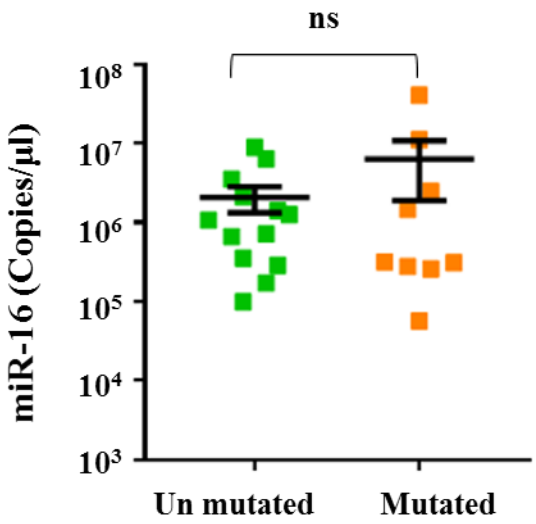

Figure 4.12 Effect of mutational status on miR-16 levels.

Comparison of miR-16 in 2 ARCTIC patient's groups, one showed unmutated gene and the other with mutated gene. The data demonstrated no significant differences between the two groups. (Mann-Whitney test). Error bars (Mean+/- SEM).

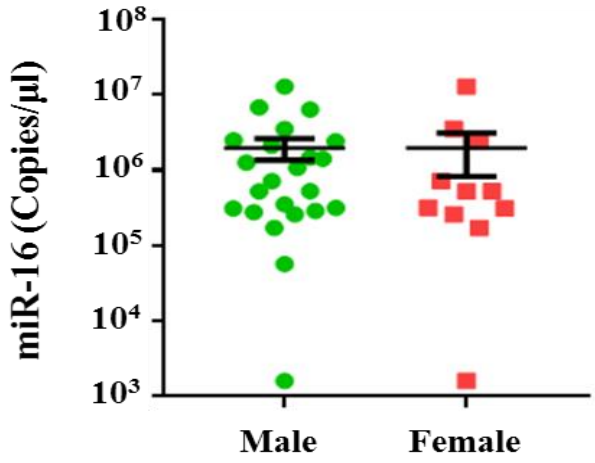

Figure 4.13 MiR-16 levels of ARCTIC patients are compared by gender.

Median and interquartile ranges are indicated. There was no significant difference (Mann–Whitney U-test) between groups. Error bars (Mean+/- SEM).

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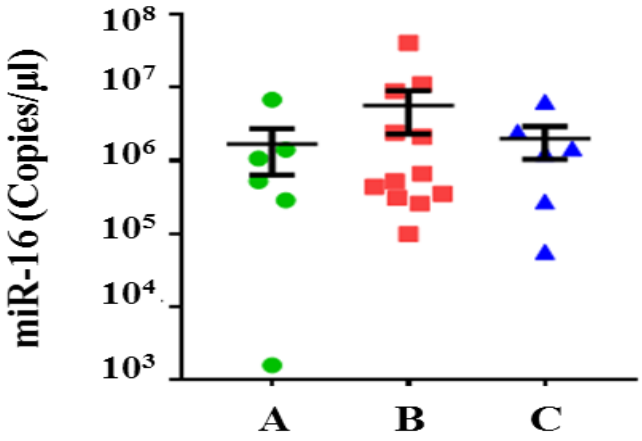

Figure 4.14 MiR-16 levels of ARCTIC patients are compared by Binet clinical stage.

Binet A indicates progressive stage A disease. Median and interquartile ranges are indicated. There was no significant difference (Mann–Whitney U-test) between groups. Error bars (Mean+/-SEM).

4.3 Discussion

4.3.1 Repository study: ARCTIC and CLEAR

In the present study, the prognostic potential of miRNAs in CLL patients was investigated. Levels of miR-363 and miR-16 in plasma was measured in two groups of patients: one was symptomatic and required treatment (ARCTIC) and the other was asymptomatic (CLEAR).

The positive finding from my study is that levels of miR-363 differ between CLEAR and ARCTIC patients. The laboratory has previously reported on the miRNA signature of CLL-EVs derived from CLL cells activated with CD40L and IL-4. There were significant changes in cellular miRNA between stimulated and unstimulated cells (Willimott and Wagner, 2012) and also enrichment of specific cellular miRNAs including miR-363, within EVs as compared with cells. These results suggest that CD40/IL-4 stimulation mediates packaging of selective miRNA within CLL-EVs, which might be distinct from the effects of BCR activation. The finding that levels of miR-363 are present in plasma of ARCTIC group at levels significantly different from CLEAR patients could suggest either that leukaemic lymphocytes release more EV than normal lymphocytes in response to stimulation in the tumour microenvironment, or simply that there are more lymphocytes and, therefore, correspondingly more EVs.

Either of these scenarios could further suggest that miR-363 is a potential biomarker for leukaemic cell activity in the microenvironment. A biomarker should be present in proportion to the tumour burden to be clinically useful and correlate with known measures of outcome such as clinical staging systems or chromosomal aberrations. We were able to obtain the clinical characteristics of ARCTIC patients and investigated these correlations.

4.3.2 **Clinical correlation**

It is sometimes clinically difficult to determine when a patient requires treatment and secondly, it may be advantageous to be able to predict when a patient needs treatment. At present, the decision is made on the basis of simple laboratory data (a rapidly rising white cell count, anaemia or thrombocytopenia), clinical data (massive lymphadenopathy or splenomegaly) or systemic symptoms (weight loss, fevers or night sweats). A biomarker would help with this decision and especially a biomarker derived from the tumour microenvironment. In order to determine whether miR-363 has potential as a biomarker, we employed clinical data collected during the course of the ARCTIC clinical trial.

Calin, Ferracin et al. (2005) demonstrated that cellular miR-16 was prognostic in CLL but plasma miRNA was not measured in this study. Moussay, Wang et al. (2011) suggest that plasma miRNA can be prognostic (including miRNA-363) but very few cases were investigated by these authors (see below).

Two miRNAs, miR-15a and miR-16-1, are located at chromosome band 13q14 and are down-regulated in the majority of patients with CLL (Calin, Ferracin et al., 2005). These genes induce apoptosis through the negative regulation of the anti-apoptotic gene BCL2 (Cimmino, Calin et al., 2005). As such, down-regulation of miR-15a/16-1 has been associated with the pathogenesis of CLL, although this remains controversial (Fulci, Chiaretti et al., 2007). Moreover, the anti-apoptotic BCL2 gene is reported to be overexpressed in 65 to 70 percent of B-cell CLLs (Korz, Pscherer et al., 2002), whereas deletions or down-regulations of miR-16-1 were reported in the same proportion of CLL samples (Migliazza, Bosch et al., 2001). It has shown that BCL2 is a target of microRNAs miR-15 and miR-16 and that down-regulation of BCL2 protein by these microRNAs triggers apoptosis (Cimmino, Calin et al., 2005).

More recently, several groups seem to have arrived at consensus that the actual percentage of cells lacking the 13q matters more than whether there is only one copy or two. Different groups come up with different number thresholds, but that is because they need to make a categorical separation of data that is by nature a continuous variable. It is probably safe to say that the more cells with 13q deletions the worse it is.

There are a number of things however, that FISH simply does not tells us. In some cases, we know whether the missing data is important, in some cases, we have yet to figure it out. FISH does not distinguish between the minimal deleted region (MDR), commonly deleted region (CDR) which contain Dleu7, or the less frequent larger 13q deletions that contain the RB protein (type II deletion). Unfortunately, FISH only tells us if there is a deletion or not - it does not tell us the size or what genes are included. The majority of CLL patients have monoallelic loss of 13q with preservation of the other allele from which miR-16 is produced. Others have demonstrated (Moussay, Wang et al., 2011) that levels of miR-16 in CLL patients plasma were 6.45+/- 0.76 (mean +/- SEM) higher than normal subjects. Because this miRNA was easily detectable and highly expressed in plasma it was used as a comparator for the expression of our test miRNAs, miR-363 and miR-374b.

Here a comparison of plasma levels of miR-16 and miR-363 was first carried out followed by an analysis of miR-363 and miR-16 survival.

Data presented in this thesis showed increased miR-16 and miR-363 levels in plasma, of patients. Of these two miRNA levels of, miR-363 levels were higher compared with miR-16 levels, but the result was not significant.

To assess the potential of using miR-16 and miR-363 as CLL biomarkers, the levels of these circulating miRNAs were compared by immunoglobulin gene mutational status. The data also did not display significant differences in miRNA levels between patients with unmated and mutated immunoglobulin genes.

Moussay, Wang et al. (2011) reported that a number of miRNAs illustrated progressive alterations in line with the severity of the disease. For instance, miR-30 levels in plasma decline as disease progresses, whereas they found that miR-363 level continues to rise as disease progresses, although they only measured levels in 6 patients. Investigating just under 100 patients in this thesis showed that while miR-363 levels are higher in patients with advanced disease as compared to asymptomatic patients, there is no association between higher levels and prognostic markers or clinical outcome.

Why do miR-363 levels not associate with prognostic markers?

This could be due to the mechanism of release of vesicles by leukaemic cells, which might reach a maximum or due to the release of vesicles with disturbing factors such as the ease or difficulty of reaching the plasma from the tissues, which might differ from individual to individual and again disturb an association between plasma level of miR-363 and clinical outcomes.

Chapter 5 Exploring the functional effects of miR-363 on leukaemic B-cells

5.1 Introduction

MiR-363 could actually be acting as a communication signal between CLL B cells and other cell types including T cells leading to increase the survival and resistant to treatments. In fact our laboratory has provided evidence that extracellular vesicles from CLL cells can be transferred to autologous T-cells and that the T-cell surface protein, CD69, is a target of miR-363 (Smallwood, Apollonio et al., 2016). However, the role of extracellular vesicles produced by CLL cells on the leukaemic cells themselves has not been investigated.

An interesting pathological characteristic associated with the CLL disease is the increase residency of leukaemic cells in tissues. To explore the effect of miR363 in this important feature of CLL pathology, we investigated the hypothesis that miR-363 controls CD69 and subsequently controls S1P1R1, therefore, CD40L, IL4 and increasing CD69 levels could be responsible for reduced S1PR1 on the cell surface. Overall this would be expected to increase residency of leukaemic cells in tissues.

To do so, miR-363 was knockdown on primary stimulated CLL cells by CD40L and IL4, and then CD 69 and S1P1R1 were constructed.

CD69 is not detected on resting circulating lymphocytes in humans, although *in vitro* cell activation showed increased induction on human T and B lymphocytes CD69 is commonly used as a marker of activated lymphocytes (Testi, D'Ambrosio et al., 1994).Recently, it has also been reported to be important in normal immune responses and lymphocyte migration, (Radulovic and Niess, 2015). In CLL, CD69 has been described to be an independent prognostic marker that significantly correlates with poor clinical and biological prognostic factors such as the number of treatment lines, the mutational status of the IGHV genes, and the expression of CD38, ZAP-70 and CD49d (D'Arena, Musto et al., 2001).

In human B cells activation by BCR, CD40, or TLR9 stimulation inhibited S1P-dependent migration, downregulated S1P1 transcription, and upregulated the expression of CD69. CD69 is a direct inhibitor of S1P1 function (Shiow, Rosen et al., 2006) by increasing internalisation of S1PR1. However, unstimulated CD69 primary B cells were

still able to migrate, although with lower efficiency, indicating that CD69 expression alone is not sufficient to block S1P1-induced migration.

In this chapter, I used confocal microscopy to determine whether CLL cells could take up EVs. The effects of CLL cell activation on miR-363 and miR-16 in CLL cells and EVs were then investigated. Next, the effects of miR-363 knockdown on viability, apoptosis and CD69 surface expression were explored. Finally, the effect of activation and miR-363 knockdown on *CD69* and *S1PR1* mRNA expression was demonstrated.

5.2 Results

5.2.1 Expression of miR-363 and miR-16 in cells and EVs and effects of activation

Our lab previously showed enrichment of miR-363 in EVs from CD40L/IL4 stimulated cells. In order to investigate the effects of B-cells receptor cross-linking, which has not been previously investigated, anti-IgM at two concentrations (10μg/ml and 20μg/ml) was employed. As comparison for miR-363, miR-16, a highly endogenously expressed miRNA was used.

Cells showed mostly significantly decreased levels of both miR-16 and miR-363 following CD40/IL-4, anti-IgM 10 μg/ml or 20 μg/ml stimulation (Mann-Whitney, $P<0.0001$) (Figure 5.1 A).

In contrast, expression of miR-363 in EVs following B-cell receptor activation by anti-IgM induced an increase in levels as compared to EVs from non-activated cells (Figure 5.1 B). MiR-363 levels in EVs following activation with anti-IgM 10 μg/ml was not significantly different from EVs from non- activated cells (Mann-Whitney, $P=0.2453$). However, there appeared to be a dose response effect with anti-IgM at 20 μg/ml causing a greater effect than anti-IgM at 10 μg/ml expression (Mann-Whitney, $P<0.0001$). EVs from CLL cells activated with CD40/IL4 showed significantly decreased levels of miR-363 compared to the expression in EVs from non-activated CLL cells (Mann-Whitney, $P<0.0001$) (Figure 5.1 B). Surprisingly CD40L activation and BCR cross-linking appear to have opposite effects on levels of miR-363.

MiR-16 expression in EVs from CLL cells activated with CD40/IL-4 or anti-IgM (10 μg/ml and 20 μg/ml) showed significantly decreased expression (Figure 5.1 B).

Next, the data on miRNA expression is presented differently in Figures 5.1 C and D MiRNA content of cells and EVs is normalized (green bars). And if there were no enrichment or depletion it would be expected that for each experimental condition there would be relatively the same amounts in cells and EVs. However, while this is

approximately true for miR-16 (Figure 5.1 C) there is evidence for enrichment of miR-363 in EVs following IgM cross-linking (Figure 5.1 D).

Overall, it seems that although activation represses levels of miR-16 and miR-363 in cells (Figure 5.1 A) there are different effects on levels in EVs. MiR-16 is repressed by all modes of activation but miR-363 is repressed by CD40L but appears to be induced by anti-IgM (Figure 5.1 B). On comparing levels in cells and EVs (Figure 5.1 C and 5.1 D) we made the assumption that if activation caused no change to levels then the fold-change observed would also not alter. This was the case for miR-16 following anti-IgM cross-linking (Figure 5.1 C) but miR-363 showed consistent enrichment.

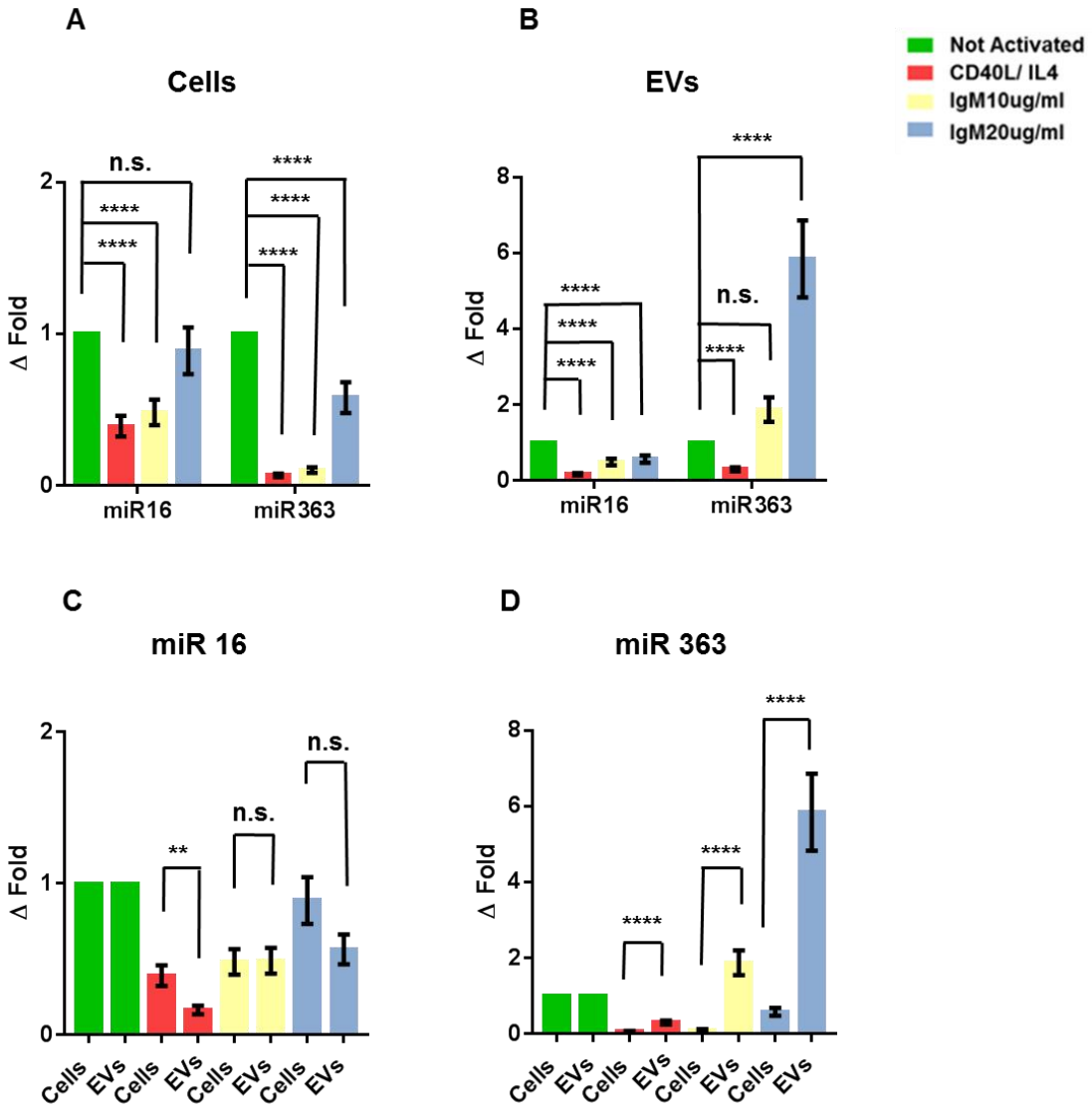

Figure 5.1 EVs from stimulated CLL cells are enriched in miR-363.

SYBER GREEN Real Time PCR **(**Applied Biosystems, #4398965) were carried out to detect. (A) miR-363 and miR-16 levels in cells from patients. Cells were either not activated (green bars), activated with CD40L/IL4 (red bar) or Anti-IgM at 10ug/ml (yellow bar) or 20 ug/ml (blue bar). n=3. Treated cells showed significant differences in miR-363 and miR-16 levels (Mann-Whitney tests). (B) miR-16 showed decreased expression in stimulated CLL- EVs compared to paired parental cells and control CLL-EVs (Mann Whitney test, P =0.1). (C and D) Comparing miR-16 and miR-363 levels in cells and EVs. The fold-change was not significant for miR-16 following anti-IgM cross-linking (C), but miR-363 showed consistent enrichment (D). Error bars (Mean +/- SEM).

5.2.2 **Do Leukemic B- Cells take up EVs?**

CLL cells must be able to take up EVs In order to have effects on cellular function. Therefore, to investigate whether leukemic B- cells take up the EVs, the particles were labelled with PKH67 dye and incubated for 24 hours with two different culture conditions of CLL cells, activated with CD40L/IL4 and not activated,. Then images were acquired by confocal microscopy. Images were obtained at time =0 just after EVs were added to the cells and at 24 hours. The time= 0 control suggested that there was little non-specific binding of EVs to cells. At time=24 hours there was evidence of EV uptake (intracellular green fluorescent material). There also appears to be binding to the surface membrane (green fluorescent rings) for some cells, which might represent non-specific binding although the explanation is not clear. Therefore, the results at indicated that both activated and non-activated leukemic B-cells took up the EVs (Figure 5.2).

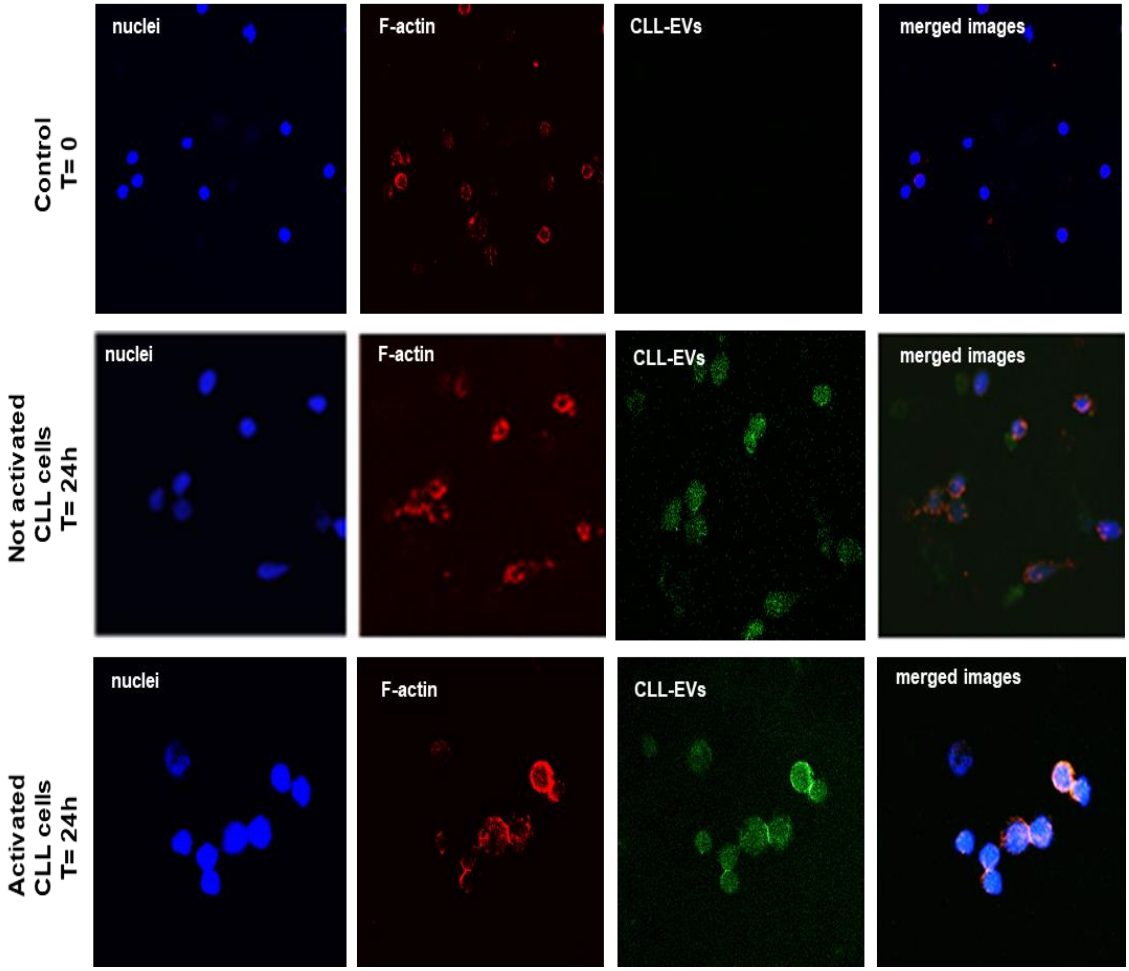

Figure 5.2 CLL- cells take up EVs. EVs from CD40/IL-4–stimulated CLL cells enter B cells.

B cells from CLL patients were incubated with fluorescently PKH67-labeled CD40L/IL-4 CLL-EVs for the indicated times (T=0 and T=24) before washing and fixation. EV transfer studies were followed by confocal imaging. The confocal images show uptake of PKH67-labeled CLL-EVs into unstimulated and stimulated (CD40/IL-4) B cells at 24 hours. F-actin stained with rhodamine phalloidin (red), PKH67-labeled CD40L/IL-4 CLL-EVs (green), and B-cell nuclei DAPI (blue). Single colour images are presented with a merged image (right side of image panels). Technical assistance was obtained from Dr. David Read, MRC Toxicology Unit.

5.2.3 Effects of miR-363 on viability and apoptosis

In order to determine the effects of miR-363 on viability of CLL cells, an ATP luminescence assay (CellTiter Glo, Promega) was used to determine the number of viable cells in culture following miR-363 knockdown. Knockdown efficiencies were good (85%) as described in Materials and Methods (Page 58). Neither activation nor miR-363 knockdown altered cell viability (Figure 5.3 A). Activation made only a very minor difference to ATP luminescence values and this is likely to be because of the short (24 hours) period in culture.

With regards to apoptosis, measured as annexin V binding, the results revealed that miR-363 knockdown caused a small decrease in apoptosis in non-activated CLL cells, compared to activate CLL cells, However, the differences were not significant (Mann Whitney, P= 0.6041) (Figure 5.3 B).

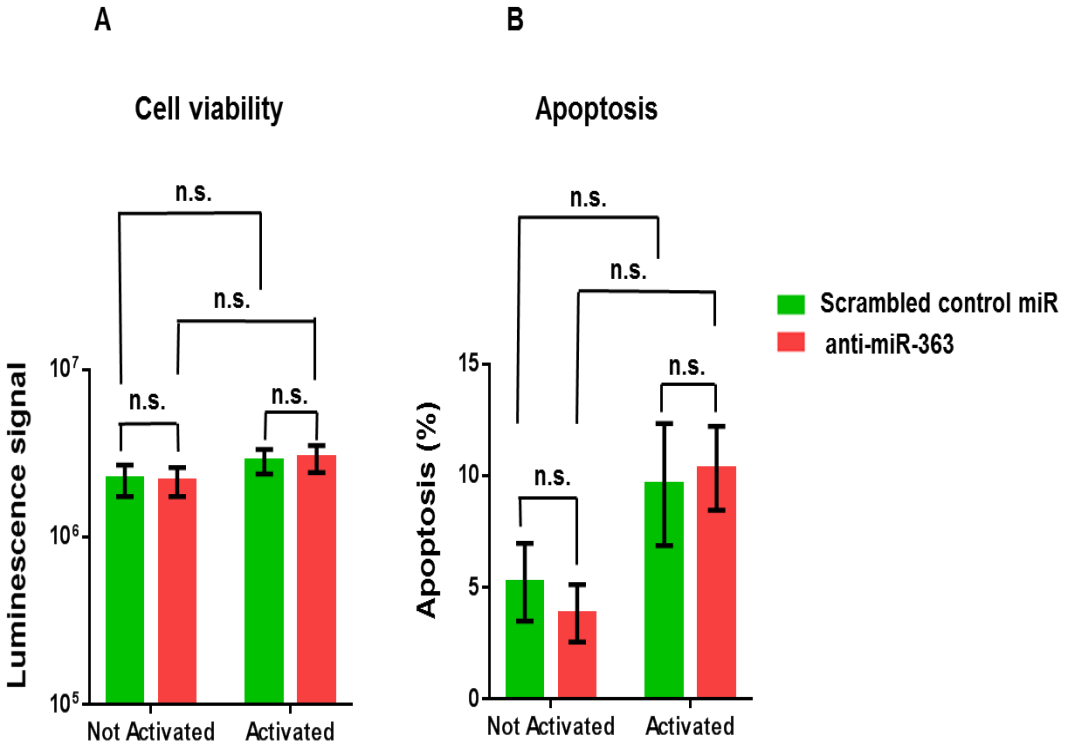

Figure 5.3 Effects of miR-363 on cell viability and apoptosis of activated and not activated CLL cells.

(A) ATP luminescence assay of lysates from activated and not activated CLL cells following transfection with anti-miR-363. For comparison, a negative control scrambled oligonucleotide was used in activated and not activated cells. Neither activation nor miR-363 knockdown produced significant differences in cell viability (Mann-Whitney test). (B) Apoptosis (annexin V binding) in activated and not activated CLL cells with either scrambled miRNA or anti-MiR-363. Neither activation nor miR-363 knockdown produced significant differences in apoptosis (Mann-Whitney test). Error bars (Mean +/- SEM) n= 3 primary CLL cells samples.

5.2.4 Effect of activation and miR-363 knockdown on CD69 and S1PR1 mRNA expression

CD69 and S1PR1 have important roles in trafficking of lymphocytes (Cyster and Schwab, 2012). In order to determine the effect of activation and miR-363 knockdown on the expression of *CD69* and *S1PR1* mRNA in CLL cells, we used real-time quantitative PCR (RT-qPCR).

Firstly, *CD69* mRNA expression was investigated. Neither activation nor miR-363 knockdown appeared to cause significant differences to *CD69* mRNA levels. (Figure 5.4 A).

In contrast, *S1PR1* mRNA expression was significantly repressed by knockdown (~70%) in activated and non-activated CLL cells. In non-activated CLL cells, *S1PR1* mRNA expression showed significantly decreased levels following miR-363 knockdown compared to the expression in in those non-activated CLL cells treated with a negative control scrambled oligonucleotide (Mann Whitney, $P<0.0079$). Similarly, activated CLL cells demonstrated significantly decreased levels of *S1PR1* mRNA with miR-363 knockdown compared to control (Mann Whitney, $P<0.0079$), although activation did not produce any extra effect. None of the differences observed were statistically significant (Figure 5.4 B).

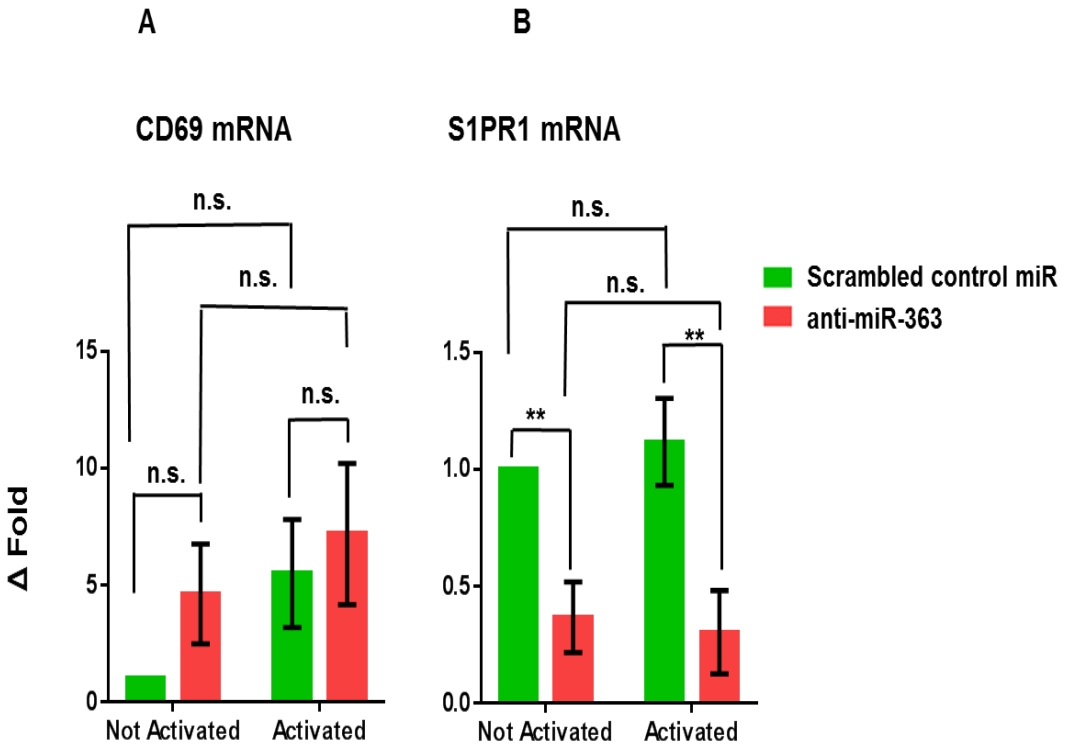

Figure 5.4 Effect of miR-363 knockdown and activation on CD69 and S1PR1 mRNA expression.

(A) Fold change of CD69 mRNA expression following miR-363 knockdown (red bars) and without knockdown (green bars) in non-activated cells and cells activated by CD40 cross-linking and IL4. There is no significant difference in Ct values of CD69 mRNA expression between conditions. CD69 mRNA in Not activated-anti-miR363 and +anti-miR, (Mann Whitney, P=0.22). CD69 mRNA in Activated -anti-miR 363 and + anti-miR (Mann Whitney, P=0.54). B) Fold change of S1PR1 mRNA expression following miR-363 knockdown (red bars) and without knockdown (green bars) in non-activated cells and cells activated by CD40 cross-linking and IL4. MiR-363 knockdown decreased the expression of S1PR1 mRNA in activated and not activated cells.S1PR1 mRNA in not activated-antimiR and +anti-miR (Mann Whitney, P= 0.54). S1PR1 mRNA in activated -anti-miR and + anti-miR (Mann Whitney, P= 0.42). Error bars Mean +/- SEM of five primary CLL cells samples.

5.2.5 Effects of miR-363 knockdown on CD69 expression, Is CD69 a target of miR-363 in CLL

In order to investigate the role of miR-363 on CD69 and S1PR1 expression, miR-363 was knocked down in CLL cells activated with CD40L/IL4. Expression levels of CD69 and S1PR1 were measured using flow cytometry at 48 hours after transfection.

CD40L/IL-4 activation induced CD69 expression but, there was no significant differences on expression levels between control and CLL cells treated following miR-363 knock down (Mann Whitney P= 0.1) (Figure 5.6 A).

Although others have detected S1PR1 on the surface of CLL cells (Till, Pettitt et al., 2015), it was hardly detectable in the experiments reported here (Figure 5.6 B).

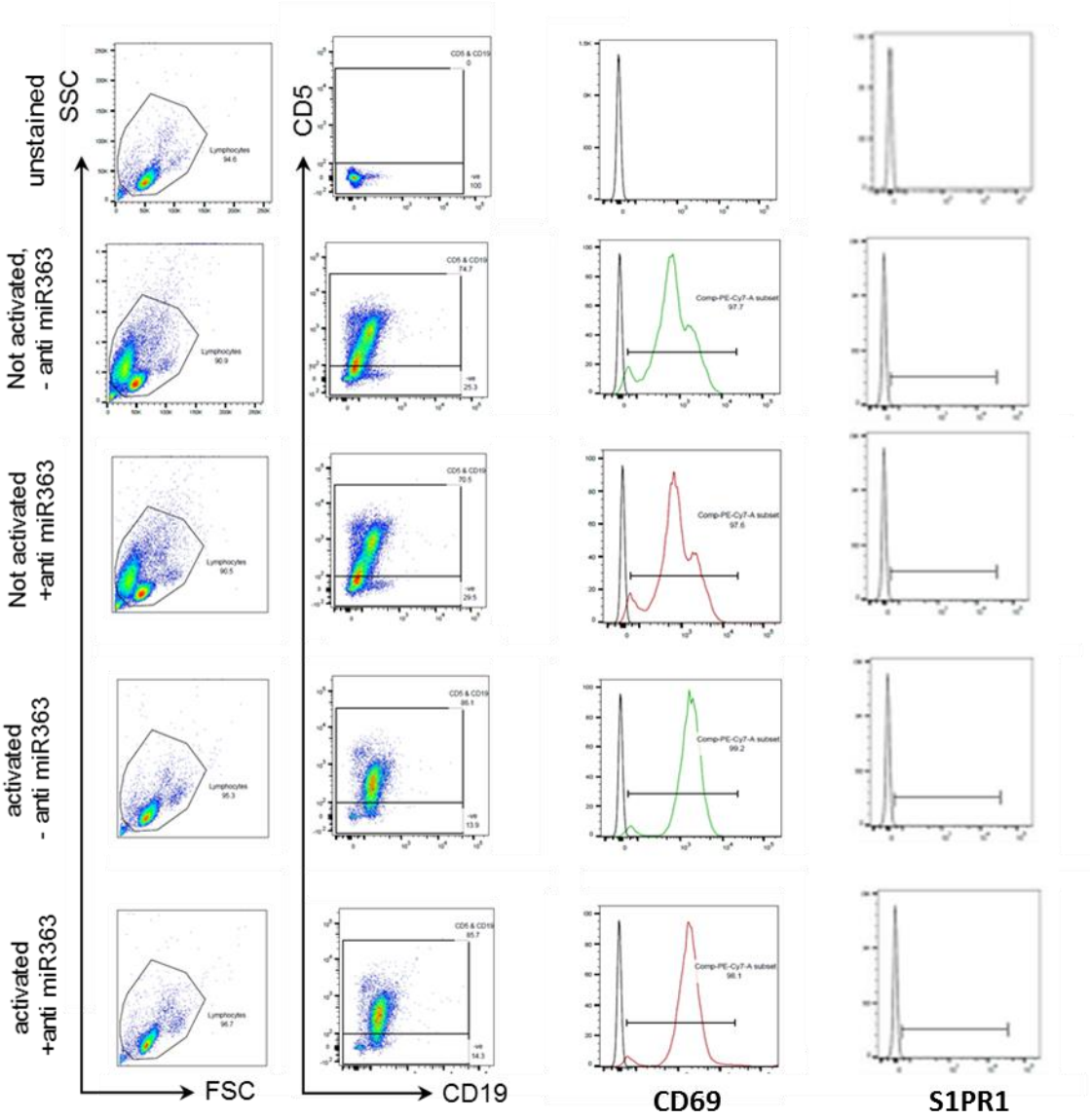

Figure 5.5 Flow cytometric analysis demonstrating expression of CD69 and S1PR1 in activated CLL cells from one patient following miR-363 knockdown.

Dot plots show gating strategy. Histograms to the right show CD69 and S1PR1 levels (scrambled oligonucleotide green lines and anti-miR-363 red lines). Unstained cells (negative control) are represented by black lines. The FACSAria II from Becton Dickinson (BD Biocsiences, San Jose, USA) The data was analysed by FlowJo (BD Biocsiences, San Jose, USA.

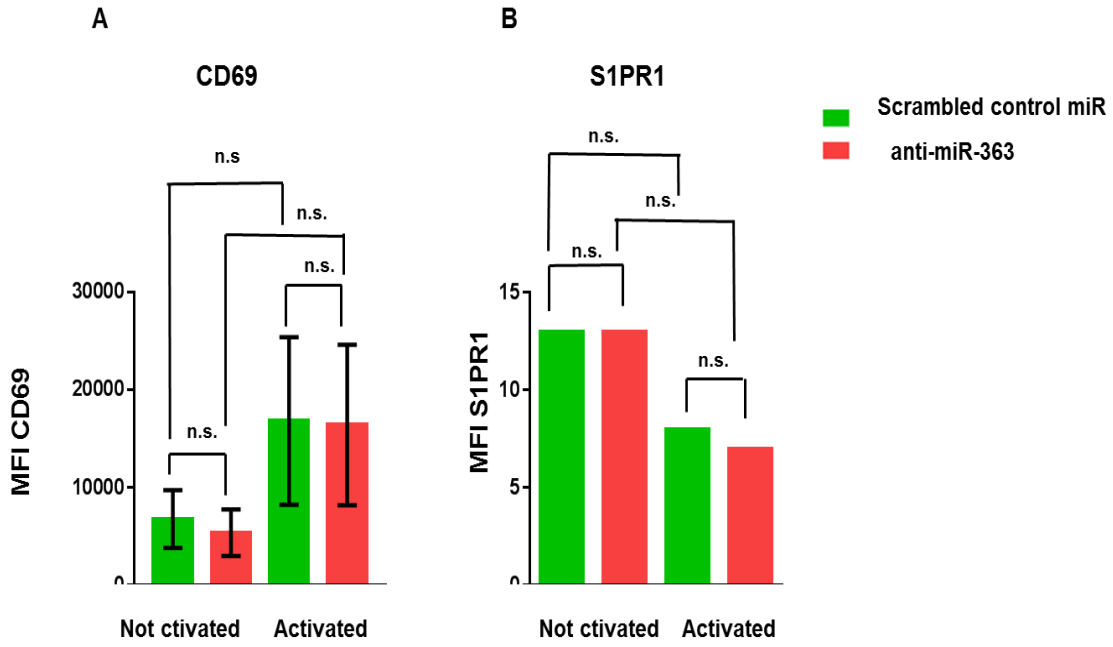

Figure 5.6 CD40/IL-4 stimulation upregulates the expression of CD69 but miR-363 knockdown has no effect.

(A) Flow cytometry analysis revealing that incubation of primary CD5+ CD19+ B cells following CD40/IL-4 stimulation increased CD69 compared to unstimulated CLL cells (Mann Whitney P= 0.12), miR- 363 knockdown (red bar) does not have effects on CD69 expression in activated CLL cells (Mann Whitney, P> 0.9). (B) S1PR1 was downregulated following CD40/IL-4 stimulation (Mann Whitney, P= 0.1), miR- 363 knockdown (red bar) does not have effects on S1PR1 expression in activated CLL cells (Mann Whitney, P> 0.1). Error bars indicate mean +/- SEM of five primary CLL cells samples.

5.3 Discussion

5.3.1 Expression of miR- 363 and miR-16 in cells and EVs and effects of activation

MicroRNAs are an important for normal gene regulation and cancer (Lu, Getz et al., 2005, Xiao and Rajewsky, 2009).The laboratory has previously demonstrated that there are changes in expression of specific cellular miRNA on CD40L/IL4 stimulation (Willimott and Wagner, 2012) and subsequently we showed enrichment of specific miRNA in extracellular vesicles (EVs) as compared to the intracellular pool (Smallwood, Apollonio et al., 2016). Similar observations have been made by other researchers (Farahani, Rubbi et al., 2015). The role of one of these, miR-363, in autologous T-cells was investigated and the principal findings were that this miRNA is required for normal immune synapse formation and motility. There is also potential for EVs and their miRNA cargo in regulation of leukaemic B-cell functions but his has not been previously investigated.

In this chapter changes in EV expression of miR-363 following B-cell receptor activation by anti-IgM are investigated. Anti-IgM caused an increase in miR-363 levels in EVs as compared to EVs from non-activated cells. Supporting this finding there appears to be a dose dependence to the enrichment in that anti-IgM at 20 µg/ml caused a greater effect than at 10 µg/ml. Potentially BCR signalling might cause changes to EV miRNA content as significant as those caused by CD40L/IL4.

If EVs do have functional effects on CLL cells these particles must be taken up by the leukaemic cells. This has not been investigated before.

Exocytosis implies the fusion of the membrane of secretion granules with, and the insertion into, the plasma membrane of other cells. The insertion is temporary in that the inserted membrane is eventually removed. One study indicated that the removed membrane is not destroyed but recycled within the cell and reused (Meldolesi and Ceccarelli, 1981). It is established that EVs form part of the machinery for intercellular communication. However, unlike some modes of intercellular communication, which involve direct cell-cell contact, EVs are a “scatter gun” method in which the vesicles and

their cargo of mRNA, protein and miRNA can perturb cells in the neighbourhood of the same and different lineages and can also potentially be taken up by the cell that released them. This allows for great complexity and there may be many factors in the microenvironment, both physical and biochemical barriers, which modify the distribution of EVs to make them a more targeted means of communication.

Confocal laser scanning microscopy combines optical and electronic processes to scan planes at a given depth. This type of microscopy can, therefore, detect signals specifically within cells. Uptake of fluorescently labelled EVs by CLL cells was shown for the first time.

Because CD69 was demonstrated to be a miR-363 target in T-cells the effects of miR-363 knockdown on CD69 were investigated. CD40L/IL-4 activation induced CD69 expression but, although good knockdown was achieved, there was no significant effect on expression levels. It appears that the regulation of CD69 is different in T-cells and B-cells and that these two cell types have different requirements for miR-363. Sphingosine-1-phosphate and its principal receptor S1PR1 have a key role in regulating lymphocyte trafficking (Cyster and Schwab, 2012). One of the functions of CD69 is to regulate S1PR1 (Mackay, Braun et al., 2015). There is a reciprocal relationship between these two proteins because CD69 causes increased internalisation and removal of S1PR1 (Cyster and Schwab, 2012). S1PR1 has been investigated in CLL (Patrussi, Capitani et al., 2015, Till, Pettitt et al., 2015) but S1PR1 was hardly detectable in the experiments reported here. The reasons for this difference are not clear. The detecting antibody and culture conditions were the same as those used by others (Till, Pettitt et al., 2015) but the method of cell activation might be important in causing S1PR1 induction.

Overall work presented here shows that miR-363 is enriched in CLL-EVs by anti-IgM and that CLL cells are able to take up EVs. However, CD69 does not seem to be a miR-363 target in CLL cells although it is in T-cells.

Chapter 6 General discussion

6.1 Increased numbers of circulating EV in CLL

One of the principle findings of the thesis is that there are more circulating EVs in CLL patients than in normal subjects. EVs are secreted by activated CLL cells (Willimott and Wagner, 2012, Yeh, Ozer et al., 2015). Normal B-cells are principally activated by engagement of the B-cell receptor by antigen, which may be on follicular dendritic cells or macrophages, and through encounter with T-cells. These methods of activating B-cells take place within the normal lymph node microenvironment. It is likely that CLL cell activation takes place in the tumour microenvironment (TME), which could be either the lymph node or bone marrow. The TME is believed to play a significant role in proliferation and survival of CLL cells and promotes resistance to conventional chemotherapy.

In this thesis, I showed that comparison of amounts of EVs between healthy volunteers and patients with CLL shows significantly increased numbers of the plasma EVs in CLL patients compared to normal subjects. This result is consistent with other's demonstrating that CLL patients showed significant higher levels of EVs in plasma than normal subjects (Yeh, Ozer et al., 2015). Therefore, it seems that increased numbers of circulating EVs is a feature of CLL and might be a feature of other lymphoproliferative disorders. There is a further possibility which is that the number of circulating EVs correlates with the number of activated i.e. proliferating cells, of leukaemic cells within the tissues. If this were the case it would be important because, apart from biopsy, which is invasive, there is at present no simple way to determine the state of CLL cells within tissues. The tissue microenvironment causes changes to proliferation and enhances ability of the leukaemic cells to survive partly through changes to BCL2 family proteins (Kater, Evers et al., 2004, Willimott, Baou et al., 2007) and assays derived from peripheral blood might be helpful to determine timing of treatment, and response to treatment. Conceivably high grade transformation (Richter's syndrome) may also produce changes to EVs detectable in the circulation.

There are confounding factors in using peripheral blood to draw conclusions about the state of leukaemic cells in the tissues. There is the potential for the large number of circulating vesicles in CLL patients to be correlated with numbers of leukemic-B cells in

the peripheral blood. However our results published in Smallwood, Apollonio et al. (2016) demonstrated that EV numbers were not significantly associated with absolute lymphocyte or platelet counts. These data are also in agreements with other's work (Ghosh, Secreto et al., 2010, Yeh, Ozer et al., 2015) and suggest that EV levels in CLL patients do not increase in parallel with numbers of circulating leukaemic cells.

6.2 Is there a disease specific composition of circulating EVs?

MiRNAs are an important component of EVs molecular cargo and recent work has illustrated a disease-associated EV miRNA signature in CLL (Farahani, Rubbi et al., 2015, Yeh, Ozer et al., 2015). Secreted EVs could have autocrine or paracrine effects. Several studies have suggested paracrine effects of CLL EVs on stromal cells (Ghosh, Secreto et al., 2010, Farahani, Rubbi et al., 2015, Paggetti, Haderk et al., 2015, Haderk, Schulz et al., 2017) while our laboratory has demonstrated that CLL derived EVs can modify $CD4^+$ T-cell function (Smallwood, Apollonio et al., 2016). Therefore, different lines of work converge on the notion that CLL cell-derived EVs are able to regulate and reprogramme the tumour microenvironment. In this thesis, I showed that EVs can be taken up by CLL cells themselves but was unable to demonstrate significant effects on proliferation or apoptosis. However, it seems that there are CLL specific miRNAs associated with EVs from activated primary CLL cells. In particular there was an enrichment of specific cellular miRNAs including miR-363 within EVs produced from CD40L/IL-4 activated CLL cells compared to control EVs from non-activated CLL cells. Others Farahani, Rubbi et al. (2015) show that miR-202-3p is packaged into CLL exosomes and causes enhanced expression of 'suppressor of fused', a Hedgehog (Hh) signalling intermediate in CLL cells providing an example of autocrine signalling.

The specific miRNA cargo of circulating EVs has been investigated and a distinct exosome microRNA signature, including miR-29 family, miR-150, miR-155 and miR-223 has been identified. Interestingly the miR-29 family has been associated with development of CLL in animal models (Santanam, Zanesi et al., 2010) while miR-155, which we showed to be enriched in CLL cells after CD40L/IL4 stimulation and which is expressed in cells within lymph node proliferation centres (Willimott and Wagner, 2012),

is also associated with B-cell receptor signalling and clinically aggressive disease (Cui, Chen et al., 2014)and may be prognostic (Ferrajoli, Shanafelt et al., 2013).Others have characterised circulating miRNA (Moussay, Wang et al., 2011)and shown disease specific plasma signatures, which included higher levels of miR-363 and miR-708 in CLL than in hairy cell leukaemia without being significantly different from levels in plasma of normal subjects. These authors go on to suggest that miR-363 levels in CLL plasma associate with disease progression (Moussay, Wang et al., 2011). Therefore there is likely to be a disease specific circulating miRNA signature but in a disease as heterogeneous as CLL it requires definition in more detail.

The Wagner laboratory showed that specific miRNA are enriched in CD40L/IL4 activated CLL cells (Willimott and Wagner, 2012) and also in CLL EVs Smallwood, Apollonio et al. (2016) and others have carried out similar experiments following B-cell receptor stimulation (Yeh, Ozer et al., 2015). MiR-363 was one of the most highly enriched in CD40L/IL4 stimulated CLL EVs and I investigated this further in the thesis.

6.3 Is miR-363 in plasma protein or vesicle bound fractions in CLL?

Our recent study (Smallwood, Apollonio et al., 2016) has demonstrated that specific miRNAs associated with EVs from activated primary CLL cells. Predominately, there was an increase of specific cellular miRNAs including miR-363 within EVs secreted from CD40L/IL4 stimulated CLL cells compared with control EVs from non-stimulated CLL cells (Willimott and Wagner, 2012). These results suggest that CD40/IL4 stimulation mediates packaging of selective miRNA within CLL-EVs, which might be distinct from the effects of BCR activation.

In the light of experiments showing that circulating miRNA could be detected in either plasma protein or particle i.e. EV, fractions (Arroyo, Chevillet et al., 2011) wished to find out which fractions contained circulating miR-363 in patients and normal subjects. Therefore, size exclusion chromatography was used to separate plasma protein from particle fractions and then RNA was extracted from the fractions and RT-PCR was carried out. The analysis of miRNA distribution between particle and plasma protein fractions in

CLL revealed that a greater proportion of the miR-16 and miR-363 appeared mainly in late eluting fractions i.e. vesicle bound fractions, in CLL patients (Alharthi, Beck et al., 2018) This suggests, for the first time, the hypothesis that disease alters the distribution of miRNA between plasma protein and vesicle fractions.

The specific changes to the distribution of two miRNA (miR-16 and miR-363) in CLL patients i.e. in normal subject these miRNA are found almost exclusively in plasma protein fractions but in CLL there are increased amounts in the vesicle fractions, is consistent with the hypothesis that a proportion of circulating miR-363 is derived from EVs produced by stimulated CLL cells in the TME. Therefore, an extension to the work described in this thesis is to investigate the distribution of miRNA between plasma protein and vesicle fractions in health and disease. This might provide diagnostic or prognostic information. Circulating miRNA are attractive biomarkers as they can be measured simply and following a relatively non-invasive blood tests. Indeed levels of miRNA have been found to be prognostic in certain cancers (Kosaka, Iguchi et al., 2010, Moussay, Wang et al., 2011, Cheng, Mitchell et al., 2013, Grasedieck, Sorrentino et al., 2013, Jones, Nourse et al., 2014) ,and analysis of levels in plasma or vesicle fractions may add to the precision of these techniques.

There is interest in microfluidic technology to analyse microvesicles (Skog, Wurdinger et al., 2008) and it may, therefore, be feasible to adapt this approach to provide routine laboratory testing.

6.4 Is there potential for circulating EVs or their miRNA content for diagnosis or prognosis?

Sometimes, it is clinically difficult to decide when a CLL patient needs treatment. Also it may be important to determine whether a patient is responding to treatment. Currently, simple laboratory data (a rapidly rising white cell count, anaemia or thrombocytopenia), clinical data (massive lymphadenopathy or splenomegaly) or systemic symptoms (weight loss, fevers or night sweats) are used to make clinical decisions with some refinement being added by the detection of cytogenetic abnormalities, especially 17p deletion.

A biomarker derived especially from the tumour microenvironment would assist with this decision. In order to demonstrate whether miR-363 has potential as a biomarker, we employed clinical data gathered during the course of the ARCTIC clinical trial.

In this thesis, plasma levels of miR-363 and miR-374b were initially measured in cohorts of patients and normal subjects. I found that mean miR-363 levels were elevated in patients although there was considerable overlap with levels found in normal subjects. A repository study using material from patients entered into the ARCTIC and CLEAR clinical trials showed that there was no association with patient survival.

It is interesting that cellular miR-16 levels are associated with clinical outcome (Calin, Ferracin et al., 2005). For this miR-16, higher levels are associated with poor prognostic factors (unmutated immunoglobulin gene status and ZAP70 expression). MiR-363 was found to be enriched in CD40L/IL-4 activated CLL (Willimott and Wagner, 2012) and it has also been suggested that miR-363 levels are higher in CLL than in hairy cell leukaemia, another low grade B-cell lymphoproliferative disorder (Moussay, Wang et al., 2011). The same authors additionally suggested that miR-363 "showed progressive changes along with the severity of the disease" such that levels increased in association with Rai clinical stage. Calin, Ferracin et al. (2005) described cellular miR-16 as being prognostic in CLL but plasma miRNA was not measured in this study. Moussay, Wang et al. (2011) proposed that plasma miRNA can be prognostic (including miR-363) but very few patients were investigated by these authors. In this study, a comparison of miR-16 and miR-363 levels in plasma was first conducted followed by an analysis of miR-363 and miR-16 survival.

The prognostic potential of plasma miRNAs in CLL patients was investigated by a repository study. Levels of miR-363 and miR-16 in plasma was measured in two groups of patients: one was symptomatic and required treatment (ARCTIC) and the other was asymptomatic (CLEAR). Results showed increased plasma levels of miR-16 and miR-363 in patients' samples as compared to normal subjects. There was no significant difference between miR-363 and miR-16 levels.

To assess the potential of using miR-16 and miR-363 as CLL biomarkers, the levels of these circulating miRNAs were compared by immunoglobulin gene mutational status.

The data also did not display significant differences in miRNA levels between patients with unmated and mutated immunoglobulin genes.

The positive finding from my study is that levels of miR-363 differ between CLEAR and ARCTIC patients and consistent with the pilot study I carried out in smaller groups of patients and normal subjects. The finding that levels of miR-363 are present in plasma of ARCTIC group at levels significantly different from CLEAR patients suggests that disease requiring treatment is characterised by higher levels of this miRNA. Biologically it could suggest either that CLL cells secrete more EV than normal lymphocytes in response to stimulation in the tumour microenvironment, or simply that there are more lymphocytes and, therefore, correspondingly more EVs. However, there was no association between miR-363 levels and outcome.

Moussay, Wang et al. (2011) stated that a number of miRNAs demonstrated progressive changes associated with the development of the tumour. For example, plasma levels of miR-30 decrease as disease progresses, while miR-363 level keeps raising as disease progresses, although they only measured levels in 6 cases. Studying just under 100 cases in this thesis it was demonstrated that while miR-363 levels are higher in patients with advanced disease as compared to asymptomatic patients, there is no links between higher levels and prognostic markers or clinical outcome. Therefore, miR-363 appears to be a marker of advanced disease but levels do not associate with other known prognostic markers being used to predict clinical outcome such as mutational status, Binet stage or gender. Therefore, miR-363 might be used as a marker for diagnosis but clearly this is of limited clinical usefulness. Further work will need to be carried out to assess whether miR-363 levels allow the distinction of CLL from other low grade lymphoproliferative conditions such as marginal zone lymphoma with which there is sometimes diagnostic difficulty. Another clinical area in which circulating miR-363 levels may be useful is in deciding whether monoclonal B-cell lymphocytosis or early stage (Binet stage A) CLL is progressing.

Speculatively miR-363 levels do not correlate with prognosis due to, the kinetics of secretion of EVs by CLL cells, which might reach a maximum level early as disease

progresses i.e. the relationship between miRNA level and disease is only linear for a short period of time.

Another possibility is that there may be patient-specific confusing factors such as the ease or difficulty of reaching the plasma from the tissues and this would again perturb a relationship between plasma level and clinical outcome.

6.5 CD69 appears to be regulated differently in CLL cells and T-cell

In previous laboratory work, CD69 was shown to be a miR-363 target in T-cells (Smallwood, Apollonio et al., 2016), therefore, in this thesis, we investigated the effects of miR-363 knockdown on CD69. CD40L/IL4 stimulation enhanced CD69 expression but, although good knockdown was achieved, there was no significant effect on expression levels. This is surprising but it seems that the regulation of CD69 is different between T-cells and B-cells and that these two cell types have different uses for miR-363.

Sphingosine-1-phosphate and its principal receptor S1PR1 have a significant role in regulating lymphocyte trafficking (Cyster and Schwab, 2012). One effect of CD69 is to control S1PR1 (Mackay, Braun et al., 2015). There is a cross relationship between these two proteins because CD69 causes increased internalisation and removal of S1PR1(Cyster and Schwab, 2012). S1PR1 has been investigated in CLL (Patrussi, Capitani et al., 2015, Till, Pettitt et al., 2015) but S1PR1 was hardly detectable in the experiments reported here. The reasons for this difference are not clear. The detecting antibody and culture conditions were the same as those used by others (Till, Pettitt et al., 2015), but the method of cell activation might be important in causing S1PR1 induction.

Overall results showed here reveals that miR-363 is increased in CLL-EVs. However, CD69 does not seem to be a miR-363 target in CLL cells although it is in T-cells.

6.6 Targets of miR-363 other than CD69

Recent studies show that the expression alterations of miR-363 always lead to tumorigenesis. The abnormal overexpressed miR-363 can act as oncogenes through downregulation of tumour suppressor genes, whereas lowly expressed miR-363 can act as tumour suppressors by negatively regulation of oncogenes (Esquela-Kerscher and Slack, 2006). The top predicted targets of human miR-363 (TargetScan v7.0) sorted by total context score is shown in the table 6.1. CD69 is the target with the highest context score, which is why we focused on this study.

Although, none of the other genes in the table 6.1 look especially interesting, there are target genes of miR-363 have been suggested in different types of human cancers. MiR-363 has been described as a tumour suppressor in various types of cancer. For example, upregulation of miR-363 downregulated colorectal carcinogenesis through directly targeting the GATA6/Lgr5 pathway (Tsuji, Kawasaki et al., 2014).

LGR5 is a member of the leucine-rich repeat containing G-protein-coupled receptors (LGRs) and is known as a target of Wnt signalling (Van der Flier, Sabates-Bellver et al., 2007).

Wnt signalling plays a crucial role in cell proliferation and tissue homeostasis. LGR5 has been utilized as a stem cell sign of tissues where Wnt signalling has a key role in the promotion. Furthermore, LGR5 has been identified to be overexpressed in some cancer tissues, such as basal cell carcinomas, hepatocelluar carcinomas, ovarian tumours and colorectal tumours (McClanahan, Koseoglu et al., 2006, Tanese, Fukuma et al., 2008).

GATA6, the members of the GATA family of zinc finger transcription factors, plays important roles in the development, regulation of differentiation and control of cell proliferation and movement. It has been demonstrated that GATA6 is expressed in proliferating cells in the intestinal crypts and is needed for crypt cell proliferation and migration (Gao, Sedgwick et al., 1998, Molkentin, 2000). Targeted the GATA6 gene in mice results in early embryonic deadliness due to the lack of endoderm differentiation (Morrisey, Tang et al., 1998, Koutsourakis, Langeveld et al., 1999, Decker, Goldman et al., 2006).

Tsuji et al. (2014) showed that LGR5 is vital for colorectal tumourigenesis. In addition, they reveal that downregulation of miR-363 leads to upregulation of GATA6, which in turn enhances LGR5 to cause colorectal cancer. These findings provided important insight into the key role of miR-363 in tumourigenicity of colorectal cancer cells through directly targeting the GATA6-LGR5 pathway.

In hepatocellular carcinoma (HCC), downregulation of miR-363 played a key role in S1PR1-induced cell proliferation. S1PR1, a direct signalling molecule bound with sphingosine 1-phosphate (S1P), triggers many downstream responses involved in cell proliferation, migration, anti-apoptosis and angiogenesis (Lee, Deng et al., 2010, Deng, Liu et al., 2012, Liang, Nagahashi et al., 2013). S1P is a small signalling molecule in plasma, formed by sphingosine kinases 1 and 2 (SphK1 and SphK2) phosphorylating the sphingosine (Pyne, El Buri et al., 2018). S1PR1, a mediator of sphingosine 1-phosphate (S1P), a member of five G-protein-coupled receptors (S1PR1– 5). It has shown that miR-363 targeted S1PR1 to decrease HCC cells proliferation by directly targeting.(Zhou, Huang et al., 2014). They also, found that miR-363 was a novel regulator of S1PR1, through binding to 3′-UTR of S1PR1 mRNA. Down expression of S1PR1 regulated by miR-363 could not only significantly affect the signalling pathway about cell proliferation but also inhibit HCC cell proliferation. Moreover, Zhou et al. (2014) confirmed the binding site of miR-363 on the 3′-UTR of S1PR1 mRNA. These findings demonstrate that miR-363 acting as a tumour suppressor by down-expression of S1PR1 in HCC.

In contrast to antitumor functions, miR-363 also acts as an oncogene in many kinds of cancers. For instance, in non-small cell lung cancer (NSCLC) cells upregulation of miR-363 decreased HMGA2 expression at both mRNA and protein level (Jiang, Cao et al., 2018). Bioinformatics analysis demonstrated that HMGA2, a member of the high mobility group A proteins, is a non-histone chromatin-binding protein was a target of miR-363. In addition, luciferase reporter assays illustrated that the 3′UTR of HMGA2 could be directly targeted by miR-363-3p. Moreover, HMGA2 mRNA was remarkably upregulated in non-small cell lung cancer (NSCLC) tissues and inverse correlated with miR-363-3p expression. Finally, the biological functions of HMGA2 under expression

were similar to the effects applied by miR-363 in NSCLC cells, also suggesting that HMGA2 is a key target of miR-363-in NSCLC (Jiang, Cao et al., 2018).

In gastric cancer, exogenous miR-363 enhanced cell growth, viability, progression, EMT and tumour formation of gastric cancer via directly targeting MBP-1 (Chen, Lu et al., 2015). C-Myc promoter binding protein 1 (MBP-1) is a negative regulator of c-*myc* expression and expressed in normal tissues (Hsu, Wang et al., 2014). It is synthesised by alternative translation initiation of α-enolase gene (Feo, Arcuri et al., 2000, Subramanian and Miller, 2000). Both MBP-1 and α-enolase are involved in controlling tumorigenesis including gastric cancer. They play role in regulating EMT (Hsu, Hsieh et al., 2009).

Taken together, these studies revealed that the expression behaviours and functional roles of miR-363 in human cancers may be different which mostly depending on the type of tissue and their target genes. Therefore, illustration of miR-363 target gene is important for identifying its biological functions in human cancer development.

Table 6.1 The top predicted targets of human miR-363 (TargetScan v7.0)

Target gene	Gene name	Conserved sites				Poorly Conserved Sites				Context Score	Aggregate P_{CT}
		total	8mer	7mer-m8	7mer-1A	total	8mer	7mer-m8	7mer-1A		
CD69	CD69 molecule	3	3	0	0	0	0	0	0	-1.49	0.96
SLC12A5	solute carrier family 12, (potassium-chloride transporter) member 5	3	2	0	1	1	0	0	1	-1.12	> 0.99
FNIP1	folliculin interacting protein 1	2	2	0	0	0	0	0	0	-1.05	0.97
ACTC1	actin, alpha, cardiac muscle 1	1	0	1	0	3	1	1	1	-1.02	0.79
MAN2A1	mannosidase, alpha, class 2A, member 1	2	1	1	0	1	0	1	0	-0.95	0.9
FBXW7	F-box and WD repeat domain containing 7	2	1	1	0	1	0	0	1	-0.93	0.9
PTAR1	protein prenyltransferase alpha subunit repeat containing 1	1	1	0	0	2	0	1	1	-0.88	0.97
RBM47	RNA binding motif protein 47	3	1	2	0	0	0	0	0	-0.82	0.89
IQWD1	IQ motif and WD repeats 1	1	1	0	0	1	0	1	0	-0.81	0.48
PCDH11X	protocadherin 11 X-linked	2	2	0	0	0	0	0	0	-0.8	< 0.1
PCDH11Y	protocadherin 11 Y-linked	1	1	0	0	1	1	0	0	-0.8	< 0.1
SYN2	synapsin II	2	2	0	0	0	0	0	0	-0.8	> 0.99
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	1	1	0	0	2	0	1	1	-0.79	0.93
MAP2K4	mitogen-activated protein kinase kinase 4	2	2	0	0	0	0	0	0	-0.79	> 0.99
MYO1B	myosin IB	2	1	1	0	0	0	0	0	-0.79	0.99
CPEB3	cytoplasmic polyadenylation element binding protein 3	4	1	0	3	0	0	0	0	-0.74	> 0.99
TMF1	TATA element modulatory factor 1	1	1	0	0	1	0	0	1	-0.73	0.97
PDZD2	PDZ domain containing 2	1	1	0	0	1	0	1	0	-0.71	0.97
SLC17A6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	1	0	1	0	2	0	1	1	-0.7	< 0.1
PHLPPL	PH domain and leucine rich repeat protein phosphatase-like	2	1	1	0	1	0	1	0	-0.69	0.86
ANP32E	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	1	0	1	0	1	1	0	0	-0.69	0.71
B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	2	0	1	1	1	0	0	1	-0.68	0.93
USP28	ubiquitin specific peptidase 28	1	1	0	0	1	0	1	0	-0.68	0.94
LHFPL2	lipoma HMGIC fusion partner-like 2	2	2	0	0	0	0	0	0	-0.68	> 0.99
RAB23	RAB23, member RAS oncogene family	2	1	1	0	0	0	0	0	-0.68	0.9
SNAPC1	small nuclear RNA activating complex, polypeptide 1, 43kDa	1	1	0	0	1	0	0	1	-0.68	0.68
RNF38	ring finger protein 38	2	1	1	0	1	0	0	1	-0.67	0.78

6.7 Involvement of miR-363 in cancers other than CLL

Previous studies indicated that expression levels of microRNAs (miRNAs) may be significantly correlated with diagnosis, treatment and prognosis in human cancer (Chen, Ba et al., 2008, Li, Liu et al., 2016, Vychytilova-Faltejskova, Radova et al., 2016). Growing evidence has revealed that miRNAs are abnormally expressed in different types of human cancer, such as bladder cancer (Shin, Park et al., 2016), gastric cancer (Qiu, Zhu et al., 2017), colorectal cancer (Chandrasekaran, Sathyanarayanan et al., 2016), glioma (Feng, Kuai et al., 2016) and NSCLC (Huang, She et al., 2016). Abnormal expression of miRNAs may be associated with cancer proliferation, migration, invasion and chemotherapy resistance, via the regulation of their target genes (He, Fang et al., 2019).

In the present study, miR-363 was found significantly higher in plasm of patients with CLL as compared to normal subjects but roles for this specific miRNA have been suggested in other cancers, for examples, in gallbladder (Wang, Zhang et al., 2016), colorectal (Hu, Min et al., 2016), gastric (Zhang, Sheng et al., 2016), and head and neck (Chapman, Wald et al., 2015), as well as in breast cancer (Zhang, Li et al., 2014).

Colorectal Cancer

In colorectal cancer, miR-363 exhibited a reduced expression in tumour tissues as compared to normal tissues. In line with this observation, upregulation of miR-363 inhibited colorectal carcinogenesis through directly targeting the GATA6/Lgr5 pathway (Tsuji, Kawasaki et al., 2014). Moreover, *in vitro* and *in vivo* experiments revealed that reduced expression of miR-363 enhanced cell migration, invasion and epithelial-mesenchymal transition (EMT) of colorectal cancer via blockade of SOX4 (Hu, Min et al., 2016). EMT is an essential process in cancer metastasis and this study suggests that miR-363 may have a role in its regulation in colorectal carcinoma.

Hepatocellular Carcinoma

Ou, Zhai et al. (2015) showed that miR-363 was lower in hepatocellular carcinoma (HCC) tissues successfully treated with cisplatin-based chemotherapy. MiR-363 overexpression

overcame cisplatin resistance in cisplatin-resistant HepG2 cells through downregulation of the pro-survival Bcl-2 family member, Mcl-1.

Others also investigated the biological function and mechanism of miR-363 in the regulation of HCC progression. They indicate that miR-363 was downregulated in HCC cell lines and tissues, and a low expression level of miR-363 was associated with tumour differentiation, TNM stage and lymph node metastasis. Induced overexpression of miR-363 significantly suppressed HCC cell proliferation, migration, invasion and decreased epithelial-mesenchymal transition (EMT) in vitro, as well as inhibited tumour growth in vivo (Wang, Chen et al., 2017). Analysis of the mechanisms of miR-363 in HCC also showed that this miRNA functions as a tumour suppressor by negatively regulating the transcription factor, E2F3 (Ye, Zhang et al., 2017).

Osteosarcoma

In osteosarcoma, miR-363 expression level was decreased in tumour tissues and cell lines. Reduced miR-363 expression was correlated with tumour size, clinical stage and distant metastasis. MiR-363-3p re-expression suppressed cell growth and metastasis through downregulation of MAP2K4 (Li, Liu et al., 2015).

Lung Cancer

Recent studies revealed that reduced miR-363 expression was correlated with tumour node metastasis classification and distant metastasis of non-small cell lung cancer (NSCLC) patients. Remarkably, miR-363-3p re-expression significantly suppressed cell proliferation and invasion of NSCLC. It has been demonstrated that (high mobility group AT-hook 2) HMGA2 was a direct target gene of miR-363 (Jiang, Cao et al., 2018) in this disease. Another study showed that decreased miR-363 expression enhanced gemcitabine resistance in NSCLC cells via regulation of CUL4A (Bian, Zhou et al., 2019).

Furthermore, cell cycle analysis showed miR-363 can induce S phase arrest by downregulating cyclin-D1 (CCND1) and upregulating cyclin-dependent kinase-2 (CDK2) in lung adenocarcinoma cells. Additionally, miR-363 enhances cell apoptosis, whereas miR-363 inhibitor prevents apoptosis and results in downregulation of Bax and

Bak, proteins which are members of the Bcl-2 family and whose oligomerisation at the outer mitochondrial membrane is an essential step in the initiation of apoptosis.

The anti-proliferative function of miR-363 toward lung cancer cells may be contributed to by its ability to inhibit the activation of the mTOR and ERK signalling pathways. The same group also had identified PCNA as a specific target of miR-363. In lung adenocarcinoma miR-363 was demonstrated suppresses tumour growth by targeting PCNA.

Roles in other cancers

In addition, miR-363 appears to play a tumour suppressor role in ovarian cancer progression by inhibiting NOB1 (Lin, Xu et al., 2017). Recent findings demonstrated that downregulation of miR-363 may be involved in the development of osteosarcoma via regulation of PDZD2 (He, Fang et al., 2019). It has been reported that miR-363 plays an important role in the increase of gastric carcinogenesis via targeting MBP-1.(Hsu, Wang et al., 2014). Moreover, (Song, Yan et al., 2015) showed low levels of miR-363 in Gastric Cancer (GC) tissues and cells. Induced expression of miR-363 inhibited cell growth and migration of GC cells and vice versa by targeting NOTCH1 gene. In neuroblastoma GRP-R-mediated tumourigenicity and increased metastatic potential are regulated, in part, by miR-335 and miR-363 (Qiao, Lee et al., 2013).

MiR-363 was also revealed as a novel prognostic factor in acute myeloid leukaemia (AML) patients undergoing chemotherapy. High miR-363 level was positively correlated with the amounts of leukaemogenic transcription factors, including Myb, RUNX3, GATA3, IKZF3, ETS1 and MLLT3. Remarkably, they found that the in silico predicted target genes (EZH2, KLF6 and PTEN) of miR-363 were downregulated in association with high miR-363 expression (Zhang, Zhang et al., 2019).

Together, these data suggest that the important role of miR-363 in human cancer. This could open a new way of investigation of disease and developments in microfluidic technology. Clearly, future studies will need to determine the precise different distributions of miR-363 between vesicles and plasma protein for patient benefit.

6.8 Reflection on the results and potential future experiments

Work from others and ourselves suggested that miR-363 might be a biomarker derived from the microenvironment. This is important because CLL cell behaviour in the microenvironment is important in determining clinical characteristics. However, while we found that miR-363 expression associated with disease as compared to normal it did not associate with prognostic markers. This implies that it is produced in excess by leukaemic cells but not in a manner that associates with the biological characteristics of the disease.

A plasma based marker would be useful for assessing responses to treatment and potentially miR-363 may have a role here. One of the most interesting findings is that the proportion of miR-363 in the vesicle bound fraction appears to increase in patients as compared to normal subjects. This might reflect increased vesicle production in disease due to the number of leukaemic cells and may therefore indirectly reflect the bulk of disease. This might be "beneficial" to the leukaemic cell in order to manipulate the microenvironment to produce a more favourable setting for leukaemic cell growth.

The observation needs confirmation by extension to other miRNA and other cancers maybe by carrying out miRNA microarrays on fractionated plasma samples from different cancers and normal subjects. If true then analysis of vesicle bound miRNA could provide an aid to diagnosis and also assess responses to treatment.

Functional experiments were carried out to investigate effects of miR-363 on CLL cells having previously (Smallwood et al., 2016) investigated autologous T-cells. It was proved by confocal microscopy that CLL cells can take up EVs, but no clear functional results were obtained. In particular, there appeared to be no effect on CD69. One issue might be that knockdown achieved by siRNA may be too transient and not de ep enough to produce functional effects. Future work could reassess this by investigation of CLL cell lines which may be more tractable. For example CRISPR/Cas9 could be used to disrupt the miR-363 locus in MEC1 cells to allow analysis of growth, EV content and CD69 metabolism.

6.9 Final Conclusion

Circulating miRNA are attractive candidate biomarkers. Although I showed that miR-363 levels are higher in CLL patients with active disease, the amounts of this miRNA did not correlate with clinical outcome. However, a potentially important finding is that the distribution of miR-363 between plasma protein and particle fractions in the circulation differs between patients and normal subjects. This could open a new line of investigation of disease and developments in microfluidic technology might make it feasible to measure levels in plasma protein and particle fractions separately for patient benefit.

Appendices

Appendix I

Publications completed during the course of this dissertation:

1- Alharthi, A., D. Beck, D. R. Howard, P. Hillmen, M. Oates, A. Pettitt and S. D. Wagner (2018). "An increased fraction of circulating miR-363 and miR-16 is particle bound in patients with chronic lymphocytic leukaemia as compared to normal subjects." BMC Res Notes **11**(1): 280.

2- Smallwood, D. T., B. Apollonio, S. Willimott, L. Lezina, A. Alharthi, A. R. Ambrose, G. De Rossi, A. G. Ramsay and S. D. Wagner (2016). "Extracellular vesicles released by CD40/IL-4-stimulated CLL cells confer altered functional properties to CD4+ T cells." Blood 128(4): 542-552.

Appendix II

A copy of the ethical approval for the present study from the Leicestershire Local Research Ethics Committee One in Leicester, UK (Section 2.1.1).

Page 1

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT

Director: Professor D Rowbotham
Assistant Director: John Hampton
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Leicester General Hospital
Gwendolen Road
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30 August 2006

Professor Martin Dyer
Professor of Haemato-Oncology
Haematology and Cancer Services
Osborne Building
Leicester Royal Infirmary
Leicester
LE1 5WW

Dear Professor Dyer

ID: 09723 **Establishment of a Haematological Malignancies Tissue Bank.**

LREC Ref: 06/Q2501/122 **MREC Ref: n/a**

Sponsor	University Hospitals of Leicester NHS Trust
Funder	Leukaemia Research
Funder	Lymphoma Research Fund (USA)
Funder	MRC

Please note that Trust Indemnity ceases 10/07/2026

We have now been notified by the Ethics Committee that this project has been given a favourable opinion by the Ethics Committee (please see the attached letter from the Ethics Committee).

Since all other aspects of your UHL R+D notification are complete, I now have pleasure in confirming full approval of the project on behalf of University Hospitals of Leicester NHS Trust.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your notification form.

The project is also now covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

Please make sure if you or other researchers have an honorary contract with the Trust that this stays within date whilst working on the research study.

If you want to extend the study's end date you will have to submit an annual report available through the R&D website which will be forwarded and noted by the Trust and the relevant Ethics Committee. This allows you to continue working on the study under the previous arrangements covered by Trust Indemnity. Please note ethics approval is only granted until the proposed end date as reflected in A3 of the COREC form. You are no longer indemnified pass this date unless you have submitted the annual report form detailing this extension.

In the meantime, in order to keep our records up to date, could you please notify the Research Office if

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there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

Below is a list of the Researchers Approved to work on this Application within UHL

Professor Martin Dyer	Consenting Doctor
Dr B Kennedy	Consenting Doctor
Dr Renata Walewska	Consenting Doctor

Yours sincerely

Professor David Rowbotham
Director for Research & Development

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University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT

Director: **Professor D Rowbotham**
Assistant Director: **John Hampton**
R&D Manager: **Carolyn Burden**

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226

19/02/2009

Prof Martin JS Dyer
Hodgkin Building
Lancaster Road
University of Leicester
PO BOX 138, Lancaster Road
LE19HN
UK

Dear Prof Martin JS Dyer

Ref: UHL 09723
Title: Establishment of a Haematological Malignancies Tissue Bank.
Project Status: Project Approved
End Date: 10/07/2026

Thank you for submitting documentation for Non-Substantial amendment addition of a member of staff for the above study.

I confirm that the amendment has the approval of the University Hospitals of Leicester NHS Trust R&D Department and may be implemented with immediate effect.

The documents received are as follows:

Document Name	Version Number	Date
Curriculum Vitae Dr Simon Wagner		11/02/2009
GCP Certificate Dr Simon Wagner		05/02/2009

Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments should be followed. Failure to do so may invalidate the approval of the study at this trust.

Please ensure that all documentation and correspondence relating to this amendment are filed appropriately in the relevant site file.

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Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q2501/122	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Carl Edwards/Ms Linda Ellis
Chair/Research Ethics Manager

Email: linda.ellis@rushcliffe-pct.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: University Hospitals of Leicester NHS Trust

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NHS

National Research Ethics Service

Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839368
Fax: 0115 8839294

04 March 2011

Professor Martin Dyer
Professor of Haemato-Oncology and Honorary Consultant Physician
University Hospitals Leicester NHS Trust
MRC Toxicology Unit
Leicester University
LE1 9HN

Dear Professor Dyer

Study title: **Establishment of a Haematological Malignancies Tissue Bank.**
REC reference: **06/Q2501/122**
Amendment number: **5**
Amendment date: **01 February 2011**

The above amendment was reviewed on 04 March 2011 by the Sub-Committee in correspondence.

Ethical opinion

To expand the current project to include use of tissue for analysis and short term (up to 96 hours culture) without prior storage

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Consent Form	6	27 January 2011
Participant Information Sheet	5	28 January 2011
Protocol	3.0	28 January 2011
Notice of Substantial Amendment (non-CTIMPs) - to expand the current project to include the use of tissue for analysis	5	01 February 2011
Covering Letter		31 January 2011

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

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Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1

Attendance at Sub-Committee of the REC meeting on 04 March 2011

Name	*Profession*	*Capacity*
Mr John Baker	Radiation Protection Advisor and Senior Lecturer (retired)	Lay
Dr Carl Edwards	Senior Research Fellow	Lay

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R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q2501/122: **Please quote this number on all correspondence**

Yours sincerely

Dr Carl Edwards
Chair

E-mail: lisa.gregory@nottspct.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Sponsor/R&D office for NHS care organisation at lead site - UHL*

Appendix III

ARCTIC Clinical information.

Patient ID	Age	gender	VH mutation status	VH Mutation %	FISH Cytogenetics		Binet Stage
					11q del	17p del	
1	61	M	M	93.2	N	N	C
2	54	M	U	100	Y	N	B
3	61	F	U	99.7	N	N	B
4	74	F	-		N	N	B
5	70	M	M	94.6	Y	N	C
6	80	M	U	98.3	N	N	C
7	74	M	U	100	N	N	B
8	61	F	U	100	N	N	A
9	61	F	-	-	N	N	A
10	61	M	-	-	-	-	B
11	52	M	U	100	N	N	B
12	72	M	-	-	N	N	B
13	63	M	U	100	N	N	C
14	66	F	M	92.8	N	N	A
15	50	M	U	98.4	N	_	A
16	48	M	M	94	N	N	C
17	79	M	U	98	Y	N	A
18	53	F	-	-	N	N	B
19	59	F	M	88.8	N	N	C
20	63	F	U	100	Y	N	C
21	64	F	M	91.7	N	N	B
22	61	F	M	90.8	N	N	A
23	61	M	M	91.9	N	N	C
24	62	F	U	100	N	Y	C
25	49	M	-	-	-	-	B
26	68	F	M	93	N	N	A
27	59	M	U	100	N	N	B
28	46	M	M	97.2	N	N	B
29	55	M	U	99.6	N	N	A
30	75	F	U	100	N	N	C
31	67	M	U	100	N	N	A
32	62	M	U	100	N	N	A
33	68	M	U	100	N	N	C
34	67	M	M	88.6	N	Y	B
35	62	M	U	99.6	N	N	B
36	53	M	U	100	N	N	A
37	68	M	M	92.9	N	N	C
38	67	F	U	98.3	Y	N	B
39	69	M	U	100	N	Y	C
40	71	M	U	99.3	N	N	A
41	61	F	U	99.7	N	N	A
42	52	M	M	93.9	N	N	C

Continue

Patient ID	Age	gender	VH mutation status	VH Mutation %	FISH Cytogenetics		Binet Stage
					11q del	17p del	
43	60	M	U	99.6	N	N	A
44	65	M	-	-	N	N	B
45	49	M	M	94.7	N	N	B
46	63	M	M	93.1	-	-	C
47	46	M	U	99.7	N	N	A
48	61	M	M	88.5	N	N	B
49	65	M	U	100	N	N	B
50	73	M	M	95.2	N	_	B
51	48	F	M	95.9	N	N	B
52	54	M	U	100	N	N	B
53	53	M	M	90.7	N	N	C
54	63	M	U	100	N	N	A
55	59	M	M	97.6	N	N	B
56	61	M	M	95.7	N	N	B
57	58	M	U	99.7	Y	N	B
58	61	F	U	98	N	N	B
59	65	M	U	100	N	N	C
60	74	M	U	100	N	N	B
61	66	M	U	99.6	Y	N	B
62	64	M	U	100	N	N	B
63	66	F	U	99.2	Y	N	C
64	59	F	U	100	N	N	B
65	65	M	-	-	N	N	B
66	63	M	M	96.9	N	N	C
67	66	M	M	94.3	N	Y	B
68	72	M	M	97	N	N	B
69	72	M	M	97.6	Y	N	C
70	52	M	M	95.9	N	N	C
71	49	M	U	99.2	Y	N	C
72	57	M	U	100	N	N	A
73	70	M	U	98.6	Y	N	A
74	65	F	U	100	N	N	C
75	58	M	U	100	Y	N	B
76	64	M	M	94.4	N	N	B
77	77	F	U	100	Y	N	C
78	64	M	M	95.5	N	N	C
79	67	F	U	100	N	N	A
80	62	F	-	-	N	N	B
81	67	M	U	100	N	N	C
82	41	F	-	-	N	N	C
83	66	F	M	94.3	N	N	B
84	75	M	U	100	N	N	B

Continue

Patient ID	Age	gender	VH mutation status	VH Mutation %	FISH Cytogenetics		Binet Stage
					11q del	17p del	
85	63	F	M	89.2	N	N	B
86	56	M	U	100	N	N	C
87	51	M	U	98.8	N	N	B
88	68	M	U	100	Y	N	B
89	66	M	M	94.7	N	N	C
90	61	M	-	-	N	N	A
91	72	M	U	100	N	N	C
92	64	M	M	94.2	N	N	B

Appendix IV

The different proportions of miRNA in particles or plasma protein fraction in healthy Volunteers.

miR-16 copies number across plasma fractions

HV	1	4	5	7	9	10	13	16	17	19	21	22	25	29	33	37
1	487.3834	NA	323.2645	NA	5778.975	NA	10493.66	NA	36359.8	99664.93	344222	NA	NA	NA	NA	NA
2	148.8825	83.45255	NA	108.9193	NA	1669.835	928.9483	3180.992	NA	16063.64	7835.92	8869.87	289.1454	NA	NA	NA
3	NA	NA	NA	NA	202.1964	NA	302.0911	NA	909.8838	24071	64629.43	NA	20999.54	101.0982	NA	9139.759

miR-363 copies number across plasma fractions

HV	1	4	5	7	9	10	13	16	17	19	21	22	25	29	33	37
1	NA	NA	NA	NA	NA	NA	NA	1400000	NA	4750050	2182357	2282800	798980	NA	NA	NA
2	NA	NA	NA	NA	NA	128000	NA	124680	NA	326900.1	103900	272400	83120	NA	NA	NA
3	NA	NA	NA	NA	NA	NA	334000	NA	NA	997480	1799800	906800	495919.9	NA	NA	NA

miR-142 copies number across plasma fractions

HV	1	4	5	7	9	10	13	16	17	19	21	22	25	29	33	37
1	481.0556	NA	1558.342	NA	5123.151	NA	4705.144	NA	2852.244	NA	963.6005	NA	391.5299	473.4434	NA	NA
2	239.6453	379.272	NA	310.4949	NA	3020.436	648.7002	1555.545	NA	709.8781	NA	394.8543	417.2683	NA	NA	NA
3	3996.764	NA	267.3145	NA	42392.31	NA	8296.171	NA	32884.4	NA	12928.84	NA	9454.926	6749.986	190.7707	598.2193

let-7a copies number across plasma fractions

HV	1	4	5	7	9	10	13	16	17	19	21	22	25	29	33	37
1	179.744	NA	383.2763	NA	4297.689	NA	4843.92	NA	968.4005	NA	586.558	NA	575.2214	471.3538	3255.705	NA
2	1470.24	5508.547	NA	NA	NA	11562.09	NA	3617.229	NA	995.6669	NA	4120.967	227.5112	NA	NA	3055.807
3	135.072	NA	NA	NA	261.5116	NA	821.7476	620.1488	162.753	NA	428.0308	620.1488	100.2624	115.0681	620.1488	2316.596

The different proportions of miRNA in particles or plasma protein fraction in CLL Patients.

miR-16 copies number across plasma fractions

	1	5	9	13	17	21	25	29	33	37
Pt 1	409.2638	272.8426	26192.8	41593.33	376992.6	953994	56205.57	4608.008	55493.15	NA
Pt 2	166.9928	111.9968	404.998	4859.999	12000.13	43899.88	7209.993	320.3291	4859.681	144.9988
Pt 3	15800	188000	56500	40000	106000	298000	39500	14000	NA	NA
Pt 4	4299.998	20400	219000	27700	238000	191000	1880.003	198.9987	683.9985	683.9985

miR-363 copies number across plasma fractions

	1	5	9	13	17	21	25	29	33	37
Pt 1	273003.8	220995	96198.1	112005.9	340989.3	1229989	112995.4	27199.02	NA	NA
Pt 2	NA	178996	NA	NA	1099995	597008.9	176000	NA	NA	NA
Pt 3	15800	188000	56500	40000	106000	298000	39500	14000	NA	NA
Pt 4	NA	NA	129000	NA	93000	59100.01	NA	NA	NA	NA

miR-142 copies number across plasma fractions

	1	5	9	13	17	21	25	29	33	37
Pt 1	331.1271	786.8932	2910.035	3104.783	2944.815	316.425	248.9488	328.7133	NA	NA
Pt 2	907.9496	577.8741	7419.439	25076.06	37089.45	15982.04	6881.251	1640.698	1064.76	156.8101
Pt 3	302.8629	1913.45	855.6852	1843.304	1503.19	942.7033	2888.518	507.778	475.2959	801.0471
Pt 4	772.1595	571.4971	3641.095	477.7998	936.1426	623.709	376.2937	384.4289	1414.433	1144.524

let-7a copies number across plasma fractions

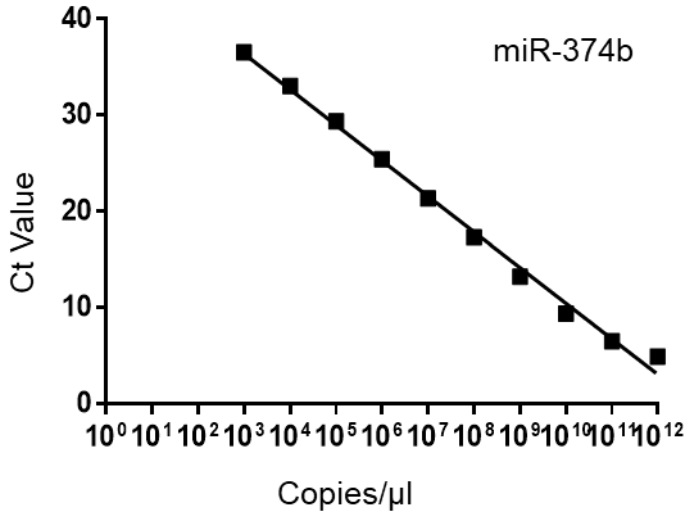
	1	5	9	13	17	21	25	29	33	37
Pt 1	16909.39	1747.129	16708.6	79900.41	8029.899	6807.208	19721.87	14.98395	NA	NA
Pt 2	NA	NA	208.479	6321.433	2708.186	1376.69	106.5721	NA	2964.775	892.8149
Pt 3	NA	213.937	538.6691	1095.605	810.5464	331.3508	31.86802	448.3769	295.7209	330.2783
Pt 4	NA	390.1998	2344.871	245.342	1100.231	633.9179	64.89394	95.06877	NA	8230.837

MiRNA copies number generated by using fractionate plasma samples from healthy volunteers n=3 (fractions: 1,4,5,7,9,10,13,16,17,19,21,22,25,29,33,37) and patients (n = 4) (fractions: 1,5,9,13,17,21,25,29, 33,37). To determine the distribution of miRNA between vesicles bound and plasma protein fractions we carried out size exclusion chromatography followed by quantitative RT-PCR. Note that fraction 13 and less represents miRNA levels with vesicles.

Appendix V

Standard curves for quantitative RT-PCR.

Standard curve was constructed for mir-374b (sequence (5'-3): AUAUAAUACAACCUGUUAAGUG). Using known number of copy of oligonucleotide as template RT-PCR was carried out and Ct values obtained. Trend line was interpolated using GraphPad Prism v6.0



References

Ahearne, M. J., S. Willimott, L. Pinon, D. B. Kennedy, F. Miall, M. J. Dyer and S. D. Wagner (2013). "Enhancement of CD154/IL4 proliferation by the T follicular helper (Tfh) cytokine, IL21 and increased numbers of circulating cells resembling Tfh cells in chronic lymphocytic leukaemia." Br J Haematol **162**(3): 360-370.

Aisenberg, A. C. and K. J. Bloch (1972). "Immunoglobulins on the surface of neoplastic lymphocytes." N Engl J Med **287**(6): 272-276.

Al-Nedawi, K., B. Meehan, J. Micallef, V. Lhotak, L. May, A. Guha and J. Rak (2008). "Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells." Nat Cell Biol **10**(5): 619-624.

Alharthi, A., D. Beck, D. R. Howard, P. Hillmen, M. Oates, A. Pettitt and S. D. Wagner (2018). "An increased fraction of circulating miR-363 and miR-16 is particle bound in patients with chronic lymphocytic leukaemia as compared to normal subjects." BMC Res Notes **11**(1): 280.

Andre, F., N. E. Schartz, M. Movassagh, C. Flament, P. Pautier, P. Morice, C. Pomel, C. Lhomme, B. Escudier, T. Le Chevalier, T. Tursz, S. Amigorena, G. Raposo, E. Angevin and L. Zitvogel (2002). "Malignant effusions and immunogenic tumour-derived exosomes." Lancet **360**(9329): 295-305.

Arroyo, J. D., J. R. Chevillet, E. M. Kroh, I. K. Ruf, C. C. Pritchard, D. F. Gibson, P. S. Mitchell, C. F. Bennett, E. L. Pogosova-Agadjanyan, D. L. Stirewalt, J. F. Tait and M. Tewari (2011). "Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma." Proc Natl Acad Sci U S A **108**(12): 5003-5008.

Aydin, S., F. Grabellus, L. Eisele, M. Mollmann, M. Hanoun, P. Ebeling, T. Moritz, A. Carpinteiro, H. Nuckel, A. Sak, J. R. Gothert, U. Duhrsen and J. Durig (2011). "Investigating the role of CD38 and functionally related molecular risk factors in the CLL NOD/SCID xenograft model." Eur J Haematol **87**(1): 10-19.

Bagnara, D., M. S. Kaufman, C. Calissano, S. Marsilio, P. E. Patten, R. Simone, P. Chum, X. J. Yan, S. L. Allen, J. E. Kolitz, S. Baskar, C. Rader, H. Mellstedt, H. Rabbani, A. Lee, P. K. Gregersen, K. R. Rai and N. Chiorazzi (2011). "A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease." Blood **117**(20): 5463-5472.

Bartels, C. L. and G. J. Tsongalis (2009). "MicroRNAs: novel biomarkers for human cancer." Clin Chem **55**(4): 623-631.

Berkova, A., Z. Zemanova, M. Trneny, J. Schwarz, J. Karban, E. Cmunt, L. Pavlistova, J. Brezinova and K. Michalova (2009). "Clonal evolution in chronic lymphocytic leukemia studied by interphase fluorescence in-situ hybridization." Neoplasma **56**(5): 455-458.

Bertilaccio, M. T., C. Scielzo, G. Simonetti, M. Ponzoni, B. Apollonio, C. Fazi, L. Scarfo, M. Rocchi, M. Muzio, F. Caligaris-Cappio and P. Ghia (2010). "A novel Rag2-/-gammac-/--xenograft model of human CLL." Blood **115**(8): 1605-1609.

Bian, W. G., X. N. Zhou, S. Song, H. T. Chen, Y. Shen and P. Chen (2019). "Reduced miR-363-3p expression in non-small cell lung cancer is associated with gemcitabine resistance via targeting of CUL4A." Eur Rev Med Pharmacol Sci **23**(2): 649-659.

Bichi, R., S. A. Shinton, E. S. Martin, A. Koval, G. A. Calin, R. Cesari, G. Russo, R. R. Hardy and C. M. Croce (2002). "Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression." Proc Natl Acad Sci U S A **99**(10): 6955-6960.

Binet, J. L., A. Auquier, G. Dighiero, C. Chastang, H. Piguet, J. Goasguen, G. Vaugier, G. Potron, P. Colona, F. Oberling, M. Thomas, G. Tchernia, C. Jacquillat, P. Boivin, C. Lesty, M. T. Duault, M. Monconduit, S. Belabbes and F. Gremy (1981). "A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis." Cancer **48**(1): 198-206.

Blair, P. A., L. Y. Norena, F. Flores-Borja, D. J. Rawlings, D. A. Isenberg, M. R. Ehrenstein and C. Mauri (2010). "CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in

healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients." Immunity **32**(1): 129-140.

Bomstein, Y., M. Yuklea, J. Radnay, H. Shapiro, F. Afanasyev, S. Yarkoni and M. Lishner (2003). "The antiapoptotic effects of blood constituents in patients with chronic lymphocytic leukemia." Eur J Haematol **70**(5): 290-295.

Boonstra, J. G., K. van Lom, A. W. Langerak, W. J. Graveland, P. J. Valk, J. Kraan, M. B. van 't Veer and J. W. Gratama (2006). "CD38 as a prognostic factor in B cell chronic lymphocytic leukaemia (B-CLL): comparison of three approaches to analyze its expression." Cytometry B Clin Cytom **70**(3): 136-141.

Brecher, M. and P. M. Banks (1990). "Hodgkin's disease variant of Richter's syndrome. Report of eight cases." Am J Clin Pathol **93**(3): 333-339.

Buning, J., D. von Smolinski, K. Tafazzoli, K. P. Zimmer, S. Strobel, M. Apostolaki, G. Kollias, J. K. Heath, D. Ludwig and A. Gebert (2008). "Multivesicular bodies in intestinal epithelial cells: responsible for MHC class II-restricted antigen processing and origin of exosomes." Immunology **125**(4): 510-521.

Burger, J. A., K. W. Li, M. J. Keating, M. Sivina, A. M. Amer, N. Garg, A. Ferrajoli, X. Huang, H. Kantarjian, W. G. Wierda, S. O'Brien, M. K. Hellerstein, S. M. Turner, C. L. Emson, S. S. Chen, X. J. Yan, D. Wodarz and N. Chiorazzi (2017). "Leukemia cell proliferation and death in chronic lymphocytic leukemia patients on therapy with the BTK inhibitor ibrutinib." JCI Insight **2**(2): e89904.

Burger, J. A. and S. O'Brien (2018). "Evolution of CLL treatment - from chemoimmunotherapy to targeted and individualized therapy." Nat Rev Clin Oncol.

Burger, J. A., N. Tsukada, M. Burger, N. J. Zvaifler, M. Dell'Aquila and T. J. Kipps (2000). "Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1." Blood **96**(8): 2655-2663.

Caligaris-Cappio, F. and P. Ghia (2008). "Novel insights in chronic lymphocytic leukemia: are we getting closer to understanding the pathogenesis of the disease?" J Clin Oncol **26**(27): 4497-4503.

Calin, G. A., C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich and C. M. Croce (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-15529.

Calin, G. A., M. Ferracin, A. Cimmino, G. Di Leva, M. Shimizu, S. E. Wojcik, M. V. Iorio, R. Visone, N. I. Sever, M. Fabbri, R. Iuliano, T. Palumbo, F. Pichiorri, C. Roldo, R. Garzon, C. Sevignani, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini and C. M. Croce (2005). "A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia." N Engl J Med **353**(17): 1793-1801.

Calin, G. A., C. G. Liu, C. Sevignani, M. Ferracin, N. Felli, C. D. Dumitru, M. Shimizu, A. Cimmino, S. Zupo, M. Dono, M. L. Dell'Aquila, H. Alder, L. Rassenti, T. J. Kipps, F. Bullrich, M. Negrini and C. M. Croce (2004). "MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias." Proc Natl Acad Sci U S A **101**(32): 11755-11760.

Carter, N. A., R. Vasconcellos, E. C. Rosser, C. Tulone, A. Munoz-Suano, M. Kamanaka, M. R. Ehrenstein, R. A. Flavell and C. Mauri (2011). "Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells." J Immunol **186**(10): 5569-5579.

Cartwright, R. A., K. A. Gurney and A. V. Moorman (2002). "Sex ratios and the risks of haematological malignancies." Br J Haematol **118**(4): 1071-1077.

Catera, R., G. J. Silverman, K. Hatzi, T. Seiler, S. Didier, L. Zhang, M. Herve, E. Meffre, D. G. Oscier, H. Vlassara, R. H. Scofield, Y. Chen, S. L. Allen, J. Kolitz, K. R. Rai, C. C. Chu and N. Chiorazzi (2008). "Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation." Mol Med **14**(11-12): 665-674.

Catovsky, D., S. Richards, E. Matutes, D. Oscier, M. Dyer, R. F. Bezares, A. R. Pettitt, T. Hamblin, D. W. Milligan, J. A. Child, M. S. Hamilton, C. E. Dearden, A. G. Smith, A. G. Bosanquet, Z. Davis, V. Brito-Babapulle, M. Else, R. Wade and P. Hillmen (2007). "Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial." Lancet **370**(9583): 230-239.

Chandrasekaran, K. S., A. Sathyanarayanan and D. Karunagaran (2016). "MicroRNA-214 suppresses growth, migration and invasion through a novel target, high mobility group AT-hook 1, in human cervical and colorectal cancer cells." Br J Cancer **115**(6): 741-751.

Chapman, B. V., A. I. Wald, P. Akhtar, A. C. Munko, J. Xu, S. P. Gibson, J. R. Grandis, R. L. Ferris and S. A. Khan (2015). "MicroRNA-363 targets myosin 1B to reduce cellular migration in head and neck cancer." BMC Cancer **15**: 861.

Chaudhry, A. and A. Y. Rudensky (2013). "Control of inflammation by integration of environmental cues by regulatory T cells." J Clin Invest **123**(3): 939-944.

Chen, X., Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang and C. Y. Zhang (2008). "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases." Cell Res **18**(10): 997-1006.

Chen, Y., X. Lu, B. Wu, Y. Su, J. Li and H. Wang (2015). "MicroRNA 363 mediated positive regulation of c-myc translation affect prostate cancer development and progress." Neoplasma **62**(2): 191-198.

Cheng, H. H., P. S. Mitchell, E. M. Kroh, A. E. Dowell, L. Chery, J. Siddiqui, P. S. Nelson, R. L. Vessella, B. S. Knudsen, A. M. Chinnaiyan, K. J. Pienta, C. Morrissey and M. Tewari (2013). "Circulating microRNA profiling identifies a subset of metastatic prostate cancer patients with evidence of cancer-associated hypoxia." PLoS One **8**(7): e69239.

Chiorazzi, N. (2007). "Cell proliferation and death: forgotten features of chronic lymphocytic leukemia B cells." Best Pract Res Clin Haematol **20**(3): 399-413.

Chiorazzi, N. and M. Ferrarini (2003). "B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor." Annu Rev Immunol **21**: 841-894.

Chiorazzi, N., K. Hatzi and E. Albesiano (2005). "B-cell chronic lymphocytic leukemia, a clonal disease of B lymphocytes with receptors that vary in specificity for (auto)antigens." Ann N Y Acad Sci **1062**: 1-12.

Cimmino, A., G. A. Calin, M. Fabbri, M. V. Iorio, M. Ferracin, M. Shimizu, S. E. Wojcik, R. I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini and C. M. Croce (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." Proc Natl Acad Sci U S A **102**(39): 13944-13949.

Cortez, M. A., C. Bueso-Ramos, J. Ferdin, G. Lopez-Berestein, A. K. Sood and G. A. Calin (2011). "MicroRNAs in body fluids--the mix of hormones and biomarkers." Nat Rev Clin Oncol **8**(8): 467-477.

Cortez, M. A. and G. A. Calin (2009). "MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases." Expert Opin Biol Ther **9**(6): 703-711.

Cui, B., L. Chen, S. Zhang, M. Mraz, J. F. Fecteau, J. Yu, E. M. Ghia, L. Zhang, L. Bao, L. Z. Rassenti, K. Messer, G. A. Calin, C. M. Croce and T. J. Kipps (2014). "MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia." Blood **124**(4): 546-554.

Cyster, J. G. and S. R. Schwab (2012). "Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs." Annu Rev Immunol **30**: 69-94.

D'Arena, G., P. Musto, G. Nunziata, N. Cascavilla, L. Savino and G. Pistolese (2001). "CD69 expression in B-cell chronic lymphocytic leukemia: a new prognostic marker ?" Haematologica **86**(9): 995-996.

Damle, R. N., F. Ghiotto, A. Valetto, E. Albesiano, F. Fais, X. J. Yan, C. P. Sison, S. L. Allen, J. Kolitz, P. Schulman, V. P. Vinciguerra, P. Budde, J. Frey, K. R. Rai, M. Ferrarini and N. Chiorazzi (2002). "B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes." Blood **99**(11): 4087-4093.

Damle, R. N., T. Wasil, F. Fais, F. Ghiotto, A. Valetto, S. L. Allen, A. Buchbinder, D. Budman, K. Dittmar, J. Kolitz, S. M. Lichtman, P. Schulman, V. P. Vinciguerra, K. R. Rai, M. Ferrarini and N. Chiorazzi (1999). "Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia." Blood **94**(6): 1840-1847.

De La Pena, H., J. A. Madrigal, S. Rusakiewicz, M. Bencsik, G. W. Cave, A. Selman, R. C. Rees, P. J. Travers and I. A. Dodi (2009). "Artificial exosomes as tools for basic and clinical immunology." J Immunol Methods **344**(2): 121-132.

Decker, K., D. C. Goldman, C. L. Grasch and L. Sussel (2006). "Gata6 is an important regulator of mouse pancreas development." Dev Biol **298**(2): 415-429.

Del Conde, I., C. N. Shrimpton, P. Thiagarajan and J. A. Lopez (2005). "Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation." Blood **106**(5): 1604-1611.

Deng, J., Y. Liu, H. Lee, A. Herrmann, W. Zhang, C. Zhang, S. Shen, S. J. Priceman, M. Kujawski, S. K. Pal, A. Raubitschek, D. S. Hoon, S. Forman, R. A. Figlin, J. Liu, R. Jove and H. Yu (2012). "S1PR1-STAT3 signaling is crucial for myeloid cell colonization at future metastatic sites." Cancer Cell **21**(5): 642-654.

Denzer, K., M. J. Kleijmeer, H. F. Heijnen, W. Stoorvogel and H. J. Geuze (2000). "Exosome: from internal vesicle of the multivesicular body to intercellular signaling device." J Cell Sci **113 Pt 19**: 3365-3374.

Dighiero, G., K. Maloum, B. Desablens, B. Cazin, M. Navarro, R. Leblay, M. Leporrier, J. Jaubert, G. Lepeu, B. Dreyfus, J. L. Binet and P. Travade (1998). "Chlorambucil in

indolent chronic lymphocytic leukemia. French Cooperative Group on Chronic Lymphocytic Leukemia." N Engl J Med **338**(21): 1506-1514.

Ding, W. and C. S. Zent (2007). "Diagnosis and management of autoimmune complications of chronic lymphocytic leukemia/ small lymphocytic lymphoma." Clin Adv Hematol Oncol **5**(4): 257-261.

Dohner, H., S. Stilgenbauer, A. Benner, E. Leupolt, A. Krober, L. Bullinger, K. Dohner, M. Bentz and P. Lichter (2000). "Genomic aberrations and survival in chronic lymphocytic leukemia." N Engl J Med **343**(26): 1910-1916.

Dohner, H., S. Stilgenbauer, K. Fischer, M. Schroder, M. Bentz and P. Lichter (1995). "Diagnosis and monitoring of chromosome aberrations in hematological malignancies by fluorescence in situ hybridization." Stem Cells **13 Suppl 3**: 76-82.

Dragovic, R. A., C. Gardiner, A. S. Brooks, D. S. Tannetta, D. J. Ferguson, P. Hole, B. Carr, C. W. Redman, A. L. Harris, P. J. Dobson, P. Harrison and I. L. Sargent (2011). "Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis." Nanomedicine **7**(6): 780-788.

Drennan, S., A. D'Avola, Y. Gao, C. Weigel, E. Chrysostomou, A. J. Steele, T. Zenz, C. Plass, P. W. Johnson, A. P. Williams, G. Packham, F. K. Stevenson, C. C. Oakes and F. Forconi (2017). "IL-10 production by CLL cells is enhanced in the anergic IGHV mutated subset and associates with reduced DNA methylation of the IL10 locus." Leukemia **31**(8): 1686-1694.

Duhren-von Minden, M., R. Ubelhart, D. Schneider, T. Wossning, M. P. Bach, M. Buchner, D. Hofmann, E. Surova, M. Follo, F. Kohler, H. Wardemann, K. Zirlik, H. Veelken and H. Jumaa (2012). "Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling." Nature **489**(7415): 309-312.

Durig, J., P. Ebeling, F. Grabellus, U. R. Sorg, M. Mollmann, P. Schutt, J. Gothert, L. Sellmann, S. Seeber, M. Flasshove, U. Duhrsen and T. Moritz (2007). "A novel nonobese

diabetic/severe combined immunodeficient xenograft model for chronic lymphocytic leukemia reflects important clinical characteristics of the disease." Cancer Res **67**(18): 8653-8661.

Elter, T., P. Borchmann, H. Schulz, M. Reiser, S. Trelle, R. Schnell, M. Jensen, P. Staib, T. Schinkothe, H. Stutzer, J. Rech, M. Gramatzki, W. Aulitzky, I. Hasan, A. Josting, M. Hallek and A. Engert (2005). "Fludarabine in combination with alemtuzumab is effective and feasible in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: results of a phase II trial." J Clin Oncol **23**(28): 7024-7031.

Esquela-Kerscher, A. and F. J. Slack (2006). "Oncomirs - microRNAs with a role in cancer." Nat Rev Cancer **6**(4): 259-269.

Esteve, J., N. Villamor, D. Colomer, F. Bosch, A. Lopez-Guillermo, M. Rovira, A. Urbano-Ispizua, J. Sierra, E. Carreras and E. Montserrat (1998). "Hematopoietic stem cell transplantation in chronic lymphocytic leukemia: a report of 12 patients from a single institution." Ann Oncol **9**(2): 167-172.

Falay, M. and G. Ozet (2017). "Immunophenotyping of Chronic Lymphocytic Leukemia." Clin Lab **63**(10): 1621-1626.

Farahani, M., C. Rubbi, L. Liu, J. R. Slupsky and N. Kalakonda (2015). "CLL Exosomes Modulate the Transcriptome and Behaviour of Recipient Stromal Cells and Are Selectively Enriched in miR-202-3p." PLoS One **10**(10): e0141429.

Feng, F., D. Kuai, H. Wang, T. Li, W. Miao, Y. Liu and Y. Fan (2016). "Reduced expression of microRNA-497 is associated with greater angiogenesis and poor prognosis in human gliomas." Hum Pathol **58**: 47-53.

Feo, S., D. Arcuri, E. Piddini, R. Passantino and A. Giallongo (2000). "ENO1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1)." FEBS Lett **473**(1): 47-52.

Ferrajoli, A., T. D. Shanafelt, C. Ivan, M. Shimizu, K. G. Rabe, N. Nouraee, M. Ikuo, A. K. Ghosh, S. Lerner, L. Z. Rassenti, L. Xiao, J. Hu, J. M. Reuben, S. Calin, M. J. You, J. T. Manning, W. G. Wierda, Z. Estrov, S. O'Brien, T. J. Kipps, M. J. Keating, N. E. Kay and G. A. Calin (2013). "Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia." Blood **122**(11): 1891-1899.

Flores-Borja, F., A. Bosma, D. Ng, V. Reddy, M. R. Ehrenstein, D. A. Isenberg and C. Mauri (2013). "CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation." Sci Transl Med **5**(173): 173ra123.

Fulci, V., S. Chiaretti, M. Goldoni, G. Azzalin, N. Carucci, S. Tavolaro, L. Castellano, A. Magrelli, F. Citarella, M. Messina, R. Maggio, N. Peragine, S. Santangelo, F. R. Mauro, P. Landgraf, T. Tuschl, D. B. Weir, M. Chien, J. J. Russo, J. Ju, R. Sheridan, C. Sander, M. Zavolan, A. Guarini, R. Foa and G. Macino (2007). "Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia." Blood **109**(11): 4944-4951.

Fuller, S. J., E. Papaemmanuil, L. McKinnon, E. Webb, G. S. Sellick, L. P. Dao-Ung, K. K. Skarratt, D. Crowther, R. S. Houlston and J. S. Wiley (2008). "Analysis of a large multi-generational family provides insight into the genetics of chronic lymphocytic leukemia." Br J Haematol **142**(2): 238-245.

Furman, R. R., J. P. Sharman, S. E. Coutre, B. D. Cheson, J. M. Pagel, P. Hillmen, J. C. Barrientos, A. D. Zelenetz, T. J. Kipps, I. Flinn, P. Ghia, H. Eradat, T. Ervin, N. Lamanna, B. Coiffier, A. R. Pettitt, S. Ma, S. Stilgenbauer, P. Cramer, M. Aiello, D. M. Johnson, L. L. Miller, D. Li, T. M. Jahn, R. D. Dansey, M. Hallek and S. M. O'Brien (2014). "Idelalisib and rituximab in relapsed chronic lymphocytic leukemia." N Engl J Med **370**(11): 997-1007.

Gabriely, G., T. Wurdinger, S. Kesari, C. C. Esau, J. Burchard, P. S. Linsley and A. M. Krichevsky (2008). "MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators." Mol Cell Biol **28**(17): 5369-5380.

Gao, X., T. Sedgwick, Y. B. Shi and T. Evans (1998). "Distinct functions are implicated for the GATA-4, -5, and -6 transcription factors in the regulation of intestine epithelial cell differentiation." Mol Cell Biol **18**(5): 2901-2911.

Ghia, P., N. Chiorazzi and K. Stamatopoulos (2008). "Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation." J Intern Med **264**(6): 549-562.

Ghosh, A. K., C. R. Secreto, T. R. Knox, W. Ding, D. Mukhopadhyay and N. E. Kay (2010). "Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression." Blood **115**(9): 1755-1764.

Goldin, L. R. and N. E. Caporaso (2007). "Family studies in chronic lymphocytic leukaemia and other lymphoproliferative tumours." Br J Haematol **139**(5): 774-779.

Gonzales, P. A., T. Pisitkun, J. D. Hoffert, D. Tchapyjnikov, R. A. Star, R. Kleta, N. S. Wang and M. A. Knepper (2009). "Large-scale proteomics and phosphoproteomics of urinary exosomes." J Am Soc Nephrol **20**(2): 363-379.

Gould, S. J. and G. Raposo (2013). "As we wait: coping with an imperfect nomenclature for extracellular vesicles." J Extracell Vesicles **2**.

Grasedieck, S., N. Scholer, M. Bommer, J. H. Niess, H. Tumani, A. Rouhi, J. Bloehdorn, P. Liebisch, D. Mertens, H. Dohner, C. Buske, C. Langer and F. Kuchenbauer (2012). "Impact of serum storage conditions on microRNA stability." Leukemia **26**(11): 2414-2416.

Grasedieck, S., A. Sorrentino, C. Langer, C. Buske, H. Dohner, D. Mertens and F. Kuchenbauer (2013). "Circulating microRNAs in hematological diseases: principles, challenges, and perspectives." Blood **121**(25): 4977-4984.

Grever, M. R., D. M. Lucas, A. J. Johnson and J. C. Byrd (2007). "Novel agents and strategies for treatment of p53-defective chronic lymphocytic leukemia." Best Pract Res Clin Haematol **20**(3): 545-556.

Gribben, J. G. (2009). "Stem cell transplantation in chronic lymphocytic leukemia." Biol Blood Marrow Transplant **15**(1 Suppl): 53-58.

Gustafson, D., S. Veitch and J. E. Fish (2017). "Extracellular Vesicles as Protagonists of Diabetic Cardiovascular Pathology." Front Cardiovasc Med **4**: 71.

Haderk, F., R. Schulz, M. Iskar, L. L. Cid, T. Worst, K. V. Willmund, A. Schulz, U. Warnken, J. Seiler, A. Benner, M. Nessling, T. Zenz, M. Gobel, J. Durig, S. Diederichs, J. Paggetti, E. Moussay, S. Stilgenbauer, M. Zapatka, P. Lichter and M. Seiffert (2017). "Tumor-derived exosomes modulate PD-L1 expression in monocytes." Sci Immunol **2**(13).

Hallek, M. (2005). "Chronic lymphocytic leukemia (CLL): first-line treatment." Hematology Am Soc Hematol Educ Program: 285-291.

Hallek, M., B. D. Cheson, D. Catovsky, F. Caligaris-Cappio, G. Dighiero, H. Dohner, P. Hillmen, M. J. Keating, E. Montserrat, K. R. Rai and T. J. Kipps (2008). "Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines." Blood **111**(12): 5446-5456.

Hamblin, T. (2000). "Historical aspects of chronic lymphocytic leukaemia." Br J Haematol **111**(4): 1023-1034.

Hamblin, T. J., Z. Davis, A. Gardiner, D. G. Oscier and F. K. Stevenson (1999). "Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia." Blood **94**(6): 1848-1854.

Hamblin, T. J., J. A. Orchard, R. E. Ibbotson, Z. Davis, P. W. Thomas, F. K. Stevenson and D. G. Oscier (2002). "CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease." Blood **99**(3): 1023-1029.

Handunnetti, S. M., A. Polliack and C. S. Tam (2017). "Microvesicles in chronic lymphocytic leukemia: ready for prime time in the clinic?" Leuk Lymphoma **58**(6): 1281-1282.

Hayes, J., P. P. Peruzzi and S. Lawler (2014). "MicroRNAs in cancer: biomarkers, functions and therapy." Trends Mol Med **20**(8): 460-469.

He, F., L. Fang and Q. Yin (2019). "miR-363 acts as a tumor suppressor in osteosarcoma cells by inhibiting PDZD2." Oncol Rep **41**(5): 2729-2738.

Herishanu, Y., B. Z. Katz, A. Lipsky and A. Wiestner (2013). "Biology of chronic lymphocytic leukemia in different microenvironments: clinical and therapeutic implications." Hematol Oncol Clin North Am **27**(2): 173-206.

Herman, S. E., A. L. Gordon, E. Hertlein, A. Ramanunni, X. Zhang, S. Jaglowski, J. Flynn, J. Jones, K. A. Blum, J. J. Buggy, A. Hamdy, A. J. Johnson and J. C. Byrd (2011). "Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765." Blood **117**(23): 6287-6296.

Hoogeboom, R., K. P. van Kessel, F. Hochstenbach, T. A. Wormhoudt, R. J. Reinten, K. Wagner, A. P. Kater, J. E. Guikema, R. J. Bende and C. J. van Noesel (2013). "A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi." J Exp Med **210**(1): 59-70.

Houlston, R. S., G. Sellick, M. Yuille, E. Matutes and D. Catovsky (2003). "Causation of chronic lymphocytic leukemia--insights from familial disease." Leuk Res **27**(10): 871-876.

Howard, D. R., T. Munir, L. McParland, A. C. Rawstron, A. Chalmers, W. M. Gregory, J. L. O'Dwyer, A. Smith, R. Longo, A. Varghese, A. Smith and P. Hillmen (2017). "Clinical effectiveness and cost-effectiveness results from the randomised, Phase IIB trial in previously untreated patients with chronic lymphocytic leukaemia to compare fludarabine, cyclophosphamide and rituximab with fludarabine, cyclophosphamide,

mitoxantrone and low-dose rituximab: the Attenuated dose Rituximab with ChemoTherapy In Chronic lymphocytic leukaemia (ARCTIC) trial." Health Technol Assess **21**(28): 1-374.

Hsu, K. W., R. H. Hsieh, C. W. Wu, C. W. Chi, Y. H. Lee, M. L. Kuo, K. J. Wu and T. S. Yeh (2009). "MBP-1 suppresses growth and metastasis of gastric cancer cells through COX-2." Mol Biol Cell **20**(24): 5127-5137.

Hsu, K. W., A. M. Wang, Y. H. Ping, K. H. Huang, T. T. Huang, H. C. Lee, S. S. Lo, C. W. Chi and T. S. Yeh (2014). "Downregulation of tumor suppressor MBP-1 by microRNA-363 in gastric carcinogenesis." Carcinogenesis **35**(1): 208-217.

Hu, F., J. Min, X. Cao, L. Liu, Z. Ge, J. Hu and X. Li (2016). "MiR-363-3p inhibits the epithelial-to-mesenchymal transition and suppresses metastasis in colorectal cancer by targeting Sox4." Biochem Biophys Res Commun **474**(1): 35-42.

Huang, T., K. She, G. Peng, W. Wang, J. Huang, J. Li, Z. Wang and J. He (2016). "MicroRNA-186 suppresses cell proliferation and metastasis through targeting MAP3K2 in non-small cell lung cancer." Int J Oncol **49**(4): 1437-1444.

Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J. P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini and C. M. Croce (2005). "MicroRNA gene expression deregulation in human breast cancer." Cancer Res **65**(16): 7065-7070.

Jacob, A., J. D. Pound, A. Challa and J. Gordon (1998). "Release of clonal block in B cell chronic lymphocytic leukaemia by engagement of co-operative epitopes on CD40." Leuk Res **22**(4): 379-382.

Jiang, C., Y. Cao, T. Lei, Y. Wang, J. Fu, Z. Wang and Z. Lv (2018). "microRNA-363-3p inhibits cell growth and invasion of nonsmall cell lung cancer by targeting HMGA2." Mol Med Rep **17**(2): 2712-2718.

Johnson, M. L., C. Navanukraw, A. T. Grazul-Bilska, L. P. Reynolds and D. A. Redmer (2003). "Heparinase treatment of RNA before quantitative real-time RT-PCR." Biotechniques **35**(6): 1140-1142, 1144.

Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr and C. Turbide (1987). "Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)." J Biol Chem **262**(19): 9412-9420.

Jones, K., J. P. Nourse, C. Keane, A. Bhatnagar and M. K. Gandhi (2014). "Plasma microRNA are disease response biomarkers in classical Hodgkin lymphoma." Clin Cancer Res **20**(1): 253-264.

Kapsogeorgou, E. K., R. F. Abu-Helu, H. M. Moutsopoulos and M. N. Manoussakis (2005). "Salivary gland epithelial cell exosomes: A source of autoantigenic ribonucleoproteins." Arthritis Rheum **52**(5): 1517-1521.

Kater, A. P., L. M. Evers, E. B. Remmerswaal, A. Jaspers, M. F. Oosterwijk, R. A. van Lier, M. H. van Oers and E. Eldering (2004). "CD40 stimulation of B-cell chronic lymphocytic leukaemia cells enhances the anti-apoptotic profile, but also Bid expression and cells remain susceptible to autologous cytotoxic T-lymphocyte attack." Br J Haematol **127**(4): 404-415.

Kaur, H., A. Arora, J. Wengel and S. Maiti (2006). "Thermodynamic, counterion, and hydration effects for the incorporation of locked nucleic acid nucleotides into DNA duplexes." Biochemistry **45**(23): 7347-7355.

Keating, M. J., N. Chiorazzi, B. Messmer, R. N. Damle, S. L. Allen, K. R. Rai, M. Ferrarini and T. J. Kipps (2003). "Biology and treatment of chronic lymphocytic leukemia." Hematology Am Soc Hematol Educ Program: 153-175.

Khoder, A., A. Sarvaria, A. Alsuliman, C. Chew, T. Sekine, N. Cooper, S. Mielke, H. de Lavallade, M. Muftuoglu, I. Fernandez Curbelo, E. Liu, P. A. Muraro, A. Alousi, K. Stringaris, S. Parmar, N. Shah, H. Shaim, E. Yvon, J. Molldrem, R. Rouce, R. Champlin, I.

McNiece, C. Mauri, E. J. Shpall and K. Rezvani (2014). "Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD." Blood **124**(13): 2034-2045.

Klein, U., M. Lia, M. Crespo, R. Siegel, Q. Shen, T. Mo, A. Ambesi-Impiombato, A. Califano, A. Migliazza, G. Bhagat and R. Dalla-Favera (2010). "The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia." Cancer Cell **17**(1): 28-40.

Koga, K., K. Matsumoto, T. Akiyoshi, M. Kubo, N. Yamanaka, A. Tasaki, H. Nakashima, M. Nakamura, S. Kuroki, M. Tanaka and M. Katano (2005). "Purification, characterization and biological significance of tumor-derived exosomes." Anticancer Res **25**(6a): 3703-3707.

Korz, C., A. Pscherer, A. Benner, D. Mertens, C. Schaffner, E. Leupolt, H. Dohner, S. Stilgenbauer and P. Lichter (2002). "Evidence for distinct pathomechanisms in B-cell chronic lymphocytic leukemia and mantle cell lymphoma by quantitative expression analysis of cell cycle and apoptosis-associated genes." Blood **99**(12): 4554-4561.

Kosaka, N., H. Iguchi and T. Ochiya (2010). "Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis." Cancer Sci **101**(10): 2087-2092.

Koutsourakis, M., A. Langeveld, R. Patient, R. Beddington and F. Grosveld (1999). "The transcription factor GATA6 is essential for early extraembryonic development." Development **126**(9): 723-732.

Krober, A., T. Seiler, A. Benner, L. Bullinger, E. Bruckle, P. Lichter, H. Dohner and S. Stilgenbauer (2002). "V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia." Blood **100**(4): 1410-1416.

Lakkaraju, A. and E. Rodriguez-Boulan (2008). "Itinerant exosomes: emerging roles in cell and tissue polarity." Trends Cell Biol **18**(5): 199-209.

Lawrie, C. H., S. Gal, H. M. Dunlop, B. Pushkaran, A. P. Liggins, K. Pulford, A. H. Banham, F. Pezzella, J. Boultwood, J. S. Wainscoat, C. S. Hatton and A. L. Harris (2008). "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma." Br J Haematol **141**(5): 672-675.

Laytragoon-Lewin, N., E. Duhony, X. F. Bai and H. Mellstedt (1998). "Downregulation of the CD95 receptor and defect CD40-mediated signal transduction in B-chronic lymphocytic leukemia cells." Eur J Haematol **61**(4): 266-271.

Lee, H., J. Deng, M. Kujawski, C. Yang, Y. Liu, A. Herrmann, M. Kortylewski, D. Horne, G. Somlo, S. Forman, R. Jove and H. Yu (2010). "STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors." Nat Med **16**(12): 1421-1428.

Lens, M. and V. Bataille (2008). "Melanoma in relation to reproductive and hormonal factors in women: current review on controversial issues." Cancer Causes Control **19**(5): 437-442.

Levesque, M. C., M. A. Misukonis, C. W. O'Loughlin, Y. Chen, B. E. Beasley, D. L. Wilson, D. J. Adams, R. Silber and J. B. Weinberg (2003). "IL-4 and interferon gamma regulate expression of inducible nitric oxide synthase in chronic lymphocytic leukemia cells." Leukemia **17**(2): 442-450.

Li, D. and D. W. Chan (2014). "Proteomic cancer biomarkers from discovery to approval: it's worth the effort." Expert Rev Proteomics **11**(2): 135-136.

Li, P., H. Liu, Z. Wang, F. He, H. Wang, Z. Shi, A. Yang and J. Ye (2016). "MicroRNAs in laryngeal cancer: implications for diagnosis, prognosis and therapy." Am J Transl Res **8**(5): 1935-1944.

Li, X., X. Liu, J. Fang, H. Li and J. Chen (2015). "microRNA-363 plays a tumor suppressive role in osteosarcoma by directly targeting MAP2K4." Int J Clin Exp Med **8**(11): 20157-20167.

Liang, J., M. Nagahashi, E. Y. Kim, K. B. Harikumar, A. Yamada, W. C. Huang, N. C. Hait, J. C. Allegood, M. M. Price, D. Avni, K. Takabe, T. Kordula, S. Milstien and S. Spiegel (2013). "Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer." Cancer Cell **23**(1): 107-120.

Lin, K., P. D. Sherrington, M. Dennis, Z. Matrai, J. C. Cawley and A. R. Pettitt (2002). "Relationship between p53 dysfunction, CD38 expression, and IgV(H) mutation in chronic lymphocytic leukemia." Blood **100**(4): 1404-1409.

Lin, Y., T. Xu, S. Zhou and M. Cui (2017). "MicroRNA-363 inhibits ovarian cancer progression by inhibiting NOB1." Oncotarget **8**(60): 101649-101658.

Lotvall, J., A. F. Hill, F. Hochberg, E. I. Buzas, D. Di Vizio, C. Gardiner, Y. S. Gho, I. V. Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M. H. Wauben, K. W. Witwer and C. Thery (2014). "Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles." J Extracell Vesicles **3**: 26913.

Lozanski, G., N. A. Heerema, I. W. Flinn, L. Smith, J. Harbison, J. Webb, M. Moran, M. Lucas, T. Lin, M. L. Hackbarth, J. H. Proffitt, D. Lucas, M. R. Grever and J. C. Byrd (2004). "Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions." Blood **103**(9): 3278-3281.

Lu, J., G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz and T. R. Golub (2005). "MicroRNA expression profiles classify human cancers." Nature **435**(7043): 834-838.

Ludwig, J. A. and J. N. Weinstein (2005). "Biomarkers in cancer staging, prognosis and treatment selection." Nat Rev Cancer **5**(11): 845-856.

Mackay, L. K., A. Braun, B. L. Macleod, N. Collins, C. Tebartz, S. Bedoui, F. R. Carbone and T. Gebhardt (2015). "Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention." J Immunol **194**(5): 2059-2063.

Manterola, L., E. Guruceaga, J. Gallego Perez-Larraya, M. Gonzalez-Huarriz, P. Jauregui, S. Tejada, R. Diez-Valle, V. Segura, N. Sampron, C. Barrena, I. Ruiz, A. Agirre, A. Ayuso, J. Rodriguez, A. Gonzalez, E. Xipell, A. Matheu, A. Lopez de Munain, T. Tunon, I. Zazpe, J. Garcia-Foncillas, S. Paris, J. Y. Delattre and M. M. Alonso (2014). "A small noncoding RNA signature found in exosomes of GBM patient serum as a diagnostic tool." Neuro Oncol **16**(4): 520-527.

Marti, G., F. Abbasi, E. Raveche, A. C. Rawstron, P. Ghia, T. Aurran, N. Caporaso, Y. K. Shim and R. F. Vogt (2007). "Overview of monoclonal B-cell lymphocytosis." Br J Haematol **139**(5): 701-708.

Masyuk, A. I., T. V. Masyuk and N. F. Larusso (2013). "Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases." J Hepatol **59**(3): 621-625.

Matutes, E., K. Owusu-Ankomah, R. Morilla, J. Garcia Marco, A. Houlihan, T. H. Que and D. Catovsky (1994). "The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL." Leukemia **8**(10): 1640-1645.

Mauri, C. and M. Menon (2017). "Human regulatory B cells in health and disease: therapeutic potential." J Clin Invest **127**(3): 772-779.

McClanahan, T., S. Koseoglu, K. Smith, J. Grein, E. Gustafson, S. Black, P. Kirschmeier and A. A. Samatar (2006). "Identification of overexpression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumors." Cancer Biol Ther **5**(4): 419-426.

McManus, M. T. (2003). "MicroRNAs and cancer." Semin Cancer Biol **13**(4): 253-258.

Medina, P. P., M. Nolde and F. J. Slack (2010). "OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma." Nature **467**(7311): 86-90.

Medzhitov, R. (2010). "Inflammation 2010: new adventures of an old flame." Cell **140**(6): 771-776.

Meldolesi, J. and B. Ceccarelli (1981). "Exocytosis and membrane recycling." Philos Trans R Soc Lond B Biol Sci **296**(1080): 55-65.

Messmer, B. T., E. Albesiano, D. Messmer and N. Chiorazzi (2004). "The pattern and distribution of immunoglobulin VH gene mutations in chronic lymphocytic leukemia B cells are consistent with the canonical somatic hypermutation process." Blood **103**(9): 3490-3495.

Michael, A., S. D. Bajracharya, P. S. Yuen, H. Zhou, R. A. Star, G. G. Illei and I. Alevizos (2010). "Exosomes from human saliva as a source of microRNA biomarkers." Oral Dis **16**(1): 34-38.

Migliazza, A., F. Bosch, H. Komatsu, E. Cayanis, S. Martinotti, E. Toniato, E. Guccione, X. Qu, M. Chien, V. V. Murty, G. Gaidano, G. Inghirami, P. Zhang, S. Fischer, S. M. Kalachikov, J. Russo, I. Edelman, A. Efstratiadis and R. Dalla-Favera (2001). "Nucleotide sequence, transcription map, and mutation analysis of the 13q14 chromosomal region deleted in B-cell chronic lymphocytic leukemia." Blood **97**(7): 2098-2104.

Mishra, A. and M. Verma (2010). "Cancer biomarkers: are we ready for the prime time?" Cancers (Basel) **2**(1): 190-208.

Mitchell, P. J., J. Welton, J. Staffurth, J. Court, M. D. Mason, Z. Tabi and A. Clayton (2009). "Can urinary exosomes act as treatment response markers in prostate cancer?" J Transl Med **7**: 4.

Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B.

Martin and M. Tewari (2008). "Circulating microRNAs as stable blood-based markers for cancer detection." Proc Natl Acad Sci U S A **105**(30): 10513-10518.

Molica, S. (2006). "Sex differences in incidence and outcome of chronic lymphocytic leukemia patients." Leuk Lymphoma **47**(8): 1477-1480.

Molkentin, J. D. (2000). "The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression." J Biol Chem **275**(50): 38949-38952.

Morrisey, E. E., Z. Tang, K. Sigrist, M. M. Lu, F. Jiang, H. S. Ip and M. S. Parmacek (1998). "GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo." Genes Dev **12**(22): 3579-3590.

Morton, L. M., S. S. Wang, S. S. Devesa, P. Hartge, D. D. Weisenburger and M. S. Linet (2006). "Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001." Blood **107**(1): 265-276.

Moudgil, K. D. and D. Choubey (2011). "Cytokines in autoimmunity: role in induction, regulation, and treatment." J Interferon Cytokine Res **31**(10): 695-703.

Moussay, E., K. Wang, J. H. Cho, K. van Moer, S. Pierson, J. Paggetti, P. V. Nazarov, V. Palissot, L. E. Hood, G. Berchem and D. J. Galas (2011). "MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **108**(16): 6573-6578.

Mraz, M., L. Chen, L. Z. Rassenti, E. M. Ghia, H. Li, K. Jepsen, E. N. Smith, K. Messer, K. A. Frazer and T. J. Kipps (2014). "miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1." Blood **124**(1): 84-95.

Nicoloso, M. S., T. J. Kipps, C. M. Croce and G. A. Calin (2007). "MicroRNAs in the pathogeny of chronic lymphocytic leukaemia." Br J Haematol **139**(5): 709-716.

Noone, A. M., K. A. Cronin, S. F. Altekruse, N. Howlader, D. R. Lewis, V. I. Petkov and L. Penberthy (2017). "Cancer Incidence and Survival Trends by Subtype Using Data from the Surveillance Epidemiology and End Results Program, 1992-2013." Cancer Epidemiol Biomarkers Prev **26**(4): 632-641.

Norin, S., E. Kimby and J. Lundin (2010). "Tumor burden status evaluated by computed tomography scan is of prognostic importance in patients with chronic lymphocytic leukemia." Med Oncol **27**(3): 820-825.

O'Brien, S. M., H. M. Kantarjian, J. Cortes, M. Beran, C. A. Koller, F. J. Giles, S. Lerner and M. Keating (2001). "Results of the fludarabine and cyclophosphamide combination regimen in chronic lymphocytic leukemia." J Clin Oncol **19**(5): 1414-1420.

Oka, A., S. Ishihara, Y. Mishima, Y. Tada, R. Kusunoki, N. Fukuba, T. Yuki, K. Kawashima, S. Matsumoto and Y. Kinoshita (2014). "Role of regulatory B cells in chronic intestinal inflammation: association with pathogenesis of Crohn's disease." Inflamm Bowel Dis **20**(2): 315-328.

Olkhanud, P. B., B. Damdinsuren, M. Bodogai, R. E. Gress, R. Sen, K. Wejksza, E. Malchinkhuu, R. P. Wersto and A. Biragyn (2011). "Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells." Cancer Res **71**(10): 3505-3515.

Oscier, D., C. Fegan, P. Hillmen, T. Illidge, S. Johnson, P. Maguire, E. Matutes and D. Milligan (2004). "Guidelines on the diagnosis and management of chronic lymphocytic leukaemia." Br J Haematol **125**(3): 294-317.

Ou, Y., D. Zhai, N. Wu and X. Li (2015). "Downregulation of miR-363 increases drug resistance in cisplatin-treated HepG2 by dysregulating Mcl-1." Gene **572**(1): 116-122.

Paggetti, J., F. Haderk, M. Seiffert, B. Janji, U. Distler, W. Ammerlaan, Y. J. Kim, J. Adam, P. Lichter, E. Solary, G. Berchem and E. Moussay (2015). "Exosomes released by

chronic lymphocytic leukemia cells induce the transition of stromal cells into cancer-associated fibroblasts." Blood **126**(9): 1106-1117.

Panayiotidis, P., D. Jones, K. Ganeshaguru, L. Foroni and A. V. Hoffbrand (1996). "Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro." Br J Haematol **92**(1): 97-103.

Patrussi, L., N. Capitani, V. Martini, M. Pizzi, V. Trimarco, F. Frezzato, F. Marino, G. Semenzato, L. Trentin and C. T. Baldari (2015). "Enhanced Chemokine Receptor Recycling and Impaired S1P1 Expression Promote Leukemic Cell Infiltration of Lymph Nodes in Chronic Lymphocytic Leukemia." Cancer Res **75**(19): 4153-4163.

Petlickovski, A., L. Laurenti, X. Li, S. Marietti, P. Chiusolo, S. Sica, G. Leone and D. G. Efremov (2005). "Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells." Blood **105**(12): 4820-4827.

Pilzer, D., O. Gasser, O. Moskovich, J. A. Schifferli and Z. Fishelson (2005). "Emission of membrane vesicles: roles in complement resistance, immunity and cancer." Springer Semin Immunopathol **27**(3): 375-387.

Plunkett, W., V. Gandhi, P. Huang, L. E. Robertson, L. Y. Yang, V. Gregoire, E. Estey and M. J. Keating (1993). "Fludarabine: pharmacokinetics, mechanisms of action, and rationales for combination therapies." Semin Oncol **20**(5 Suppl 7): 2-12.

Pritchard, C. C., H. H. Cheng and M. Tewari (2012). "MicroRNA profiling: approaches and considerations." Nat Rev Genet **13**(5): 358-369.

Pyne, N. J., A. El Buri, D. R. Adams and S. Pyne (2018). "Sphingosine 1-phosphate and cancer." Adv Biol Regul **68**: 97-106.

Qiao, J., S. Lee, P. Paul, L. Theiss, J. Tiao, L. Qiao, A. Kong and D. H. Chung (2013). "miR-335 and miR-363 regulation of neuroblastoma tumorigenesis and metastasis." Surgery **154**(2): 226-233.

Qiu, X., H. Zhu, S. Liu, G. Tao, J. Jin, H. Chu, M. Wang, N. Tong, W. Gong, Q. Zhao, F. Qiang and Z. Zhang (2017). "Expression and prognostic value of microRNA-26a and microRNA-148a in gastric cancer." J Gastroenterol Hepatol **32**(4): 819-827.

Rabinowits, G., C. Gercel-Taylor, J. M. Day, D. D. Taylor and G. H. Kloecker (2009). "Exosomal microRNA: a diagnostic marker for lung cancer." Clin Lung Cancer **10**(1): 42-46.

Radulovic, K. and J. H. Niess (2015). "CD69 is the crucial regulator of intestinal inflammation: a new target molecule for IBD treatment?" J Immunol Res **2015**: 497056.

Rai, K. R., B. L. Peterson, F. R. Appelbaum, J. Kolitz, L. Elias, L. Shepherd, J. Hines, G. A. Threatte, R. A. Larson, B. D. Cheson and C. A. Schiffer (2000). "Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia." N Engl J Med **343**(24): 1750-1757.

Rai KR, S. A., Cronkite EP, Chanana AD, Levy RN, Pasternack BS. (2016). " Clinical staging of chronic lymphocytic leukemia. ." Blood **128**(17): 2109.

Rai, K. R., A. Sawitsky, E. P. Cronkite, A. D. Chanana, R. N. Levy and B. S. Pasternack (1975). "Clinical staging of chronic lymphocytic leukemia." Blood **46**(2): 219-234.

Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. Melief and H. J. Geuze (1996). "B lymphocytes secrete antigen-presenting vesicles." J Exp Med **183**(3): 1161-1172.

Rassenti, L. Z., S. Jain, M. J. Keating, W. G. Wierda, M. R. Grever, J. C. Byrd, N. E. Kay, J. R. Brown, J. G. Gribben, D. S. Neuberg, F. He, A. W. Greaves, K. R. Rai and T. J. Kipps (2008). "Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia." Blood **112**(5): 1923-1930.

Ratajczak, J., K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak and M. Z. Ratajczak (2006). "Embryonic stem cell-derived microvesicles reprogram hematopoietic

progenitors: evidence for horizontal transfer of mRNA and protein delivery." Leukemia **20**(5): 847-856.

Robak, T. and P. Robak (2013). "BCR signaling in chronic lymphocytic leukemia and related inhibitors currently in clinical studies." Int Rev Immunol **32**(4): 358-376.

Rosen, A., F. Murray, C. Evaldsson and R. Rosenquist (2010). "Antigens in chronic lymphocytic leukemia--implications for cell origin and leukemogenesis." Semin Cancer Biol **20**(6): 400-409.

Rosenwald, A., A. A. Alizadeh, G. Widhopf, R. Simon, R. E. Davis, X. Yu, L. Yang, O. K. Pickeral, L. Z. Rassenti, J. Powell, D. Botstein, J. C. Byrd, M. R. Grever, B. D. Cheson, N. Chiorazzi, W. H. Wilson, T. J. Kipps, P. O. Brown and L. M. Staudt (2001). "Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia." J Exp Med **194**(11): 1639-1647.

Santanam, U., N. Zanesi, A. Efanov, S. Costinean, A. Palamarchuk, J. P. Hagan, S. Volinia, H. Alder, L. Rassenti, T. Kipps, C. M. Croce and Y. Pekarsky (2010). "Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression." Proc Natl Acad Sci U S A **107**(27): 12210-12215.

Sarkar, J., D. Gou, P. Turaka, E. Viktorova, R. Ramchandran and J. U. Raj (2010). "MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration." Am J Physiol Lung Cell Mol Physiol **299**(6): L861-871.

Sarvaria, A., J. A. Madrigal and A. Saudemont (2017). "B cell regulation in cancer and anti-tumor immunity." Cell Mol Immunol **14**(8): 662-674.

Saunderson, S. C., P. C. Schuberth, A. C. Dunn, L. Miller, B. D. Hock, P. A. MacKay, N. Koch, R. W. Jack and A. D. McLellan (2008). "Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor." J Immunol **180**(12): 8146-8152.

Schetelig, J., M. Schaich, K. Schafer-Eckart, M. Hanel, W. E. Aulitzky, H. Einsele, N. Schmitz, W. Rosler, M. Stelljes, C. D. Baldus, A. D. Ho, A. Neubauer, H. Serve, J. Mayer,

W. E. Berdel, B. Mohr, U. Oelschlagel, S. Parmentier, C. Rollig, M. Kramer, U. Platzbecker, T. Illmer, C. Thiede, M. Bornhauser and G. Ehninger (2015). "Hematopoietic cell transplantation in patients with intermediate and high-risk AML: results from the randomized Study Alliance Leukemia (SAL) AML 2003 trial." Leukemia **29**(5): 1060-1068.

Schwarzenbach, H., N. Nishida, G. A. Calin and K. Pantel (2014). "Clinical relevance of circulating cell-free microRNAs in cancer." Nat Rev Clin Oncol **11**(3): 145-156.

Seiler, T., H. Dohner and S. Stilgenbauer (2006). "Risk stratification in chronic lymphocytic leukemia." Semin Oncol **33**(2): 186-194.

Sellick, G. S., D. Catovsky and R. S. Houlston (2006). "Familial chronic lymphocytic leukemia." Semin Oncol **33**(2): 195-201.

Seow, Y. and M. J. Wood (2009). "Biological gene delivery vehicles: beyond viral vectors." Mol Ther **17**(5): 767-777.

Shanafelt, T. D., D. Jelinek, R. Tschumper, S. Schwager, G. Nowakowski, G. W. DeWald and N. E. Kay (2006). "Cytogenetic abnormalities can change during the course of the disease process in chronic lymphocytic leukemia." J Clin Oncol **24**(19): 3218-3219; author reply 3219-3220.

Shaw, H. M., G. W. Milton, G. Farago and W. H. McCarthy (1978). "Endocrine influences on survival from malignant melanoma." Cancer **42**(2): 669-677.

Shin, S. S., S. S. Park, B. Hwang, B. Moon, W. T. Kim, W. J. Kim and S. K. Moon (2016). "MicroRNA-892b influences proliferation, migration and invasion of bladder cancer cells by mediating the p19ARF/cyclin D1/CDK6 and Sp-1/MMP-9 pathways." Oncol Rep **36**(4): 2313-2320.

Shiow, L. R., D. B. Rosen, N. Brdickova, Y. Xu, J. An, L. L. Lanier, J. G. Cyster and M. Matloubian (2006). "CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs." Nature **440**(7083): 540-544.

Skog, J., T. Wurdinger, S. van Rijn, D. H. Meijer, L. Gainche, M. Sena-Esteves, W. T. Curry, Jr., B. S. Carter, A. M. Krichevsky and X. O. Breakefield (2008). "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers." Nat Cell Biol **10**(12): 1470-1476.

Slack, F. J. and J. B. Weidhaas (2008). "MicroRNA in cancer prognosis." N Engl J Med **359**(25): 2720-2722.

Smallwood, D. T., B. Apollonio, S. Willimott, L. Lezina, A. Alharthi, A. R. Ambrose, G. De Rossi, A. G. Ramsay and S. D. Wagner (2016). "Extracellular vesicles released by CD40/IL-4-stimulated CLL cells confer altered functional properties to CD4+ T cells." Blood **128**(4): 542-552.

Sokolova, V., A. K. Ludwig, S. Hornung, O. Rotan, P. A. Horn, M. Epple and B. Giebel (2011). "Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy." Colloids Surf B Biointerfaces **87**(1): 146-150.

Song, B., J. Yan, C. Liu, H. Zhou and Y. Zheng (2015). "Tumor Suppressor Role of miR-363-3p in Gastric Cancer." Med Sci Monit **21**: 4074-4080.

St Laurent, G., C. Wahlestedt and P. Kapranov (2015). "The Landscape of long noncoding RNA classification." Trends Genet **31**(5): 239-251.

Stahlhut, C. and F. J. Slack (2013). "MicroRNAs and the cancer phenotype: profiling, signatures and clinical implications." Genome Med **5**(12): 111.

Stevenson, F. K., S. Krysov, A. J. Davies, A. J. Steele and G. Packham (2011). "B-cell receptor signaling in chronic lymphocytic leukemia." Blood **118**(16): 4313-4320.

Stilgenbauer, S., L. Bullinger, P. Lichter and H. Dohner (2002). "Genetics of chronic lymphocytic leukemia: genomic aberrations and V(H) gene mutation status in pathogenesis and clinical course." Leukemia **16**(6): 993-1007.

Stilgenbauer, S., B. Eichhorst, J. Schetelig, S. Coutre, J. F. Seymour, T. Munir, S. D. Puvvada, C. M. Wendtner, A. W. Roberts, W. Jurczak, S. P. Mulligan, S. Bottcher, M. Mobasher, M. Zhu, M. Desai, B. Chyla, M. Verdugo, S. H. Enschede, E. Cerri, R. Humerickhouse, G. Gordon, M. Hallek and W. G. Wierda (2016). "Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 study." Lancet Oncol **17**(6): 768-778.

Subramanian, A. and D. M. Miller (2000). "Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-myc protooncogene." J Biol Chem **275**(8): 5958-5965.

Svensson, K. J., H. C. Christianson, A. Wittrup, E. Bourseau-Guilmain, E. Lindqvist, L. M. Svensson, M. Morgelin and M. Belting (2013). "Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1." J Biol Chem **288**(24): 17713-17724.

Tanese, K., M. Fukuma, T. Yamada, T. Mori, T. Yoshikawa, W. Watanabe, A. Ishiko, M. Amagai, T. Nishikawa and M. Sakamoto (2008). "G-protein-coupled receptor GPR49 is up-regulated in basal cell carcinoma and promotes cell proliferation and tumor formation." Am J Pathol **173**(3): 835-843.

Tang, D., Y. Shen, M. Wang, R. Yang, Z. Wang, A. Sui, W. Jiao and Y. Wang (2013). "Identification of plasma microRNAs as novel noninvasive biomarkers for early detection of lung cancer." Eur J Cancer Prev **22**(6): 540-548.

Taylor, D. D. and C. Gercel-Taylor (2008). "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer." Gynecol Oncol **110**(1): 13-21.

Taylor, D. D., H. D. Homesley and G. J. Doellgast (1980). "Binding of specific peroxidase-labeled antibody to placental-type phosphatase on tumor-derived membrane fragments." Cancer Res **40**(11): 4064-4069.

Taylor, D. D., K. S. Lyons and C. Gercel-Taylor (2002). "Shed membrane fragment-associated markers for endometrial and ovarian cancers." Gynecol Oncol **84**(3): 443-448.

Testi, R., D. D'Ambrosio, R. De Maria and A. Santoni (1994). "The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells." Immunol Today **15**(10): 479-483.

Thery, C., K. W. Witwer and E. Aikawa (2018). "Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines." J Extracell Vesicles **7**(1): 1535750.

Till, K. J., A. R. Pettitt and J. R. Slupsky (2015). "Expression of functional sphingosine-1 phosphate receptor-1 is reduced by B cell receptor signaling and increased by inhibition of PI3 kinase delta but not SYK or BTK in chronic lymphocytic leukemia cells." J Immunol **194**(5): 2439-2446.

Tinhofer, I., I. Marschitz, M. Kos, T. Henn, A. Egle, A. Villunger and R. Greil (1998). "Differential sensitivity of CD4+ and CD8+ T lymphocytes to the killing efficacy of Fas (Apo-1/CD95) ligand+ tumor cells in B chronic lymphocytic leukemia." Blood **91**(11): 4273-4281.

Tsuji, S., Y. Kawasaki, S. Furukawa, K. Taniue, T. Hayashi, M. Okuno, M. Hiyoshi, J. Kitayama and T. Akiyama (2014). "The miR-363-GATA6-Lgr5 pathway is critical for colorectal tumourigenesis." Nat Commun **5**: 3150.

Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee and J. O. Lotvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat Cell Biol **9**(6): 654-659.

Valenti, R., V. Huber, P. Filipazzi, L. Pilla, G. Sovena, A. Villa, A. Corbelli, S. Fais, G. Parmiani and L. Rivoltini (2006). "Human tumor-released microvesicles promote the

differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes." Cancer Res **66**(18): 9290-9298.

Van der Flier, L. G., J. Sabates-Bellver, I. Oving, A. Haegebarth, M. De Palo, M. Anti, M. E. Van Gijn, S. Suijkerbuijk, M. Van de Wetering, G. Marra and H. Clevers (2007). "The Intestinal Wnt/TCF Signature." Gastroenterology **132**(2): 628-632.

Vigorito, E., K. L. Perks, C. Abreu-Goodger, S. Bunting, Z. Xiang, S. Kohlhaas, P. P. Das, E. A. Miska, A. Rodriguez, A. Bradley, K. G. Smith, C. Rada, A. J. Enright, K. M. Toellner, I. C. Maclennan and M. Turner (2007). "microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells." Immunity **27**(6): 847-859.

Vychytilova-Faltejskova, P., L. Radova, M. Sachlova, Z. Kosarova, K. Slaba, P. Fabian, T. Grolich, V. Prochazka, Z. Kala, M. Svoboda, I. Kiss, R. Vyzula and O. Slaby (2016). "Serum-based microRNA signatures in early diagnosis and prognosis prediction of colon cancer." Carcinogenesis **37**(10): 941-950.

Waaler, E. (2007). "On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. 1939." APMIS **115**(5): 422-438; discussion 439.

Waldron, I. (1983). "Sex differences in illness incidence, prognosis and mortality: issues and evidence." Soc Sci Med **17**(16): 1107-1123.

Wang, G. J., Y. Liu, A. Qin, S. V. Shah, Z. B. Deng, X. Xiang, Z. Cheng, C. Liu, J. Wang, L. Zhang, W. E. Grizzle and H. G. Zhang (2008). "Thymus exosomes-like particles induce regulatory T cells." J Immunol **181**(8): 5242-5248.

Wang, S. H., W. J. Zhang, X. C. Wu, M. Z. Weng, M. D. Zhang, Q. Cai, D. Zhou, J. D. Wang and Z. W. Quan (2016). "The lncRNA MALAT1 functions as a competing endogenous RNA to regulate MCL-1 expression by sponging miR-363-3p in gallbladder cancer." J Cell Mol Med **20**(12): 2299-2308.

Wang, Y., T. Chen, H. Huang, Y. Jiang, L. Yang, Z. Lin, H. He, T. Liu, B. Wu, J. Chen, D. W. Kamp and G. Liu (2017). "miR-363-3p inhibits tumor growth by targeting PCNA in lung adenocarcinoma." Oncotarget **8**(12): 20133-20144.

Webber, J., V. Yeung and A. Clayton (2015). "Extracellular vesicles as modulators of the cancer microenvironment." Semin Cell Dev Biol **40**: 27-34.

Wiestner, A. (2012). "Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia." Hematology Am Soc Hematol Educ Program **2012**: 88-96.

Wiestner, A., A. Rosenwald, T. S. Barry, G. Wright, R. E. Davis, S. E. Henrickson, H. Zhao, R. E. Ibbotson, J. A. Orchard, Z. Davis, M. Stetler-Stevenson, M. Raffeld, D. C. Arthur, G. E. Marti, W. H. Wilson, T. J. Hamblin, D. G. Oscier and L. M. Staudt (2003). "ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile." Blood **101**(12): 4944-4951.

Willimott, S., M. Baou, K. Naresh and S. D. Wagner (2007). "CD154 induces a switch in pro-survival Bcl-2 family members in chronic lymphocytic leukaemia." Br J Haematol **138**(6): 721-732.

Willimott, S. and S. D. Wagner (2012). "Stromal cells and CD40 ligand (CD154) alter the miRNome and induce miRNA clusters including, miR-125b/miR-99a/let-7c and miR-17-92 in chronic lymphocytic leukaemia." Leukemia **26**(5): 1113-1116.

Woyach, J. A., A. S. Ruppert, D. Guinn, A. Lehman, J. S. Blachly, A. Lozanski, N. A. Heerema, W. Zhao, J. Coleman, D. Jones, L. Abruzzo, A. Gordon, R. Mantel, L. L. Smith, S. McWhorter, M. Davis, T. J. Doong, F. Ny, M. Lucas, W. Chase, J. A. Jones, J. M. Flynn, K. Maddocks, K. Rogers, S. Jaglowski, L. A. Andritsos, F. T. Awan, K. A. Blum, M. R. Grever, G. Lozanski, A. J. Johnson and J. C. Byrd (2017). "BTK(C481S)-Mediated Resistance to Ibrutinib in Chronic Lymphocytic Leukemia." J Clin Oncol **35**(13): 1437-1443.

Xiao, C. and K. Rajewsky (2009). "MicroRNA control in the immune system: basic principles." Cell **136**(1): 26-36.

Yanaba, K., J. D. Bouaziz, K. M. Haas, J. C. Poe, M. Fujimoto and T. F. Tedder (2008). "A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses." Immunity **28**(5): 639-650.

Ye, J., W. Zhang, S. Liu, Y. Liu and K. Liu (2017). "miR-363 inhibits the growth, migration and invasion of hepatocellular carcinoma cells by regulating E2F3." Oncol Rep **38**(6): 3677-3684.

Yeh, Y. Y., H. G. Ozer, A. M. Lehman, K. Maddocks, L. Yu, A. J. Johnson and J. C. Byrd (2015). "Characterization of CLL exosomes reveals a distinct microRNA signature and enhanced secretion by activation of BCR signaling." Blood **125**(21): 3297-3305.

Younes, A., V. Snell, U. Consoli, K. Clodi, S. Zhao, J. L. Palmer, E. K. Thomas, R. J. Armitage and M. Andreeff (1998). "Elevated levels of biologically active soluble CD40 ligand in the serum of patients with chronic lymphocytic leukaemia." Br J Haematol **100**(1): 135-141.

Zhang, H., N. Zhang, R. Wang, T. Shao, Y. Feng, Y. Yao, Q. Wu, S. Zhu, J. Cao, H. Zhang, Z. Li, X. Liu, M. Niu and K. Xu (2019). "High expression of miR-363 predicts poor prognosis and guides treatment selection in acute myeloid leukemia." J Transl Med **17**(1): 106.

Zhang, L., C. A. Valencia, B. Dong, M. Chen, P. J. Guan and L. Pan (2015). "Transfer of microRNAs by extracellular membrane microvesicles: a nascent crosstalk model in tumor pathogenesis, especially tumor cell-microenvironment interactions." J Hematol Oncol **8**: 14.

Zhang, P. F., L. L. Sheng, G. Wang, M. Tian, L. Y. Zhu, R. Zhang, J. Zhang and J. S. Zhu (2016). "miR-363 promotes proliferation and chemo-resistance of human gastric cancer via targeting of FBW7 ubiquitin ligase expression." Oncotarget **7**(23): 35284-35292.

Zhang, R., Y. Li, X. Dong, L. Peng and X. Nie (2014). "MiR-363 sensitizes cisplatin-induced apoptosis targeting in Mcl-1 in breast cancer." Med Oncol **31**(12): 347.

Zhou, H., A. Cheruvanky, X. Hu, T. Matsumoto, N. Hiramatsu, M. E. Cho, A. Berger, A. Leelahavanichkul, K. Doi, L. S. Chawla, G. G. Illei, J. B. Kopp, J. E. Balow, H. A. Austin, 3rd, P. S. Yuen and R. A. Star (2008). "Urinary exosomal transcription factors, a new class of biomarkers for renal disease." Kidney Int **74**(5): 613-621.

Zhou, P., G. Huang, Y. Zhao, D. Zhong, Z. Xu, Y. Zeng, Y. Zhang, S. Li and F. He (2014). "MicroRNA-363-mediated downregulation of S1PR1 suppresses the proliferation of hepatocellular carcinoma cells." Cell Signal **26**(6): 1347-1354.

Noone AM, Howlader N, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2015, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2015/, based on November 2017 SEER data submission, posted to the SEER web site, April 2018.

(https://www.sigmaaldrich.com/life-science/protein-sample-preparation/extracellular-vesicle-preparation.html).