UNIVERSIDADE DO ALGARVE

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CONTRIBUTION OF THE INTRACELLULAR CHOLESTEROL LEVELS FOR THE EXPRESSION OF VEGF-C AND VEGFR-3 IN ACUTE MYELOID LEUKEMIA

DISSERTAÇÃO DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS

SUPERVISÃO EXTERNA: DOUTORA ANA MAGALHÃES, DOUTOR SÉRGIO DIAS

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam das listagens de referências incluídas.

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ABBREVIATIONS

AML Acute Myeloid Leukemia

APOA-I Alipoprotein A1

Bcl-2 B-cell lymphoma 2

bFGF basic Fibroblast Growth Factor

BM Bone Marrow

CD36 Cluster of differentiation 36

dNTPs Deoxyribonucleoside triphosphates

ELISA Enzyme Linked Immunosorbent Assay

ER Endoplasmatic Reticulum

FAB Frensh-American-British

FGF-1 Fibroblast Growth Factor 1

FLT-1 fms-like tyrosine kinase 1

FLT-4 fms-like tyrosine kinase 4

GC Golgi Complex

G-CSF Granulocyte-Colony-Stimulating Factor

HDL High-density lipoprotein

HGF Hepatocyte Growth Factor

HMG-coAR 3-hydroxy-3-methylglutaryl-coenzyme A redutase

HRP Horseradish peroxidase enzyme

HSC Hematopoietic Stem cell

HSPG Heparan Sulfate-Proteoglycans

KDR kinase domain receptor

LDL Low-density lipoprotein

LDLR Low-density lipoprotein Receptor

LMA Leucemia Mielóide Aguda

LXR Liver X Receptors

MO Medula Óssea

MβCD Methyl-β-Cyclodextrin

NP Neuropilin

O/N Overnight

PBS Phosphate buffered saline

PD ECGF Platelet derived endothelial cell growth factor

PDGF Platelet derived Growth Factor

PFA paraformaldehyde

PGE_1 prostaglandin 1

PGE_2 prostaglandin 2

qPCR quantitative Polimerase Chain Reaction

RNase Out Ribonuclease inhibitor

mRNA messenger RNA

cDNA complementary DNA

RPM Revolutions per minute

RTK Receptor Tyrosine Kinase

SDS Sodium docedyl sulfate

SDS-PAGE Sodium docedyl sulfate polyacrilamide gel electrophoresis

SRB1 Scavenger receptor B1

SREBPs Sterol Regulatory Element binding proteins

TBS Tris Buffered Saline

TGF-α Transforming Growth Factor α

TGF-β Transforming Growth Factor β

VEGF Vascular Endothelial Growth Factor

VEGFR Vascular Endothelial Growth Factor Receptor

WHO World Health Organization

RESUMO

A Leucemia mieloide aguda (LMA) é um tipo de cancro que ocorre nas células hematopoiéticas da linhagem mieloide, devido a alterações malignas no processo normal de hematopoiese na medula óssea (MO). Variadas alterações genéticas estão na origem do processo de transformação das células hematopoiéticas mielóides em células malignas, denominadas por blastos ou mieloblastos. Estas células sofrem uma paragem no ciclo celular e adquirem a capacidade de proliferar indefinidamente assim como a capacidade de resistência à apoptose, permanecendo num estado indiferenciado e extremamente proliferativo. A elevada proliferação de mieloblastos leva à sua acumulação na MO e posteriormente no sangue periférico, alterando a hematopoiese normal, o que leva a um défice na produção das células do sangue e consequentemente a desordens como trombocitopenia, anemia ou granulocitopenia. Um dos maiores desafios que esta patologia apresenta actualmente é a resistência à quimioterapia.

Vários fatores têm sido indicados como relevantes na aquisição de resistência aos fármacos utilizados atualmente na terapia, por parte das células leucémicas. Neste trabalho é focada a família de factores de crescimento VEGF (*Vascular Endothelial Growth Factors*), nomeadamente o VEGF-C e o seu receptor VEGFR-3. Alguns sub-tipos de LMA expressam VEGFR-3 e produzem VEGF-C, estando colocada a hipótese da existência de um loop autócrino que estará a modelar a sobrevivência de células leucémicas. Inicialmente indicado como promotor de linfangiogénese durante o desenvolvimento embrionário, a sinalização do VEGF-C através do VEGFR-3 tem sido mais recentemente relacionada com a progressão e agressividade de vários tipos de cancro, estando ligada à proliferação, sobrevivência e consequente resistência a drogas citotóxicas pelas células de LMA. Estudos em LMA pediátrica estabeleceram que o aumento dos níveis de VEGF-C endógeno nas células leucémicas está relacionado com a diminuição da resposta aos fármacos, revelando uma influência do factor na sobrevivência destas células. Ao nível molecular, a sinalização de VEGF-C demonstrou induzir a expressão do factor anti-apóptotico Bcl-2, que está sobre expresso em variados tumores e está relacionado com o processo de tumorigénese e resistência à quimioterapia.

Adicionalmente, o colesterol intracelular tem demonstrado proteger células de LMA dos efeitos citotóxicos dos fármacos e estas células apresentam um metabolismo de colesterol desregulado, com aumento dos níveis de colesterol intracelular após exposição aos fármacos.

A sinalização através do VEGFR-1 é mediada pelos níveis de colesterol intracelular em células de LMA, e elevados níveis de colesterol aumentam a activação deste receptor, contribuindo para a expansão e desenvolvimento da doença, o que sugere uma relação entre os níveis de colesterol celular e receptores da família VEGF. Uma vez que também o receptor VEGFR-3 está implicado na progressão de LMA, é importante compreender de que forma o colesterol celular estará a modelar a sua expressão, assim como de que forma poderá estar a modelar a produção do seu ligando VEGF-C, nas células leucémicas. A existência de uma modulação da produção de VEGF-C nas células pelo colesterol poderá sugerir um envolvimento deste na indução da expressão do factor anti-apóptotico Bcl-2 pelo VEGF-C, e subsequente resistência à quimioterapia.

O objectivo do presente estudo passa por compreender de que forma o colesterol intracelular está a afectar a expressão dos factores VEGF-C, VEGFR-3 e Bcl-2 em células leucémicas, no sentido de compreender melhor a importância dos níveis de colesterol para estas células, e que relevância poderá ter para a actividade biológica destes factores. Como modelo de estudo para LMA foram utilizadas duas linhas celulares, HEL (*Human Erythroleukemia*) e THP-1 (*Human Monocytic Leukemia*), e a estratégia utilizada passou pelo enriquecimento e pela remoção parcial de colesterol destas células com complexos de colesterol + Metil-β-Ciclodextrina e Metil-β-Ciclodextrina, respectivamente. O tratamento das células com Metil-β-Ciclodextrina reduz a viabilidade das células e por isso foram utilizadas baixas concentrações (0.2 mM) durante períodos de tempo de 4 horas. Os níveis de colesterol das células foram sempre medidos após a exposição aos complexos de colesterol + Metil-β-Ciclodextrina e Metil-β-Ciclodextrina para confirmar a eficiência do respectivo enriquecimento e parcial remoção. Os níveis de expressão de VEGFR-3, VEGF-C e Bcl-2 foram analisados nas duas condições de colesterol celular por PCR quantitativo (PCRq); os níveis de VEGF-C foram ainda analisados por Western-blotting e os de VEGFR-3 por citometria de fluxo.

Os resultados preliminares deste estudo demonstram que o enriquecimento das células leucémicas com colesterol promove a diminuição dos níveis de mRNA VEGFR-3, enquanto que a remoção parcial de colesterol promove um aumento dos níveis de mRNA do VEGF-C. Não foram detectadas diferenças na expressão de Bcl-2, o que demonstra que esta parece não ser afectada pelos níveis de colesterol celular. A expressão de VEGFR-3 através da análise por citometria de fluxo não demonstrou diferenças entre as duas condições de colesterol.

Em suma, estes resultados demonstram um aumento do VEGF-C endógeno quando é removido colesterol das células leucémicas, indicando uma modelação da sua produção pelos níveis de colesterol celular. Os níveis de VEGFR-3 são afectados pelas alterações de colesterol apenas ao nível do mRNA, o que indica que a modelação de VEGFR-3 pelo colesterol é feita ao nível do mRNA. No entanto, são necessárias mais abordagens para compreender que funções celulares estão a ser modeladas pelas alterações de expressão verificadas, nomeadamente ensaios funcionais *in vitro* para analisar a proliferação celular e a resistência à apoptose, no sentido de investigar que relação poderá existir entre o que foi observado e a resistência à quimioterapia. Este trabalho deverá assim contribuir para uma melhor compreensão de como as vias de sinalização do VEGF-C são reguladas na leucemia. Uma vez que a expressão de VEGF-C tem sido associada à resistência à quimioterapia é importante explorar as questões obtidas neste estudo, para uma melhor compreensão de como as células de LMA resistem aos fármacos citotóxicos.

Palavras-chave: leucemia mielóide aguda; colesterol celular; VEGF-C; VEGFR-3; resistência à quimioterapia; Methyl-β-Cyclodextrin; Bcl-2; apoptose

ABSTRACT

Some subsets of Acute Myeloid Leukemia (AML) cells express the Vascular Endothelial Receptor 3 (VEGFR-3), a receptor for the Vascular Endothelial Growth Factor-C (VEGF-C). The VEGFR-3/VEGF-C axis has been related to many cancers, including leukemia, due to the promotion of cell proliferation, chemotherapy resistance and tumor aggressiveness. At the molecular level VEGF-C signaling has been shown to induce the expression of the Bcl-2 anti-apoptotic factor. Cholesterol has been reported to modulate the expression of VEGFR's in AML. Here, we hypothesize that intracellular cholesterol levels could modulate signaling through the VEGFR-3/VEGF-C axis, contributing towards increased leukemia aggressiveness. To test this hypothesis we either enriched or partially depleted cholesterol from the THP-1 and HEL cell lines with Methyl-β-Cyclodextrin + cholesterol complexes and Methyl-β-Cyclodextrin alone, respectively. It was seen that Methyl-β-Cyclodextrin compromises cell viability, so we tested concentrations of 0,2 mM. In these conditions we looked at VEGFR-3, VEGF-C and Bcl-2 expression by RQ-PCR and Western Blot; VEGFR-3 expression was also assessed by flow cytometry. Our preliminary results show that cholesterol enrichment of cells leads to a decrease of VEGFR-3 mRNA levels, while cholesterol depletion leads to an increase of VEGF-C mRNA levels. No differences in Bcl-2 have been detected. We are now confirming these results at the protein level. By flow cytometry VEGFR-3 expression didn't show differences between conditions. Subsequent experiments will include in vitro functional assays to assess cell viability, proliferation and resistance to apoptosis. This work may contribute to better understand how VEGF-C signaling pathways are regulated in leukemia. As VEGF-C expression has been associated with resistance to chemotherapy by AML cells, this work may contribute to better understand how resistance is achieved.

Key words: acute myeloid leukemia; cellular cholesterol; VEGF-C; VEGFR-3; chemotherapy resistance; Methyl-β-Cyclodextrin; Bcl-2; apoptosis

INTRODUCTION

1. HEMATOPOIESIS

1.1 Hematopoietic Process

Hematopoiesis is a continuous process whereby the blood cells are formed and it takes place, in adulthood, mainly in the bone marrow (BM). The Hematopoietic Stem Cell (HSC) is the multipotent common precursor of all blood cells formed, making hematopoiesis a hierarchically organized process, starting with a precursor cell that will give rise to all lineages of hematopoietic cells. (Sashida and Iwama *et al.* 2012) (Seke Etet *et al.* 2013) *et. al* 2012)

Hematopoietic Stem cells (HSCs) have the capacity of self-renewal and differentiation, giving rise to daughter stem cells to maintain the stem cell pool while still contributing to the pool of differentiating cells. Throughout the differentiation process these cells give rise to various intermediate progenitor cells that undergo a gradual commitment to each of the blood cell lineages, until a mature blood cell is formed (Doulatov *et al.* 2012). HSCs originate two major intermediate precursors which give rise to the two major hematopoietic cell lineages: myeloid and lymphoid. The lymphoid lineage yields T, B and NK (Natural Killer) lymphocytes and the myeloid lineage originates granulocytes (basophils, eosinophils, neutrophils), monocytes (dendritic cells and macrophage), erythroblasts (erythrocytes) and megakaryocytes (platelets) (Figure 1).

As just mentioned HSCs reside at the apex of hematopoietic hierarchy and are modulated by different cellular and molecular mechanisms, to form mature blood cells. These comprise intrinsic genetic processes and bone marrow microenvironment derived factors. The interaction between blood cellsnd their microenvironment determines essential cellular processes such as proliferation, differentiation or apoptosis. (Doulatov *et al.* 2012) (C. Smith *et al.* 2003)

1.2 Malignant Hematopoiesis and Leukemia

The regulated process of hematopoiesis can fail causing malignant transformation of blood forming cells. Normal hematopoietic progenitors can be transformed into malignant cells due to mutations in genes encoding critical regulatory pathways like the ones which regulate cell differentiation and maturation (Seke Etet *et al.* 2013).

When a hematopoietic progenitor cell transforms into a malignant cell it acquires higher abilities of proliferation and survival, giving rise to a hematological neoplasm. Hematological malignancies can be derived from hematopoietic progenitors developing in the lymphoid or myeloid lineage or from multipotent hematopoietic stem cells (HSCs) (Lowenberg *et al.* 1999).

Neoplastic proliferations of cells from the hematopoietic lineage may originate leukemias, which can be broadly divided into two main groups, according to the two major lineages that derive from the multipotent HSC: Lymphoid leukemias which affect B, T and NK lymphocytes lineage; and Myeloid leukemias which affect granulocytic, erythroid and megakaryocytic lineages (Estey *et al.* 2012).

In addition to this simplified classification which results from the hematopoietic lineage affected, leukemia may also be further divided into acute leukemia or chronic leukemia, based on the rates of disease progression (acute leukemias progress from the BM to the peripheral blood in less time than chronic leukemias) and other molecular and cellular features (beyond the scope of the present Thesis) (Table 1).

TABLE 1 – Four major types of Leukemia. A simplified view.

Cell lineage	Chronic	Acute
Myeloid Leukemia	Chronic Myeloid Leukemia	Acute Myeloid Leukemia
Lymphocytic Leukemia	Chronic lymphocytic Leukemia	Chronic Myeloid Leukemia

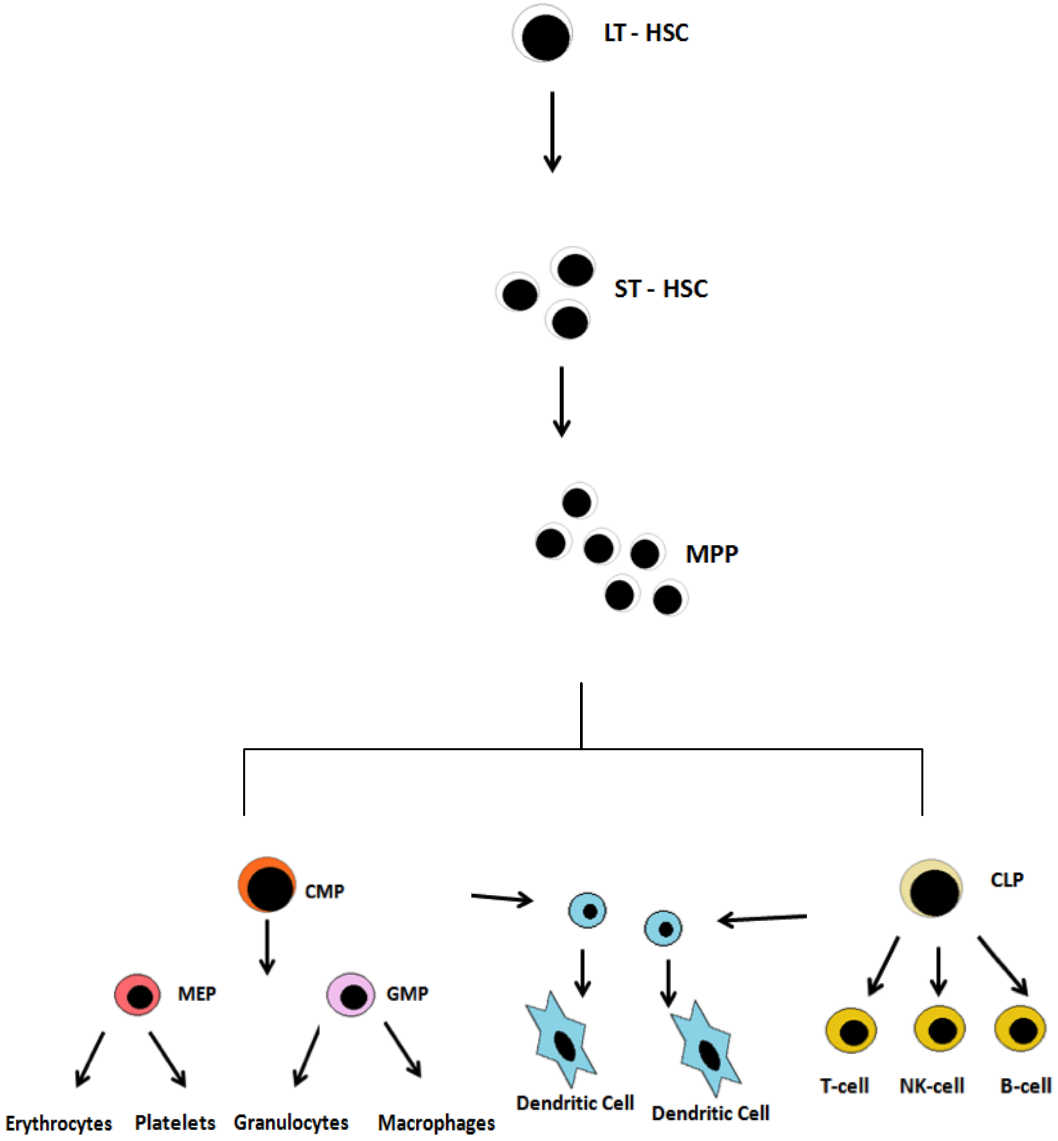


FIGURE 1 – Schematic representation of hematopoiesis. This scheme represents, in a simplified manner, hematopoietic process steps during the generation of specific blood lineages from a common HSC. HSC can be broadly divided into two cellular subsets: LT-HSC (Long term HSC) and ST-HSC (Short term HSC). LT-HSC cells have the capacity to undergo self-renewal indefinitely, while ST-HSC have the capacity to undergo self-renewal. ST-HSC give rise to MPP (Multipotent Progenitors) which originate the two major blood cell lineages: Myeloid and Lymphoid derived from CMP (Common Myeloid Progenitors) and CLP (Common lymphoid progenitors), respectively. Dendritic cells may be originated both from myeloid and lymphoid lineages.

2. ACUTE MYELOID LEUKEMIA (AML)

AML is a clonal hematopoietic disorder characterized by a group of genetic changes affecting a hematopoietic stem cell or a myeloid-specific progenitor cell. This changes lead to an undifferentiated and proliferative stage of myeloid cells, which become resistant to apoptosis. Cells which undergo cell cycle arrest and remain undifferentiated, instead of continuing the process of cell differentiation into a mature blood cell, are called blast cells. Higher proliferation and survival of blasts cells leads to their accumulation in the BM and expansion into the peripheral blood (Lowenberg *et al.* 1999).

Blast cells are also found in healthy people, around five percent of the cells of a normal BM are blast cells, however, these cells do eventually continue to mature until becoming fully differentiated and functional, which does not occur in individuals with AML. Higher proliferation and survival of blasts cells leads to an accumulation in the bone marrow and peripheral blood. The blast percentage commonly used to diagnosis AML is around twenty percent in the peripheral blood and BM (Lowenberg *et al.* 1999).

The BM infiltration with blasts alters normal hematopoiesis, causing a decrease in the production of mature myeloid cells and therefore a decrease of these cells in peripheral blood, leading to thrombocytopenia, anemia, granulocytopenia and eventually serious infections and hemorrhage (Huang *et al.* 2013).

AML can occur in patients of any age but is the most frequent type of leukemia in adults, having the lowest survival rate between all other sub-types and being primarily a disease of late adulthood. It is unusual a diagnosis of AML in patients younger than 40 years old and usually the mean age of diagnosis is 65 years old (Deschler and Lübbert *et al.* 2006). The increased incidence of AML along aging is due largely to the existence of other hematopoietic disorders that can progress to leukemia, like myelodysplasic-related changes, which become more common in older patients (Lowenberg *et al.* 1999). To summarize, AML covers a heterogeneous group of myeloid

leukemias, which differ in the lineage that is affected, and also the underlying cause, age of occurrence, and clinical prognosis.

2.1 AML pathogenesis

AML usually develops after an accumulation of specific mutations or translocations and other types of genetic alterations along time. These changes can be due to a variety of factors or an arrangement between those factors (Lowenberg *et al.* 1999).

There are several risk factors that can trigger leukemogenesis. These include age, prior hematological disorders, genetic disorders, exposures to viruses or chemical and radiation, as well as chemotherapy. These factors can alter in many ways, as referred, a hematopoietic progenitor cell line, triggering the leukemogenesis process (Lowenberg *et al.* 1999) (Deschler and Lübbert *et al.*2006).

Each sub-type of AML may have different casual agents and underlying mechanisms, suggesting that a particular molecular alteration or mutation may be caused by a particular agent, although in most cases AML is developed de novo, without evidence of exposure to any leukemogenic agent *(Deschler and Lübbert et al.* 2006).

2.2 AML diagnosis and classification

As already mentioned AML is a heterogeneous disease and can be classified in several sub-types according to different parameters. This classification is part of the disease's diagnosis and plays an important role in the decision of which treatment patient must be subjected to, and it is also an important predictor of disease's prognosis.

There are actually two major systems of AML classification based on four main methods of blast cells analysis: morphology, staining, molecular analysis and flow cytometry (see tables 2 and 3).

The French-American-British (FAB) system (Table 2) comprises the traditional classification of acute leukemia, based on morphologic characteristics and cytochemical studies of blast cells. The FAB system divides AML in 8 sub-types (M0 to M7) which differ according to the degree of maturation of leukemic blasts (Abdul-hamid *et al.* 1999).

TABLE 2 – French-American-British (FAB) classification system of AML.

M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal differentiation
M2	Acute myeloblastic leukemia with differentiation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Recently, with new and more advanced techniques of molecular analyses and with the principle that classification of leukemia should not only be based on morphologic means, the World Health Organization (WHO) created another classification system for AML. This is based more on the genetic heterogeneity of the disease (Table 3), attempting to clearly define blast cells and elucidate what specific issues are beyond their capacity of rapid and efficient replication (Vardiman *et al.* 2002) (Lowenberg *et al.*1999).

The WHO system divides AML into 4 sub-groups: 1) AML with recurrent genetic abnormalities 2) AML with multi lineage dysplasia 3) therapy and myelodysplasic syndromes related AML 4) not otherwise categorized AML (this one reflects the FAB system classification M0 to M7) and corresponds to that cases where molecular genetics does not contribute to incorporate any of the other earlier (Lowenberg *et al.*1999) (Vardiman *et al.* 2002) (Vardiman *et al.* 2009).

To do a successful AML diagnosis it is essential an integration of clinical,hematological, morphologic, immunophenotypic and genetic features in a systematic way (Lowenberg *et al.* 1999) (M. Smith *et al.* 2004).

TABLE 3 – 2008 WHO classification of AML (Abdul-hamid ***et al.*** 1999) (Vardiman ***et al.*** 2002) (Vardiman ***et al.*** 2009)

Acute myeloid leukemia with recurrent genetic abnormalities	➢ AML with t(8;21)(q22;q22), (AML1/ETO) ➢ AML with abnormal bone marrow eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22), (CBF/MYH11) ➢ Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12), (PML/RAR) and variants ➢ Acute myeloid leukemia with 11q23 (MLL) abnormalities
Acute myeloid leukemia with multilineage dysplasia	➢ Following MDS or MDS/MPD ➢ Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages
Acute myeloid leukemia and myelodysplastic syndromes, therapy related	➢ Alkylating agent/radiation–related type ➢ Topoisomerase II inhibitor–related type (some may be lymphoid) ➢ Others
Acute myeloid leukemia, not otherwise categorized (NOS)	➢ Acute myeloid leukemia minimally differentiated ➢ Acute myeloid leukemia without maturation ➢ Acute myeloid leukemia with maturation ➢ Acute myelomonocytic leukemia ➢ Acute monoblastic/acute monocytic leukemia ➢ Acute erythroid leukemia ➢ Acute megakaryoblastic leukemia ➢ Acute basophilic leukemia ➢ Acute panmyelosis with myelofibrosis ➢ Myeloid sarcoma

3. VASCULAR ENDOTHELIAL GROWTH FACTORS AND TUMOR ANGIOGENESIS

Angiogenesis is the growth of new blood vessels from pre-existing ones by sprouting of endothelial cells. The angiogenic process is tightly regulated by several proangiogenic and antiangiogenic factors, which establish the balance that regulates blood vessel formation. In a normal adult the vasculature is remarkably quiescent and angiogenesis occurs only in some physiological conditions like the female reproductive cycle (ovulation, menstruation, implantation, pregnancy) or wound healing. Angiogenesis in adults is therefore largely restricted to pathological situations, such as diabetic retinopathy, rheumatoid arthritis, psoriasis and tumor growth (Hanahan and Folkman *et al.* 1996).

Many factors with angiogenic activity have been identified to date. The first to be identified were the fibroblast growth factors (FGF-1 and bFGF), others include the transforming growth factors (TGF-α and TGF-β), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-alpha (TGF-α), platelet-derived endothelial growth factor (PD-ECGF), interleukin-8 and prostaglandins (PGE_1 and PGE_2). However, the vascular endothelial growth factor (VEGF), actually known as VEGF-A, because it acts in most situations that require angiogenesis, is one of the most important factors modulating angiogenesis (Ute Modlich and Roy Bicknell).

VEGF belongs to the Vascular Endothelial Growth Factors (VEGFs) family of secreted polypeptides, which are central regulators of vasculogenesis during embryonic development and angiogenesis in the adult life. The mammalian VEGF family consists of six VEGF ligands which arise as different splice variants and processed forms with different biological activities: VEGF-A commonly referred as VEGF), VEGF-B, VEGF-C, VEGF-D, the viral homolog VEGF-E and placenta growth factor (PLGF). These ligands are described to signal through three tyrosine kinase receptors designated as VEGF receptor-1 (also known as FLT1), VEGF receptor-2 (also known KDR) and VEGFR-3 (also known FLT4) and also through co-receptors such as heparan sulphate proteoglycans (HSPGs) and neuropilins (NPs). This happens in an overlapping way with different affinities and selectivities (Olsson *et*

al. 2006) (Ellis and Hicklin *et al*. 2008) (Schmidt and Carmeliet *et al*. 2011). VEGF-A binds to VEGFR-1 and VEGFR-2; PLGF and VEGF-B binds to VEGFR-1; VEGF-C and VEGF-D bind to VEGFR-3. VEGF-C and –D, depending on the species and due to proteolytic processing may bind to VEGFR-2, however with lower affinity than to VEGFR-3. This variety of VEGF ligands to different VEGFRs provides distinct binding specificities which contribute to their diversity of biological function (Olsson *et al*. 2006).

It is now well recognized that angiogenesis is essential for the growth of solid tumors. The angiogenic cascade leading to tumor vascularization can be divided into 2 general phases: the pre vascular phase, where a tumor remains in a dormant state with a small size, and the vascular phase where tumor is able to recruit its own vasculature, which occurs when its size is increased and the levels of hypoxia are higher. Only after a tumor acquires its own blood supply can it expand in size (Schmidt *et al.* 2011).

VEGF mediates numerous changes within the tumor vasculature, including endothelial cell proliferation, migration, invasion, survival, chemotaxis of bone marrow-derived progenitor cells, vascular permeability and vasodilation (Ellis and Hicklin *et al*. 2008). Overexpression of VEGF is therefore associated with tumor progression and poor prognosis in many tumor systems as colorectal carcinoma, breast cancer, prostate cancer, lung cancer and melanoma (Hicklin and Ellis *et al*. 2005). There are actually several anti angiogenic cancer therapy approaches conduced in order to inhibit VEGF signaling (Ellis and Hicklin *et al*. 2008).

3.1 Angiogenesis in Acute Myeloid Leukemia

Initially angiogenesis was considered to not, or only minimally contribute to the pathogenesis of non-solid tumors, like leukemias. Actually it has become evident that the microvasculature of the BM plays an important role in hematopoiesis both in health and in disease, and a role for angiogenesis in the

pathophysiology of hematological malignancies has been suggested, highlighting the importance of the BM microenvironment in regulating hematopoiesis.

Several studies demonstrated an increase of angiogenesis in several hematological malignancies, such as multiple myeloma, leukemias and myelodysplasic syndromes. For example, an increased microvessel density in the bone marrow of patients with AML has been observed, suggesting an important role for angiogenesis in disease development and progression (Padró *et al.* 2013). Angiogenesis is stimulated upon infiltration of leukemic blasts in the BM (Schmidt *et al.* 2011) and both leukemic blasts and cells from the BM microenvironment secrete angiogenic growth factors and mediators (Ayala *et al.* 2009). In AML the paracrine exchange of growth factors between blasts and endothelial cells is believed to contribute to disease pathogenesis while the bone marrow angiogenesis is promoted by growth factors released by the leukemic blasts. The VEGF family of growth factors has been indicated as one of the most important inducer of angiogenesis and several members have been shown to be constitutively expressed by AML blasts (Loges *et al.* 2005).

In addition to release of VEGF to stimulate angiogenesis, subsets of leukemic cells also express its receptors - VEGFR-1, VEGFR-2, VEGFR-3 (Fragoso *et al.* 2006) - and some studies in human leukemias showed that VEGFRs may be essential for tumor cell growth by promoting VEGF/VEGFR autocrine loops (Hicklin *et al.* 2005) such as the VEGF/VEGFR-2 autocrine loop which support leukemic cell survival and migration (Dias *et al.* 2000).

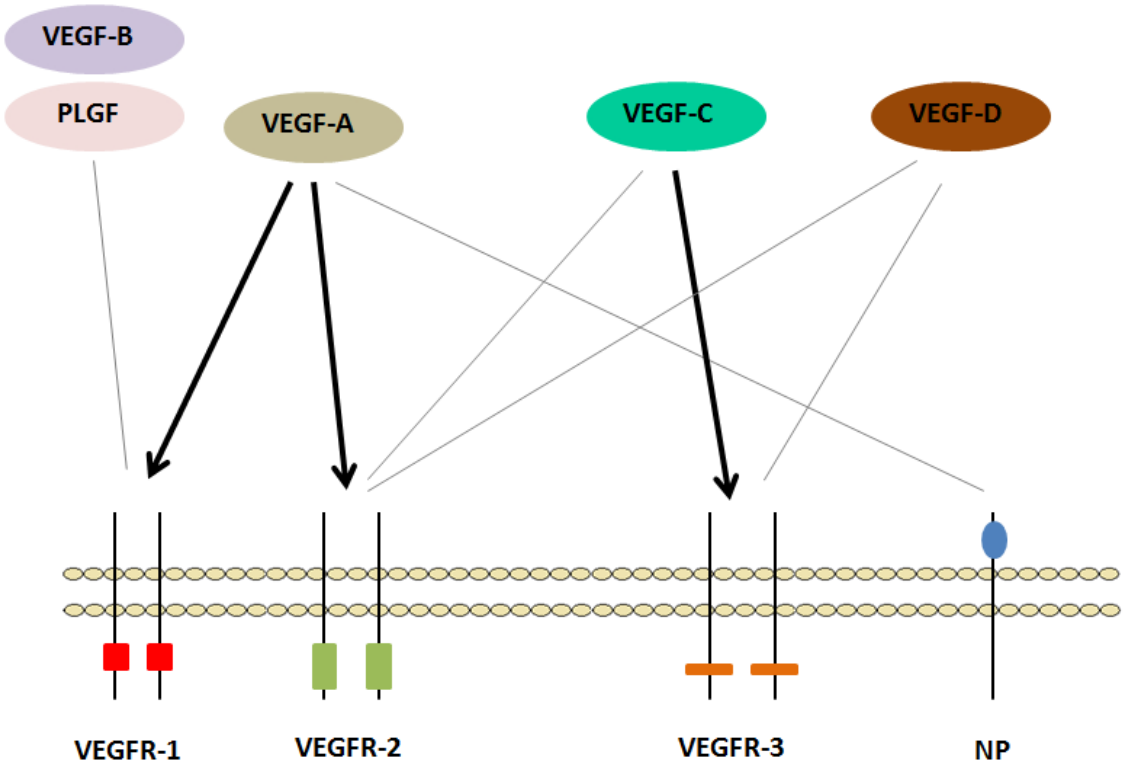


FIGURE 2 –VEGF/VEGFRs signaling axis involved in leukemia growth are represented with bold arrows. VEGF-A signalized through VEGFR-1 and VEGFR-2 promoting angiogenic and vasculogenesis stimulus; VEGF-C signaling through VEGFR-3 are involved in lymphangiogenic stimulus, mainly during embryonic development. In addition to their well established functions in angiogenesis and lymphangiogenesis, this signaling axis is suggested to be also related to leukemia progression and chemotherapeutic resistance by establishment of autocrine/paracrine angiogenic loops.

3.2 VEGF-C/VEGFR-3 signaling axis in AML

Besides VEGF-A, another member of VEGF family – VEGF-C - has been linked to the survival and proliferation of leukemic blasts in subsets of acute leukemias, due to stimulation of proangiogenic factors production and autocrine/paracrine signaling (Dias *et al.* 2002).

VEGF-C is characterized mainly as a lymphangiogenic and angiogenic growth factor, signaling through VEGFR-3 (FLT-4) with greater, or VEGFR-2 (KDR), with

lower binding affinity, respectively (Dias *et al*. 2002). Intracellular VEGF-C undergoes proteolytic processing leading to a variety of precursor forms; during the process VEGF-C acquires the ability to bind and to activate VEGFR-2, and increases its affinity and activating properties towards VEGFR-3 (Figure 3). It has been proposed that biosynthesis of VEGF-C as a precursor prevents unnecessary angiogenic effects raised by VEGFR-2 signaling, and allows VEGF-C to signal preferentially via VEGFR-3 (Joukov *et al.* 1997).

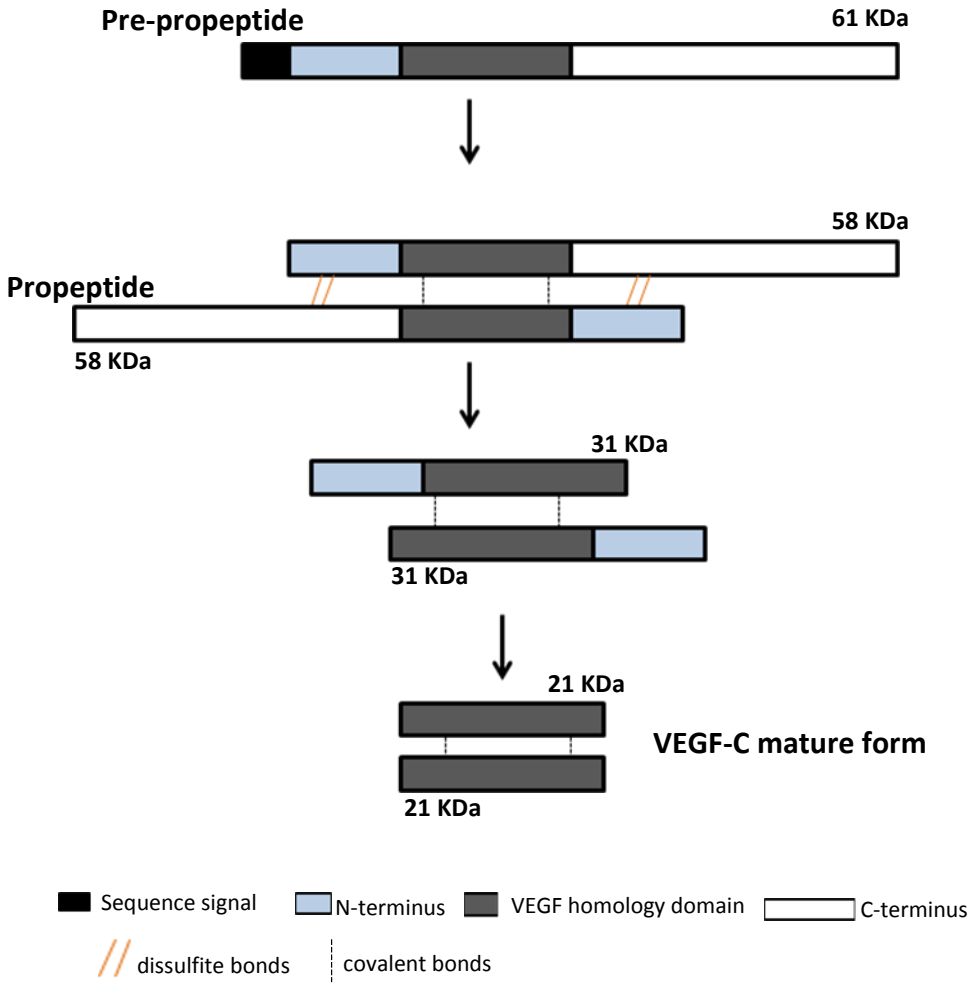


FIGURE 3 – Squematic model of the proteolytic processing of VEGF-C. Adapted from (Joukov *et al.* 1997).

VEGF-C expression has been linked to many tumor events, namely intratumoral lymphangiogenesis and metastasis, being reported as an important factor in tumor development and progression, particularly by signaling through VEGFR-3. The VEGF-C/VEGFR-3 signaling axis also exerts direct effects on cancer cells leading to tumor progression (Su *et al.* 2007).

In AML, VEGF-C has been indicated as a relevant modulator of leukemic cell proliferation, migration and also chemotherapeutic resistance through a VEGF-C/VEGFR-3 paracrine loop. Leukemic blast cells release proangiogenic growth factors in the bone marrow which stimulate the BM endothelium, resulting in production of VEGF-C by endothelial cells. VEGF-C in turn signals through VEGFR-3 expressed in leukemic blasts, creating a paracrine angiogenic loop which promotes leukemia survival and proliferation. However, it is also hypothesized the existence of an autocrine loop since certain subsets of leukemias release VEGF-C.

Finally, studies in pediatric AML reported that mRNA levels of VEGF-C in primary pediatric leukemic cells are closely related to increased *in vitro* drug resistance, and a longer time to kill most blast cells *in vivo* upon drug exposure, revealing an influence of endogenous VEGF-C in leukemic cell survival. Whether this VEGF-C acts in an autocrine or paracrine loop is not known (De Jonge *et al.* 2008).

3.3 VEGF-C and AML cell resistance to apoptosis: Bcl-2 family

Bcl-2 family proteins are key regulators of the mitochondrial or intrinsic apoptotic pathway, inducing or preventing the release of apoptogenic proteins to determine the fate of the cell after receiving a physical or chemical insult. All members of this family are involved in the regulation of cell death, with some of them being anti-apoptotic, like Bcl-2 itself, and other being proapoptotic. The antiapoptotic protein Bcl-2 is overexpressed in many tumor cells promoting tumorigenesis and chemotherapy resistance by inhibition of apoptosis (Marzo *et al*. 2008).

Several studies link VEGF family and Bcl-2 expression in AML, pointing this factor as one of the main effectors of VEGF-induced survival of leukemic cells. Both VEGF-A and VEGF-C have been shown to induces Bcl-2 expression on primary leukemias and cell lines (Dias *et al*. 2002).

4. CHOLESTEROL AND ACUTE MYELOID LEUKEMIA (AML)

4.1 Cellular cholesterol: physiological significance and homeostasis

Cellular cholesterol is an important membrane component in higher eukaryotes due to its role in the generation of a semipermeable barrier between cellular compartments and in the regulation of membrane fluidity. Cholesterol is enriched in the plasma membrane, where it normally accounts for 20-25% of the lipid molecules. Cholesterol is also a relevant factor in the modulation of the functions of membrane proteins, participating in several membrane trafficking and transmembrane signaling processes. All nucleated cells can synthesize cholesterol from acetyl CoA through the mevalonate pathway; this is called the *de novo* cholesterol synthesis, one of the two sources of cellular cholesterol. Another source is the dietary intake of cholesterol obtained with food (Ikonen *et al*. 2008), the dietary cholesterol is uptaken by the cells through Low Density Lipoprotein LDL, which is the major cholesterol carrier molecule in the plasma (Goldstein and Brown *et al*. 1974).

Cholesterol homeostasis is achived by coordinated transcriptional control of cholesterol-regulating genes (Banker *et al.* 2004). The synthesis of cellular cholesterol, uptake and processing reactions are mainly regulated by two nuclear receptors systems: sterol regulatory element binding proteins (SREBPs), activated in the presence of poor cellular cholesterol conditions, and liver X receptors (LXRs), activated when there is an excess of cellular cholesterol (Ikonen *et al*. 2008).

SREBP is located in the endoplasmatic reticulum (ER) and in conditions of low cellular cholesterol levels is carried to the Golgi complex (GC) where undergoes proteolytic processing, the resulting fragment is transported to the nucleus activating transcription of genes such as HMG-CoA and LDLR, responsible for *de novo* cholesterol biosynthesis and uptake of cholesterol by the cell, respectively (Ikonen *et al*. 2008).

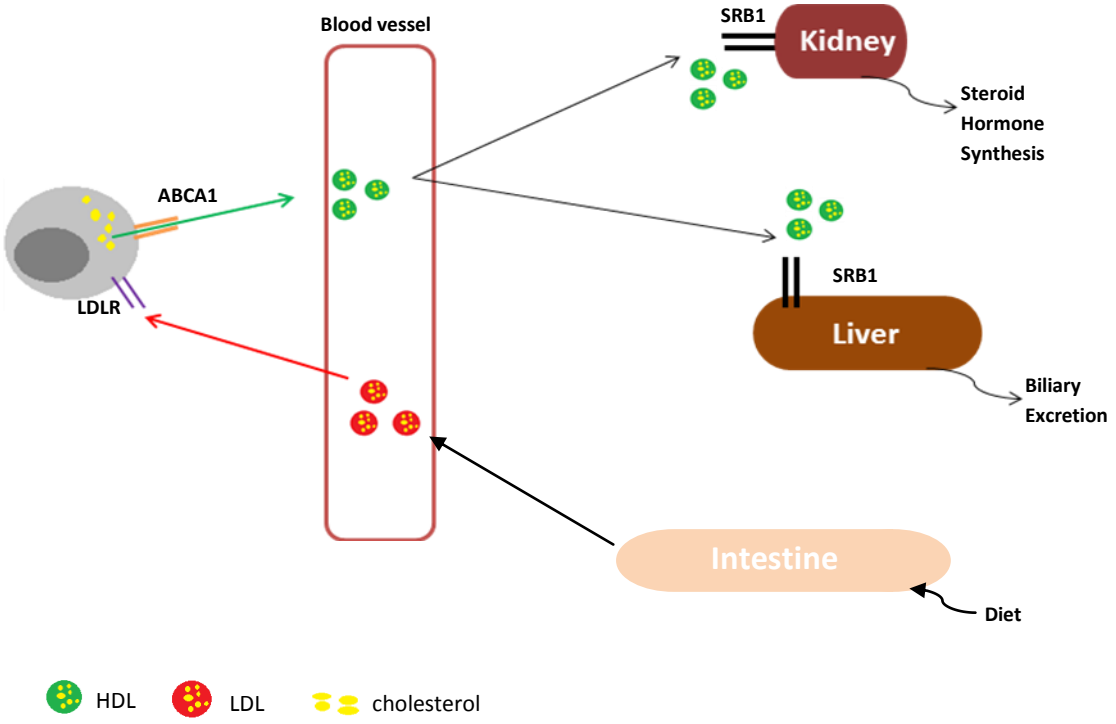


FIGURE 3 – Squematic representation of cholesterol efflux/influx. LDL transports dietary cholesterol in the plasma and it is uptake by cells through LDLR, whereas HDL includes excess cholesterol excreted by cells and delivers to steroidogenic cells in the kidneys and hepatocytes in the liver, through SRB1 receptor, for steroid hormone synthesis and biliary excretion, respectively.

LXRs are activated with excess of cholesterol but the crosstalk between their activation and membrane cholesterol levels is not well understood. However, it is established that higher cholesterol levels promote an increase in ABC transporters in plasma membrane, such as ABCA1, which allows cholesterol efflux from the

cells. ABCA1 is a key modulator of cellular cholesterol export, acting as a mediator in HDL particle formation and maintenance of its plasma levels. The binding of APOA-I to the ABCA1 transporter triggers a multi-step process by which lipids as cholesterol are transferred to the APOA-I to generate HDL precursors. Then, HDL enters the circulation and in the liver (hepatocytes) and in the kidney (steroidogenic cells) scavenger receptor B1 (SRB1) are responsible for the uptake of cholesterol contained in HDL particles, for conversion to bile acids and for steroid hormone synthesis, respectively (Hoang *et al.* 2013) (Ikonen *et al* 2008).

4.2 Cholesterol, lipid rafts and cancer

Tumor cells metabolism has shown to be altered in many cancers and it has begun to emerge a link between cholesterol levels and disease progression, aggressiveness and prognostic. Cholesterol is considered to have survival effects on cells and this may be related with the accumulation of cholesterol in lipid rafts domains at the plasma membrane (Scheinman *et. al* 2013). Lipid rafts consist of dynamic assemblies of cholesterol and sphingolipids known as lipid micro-environments on the cell surface that contain a set of proteins. Lipid rafts can change their size and composition in response to intra or extracellular stimulus favoring specific protein-protein complexes interaction and resulting in the activation of signaling cascades (Simons *et al.* 2000).

Lipid rafts are composed by subsets of caveolae, cell surface invaginations formed from lipid rafts by polymerization of caveolins – hairpin-like palmiotoylated integral membrane proteins that tightly bind cholesterol. Lipid rafts have been implicated in various physiological cellular processes (Murai *et al.* 2012) but signal transduction may be the most important role of lipid rafts at cell surface (Simons and Toomre *et al.* 2000). Many proteins, such as receptor tyrosine kinase family members (RTK), involved in signal transduction precisely exhibit their functions through lipid rafts (Patra *et al.* 2008). Changes in the molecular structure of lipid rafts domains may cause aberrant signaling (Patra *et al.* 2008) and because

of that, lipid rafts have been implicated in signaling pathways and in cancer progression (Murai *et al.* 2012).

4.3 Cholesterol in AML

In AML cells, cholesterol metabolism is deregulated (Li *et al.* 2003). Leukemic cells from patients have a higher uptake of LDL as compared to mononuclear blood cells and an increased HMG-CoA reductase (HMG-CoAR) activity (Banker et al. 2004). Compared with normal cells, cultured AML cells overexpress the genes for LDLR and HMG-CoAR (Vitols *et al.* 1994). Tumor cells are characterized by rapid proliferation, and presumably one of the advantages of being cholesterol enriched is the requirement of cholesterol for new membrane synthesis (Li *et al.* 2003). Cholesterol levels are also reported to increase in AML cells that are treated in vitro with sub lethal doses of radiation or chemotherapeutics but when cholesterol biosynthesis is inhibited with a HMG-CoAR inhibitor (mevastatin) or upon LDL deprivation by serum starvation, AML cells become sensitize to radiation and chemotherapeutic drugs (Banker *et al.* 2004). This suggests a protective role for cellular cholesterol and exogenous LDL against the cytotoxic effects chemotherapy.

4.4 Cholesterol influence in VEGF/VEGFRs signaling: AML

Lipid rafts work as complex signaling centers in cellular membranes and its function has been related to VEGF signaling, namely with VEGF signaling through VEGFR-2 in endothelial cells (Labrecque *et al.* 2003) and VEGFR-1 in leukemic cells (Casalou *et al.* 2011) suggesting a role for this cholesterol-rich membrane domains on VEGF signaling.

VEGFR-2 is reported to be located at caveolae sub-domains of lipid rafts, associated with the caveolin-1 protein in endothelial cells (Labrecque *et al.* 2003) as well as VEGFR-1 in subsets of AML cells (Casalou *et al.* 2011). VEGFR-1 location

in caveolae is promoted by stimulation of VEGF/PLGF ligand, and it was also seen that membrane cholesterol levels influence this location and receptor functions. Cholesterol extraction from AML cell lines with Methyl-β-Cyclodextrin abolishes VEGFR-1 activation induced by PLGF and in contrast, cholesterol enrichment with cholesterol included in Methyl-β-Cyclodextrin increases receptor expression and activation in presence or absence of the PLGF ligand. Increased VEGFR-1 activation in higher cellular cholesterol conditions may contribute to AML expansion and development, once signaling through VEGFR-1 is related to augmented cell viability, migration and VEGF production, emphasizing a link between cholesterol avidity and acute leukemias (Casalou *et al.* 2011).

AIMS

Some subsets of AML express ligands and receptors of the VEGF family and VEGF signaling has been shown to promote leukemic cell proliferation and survival leading to AML progression and aggressiveness. Among VEGF members, VEGF-C is particularly important, as it´s expression has been linked to the resistance to chemotherapy by AML cells. As resistance to chemotherapy in one of the main problems AML patients have to deal with, identifying factors that promote VEGF-C production and signaling could help in the search for better treatments for this disease.

Cholesterol has also pro-proliferation and pro-survival effects on AML cells. Additionally it is involved in the modulation of VEGFR-1 at the cellular membrane, influencing its activity. For this reason we hypothesized that cholesterol may affect VEGF-C expression and signaling.

To answer that we had three main goals:

1) To enrich and partially deplete intracellular cholesterol levels in AML cells
2) To analyze whether intracellular cholesterol content affects VEGFC and VEGFR-3 expression
3) To analyze whether intracellular cholesterol content affects Bcl-2 expression as Bcl-2 is a VEGFC signaling effector that promotes AML cell survival

REFERENCES

Sashida, G. & Iwama, A. Epigenetic regulation of hematopoiesis. *International journal of hematology* **96**, 405–12 (2012)

Seke Etet, P. F. *et al.* Normal hematopoiesis and hematologic malignancies: Role of canonical Wnt signaling pathway and stromal microenvironment. *Biochimica et biophysica acta* **1835**, 1–10 (2013)

Doulatov, S., Notta, F., Laurenti, E. & Dick, J. E. Hematopoiesis: a human perspective. *Cell stem cell* **10**, 120–36 (2012)

Smith, C. Hematopoietic stem cells and hematopoiesis. *Cancer control : journal of the Moffitt Cancer Center* **10**, 9–16 (2003)

Trujillo, A., McGee, C. & Cogle, C. R. Angiogenesis in acute myeloid leukemia and opportunities for novel therapies. *Journal of oncology* **2012**, 128608 (2012).

Lowenberg, B., Downing, J. R. & Burnett, A. Acute Myeloid Leukemia. *New England Journal of Medicine* **341**, 1051–1062 (1999)

Estey, E. H. Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *American journal of hematology* **87**, 89–99 (2012)

Huang, C.-W. *et al.* Clinical presentation. *Thorax* 1051–1062 (2013)

Deschler, B. & Lübbert, M. Acute myeloid leukemia: epidemiology and etiology. *Cancer* **107**, 2099–107 (2006)

Abdul-hamid, G. Classification of Acute Leukemia. (1999)

Vardiman, J. W. *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114**, 937–51 (2009)

Vardiman, J. W., Harris, N. L. & Brunning, R. D. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* **100**, 2292–302 (2002)

Smith, M. *et al.* Adult acute myeloid leukaemia. *Critical reviews in oncology/hematology* **50**, 197–222 (2004)

Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353–64 (1996)

Ute Modlich and Roy Bicknell, Angiogenesis (Chapter 17), *The Cancer Handbook.*

McMahon, G. VEGF receptor signaling in tumor angiogenesis. *The oncologist* **5, Suppl 1**, 3–10 (2000)

Olsson, A.-K., Dimberg, A., Kreuger, J. & Claesson-Welsh, L. VEGF receptor signalling - in control of vascular function. *Nature reviews. Molecular cell biology* **7**, 359–71 (2006)

Hicklin, D. J. & Ellis, L. M. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23**, 1011–27 (2005)

Schmidt, T. & Carmeliet, P. Angiogenesis: a target in solid tumors, also in leukemia? *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* **2011**, 1–8 (2011)

Ellis, L. M. & Hicklin, D. J. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nature reviews. Cancer* **8**, 579–91 (2008)

Padró, T. *et al.* Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. 2637–2644 (2000)

Ayala, F., Dewar, R., Kieran, M. & Kalluri, R. Contribution of bone microenvironment to leukemogenesis and leukemia progression. *Leukemia* **23**, 2233–41 (2009)

Loges, S. *et al.* Analysis of concerted expression of angiogenic growth factors in acute myeloid leukemia: expression of angiopoietin-2 represents an independent prognostic factor for overall survival. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23**, 1109–17 (2005)

Dias, S. *et al.* Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *The Journal of clinical investigation* **106**, 511–21 (2000)

Fragoso, R. *et al.* VEGFR-1 (FLT-1) activation modulates acute lymphoblastic leukemia localization and survival within the bone marrow, determining the onset of extramedullary disease. *Blood* **107**, 1608–16 (2006)

Dias, S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. *Blood* **99**, 2179–2184 (2002)

Dias, S. VEGF165 promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* **99**, 2532–2540 (2002)

Joukov, V. *et al.* Proteolytic processing regulates receptor specificity and activity of VEGF-C. *The EMBO journal* **16**, 3898–911 (1997)

Su, J.-L. *et al.* The role of the VEGF-C/VEGFR-3 axis in cancer progression. *British journal of cancer* **96**, 541–5 (2007)

de Jonge, H. J. M. *et al.* Endogenous vascular endothelial growth factor-C expression is associated with decreased drug responsiveness in childhood acute myeloid leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 924–30 (2008)

Marzo, I. & Naval, J. Bcl-2 family members as molecular targets in cancer therapy. *Biochemical pharmacology* **76**, 939–46 (2008)

Ikonen, E. Cellular cholesterol trafficking and compartmentalization. *Nature reviews. Molecular cell biology* **9**, 125–38 (2008)

Goldstein, J. L., Brown, M. S. & Brown, S. Binding and Degradation of Low Density Fibroblasts. J. Biol. Chem. 249:5153-5162. (1974)

Li, H. Y., Appelbaum, F. R., Willman, C. L., Zager, R. a & Banker, D. E. Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. *Blood* **101**, 3628–34 (2003)

Hoang, J.-J., Baron, S., Volle, D. H., Lobaccaro, J.-M. a & Trousson, A. Lipids, LXRs and prostate cancer: are HDACs a new link? *Biochemical pharmacology* **86**, 168–74 (2013)

Scheinman, E. J., Rostoker, R. & Leroith, D. Cholesterol affects gene expression of the Jun family in colon carcinoma cells using different signaling pathways. *Molecular and cellular endocrinology* **374**, 101–7 (2013)

Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nature reviews. Molecular cell biology* **1**, 31–9 (2000)

Murai, T. The role of lipid rafts in cancer cell adhesion and migration. *International journal of cell biology* **2012**, 763283 (2012)

Patra, S. K. Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochimica et biophysica acta* **1785**, 182–206 (2008)

Li, H. Y., Appelbaum, F. R., Willman, C. L., Zager, R. a & Banker, D. E. Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. *Blood* **101**, 3628–34 (2003)

Vitols, S., Norgren, S., Juliusson, G., Tatidis, L. & Luthman, H. Multilevel regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in normal and leukemic cells. *Blood* **84**, 2689–98 (1994)

Labrecque, L. *et al.* Regulation of Vascular Endothelial Growth Factor Receptor-2 Activity by Caveolin-1 and Plasma Membrane Cholesterol. **14**, 334–347 (2003)

Casalou, C. *et al.* Cholesterol regulates VEGFR-1 (FLT-1) expression and signaling in acute leukemia cells. *Molecular cancer research : MCR* **9**, 215–24 (2011)

MATERIAL AND METHODS

CELL CULTURE

HEL and THP1 cell lines were used as models for human erythroleukemia and human monocytic leukemia, respectively, both subsets of AML. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% L-Glutamine and 1% streptomycin/penicillin. HUVEC cells were used to perform qPCR experiments, and were cultured in EBM2 containing 5% FBS in a flask coated with gelatin 0.2%.

CELL VIABILITY

Cell viability was assessed through Trypan blue exclusion method. 10 µl of cells were mixed with 2µl of Trypan Blue in triplicate. Cells stained with blue (death cells) were then counted.

MβCD AND MβCD + CHOLESTEROL COMPLEXES SOLUTIONS

MβCD and MβCD + cholesterol complexes solution were done with base on (Klein *et. al* 1995) and used, respectively, to remove and to enrich cells with cholesterol. For MβCD solution, MβCD (Sigma-Aldrich) was dissolved into 1x PBS with constant stirring at 80ºC and when solution has reached this temperature a stock solution of isopropanol/chloroform (2:1) was added. For MβCD + cholesterol complexes the same procedure was follow but cholesterol was previously dissolved into isopropanol/chloroform (2:1) solution and then added to the MβCD dissolved into PBS at 80ºC. As a control solution for the experiment execution isopropanol/chloroform (2:1) was dissolved into 1x PBS. All these three solutions were stored and managed at room temperature.

ENRICHMENT AND DEPLETION OF CELLULAR CHOLESTEROL CONTENT FROM CELLS

HEL and THP-1 cell lines at $0.6x10^6$ cell/mL concentration were plated in serum-free RPMI 1640 medium for about 12 hours and then treated with MβCD (from 0.2 mM to 10 mM), MβCD + cholesterol complexes (0.2 mM MβCD + 0.02 mM cholesterol) and isopropanol/chloroform (2:1) for time periods of 4 to 24 hours, according with the

different experiments, at 37ºC. Cells were then collected into eppendorfs and centrifuge at 1200 RPM for 5 minutes. Supernatants were removed and cellular pellets stored at -20 ºC or -80 ºC depending on the future analyzes.

QUANTIFICATION OF CELLULAR CHOLESTEROL CONTENT

Cellular cholesterol content was measured by Amplex® Red cholesterol Kit (Invitrogen), a fluorometric method for the sensitive quantification of cholesterol. First, several dilutions of cholesterol reference standard (2 mg/mL) were prepared in order to obtain a standard curve: 0µg/mL, 1µg/mL, 2µg/mL, 4µg/mL and 8µg/mL. Cholesterol reference standard was diluted into 1x Reaction Buffer (0.5M potassium phosphate, pH 7.4, 0.25M NaCl, 25mM cholic acid, 0.5% Triton® X-100) as well as cellular pellets to proceed with cell lysis. 50 µl of each cholesterol standard dilution and cellular sample were plated into a 96 wells plate and then 50µl of 300 µM Amplex® Red Reagent (2 U/mL horseradish peroxidase, 2U/mL cholesterol oxidase and 0.2U/mL cholesterol esterase) was added to each plate micro well for the reaction beginning. Plate was incubated for 30 minutes at 37ºC, protected from light, and then absorbance was measured at 573 nm (Infinite® M200, Tecan).

PROTEIN QUANTIFICATION

Protein concentrations were determined using the Bio-Rad Laboratories DC protein assay kit, a colorimetric assay for protein concentration after detergent solubilization. Using BSA (100 mg/mL) as a protein reference several dilutions were done in order to obtain a standard curve: 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL and 1.6 mg/mL. BSA reference and cellular pellets were diluted into Reaction Buffer 1x or into RIPA buffer composed by 50mM Tris, pH 7.4, 150mM NaCl, 5mM EDTA, 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate, 1 pellet protease inhibitors (Roche), 1M NaF and 100mM Na_3VO_4 to proceed with cell lysis. 5 µl of each cellular sample and BSA standard dilution was plate into a 96 wells micro plate and then 25µl of Reagent A', composed by Reagent A (Sodium hydroxide) and Reagent S (Sodium dodecyl sulfate), was added to each micro well followed by 200µl of Reagent B (Folin reagent). Plate

was incubated for 5 minutes at room temperature and then absorbance was measure at 750 nm (Infinite® M200, Tecan).

RNA EXTRACTION

RNA was extracted using TRIzol® reagent method which maintains the integrity of RNA during cellular components disruption. 500 µl of TRI® reagent (Sigma-Aldrich) was added to each cellular pellet for sample homogenization, then chloroform (200µl/mL TRIzol®) was added and samples centrifuged at 14000 RPM for 20 minutes at 4ºC.The top aqueous phase of each tube, containing the RNA was transferred to new tubes and mixed with isopropanol (0.5µl/µl TRIzol®) and kept O/N at -20 ºC. Tubes were then centrifuged at 14000 RPM for 20 minutes at 4ºC to precipitate RNA, supernatants were removed and the pellets were washed in ethanol 80% (in DEPC-treated water). Pellets were resuspendend into DEPC-treated water and quantification was performed on NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The majority of these steps were performed on ice to prevent RNA degradation.

cDNA SYNTHESIS

The cDNA synthesis for quantification of transcripts was performed from 500 ng of total RNA using Random Primers (Invitrogen™), 10 mM dNTPs and DEPC-treated water up to 12 µl. This mix was incubated 5 minutes at 65 ºC and then a mix containing 5x First Strand Buffer (Invitrogen™), 0.1M DTT (Invitrogen™) and RNAse Out (Invitrogen™) was added and. Tubes were incubated at 25 ºC for 2 minutes and then 1µl of SuperScript enzyme (Invitrogen™) was added and incubated at 25 ºC for 15 minutes, followed by 50 ºC during 50 minutes and 70ºC for 15 minutes. In the end of these reactions cDNA was diluted into H_2O redistilled (1:3).

QUANTITATIVE PCR (qPCR) FOR mRNA QUANTIFICATION

Levels of mRNA were measured by qPCR through ViiA™ 7 Real time PCR system (Applied Biosystems) in 392-well plates, using SYBR-Green. This method allows the

amplification and quantification of a target gene, obtained through emission of a fluorescent dye that intercalates into de DNA strands. Relative expression was calculated using the comparative method $2^{-\Delta Ct}$ and sample normalization was performed with 18S mRNA expression. For the amplification reaction a 8.3 µl mix containing SYBR® Green (Applied Biosystems), 10 µM of both forward (FWD) and reverse (REV) primers, H_2O redistilled and 1 µl of cDNA was prepare per well plate. The sequences of the primers used in this study are listed in TableS1 on Supplementary information section.

PROTEIN EXTRACTION AND WESTERN-BLOTTING

Analysis of protein content in cells subjected to MβCD treatments was performed by western-blotting.

Cellular pellets were ressuspendend into RIPA buffer, maintained on ice for 30 minutes to perform cell lysis and then centrifuged at 13400 RPM for 20 minutes at 4 ºC. Supernatants (containing protein) were stored into new tubes and protein levels were measured.

Proteins were separated by sodium-docedyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE), this method allows the sorting of proteins by their molecular weight and charge. SDS-PAGE gel composition and porosity is chosen based on the specific weight of the target proteins to be analyzed. Here, a 15% gel was used since we wanted to separate proteins of 21-61 KDa size. This gel is composed of two acrylamide mixes: the resolving or separating gel (Table S6), and, on gel top, a stacking gel (Table S7) composed with pores that compresses proteins in a thin starting zone.

Before sample loading into the gel proteins were denatured through the addition of Laemli Buffer with 10% β-mercaptoethanol to an equal protein volume (equivalent to 11µg) and heat for 10 minutes at 95 ºC. β-mercaptoethanol denatures protein via its ability to clive disulfide bonds, and therefore disrupts both the tertiary and the quaternary structure of proteins. Then, samples, and 7µl of protein ladder (Precision Plus Protein™, Bio-Rad) with molecular weights standard, were loaded in the gel, and run at 140V for about 2 hours in an electrophoresis equipment (Mini Trans-Blot®

Electrophoretic Transfer Cell, Bio-Rad) filled with Running Buffer pH 8.3 (25 mM Tris, 190 mM Glycine and 0.1% SDS). Proteins are denatured in the presence of a detergent, SDS in this case, being coated with a negative charge and, therefore, they will run to the positive pole when electric current is applied to the gel.

Next steps comprises the transfer of the proteins contained in the gel to a nitrocellulose membrane, by electric current, and the exposition of proteins on thin surface of the membrane for detection. This transfer was also performed with the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) filled with Transfer Buffer (250 mM Tris, 1250 mM Glycine, 10% methanol), O/N, 4ºC, at 40V. After transfer, membrane was washed three times, during 10 minutes, with TBST and a blocking step was done to prevent the non-specific interaction of the antibody. Membrane was incubated with 5% milk in TBST (Table S12) for 1 hour at room temperature with shaking (VWR® Microplate shaker). Then the membrane was incubated with primary antibody rabbit anti-human VEGF-C (1:1000; Invitrogen™) diluted into TBST O/N, at 4ºC, shaking. After washing membrane three times with TBST, the membrane was then incubated with the secondary antibody anti-rabbit IgG conjugated with horseradish proxidase (HRP) enzyme (1:5000; W4018, Promega) for 2 hours at room temperature with shaking, and finally again washed three times with TBST.

At the end, the bands corresponded to the specific protein (VEGF-C) were detected by incubating the membrane with the substrate for HRP enzyme (Pierce ECL Western blotting substrate, Thermo Scientific) and osbserved in a Bio-Rad molecular image system (Chemidoc XRS^{+}) with Image Lab software. The bands obtained were quantified through optical density with ImageJ software.

The procediment described above was also performed to detect β-actin, the internal protein control used in these work with the primary antibody goat anti-human β-actin (1:5000; Santa Cruz Biotechnology) and the secondary antibody donkey anti-goat IgG conjugated with HRP enzyme (1:5000; sc-2020; Santa Cruz Biotechnology).

FLOW CYTOMETRY

Flow cytometry was used to analyze the pattern of VEGFR-3 expression at cell surface. After exposure to treatments with MβCD, MβCD + cholesterol complexes and isopropanol/chloroform (2:1) cells at $0.5x10^6$ were collected, centrifuged for 5 minutes, 1200 RPM at room temperature and then washed twice with 1x PBS. Cells were then fixed through the addition of 300µl 4% PFA (paraformaldehyde) followed by 15 minutes incubation at room temperature. After wash twice with 1x PBS samples were incubated with the primary antibody rabbit anti-VEGFR-3 (1:100; ab27278; Abcam) diluted into PBS/0.5% BSA and blank samples were incubated with PBS/0.5% BSA (Table S13), for around 60 minutes at 4ºC. Next samples were incubated with the secondary antibody 488 donkey anti-rabbit (1:400; A21206; Alexa Fluor®), after washing twice with 1xPBS, for 60 minutes at 4ºC. Finally, samples were washed as described previously, ressupend into 1x PBS and then the results accquired with BD FACSCalibur™ flow cytometer (BD Biosciences). The final data was analyzed with FlowJo software.

STATISTIC ANALYSIS

Statistical analysis was performed with GraphPad® Prism 5.0 software. The statistical significance was determined using student's T-test ($p > 0.05$) and error bars represent the standard error of the mean.

RESULTS

Depletion of cellular cholesterol with MβCD in THP-1 and HEL cell lines

One of the aims of this study was to understand if cholesterol depletion affected the expression of factors related to leukemia cell survival. Methyl-β-cyclodextrin (MβCD) has been largely used in recent years to manipulate the cholesterol composition of cellular membranes, being capable of partially deplete cholesterol from cells. For this, as a first approach, we treated HEL and THP-1 cells with 0,2 mM MβCD for 4 hours and evaluated intracellular cholesterol content using Amplex© Red Cholterol Kit (Invitrogen). As it can be seen in Figure 1A, in these conditions, a decrease in intracellular cholesterol content is observed in both cell lines, however with a small sample size (n=3) this is not statistically significant.

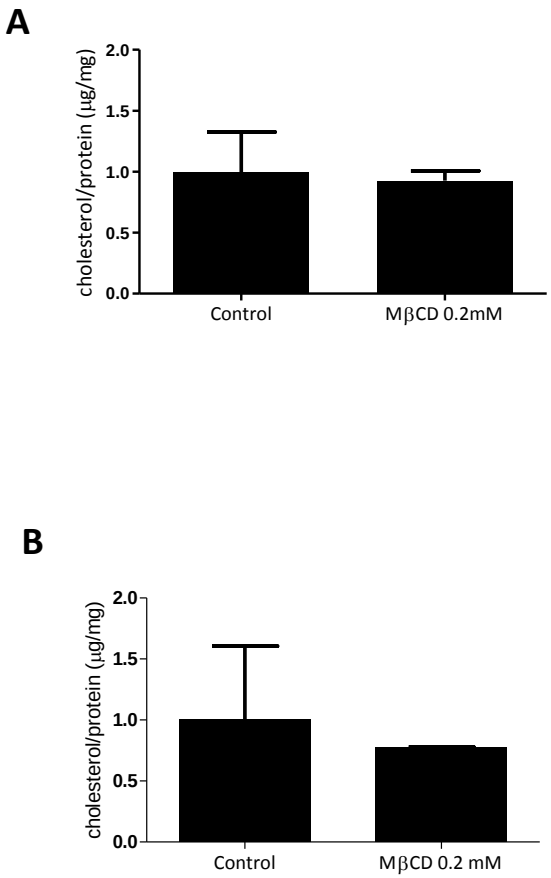


FIGURE 1 – Lower concentration of MβCD deplets cholesterol from cells. (A), (B) Cholesterol depletion in HEL and THP-1 cell line, respectively. Cholesterol levels were measured using Amplex© Red Cholesterol Kit in relation to protein levels. n=3

Increasing MβCD concentrations were then used in order to decrease intracellular cholesterol content. In figure 2 we can observe that using MβCD concentration from 1mM to 10 mM results in a more efficient cholesterol depletion than with 0.2 mM in HEL cell line (Figure 2A). In THP-1 cell line (Figure 2B) there is a trend to decrease of cholesterol levels with 1mM, but it is not statistically significant. However 2mM MβCD depletes cholesterol significantly from THP-1 cells.

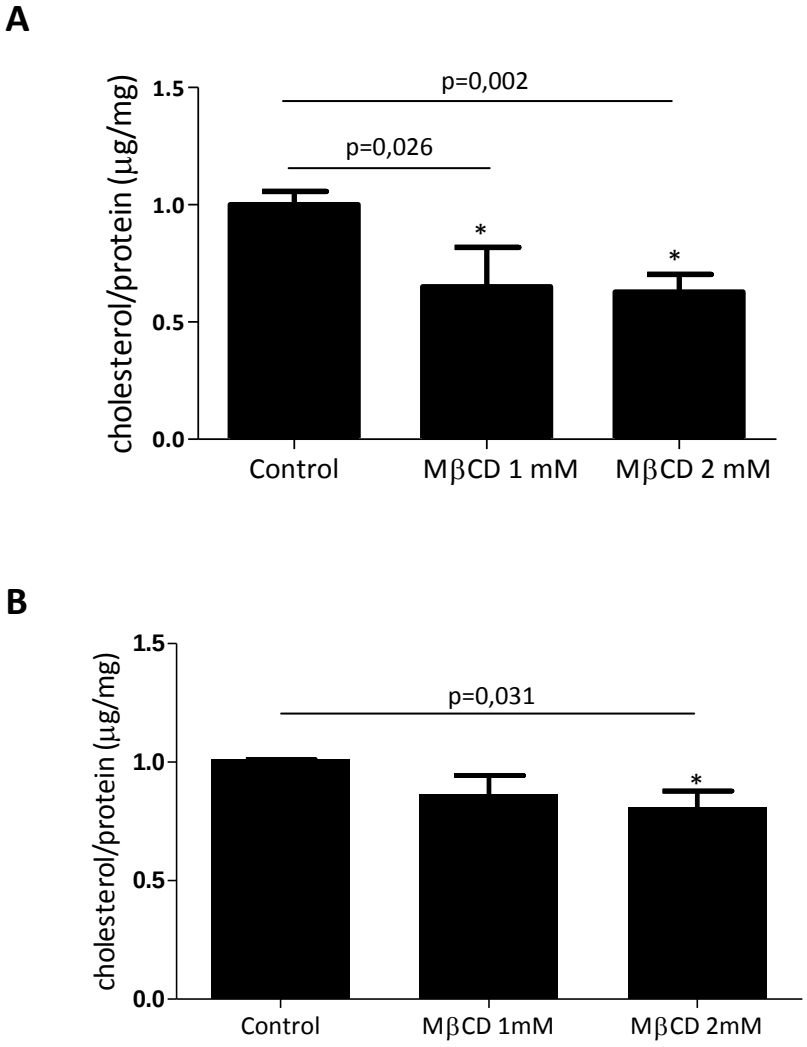


FIGURE 2 – 2mM of MβCD partially depletes cholesterol in both cell lines. (**A),** (**B)** Cholesterol depletion in HEL and THP-1 cell line, respectively. Cholesterol levels were measured using Amplex© Red Cholesterol Kit (Invitrogen) in relation to protein levels. n=3

With the intent of experimenting even higher concentrations of MβCD, to maximize cholesterol removal, 5mM and 10 mM concentrations were tested also in both cell lines. It was verified that 5mM of MβCD reduces cholesterol levels by half in relation to control and that 10 mM of MβCD reduces even more, however there is no significant difference in depleted cholesterol levels between these two conditions but a further decrease in 10 mM is seen, but this is not statistically significant in relation to 5mM. Both concentrations decrease cholesterol in relation to control samples.

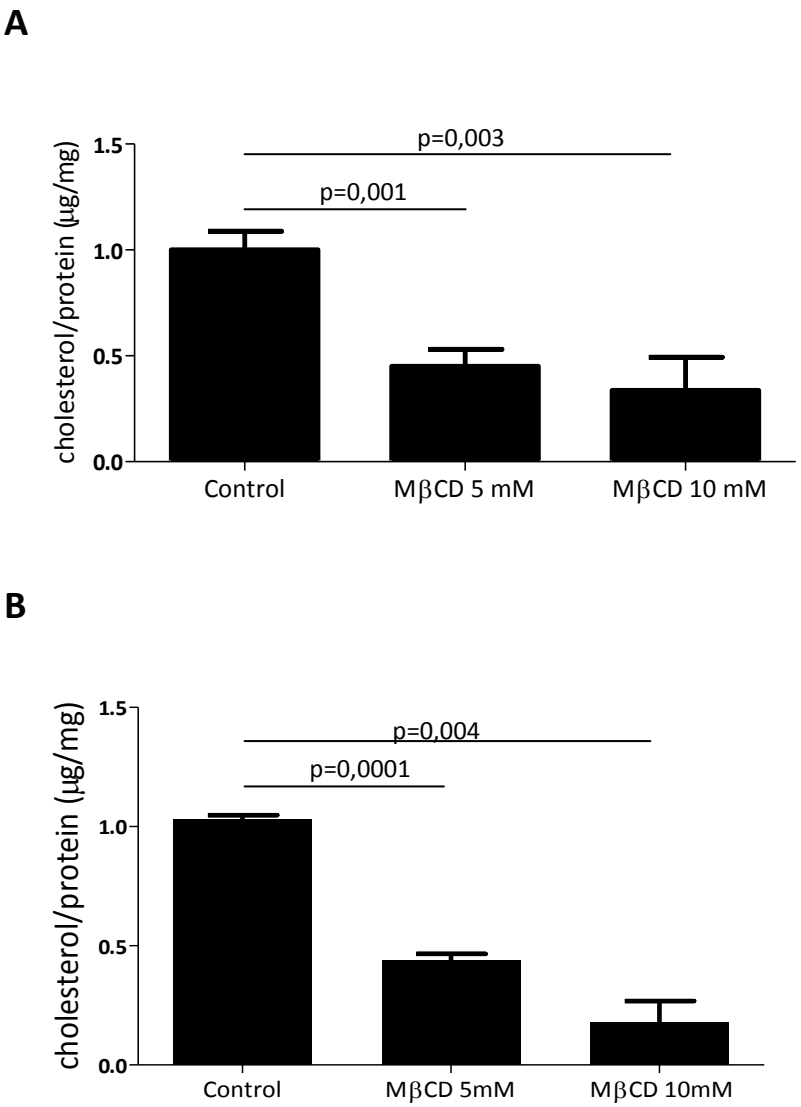


FIGURE 3 – Depletion of cellular cholesterol with MβCD is concentration dependent. (**A),** (**B)** Cholesterol depletion in HEL and THP-1 cell line, respectively. Cholesterol levels were measured using Amplex© Red Cholesterol Kit in relation to protein levels. n=3

These results confirm that MβCD depletes cholesterol from cells efficiency in a concentration-dependent manner.

Enrichment of cellular membrane with cholesterol through MβCD + cholesterol complexes in THP-1 and HEL cell lines

MβCD is also recognized as a vehicle for hydrophobic molecules, forming inclusion complexes with several compounds (Del Valle *et al.* 2004) and has higher affinity for encapsulating sterols, in particular cholesterol (Kilsdonk *et al.* 1995). Since, as in addition to deplete cholesterol in cells we also aimed at increasing intracellular cholesterol content, we used MβCD has a vehicle to delivery cholesterol to cellular membranes in HEL and THP-1 cell lines. By using 0.2 mM of MβCD + cholesterol complexes (0.02 mM of cholesterol) we were able to increase cholesterol content by around two fold in both cell lines. (Figure 4A and B).

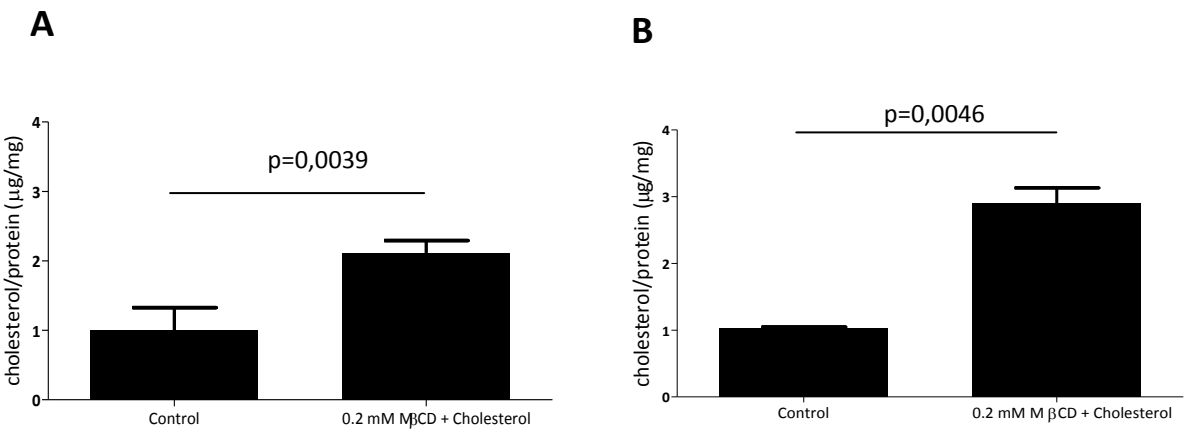


FIGURE 4 – Low concentrations of MβCD are capable of transport and enrich cellular membrane with cholesterol. (**A),** (**B)** Cholesterol enrichment in HEL and THP-1 cell line, respectively, after 4 hours treatment. n=3

MβCD reduces cell viability

As a molecule or molecular carrier that interferes with the normal lipid equilibrium of cellular membranes by depleting or adding cholesterol, it is important to analyze the effects that MβCD and MBCD+cholesterol complexes have in cell viability.

For this, cells were exposed to the different concentrations of MβCD previously tested for different periods of time (4, 10 and 24 hours) and it was observed that cell viability is reduced with increasing concentration of MβCD and long times of exposure (Figure 5). From 1mM to 10 mM a proportional decrease in percentage of viable cells is observed throughout time, except for the 0,2mM MβCD condition. This viability reduction is demonstrated in both HEL (Figure 5A) and THP-1 cells (Figure 5B).

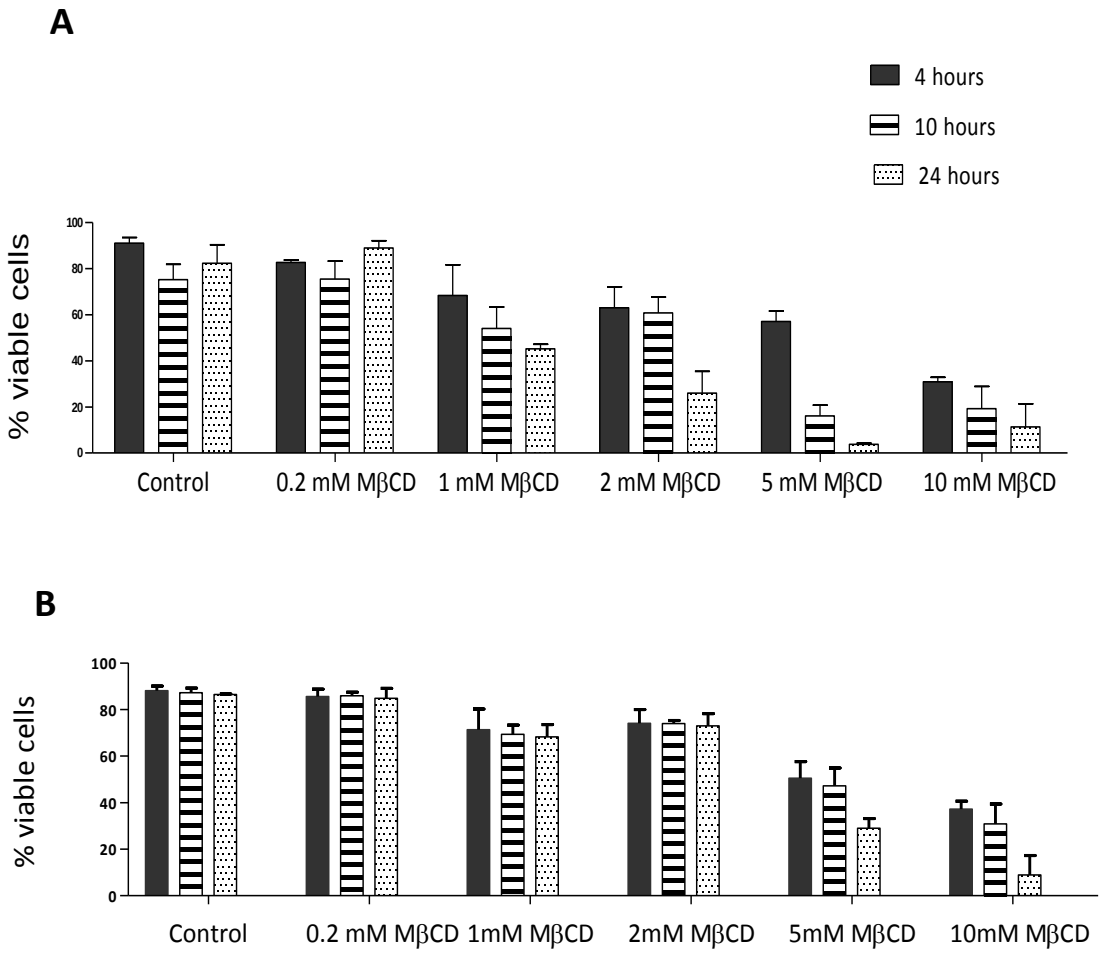


FIGURE 5 - Higher concentrations of MβCD and longtime exposures reduces cell viability. (A) HEL cell line and **(B)** THP-1 cell line percentage of viable cells with different MβCD concentrations. Cell viability was assessed by Tripan Blue Exclusion. n=3

0.2 mM MβCD + cholesterol complexes minimally affect cell viability

Next we analyzed cell viability with 0.2 mM MβCD + cholesterol complexes and observed around 80% of viable cells in both HEL and THP-1 cell line after 4, 10 and 24 hours of exposure. Noticeably, there is also a small decrease in cell viability after 10 and 24 hours in both cell lines and also in the control.

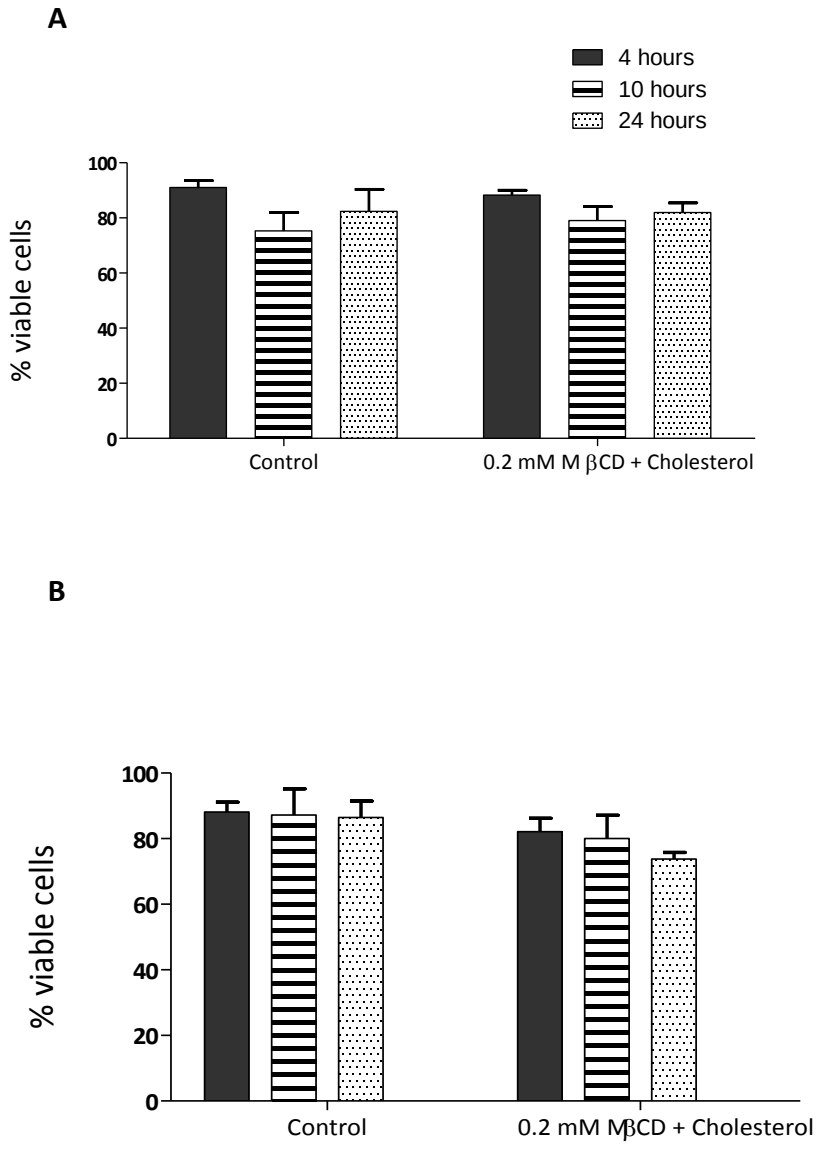


FIGURE 6 – Complexes of 0.2mM MβCD and cholesterol do not interfere with cell viability . (A) HEL cell line and **(B)** THP-1 cell line percentage of viable cells after treatment with 0.2 mM MβCD + 0.02mM Cholesterol complex during different exposure periods. Cell viability was assessed by Tripan Blue Exclusion. n=3

The ribosomal subunit 18 S is an adequate housekeeping gene to use as internal control to quantify mRNA by quantitative PCR in AML cell lines that have been exposed to MβCD and MBCD+ cholesterol complexes

To access reliable and accurate gene expression data by quantitative PCR (qPCR) it is essential to do the gene expression normalization against internal control genes taken as references, this are usually housekeeping genes. Good internal controls are genes which expression does not undergo variations due to different cell treatments, cellular environment conditions, drugs or biological factors. Since the treatment of cells with MβCD was the basis of our experiments we wanted to check among 3 initial selected housekeeping genes, which would remain more stable upon treatment. The genes selected were 18S, BMG1 and HPTR, as they are regularly used in the lab for AML cells. Gene expression was analyzed comparing control and HEL cells treated with 0.2 mM MβCD and 0.2 mM MβCD + Cholesterol, and it was observed that 18S has the more stable amplification curve and furthermore, it amplified during the first few cycles of the qPCR reaction.

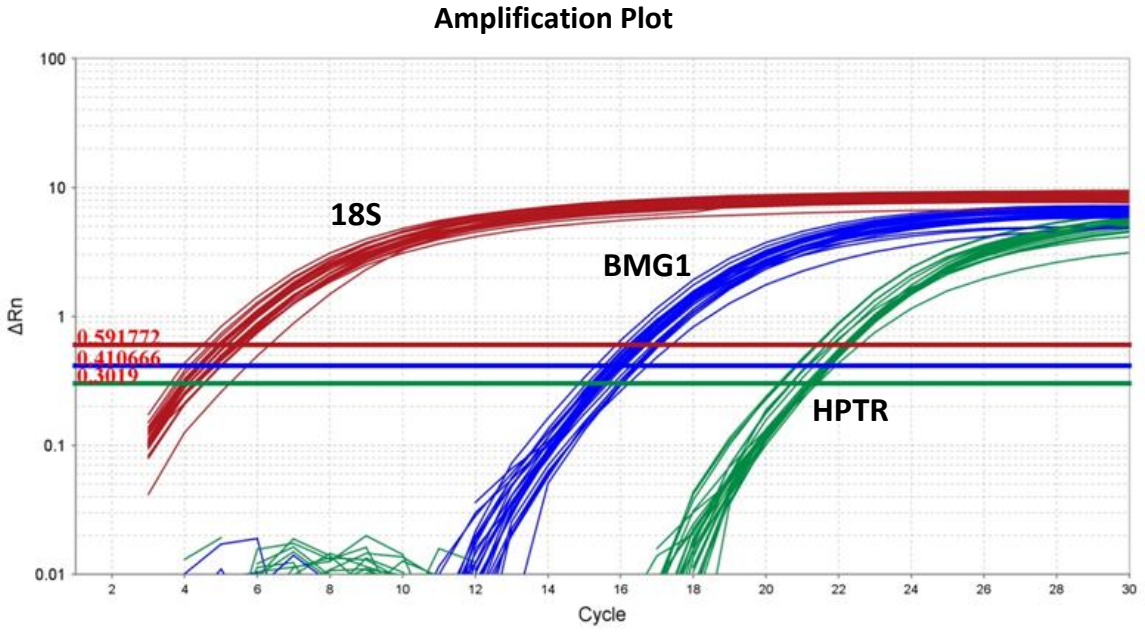
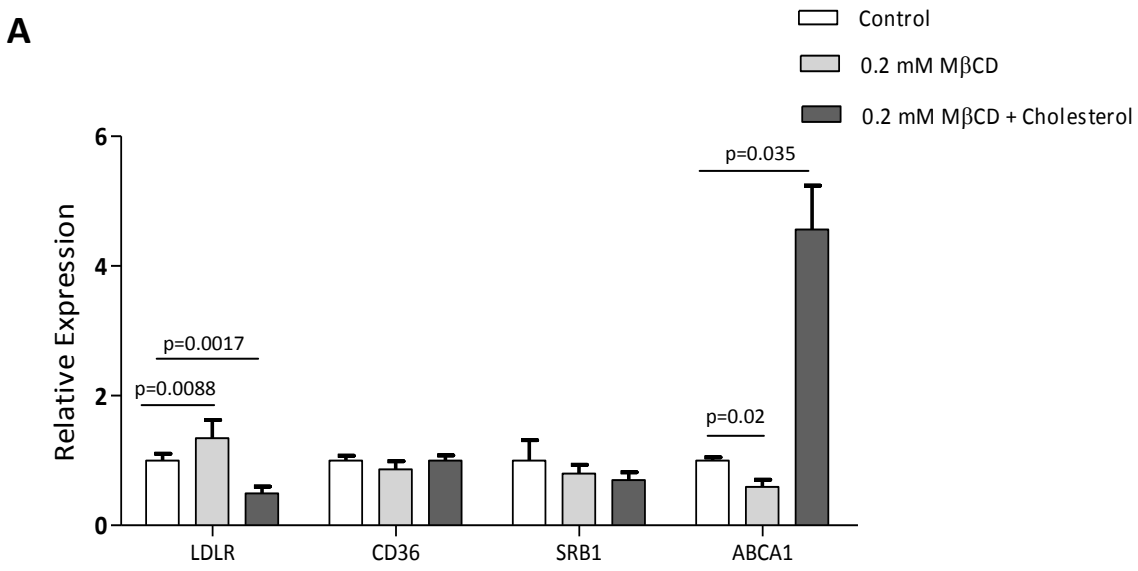


FIGURE 7 – 18S has the more stable amplification curve in relation to the other genes analyzed in HEL cell line. Amplification plot image was get and adapted from ViiA™ 7 Real-Time PCR software. 18S corresponds to red curve, BMG1 to blue curve and HPTR to green curve. Each curve line from each amplification curve corresponds to one sample, showing more stable amplification curves with 18S.

Cholesterol depletion and enrichment modulate the expression of several cholesterol homeostasis-related genes

Several genes regulate intracellular cholesterol homeostasis. To assess if cells are sensing cholesterol changes, we analysed by qPCR the expression of several cholesterol related genes including cholesterol receptors and transporters.

HEL and THP-1 cell lines were subjected to treatments with MβCD and MβCD + cholesterol complexes and, as a first approach, the 0.2 mM concentration was used to deplete and to enrich cholesterol, respectively. As it can be seen in figure 8, in relation to control, LDLR mRNA expression increases when MβCD is added to cells and decreases in MβCD + cholesterol conditions. This is true for both cell lines used. In another hand modified LDL receptors such as CD36 and SRB1 mRNA expression show no differences between the different conditions for the HEL cell line, while in THP-1 cells CD36 is increased in both conditions. Finally, ABCA1 mRNA expression is significantly decreased in cells treated with MβCD and significantly increased in cells treated with MβCD + cholesterol, in both cell lines.



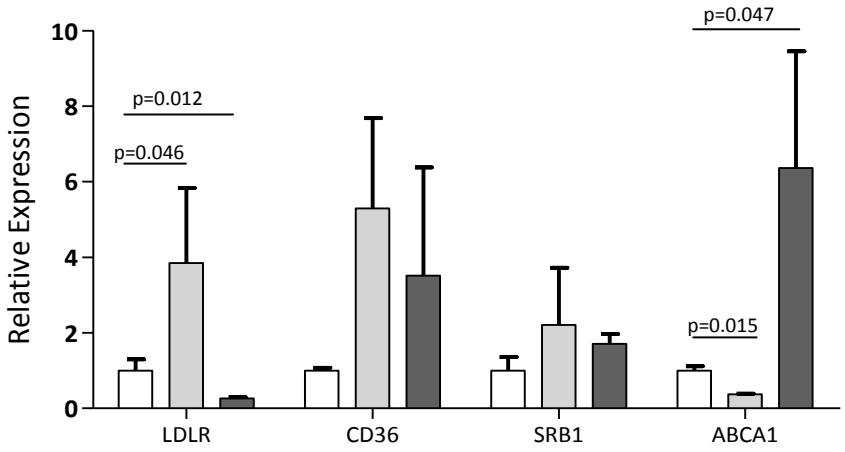


FIGURE 8 – Cells are sensible to changes in membrane cellular cholesterol. qPCR for several cholesterol modulator genes in HEL cell line **(A)** and THP-1 cell line **(B)** genes after 4 hours treatment with 0.2 mM MβCD and 0.2 mM MβCD + 0.02 mM cholesterol complexes. mRNA expression is relative to mRNA expression of control samples. n=3

These observations suggest that cells are sensible to the alterations caused by 0.2 mM MβCD, this concentration depletes low levels of cholesterol from cells but it is enough to induce alterations in cholesterol homeostasis-related genes at the mRNA level. In addition, we confirm that a four-hour treatment with the same concentration of MβCD + cholesterol complexes is able to increase the cholesterol content of cells as alterations at the level of mRNA of receptors associated with cholesterol uptake and efflux are altered.

Bcl-2 mRNA expression is not associated with intracellular cholesterol levels

The Bcl-2 family of proteins has been related to AML, mainly by acting as an anti-apoptotic factor leading to cell survival and resistance to chemotherapy. We hypothesized that the molecular mechanisms involved in Bcl-2 expression in AML could be modulated by cellular cholesterol levels and therefore analyzed the

expression of Bcl-2 mRNA by qPCR. As it can be seen in figure 9A, Bcl-2 mRNA expression in both HEL and THP-1 cells is not change when cholesterol is either depleted or enriched.

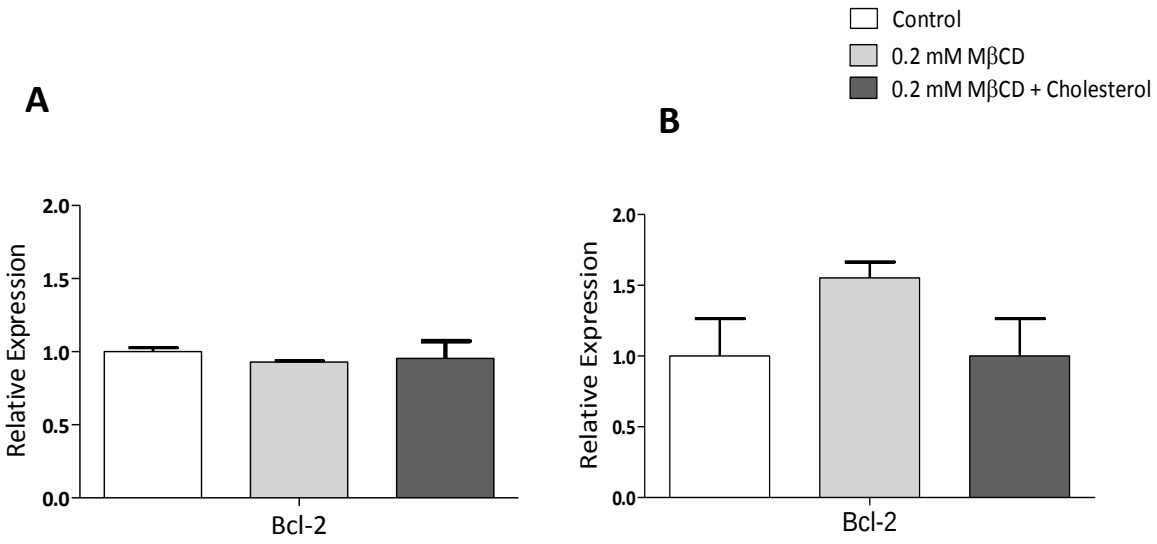


FIGURE 9 – Bcl-2 expression is not regulated by cellular cholesterol levels but it seems to increase in THP-1 cells treated with MβCD. qPCR for **(A)** HEL and **(B)** THP-1 cell lines to determine Bcl-2 mRNA expression after 4 hours treatment with 0.2 mM MβCD and 0.2 mM MβCD + cholesterol complexes. mRNA expression is relative to mRNA expression of control samples. n=3

VEGF-C mRNA expression increases in cells treated with 0.2 mM of MβCD

VEGF-C, acting through the VEGFR-3, is involved in signaling pathways that lead to cell proliferation and AML progression. Here we investigated at mRNA level if its endogenous expression is related with membrane cholesterol levels, analyzing VEGF-C expression in lower and higher cholesterol conditions by qPCR in both HEL and THP-1 cell lines. Interestingly, it was observed that VEGF-C mRNA expression is increased in relation to control when HEL cells are treated with 0.2 mM MβCD (Figure 10). In another hand no significant differences are observed when cells are treated with MβCD + cholesterol complexes. In THP-1 (Figure 10B) VEGF-C expression is increased in both high and low cholesterol conditions when compared to control.

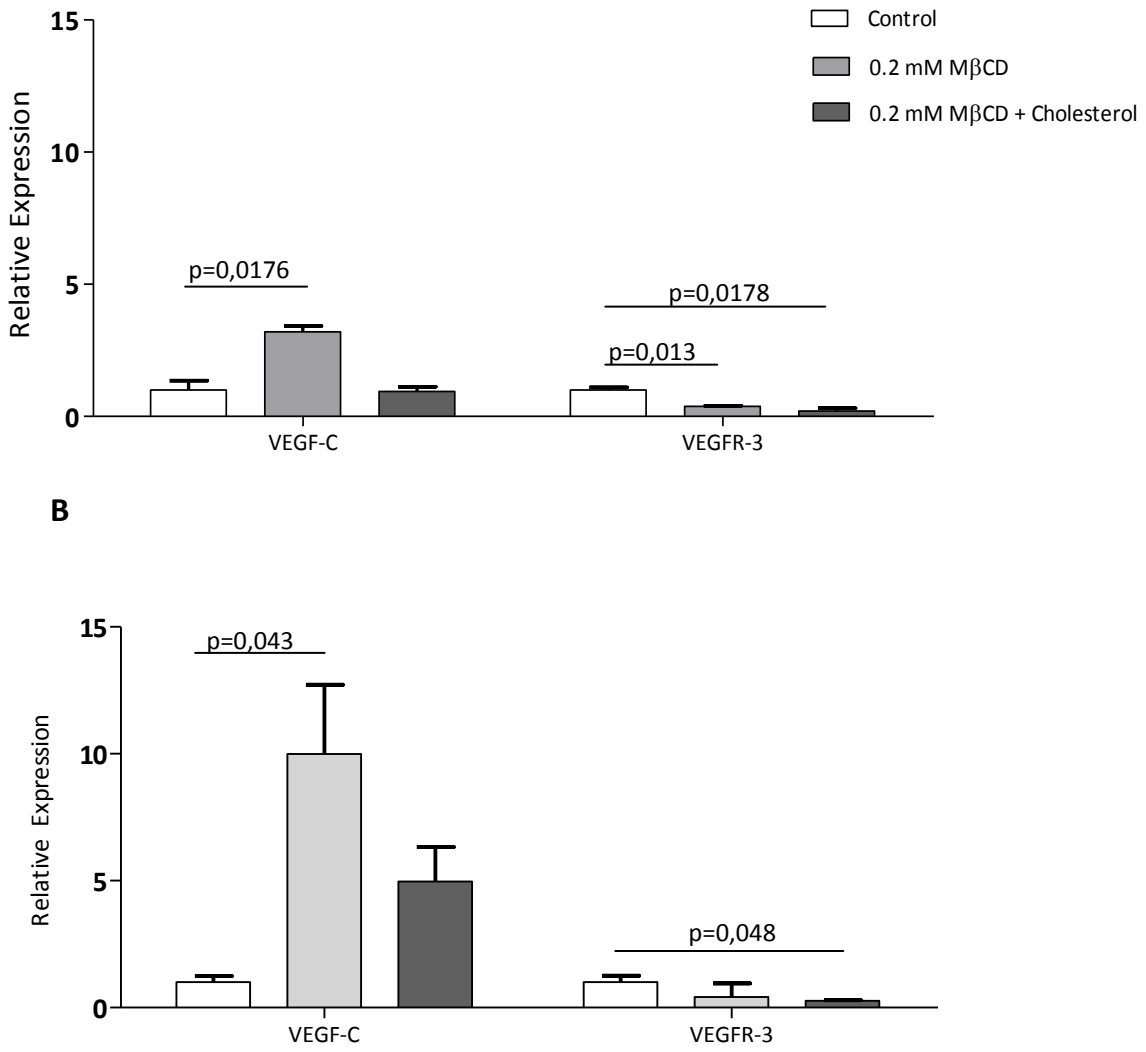


FIGURE 10 – VEGF-C is increased in low cellular cholesterol levels and VEGFR-3 is decreased in both low and high cellular cholesterol levels. qPCR for HEL cell line **(A)** and THP-1 cell line **(B)** to determine mRNA expression of VEGF-C and VEGFR-3 after 4 hours treatment with 0.2 mM MβCD and 0.2 mM MβCD + 0.02 mM cholesterol complexes. mRNA expression is relative to mRNA expression of control samples. n=3

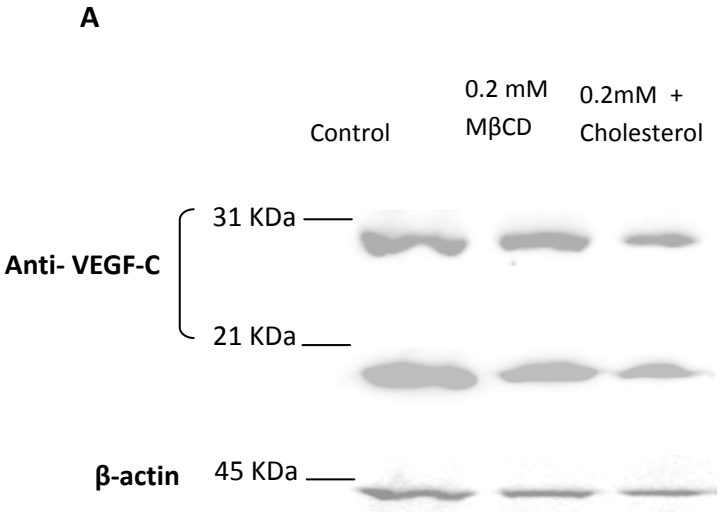
VEGFR-3 mRNA expression decreases in both conditions of depletion and enrichment of cellular cholesterol with MβCD

In addition to VEGF-C, the expression of VEGFR-3 mRNA was also analysed. In the two cell lines we could see that the expression of the receptor decreases in both poor and rich cholesterol conditions in HEL cells (Figure 10A). In the THP-1 cell line VEGFR-3

mRNA expression is decreased in high cholesterol levels and this is statistically significant whereas the decrease observed with low cholesterol levels it is not (Figure 10B). Taken together these data suggest that VEGF-C expression at the mRNA level increases when cells have low concentrations of cholesterol while it is not clear so far whether the changes at the VEGFR-3 mRNA levels are related to the alterations at cholesterol content levels of cells, or to a non-cholesterol-associated effect of MβCD.

VEGF-C protein levels increase with lower intracellular cholesterol in one of the two AML cell lines studied

To see whether the alterations we observe at the mRNA level are also observed at the protein level we investigated VEGF-C protein expression by Western Blot. The antibody used to detect VEGF-C is human specific and it may detect 3 different proteolytic processed variants but in our hands only two of them were detected consistently.



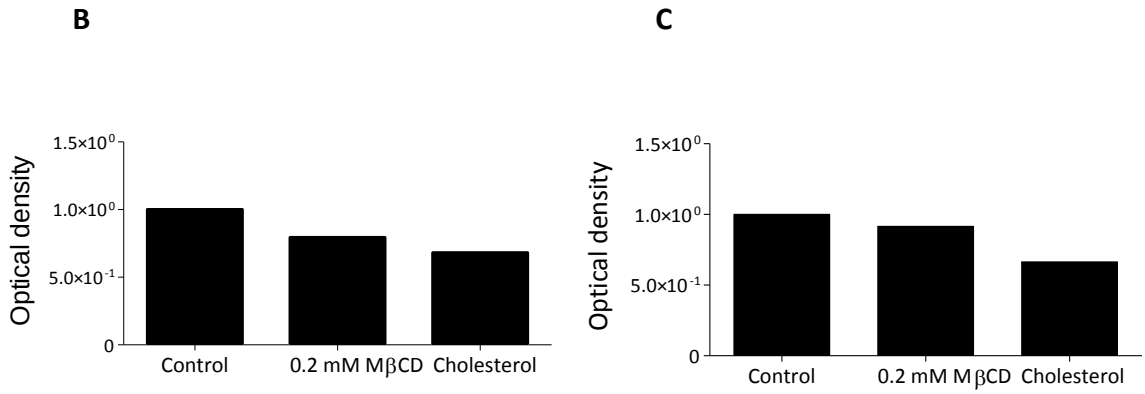
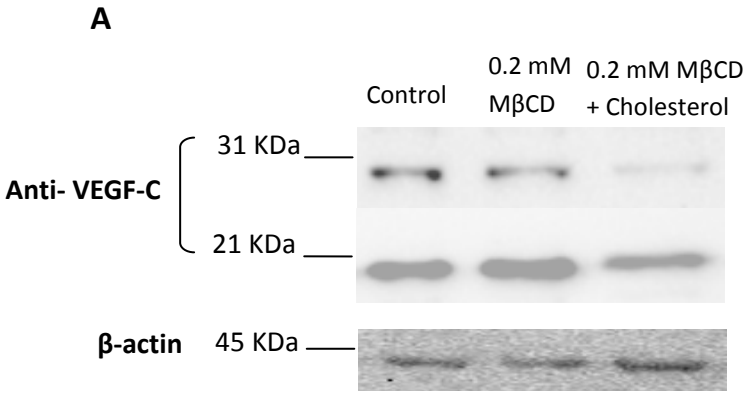


FIGURE 11 – VEGF-C protein levels tend to decrease when treated with 0.2 mM MβCD and 0.2 mM MβCD + cholesterol complexes in HEL cells. (A) Western-Blotting for VEGF-C detection in HEL cells. **(B)** Optical density for 21 KDa VEGF-C variant form (mature form) and for **(C)** 31 KDa VEGF-C variant form. n=1

In figure 11 and 12, the bands represented correspond to different VEGF-C processed forms originated during proteolytic process, the 21 KDa band may correspond to the mature form of VEGF-C that is secreted by cells and the 31 KDa band may correspond to the processing form just prior to the fully mature.

In the HEL cell line (Figure 10A) VEGF-C variants show a tendency to decrease in both lower and higher cholesterol conditions. In the THP-1 cell line (Figure 12) the VEGF-C mature form show a tendency to increase with lower cellular cholesterol levels and tend to decrease in higher cholesterol conditions, however this observed trend has no statistic significance. 31KDa VEGF-C variant is decreased in higher cholesterol conditions, with statistical significance in THP-1 cells.



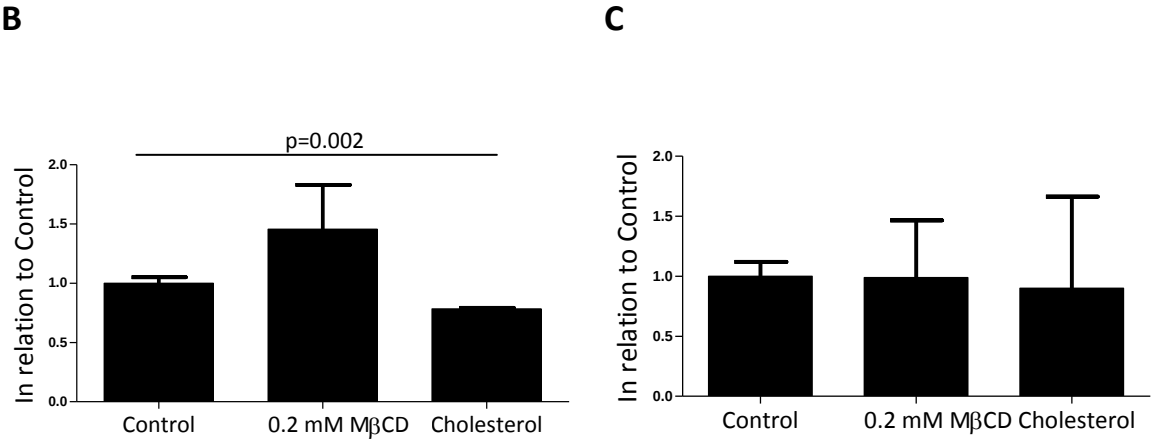


FIGURE 12 – VEGF-C protein levels increase upon cholesterol depletion and decrease with cholesterol enrichment in THP-1 cells. (A) Western-blotting for VEGF-C detection in THP-1 cells. **(B)** Optical density for 31 KDa VEGF-C variant (mature form) and for **(C)** 21 KDa VEGF-C variant. n=3

VEGFR-3 levels at the cellular membrane are not affected by changes in intracellular cholesterol

VEGFR-3 is a receptor located at the cellular membrane, to look at the expression of VEGFR-3 at the plasma membrane, we used a specific antibody against VEGFR-3 and flow cytometry analysis. We did this for the THP-1 cell line.

We were able to observe that the entire population of THP-1 cells expresses VEGFR-3 and that both in high and low cholesterol conditions cells express equivalent amount of the receptor at the membranes (Figure 13A). As shown by the plots in Figure 13B, looking at mean intensity fluorescence values in relation to control it is clear that VEGFR-3 remains the same expression level despite the alterations of cholesterol content.

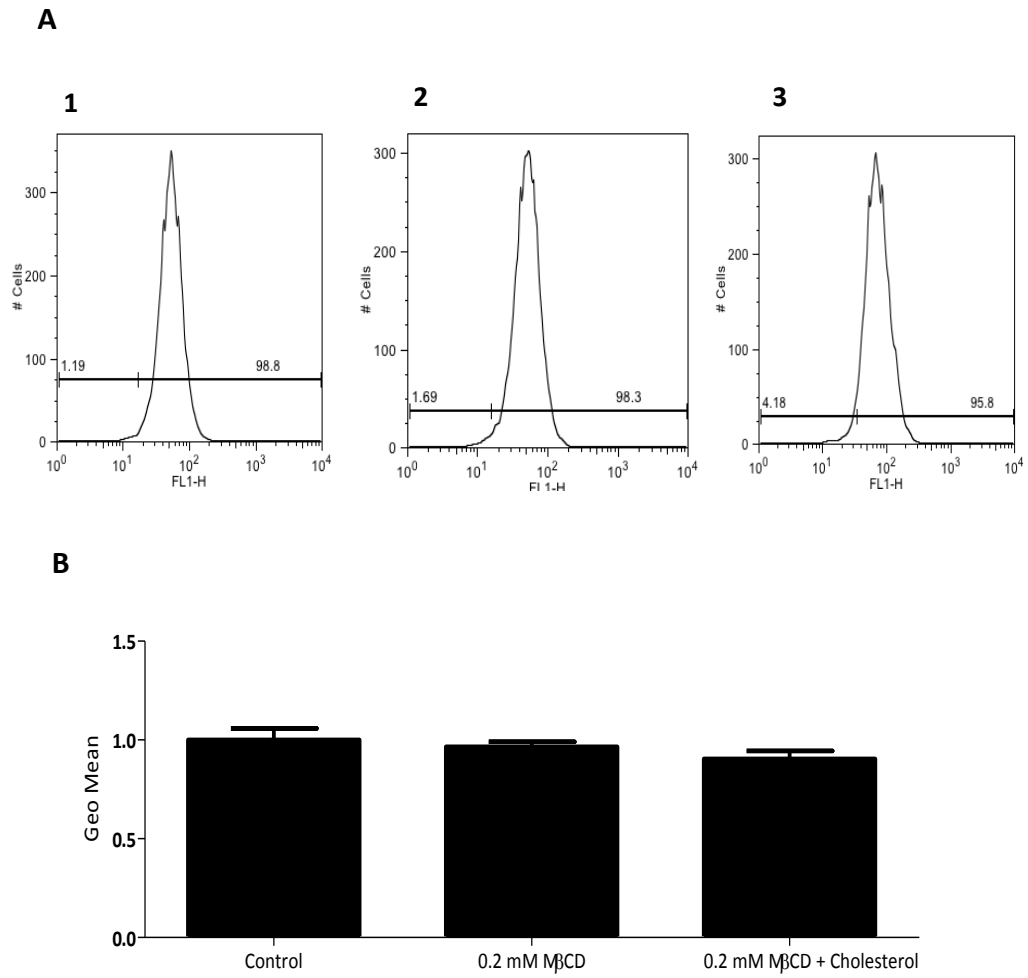


FIGURE 13 – VEGFR-3 expression in cellular membrane does not undergo significant alterations with different cholesterol levels. (A) Representative histograms with percentage of positive cells, for control samples (A1) and each cholesterol condition (A2-3) **(B)** Geometric mean values from flow cytometry correspondent to mean intensity of fluorescence in each condition. n=3

The expression of VEGF-C and VEGR-3 is much lower in AML than in endothelial cells

With the intent of understanding how much VEGF-C and VEGFR-3 are expressed in AML cells we analyzed the expression of this factors in THP-1 cell line and compared it with levels of expression in primary endothelial cells, namely in Human Umbilical Endothelial Cells (HUVEC).

As it can be seen in figure 14 we verify that leukemic cell express minimal amounts of VEGF-C and VEGFR-3 in comparison with HUVEC. HUVEC express higher levels of VEGFR-3 mRNA in comparison with VEGF-C.

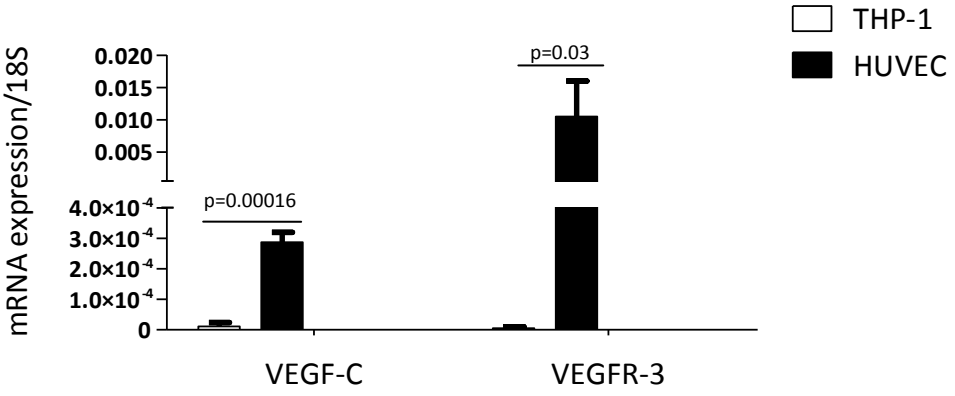


FIGURE 14 – Endothelial cells express higher levels of VEGFR-3. qPCR for VEGF-C and VEGFR-3 mRNA expression in leukemic and endothelial cells. mRNA expression is relative to internal control gene expression 18S. n=3

Bcl-2 mRNA levels are higher in acute leukemia cells

As referred previously high expression of Bcl-2 is related with malignant cells, so next we compare Bcl-2 mRNA expression levels in THP-1 cells with HUVEC by RQ-PCR.

Bcl-2 mRNA expression is significantly higher in AML cells comparing with HUVEC, reinforcing the relevant role of Bcl-2 as an anti apoptotic factor in leukemic cell survival.

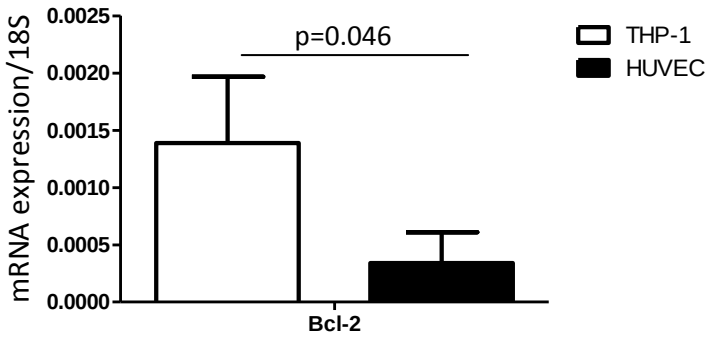


FIGURE 14 – AML cells express higher Bcl-2 mRNA. qPCR for Bcl-2 mRNA expression in leukemic and endothelial cells. mRNA expression is relative to internal control gene expression 18S. n=3

REFERENCES

Del Valle, E. M. M. Cyclodextrins and their uses: a review. *Process Biochemistry* **39**, 1033–1046 (2004)

Kilsdonk, E. P. *et al.* Cellular cholesterol efflux mediated by cyclodextrins. *The Journal of biological chemistry* **270**, 17250–6 (1995)

DISUSSION AND FUTURE PERSPECTIVES

The purpose of this work was to address if cholesterol is a regulator of the expression of VEGF-C and its receptor VEGFR-3, in AML cells.

Cholesterol is an important component of cellular membranes and plays a fundamental role in cell signaling (Wymann *et. al* 2008). Presently its role in hematological malignancies is not completely understood neither is its role in tumor angiogenesis mechanisms that contribute to disease aggressiveness; nevertheless, many studies including some from our Lab point towards cellular cholesterol and plasma cholesterol involvement in hematological cancer progression. In the present work we focused our attention on how may cellular cholesterol levels contribute to regulate the production of VEGF-C by leukemic cells.

Either alone or in conjugation with cholesterol MβCD has been extensively used to study cellular modifications cause by the disruption of normal lipid homeostasis of the membrane. It is a well-accepted tool to modify the structure and function of the plasma membrane (Upadhyay *et al.* 2006) but it is important to consider the impact that MβCD and MβCD + cholesterol complexes have in cell viability. Because of this, for each cell type different times of exposure and different concentrations should be tested. Some reports mention that under conditions of very low cholesterol, after long exposures to MβCD or high concentrations, cells typically lose their morphology which may promote the loss of cell viability (Zidovetzki *et. al* 2007).

First we investigated how MβCD impacted cell viability in cells and the patterns of cholesterol depletion and enrichment in an effort to define our experiments based on an approach that positively correlates cell viability with efficient cholesterol alterations in cellular membrane. Both AML cell lines used presented cell susceptibility to MβCD concentrations above 1mM and cells treated with higher concentrations show a faster viability decrease over time (Figure 5). This confirms that cell cholesterol depletion with this molecule has to be adjusted in terms of exposure and concentration, otherwise cell viability is impaired. As 0.2 mM showed to keep cell viability almost constant in time, it was our first choice to deplete cholesterol. MβCD + cholesterol complexes were also used at a low MβCD concentrarion (0.2mM) which can explain the maintainance of cell viability for long periods of time (Figure 6). Cholesterol concentration of 0.02 mM was efficient in enriching cells with cholesterol, which together with an ideal concentration of MβCD proved to be a good

mechanism for cholesterol delivery to cells (Figure 4). Based on this data and previously identical studies (Casalou *et al.* 2011), 4 hours of exposure was chosen as the most suitable time point for both enrichment and depletion of the cellular cholesterol.

Changes at the expression levels of a set of genes that regulate cholesterol homeostasis are a good marker to address whether cholesterol content changes inside cells. LDLR and ABCA1 are key regulators of cellular cholesterol influx and efflux, respectively. When cells detect low levels of cholesterol LDLR mRNA expression is activated in order to increase the efficiency of cholesterol uptake and when there is cholesterol excess ABCA1 mRNA expression is activated in order to increase cholesterol exportation at the membrane. Another complex key steps occur in regulation of cholesterol homeostasis but here our goal was just to verify if cells are responding as expected to induced cholesterol disruptions. It was seen that with 4 hours exposure to the lower concentration of 0.2 mM MβCD, cells respond significantly (Figure 8). We assumed then that other changes related with cholesterol modulation should be also detected, and 0.2mM MβCD was the concentration chosen to perform our experiments.

One mechanism by which VEGF-C leads to tumor cell survival and resistance to chemotherapy is by increasing the expression of the anti-apoptotic gene Bcl-2 *(No et al*. 1999) (Dias *et al.* 2002).

Here we wanted to assess whether cholesterol was required for the VEGF-C induced expression of Bcl-2 in leukemic cells. No differences in Bcl-2 mRNA expression were however found when intracellular cholesterol was either depleted or enriched. These results suggest that cholesterol levels don't affect the expression of Bcl-2 at the mRNA level (Figure 9). MβCD efficiently removes cholesterol from the plasma membrane and it may also affect cholesterol levels in intracellular membranes (Zidovetzki *et al.* 2007). The anti-apoptotic Bcl-2 protein is embedded in the nuclear envelope, endoplasmatic reticulum and outer mitochondrial membrane (Youle *et al.* 2008) and whether MβCD mediated cholesterol changes can achieve these sub-cellular locations in order to promote Bcl-2 activity alteration was not analyzed. Assuming that the cell function is affected by the variation of cholesterol levels Bcl-2 modulation at mRNA level seems to be independent of cholesterol levels. In one cell line we did detect a tendency for Bcl-2 to be increased in the presence of MβCD,

however this was not stastistically significant (Figure 9B). This may suggest some relationship between cholesterol depletion and levels of Bcl-2 expression but either increasing the number of samples or the exposure of cells to MβCD will be required to confirm it. We tried to assess Bcl-2 protein levels by western blot to confirm these data with no success, maybe due to problems with the chosen anti-Bcl-2 antibody (data not shown).

Some subsets of acute myeloid leukemias express VEGFR-3 and it is assumed that VEGF-C signaling through this receptor leads to leukemic cell proliferation and survival. It was reported that VEGFR-1 expression and signaling is linked to cholesterol levels in acute leukemia (Casalou *et al.* 2011) highlighting the importance of cellular cholesterol levels in regulating the VEGF family of proteins, namely in acute leukemia. In the context of our study, we assessed if cellular cholesterol levels in the plasmatic membrane are modulating VEGFR-3 expression in AML by depletion and enrichment of cholesterol. First, by qPCR a decrease in VEGFR-3 mRNA expression was verified with both low and high cholesterol conditions in both cell lines that were used with a residual trend to be more sub-expressed in cholesterol enriched cellular environment (Figure 10). The fact that increase and decrease of intracellular cholesterol content give the same outcome, suggest that either VEGFR-3 expression is very sensitive to cholesterol changes or the effect seen is not related to cholesterol but to any other effect of MβCD. The observations seen at the VEGFR-3 mRNA expression levels although do not correspond to our flow cytometry results which points to the expression maintenance of VEGFR-3 in both cholesterol conditions although only THP-1 cell line has been tested for flow cytometry (Figure 13). The discrepancy between the data obtained at the mRNA and protein level could be explained by a big stability of the VEGFR-3 receptor protein. As we chose the same time point to address both mRNA and protein expression it is possible that the changes detected at the RNA level had no time to change at the protein level.

VEGFR-1 is known to be located at caveolae cholesterol rich domains in plasma membrane in leukemic cells (Casalou *et al.* 2011) and the presence of VEGFR-3 in these caveolae domains has also been reported, but in endothelial cells membrane (Galvagni *et al.* 2007), which suggest that VEGFR-3 may also localize at caveolae in leukemic cells. Caveolae maintenance is modulated by cholesterol levels in membrane and its disruption may affect receptor function and downstream signaling (Frank *et al.* 2006). It is important as a future

approach to address if VEGFR-3 is also located at caveolae in acute leukemia cells because cholesterol disruption in lipid rafts could lead to impaired expression of this receptor in case of poor amounts or in case of higher amounts of cholesterol, which could suggest a need for a strong balance in cholesterol levels for gene expression of VEGFR-3.

VEGF-C undergoes proteolytic processing in cells forming a set of processed variants that are secreted. In this work we were able to detect by western blot two VEGF-C forms of different sizes. Both cell lines showed a band of 31 KDa, that may correspond to the processed form prior to the mature form, and a band of 21 KDa that may correspond to the mature form (Joukov *et al.* 1997). Depleting cholesterol from cells with MβCD led to an increase of VEGF-C mRNA levels which was also observed at protein level by Western Blot in both cells lines (Figure 11 and 12). Enrichment of cholesterol content with MβCD + cholesterol complexes reduces VEGF-C protein levels in both cell lines, with greater significance in THP-1 cell line. This is not observed at mRNA levels, where VEGF-C mRNA expression does not change in HEL cell line. In THP-1 cell line there is a tendency to VEGF-C mRNA expression increase in higher cholesterol levels however this is not statistically significant. Processing mechanisms such as post translational modifications and proteolytic processing may be behind these differences between VEGF-C mRNA and protein levels, however it seems clear that depletion and enrichment of cholesterol levels with MβCD and MβCD cholesterol complexes, respectively, are somehow interfering with VEGF-C production in AML cells.

In order to investigate the release of VEGF-C, AML cells were treat with 0.2 mM MβCD and 0.2 mM + cholesterol complexes during four hours and ELISA assay were performed but due to very low amounts of VEGF-C in cell supernatants no consistent results were achieved (data not shown), a larger time point and superior cell concentration should be used in future experiments.

Increased VEGF-C production is evident when cells are treated with 0.2 mM MβCD which could be related with the decrease in VEGFR-3 mRNA levels. It would be interesting in the future to investigate the expression levels of VEGF-D, another VEGF-C receptor. It should be noted that all this observations were made with a four hours time point which does not invalidate that the low production of VEGFR-3 mRNA will not be reflected later at the

membrane level, the same for VEGF-C, whose increases at protein level may be manifested more significantly at later time points.

As a general conclusion our preliminary results suggest that enrichment and depletion of cholesterol with 0.2 mM MβCD regulate VEGF-C and VEGFR-3 expression. This work opens new questions that need to be explored, namely if the higher production of VEGF-C showed here in cholesterol depletion conditions is involved with higher proliferation and survival of AML cells, and whether it may be related with an autocrine loop that promotes disease promotion. Functional assays with several chemotherapeutic drugs may also be an useful approach to test cell resistance and if it is linked to the expression changes verified. It is also important to evaluate the effects of the method used here to induce cellular cholesterol changes, since MβCD can be interfering with other cellular functions that subsequently change VEGF-C and VEGFR-3 expression. Addition of LDL to cell medium may mimic in a better way the increase of cellular cholesterol and all the cellular responses related to that. Similarly the use of lipoprotein depletion media or HDL may be an alternative way for reducing cellular cholesterol levels.

Taken together, these results do not fully support our initial hypothesis suggesting that higher cholesterol levels might be related with higher expression of VEGF-C by AML cells, but open new possibilities to be explored in order to understand which mechanisms are behind the observations made here and whether these are involved in AML pathogenesis.

REFERENCES

Wymann, M. P. & Schneiter, R. Lipid signalling in disease. *Nature reviews. Molecular cell biology* **9**, 162–76 (2008)

Upadhyay, A. K. *et al.* Methyl-beta-cyclodextrin enhances the susceptibility of human breast cancer cells to carboplatin and 5-fluorouracil: involvement of Akt, NF-kappaB and Bcl-2. *Toxicology and applied pharmacology* **216**, 177–85 (2006)

Zidovetzki, R. & Levitan, I. Use of cyclodextrins to manipulate plasma membrane cholesterol content : Evidence , misconceptions and control strategies. **1768**, 1311–1324 (2007)

Casalou, C. *et al.* Cholesterol regulates VEGFR-1 (FLT-1) expression and signaling in acute leukemia cells. *Molecular cancer research : MCR* **9**, 215–24 (2011)

No, J. E., Christensen, J., Mooney, D. J. & Polverini, P. J. Vascular Endothelial Growth Factor (VEGF) -Mediated Angiogenesis Is Associated with Enhanced Endothelial Cell Survival and Induction of Bcl-2 Expression. **154**, 375–384 (1999)

Dias, S. VEGF165 promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* **99**, 2532–2540 (2002)

Dias, S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. *Blood* **99**, 2179–2184 (2002)

Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews. Molecular cell biology* **9**, 47–59 (2008)

Su, J.-L. *et al.* The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. *Cancer cell* **9**, 209–23 (2006)

Galvagni, F. *et al.* Vascular endothelial growth factor receptor-3 activity is modulated by its association with caveolin-1 on endothelial membrane. *Biochemistry* **46**, 3998–4005 (2007)

Frank, P. G. *et al.* Caveolin-1 and regulation of cellular cholesterol homeostasis. *American journal of physiology. Heart and circulatory physiology* **291**, H677–86 (2006)

Joukov, V. *et al.* Proteolytic processing regulates receptor specificity and activity of VEGF-C. *The EMBO journal* **16**, 3898–911 (1997)

SUPPLEMENTARY INFORMATION

TABLE S1 – qPCR primer sequences

PRIMER	SEQUENCE 5' TO 3'
HUMAN ABCA1 FWD	GATCTGGAGGAGCAGTTAGG
HUMAN ABCA1 RV	GAGTTGAGGTTGGCCTGTTC
HUMAN BCL-2 FWD	TTCTTTGAGTTCGGTGGGGTC
HUMAN BCL-2 RV	TGCATATTTGTTTGGGGCAGG
HUMAN CD36 FWD	GGTGTGGTGATGTTTGTTGC
HUMAN CD36 RV	CAGGGCCTAGGATTTGTTGA
HUMAN LDLR FWD	GATACCAAGGGCGTGAAGAC
HUMAN LDLR RV	AAGCCATGAACAGGATCCAC
HUMAN VEGF-C FWD	GATCTGGAGGAGCAGTTAGG
HUMAN VEGF-C RV	GAGTTGAGGTTGGCCTGTTC
HUMAN VEGFR-3 FWD	ACCAAACAAGGAGCTGGATG
HUMAN VEGFR-3 RV	ATTTCTGGGGCAGGTTCTTT
HUMAN 18S FWD	GCCCTATCAACTTTCGATGGTA
HUMAN 18S RV	CCGGAATCGAACCCTGAATT
HUMAN SRB1 FWD	TCCTCACTTCCTCAACGCTG
HUMAN SRB1 RV	TCCCAGTTTGTCCAATGCC

TABLE S2 – Methyl-β-Cyclodextrin + cholesterol

MβCD	0.5 g
Cholesterol	0.030 g
Isopropanol/choloroform (2:1)	200 µl
1x PBS	5.5 mL

TABLE S3 – Methyl-β-Cyclodextrin

MβCD	0.5 g
Isopropanol/choloroform (2:1)	200 µl
1x PBS	5.5 mL

TABLE S4 – isopropanol/chloroform (2:1)

Isopropanol/Chloroform (2:1)	200 µl
1x PBS	5.5 mL

TABLE S5 – RIPA buffer

Tris 50 mM pH 7.4	500 µl
NaCl 150 mM	300 µl
EDTA 5mM	100 µl
NP-40 1%	100 µl
SDS 0.1%	10 µl
Sodium deoxycholate 0.5%	0.05 g
Na_3VO_4 1 mM	20 µl
1mM NaF10 mM	20 µl
H_2O	10 mL

TABLE S5 – 5x Laemli Buffer

Tris-Base 500 mM	2 mL
Bromophenol Blue	4 mL
Glycerol	2 mL
SD 10%	0.2g

TABLE S6 – 10% poly-acrilamide gel

Acrylamide 30%	2.1 mL
Tris 1.5 M pH 8.8	2.7 mL
10% SDS	80 µl
10% APS	40 µl
TEMED	4 µl
H_2O	3.2 mL

TABLE S7 – stacking Gel

Acrylamide 30%	**0.35 mL**
Tris 0.5M pH 6.8	0.75 mL
SDS 10%	30 µl
APS 10%	30 µl
TEMED	3 µl
H_2O	1.84 mL

TABLE S8 – Running Buffer 10x

Tris 25mM	**0.30 g**
Glycine 190mM	14.2 g
H_2O	1 L (Adjust pH 8.3)

TABLE S9 - Running Buffer 1x

Running Buffer 1x	100 mL
SDS 0.1%	1 mL
H_2O	900 mL

TABLE S10 – Transfer Buffer 10x

Tris 250 mM	30.2 g
1250 mM	93.83 g
H_2O	1 L

TABLE S11 – Transfer Buffer 1x

Transfer Buffer 1x	100mL
Methanol	100 mL
H_2O	800 mL

TABLE S12 – 10x TBS

Trizma HCl	24.23 g
NaCl	80.06 g
H_2O	1 L (AdjustpH 7.6)

Table S12 – 1x TBS-T (Tween-20)

TBS 10x	100 mL
H_2O	900 mL
Tween-20	1 mL

Table S13 – PBS/BSA

PBS 1x	100 mL
BSA 1%	0.5 g