FACULDADE DE MEDICINA
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CHRACTERIZATION AND QUANTIFICATION OF SYSTEMIC CHANGES AS INDICATORS OF TUMOR PROGRESSION: CHRONIC LYMPHOCYTYC LEUKEMIA

Telma Cristina Lourenço Carrilho

Mestrado em Investigação Biomédica
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DISSERTAÇÃO ORIENTADA PELO DOUTOR SÉRGIO JERÓNIMO RODRIGUES DIAS E PELA DOUTORA ROSA CRISTINA SIMÕES FERNANDES

2011

This study was performed in CIPM (Centro de Investigação em Patobiologia Molecular) in IPOLFG (Instituto Português de Oncologia de Lisboa Francisco Gentil) in the angiogenesis laboratory.

Dissertation presented to the Faculty of Medicine, University of Coimbra, to the attainment of Master Degree in Biomedical Research, with supervision of Doctor Sérgio Dias and Doctor Rosa Fernandes.

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Agradecimentos

Em primeiro lugar quero agradecer ao Doutor Sérgio Dias pela oportunidade que me deu de integrar o grupo de angiogénese e de realizar esta tese de mestrado e pela confiança que depositou em mim.

Agradeço à Doutora Rosa Fernandes pela disponibilidade que sempre demonstrou e pela ajuda.

Gostaria de agradecer ao Doutor Paulo Pereira por ter aceite a minha candidatura a este mestrado pela disponibilidade e ao Doutor Steve Catarino pela disponibilidade e apoio prestado.

Quero agradecer à Doutora Maria Gomes da Silva pela disponibilidade, dedicação e contribuição essencial para este estudo, pois sem ela não seria possível.

Gostaria de agradecer à Ana Costa, que esteve sempre disponível para me ajudar dentro e fora do laboratório e que foi incansável, pelos conhecimentos que me transmitiu e pelo apoio, incentivo e dedicação.

Agradeço Ana Sofia Cachaço pela ajuda nos ELISA e pela disponibilidade e apoio, agradeço ao Francisco Caiado pela ajuda na citometria e pelo empenho, incentivo e disponibilidade.

Agradeço aos restantes colegas do grupo de Angiogénese, Ana Magalhães, Tânia, Leonor, Germana, Jacinta, Fernanda Silva, Catarina Santos, Ana Bastos, Daniela, Joana, Inês e Sofia pela disponibilidade, ajuda e pela boa disposição de sempre.

Agradeço também à Ana Lúcia, Jú, Sidónia, Lara Neto pela ajuda e disponibilidade sempre que precisei.

Agradeço também à equipa técnica da unidade de Citometria pela ajuda.

Gostaria de agradecer a todos os doentes que aceitaram participar neste estudo doando um pouco do seu sangue.

Quero agradecer aos meus pais pela oportunidade de realizar este mestrado sem eles não teria sido possível e por acreditarem nas minhas capacidades: à minha mãe por toda a força que me transmite, pela paciência e apoio; ao meu pai pelo apoio, incentivo e motivação.

Gostaria de agradecer ao meu namorado, Fábio, pela paciência e apoio.

Agradeço à avó Benvinda pelo apoio, compressão e paciência, à avó Médes e ao avô Domingos pelo apoio.

Quero fazer um agradecimento especial ao avô Zé por sempre ter acreditado que eu iria conseguir chegar até aqui e por toda a força que me fez acreditar que eu tinha.

Abbreviations

ALL (Acute Lymphocytic Leukemia)

BCL-2 (B-Cell Lymphoma 2)

BM (Bone Marrow)

BSA (Bovine Serum Albumin)

CIPM (Centre for Research in Molecular Pathobiology)

CLL (Chronic Lymphocytic Leukemia)

CML (Chronic Myeloid Leukemia)

Cp (Crossing point)

CSL (Ubiquitous Transcription Factor)

CXCR4 (Chemokine C-X-C motif Receptor 4)

DMSO (Dimethyl Sulfoxide)

ECM (Cell- Extracellular Matrix)

ELISA (Enzyme-Linked Immunosorbent Assay)

EPCs (Endothelial Progenitor Cells)

FACS (Fluorescence-Activated Cell Sorting)

FBS (Fetal Bovine Serum)

HDL (High-Density Lipoprotein)

HES (Hairy/Enhancer of Split)

HEY (HES-related repressor proteins)

HIF (Hypoxia-Inducible Factor)

HSP (Heparin Sulphate Proteoglycans)

IgE (Immunoglobulin E)

IgHv (Immunoglobulin heavy-chain)

IL (Interleukin)

IPOLFG (Instituto Português de Oncologia de Lisboa Francisco Gentil)

KDR (Kinase Insert Domain Receptor)

MCL-1 (Myeloid Cell Leukemia Sequence 1)

miRISC (miRNA-containing RNA-Induced Silencing Complex)

miRNAs (microRNAs)

MMP (Matrix Metalloproteinase)

MVs (microvesicles)

NHL (Non-Hodgkin Lymphoma)

NICD (Notch Intracellular Domain)

PB (Peripheral Blood)

PBMNC (Peripheral Blood Mononucleated Cells)

PIGF (Placental Growth Factor)

pre-miRNA (precursor of miRNAs)

qPCR (quantitative real time Polymerase Chain Reaction)

sCD23 (soluble CD23)

SCF (Stem Cell Factor)

SD (Standard Deviation)

SDF-1(Stromal cell-Derived Factor-1)

SLL (Small Lymphocytic Lymphoma)

sTK (serum Thymidine Kinase)

sβ_2m (serum β_2-microglobulin)

TNF-alpha (tumor necrosis factor-alpha)

uPA (Urokinase Type of Plasminogen Activator)

UTR (untranslated regions)

VEGF (Vascular Endothelial Growth Factor)

VEGFR-1/2/3 (Vascular Endothelial Growth Factor Receptor-1/2/3)

WHO (World Health Organization)

Zeta-chain-Associated Protein kinase 70 (ZAP-70)

Resumo

A Leucemia Linfocítica Crónica (LLC) é um tumor hematológico de células B maduras e é o tipo de leucemia com maior prevalência nos países ocidentais. Esta leucemia caracteriza-se por uma acumulação de pequenos linfócitos B maduros no sangue e na medula óssea, mas que também pode infiltrar os gânglios linfáticos, o fígado e o baço. Os sintomas habitualmente apresentados nesta patologia são fadiga, anemia hemolítica auto-imune, infecções, espleomegália, hepatomegália e adenopatias, no entanto, a maioria dos doentes são assintomáticos **(WHO classificativo, 2008).**

O diagnóstico da LLC faz-se com base numa contagem de linfócitos B ≥ $5x10^9/L$ no sangue. Imunofenotipicamente apresenta uma expressão positiva de CD5, CD19, CD22 e níveis baixos de CD20 e CD79b **(Cramer and Hallek, 2011)**.

Esta doença classifica-se em diferentes grupos de prognóstico com base nos sistemas de classificação por estadios de Rai (**Rai *et al.*, 1975)** ou Binet (**Binet *et al.*, 1981)**.

A classificação de Rai começa no estadio 0 e termina no estadio IV, enquanto que a classificação de Binet começa no estadio A e termina no estadio C e foi esta a utilizada neste estudo para classificar os doentes. Sendo que ao estadio A de Binet corresponde o estadio 0 de rai, ao estadio B os estadios I e II e ao estadio C o estadio II e IV **(Cramer and Hallek, 2011)**.

A LLC tem um comportamento bastante heterogéneo podendo ir de um bom prognóstico quando o doente se encontra num estadio indolente da doença e não necessita de tratamento até um mau prognóstico no qual a doença se manifesta de forma agressiva e é necessário recorrer a tratamentos. Esta doença pode passar rapidamente de um estadio indolente para um estadio mais agressivo, o que torna imperativo que se estabeleçam factores de prognóstico viáveis e credíveis que possam ajudar na classificação dos diferentes estadios em que os doentes se encontram.

Segundo a OMS (Organização mundial de saúde) a LLC e o linfoma linfocítico de células B (um linfoma não-Hodgkin) são diferentes manifestações clinicas da mesma doença. A designação de linfoma aplica-se quando há ocorre um aumento do tamanho dos linfócitos B e da proliferação celular, infiltração da medula óssea e/ou de gânglio linfáticos e desaparece a Linfocitose apresentando uma redução do numero de linfócitos para $< 5x10^9/L$ no sangue.

Com a finalidade de melhor compreender e tentar encontrar novos factores de prognóstico de forma a melhor definir os diferentes estadios da LLC, o principal objectivo deste estudo foi definir processos moleculares e celulares que permitam prever a progressão da LLC. Tendo como objectivos específicos relacionar a percentagem de células leucémicas com a percentagem de células que têm origem na medula óssea e com os diferentes estadios da doença recorrendo à citometria de fluxo. Relacionar os diferentes estadios da doença com níveis circulantes de factores angiogénicos (VEGF) e quimiotácticos (SDF-1), medidos com kits de ELISA, presentes no plasma do sangue de doentes com LLC e linfoma linfocítico. Para além disso pretende-se caracterizar a nível molecular as células leucémicas presentes no sangue periférico dos doentes através de técnicas de PCR quantitativo. De forma a analisar a expressão de genes anti-apoptóticos (BCL-2 e MCL-1), de membros da via Notch (Jagged2 e HEY-2) e de miRNAs presentes nas células mononucleadas destes doentes e os níveis de miRNAs circulantes no plasma do doentes.

As amostras de sangue periférico utilizadas neste estudo são de doentes com LLC e linfoma linfocítico que nunca tinham sido tratados e foram obtidas com o consentimento informado dos doentes, segundo as regras do IPOLFG.

O primeiro parâmetro analisado neste estudo foi a percentagem de diferentes populações celulares que têm origem na medula óssea e a sua relação com a percentagem de células leucémicas nas amostras de sangue dos doentes com LLC e linfoma linfocítico. Verificou-se que existem mais células leucémicas em circulação nos estadios mais agressivos da doença. No entanto, devido ao reduzido número de

amostras não foi possível alcançar resultados relevantes estatistiscamente entre as diferentes varáveis. Em geral, o aumento de células leucémicas em ciurculação relaciona-se com o aumento das células com origem na medula óssea.

Os segundos parâmetros estudados foram os níveis circulantes de VEGF e SDF-1 no plasma obtido das amostras de sangue dos doentes. O VEGF é um factor angiogénico que desempenha um papel importante na LLC, neste estudo verificou-se um aumento dos níveis de VEGF no estadio menos agressivo da LLC (estadio A). Estes resultados estão de acordo com os resultados descritos por Molica et al. (2002).

Quanto ao SDF-1 é um factor quimiotáctico envolvido na mobilização das células leucémicas da LLC através da corrente sanguínea. Os resultados obtidos neste estudo mostraram um aumento dos níveis de SDF-1 nos doentes comparativamente aos controlos saudáveis, o que coincide com os dados publicados por Barretina et al. (2003).

O terceiro parâmetro estudado foi a expressão de genes anti-apoptóticos, o BCL-2 e o MCL-1. As células leucémicas da LLC são células com resistência à apoptose, aumentando assim a sua capacidade de sobrevivência.

Por isso é importante estudar o papel da apoptose nestas células. Neste estudo observou-se um aumento da expressão do BCL-2 e do MCL-1 nas amostras de LLC quando comparadas com as amostras de linfoma linfocítico.

O quarto parâmetro estudado foi a expressão de membros da via Notch, Jagged2 e HEY-2, uma vez que esta via desempenha um papel na patogénese da LLC, como foi recentemente publicado **(Rosati *et al.*, 2009).** Neste estudo verificou-se um aumento da expressão do Jagged 2 nas amostras de linfoma linfocítico e nos estadios mais agressivos da doença (estadios B e C), enquanto que a expressão do HEY-2 não revelou diferenças significativas entre os estadios da doença.

Os últimos parâmetros estudados foram a expressão de vários miRNAs (microRNAs) miR-155, miR-15a and miR-16-1, miR-21, miR-101, miR-223, miR-221, miR-34a, miR-

34c, miR-141, miR-210, miR-942 e miR-380, nas células mononucleadas isoladas do sangue dos doentes e os níveis destes miRNAs circulantes no plasma dos doentes.
De todos estes, destacam-se os miR-942, miR-380 e miR-101 pela sua capacidade de distinção entre a LLC e linfoma linfocítico, quando são detectados no plasma. Estando os níveis deste miRNAs circulantes aumentados na amostras de LLC.

Em conclusão, os resultados deste estudo sugerem que podem ser usados diferentes marcadores celulares e moleculares para classificar doentes com LLC em diferentes estadios. Esclarecendo um pouco os complexos processos moleculares entre diferentes genes e vias de sinalização, que em conjunto contribuem para a progressão desta doença maligna e fatal.

Palavras chave: Leucemia Linfocítica Crónica (LLC), progressão da doença, apoptose, miRNAs

Abstract

Chronic Lymphocytic Leukemia (CLL) is a mature B-cell malignancy characterized by the accumulation of small B lymphocytes with mature appearance and peripheral blood (PB) and bone marrow (BM) are usually involved. CLL is classified into prognostic groups, some patients could have an indolent disease, while other patients could develop an aggressive disease that requires early therapy. Among these prognostic groups, CLL and SLL are different clinical manifestations of the same disease according to World Health Organization (WHO). Because of that, it is important to establish molecular or cellular criteria (biomarkers) that allow predicting patient staging or progression.

For this purpose, we used several techniques and molecular approaches, as follows: flow cytometry to classify the disease stages using well established markers; the same technique was used to detect and quantify circulating Bone marrow-derived cells; circulating angiogenic Vascular Endothelial Growth Factor (VEGF) and chemotactic Stromal cell-Derived Factor-1 (SDF-1) factors were quantified by Enzyme-Linked Immunosorbent Assay (ELISA); apoptosis-related and notch pathway-related genes and microRNA signatures were obtained and quantified using quantitative real time Polymerase Chain Reaction (qPCR).

In this study it was found that there are more leukemic cells in circulation in more aggressive stages of CLL. The reduced patient number did not allow reaching statistical significance (or correlations) between the different variables, although there were several tendencies. In general, the presence of increased circulating leukemia cells correlated with the presence of bone marrow-derived progenitors and with VEGF levels, for instance.

The anti-apoptotic genes, B-cell Lymphoma 2 (BCL-2) and myeloid cell leukemia sequence 1 (MCL-1) showed increased expression in CLL patients. The more

aggressive stages of CLL showed increase BCL-2, suggesting that these cells have a higher resistance to apoptosis. However this was not observed with MCL-1, where no difference was observed between CLL stages.

The Notch ligand Jagged2 showed a higher expression in SLL and in more aggressive stages of CLL which suggested its expression may be associated with aggressiveness and resistance to apoptosis.

The levels of circulating miRNAs miR-210, -380 and -101 (plasma-derived) could discriminate between CLL and SLL, being higher in CLL patients.

With the results presenting in the current study, it was also possible establish different profiles using circulating miRNAs expression between the less and more aggressive stages of CLL.

Taken together, our results suggest different cellular and molecular markers may be used to classify (and stratify) CLL patients at different stages, and more importantly, shed some light into the complex molecular cross-talk between different gene and signaling pathways, which together contribute towards the onset progression of this malignant and fatal disease.

Key words: Chronic lymphocytic leukemia (CLL), disease progression, apoptosis, miRNAs

Aims

The main aim of the present study was to define the molecular and cellular processes that allow predicting CLL progression.

In detail, CLL progression stages were correlated with the levels of circulating bone marrow-derived cell populations as well as with angiogenic and chemotatic factors present in the PB of CLL patients.

Another aim of the present study was to contribute towards the molecular characterization of circulating CLL cells and correlate it with CLL progression.

Introduction

1. Chronic Lymphocytic Leukemia

1.1 Characterization and diagnosis criteria of Chronic Lymphocytic Leukemia

Lymphocytic leukemia is a type of leukemia affecting circulating lymphocytes, a subtype of white blood cells, Most lymphocytic leukemias involve a particular subtype of lymphocytes, the B cells. Clinically, lymphocytic leukemia is commonly divided, by the stage of maturation at which the clonal (neoplastic) lymphoid population stopped maturing, into two main categories, the acute lymphocytic leukemia (ALL) and the chronic lymphocytic leukemia (CLL).

CLL is the most prevalent form of adult leukemia in western countries with an incidence of 2-6 cases per 100,000 person per year and increasing with age with a frequency of (12.8/100,000) from 65 years **(WHO classification, 2008).** In the U.S.A., CLL has an incidence of 4.1 cases per 100,000 inhabitants and there are more than 15,000 new cases per year **(Cramer and Hallek, 2011).** In Europe, CLL has an incidence of 3.79 cases per 100,000 inhabitants **(Sant *et al.*, 2010).** This form of leukemia is less common in African and Asian people, being particularly prevalent in North America and Europe. The median age of CLL diagnosis is between 67 and 72 years and men are more affected by this disease. The proportion of young patients diagnosed with early-stage CLL is increased probably because an increase in the frequency of blood tests **(Cramer and Hallek, 2011).**

The underlying etiology and pathology of CLL is yet unknown. Evidence suggests that genetic factors and antigenic stimulation are involved in the characteristic overproduction of mature B lymphocytes. In CLL, peripheral blood (PB) and bone marrow (BM) are usually involved and lymph nodes and liver and spleen are also

typically infiltrated **(WHO classification, 2008).** The common symptoms are fatigue, autoimmune hemolytic anemia, infections, splenomegaly, hepatomegaly and lymphadenopathy. Most patients are asymptomatic at diagnosis **(Zenz *et al.*, 2010; WHO classification, 2008).**

In fact, the diagnosis of CLL is established by blood count of ≥ $5x10^9$/L B lymphocytes with a CLL phenotype. CLL cells usually show expression of surface antigen CD5, B-cell surface antigens CD19, CD22 and low levels of CD20 and CD79b **(WHO classification, 2008).**

The accumulation of a monoclonal population of $CD5^+/CD19^+$ B cells or leukemic cells is a feature of this disease in both prognostic groups aggressive CLL and indolent CLL **(Cramer and Hallek, 2011).**

CLL is classified into different prognostic groups based on the staging systems developed by Rai **(Rai *et al.*, 1975)** and Binet (**Binet *et al.*, 1981).**

The Rai classification starts with Stage 0 and ends with the stage IV **(Table 1)** and the Binet classification starts with A stage and ends with C stage **(Table 2)**. The median survival for patients in Binet stage A or Rai stage 0 is over 10 years, for patients in stage B or I-II is 5-7 years and 2-3,5 years for patients who are in stage C or III-IV **(Cramer and Hallek, 2011).**

Table 1 – Clinical Rai classification of CLL stages (adapted from Cramer and Hallek, 2011

Table 1 | Clinical staging system according to Rai *et al.* (1975)[11]

Stage	Lymphadenopathy	Hepatomegaly or splenomegaly	Hemoglobin (g/dl)	Platelets (platelets/µl)
0	None	None	≥11	≥100,000
I	≥1	None	≥11	≥100,000
II	Irrelevant	≥1	≥11	≥100,000
III	Irrelevant	Irrelevant	<11	≥100,000
IV	Irrelevant	Irrelevant	Irrelevant	<100,000

Table 2 - Clinical Binet classification of CLL stages (adapted from Cramer and Hallek, 2011)

Table 2 | Clinical staging system according to Binet *et al.* (1981)[12]

Stage	Number of involved areas (palpable in clinical examination)*	Hemoglobin (g/dl)	Platelets (platelets/μl)
A	<3	≥10	≥100,000
B	≥3	≥10	≥100,000
C	Irrelevant	<10	<100,000

*Cervical lymph nodes (submandibular, supraclavicular, infraclavicular, occipital, preauricular, retroauricular, and oropharyngeal), axillary lymph nodes, inguinal lymph nodes, liver or spleen.

The first prognostic markers used to complement the clinical staging systems were based on the morphology of CLL cells in PB. There must be ≥ $5x10^9$/L monoclonal lymphocytes with a CLL phenotype in the PB. To establish the diagnosis of CLL lymphocytosis needs to be present for at least 3 months, in part to separate CLL from B cell Small Lymphocytic Lymphoma (SLL) classification **(WHO classification, 2008).** BM aspirate or biopsies are normally not required to make CLL diagnosis. However because CLL is a BM disease, sometimes it is appropriate to evaluate its involvement. In that case, to establish a CLL diagnosis BM aspirate smears must show the presence of > 30% lymphoid cells in the nucleated cell fraction of the BM **(Cheson *et al.*, 1996).**

1.2 Molecular markers used to stage CLL

The most important serum markers in patients with CLL are serum β_2-microglobulin (sβ_2m), serum thymidine kinase (sTK) and soluble CD23 (sCD23). In CLL, elevated sβ_2m levels are correlated with advanced disease stage and BM infiltration. The cellular enzyme sTK is a useful marker for proliferative activity and is associated with rapid disease progression and advanced disease stage. CD23 is a receptor for Immunoglobulin E (IgE) with low affinity that is expressed on mature B cells in physiological conditions, but also on antigen-presenting cells and platelets.

sCD23 correlates with diffuse BM infiltration, disease progression in early-stage CLL and with reduced survival **(Saka *et al.*, 2006).**

CD38 and Zeta-chain-associated protein kinase 70 (ZAP-70) are two powerful prognostic factors in CLL. CD38 is a transmembrane glycoprotein regulated by the tumor microenvironment and it plays a diverse set of roles on lymphocytes. Besides its function as a plasma membrane signaling receptor, CD38 can function as an enzyme. Elevated expression of CD38 is associated with advanced disease stages, higher incidence of lymphadenopathy and hepatomegaly **(Schroers *et al.*, 2005)**. ZAP-70 is a signaling protein in the T-cell receptor complex that is expressed on CLL cells but not on normal B cells **(Chen *et al.*, 2002).** ZAP-70 expression is related with adverse prognosis factors in CLL **(Cramer and Hallek, 2011).**

During the development and differentiation of normal B lymphocytes there are multiple changes on immunoglobulin genes, namely mutations in Immunoglobulin heavy-chain (IgHv) genes. CLL patients without IgHv mutations have a worse prognosis, developing an aggressive disease that requires early therapy, while patients with mutations in IgHv gene have a more stable indolent disease, with no benefits from chemotherapy **(Cramer and Hallek, 2011).**

In approximately 80% of patients with CLL have one or more cytogenetic abnormalities. These alterations can be deletions in chromosome 13, 17 or 11 or trisomy of chromosome 12 **(Döhner *et al.*, 2000).**

The most frequent genomic aberrations in CLL is the deletion of 13q14 region. In this region are located two microRNAs (miRNAs) genes, miR-15a and miR-16-1, that have been implicated in CLL pathogenesis (**Calin *et al.*, 2002**). Also, approximately one-third of patients with advanced CLL have 11q22-q23 deletions (**Austen *et al.*,2005**) and the ATM gene is localized in this region. In 4-9% of CLL patients are found 17p13

deletions, a region where the tumor suppressor TP53 gene, which encodes protein p53, is located **(Zenz *et al.*, 2008).**

Although numerous prognostic factors have been identified for CLL, most of them have not been recommended for routine clinical practice **(Cramer and Hallek, 2011).**

1.3 CLL biology: microenvironment

CLL cells have the capability to interact and modify their microenvironment, which consists of T cells, other hematopoietic cell, stromal cells, adhesion molecules (integrins, immunoglobulins, selectins), chemokine receptors (as chemokine C-X-C motif receptor 4 - CXCR4), **(Jaksic *et al.*, 2010)** chemokines (CCL22 and CCL-17) **(Ghia *et al.*, 2002)** and also angiogenic factors including vascular endothelial growth factor (VEGF) , tumor necrosis factor-alpha (TNF-alpha) and matrix metalloproteinase (MMP)-9 **(Aref *et al.*, 2007)** and stromal cell-derived factor (SDF-1) derived from BM stromal cells **(Gabrilove, 2001).**

CLL cells rapidly undergo apoptosis when they are removed from patients, thus, CLL cells appear to recruit accessory cells and create a microenvironment that supports their own survival **(Zenz et al., 2010).** CLL cells not only respond to chemokines, but also secrete chemokines **(Burger, 2010).** CLL cells can respond to signals delivered by T-cells, which can promote CLL cells survival and proliferation **(Burger *et al.*, 2009).** CLL cells can recruit CD4+ T-cells that induce chemokines, including CCL22 **(Ghia *et al.*, 2002).** In CLL patients, CCL3 and CCL4 are critical chemokines for T-cell recruitment to lymphoid tissues, which can produce CLL-T-cell interactions in the lymph nodes microenvironment and may participate in proliferation of CLL cells **(Burger *et al.*, 2009).**

In their appropriate microenvironment, CLL cells have an apparent survival advantage which is consistent with a defect in apoptosis **(Cramer and Hallek, 2011).** CLL pathogenesis involves proliferation and survival of the malignant cells which depends

of specific cell types and soluble factors in microenvironmental niches**. (Pleyer *et al.*, 2009).**

1.4 Small Lymphocytic Lymphoma

SLL is a mature B-cell non-Hodgkin lymphoma (NHL). CLL and SLL are different clinical manifestations or stages of the same disease according to WHO (World Health Organization) classification of Tumors of Hematopoietic and Lymphoid tissues (4th edition, 2008) **(WHO classification, 2008).** The similarity of membrane phenotypes between CLL and SLL provided evidence that the two are different tissue expressions of the same disease **(Batata *et al.*, 1992).**

The SLL designation is used in cases when CLL shows an increase in cell size, proliferative activity, lymph nodes and BM infiltration and < $5x10^9$ B cells/L in PB circulation (this cut-off is used to separate CLL from SLL classification **(WHO classification, 2008).** Given the distinct clinical presentation and outcome, there has been a continued interest in defining molecular features that allow distinguishing CLL from SLL.

2. Tumor Biology: Neovessel formation

2.1 Angiogenesis and Vasculogenesis

Neovessel formation is a very complex process that involves multiple cell types and environmental signals allowing tissue oxygenation, nutrition and metabolic waste product removal. Angiogenesis is a regulated process responsible for the development of new blood vessels from pre-existing ones **(Sullivan and Brekken, 2010)** while, vasculogenesis is the *de novo* formation of blood vessels via direct differentiation of endothelial progenitor cells (EPCs) into vessel incorporated endothelial cells **(Letilovic *et al.*, 2006)**.

Neoplastic growth is both angiogenesis and vasculogenesis-dependent. The increase in tumor mass during the initial stages of tumor growth results in the establishment of a hypoxic environment, which contribute for the production of pro-angiogenic growth factors and the beginning of the "angiogenic switch" **(Hanahan and Folkman., 1996).** The "angiogenic switch" is a key step in tumor development that occurs when levels of angiogenesis endogenous activators increase relatively to the levels of endogenous inhibitors changing the balance of angiogenic mediators and stimulating angiogenesis **(Sullivan and Brekken, 2010).** The process of angiogenesis also depends on cell-cell and cell- extracellular matrix (ECM) interactions **(Pepper, 2001).**

Assessment of angiogenesis can be done via different methods. The measurement of angiogenic factors levels using enzyme-linked immunosorbent assay (ELISA) allows the quantification of angiogenic factors on serum, plasma, bone marrow or purified lysates of tumor cells. Other indirect method to evaluate angiogenesis is the quantification of the number of EPCs in PB. Indeed a positive relation between the number of circulating EPCs and the number of tumor vessels has been proposed in different tumor models. Furthermore under the influence of angiogenic factors the number of EPCs is increased, as reported in CLL and other hematological malignancies **(Letilovic *et al.*, 2006).**

2.2 Angiogenesis in hematological malignancies

Until recently, it was believed that angiogenesis was not relevant in hematological malignancies as it is in solid tumors, mainly due to the fact that the BM and lymph nodes are the principal sites of tumor accumulation in these diseases **(Rajkumar *et al.*, 2002; Jaksic *et al.*, 2001).**

Perez-Ataide *et al*. (1997) was the first group to reported the higher density in bone marrow microvessel in patients with acute lymphoblastic leukemia **(Perez *et al.*,1997).** A more recent study performed by Peterson and Kini (2001) demonstrated a positive

correlation between the microvessel count and the clinical Rai stage of CLL, patients with higher microvessels counts were more likely to have advanced diseases **(Peterson and Kini*,* 2001).**

2.3 Angiogenesis in CLL

CLL growth and expansion are influenced by angiogenesis through powerful interactions between endothelial and leukemic cells. Endothelial cells exposed to an angiogenic factor produced by leukemic cells, increase the expression of several hematopoietic growth factors (as stem cell factor – SCF) and cytokines (as interleukin (IL)-6). These cytokines stimulate the proliferation and migration of malignant cells, via paracrine interactions **(Ribatti *et al.*, 2001).** The pro-angiogenic environment found in CLL could be just a reflection of high numbers of leukemic cells, which can produce high levels of angiogenic factors. When the levels of BM angiogenesis are adjusted to the number of cells in BM, angiogenic factors are elevated in patients with CLL compared to healthy controls **(Krejcí *et al.,* 2001).**

Another role of increased angiogenesis in hematologic diseases is to allow the dissemination of malignant cells throughout the body. New formed vessels have increased permeability by the influence of angiogenic factors such as VEGF **(Till *et al.,* 2005).**

2.4. VEGF

The angiogenic factor VEGF or VEGF-A is a major growth factor produced by cells under hypoxia conditions occurring during angiogenesis. There are several members of VEGF family: VEGF-B, VEGF-C and Placental Growth Factor (PlGF), but VEGF-A (or just VEGF) is the most commonly found in most malignancies. VEGF

ligands interact with the vascular endothelial growth factor receptors -1, 2 and 3 (VEGFR-1/2/3). VEGF-A gene contains 8 exons separated by 7 introns, which give origin to four principal isoforms generated by alternative splicing. $VEGF_{121}$ and $VEGF_{165}$ are free soluble proteins, while $VEGF_{189}$ and $VEGF_{206}$ are found as insoluble forms bounded to the ECM, although $VEGF^{165}$ also could appear bound to the ECM **(Podar and Anderson, 2005; Catena *et al.,* 2010).**

VEGF has heparin sulphate proteoglycans (HSP) binding domains, which have strong affinity for proteoglycans established on cell plasma membranes or inside the ECM. Release of VEGF from the ECM and cell membrane allows VEGF – mediated activity and signalling. The proteolytic release of VEGF is mediated by the extracellular proteases Plasmin, Urokinase Type of Plasminogen Activator (uPA) and MMPs **(Bergers *et al.*, 2000; Sullivan and Brekken, 2010).**

During embryogenesis, hematopoietic and endothelial progenitor cells (termed angioblasts) are thought to be originated from the same precursor cell, the hemangioblast. Because of that, numerous pathways are shared by hematopoietic and vascular cells, being VEGFR-2 (also known as Kinase insert domain receptor - KDR)/VEGF pathway one of them. KDR binds to soluble factors like VEGF which improves proliferation and migration of endothelial cells **(Ziegler *et al.*, 1999; Neufeld *et al.*, 1999).** In some haematological malignancies, KDR is not only express by endothelial cells but is also express by multipotent hematopoietic stem cells and was proved that leukemic cells also express this receptor **(Fiedler *et al.*, 1997).** Because of that, maybe the transformation of hematopoietic cells into leukemic cells is connected with KDR expression **(Dias *et al.*, 2000).** Some papers report KDR as being the most important receptor for inducing cellular signals for proliferation and differentiation of endothelial cells although VEGFR-1 also plays important roles in subsets of malignant leukemia cells **(Fragoso *et al.*, 2006).**

Dias et al. (2000) showed that leukemic cells produce VEGF and express VEGF receptors like KDR. They also proved, for the first time, that VEGF receptors provide functional signals, increasing proliferation and MMP activation on leukemia cells, similar to the effect observed in endothelial cells. This study was the first to show that the blockade of VEGF-KDR interactions decreased leukemic cells growth, survival and metastisation. High levels of VEGF in PB of leukemic patients may be connected with migration of leukemic cells to BM extravascular space **(Dias *et al.*, 2000).** VEGF improves proliferation of endothelial cells and production of hematopoietic growth factors and also promotes leukemic cell proliferation and growth generated by KDR binding **(Dias *et al.*, 2000).**

2.4.1 The role of VEGF in CLL

Angiogenesis plays an important role in the pathogenesis of CLL. CLL cells produce angiogenic factors, such as VEGF which have an antiapoptotic effect on CLL cells and have a positive correlation with B-cell lymphoma 2 (BCL-2), an antiapoptotic gene **(Letilovic *et al.*, 2006).**

Increased plasma levels of VEGF were detected in patients with CLL. VEGF is a predictor of outcome in patients with CLL **(Faderl *et al.*, 2002; Molica *et al.*, 2002).**

The diagnosis and prognostic implications of the angiogenic phenotype should be used as a prognostic tool to help the classification of patients with CLL in Rai or Binet stages **(Letilovic *et al.*, 2006).**

3. Biology of CLL: The role of Stromal cell-derived factor-1 in CLL

Stromal cell-derived factor-1 (SDF-1) is the ligand for the chemokine receptor CXCR4 that plays an important role in mobilization of normal lymphocytes, monocytes, hematopoietic stem cells and progenitor cells. SDF-1 is produced by BM stromal cells and acts like a chemoattractant supporting the homing of stem cells **(Möhle *et al.*, 1999).**

The CXCR4/SDF-1 system has been reported as an important regulator of the trafficking of CLL cells between blood, lymphoid organs and BM. CLL cells not only respond to chemokines secreted in the microenvironment, but also secrete chemokines **(Burger, 2010).**

It has been reported that CXCR4 is overexpressed on circulating CLL cells compared to cells from healthy controls **(Möhle *et al.*, 1999; Barretina *et al.*, 2003).** However, CLL patients showed lower SDF-1 plasma levels compared to the control group. So, no correlation was found between CXCR4 and SDF-1 expression and the clinical stage of the disease **(Barretina *et al.*, 2003).**

Ghobrial et al. (2004) was the first group to describe a strong association of Rai stages with CXCR4 expression levels in CLL cells. They showed a significant increase in the expression of CXCR4 in CLL cells from patients with advanced Rai stages of the disease. They also showed that circulating CLL cells had higher expression of CXCR4 than B lymphocytes in lymph nodes, which suggest that CXCR4 could be down-regulated when malignant B cells enter the lymph nodes **(Ghobrial *et al.*, 2004).**

4. Biology of CLL: evidence for circulating BM-derived cells

The BM is the major hematopoietic organ and a primary lymphoid tissue, responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes and platelet and is served by numerous blood vessels **(Travlos, 2006; Caligaris-Cappio *et al.*,1992; Ghia *et al.*, 2002).**

A great variety of cells are present in the BM, the majority are hematopoietic cells but non-hematopoietic cells are also present, including osteoblasts and osteoclasts, fibroblasts and endothelial cells **(Wilkins, 1992).** Some of these BM-derived cells were investigated in the present study.

4.1 Endothelial Progenitor Cells

It is now accepted that a reduced but significant part of tumor endothelial cells derive from EPCs, which were released from the BM and recruited to the sites of new blood vessel formation. EPCs can contribute to tumor blood vessel formation via two distinct processes: direct differentiation into vessel incorporated endothelial cells or paracrine/justacrine interaction and activation of pre-existing endothelial cells **(Urbich and Dimmeler, 2004).**

The identity and origin of EPCs, has not been completely determined but there are some consensual surface markers used to characterize these cells. The first EPCs phenotype was defined by *Asahara et al* (1997) that characterized EPCs as peripheral PBMNC expressing hematopoietic stem cell marker (CD34) and an endothelial-specific marker (KDR) . Since this seminal paper the molecular definition of EPCs has been under intense scrutiny and other markers have been considered essential in the definition of EPCs, namely CD133 **(Asahara *et al.*, 1997; Peichev *et al.*, 2000).**

However, BM- and PB-derived $CD133^{+}/KDR^{+}$ cells have been shown to behave as EPCs, thus being sufficient to define this cell population **(Urbich and Dimmeler, 2004;**

Shantsila *et al.*, 2008). CD133 is a highly conserved antigen with unknown biological activity, being and a more immature hematopoietic stem cell marker expressed on hematopoietic stem cells but not in mature endothelial cells and monocytic cells. Thus, $CD133^{+}KDR^{+}$ reflects an immature progenitor cells population while $CD34^{+}KDR^{+}$ may also represent cells of the wall vessel (more mature stage) **(Urbich and Dimmeler, 2004).**

The presence of circulating EPC has been correlated with angiogenic activity during tumor growth. Several growth factors are involved in regulation of endothelial differentiation, proliferation, migration and formation of functional vessels, being VEGF a strong stimulus for the recruitment of BM-derived EPC and one of the major inducers of vasculogenesis and angiogenesis **(Gehling *et al.*, 2000).**

EPCs involvement in pathological and physiological angiogenesis, has been growing the interest in EPCs for therapeutic purposes and providing an attractive marker to disease progression **(Igreja *et al.*, 2008).** Several pre-clinical tumor models demonstrated the relevance of monitoring the levels of EPCs during the progession of diseases, like in multiple myeloma and non-small cell lung cancer **(Zhang *et al.,* 2005; Dome *et al.*, 2006).** In lymphoma patients there is an increase of EPCs and a positive correlation with the circulating VEGF levels, demonstrating the relevance of measuring EPC as biomarker for disease progression **(Igreja *et al.*, 2007; Becker and Jordan, 2011).**

4.1.1 Homing and differentiation of EPCs

In order to play their role on blood vessels formation, EPC need to accomplish 4 steps: mobilization, homing (involved chemoattraction, adhesion, transendothelial migration), invasion and differentiation **(Figure 1) (Urbich and Dimmeler, 2004).**

The mobilization of stem cells in the BM is determined by local microenvironment, the stem cell niche, which consists of fibroblasts, osteoclasts and endothelial cells.

Physiologically, ischemia is the predominant signal to induce mobilization of EPCs from the BM and is regulated by VEGF and SDF-1. These growth factors are released to the circulation and induce mobilization of progenitor cells **(Urbich and Dimmeler, 2004).** Homing of EPCs involves adhesion of progenitor cells to endothelial cells activated by cytokines. Chemoattraction may be the most important event to allow the recruitment of progenitor cells to the ischemic location. Overexpression of SDF-1 has been shown to increase stem cell homing and incorporation into ischemic tissues. VEGF levels are increased during ischemia and act also as a chemoattractive factor to EPCs. Finally, the differentiation of EPCs into endothelial cells may be important for functional integration in the vessels. VEGF and its receptors play an important role for stimulating endothelial differentiation in the embryonic development **(Urbich and Dimmeler, 2004).**

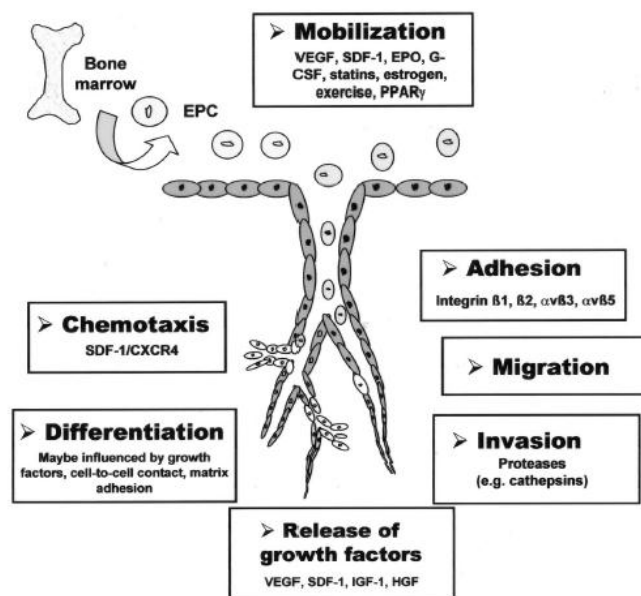


Figure 1 - EPC Homing and Differentiation (Adapted from Urbich and Dimmeler, 2004)

4.2 Other circulating BM-derived cells in CLL

4.2.1 CD11b myeloid cells

CD11b is a transmembrane molecule that belongs to the α-subunit integrin receptor family, important in cellular adhesion, migration and cell survival. It is frequently involved in cancer development and therapy resistance, making integrins attractive candidates for treatment approaches in oncology **(Rhein *et al.*, 2010).**

In immunophenotyping, CD11b is a leukocyte-specific receptor and is not exclusively, but mainly expressed in myelomonocytic cells (monocyte/macrophages, granulocytes and natural killer cells). Thus, CD11b is expressed mainly on myeloid lineage **(Rhein *et al.*, 2010).** Since 1987, it has also been described on malignant CLL cells, although, this surface marker is not found commonly in blood or tissue B cells **(Morabito *et al.*, 1987).**

4.2.2 Progenitor cells

CD34 is a well-known marker for progenitor cells in blood vessels and stromal tissues. $CD34^+$ cells or progenitor cells were reported as been increased in CLL patients compared with healthy controls. A higher percentage of $CD34^+$ cells was detected in stages B and C as compared to stage A, which could be explained by the higher number of circulating cells in advanced stages of the disease **(Berger *et al.*, 1999).**

4.2.3 $CXCR4^+$ cells population in CLL

$CXCR4^+$ cells are a population of cells with chemotactic activity that allows cell mobilization. As described on section 2.5, CLL cells show significant increase of CXCR4 expression.

5. Biology of CLL: apoptosis

CLL is a human malignancy caused predominantly by defective apoptosis **(Cramer and Hallek, 2011; Kitada and Reed, 2004; Moshynska *et al.*, 2004).**

Cancer cells develop the capability to resist to apoptosis through the upregulation of anti-apoptotic proteins and downregulation of proapoptotic proteins (among other mechanisms). The B-cel lymphoma 2 (BCL-2) family of proteins includes both anti-apoptotic and pro-apoptotic members (the central regulators of apoptosis). There are six anti-apoptotic members which are BCL-2, BCL-xl, BCL-B, BCL-W, BFL-1 and MCL-1 **(Placzek *et al.*, 2010).**

In CLL, BCL-2 is expressed at elevated levels, correlated with short patient survival and aggressive disease. The mechanisms responsible for the high levels of BCL-2 in CLL are not well understood **(Hanada *et al.*, 1993).** High levels of myeloid cell leukemia sequence 1 (MCL-1) have been correlated with advanced forms of CLL. **(Kitada and Reed 2004; Placzek *et al.*, 2010)** MCL-1 contributes to the longevity of CLL cells and higher expression in CLL has been related with resistance to chemotherapy **(Moshynska *et al.*, 2004).** Exogenous signals provided by lymphokines, cytokines, chemokines and cell adhesion molecules have been reported to induce MCL-1 expression in normal and malignant B cells. These findings show that the microenvironment is connected with MCL-1 expression. Some studies also described that MCL-1 is required for survival of hematopoietic progenitor cells. MCL-1 is first expressed in lymphoid development and later in the maintenance of mature lymphocytes **(Opferman *et al.*, 2003).**

6. Biology of CLL: involvement of the Notch pathway

6.1 Description of Notch Pathway

Notch is a transmembrane hetero-dimeric receptor family containing four members: Notch1, Notch2, Notch3 and Notch4. In humans, there are five ligands for these receptors: Delta-Like 1, Delta-Like 3, Delta-Like 4, Jagged 1 and Jagged 2.

Notch pathway target genes can be generally divided into two distinct gene families, the basic helix-loop-helix proteins Hairy/Enhancer of Split (HES) and Hes-related repressor proteins (HEY). In particular HES1, HES5, HES7, HEY1, HEY2 and HEYL genes are direct targets of the Notch pathway **(Nemir *et al.*, 2006).** These genes function as transcriptional repressors and HEY appears to be important in the development of vascular tissue **(Iso *et al.*, 2003).** In a physiological condition, binding of the Notch ligand to its receptor initiates Notch signaling by releasing the Notch intracellular domain (NICD) through proteolytic cleavage by α-secretase and γ-secretase. Then, the NICD translocates into the nucleus where it modulates gene expression by binding to a ubiquitous transcription factor (CSL). This binding recruits transcription activators to the CSL complex and converts it into a transcriptional activator **(Figure 2) (Shih and Wang, 2007)**.

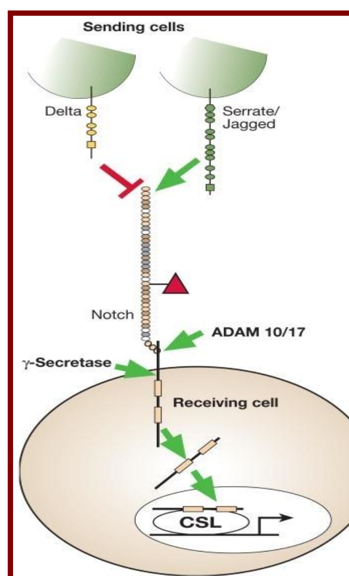


Figure 2- Notch signaling (adapted from Shih and Wang, 2007)

The physiologic functions of Notch signalling are the maintenance of stem cells, specification of cell fate and regulation of differentiation of multiple cell types both during development and in adulthood **(Artavanis-Tsakonas *et al.,* 1999).** In cancer, Notch pathway activation has been mostly associated with poor prognosis. In particular molecular processes that increase Notch activation such as chromosomal translocation, point mutations and chromosomal amplification of the Notch receptor loci, have been shown to lead to cancer progression **(Shih and Wang, 2007).**

6.2 Notch pathway in CLL

The deregulation of Notch pathway has been proposed to contribute to the biology of CLL. In this disease, overexpression of Notch2 seems to be involved in increased CLL cell survival **(Hubmann *et al.*, 2002).** In contrast, some authors reported that Notch is not a survival factor in CLL cells because it is not activated in these cells. When CLL cells were compared with normal B cells, similar levels of Notch 1 and Notch2 and their ligands were found **(Hadju *et al.*, 2007).** However, these authors reported that Delta-Like 1 was not expressed by normal B-cells but was detected in some CLL samples at low levels. They also found that HES-1 expression levels in normal B-cells were higher than in CLL cells, although a statistical correlation between CLL stage and HES-1 levels was not found **(Hadju *et al.*, 2007).**

On the other hand, Rosati et al (2009), reported that Notch signaling is activated in CLL cells. They showed that CLL cells but not normal cells express Notch1 and Notch2 as well as their ligands Jagged1 and Jagged2. In that study, Notch signaling activation was identified as a novel mechanism of CLL cell apoptosis resistance and survival capability **(Rosati *et al.*, 2009).**

7. MicroRNAs

7.1 What are microRNAs?

microRNAs (miRNAs) are short (19-25 nucleotides), single-stranded, noncoding RNAs that regulate the expression of hundreds of genes targeting the 3'-untranslated regions (3'-UTR). This results in degradation of mRNA or inhibition of translation. The miRNAs may regulate up to 30% of the protein-coding genes in the human genome **(Lewis *et al.*, 2005)** involved in several processes including development, hematopoiesis, organogenesis, apoptosis, cell proliferation and tumorigenesis **(Kim *et al.*, 2005a,b).** miRNAs can also function as tumor suppressors or oncogenes (oncomirs) **(Croce, 2009).**

7.2 miRNA biogenesis

The miRNAs genes are often located in clusters that may be transcribed from polycistronic transcriptional units. miRNAs genes are transcribed by RNA polymerase II (Pol II) into primary miRNAs **(Lee *et al.*, 2004).** The primary miRNAs are then processed by the nuclear RNase, Drosha, to release the precursor of miRNAs (pre-miRNA) and exported to the cytoplasm by the Exportin-5-RanGTP **(Yi *et al.*, 2003).** In the cytoplasm, Dicer RNase III recognizes the short 3'overhang made by Drosha and cleaves the pre-miRNAs into *ca*. 22 nt miRNAs duplexes **(Lee *et al.*, 2003).** Mature miRNAs are then incorporated into miRISC (miRNA-containing RNA-induced Silencing complex) and available to bind regulatory UTRs of the mRNAs.An alternative miRNA biogenesis mechanism has been identified, where the miRNA precursors reside in introns. In these intronic miRNAs, named mirtrons, the 3' end of the stem-loop precursor structure coincides with the 3' splice site, and is cleaved by nuclear pre-mRNA splicing rather than by Drosha **(Berezikov *et al.*, 2007; Okamura *et al.*, 2007; Ruby *et al.*, 2007).**

7.3 miRNAs role in CLL and other hematological malignancies

Expression profiling of miRNA emerged as a powerful tool and had been used with several purposes. miRNA expression profiles could identify the differentiation state of tumors and were considered more successful than messenger RNA expression in classifying poorly differentiated tumors. Although miRNA expression profiling could be used to discriminate between normal and malignant tissue, they are also useful to distinguish certain types or subtypes of cancer, distinguishing patients with good versus poor prognosis or even used to identify oncomirs **(Lu *et al.*, 2005).**

In CLL the miR-15a and miR-16 are important in prognosis: patients with good prognosis showed down-regulation of miR-15a and miR-16, whereas bad prognosis was associated with down-regulation of miR-29 **(Calin *et al.*, 2002, 2004).** These miRNAs reside in a cluster that is located in the 13q14.3 chromosome region, which is frequently deleted in CLL patients, and could explain the loss or down-regulation of these miRNA in CLL patients **(Calin *et al.*, 2002).** The down-regulation of the miR-15a/16-1 resulted in an increase of cancer-related genes involved with apoptosis and cell cycle **(Calin *et al.,* 2008).** The miR-21, miR-150 and miR-155 were shown to be up-regulated in CLL **(Fulci *et al.*, 2007)**, while miR-181a, let-7a and miR-30d were down-regulated **(Marton *et al.*, 2008).**

Other hematological tumors benefits with the discovery of miRNA as biomarkers for diseases. miRNA signatures were associated with cytogenetic abnormalities in AML and the high expression of miR-191 and miR- 199a correlated with patients having poor prognosis **(Garzon *et al.*, 2008).**

Several miRNA were differentially expressed between AML and ALL, where miR-128a, miR-128b, miR-223 and let-7b were the most significant and discriminatory. It was reported that some AML patients showed up-regulation of miR-155, which may inhibit

the expression of genes implicated in hematopoietic development and disease **(O'Connell *et al.*, 2008).**

In chronic myeloid leukemia (CML) was reported that CD34$^+$ cells from patients in chronic phase, the oncomir- 1 comprising seven miRNA (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b-1, miR- 20a, and miR-92a-1) was up-regulated compared to blast phase **(Venturini *et al.*, 2007)**.

7.4 The circulating miRNAs and their prognostic value

miRNAs were recently found to exist in the peripheral circulation, either inside microvesicles (MVs) (**Hunter *et al.*, 2008)** but also associated to high-density lipoprotein (HDL) **(Vickers *et al.*, 2011).** In fact, circulating miRNAs have prognostic value, as shown for miR-141 in colon cancer **(Cheng *et al.*, 2011)** and prostate cancer **(Mitchell *et al.*, 2008)** or miR-21 that predict poor survival in colon rectal cancer **(Asangani *et al.*, 2008).** In CLL, circulating miRNAs also have been reported as potential biomarkers on diagnosis and classification of CLL. Moussay et al., described that levels of miR-150 in plasma of CLL patients correlated with stages of the disease **(Moussay *et al.*, 2010).**

Circulating miRNAs are stable and can be detected directly in serum and plasma samples **(Chen *et al.*, 2008).** Serum contains many miRNAs derived from various tissue/organs and recent findings showed that serum miRNA expression can be used as a novel serum biomarker providing more sensitive and specific tests than those usually used for diagnosis of cancer and other diseases **(Chen *et al.*, 2008).**

Since the discovery that miRNAs could circulate inside MVs, studies begin to emerge to unravel the role of these MVs in the cell-to-cell communication. The importance of communication between tumor cells and their environment through MVs has been

recently demonstrated. It has been show that MVs contain miRNAs that having the ability to migrate from BM, where MVs are released from mesenchymal stem cells to another organs thought blood vessels. These miRNAs are functional and this is a programmed communication between cells, regulated by the release of factors that influence cell fate, function and plasticity. Recently, MVs have been described as a strong paracrine mechanism that could re-direct cell fate via the active transfer of functional mRNAs and miRNAs **(Collino *et al.*, 2010).**

7.5 The role of specific miRNAs used in this study in CLL and other cancers

The miRNAs used in the current project for profiling studies were selected based on the previous study performed in our lab **(Teixeira, 2009)** and their importance already described in the literature. Teixeira has performed microarrays of miRNAs of CLL and SLL samples pools and results showed specific miRNAs in which expression is differentially expressed in CLL pools versus SLL pool **(Teixeira, 2009)**. Therefore, two miRNAs, miR-942 and miR-380 were selected in the current project to test expression in individual patients. The other miRNAs selected for profiling studies were miRNAs from the miR-15/16 cluster (miR-15a, miR-16-1), miR-155, miR-21, miR-101, miR-223, miR-221, miR-222, miRNAs form the miR-34 family (miR-34a, miR-34c), miR-141 and miR-210.

The miR-942 was previously identified as differentially expressed between PBMNC of CLL and SLL pools of samples by miRNA microarrays (Exiqon), where its expression levels were higher in the CLL pool than in SLL pool **(Teixeira, 2009).** A literature search for miR-942 returned only sequence data, with no known function to date.

The miR-380 was identified from the previous study in our lab, where it showed to have higher expression in SLL samples, and in samples with more percentage of EPC

(Teixeira, 2009). This miRNA, along with miR-942 was identified in by miRNA microarrays (Exiqon), using CLL and SLL pools RNA from P. To date, no targets were reported in the literature.

As already described, the cluster miR-15-16-1, of miR-15a and miR-16-1 is localized in the chromosome 13 (13q14.3), in a region frequently deleted in CLL. As this miRNA cluster regulates post-transcriptionally the anti-apoptotic protein BCL-2, the absence of the cluster miR-15-16-1 results in an aberrantly increase of BCL-2, and therefore cells highly resistant to apoptosis **(Calin *et al.*, 2002).** Other known targets of the miR-15a and miR-16-1 are CNOT6L, USP15, PAFAH1B1, ESRRG genes involved in CLL **(Calin *et al.*, 2005).**

The miR-155 is an oncogene, often over-expressed in solid tumors, as lung, colon and breast cancer, and in hematological cancers as AML **(Nana-Sinkam and Croce, 2010)** and CLL **(Fulci *et al.*, 2007).** This miRNA is known to regulate ZNF537, PICALM, RREB1, BDNF and QKI **(Calin *et al.*, 2005).**

The miR-21 was described as an oncogene which plays a key role in resisting programmed cell death in cancer cells and that targeting apoptosis is a viable therapeutic option against cancers expressing miR-21 **(Buscaglia and Li., 2011).** miR-21 was described as been involved in leukemia drug resistance by regulation of PTEN gene, a tumor suppressor **(Bai *et al.*, 2011)** it was also been reported a down-regulation of PTEN and PDCD4 genes in other leukemias, as NK-cell lymphoma/leukemia (**Asangani *et al.*, 2008; Yamanaka *et al.*, 2009**). In CLL miR-21 targets the CCND2 and DPH1 transcripts, where CCND2 was reported to control cell cycle progression in CLL cells while DPH1 is a candidate tumor suppressor gene in ovarian and breast cancers and is localized telomerically to TP53 **(Rossi *et al.*, 2010).**

The miR-101 is frequently down-regulated in cancers, as in glioblastoma **(Smits *et al.*, 2010),** where it promotes apoptosis through targeting MCL-1 **(Su *et al.*, 2009).** It was also reported as a target for the proto-oncogene MCYCN **(Buechner *et al.*, 2011).**

The expression of miR-223 is overexpressed in gastric carcinoma **(Li *et al.*, 2011)** and this miRNA is known to regulate PTBP2, SYNCRIP, WTAP and FBXW7 transcripts **(Calin *et al.*, 2005).** In CLL, mir-223 expression decreased with progression from Binet stage A the less aggressive stage of CLL to C, the most aggressive stage of CLL **(Stamatopoulos *et al.*, 2009).**

The expression of the miRNAs miR-221 and miR-222, belonging to miR-221/222 cluster, is overexpressed in solid tumors as non-small cell lung cancer **(Chen *et al.*, 2008; Calin and Croce, 2006)** and also in CLL **(Moussay *et al.*, 2010).** The miR-221 and miR-222 regulate the tumor suppressor p27, therefore the aberrant increase of miR-221 and miR-222 lead to a decrease of the p27, resulting in an abnormally high cell-cycle progression **(Nana-Sinkam and Croce, 2010).** miR-221 is known to regulate HECTD2, CDKN1B, NOVA1, ZFPM2 and PHF2 transcripts **(Calin *et al.*, 2005).**

The miR-34 family is composed by miR-34a, miR-34b and miR-34c. These genes are up-regulated by p53, and their over-expression causes senescence, apoptosis or cell cycle cessation by regulating proteins such as BCL2. The TP53, located at the 17p13.1 locus, encodes the tumor-supressor protein p53 that plays a central role in regulating the cell cycle and apoptosis. The miR-34a had been described as promoting apoptosis in prostate cancers and neuroblastomas **(Cannel and Bushell, 2010).** CLL patients with loss of TP53 through 17p deletions are resistant to treatment and have poor clinical outcomes **(Ward *et al.*, 2011).** Consequently in CLL, the abnormal expression of miR-34a is associated with patients having p53 mutations or 17p13.1 deletions, resulting in lower miR-34a expression **(Nana-Sinkam and Croce, 2010).**

The miR-34c also functions as a tumor-suppressor through the inhibition of growth and invasion of cancer cells. This miRNA has been described as down-regulated in solid tumors **(Hagman *et al.*, 2010; Cai *et al.*, 2010; Cannel and Bushell, 2010).** This miRNA was reported as a tumor-suppressor by repressing MYC translation, an oncogene that regulate cell proliferation and is frequently deregulated in human malignancies **(Cannel and Bushell, 2010).**

The expression of the miR-141 was reported in epithelial tumors **(Mitchell *et al.*, 2008).** The levels of circulating miR-141 were reported to have prognostic value in colon cancer **(Cheng *et al.*, 2011).**

The miR-210 is a hypoxia-regulated miRNA, which induction is directly related with hypoxic response in both normal and malignant cells **(Devlin *et al.*, 2011)** and has an important role as a tumor-suppressor with effects on cancer cell proliferation **(Tsuchiya *et al.*, 2011)**. The miR-210 has been reported as a prognostic factor in solid tumors, as breast cancer **(Rothé *et al.*, 2011)** and the circulating miR-210 was also identified as prognostic marker in pancreatic cancer patients **(Ho *et al.*, 2010).**

miR-210 have been named “master miRNA of the hypoxic response” by Ivan et al., (2008), and due to its importance, many studies have been conducted to find targets of miR-210 **(Ivan et al., 2008).** This miRNA is involved in regulation of many cellular processes as migration and adhesion (CLASP2, MDGA1, P4HB, PTPN1, NCAM1), cell cycle (ACVR1, APC, CDK10, E2F3, SERTAD2), differentiation (Ephrin A3, BDNF, ACVR1) and others. Other miR-210 targets are NPTX1, RAD52, ACVR1, MNT, CASP8AP2, FGFRL1, HOXA1 and HOXA9 **(Fasanaro *et al.*, 2008; Fasanaro *et al.*, 2009; Chan *et al.*,2009; Crosby *et al.*,2009; Giannakakis *et al.*,2008; Kim *et al.*,2009; Mizuno *et al.*,2008; Pulkkinen *et al.*,2008; Zhang *et al.*,2009).**

Methods

1. Patients Classification and sample collection

PB was collected from patients with CLL/SLL prior treatment, after informed consent of each patient and as according IPOFG guidelines. Patients were grouped accordingly to standard diagnosis criteria by the Hemato-oncology department at IPOFG. Accordingly patients were grouped into CLL or SLL groups. CLL patients were further classified in stage A, B or C according to the clinical stage of the disease under Binet classification. In this study were used 19 samples from patients with CLL in stage A, 6 samples from 6 patients with CLL in stage B or C and 8 samples from patients with SLL. Patients were also classified according to sex, age and cytogenetic abnormalities. Patients included in the present study were not under treatment **(supplementary table 1).**

From a total of 33 samples used in the present study, 11 were received during this project while 22 samples were collected previously **(Teixeira, 2009).** All samples were collected and processed under the same conditions.

1.1 Sample Processing: plasma and cells collection

The plasma was isolated by centrifugation of the blood sample at 453g for 10 min at 4ºC and stored at -70ºC. PBMNC were isolated by density gradient centrifugation with Lymphoprep (Axis-Shield, Rodelokka, Oslo, Norway). PBMNC were then counted and separated for different analyses: $2x10^6$ cells were resuspended with PBS-0,5% bovine serum albumin (BSA) for Flow Cytometry technique, $2x10^6$ cells were stored in TRIZOL reagent (Invitrogen) for further RNA extraction and the remaining cells were frozen at -70ºC in a mixture of cell culture medium (RPMI, 40%) with fetal bovine serum (FBS, 50%) and dimethyl sulfoxide (DMSO, 10%).

2. Flow Cytometry

To analyze cell populations by fluorescence-activated cell sorting (FACS), approximately $2x10^6$ PBMNC of each sample were incubated for 45 minutes with fluorescent conjugated antibodies, with rotation at 4ºC. After that the cells were washed twice in PBS-0,5%, BSA with 2 centrifugations of 5 minutes at 135 g for 5 minutes. At that point 100.000 PBMNC, resuspended in 200 µl of PBS-0,5%, BSA, were acquired. Flow cytometry was done in FACSCalibur (BD Biosciences) and analysis was done with the use of CellQuest software.

To identify populations of BM circulating cells immunofluorescent staining was performed, according to the manufacter´s instructions, with the use of the following fluorescent conjugated antibodies: **(supplementary table 2).**

The myeloid population was defined by CD11b expression, the population of cells with chemotactic activity was defined by CXCR4 and the population of progenitor cells was defined by CD34. The population of myeloid cells with chemotactic capability was thus defined by CD11b and CXCR4 markers and the EPC population was isolated using the CD133 and KDR markers according to previously reported **(Urbich and Dimmeler, 2004; Shantsila *et al.*, 2008).** The population of progenitor cells with chemotactic capability was defined by CD34 and CXCR4 markers and the population of myeloid progenitor cells was defined by CD34 and CD11b markers. 11 samples were analyzed (7 samples of CLL stage A, 2 samples of CLL B or C and 2 samples of SLL).

3. Enzyme-Linked Immunosorbent Assay (ELISA)

3.1 VEGF

The VEGF levels were measured in undiluted plasma of 11 PB samples (9 CLL and 2 SLL). Five controls from healthy controls (with ages between 23 and 32 years) were included in this experiment.

To avoid protein degradation, 0,1% of protease Inhibitor (complete, Mini, EDTA-free Roche, Mannheim, Germany), was added to the samples. The VEGF ELISA was performed according to the manufacturer's protocol (Calbiochem, Dalmstadt, Germany). All samples were assessed in duplicate.

The VEGF levels of 22 samples (16 CLL and 6 SLL) obtained previously **(Teixeira, 2009)** were included in the analysis. The protocols used in the present project and in that previous study were identical and therefore could be compared. We chose to add those additional data to improve the statistical significance of the data.

3.2 SDF-1

SDF-1 levels were measured in undiluted plasma of 11 PB samples (9 CLL and 2 SLL). Five controls from healthy controls (with ages between 23 and 32 years) were included in this experiment. All samples were treated as mentioned above in section 3.1.

4. RNA Extraction

4.1 Extraction and quantification of total RNA from PBMNC

Total RNA was extracted from the PBMNC using TriZol Reagent (Invitrogen) according to manufacturer's instructions. The RNA concentrations were measured on a NanoDrop Spectrophotometer ND-1000 (NanoDrop), in which surface tension is used to hold a column of liquid sample in place while a measurement is made. The purity of the sample is given by the ratio absorbance at 260 and 280 nm. A ratio of 1.8 indicates pure DNA, whereas a ratio of 2.0 indicates pure for RNA. Ratios below these values are indicative of protein, phenol or other contaminants that absorb light near 280 nm. For RNA measurements, the ratio of the sample absorbance at 260 and 230 nm is also a useful parameter to reveal the presence of contaminants. A ratio 260/230 of 2.0 indicates that RNA sample is free of contaminants.

4.2 miRNA Extraction from Plasma

The miRNAs were isolated from 250 µl of plasma using a method that combines Qiazol (QIAGEN, Austin, Texas, U.S.A.) and Chloroform extraction with and further enrichment of the small RNAs (miRNAs) with the miRNeasy Mini Kit (QIAGEN, Austin, Texas, U.S.A.). The RNA was stored at -70ºC until use. The detailed protocol is in the Appendix 1.

5. cDNA Synthesis

5.1 cDNA Synthesis for analysis of expression of coding genes

The first strand of cDNA was synthesised by combining RNA (*ca*. 1 µg), 50 ng/µl of random-hexamer primer, 2 µl of 10 mM dNTP mix and water in a 12 µl volume. The reaction was incubated at 65ºC for 5 min and then placed on ice. A master mix was

prepared, containing: 4 µl 5 x cDNA synthesis buffer, 1 µl 0.1 M DTT, 1 µl RNase-OUT, 1 µl DEPC-treated water and 1 µl ThermoScriptTM enzyme. Eight µl of the master mix were added to each tube containing the RNA mixture and incubated as follows: 10 min at 25ºC, 45 min at 50ºC and 5 min at 85ºC. Synthesized cDNA was stored at -20ºC.

5.2 cDNA synthesis for analysis of miRNA levels

cDNA synthesis for analysis of miRNA cDNA was synthesized from ca. 500 ng of RNA isolated from plasma samples using the NCode™ miRNA first-strand synthesis (Invitrogen™). This method provides the polyadenylation of mature microRNAs of total RNA using poly A polymerase and ATP. Following polyadenylation, SuperScriptTM III RT and a specially-designed Universal RT Primer provided are used to synthesize cDNA from the tailed miRNA population, as described by the manufacturer's.

6. Quantitative RT-PCR (qPCR)

The quantification of coding and non-coding genes (miRNAs) was carried out in a Light Cycler 480 System (Roche, Mannheim, Germany) instrument in 384-well plates. In the Light Cycler , the threshold of detection is defined as Cp (crossing point), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal **(Millon *et al.*, 2005).** All samples were used in triplicate. Negative controls were water (no cDNA). A total of 48 cycles were used and the thermocycling parameters were: 5 minutes at 95ºC, 30 seconds at 60 to 95ºC, 1 minute and 5 seconds at 65 to 97ºC and 30 seconds at 40ºC. The sequences of all primers and the miRNAs used in this study, are listed in the **(supplementary table 3).** The primers were designed in the Primers Express software (Applied Biosystems) with parameters optimized for quantitative real time polymerase chain reaction (qPCR).

6.1 Quantification of coding genes

Two µl of diluted cDNA (1:2) was used as template in 15µL qPCR reactions with 10 µM of each primer and SYBR Green I Master (Roche). The human 18S rRNA was used as endogenous control to normalize RNA input levels and mean of ΔCp values of 5 healthy controls (with ages between 23 and 32 years) was used as a calibrator. Relative fold changes of gene expression were assed using 2^(-ΔΔCp) comparative method, as previously described **(Livak and Schmittgen, 2001).**

6.2 Quantification of miRNAs

6.2.1 Quantification of miRNAs isolated from blood plasma

To quantify miRNAs, two µl of diluted cDNA (1:1,5) prepared as described previously (section 5.2) were used as template in 15µL qPCR reactions with 10 µM of each primer (Universal qPCR primer and a miRNA-specific forward primer) and SYBR Green I Master mix (Roche). The forward primer in qPCR is specific for the miRNA sequence of interesting which the DNA oligo (primer) is identical to the entire mature miRNA sequence, where the Uracil bases are replaced by Thymine bases (supplementary table 3). The miRNA sequences were extracted from the miRbase **(Kozomara and Griffiths-Jones, 2011)**.

Normalization of RNA input levels was performed using mean of ΔCp values of three human miRNAs (miR-363*, miR-191, miR-223*) that had no variance in expression between CLL and SLL samples and controls. Results were presented as 2^(-ΔCp) as previously described **(Vasilescu *et al.*, 2009)**.

6.2.2 Quantification of miRNAs isolated from PBMNC

The quantification of miRNAs isolated from cells is identical to described above in section 6.2.1, for plasma (two µl of diluted cDNA (1:1,5) was used as template in 15µL qPCR reactions with 10 µM of each primer and SYBR Green I Master (Roche). However, the normalization was performed with human small nuclear gene U6 expression and miRNA relative expression was calculated using the ΔCp method. Results were presented as 2^(-ΔCp) as previously described **(Swarbrick *et al.*,2011)**.

7. Statistical analysis

Statistical significant differences were assessed by unpaired two-tailed Student's t test and statistical significance was defined as $p<0.05$. Correlation analyses were calculated using the Pearson correlation test. Statistical analyses and graphs were obtained using GraphPad Prism v. 5.0 (GraphPad Software, CA, USA).

Results

1. Circulating BM cells

In order to quantify circulating cells of the BM and relate it with disease progression, the quantification of populations present in PBMC isolated from PB of 7 CLL and 2 SLL patients were performed by flow cytometry technique **(Table 3).**

Table 3 - Percentage of circulating BM cells identified in each sample analyzed by flow cytometry.

Stages	Samples	CD11b+	CXCR4+	CD34+	CD11b/ CXCR4+	CD133/ KDR+	CD34/ CXCR4+	CD34/ CD11b+
CLL A	CLL35	24.17%	8.64%	8.62%	8.32%	0.00%	………	………
	CLL36	6.14%	2.27%	1.38%	1.11%	0.11%	0.22%	0.19%
	CLL39	7.58%	4.14%	5.41%	3.40%	0.02%	0.25%	0.10%
	CLL40	2.08%	2.02%	3.37%	1.43%	0.00%	0.19%	0.05%
	CLL41	4.12%	0.01%	0.07%	0.02%	0.00%	0.01%	0.00%
	CLL42	4.66%	5.78%	0.85%	3.51%	0.06%	0.29%	0.00%
	CLL43	1.25%	0.52%	0.27%	0.68%	0.00%	0.03%	0.01%
CLL B	CLL38	40.28%	54.59%	0.83%	0.79%	0.00%	1.29%	1.04%
CLL C	CLL34	1.47%	1.25%	1.02%	1.01%	0.01%	………	………
SLL	CLL37	60.27%	41.11%	0.80%	9.92%	0.00%	10.57%	10.40%
	CLL44	6.38%	6.79%	0.31%	4.15%	0.00%	1.07%	0.38%

The population of progenitor cells was defined as $CD34^+$ cells, the myeloid population was defined as $CD11b^+$ cells and the population of cells with chemotactic capability was defined as $CXCR4^+$ cells. The population of myeloid cells with chemotactic capability was defined as $CD11b^+$/$CXCR4^+$ cells, the population of myeloid progenitor cells was defined as $CD34^+$/$CD11b^+$ cells, the population of progenitor cells with chemotactic capability was defined as $CD34^+$/$CXCR4^+$cells and the population of EPCs was defined as $CD133^+$/KDR^+ cells.

1.1 Percentage of Leukemic cells in CLL and SLL

The Leukemic population, defined together with Hemato-Oncology Department, is presented in the following table **(Table 4).**

Table 4 - Percentage of Leukemic cells of each sample analyzed by flow cytometry.

Stages	Samples	Leukemic Cells
CLL A	CLL35	35.00%
	CLL36	65.00%
	CLL39	50.00%
	CLL40	40.00%
	CLL41	48.00%
	CLL42	40.00%
	CLL43	57.00%
CLL B	CLL38	94.00%
CLL C	CLL34	92.00%
SLL	CLL37	90.00%
	CLL44	24.00%

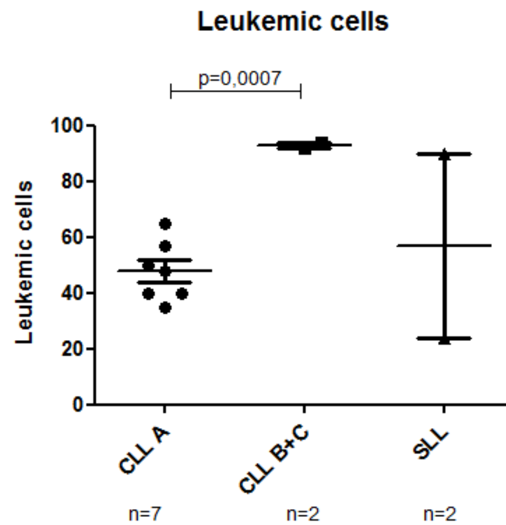


Figure 3 - Percentage of Leukemic cells in CLL (stage A and stages B or C) and SLL, analyzed by flow cytometry. Error bars represent standard deviation (SD).

To investigate the relation between disease stages and the percentage of malignant cells in circulation, the percentage of Leukemic cells was quantified in 11 samples (9 CLL and 2 SLL).

From the results presented in Table 4 and Figure 3, it can be observed that samples of CLL in stage B or C (93.00%) had, on average, a higher percentage of leukemic cells in circulation when compared with stage A (47.86%). Although on average the SLL as a 57.00% of leukemic cells, the number of samples analyzed was small, and percentages of leukemic cells in these two samples was not similar (90.00% and 24.00%). Therefore, a higher number of samples should be analyzed to compare with CLL samples.

1.2 Individual analysis of Circulating BM cells – examples

From the total of 11 samples analyzed by Flow Cytometry, it was observed that the percentages of cells populations differed between samples. In order to clarify these differences we look carefully for 4 of these samples, as examples (CLL34, CLL38, CLL39 and CLL44).

It was observed that in CLL39, a sample of CLL stage A, 50 % of the total PBMNC was leukemic cells, this sample had 0.02% of EPCs, 4.14% of $CXCR4^{+}$ cells and 7.58% of $CD11b^{+}$ cells. The levels of this samples of VEGF and SDF-1 was 12,89 and 657,70 pg/ml, respectively, as it as will be presented later in this study **(Tables 3 and 4).**

In CLL38, a sample of CLL stage B, 92 % of the total PBMNC was comprised by leukemic cells, this sample had 0.0% of EPCs, 54.59% of $CXCR4^{+}$ cells and 40.28% of $CD11b^{+}$ cells.

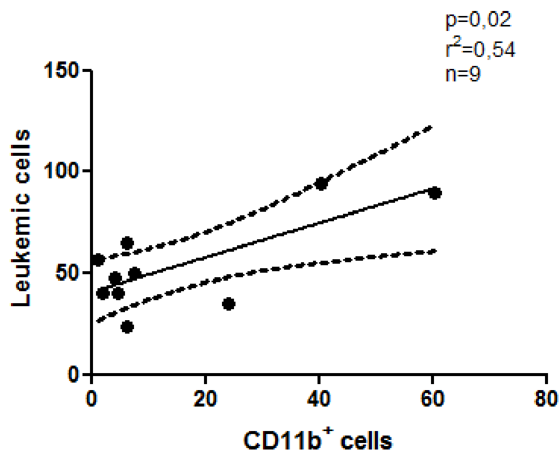
In CLL34, a sample of CLL stage C, 94 % of the total PBMNC was leukemic cells, this sample had 0.01% of EPCs, 1.25% of $CXCR4^{+}$ cells and 1.47% of $CD11b^{+}$ cells.

In CLL44, a sample of SLL, 24 % of the total PBMNC was leukemic cells, this sample had 0.0% of EPCs, 6.79% of $CXCR4^{+}$ cells and 6.38% of $CD11b^{+}$ cells.

1.3 Correlations between circulating Leukemic cells and circulating BM Cells

In order to investigate the relation between BM circulating cells and Leukemic cells, a correlation analysis using Pearson Test was done. ($p<0,05$).

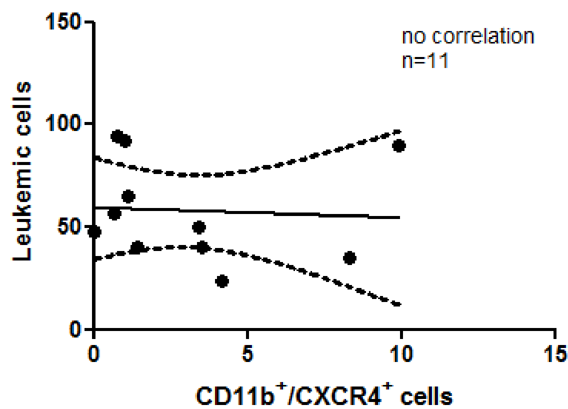
The data suggest that $CD11b^{+}$ cells (myeloid population) and Leukemic cells had a positive correlation between 10 samples **(Figure 4).**



CLL A	n=6
CLL B	n=1
SLL	n=2

Figure 4 - Correlation analysis between percentage of Leukemic cells and CD11b$^+$ cells analyzed by flow cytometry. Pearson test was performed to evaluate correlations.

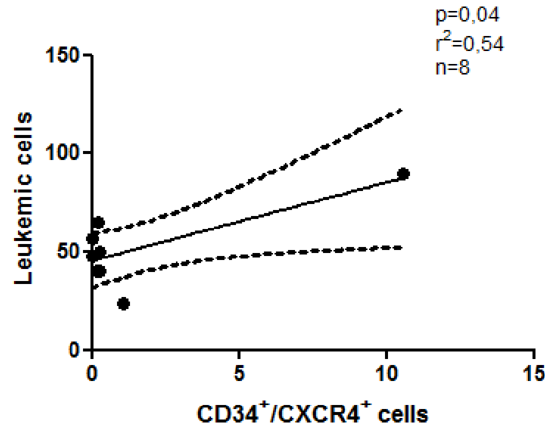
It was not possible to establish a correlation between Leukemic cells and CD11b$^+$/CXCR4$^+$ cells (myeloid population with chemotactic capability) in the 11 samples analyzed (9 CLL and 2 SLL) **(Figure 5).**



CLL A	n=7
CLL B	n=2
SLL	n=2

Figure 5 - Correlation analysis between percentage of Leukemic cells and CD11b$^+$/CXCR4$^+$ cells analyzed by flow cytometry, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

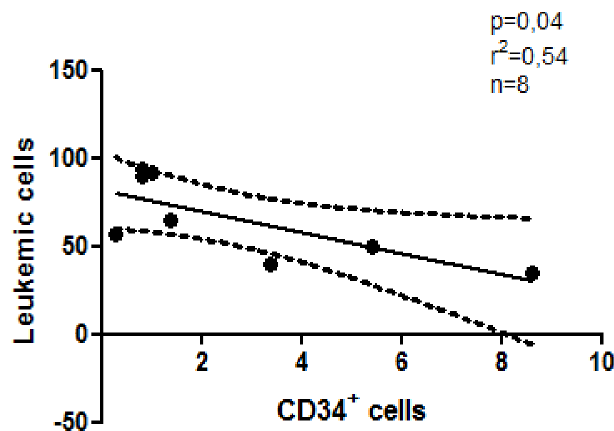
It was found a positive correlation in 8 samples of CLL and SLL between CD34$^+$/CXCR4$^+$ cells (progenitor cells with chemotactic capability) and Leukemic cells **(Figure 6).**



CLL A	n=6
CLL B	n=0
SLL	n=2

Figure 6 - Correlation analysis between percentage of Leukemic cells and CD34$^+$/CXCR4$^+$ cells analyzed by flow cytometry, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

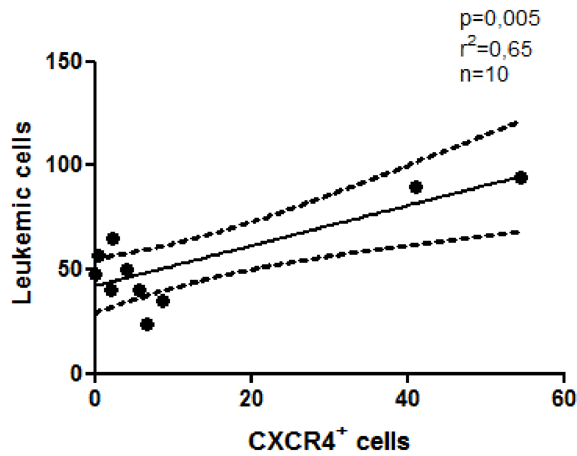
In 8 samples of CLL and SLL a positive correlation was verified between CD34$^+$ cells (progenitor cells population) and Leukemic cells **(Figure 7).**



CLL A	n=5
CLL B	n=2
SLL	n=1

Figure 7 - Correlation analysis between percentage of Leukemic cells and CD34$^+$ cells analyzed by flow cytometry, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

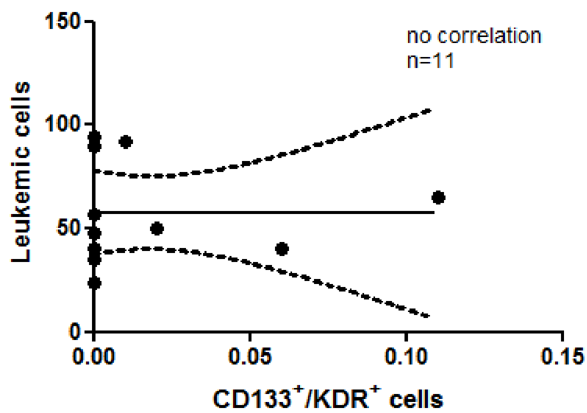
It was detected a positive correlation in 10 samples of CLL and SLL, between CXCR4$^+$ cells (cell population with chemotactic capability) and Leukemic cells **(Figure 8).**



CLL A	n=7
CLL B	n=1
SLL	n=2

Figure 8 - Correlation analysis between percentage of Leukemic cells and $CXCR4^{+}$ cells analyzed by flow cytometry, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

It was not possible establish a correlation between Leukemic cells and $CD133^{+}/KDR^{+}$ cells (myeloid population with chemotactic capability) in 11 samples (9 CLL and 2 SLL) **(Figure 9).**



CLL A	n=7
CLL B	n=2
SLL	n=2

Figure 9 - Correlation analysis between percentage of Leukemic cells and $CD133^{+}/KDR^{+}$ cells analyzed by flow cytometry, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

2. Levels of VEGF in CLL versus SLL

The levels of circulating VEGF measured in patients plasma, were evaluated and related with disease stages, in 19 samples of CLL, 4 samples of SLL and 4 normal controls. The results are presented in the following sections.

The circulating levels of VEGF of normal controls was not detected (0.00 pg/ml), whereas in CLL (38.42 pg/ml) the VEGF levels were significantly higher, in average, than in SLL (21.34 pg/ml) (p=0,0299 (**Figure 10A**). There were not found statistical significant differences between less aggressive stage of CLL (stage A) and the most aggressive stages of CLL (stages B and C), but in average, the both stages of CLL, had statistically higher levels of VEGF than SLL (**Figure 10B**).

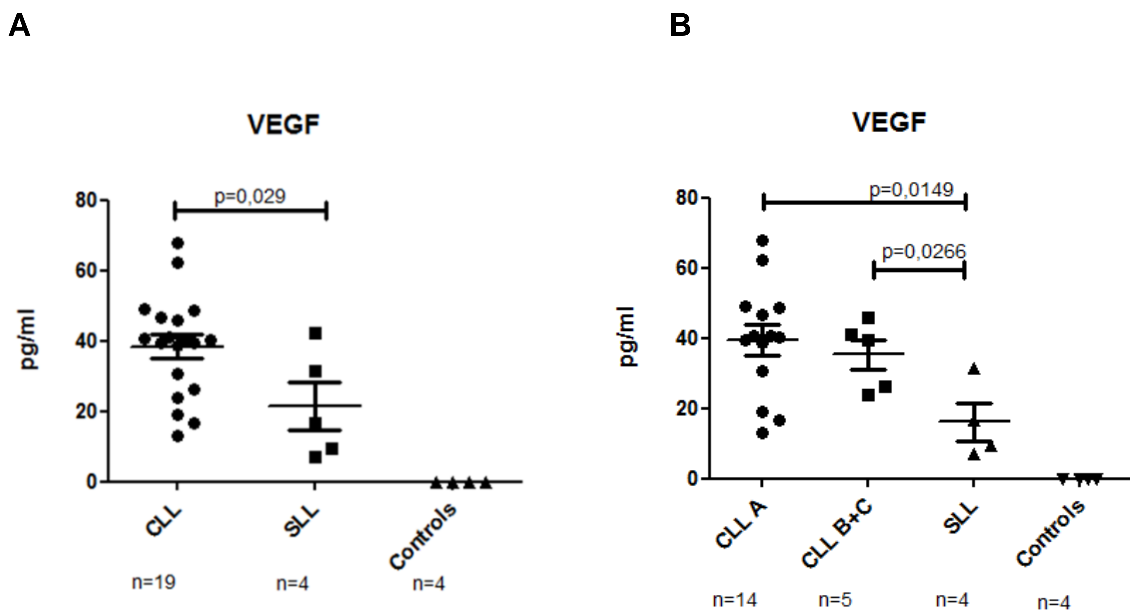
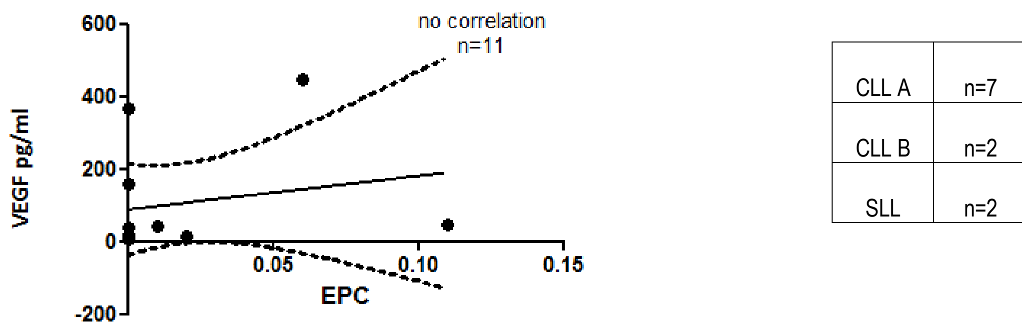


Figure 10 – VEGF levels in CLL (stage A and stages B or C) and SLL, measured by ELISA technique. P values were obtained from Unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

2.1 Correlation between circulating EPC and VEGF levels

To identify a relation between circulating EPCs (CD133$^+$/KDR$^+$ cells) and VEGF levels, it was done a correlation analysis using Pearson Test. (p<0,05). It was not found any correlation between VEGF levels and circulating EPCs in a panel of 11 samples of CLL and SLL patients **(Figure 11).**



CLL A	n=7
CLL B	n=2
SLL	n=2

Figure 11 - Correlation analysis between percentage of EPCs (CD133$^+$/KDR$^+$ cells) analyzed by flow cytometry and VEGF levels measured by ELISA technique, in CLL and SLL samples.Pearson test was performed to evaluate correlations.

3. Levels of SDF-1 in CLL versus SLL

It were evaluated the levels of circulating SDF-1 measured in patients plasma with ELISA kit and related it with disease stages, in 9 samples of CLL, 2 samples of SLL and 4 normal controls. The results are presented in the following sections.

SFD-1 levels were higher, in average, in SLL (1107.00 pg/ml) than in CLL (851.60 pg/ml) samples although this difference was not statistically relevant (Figure 12A). Likewise, there was no statistically differences between SDF-1 levels of healthy

controls (1035.00 pg/ml) and CLL or SLL patients **(Figure 12A).** The Levels of SDF-1 was not statistically different between CLL stage A and CLL stage B and C, although the levels of SDF-1 were higher in CLL stage A **(Figure 12B).**

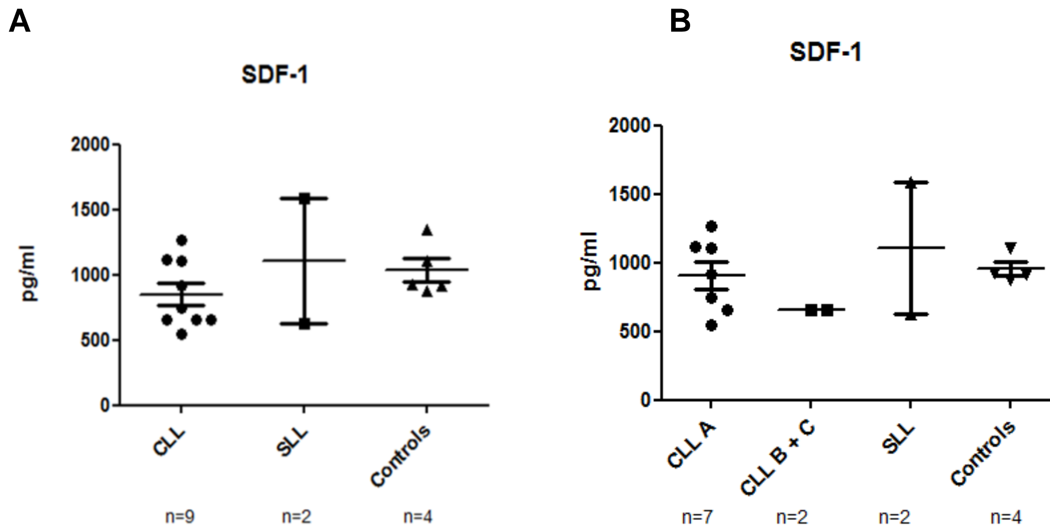
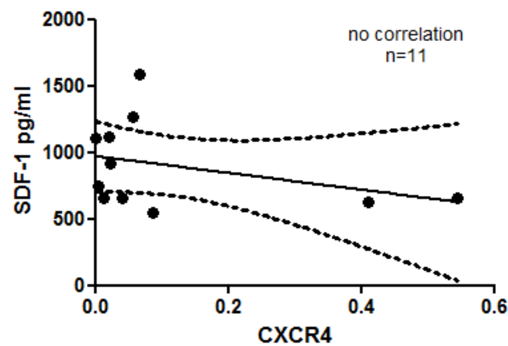


Figure 12 – SDF-1 levels in CLL (stage A and stages B or C) and SLL, measured by ELISA technique. P values were obtained from Unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

3.1 Correlation between CXCR4 + population and SDF-1 levels

To identify a relation between circulating CXCR4^{+} cells and SDF-1 levels, it was done a correlation analysis using Pearson Test. ($p<0,05$). It was not found any correlation between SDF-1 levels and CXCR4^{+} cells in a panel of 11 samples of CLL and SLL patients **(Figure 13).**



CLL A	n= 7
CLL B	n=2
SLL	n=2

Figure 13 - Correlation analysis between percentage of $CXCR4^{+}$ cells analyzed by flow cytometry and SDF-1 levels measured by ELISA technique, in CLL and SLL samples. Pearson test was performed to evaluate correlations.

4. Expression of anti-apoptotic genes in CLL and SLL

One of the hallmarks of CLL is the resistance of the leukemic cells to apoptosis, therefore the expression of two apoptosis-related genes (BCL-2 and MCL-1) was investigated. To quantify the levels of expression of BCL-2 and MCL-1, and relate it with disease stage and progression, qPCR analysis was performed. The results are presented in the following sections.

4.1 Expression of BCL-2 in CLL versus SLL

The quantification of the anti-apoptotic gene BCL-2 expression was performed on a panel of 15 samples of CLL and 6 samples of SLL. The BCL-2 expression was expressed at higher levels, in average, in CLL samples ($2{,}06\times10^{-4}$) than in SLL samples ($5{,}6\times10^{-5}$) and this difference was statistically significant ($p=0{,}014$) **(Figure 14A)**.

When comparing the CLL stages, a higher level of BCL-2 transcript was observed in the most aggressive stages of CLL (stages B and C) when compared with the less aggressive stage of CLL (stage A). The BCL-2 transcript levels were reduced in SLL when compared with all stages of CLL (CLLA and SLL p=0,033; CLLB and SLL p=0,002) **(Figure 14B).**

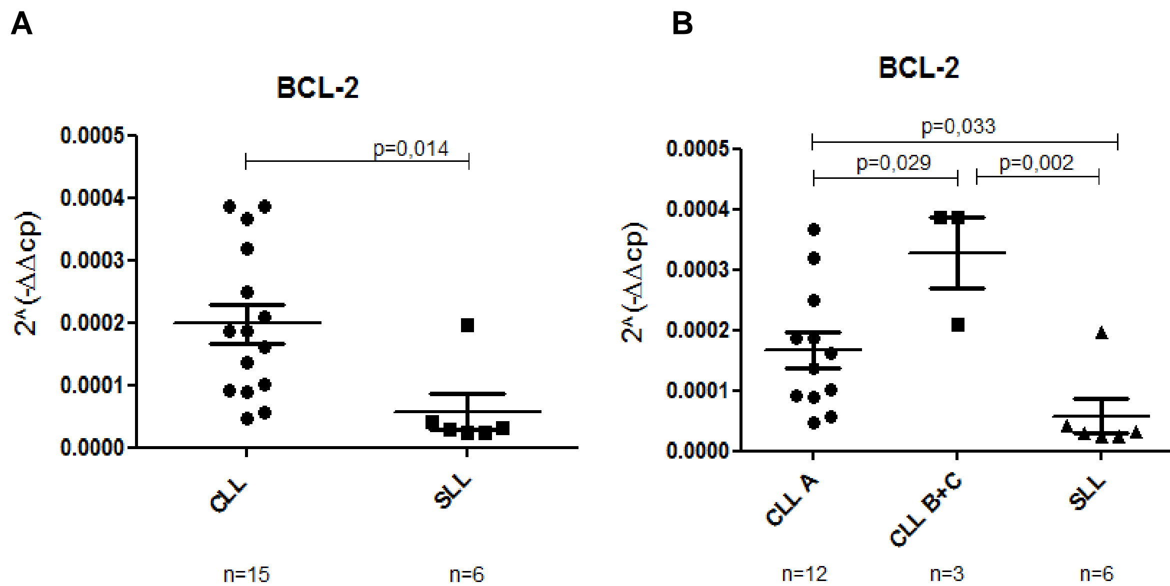


Figure 14 – BCL-2 expression in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using 18S ribosomal RNA and fold changes were calculated relative to the average expression in cells of healthy controls using the 2^(-ΔΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

4.2 Expression of MCL-1 in CLL versus SLL

The analysis of MCL-1 gene expression, an anti-apoptotic gene of the BCL-2 family, was performed on a panel of 13 CLL and 5 SLL. MCL-1 expression showed a statistically significant increase in average, in CLL ($5,37x10^{-2}$) than in SLL samples ($1,98x10^{-2}$) (p=0,021). **(Figure 15A)** The MCL-1 levels of expression were reduced in SLL than in CLL stages (stage A and stages B and C) **(Figure 15B).**

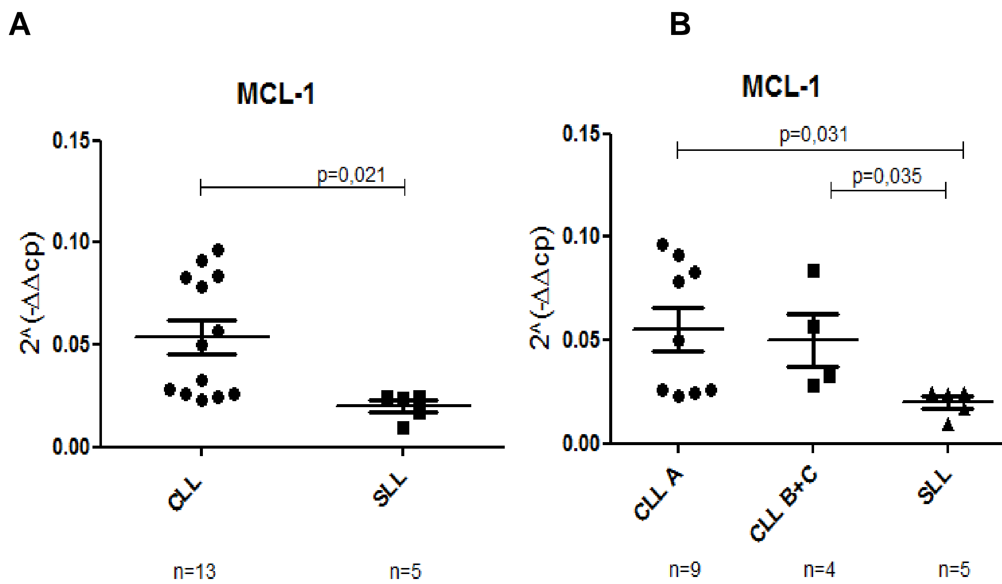
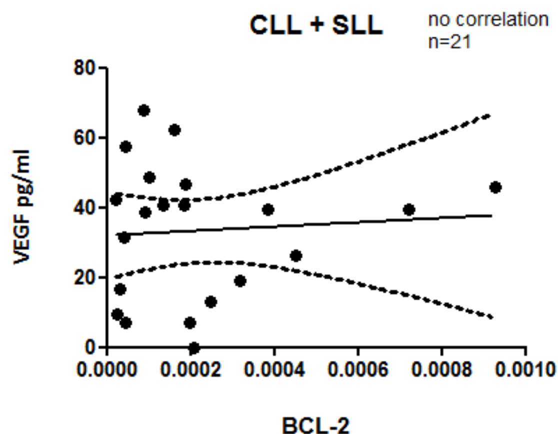


Figure 15 – MCL-1 expression in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using 18S ribosomal RNA and fold changes were calculated relative to the average expression in cells of healthy controls using the 2^(-ΔΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

4.3 Association between VEGF levels and BCL-2 expression

Previous studies have described a negative correlation between VEGF levels and BCL-2 expression in patients with CLL **(Bairey *et al.*, 2001)**, therefore this association was investigated on the present study using a Pearson´s correlation (p<0,05).

It was not possible to establish a correlation between circulating VEGF levels and BCL-2 expression in the 21 samples analyzed (15 CLL and 6 SLL) **(Figure 16).**



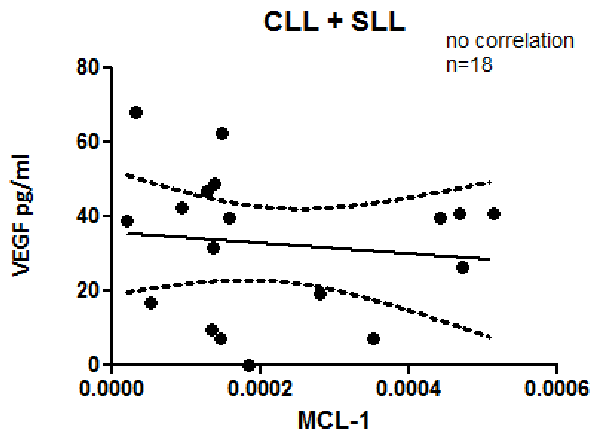
CLL A	n= 11
CLL B	n= 4
SLL	n= 6

Figure 16 - Correlation analysis between BCL-2 expression analyzed by qPCR and VEGF levels measured by ELISA technique in CLL and SLL samples. Pearson test was performed to evaluate correlations.

4.4 Association between VEGF levels and MCL-1 expression

As a correlation between VEGF levels and MCL-1 expression in patients with CLL was previously reported **(Véronèse *et al.*, 2009)**, this association was investigated on the present study using a Pearson´s correlation (p<0,05).

It was not observed a correlation between circulating VEGF levels and MCL-1 expression in the 18 samples of CLL and SLL analyzed **(Figure 17).**



CLL A	n= 11
CLL B	n= 2
SLL	n= 4

Figure 17 - Correlation analysis between MCL-1 expression analyzed by qPCR and VEGF levels measured by ELISA technique in CLL and SLL samples. Pearson test was performed to evaluate correlations.

5. Expression of Notch Pathway genes in CLL and SLL

To further characterize CLL cells, the expression of genes associated with Notch pathway, specifically of Jagged2 and HEY2, was investigated. The levels of expression of Jagged2 and HEY-2 were quantified by qPCR in a panel of 10 CLL and 5 SLL samples.

5.1 Expression of Jagged2 in CLL versus SLL

The expression of Jagged2, a Notch ligand, was statistically higher in average, in SLL than in CLL samples (p=0,02) **(Figure 18A).** The most aggressive stages of CLL **(**stages B and C) showed a statistically increased Jagged2 expression when compared with the less aggressive stage of CLL (stage A) **(Figure 18B).**

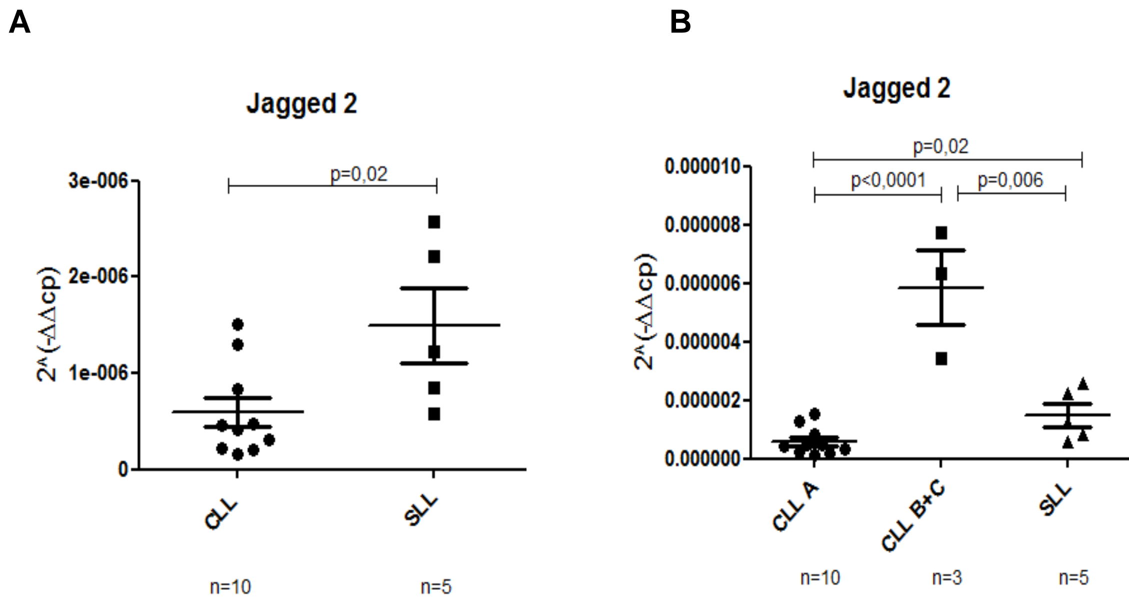


Figure 18 – Jagged2 expression in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using 18S ribosomal RNA and fold changes were calculated relative to the average expression in cells of healthy controls using the 2^(-ΔΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

5.2 Expression of HEY-2 in CLL versus SLL

The expression of HEY-2 transcript, a target of the Notch Pathway, was higher in CLL than in SLL, although this difference was not statistically significant **(Figure 19A).**

In CLL samples, the HEY-2 expression was higher in the most aggressive stages of CLL **(**stages B and C) than in the less aggressive stage of CLL (stage A), although this difference was not statistically significant **(Figure 19B).**

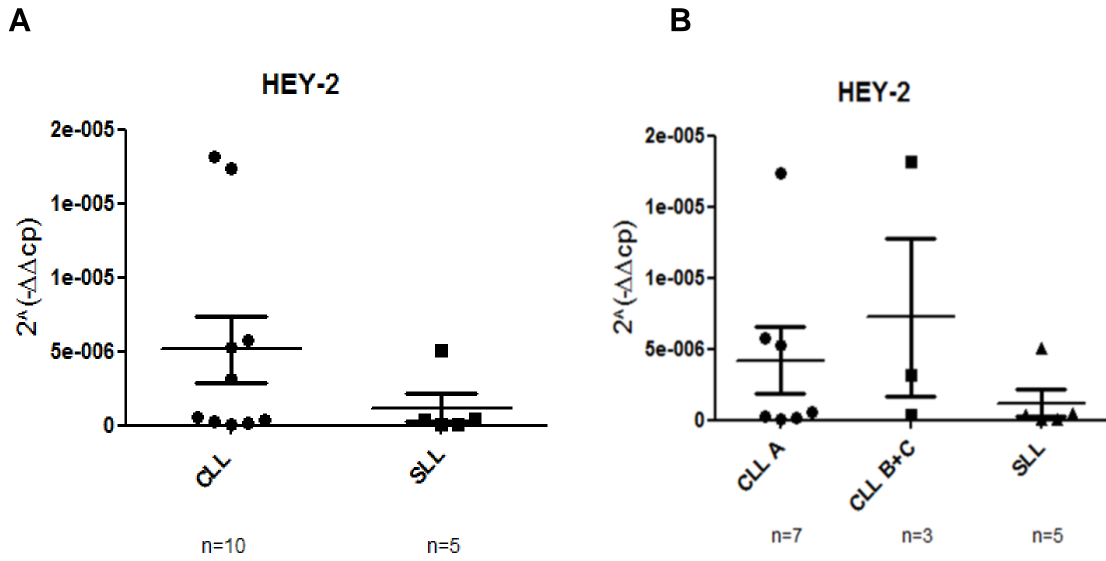


Figure 19 – HEY-2 expression in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using 18S ribosomal RNA and fold changes were calculated relative to the average expression in cells of healthy controls using the $2^{(-\Delta\Delta Cp)}$ method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6. miRNA levels in PB: plasma versus cells

6.1 Expression of miR-155 in CLL versus SLL

There are several reports showing and increased expression of miR-155 in solid tumors, as lung, colon and breast cancer, and in hematological cancers as AML **(Nana-Sinkam and Croce, 2010)** and CLL **(Fulci *et al.*, 2007).**

6.1.1. Circulating levels of miR-155

In this study, it was found that circulating levels of miR-155 in plasma ,were in average higher in CLL patients than in SLL (**Figure 20A**). When the stages of the disease were compared, it was observed that expression of miR-155 was significantly increased in less aggressive stage of CLL (stage A) than in stages B and C (p=0,015) or in SLL (p=0,009) **(Figure 20B).**

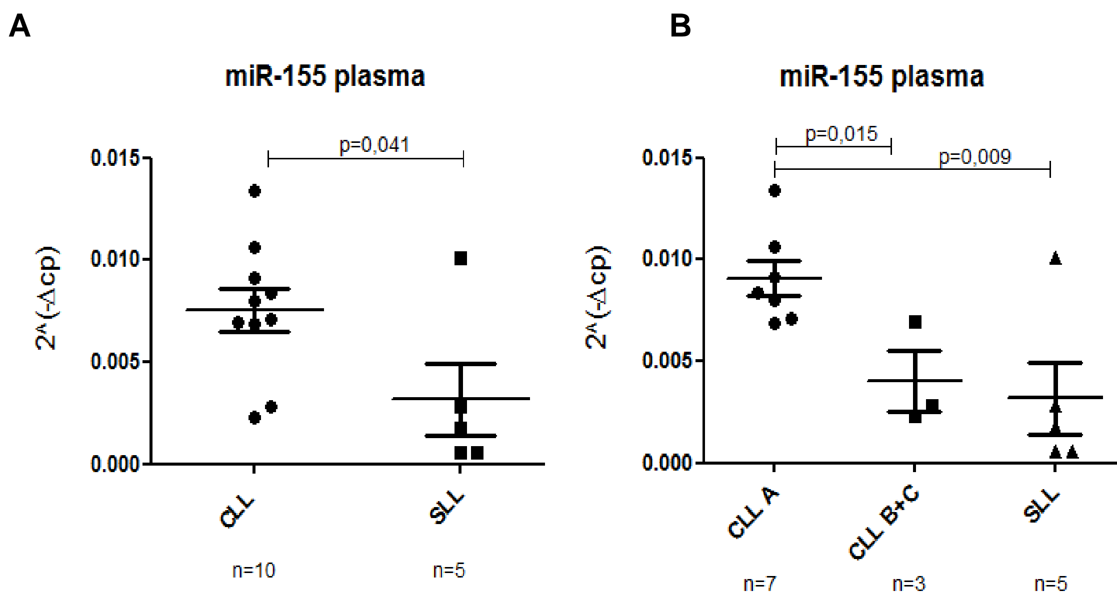


Figure 20 – miR-155 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD)

6.1.2. Expression levels of miR-155 in the PBMNC

Expression levels of miR-155 were lower, in average, in CLL than in SLL samples. However, only two SLL samples were included in this analysis (**Figure 21A)**. When stages of CLL were compared, a higher level of miR-155 was found in more aggressive stages (CLL B+C) than in CLL A **(Figure 21B).**

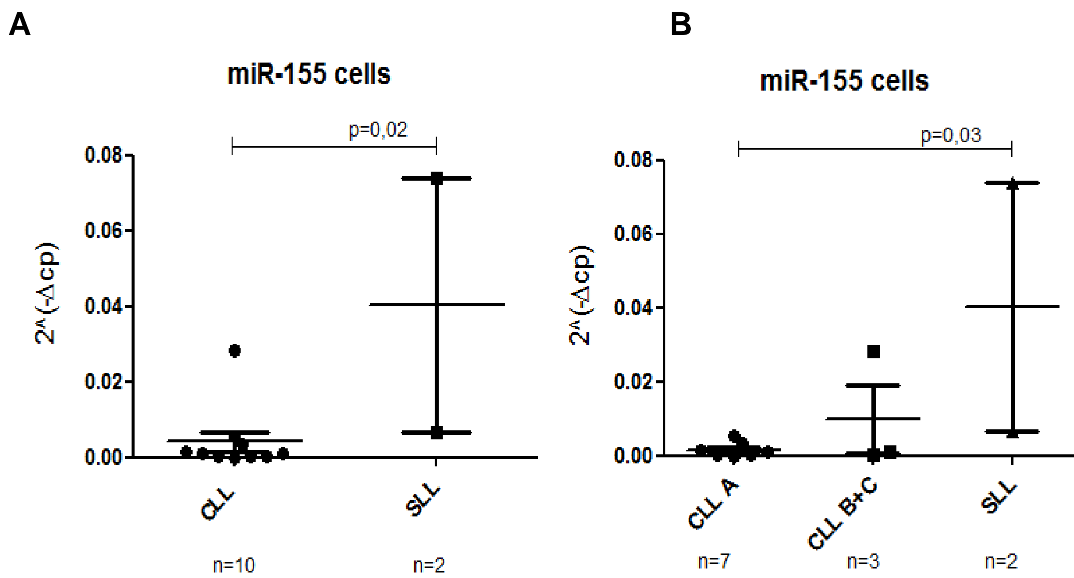
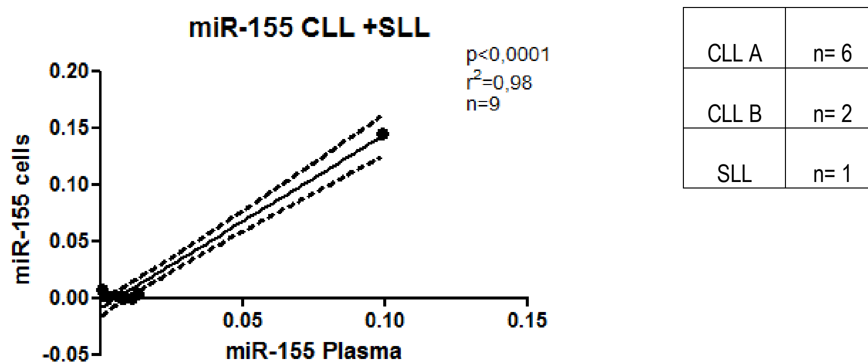


Figure 21 – miR-155 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.1.3. Correlation between miR-155 expression in cells and circulating miR-155 in plasma

A correlation between the levels of expression of miR-155 in the cells and the circulating levels in the plasma was investigated using 8 samples of CLL and 1 of SLL. A strong positive correlation between expression of miR-155 in cells and circulating levels in plasma was observed (r^2=0,98) **(Figure 22**).



CLL A	n= 6
CLL B	n= 2
SLL	n= 1

Figure 22 - Correlation analysis between miR-155 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.2 Expression of miR-15a in CLL versus SLL

The miR-15a is part of a miRNA cluster formed by 15a and 16-1 miRNAs, and is located in the chromosome 13 (13q14.3), a region frequently deleted in CLL patients. The miRNAs of the cluster miR-15a-16 are involved in the CLL pathogenesis and it was found that these miRNAs target the anti-apoptotic gene BCL-2 **(Calin et al., 2002; Calin et al.,2005).**

6.2.1 Circulating levels of miR-15a

The circulating levels of miR-15a in plasma were higher in average in CLL than in SLL samples. This difference was statistically significant (p=0.04) **(Figure 23A).** When the stages of CLL were compared, it was found an increased level of miR-15a in circulation in the less aggressive stage of CLL (stage A) than in stages B and C or with SLL **(Figure 23B).**

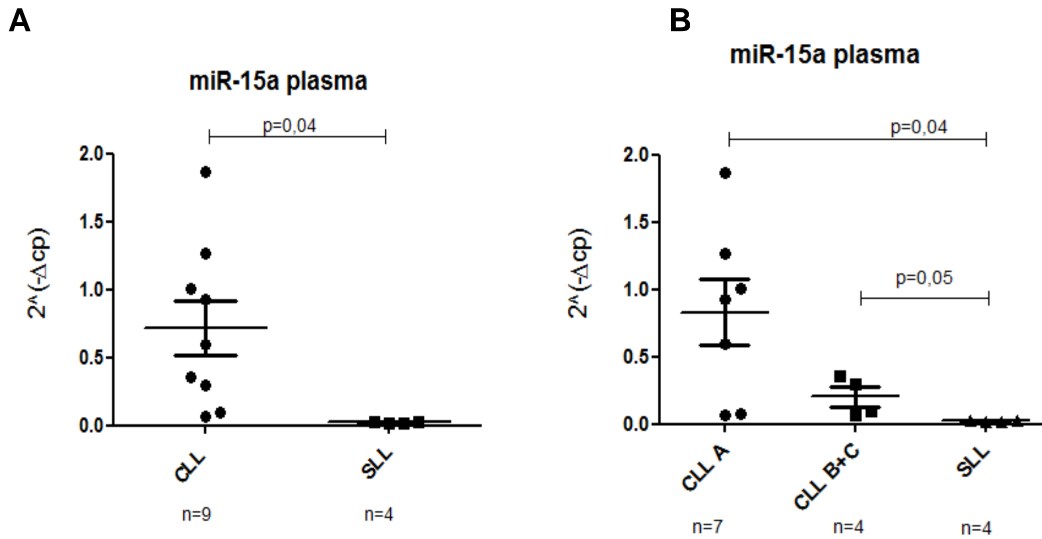


Figure 23 – miR-15a expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.2.2. Expression levels of miR-15a on total PBMNC

The expression of miR-15a in PBMNC was higher, in average, in CLL than in SLL patients **(Figure 24A)** and it was increased in less aggressive stage of CLL (stage A) than in the other disease stages, although these differences were not statistically significant **(Figure 24B).**

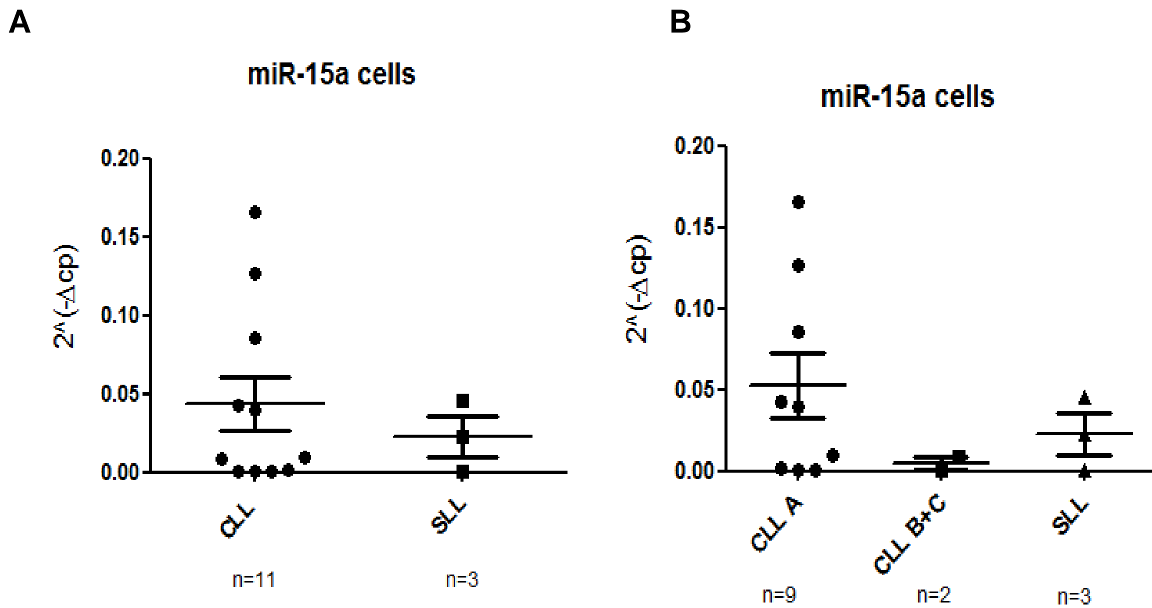
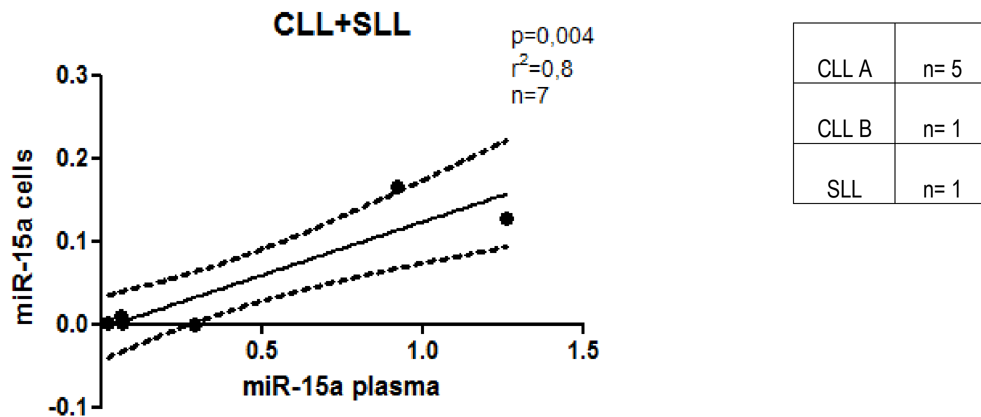


Figure 24 – miR-15a expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.2.3 Correlation between miR-15a expression in cells and circulating miR-15a in plasma

To establish a relation between levels of circulating miR-15a in plasma and expression in cells, a correlation test was performed using 6 samples of CLL and of 1 SLL. From this analysis, it was found a positive correlation (r^2=0.8) showing that samples in which cells express higher levels of miR-15a are the ones in which circulating levels of miR-15a could be observed **(Figure 25).**



CLL A	n= 5
CLL B	n= 1
SLL	n= 1

Figure 25 - Correlation analysis between miR-15a expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

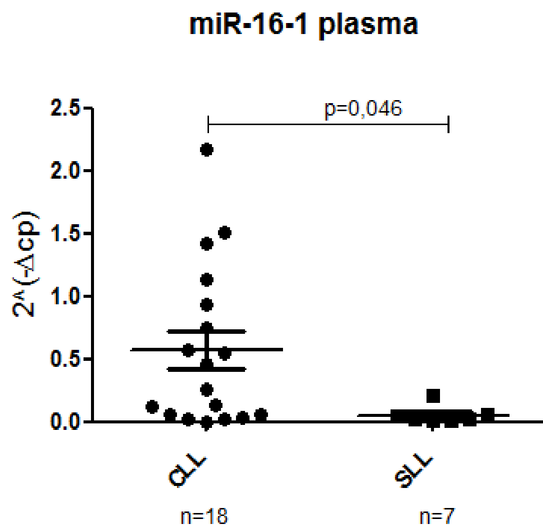
6.3 Expression of miR-16-1 in CLL versus SLL

The cluster of miR-16-1 is part of the cluster miR-15-16, and along miR-15a, also part of the same cluster, as above described, is involved in CLL pathogenesis and regulates the BCL-2 **(Calin et al, 2002; Calin et al, 2005)**

6.3.1 Circulating levels of miR-16-1

The levels of miR-16-1 in the plasma were highly increased, in average, in CLL when compared with SLL patients **(Figure 26A).** This difference was statistically significant (p=0.046). There was no statistically significant difference between disease stages of CLL. although, in average the circulating levels of miR-16-1 were lower in the more aggressive stage of CLL (CLL B+C) than in the less aggressive stage of CLL (stage A) **(Figure 26B).**

A



B

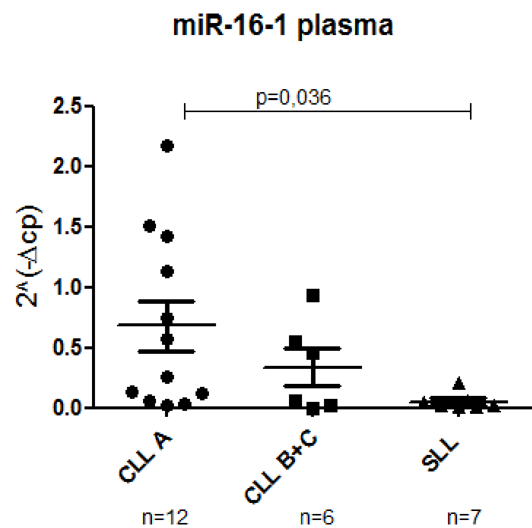


Figure 26 – miR-16-1 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.3.2 Expression levels of miR-16-1 on total PBMNC

The levels of expression of miR-16-1 were not different in average between CLL and SLL samples. However, only two samples of SLL were included in this analysis. Although the differences were not statistically significant, the levels of miR-16-1 in cells, were in average, higher in CLL (fold change=0,20) than in SLL (fold change=0,14) (**Figure 27A**). When stages of CLL were compared, a difference could be observed, where the expression levels of miR-16-1 were higher in less aggressive stage than in stages B and C or even in SLL (**Figure 27B**).

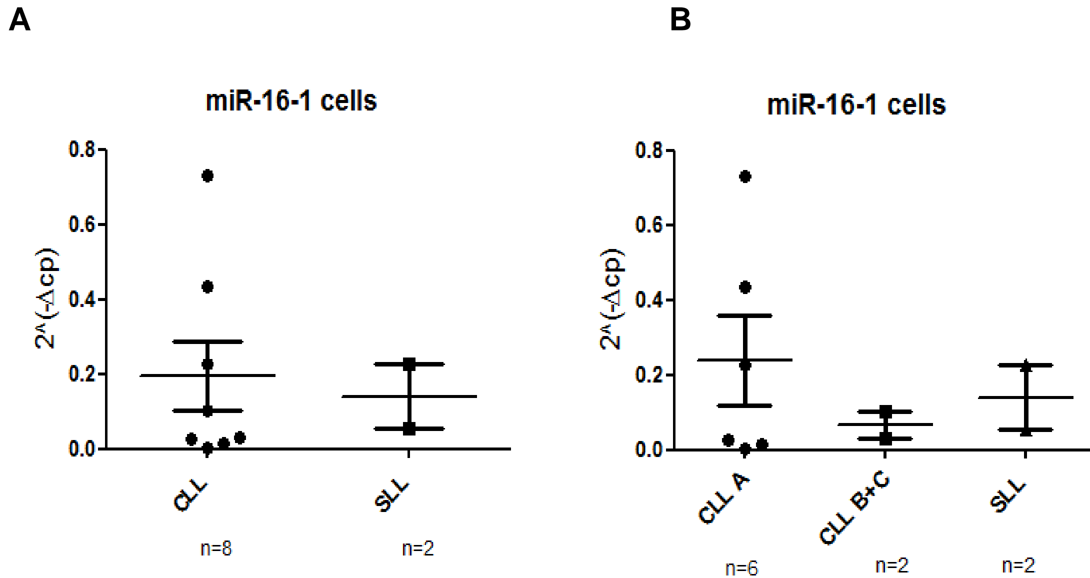


Figure 27 – miR-16-1 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.3.3 Correlation between miR-16-1 expression in cells and circulating miR-16-1 in plasma

To establish a relation between levels of circulating miR-16-1 in plasma and its expression in cells, a correlation test was performed using 7 samples of CLL and 2 of SLL. From this analysis, a positive correlation was found, although this correlation was not strong (r^2=0.67) **(Figure 28).**

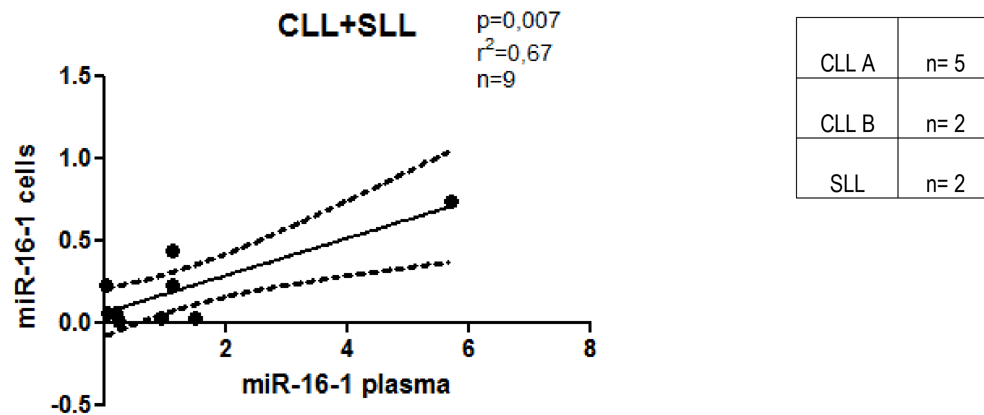


Figure 28 - Correlation analysis between miR-16-1 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.4 Expression of miR-21 in CLL versus SLL

miR-21 has been described as been involved in leukemia drug resistance by regulation of PTEN gene, a tumor suppressor **(Bai *et al.*, 2011)** it was also been reported as down-regulated of PTEN and PDCD4 genes in other leukemias, as NK-cell lymphoma/leukemia (**Yamanaka *et al.*, 2009**). Another study, reported that in CLL *CCND2* and *DPH1* are possibly targeted by miR-21, *CCND2* was reported to control cell cycle progression in CLL cells while *DPH1* is a candidate tumor suppressor gene in ovarian and breast cancers and is localized telomerically to TP53 (**Rossi et al., 2010**). miR-21 was described as an oncogene which plays a key role in resisting programmed cell death in cancer cells (**Buscaglia and Li, 2011**)

6.4.1 Circulating levels of miR-21

The levels of circulating miR-21 in plasma were increased in CLL patients when compared with SLL patients (**Figure 29A**). This difference in expression was statistically significant (p=0,049). When CLL stages were compared, a difference between more and less aggressive stages was also observed: levels of circulating miR-21 were significantly higher in less aggressive stage of CLL (stage A) than in the most aggressive stages of CLL (stages B and C) and SLL (**Figure 29B**).

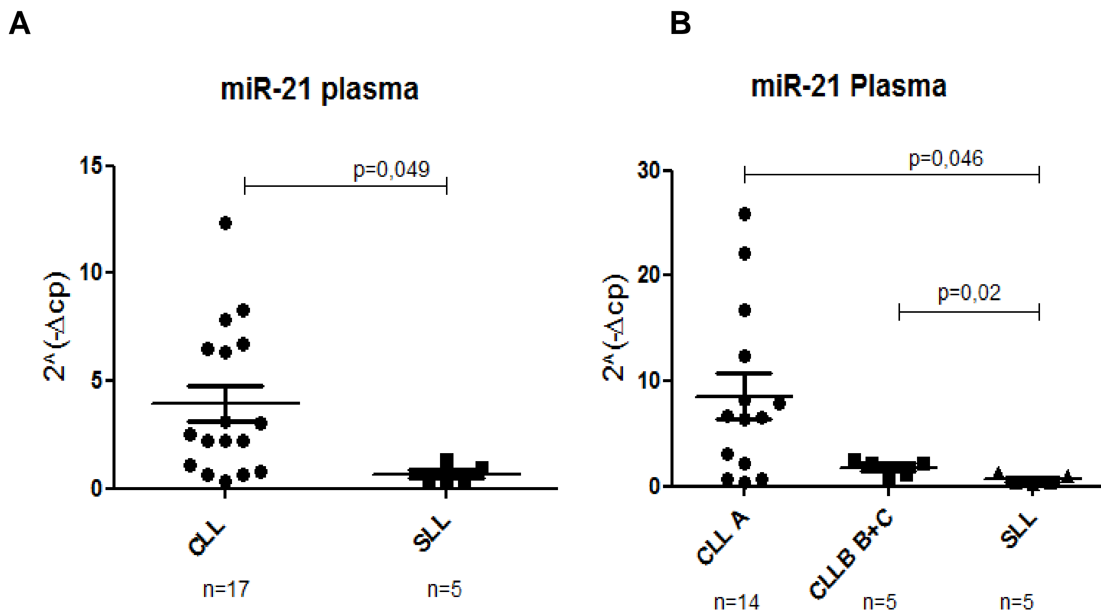


Figure 29 – miR-21 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.4.2 Expression levels of miR-21 on total PBMNC

The expression of miR-21 in cells showed a significant increase, in average, in SLL patients when compared with CLL patients **(Figure 30A).** Moreover, no differences in miR-21 expression were found between more and less aggressive stages of CLL **(Figure 30B).**

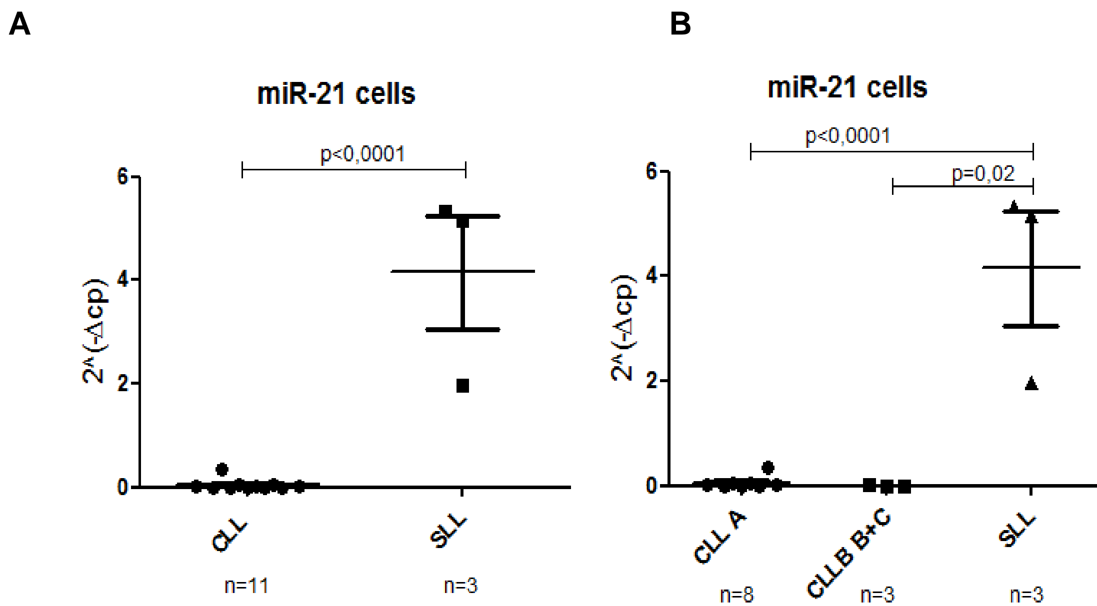
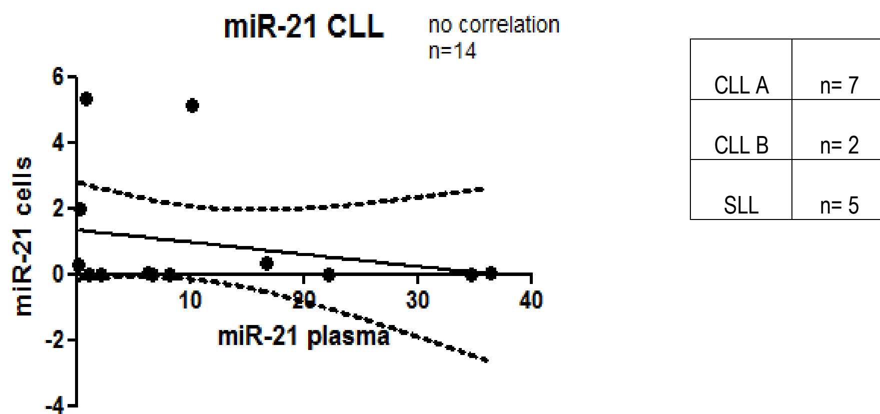


Figure 30 – miR-21 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.4.3. Correlation between miR-21 expression in cells and circulating miR-21 in plasma

It was not possible establish a correlation between circulating miR-21 levels in plasma and expression in PBMNC in the 14 samples used (9 CLL and 5 SLL) **(Fig. 31).**



CLL A	n= 7
CLL B	n= 2
SLL	n= 5

Figure 31 - Correlation analysis between miR-21 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.5 Expression of miR-101 in CLL versus SLL

The miR-101 was reported in the literature as an inducer of apoptosis and inhibitor of cell proliferation and invasion. miR-101 is frequently downregulated in cancers, as in glioblastoma **(Smits *et al.*, 2010),** gastric cancer and in hepatocellular carcinoma where promotes apoptosis through targeting MCL-1 **(Su *et al.*, 2009).**

6.5.1 Circulating levels of miR-101

The circulating levels of miR-101 in plasma were different between CLL and SLL. This difference is highly statistically significant (p=0,001) **(Figure 32A).** However, no difference was found within stages of CLL **(Figure 32B)**.

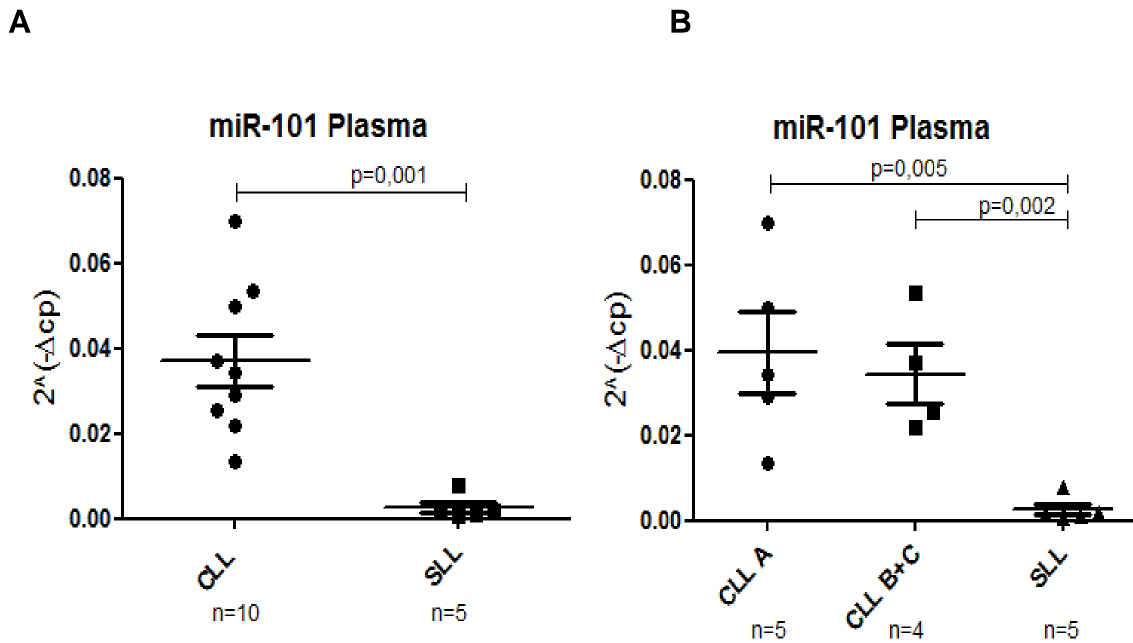


Figure 32 – miR-101 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.5.2. Expression levels of miR-101 in the PBMNC

The miR-101 expression levels were lower in CLL when compared with SLL samples, although only 2 SLL samples were included in this analysis **(Figure 33A).** No differences were found between CLL stages **(Figure 33B).**

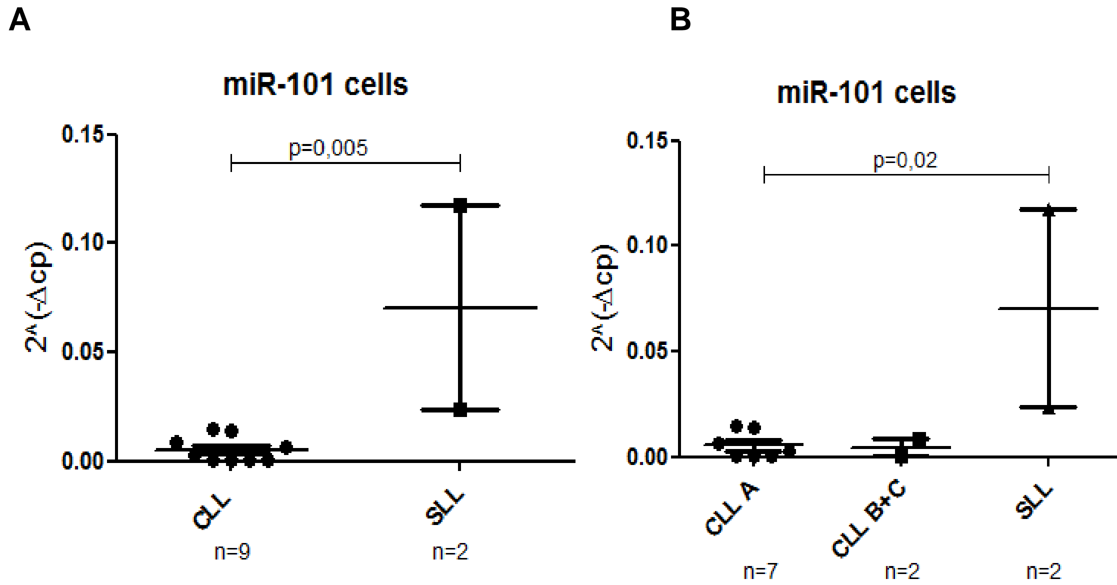


Figure 33 – miR-101 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student’s t test. Error bars represent standard deviation (SD).

6.5.3. Correlation between miR-101 expression in cells and circulating miR-101 in plasma

It was not possible establish a correlation between miR-101 expression in cells and circulating levels in plasma of CLL and SLL samples in the 5 CLL and 2 SLL samples used **(Figure 34).**

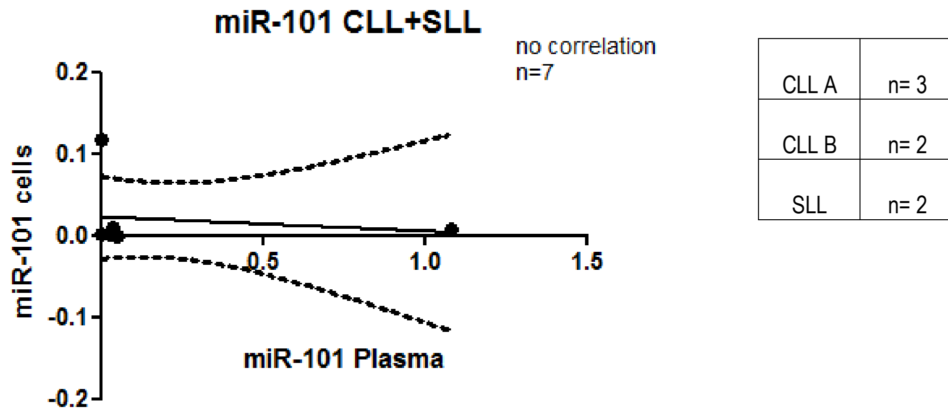


Figure 34 - Correlation analysis between miR-101 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.6 Expression of miR-223 in CLL versus SLL

In CLL, miR-223 expression decreased with progression from Binet stage A, the less aggressive stage of CLL, to stage C, the most aggressive stage of CLL. **(Stamatopoulos *et al.*, 2009).**

6.6.1 Circulating levels of miR-223

The levels of miR-223 in circulation were higher, in average, in CLL samples than in SLL, although this difference was not statistically significant **(Figure 35A).** Considering the CLL stages, it was found that there is less miR-223, in average, in circulation in the more aggressive stages (B and C) than in the less aggressive stage (stage A). However, this difference was not statistically significant. **(Figure 35B).**

A B

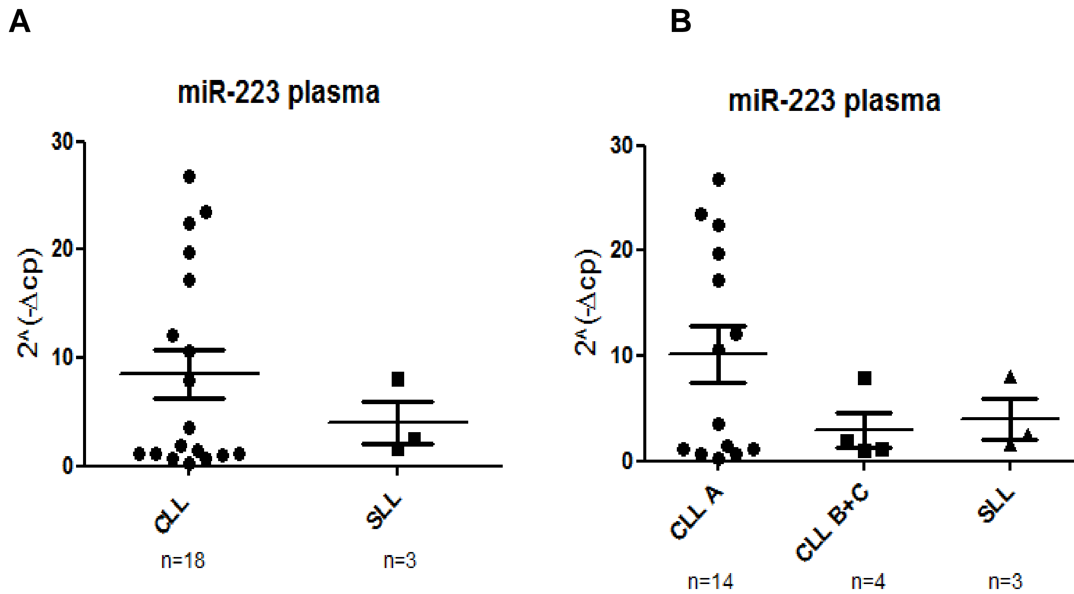


Figure 35 – miR-223 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.6.2. Expression levels of miR-223 in the PBMNC

The expression levels of miR-223 in the mononuclear cells are significantly reduced in CLL compared with SLL (p=0,01) **(Figure 36A)**. There were no differences of expression in miR-223 in the more and less aggressive stages of CLL, being all down-regulated **(Figure 36B).**

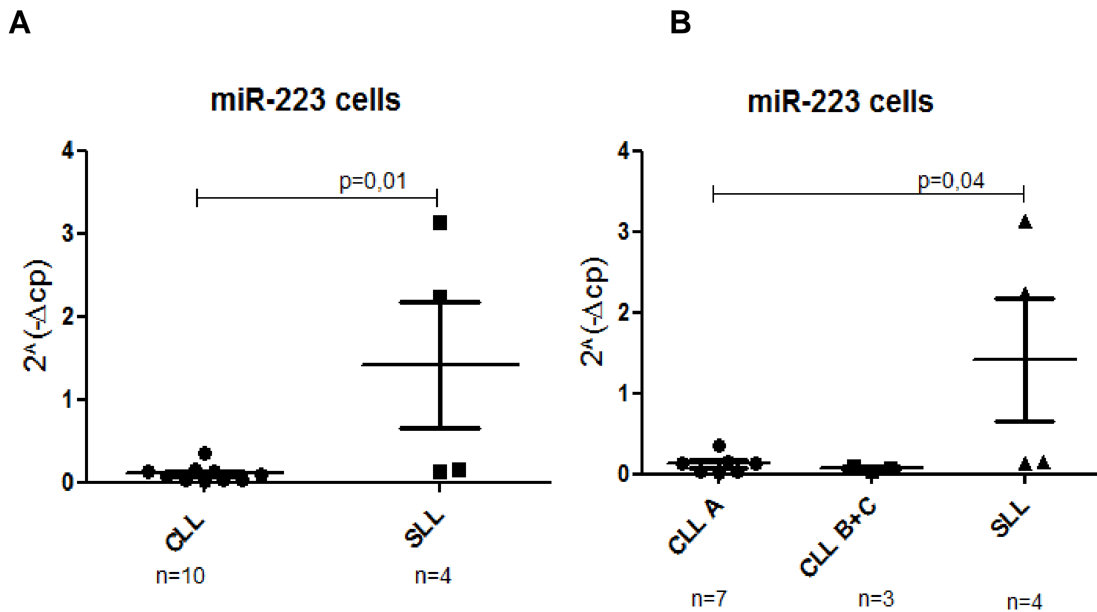


Figure 36 – miR-223 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.6.3. Correlation between miR-223 expression in cells and circulating miR-223 in plasma

A positive correlation was found between miR-223 expression in cells and circulating levels in plasma, although was not strong (r^2=0.44). This analysis included 9 samples of CLL and 4 SLL samples **(Figure 37).**

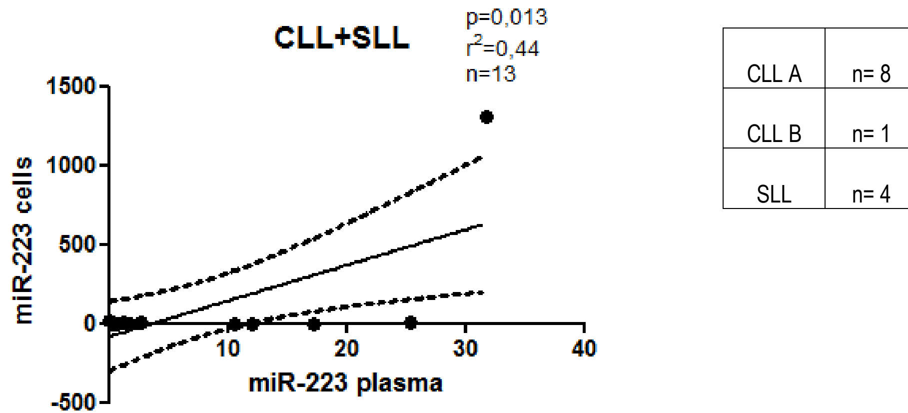


Figure 37 - Correlation analysis between miR-223 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.7 Expression of miR-221 in CLL versus SLL

The miR-221 regulates the tumor suppressor p27, through the inhibition of cell-cycle progression and was reported as having an increased expression in solid tumors, as non-small cell lung cancer, papillary thyroid carcinoma and hepatocellular carcinoma. **(Chen et al., 2008; Calin and Croce, 2006)** and also in CLL **(Moussay et al, 2010)**.

6.7.1 Circulating levels of miR-221

The miR-221 levels in circulation were higher in CLL than SLL in samples. This difference was statistically significant (p=0,04) **(Figure 38A).** miR-221 expression levels were higher in less aggressive stage of CLL (stage A) than in the most aggressive stages of CLL (stages B and C) or in SLL, although this difference only was statistically significant between CLL stage A and SLL samples **(Figure 38B).**

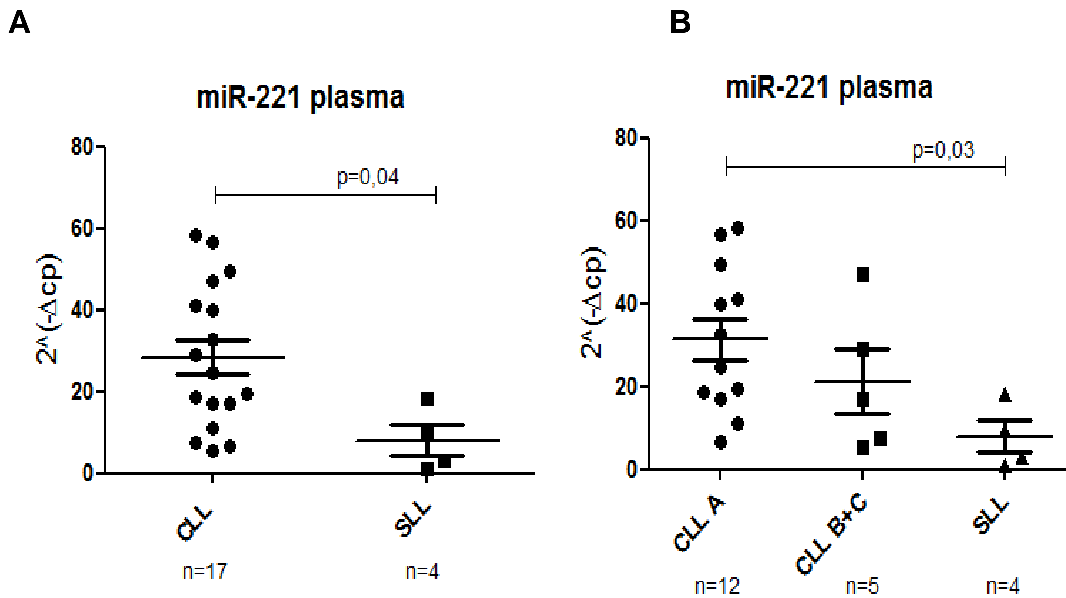


Figure 38 – miR-221 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.7.2 Expression levels of miR-221 on total PBMNC

On the total PBMNC, the miR-221 expression was higher, in average, in SLL samples when compared with CLL samples **(Figure 39A).** When stages of CLL were compared, it was found that miR-221 expression was higher in CLL stage A than in CLL stages B and C, but this difference was not statistically relevant. Only between the less aggressive stage of CLL (stage A) and SLL samples this difference was considered statistically significant **(Figure 39B).**

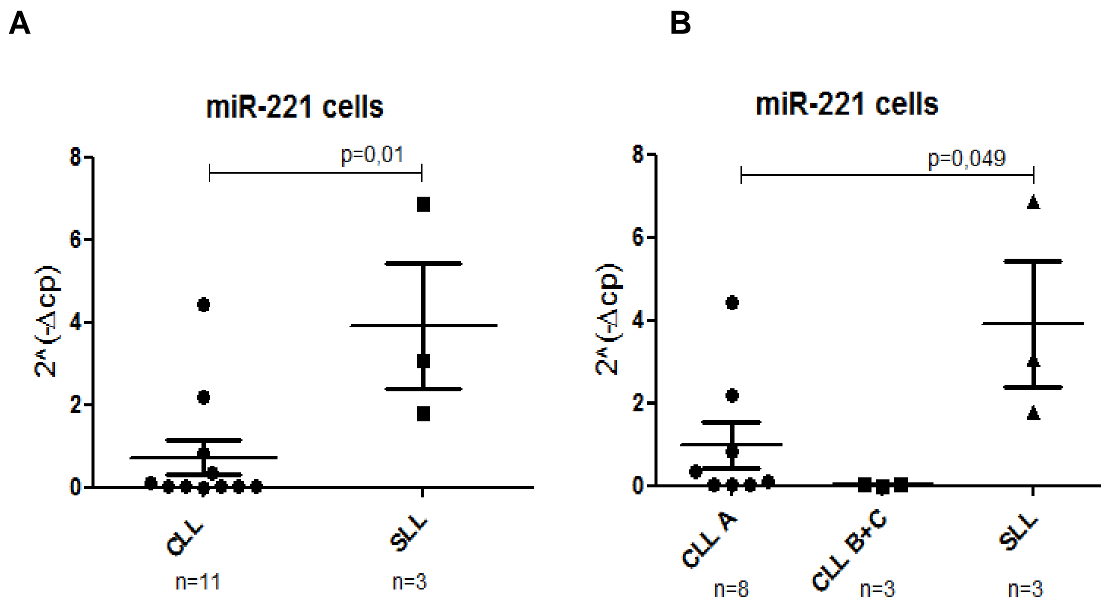
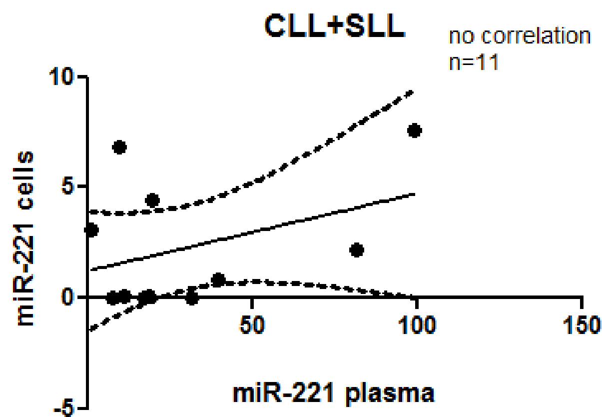


Figure 39 – miR-221 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.7.3 Correlation between miR-221 expression in cells and circulating miR-221 in plasma

It was not possible establish a correlation between miR-221 expression in cells and circulating levels in plasma. This analysis included 7 samples of CLL and 4 SLL samples **(Figure 40).**



CLL A	n= 6
CLL B	n= 1
SLL	n= 4

Figure 40 - Correlation analysis between miR-221 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.8 Expression of miR-34c in CLL versus SLL

The expression of miR-34c is induced by the tumor suppressor p53, which is encoded by the TP53 gene located on the chromosome 17 (17p13.1), a region frequented deleted in CLL patients **(Ward *et al.*, 2011).** This miRNA has been described as downregulated in some solid tumors as laryngeal carcinoma, prostate cancer **(Cai *et al.*, 2010; Hagman *et al.*, 2010),** melanoma, ovarian, colorectal, colon, breast and lung cancers **(Cannel and Bushell, 2010).** This miRNA was reported as a tumor-supressor by repressing MYC translation, an oncogene that regulate cell proliferation and is frequently deregulated in human malignancies **(Cannel and Bushell, 2010).**

6.8.1 Circulating levels of miR-34c

No differences were found in the levels of circulating miR-34c in CLL and SLL samples **(Figure 41A).** However, considering the CLL stages, miR-34c expression was higher in the most aggressive stages of CLL (stages B and C) than in less aggressive stage of CLL (stage A) and was identical to SLL, although none of these differences was statistically significant **(Figure 41B).**

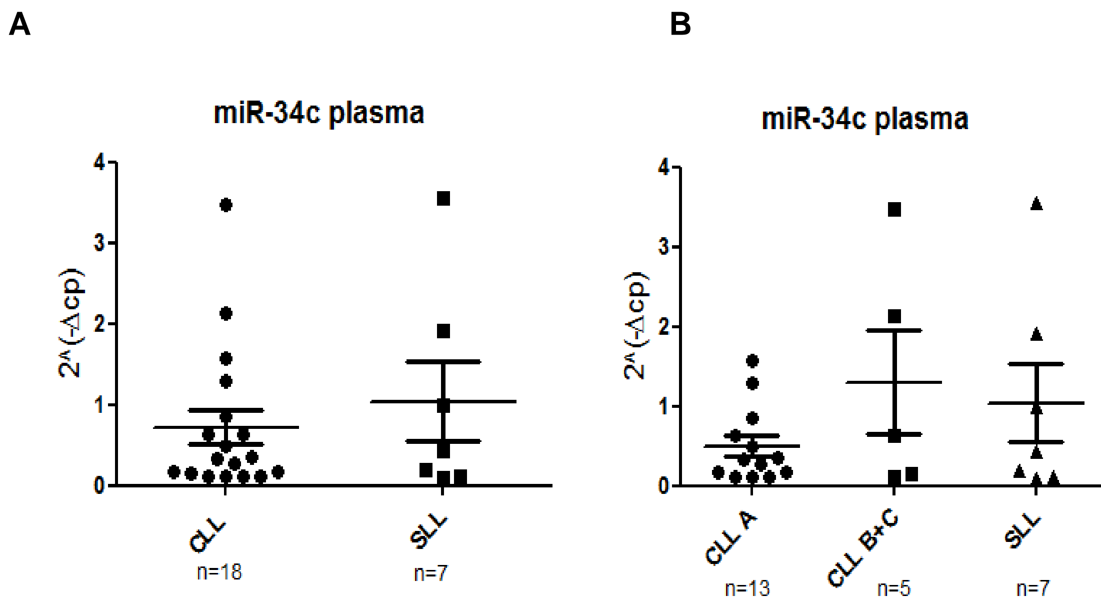


Figure 41 – miR-34c expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.8.2. Expression levels of miR-34c on total PBMNC

The expression of miR-34c on the total PBMNC of CLL samples was significantly reduced (p=0,008) when compared with SLL samples **(Figure 42A)**. However, no differences were found within stages of CLL **(Figure 42B)**.

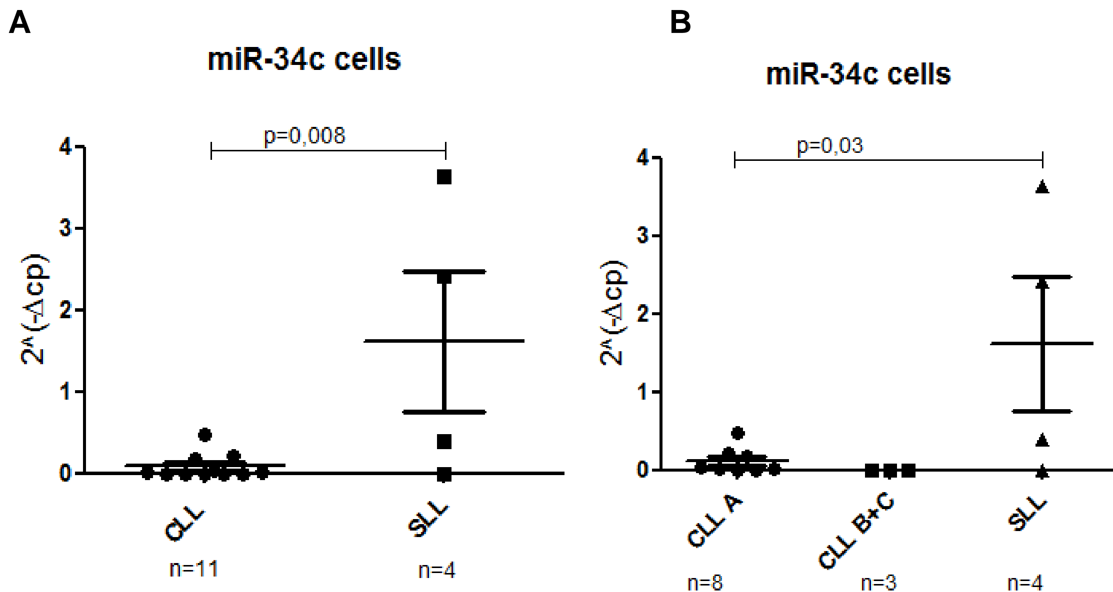


Figure 42 – miR-34c expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.8.3. Correlation between miR-34c expression in cells and circulating miR-34c in plasma

No correlation was found between miR-34c expression in cells and circulating levels in plasma. This analysis included 9 samples of CLL and 3 SLL samples **(Figure 43).**

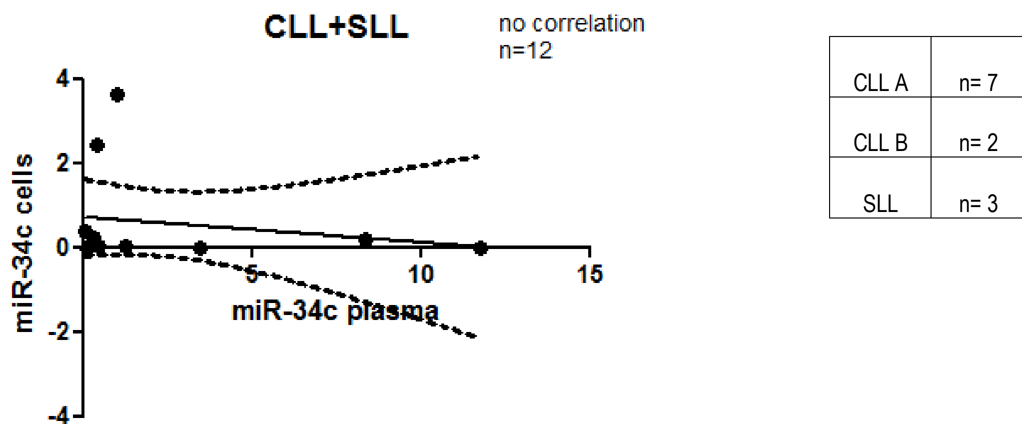


Figure 43 - Correlation analysis between miR-34c expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.9 Expression of miR-34a in CLL versus SLL

The miR-34a share the same family of miR-34c mentioned above. As miR-34c, the miR-34a is associated with p53, and was reported to be downregulated in CLL in patients with p53 mutations or 17p13.1 deletions **(Nana-Sinkam and Croce, 2010).**

6.9.1 Circulating levels of miR-34a

The CLL samples had reduced levels of circulating miR-34a than SLL, however only one sample of SLL was included in this analysis **(Figure 44A).** All the 4 CLL samples analyzed were classified as stage A.

A

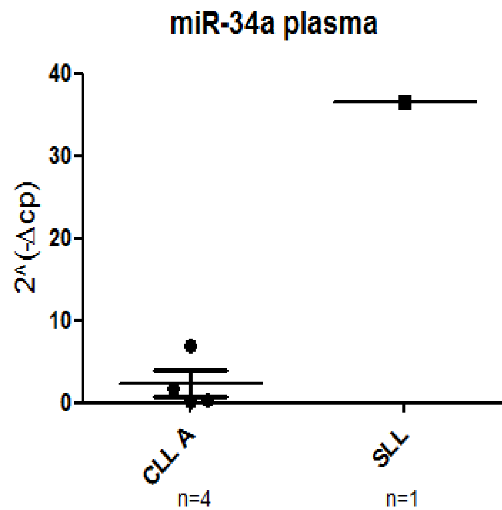


Figure 44 – miR-34a expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.9.2 Expression levels of miR-34a on total PBMNC

The miR-34a was expressed at lower levels in CLL samples when compared with SLL samples. This difference was statistically significant **(Figure 45A**). No differences in the miR-34a expression were found in CLL stage A and CLL stages B and C **(Figure 45B).**

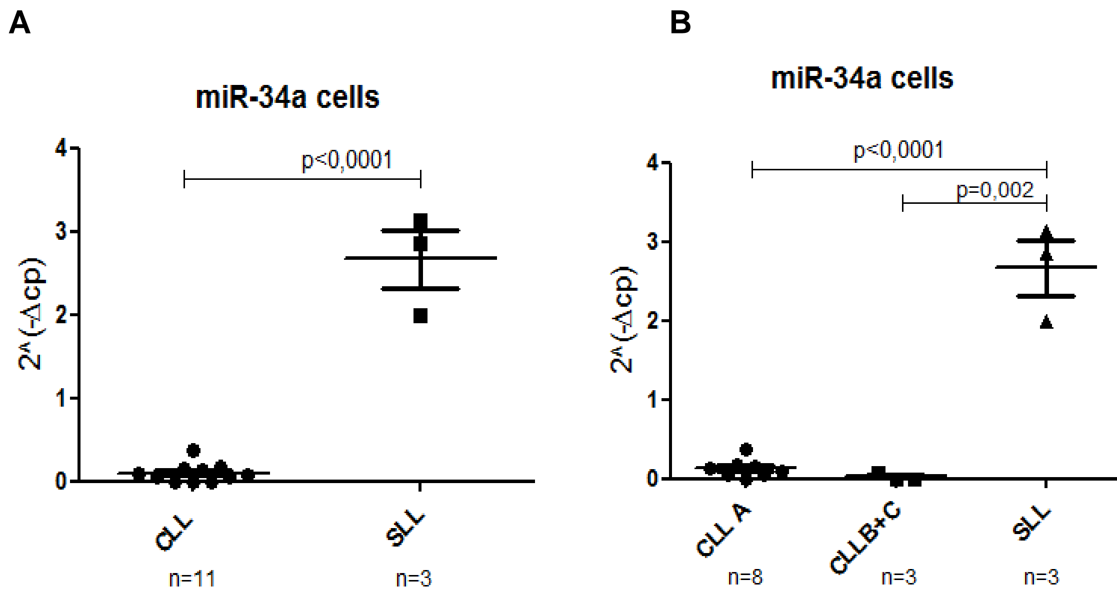


Figure 45 – miR-34a expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.9.3. Correlation between miR-34a expression in cells and circulating miR-34a in plasma

A positive correlation was found between miR-34a expression in cells and circulating levels in plasma. This analysis included 4 CLL samples from stage A only **(Figure 46).** The results showed that samples having higher level of miR-34a in circulation were the samples in which cells also expressed higher levels of miR-34a.

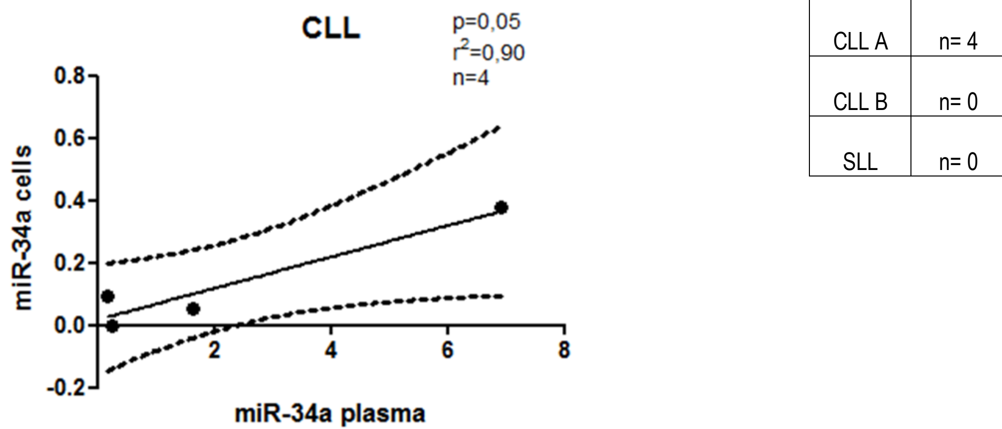


Figure 46 - Correlation analysis between miR-34a expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.10 Expression of miR-141 in CLL versus SLL

The miR-141 was expressed in epithelial tumors like breast, lung, colon and prostate cancers **(Mitchell *et al.*, 2008)**. The circulating miR-141 levels were reported to have prognostic value in colon cancer **(Cheng *et al.*, 2011).**

6.10.1 Circulating levels of miR-141

No differences were found in the circulating levels of miR-141 between CLL and SLL plasma **(Figure 47A)**, neither among the stages of CLL **(Figure 47B).**

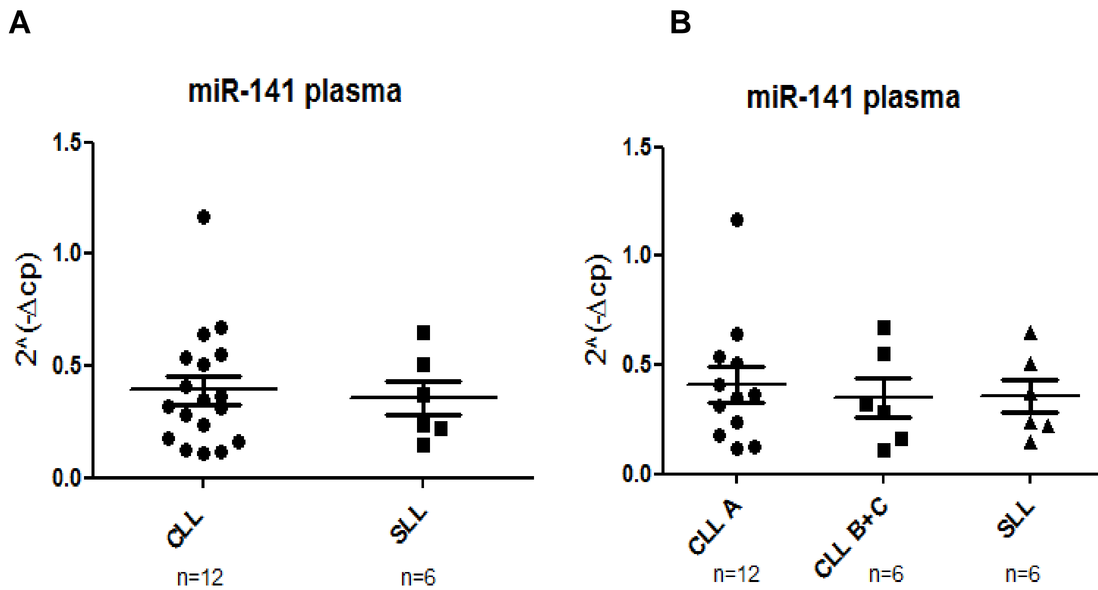


Figure 47 – miR-141 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.10.2 Expression levels of miR-141 on total PBMNC

The miR-141 expression in cells showed a significant increase in average in SLL patients when compared with CLL patients **(Figure 48A). The** SLL patients also had a higher miR-141 expression level than observed in CLL stages (stage A and CLL stages B and C), although these differences were not statistically significant **(Figure 48B).**

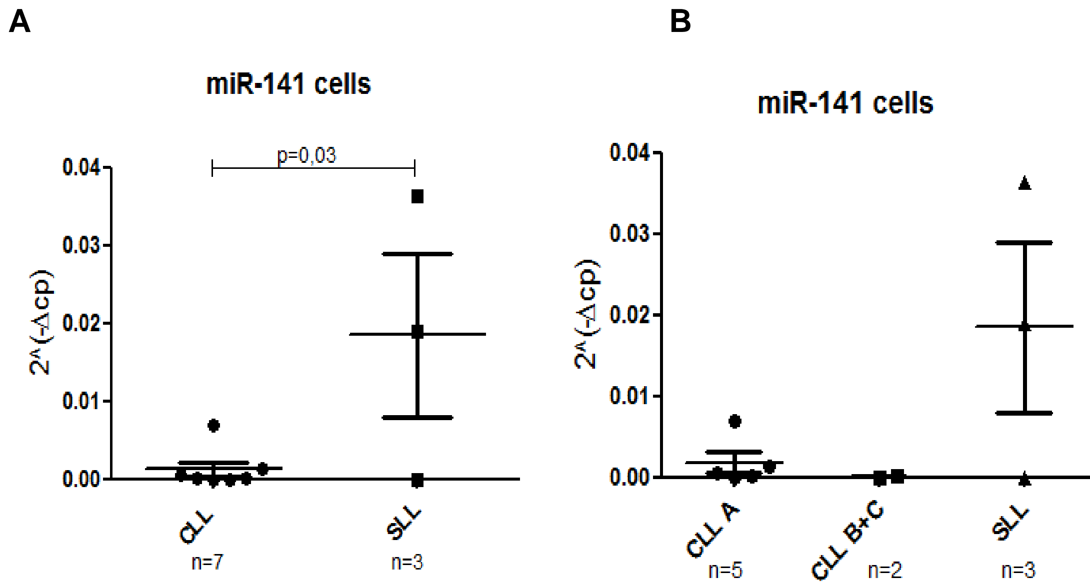
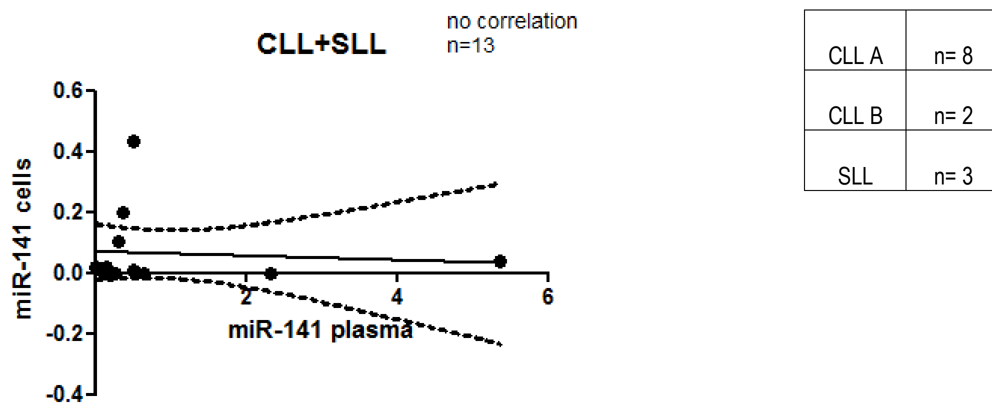


Figure 48 – miR-141 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.10.3. Correlation between miR-141 expression in cells and circulating miR-141 in plasma

It was not possible establish a correlation between miR-141 expression in cells and circulating levels in plasma. This analysis included 10 samples of CLL and 3 SLL samples **(Figure 49).**



CLL A	n= 8
CLL B	n= 2
SLL	n= 3

Figure 49 - Correlation analysis between miR-141 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.11 Expression of miR-210 in CLL versus SLL

The miR-210 has been reported as a prognostic factor in solid tumors, as breast cancer **(Rothé *et al.*, 2011)** and the circulating miR-210 was also identified as prognostic marker in pancreatic cancer patients **(Ho *et al.*, 2010).**

In a previous study performed in our lab, it was found that miR-210 levels were increased in the SLL pool of cells samples **(Teixeira, 2009).**

6.11.1 Circulating levels of miR-210

The circulating levels of mir-210 in the plasma, were significantly increased in CLL patients when compared with SLL patients **(Figure 50A).** This difference was statistically significant (p=0,03). A difference in circulating miR-210 was also observed between less and more aggressive stages of CLL: the circulating miR-210 was

significantly higher in the less aggressive stage (stage A) than in the most aggressive stages of CLL (stage B and C) **(Figure 50B).**

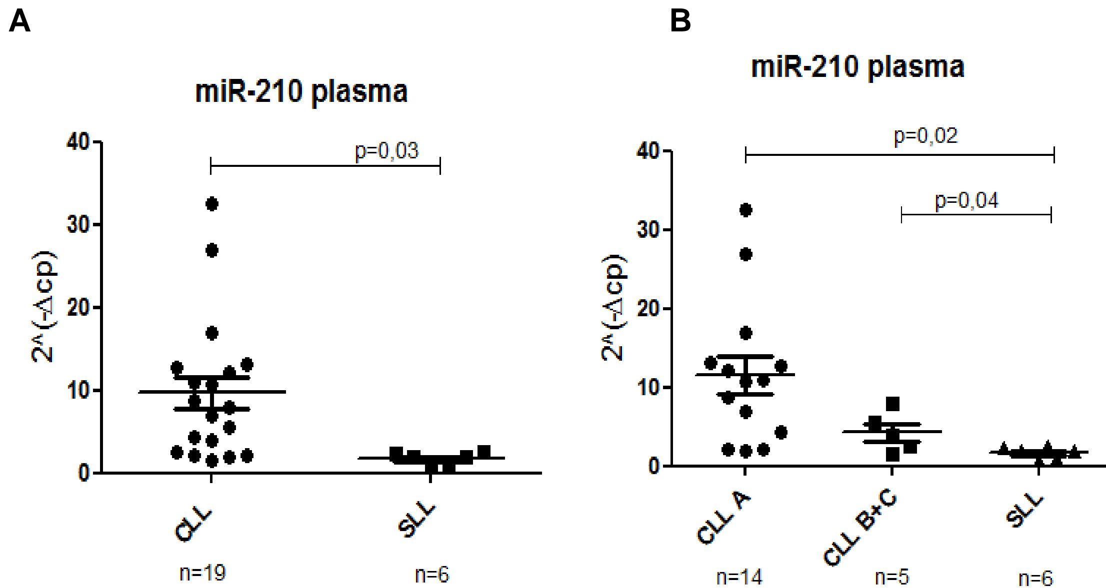


Figure 50 – miR-210 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.11.2 Expression levels of miR-210 on total PBMNC

The expression levels of miR-210 in the PBMNC of CLL patients was reduced in average when compared with SLL patients, although this difference was not statistically significant and only two SLL samples were included in this analysis **(Figure 51A).** When comparing CLL stages, it was observed that in less aggressive stage of CLL (stage A) the level of miR-210 was higher than in stages B and C, although these differences were not statistically significant (**Figure 51B).**

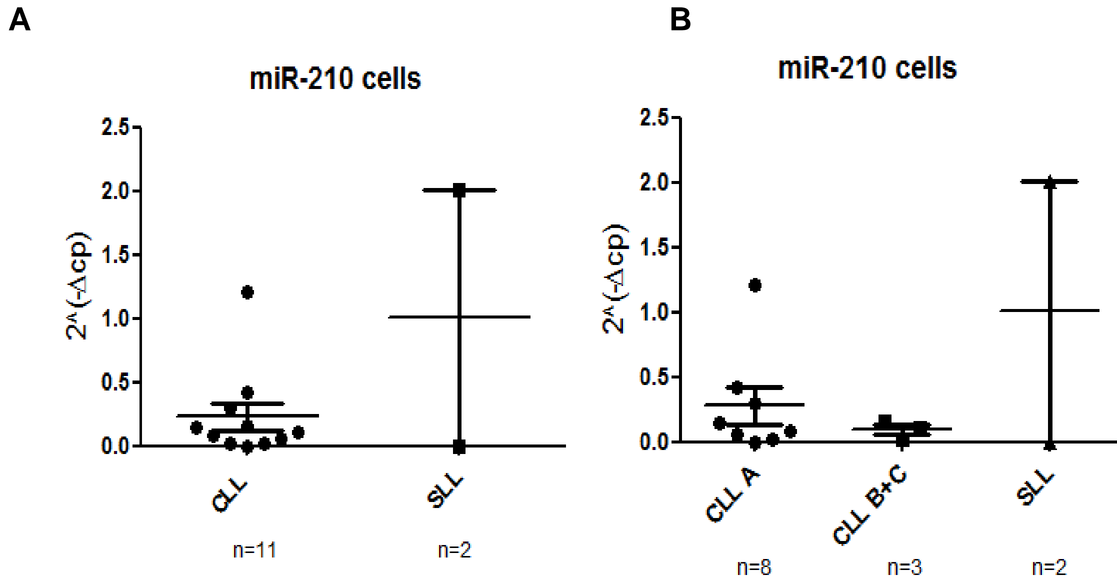


Figure 51 – miR-210 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.11.3. Correlation between miR-210 expression in cells and circulating miR-210 in plasma

It was not possible establish a correlation between miR-210 expression in cells and circulating levels in plasma of CLL and SLL samples. This analysis included 8 samples of CLL and 2 SLL samples **(Figure 52).**

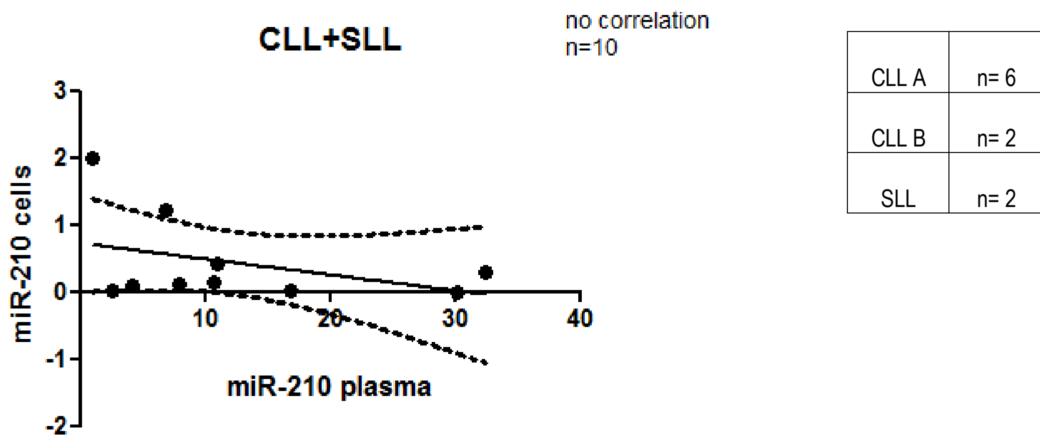


Figure 52 - Correlation analysis between miR-210 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.12 Expression of miR-942 in CLL versus SLL

The miR-942 was previously identified as differentially expressed between mononuclear cells of CLL and SLL pools of samples by miRNA microarrays, where its expression levels were higher in the CLL pool then in SLL pool **(Teixeira, 2009).** In the current project it was investigated the level of expression of the miR-942 in individual samples of CLL and SLL, either in PBMNC and blood plasma. A literature search for miR-942 returned only sequence data, with no known function to date**.**

6.12.1 Circulating levels of miR-942

The levels of miR-942 were differentially expressed in the plasma of patients having CLL and SLL, being significantly higher in CLL patients than in SLL patients (p=0,03) **(Figure 53A,B).** There was no difference between the circulating levels of miR-942 within the CLL subtypes (CLLA and CLLB+C) **(Figure 53B).**

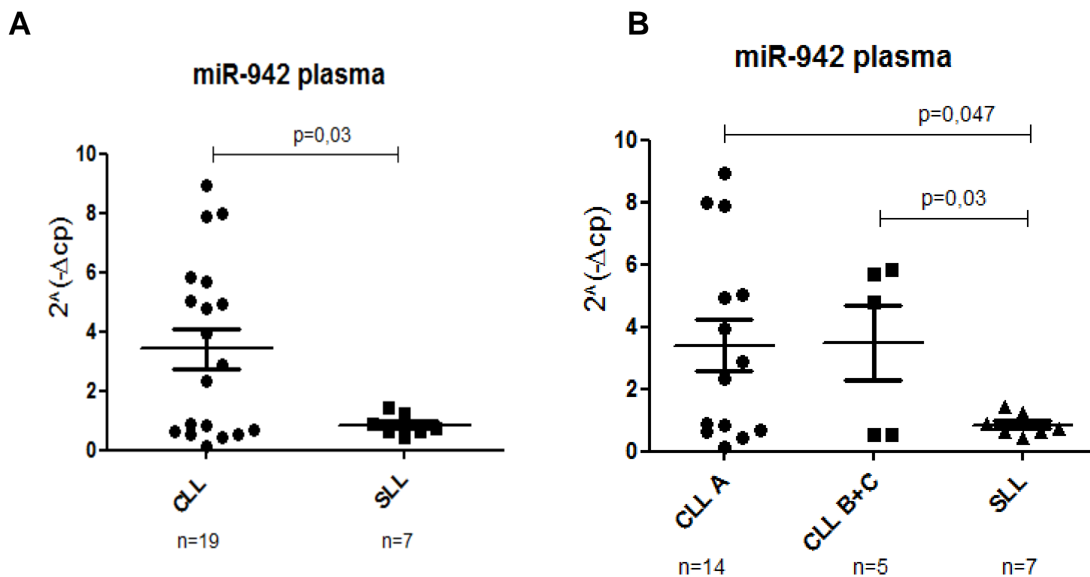


Figure 53 – miR-942 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.12.2. Levels of expression of miR-942 on total PBMNC

The level of expression of miR-942 was analyzed in PBMNC of 10 CLL samples and 3 SLL samples. Although the circulating levels of miR-942 were higher in CLL patients than in SLL, no significantly difference was found in levels of expression of miR-942 in CLL and SLL cells. Additionally, the levels of expression of miR-942 were lower, in average, in CLL (fold change =0,33) when compared with SLL ((fold change =0,86)

(Figure 54A). Considering the CLL stages, miR-942 is up-regulated in stage A when compared with CLL stages B and C but not with SLL **(Figure 54B)**, although these differences were not statistically significant. Therefore, taking together the miR-942 levels in cells and plasma, it was observed that the expression of miR-942 in PBMNC is lower in average in CLL samples than in SLL, but differences were not statistically significant, while circulating miR-942 levels are significantly higher in CLL than in SLL.

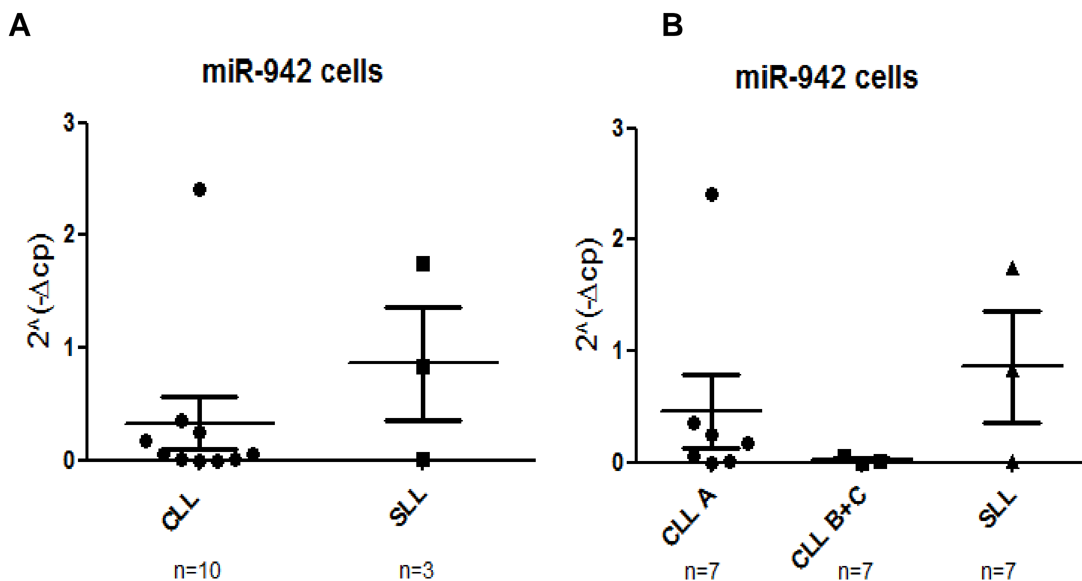
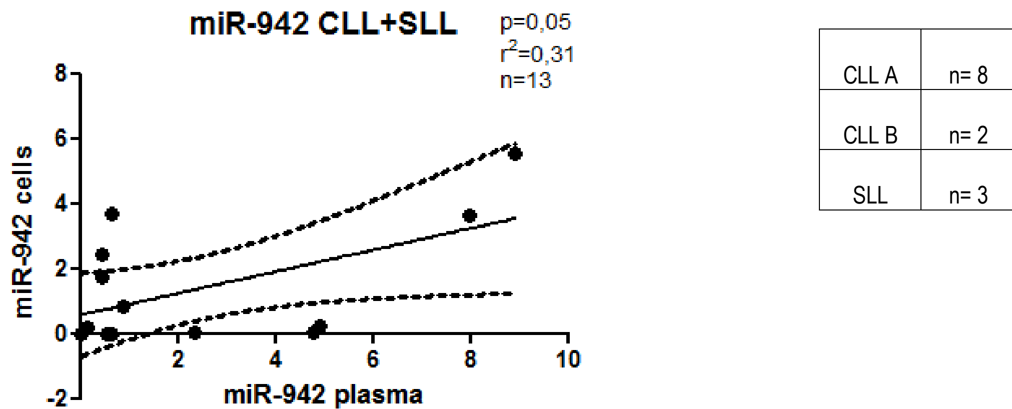


Figure 54 – miR-942 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.12.3. Correlation between miR-942 expression in cells and circulating miR-942 in plasma

A correlation between the levels of expression of miR-942 in the cells and the circulating levels in the plasma was investigated. It was observed a positive correlation between expression of miR-942 in cells and circulating levels in plasma using 10 samples of CLL and 3 of SLL **(Figure 55**). However this correlation was not strong (r^2=0,31).



CLL A	n= 8
CLL B	n= 2
SLL	n= 3

Figure 55 - Correlation analysis between miR-942 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Pearson test was performed to evaluate correlations.

6.13 Expression of miR-380 in CLL versus SLL

The miR-380 was identified from a previous study in our lab, where it showed to have higher expression in SLL samples, and in samples with higher percentages of EPCs **(Teixeira, 2009).** This miRNA, along with miR-942 was identified in by miRNA microarrays, using CLL and SLL pools RNA from PBMNC. So, no targets were identified.

6.13.1 Circulating levels of miR-380

The circulating levels of miR-380 were significantly higher in CLL patients than in SLL patients. This difference was statistically significant (p=0.0117) **(Figure 56A).** When comparing high (stage B+C) and less aggressive (stage A) CLL stages, no difference was found **(Figure 56B).**

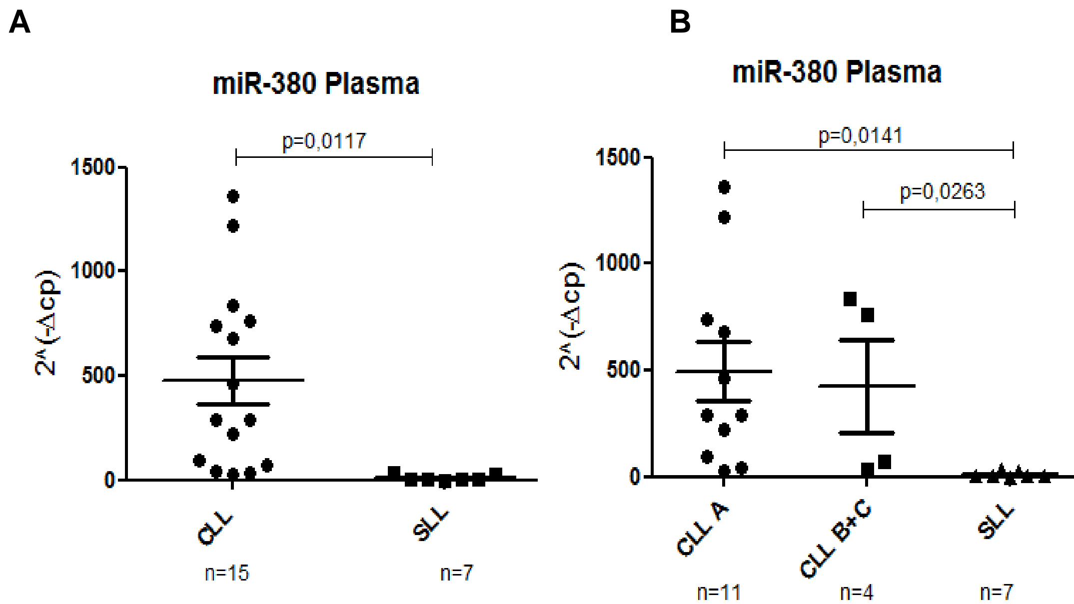


Figure 56 – miR-380 expression in the plasma of CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using the average of expression of 3 miRNAs (miR-363*, miR-191, miR-223*) and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.13.2 Expression levels of miR-380 on total PBMNC

The level of miR-380 expression in cells was statistically higher, in average, in SLL than in CLL samples (p=0,001) **(Figure 57A).** The less aggressive stage of CLL (stage A) showed a significant increase of miR-380 when compared with SLL samples. Additionally, the most aggressive stages of CLL (stages B and C) had higher level of miR-380 than SLL samples but this difference was not statistically significant. Comparing the CLL stages, the most aggressive stages of CLL (stages B and C) showed a significant increase in the miR-380 expression than CLL (stage A) **(Figure 57B).**

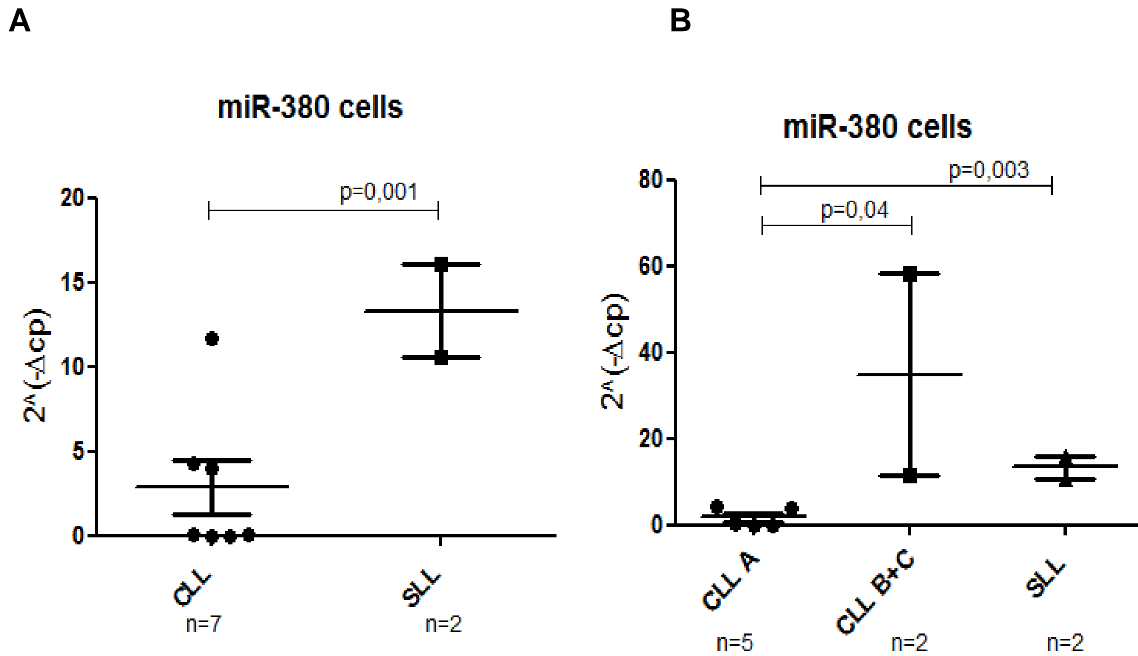
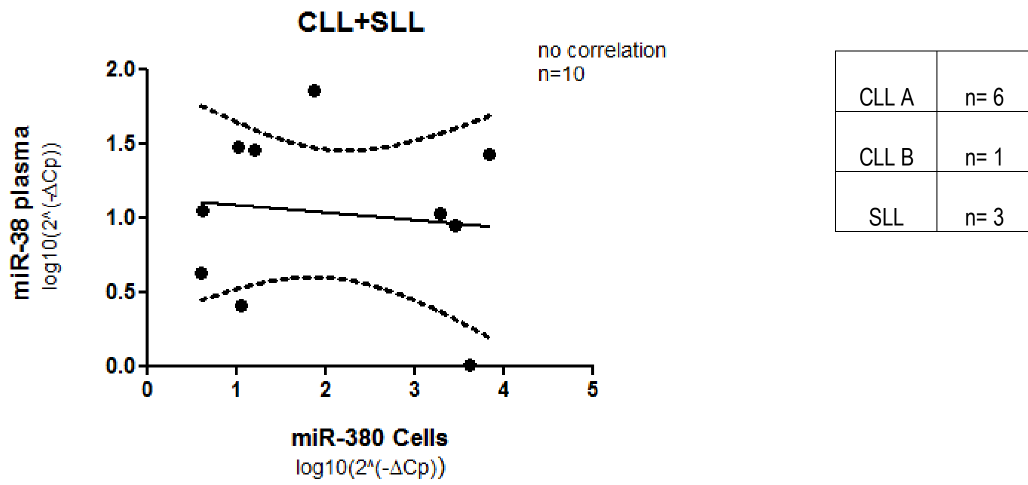


Figure 57 – miR-380 expression in cells in CLL (stage A and stages B or C) and SLL samples, analyzed by qPCR. Cp values were normalized using U6 small nuclear RNA and fold changes were calculated using 2^(-ΔCp) method. P values were obtained from unpaired two-tailed Student's t test. Error bars represent standard deviation (SD).

6.13.3. Correlation between miR-380 expression in cells and circulating miR-380 in plasma

It was not possible establish a correlation between miR-380 expression in cells and circulating levels in plasma. This analysis included 7 samples of CLL and 3 SLL samples **(Figure 58).**



CLL A	n= 6
CLL B	n= 1
SLL	n= 3

Figure 58 - Correlation analysis between miR-380 expression in cells and circulating levels in plasma of CLL and SLL samples quantified by qPCR. Log10 of the values of used. Pearson test was performed to evaluate correlations.

Discussion

CLL is classified into prognostic groups, some patients could have an indolent disease, while other patients could develop an aggressive disease that requires early therapy. CLL and SLL are different clinical manifestations or stages of the same disease according to WHO. Because of that, it is important to establish a molecular characterization of these variants of the disease, which until now remain unknown.

So, in the current study we investigated some of the molecular and cellular processes that allow to predict CLL progression.

Characterization of the leukemic population

The BM is the major hematopoietic organ and a primary lymphoid tissue **(Travlos, 2006)** and is formed for a great variety of cells, the majority are hematopoietic cells but non-hematopoietic cells are also present **(Wilkins, 1992).**

As expected, there are more leukemic cells in circulation in more aggressive stages of CLL: in the most aggressive stages of CLL, stages B and C, according to Binet classification, there is a higher percentage of leukemic cells in circulation, when compared with the CLL stage A, less aggressive. In SLL the percentage of leukemic cells was disparate in the two samples analyzed. As only two SLL samples were analyzed, no conclusions can be drawn from the results.

BM-derived populations (myeloid cells, cells with chemotactic capability, myeloid cells with chemotactic capability, myeloid progenitor cells, progenitor cells with chemotactic capability and EPCs) were quantified in the PB by flow-cytometry and their correlation with leukemic cell population in circulation was investigated. In general the correlations obtained were not strong, which may, be simply due to the low number of samples analyzed.There was a correlation between the BM derived myeloid population ($CD11b^+$) detected in the PB and the percentage of leukemic cells, regardless the stage or variant of the disease. Although this correlation was not strong, the results

could suggest that higher percentage of leukemic cells in circulation, as occurrs in more aggressive stages, lead to a higher mobilization of the myeloid cells from the BM into PB. However, when looking to the percentage of myeloid population with chemotactic ability ($CD11b^+/CXCR4^+$), no correlation was found. The role of this myeloid cell population in the circulation is not known.

There was also a correlation between the BM derived progenitor cells population (CD34+ cells) detected in the PB and the percentage of leukemic cells; between percentage of cells with chemotactic activity (CXCR4+ cells) and leukemic cells and between percentage of progenitor cells with chemotactic activity (CD34+/CXCR4+ cells) and leukemic cells.

These results showed that when the percentage of these populations of BM-derived cells increases the percentage of leukemic cells are also increased. These findings suggest that the proliferation of leukemic cells increase the proliferation of other cells, such as progenitor cells (CD34+ cells), cells with mobilization and chemotactic capability (CXCR4+) but also cells from myeloid lineage (CD11b+ cells).

In several hematological cancers, it is described the increase of progenitors in circulation **(Igreja *et al.*, 2007; Becker and Jordan, 2011).** The progenitor population in circulation is naturally very low, unless some physiological stimulus is inducted, as pregnancy, or in disease as in wound healing and cancer **(Buemy et al., 2007; Asahara et al., 1999; Zhang *et al.,* 2005; Dome *et al.*, 2006).** Intriguingly it was not observed a correlation between the number of progenitors and leukemic cells in the peripheral circulation in the present study: samples in which higher numbers of leukemic cells were identified were not always the ones that had more progenitor cells in circulation.

VEGF plays an important role in the pathogenesis of CLL **(Letilovic *et al.*, 2006)**. The levels of VEGF are usually higher in PB of leukemic pateints **(Dias et al., 2000).**

In this work the levels of VEGF in circulation could distinguish between healthy controls and leukemia patients: as expected in the healthy controls the levels of VGF were undetectable contrary to the observed in the sample patients. CLL patients had increased of VEGF levels than SLL and the higher levels of VEGF belong to CLL stage A, the less aggressive stage of the disease, although this difference was not statistically significant. These results were concordant with Molica *et al.*, this group observed high levels of VEGF in CLL stage A. **(Molica *et al.*, 2002).** However, in SLL the levels of circulating VEGF were lower than in CLL. As in the SLL variant the disease involves mainly the BM and lymph nodes infiltration, the increased levels of VEGF produced by leukemic cells may be localized in these organs and consequently not be detected in circulation. This is the opposite in CLL that is characterized by high number of leukemic cells in circulation (see section 1 the percentage of leukemic cells) and therefore higher levels of VEGF produced by these cells can be detected.

The SDF-1 is a ligand of the receptor CXCR4 and plays an important role on mobilization of CLL cells thought the body. Several reports showed the involvement of SDF-1 in the mobilization of cells from the BM niches (refs). In the present study, healthy donors showed higher levels of SDF-1 in plasma samples than CLL patients, although these differences was not statistically different and the levels of SDF-1 were not statistically different between CLL stages. However, in SLL these seems to be an increase of SDF-1 levels, but the number of samples were not sufficient to make conclusions, because only two samples of SLL were analyzed in this study. Interestingly, it was reported that B-CLL patients showed lower SDF-1 plasma levels compared to the control group**. (Barretina *et al.*, 2003)** The same group reported that there was no correlation between CXCR4 or SDF-1 expression and the clinical stage of disease. Likewise it was observed in the present study, no correlation between the

percentage of CXCR4^{+} cells and circulating SDF-1 levels. The results obtained suggest that other factors, and not only alteration in the SDF-1, should justify the BM infiltration for neoplastic cells observed in CLL and that CXCR4 could be involved in other features with malignant B cells, such as increased survival, rather than in their homing or migration to the BM. Together these results suggest that levels of SDF-1 cannot be used to reflect mobilization from the BM or even to distinguish between healthy and diseased samples. A higher number of samples would be required to infer any correlation with the circulating SDF-1 levels.

However, the results that involved SDF-1 levels may not have been reliable in this study. The R&D systems reported defects in the SDF-1 ELISA Kit used in the present study to measure circulating SDF-1 levels in plasma samples from CLL and SLL patients and healthy donor.

The key feature of CLL cells is their resistance to apoptosis conferred by overexpression of anti-apoptotic genes. Therefore, genes involved in apoptosis pathway have been extensively studied in CLL **(Hanada *et al.*, 1993; Kitada and Reed, 2004; Placzek *et al.*, 2010; Moshynska *et al.*, 2004).** In this thesis, two of these genes having anti-apoptotic role, the BCL-2 and MCL-1, were studied. The MCL-1 is an anti-apoptotic member of the BCL-2 family of apoptosis-regulating proteins.

The anti-apoptotic genes, BCL-2 and MCL-1 showed increased expression in CLL when compared with SLL patient. Because CLL patients had more cells in circulation than SLL patients, these patients had likely more cells resistant to apoptosis and consequently more expression of anti-apoptotic genes.

Interestingly, the most aggressive stages of CLL (stages B+C) were characterized by an increase of the anti-apoptotic gene (BCL-2), showing that these cells are molecularly regulated to have a higher resistance to apoptosis. These results are

consistent with those that have been described on the BCL-2 which reported that BCL-2 had elevated levels correlated with aggressive disease **(Hanada *et al.*, 1993).**

So these results suggest that apoptosis have an important role in CLL and the anti-apoptotic gene BCL-2 may be a potential biomarker between CLL stages.

However this was not observed with the MCL-1, where no difference was observed in the transcript levels between less and most aggressive stages of CLL. It was previously reported that high levels of MCL-1 expression and protein levels in CLL cells correlate with poor disease prognosis and resistance to chemotherapy **(Pepper et al., 2008).**

Additionally, it was reported that the VEGF induce the expression of MCL-1 via autocrine signaling loops in B-CLL **(Véronèse et al., 2009).** Interestingly, taken together the VEGF levels and MCL-1 expression levels it was observed an identical pattern of levels/expression across CLL A, CLL B+C and SLL, confirming the association between VEGF and MCL-1, although this correlation was not found using a Pearson´s correlation test. A higher number of samples would be necessary to confirm the correlation between VEGF and MCL-1.

Notch pathway and leukemia

It has been investigated the role of Notch signaling in tumorigenesis, and particulary, its role in CLL pathogenesis was recently reported **(Rosati *et al.*, 2009).** These authors found that Notch signaling is constitutively activated in B-CLL cells that express both Notch1 and Notch2 proteins and their ligands Jagged1 and Jagged2. Notch signaling plays a critical role in B-CLL cell survival and apoptosis resistance and suggest that it could be a novel potential therapeutic target. This feature may help cells to resist to apoptosis. Although Rosati et al. (2009) have shown the Notch activation in B-CLL cells compared to normal B-cells, the stuffy of componets of the Noch pathway in the variants of CLL (CLL, SLL) and stages (CLLA, CLLB, CLL C) was not reported. In this thesis it was observed that expression of Jagged2, that is constutively expressed in

malignant B-cells is higher in SLL than in CLL, suggesting that SLL cells may have a higher ability to bypass apoptosis. This feature was already discussed above, when discussing the higher levels of BCL-2 in SLL cells. Taking together, BCL-2 and Jagged2 levels seem to suggest that SLL cells found in the peripheral circulation have a higher resistance to apoptosis. Moreover, the levels of Jagged2 were also significantly higher in more aggressive stages of CLL (stages B+C) than less aggressive stages (Stage A). This result also confirms that expression of Jagged2 may be associated with aggressiveness and resistance to apoptosis, therefore, the levels of Jagged2 together with BCL-2 expression may be informative in terms of aggressiveness and stage of CLL. These results suggest that Notch pathway may have an important role in CLL and Notch ligands as Jagged2 could be a potential biomarker between CLL stages.

The study of the notch-related gene HEY-2 did not reveal significant differences between CLL and SLL, although a reduction in expression of this gene could be observed, in average, in the SLL samples. Reports about the association between CLL and HEY-2 expression in the literature are scarce, and so the role of this gene in CLL is not clear.

Molecular characterization by miRNAs

miRNAs are key regulators of gene expression and are critical players in tumorigenesis. In CLL, miR-15a and miR-16-1, located in the region 13q14, which is often deleted, are responsible for CLL pathogenesis **(Calin *et al.*, 2002)**. These were the first miRNAs to be reported in association to cancer.

To better characterize CLL/SLL disease stages miRNA profiling studies using mononuclear cells and plasma of the PB of CLL and SLL patients was performed. In

this thesis, the levels of the miRNAs miR-155, miR-15a and miR-16-1, miR-21, miR-101, miR-223, miR-221, miR-34a, miR-34c, miR-141, miR-210, miR-942, miR-380, in a panel of CLL and SL samples was investigated.

Considering the miRNAs isolated from plasma, from the 13 miRNAs profiled, 9 were useful to discriminate CLL from SLL. The results will be discussed in detail as follows.

The most useful miRNAs showing ability to discriminate between the two variants of the disease were miR-942, miR-380 and miR-101, when detected in the peripheral circulation, although their levels of expression in the PBMNC was also different in CLL and SLL. The levels of circulating miR-942, miR-380 and miR-101 could discriminate between the CLL and SLL, being higher in the CLL than in SLL. Although variability in circulating levels of these miRNAs was observed in CLL plasma samples, in the SLL plasma samples the level of miR-942, miR-380 and miR-101 was always strongly reduced. However, the levels of these miRNAs did not differentiate CLL Binet stages (A, B and C). In other study performed in our lab, the miRNAs miR-942, miR-380 and miR-101 were previously identified by microarrays of miRNAs (miRCURY LNA microRNA Arrays v.10.0, Exiqon) performed using CLL and SLL pools of samples. **(Teixeira, 2009).** This analysis allow the recovery of miRNAs differentially expressed in CLL versus SLL PBMNC and indeed their detection in the plasma of individual samples proved to be a useful tool to differentiate these two variants of the disease. Interestingly, although the levels of miR-942, miR-380 and miR-101 could distinguish between CLL and SLL, the pattern of quantification was inverse when detected in the cells or extracellular in the plasma. These observations of changes between cells and extracellular in the plasma levels is similar with results reported at Wang et al. **(Wang *et al.*, 2009)**; similar results were already mentioned in Moussay et al. study **(Moussay *et al.*, 2010)**. The expression in cells obtained reflects the expression of these miRNAs in all PBMNC isolated. Likewise, miRNAs found in plasma can also be shed by a variety of cells, although the majority may be produced by the cells found in the

peripheral circulation. These circulating miRNAs could be released by other cell types, such as BM stromal cells, which have the ability to provide protection for CLL cells **(Jaksić *et al.*, 2010**). This hypothesis had been already explored by Moussay et al. (2010). It has been reported that miRNAs are selectively shed, that is, the fact that cells express a miRNAs at high levels that doesn't mean that the miRNA is shed. miRNAs are released in a controlled fashion not very clear mechanism that is part of the cell to cell communication. In fact was very consistent the pattern of quantification of all these three miRNAs (miR-942, miR-380 and miR-101) in cells and plasma.

The miR-942 and miR-380 have no known targets in the literature. These miRNAs were previously identified in our lab and would be interesting to investigate their specific role in the two variants of the disease, as its expression is very specific of CLL, regardless the stage, but not SLL in plasma, while the opposite occurs in cells.

The miR-15a and miR-16-1 functions as tumor suppressor in CLL and these two miRNAs were implicated in the CLL pathogenesis, as are located in the fragile site 13q14.3, a chromosomal region that is often deleted in CLL **(Calin et al., 2002)** consequently these miRNAs showed a reduced expression in CLL samples **(Fulci *et al.*, 2007).** Further, the anti-apoptotic BCL-2 transcript was described as a target of both miR-15a and miR-16 **(Calin et al., 2002).** Due to the relevant role in CLL, the levels of these miRNAs in the plasma of CLL and SLL patients were investigated. The levels of miR-15a, miR-16-1 could significantly differentiate CLL from SLL. Moreover, the circulating levels of miR-15a were also useful to discriminate between Binet stages of CLL. The circulating levels of miR-15a are higher in the stage A (Binet), which is the less aggressive stage of CLL than in the most aggressive CLL stage. Therefore, as miR-15a targets the BCL-2, reduced levels of miR-15a are indicative of poor prognosis reflecting the anti-apoptotic state of CLL cells. Indeed, a higher level of BCL-2 in SLL (inverse to the miR-15a and miR-16-1 quantification in plasma) was observed and previously discussed. The levels of miR15a and miR-16-1 showed higher discriminative

power than expression of these miRNAs in the cells. Although the circulating miR15a and miR-16-1 levels in plasma could discriminate between CLL and SLL and correlated with BCL-2 levels, the expression of these miRNAs was not sufficient to distinguish CLL from SLL. However, the expression of these miRNAs in cells was useful to discriminate Binet stages of CLL, being lower in the most aggressive stages. This is according to described that these miRNAs regulate BCL-2, so it would be expected to be reduced in the most aggressive stages allowing an up-regulation of BCL-2 and consequently higher resistance to apoptosis.

The expression of miR-210 in cells, in hypoxia, is directly regulated by Hypoxia-inducible factor (HIF)-1α and circulating miR-210 has been identified as prognostic marker in pancreatic cancer patients **(Ho *et al.*, 2010).** In this study, the levels of circulating miR-210 were significantly higher in CLL than in SLL, as observed with miR-15a and miR-16-1. Interestingly, the pattern of miRNA quantification in cells and plasma was also the opposite, as with miR-15a and miR-16. However, contrary to these two miRNAs, miR-210 does not seem to be specific of CLL or SLL as the levels of miR-210 in the circulation could also discriminate different stages of CLL. In fact, the levels of miR-210 seem to decrease with severity of the disease. This is contrary to reported in the pancreas, that is a solid tumor and therefore may be different from hematologic tumors. However, the levels of expression in cells are according to the literature, where the most aggressive variant of SLL has the higher levels of expression. The level of expression of miR-210 in solid tumors as breast is a prognostic factor **(Rothé *et al.*, 2011)**, and its expression is directly associated to the hypoxic and more aggressive tumors. In this study, considering the stages of CLL only, the level of miR-210 is higher in the less aggressive stage. However, hypoxia in the hematological tumors may not have identical role in the solid tumors.

The miR-155 levels are increased in multiple cancers and promote B cell lymphomagenesis **(Eis *et al.*, 2005; Kluiver *et al.*, 2005)**. The elevated levels of miR-155 were described as a candidate pathogenic factor in CLL **(Li *et al.*, 2011)**. Indeed considering miR-155 expression in the cells, it was observed a increase of expression with aggressiveness of the disease. This could be observed comparing the CLL and SLL, where the more aggressive variant, SLL, express higher levels. Considering the Binet stages of the disease and SLL, all together, the level of miR-155 increased from the less to the most aggressive stages and variant of the disease. However, this pattern was not observed with the levels of circulating miR-155, which is the opposite verified in the cells. The circulating miR-155 levels decrease with severity of the disease.

The miR-21, as miR-155 presents an opposite pattern in the miRNA quantification between cells and plasma. The miR-21 is often reported as up-regulated in solid tumors as prostate **(Yaman Agaoglu *et al.*, 2011)** and hepatocellular cancer **(Li *et al.*, 2010)** and in CLL miR-21 expression levels are higher and correlated with poor prognosis and predicted overall survival **(Rossi *et al.*, 2010)**. Likewise, in this study, the expression of miR-21 in cells was higher in SLL than in CLL but no differences were observed within the Binet stages of CLL. However, miR-21 detected in the plasma was reduced in the most aggressive stages of the disease (CLL B+C) and SLL when compared with the less aggressive stage of CLL (CLLA). This results suggest that circulating miR-21 levels does not reflect the severity of the disease.
In this study, miR-223 in plasma had levels in CLL than SLL patients and was increased in less aggressive stage of CLL (stage A). This result was concordant with Stamatopoulos *et al.* (2009), where they reported that in CLL the miR-223 expression, in cells, decrease with progression from the less aggressive stage of CLL (Binet stage A) to stage C **(Stamatopoulos *et al.*, 2009),** although these results were observed in cells. On the other hand in the present study, in cells, the results are the opposite: the

higher levels of miR-223 expression were verified in SLL patients when compared with CL. However, it was possible establish a positive correlation between expression in plasma and cells in 13 samples of CLL and SLL patients.

The miR-221/222 cluster modulates the expression of p27 protein in CLL cells that may represent a regulatory loop that helps maintaining CLL cells in a resting condition **(Frenquelli, 2010).** In this study only miR-221 was analyzed. The levels of miR-221 in cells were higher in the SLL compared with CLL but within Binet stages, the level of miR-221 was reduced in the most aggressive stage. An opposite pattern of miR-221 quantification was observed in the plasma. The extracellular miR-221 levels were reduced in SLL and also decreased with aggressiveness of CLL. This is not according to reported in other cancers. For instance, in the esophageal squamous cell carcinoma, the levels of miR-221 were elevated in the patients when compared to healthy controls **(Komatsu *et al.*, 2011).** However, in this thesis, no healthy controls were compared and therefore this analysis could not be done. In CLL, miR-221 levels in circulation were not found in the literature.

The miR-34a and miR-34c are components of the same cluster. miR-34a is a transcriptional target of p53 and implicated in carcinogenesis. In CLL, miR-34, as miR-155, is up-regulated when compared to the controls **(Li *et al.*, 2011).** In this study, the miR-34a and miR-34c have the same expression pattern in the CLL and SLL, being up-regulated in the most aggressive variant SLL. Likewise, the levels of miR-34a and miR-34c were also similar within these two miRNAs and the Binet stages of CLL, where no differences could be observed. In the peripheral circulation, the levels of miR-34c are higher in SLL than in CLL and no differences were observed between the less aggressive stage of the CLL and SLL. This result suggest that extracellular miR-34c levels did not reflect the aggressiveness of the disease. Likewise, miR-34a levels in the plasma did not reflect the aggressiveness of the disease, although a reduced number

of samples was analyzed. A higher number of samples would be necessary to include in this study. The quantification of miR-34a in the plasma proved to be difficult and several samples were invalid on the detection. The difficulties that occurred in the quantification of miR-34a may be due to its low levels in the plasma suggesting that miR-34a may be not shed from the cells or it is at low levels.

In colon cancer, circulating levels of miR-141 is an independent prognostic factor for advanced colon cancer **(Cheng *et al.*, 2011).** Although there are no reports about the involvment of miR-141 in CLL, its detection was performed as a positive control for miRNA extraction from plasma and as it is implicated in metastization and progression of the disease its level was investigated in CLL. The levels of miR-141 in the plasma were identical, in average, in all stages and forms of the disease, thus circulating levels of miR-141 in CLL are not useful indicators of progression. However, in cells, miR-141 expression was higher in SLL than in CLL, although was not sufficient to discriminate the Binet stages of CLL.

With these results was also possible establish a profile to identified the less aggressive stages of CLL (stage A), in these stage several circulating miRNAs were over-expressed such as, miR-155, miR-15a, miR-16-1, miR-21, miR-101, miR-223, miR-221, miR-210 and miR-380 and a reduction of expression levels of miR-34 and miR-34c. While, the opposite expression levels of these circulating miRNAs could establish the profile of the more aggressive stages of CLL (stages B and C).

Conclusion

In conclusion the results found in this study suggest that VEGF had increased levels in the less aggressive stage of CLL (stage A). The anti-apoptotic genes, BCL-2 and MCL-1, showed an increase expression in CLL patients. It was confirmed that BCL-2 had higher expression levels in the more aggressive stages of CLL (stages B and C), as already reported in other studies.

The Notch pathway had an important role in CLL and Jagged2 seems to be a useful novel biomarker between CLL stages, which had not been reported in CLL.

miRNAs are powerful tools used in cancers and in this study prove to be very useful in CLL, particularly circulating miRNAs from plasma samples. Possible candidates to for novel biomarkers useful in distinguishing between CLL and SLL, the miR-210, miR-380 and miR-101 were identified. It was also possible to establish different profiles using circulating miRNAs expression between the less and more aggressive stages of CLL.

Future Work

In future work it will be interesting to perform *in vitro* and *in vivo* studies to validate these miRNAs, found in this study, as new potential biomarkers in a panel with more CLL and SLL samples. It will be also interesting to develop functional experiences with CLL and SLL cells in culture to test the role of BCL-2 and Jagged2 in different conditions in different stages of the disease.

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Appendixes

Appendix 1 - miRNA Extraction from Plasma

The isolation of miRNA from plasma was performed as described. The plasma (250 µl) was homogenized with 750 ul of a QIAzol (QIAGEN, Austin, Texas, U.S.A.) master mix containing the QIAzol and 1.25 ul of MS2 RNA. The homogenate was incubated at room temperature for 5 min to allow dissociation of nucleoprotein complexes. Chloroform (200 µl) was added to each sample and incubated at room temperature for 2 min and then centrifuged at 12 000 g for 15 min at 4ºC. The upper phase was then transferred to a 2 ml tube and mixed with 1.5 vol of 100% ethanol. The small RNAs were further enriched using the RNeasy Mini Spin Column (Qiagen): 750 ul of the upper phase-ethanol mixture was transferred to the column and centrifuged at 13 000 g for 30 sec and flow-through was discarded. The membrane of the column containing the small RNAs was washed with the buffers provided and as according to the manufacturers instructions. The small RNA fraction containing the miRNAs was eluted from the column membrane by centrifugation (1 min at 13 000 g) with 45 ul of nuclease-free water and RNA and stored at -70ºC until further use.

Supplementary information

supplementary table 1 - Clinical and cytogenetic characteristics of CLL and SLL.

Only patients without previous treatment were included in this study.

Sample	Age	Sex	Diagnosis	Cytogenetic
CLL 1	78	M	CLL-B stage A (Binet)	Del 13q14.3
CLL 2	61	M	CLL-B stage A (Binet)	Normal
CLL 3	78	M	CLL-B stage A (Binet)	Normal
CLL 5	71	F	CLL-B stage A (Binet)	Del 13q14.3
CLL 6	70	M	CLL-B stage A (Binet)	Normal
CLL 7	85	F	CLL-B stage A (Binet)	Del 13q14.3
CLL 8	58	F	CLL-B stage A (Binet)	Normal
CLL 9	63	F	CLL-B stage A (Binet)	Del 13q14.3
CLL 10	52	F	CLL-B stage A (Binet)	Del 13q14.3
CLL 11	64	M	CLL-B stage B (Binet)	Del 11
CLL 12	71	M	CLL-B stage B (Binet)	Del 13q14.3
CLL 13	70	M	CLL-B stage A (Binet)	Del 13q14.3
CLL 28	76	F	CLL-B stage B (Binet)	Trisomy 12
CLL 29	75	F	CLL-B stage B (Binet)	Normal
CLL 31	80	F	CLL-B stage A (Binet)	Del 17
CLL 33	63	F	CLL-B stage A (Binet)	Normal
CLL 34	61	M	CLL-B stage C (Binet)	Normal

CLL 35	76	M	CLL-B stage A (Binet)	Del 13q14.3
CLL 36	66	F	CLL-B stage A (Binet)	Normal
CLL 37	53	M	Lymphoma(SLL)	Normal
CLL 38	51	M	CLL-B stage B (Binet)	Normal
CLL 39	73	M	CLL-B stage A (Binet)	Normal
CLL 40	67	M	CLL-B stage A (Binet)	Del 13q14.3
CLL 41	80	M	CLL-B stage A (Binet)	Normal
CLL 42	58	F	CLL-B stage A (Binet)	Normal
CLL 43	64	F	CLL-B stage A (Binet)	Normal
CLL 44	79	F	Lymphoma(SLL)	Trisomy 12
SLL 1	74	M	Lymphoma(SLL)	Trisomy 12
SLL 2	89	M	Lymphoma(SLL)	Normal
SLL 3	67	M	Lymphoma(SLL)	Normal
SLL 4	76	F	Lymphoma(SLL)	------
SLL 5	66	F	Lymphoma(SLL)	Normal
SLL 6	84	F	Lymphoma(SLL)	Normal

supplementary table 2 - Antibodies and respective working dilution used to define populations of BM circulating cells by FACS.

Cell populations	Cell populations	Antibodies	Fluorochrome	Brand	Dilution
CD11b+	Myeloid	**CD11b**	APC	Biolegend	1:100
CXCR4+	Cells with chemotactic activity	**CXCR4**	FITC	R&D Systems	1:100
CD34+	Progenitor cells	**CD34**	FITC	MACS (Miltenyl Biotec)	1:100
CD11b/CXCR4+	Myeloid with chemotactic activity	**CD11b** **CXCR4**	APC FITC	Biolegend R&D Systems	1:100 1:100
CD133/KDR+	Endothelial Progenitor cells (EPC)	**KDR** **CD133**	APC PE	R&D Systems MACS (Miltenyl Biotec)	1:100 1:40
CD34/CXCR4+	Progenitor cells with chemotactic activity	**CD34** **CXCR4**	PE FITC	BD Pharmigen R&D Systems	1:40 1:100
CD34/CD11b+	Myeloid Progenitor cells	**CD34** **CD11b**	PE APC	BD Pharmigen Biolegend	1:40 1:100

supplementary table 3 - Sequences of primers and miRNAs used in this study.

To quantify miRNAs, one of the primers used in the reaction is the mature sequence of the miRNA, where the uracil was replaced by thymine.

Primers/miRNAs	**Sequence 5' – 3'**
U6 Forward	GTGCCGCTTCGGCAGCACATATAC
U6 Reverse	AAAAATATGGAACGCTTCACGAATTTG
18S Forward	GCCCTATCAACTTTCGATGGTAGT
18S Reverse	CCGGAATCGAACCCTGATT
HEY-2 Forward	TCGCCTCTCCACAACCTTCAG
HEY-2 Reverse	TGAATCCGCATGGGCAAACG
BCL-2 Forward	GGCTGGGATGCCTTTGTG
BCL-2 Reverse	CAGCCAGGAGAAATCAAACAGA
MCL-1 Forward	TGAAATCGTTGTCTCGAGTGATG
MCL-1 Reverse	GTCACAATCCTGCCCCAGTT
Jagged 2 Forward	ACAGCGCCACTTGCAACA
Jagged 2 Reverse	CAGGTGTAGTGGCCGAAAAAG
miR-101	TACAGTACTGTGATAACTGAA
miR-155	TTAATGCTAATCGTGATAGGGGT
miR-15a	TAGCAGCACATAATGGTTTGTG

miR-16-1	TAGCAGCACGTAAATATTGGCG
miR-942	TCTTCTCTGTTTTGGCCATGTG
miR-380	TATGTAATATGGTCCACATCTT
miR-34 a	TGGCAGTGTCTTAGCTGGTTGT
miR-34c	AGGCAGTGTAGTTAGCTGATTGC
miR-141	TAACACTGTCTGGTAAAGATGG
miR-221	AGCTACATTGTCTGCTGGGTTTC
miR-223	TGTCAGTTTGTCAAATACCCCA
miR-210	CTGTGCGTGTGACAGCGGCTG
miR-21	TAGCTTATCAGACTGATGTTGA