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BONE METABOLISM INVOLVEMENT IN PEDIATRIC ACUTE LYMPHOCYTIC LEUKEMIA: THE RECEPTOR ACTIVATOR NUCLEAR FACTOR KAPPA B LIGAND PATHWAY

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Sommario

Lo studio parte dall'osservazione che i pazienti pediatrici affetti da leucemia, presentano un interessamento osseo ancora prima della diagnosi e che persiste durante e dopo la terapia.
La principale via regolatrice del metabolismo osseo è quella del Receptor Activator Nuclear Factor Kappa B Ligand (RANKL), anche noto come CD254. Questo ligando esiste sia in forma solubile sia di membrana (CD254). Il CD254 è espresso soprattutto dagli Osteoblasti (OBs), le cellule responsabili della formazione dell'osso, mentre il suo recettore, RANK, dagli Osteoclasti (OCs), le cellule deputate al riassorbimento. Gli osteoclasti degradano l'osso dopo l'attivazione attraverso il legame del RANKL. Altre cellule possono esprimere il RANKL e l'alta espressione del RANKL è associata alla capacità delle cellule di indurre osteolisi. L'analisi al citofluorimetro ha dimostrato che l'espressione della forma di membrana CD254 è up-regolata nei pazienti all'esordio B LLA Common rispetto ai controlli fuori terapia o a fine mantenimento. Mediante esperimenti di co-cultura è stata studiata la capacità dei blasti di influenzare il differenziamento dei precursori degli osteoclasti. E' stata valutata quindi la capacità dei precursori CD14+ di fondersi a formare una cellula multinucleata, l'osteoclasto maturo, il quale in seguito diventa attivo e in grado di degradare la matrice ossea. La capacità di degradazione dell'osso è stata valutata usando una linea cellulare leucemica (SEM) esprimente il CD254, messa in co coltura con i CD14+. Le cellule coltivate in un terreno contenente frammenti di osso sono state in grado di degradare il substrato osseo. E' stata poi valutata la capacità di multinucleazione da parte dei blasti da BM degli esordi ed esprimenti CD254 rispetto a quella dei controlli. Si è visto che l'alta espressione del RANKL sui blasti CD19+ dei pazienti alla diagnosi induce multinucleazione dei CD14+. Inoltre studi di Gene Expression Profile (GEP) sono stati fatti per valutare se ci fosse un'alterazione dei geni coinvolti nell'osteoclastogenesi e nell'osteopetrosi e alcuni di questi sono risultati down regolati in modo significativo.
In questo network d'interazioni cellulari abbiamo voluto studiare un aspetto della comunicazione cellulare che riguarda le Microvescicole circolanti o *"Extracellular Vesicles"* (EVs). Le EVs sono state caratterizzate al citometro per l'espressione di marcatori di leucemia (CD19), piastrine (CD61) e per il metabolismo osseo (CD254). I risultati indicano che la produzione di EVs negli esordi dei pazienti B ALL è down regolata e che solo poche vescicole esprimono il CD19. Nel complesso questi dati suggeriscono che esiste un coinvolgimento diretto dei blasti nella via che regola il metabolismo osseo e che i blasti possono influenzare gli osteoclasti.

Summary

This study begins with the clinical observation of muscolo-skeletal involvement in leukaemia. Bone metabolism alterations have been reported before diagnosis, and persist during therapy and follow up. The Receptor Activator Nuclear Factor Kappa B Ligand (RANKL), also known as CD254, represents the major pathway that regulates bone metabolism. This ligand can exist either as a soluble form either as membrane form (CD254). The ligand is expressed mainly by bone forming cells osteoblasts (OBs) and its receptor RANK is expressed by bone resorbing cells osteoclasts (OCs). Upon the binding of the ligand to its receptor osteoclasts precursor are induced to fuse into a multinucleate cell, able to resorb bone substrates. Flow cytometry analysis showed that membrane CD254 was up regulated in B ALL patients at diagnosis compared with control patients (Stop therapy and maintenance).

Co-colture of CD14+ osteoclast precursors have been performed in order to assess the ability of CD14+ to fuse into multinucleate cells and eventually to resorb bone substrate. We evaluated the ability of a leukaemia cell line SEM, showing CD254 expression to influence CD14+ cells to differentiate and digest bone. Subsequently we co-coltured primary bone marrow cells from patients at diagnosis and controls and the ability to induce multinucleation of CD14+ cells were evaluated. Gene expression profile (GEP) was performed in order to see if RANKL involved pathways were altered in patients at diagnosis. We found that osteoclastogenic pathway, and osteoclast specific genes involved in osteopetrosis were down regulated.

In this context of interaction we decided to focus on the cell communication through circulating membrane vesicles. Known with the generic term of extracellular vesicles (EVs), those can be isolated from the peripheral and bone marrow plasma. Here they have been isolated and analysed by flow cytometer for specific B leukemic (CD19), platelets (CD61) and bone (CD254) markers. The results showed that the overall number of EVs in peripheral blood plasma from diagnosis was reduced compared to the controls. Only few CD19+ vesicles were present in the peripheral blood plasma of diagnosis.

In conclusion this study highlights the importance of the interaction between blasts cells and the bone metabolism, suggesting leukaemia can actively influence osteoclasts precursors.

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1. INTRODUCTION

1.1. Acute lymphocytic leukaemia: an overview

Acute lymphocytic leukaemia (ALL) is a clonal B and T disorder of the neoplastic cells characterized by an increase of slow proliferating cells in the bone marrow and other lymphoid organs. This gives rise to an accumulation of circulating monoclonal cells that escape the normal cellular process involved in cell cycle cycling, senescence and apoptosis. ALL is the most common cancer diagnosed in children and representing about 25% of cancer diagnoses among children younger than 15 years (Howlander *et al.,* 2013). Dramatic improvements in survival have been achieved in children and adolescents with cancer. For ALL, the 5-year survival rate has increased approximately to 90% for children younger than 15 years and to more than 75% for adolescents aged 15 to 19 years (Smith *et al.,* 2014; Hunger *et al.,* 2005). Childhood and adolescent cancer survivors require close follow-up because cancer therapy produce side effects that may persist or develop months or years after treatment. Risks of ALL have been identified. The primary risk factors for ALL include prenatal exposure to x-rays, postnatal exposure to high doses of radiation. Also genetic conditions associated to inherited disorders as Down syndrome, Neurofibromatosis (Stiller *et al.,* 1994), Shwachman syndrome (Wood *et al.,* 1980), Ataxia telangiectasia (Moriyama *et al.,* 2015) might induce leukemia. Inherited genetic polymorphisms and carriers of a constitutional Robertsonian translocation (Li *et al.,* 2014). Development of ALL is in most cases a multi-step process, with more than one genomic alteration required for leukaemia disease to develop. In some cases of childhood ALL, the initial genomic alteration appears to occur in utero (Taub *et al.,* 2002). ALL characterized by specific chromosomal abnormalities, with blood cells carrying at least one leukemic genomic abnormality at the time of birth, can acquire additional genomic changes postnatally (Bateman *et al.,* 2010). Genomic studies of identical twins with leukaemia further support the prenatal origin of some leukaemias (Greaves *et al.,* 2002). Today is accepted the hypothesis that additional postnatal genomic changes are needed for the development of this type of ALL and that in most cases in which a leukaemia-associated alteration is present at birth, the additional leukemogenic genomic changes do not occur and no leukaemia develops. Among children with ALL, more than 95%

attain remission, and approximately 80% of patients aged 1 to 18 years with newly diagnosed ALL treated on current regimens are expected to be long-term event-free survivors (Gaynon *et al.,* 2010). Despite the treatment advances noted in childhood ALL, numerous important biologic and therapeutic questions remain to be answered before the goal of curing every child with ALL with the least associated toxicity can be achieved. Treatment related skeletal complications such as osteonecrosis significantly contribute to short and long term disability in many survivors of pediatric hematological malignasies (Moab and Halton, 2014). In about 80% to 85% of children with ALL, the leukaemia starts in B cells. There are several subtypes of B-cell ALL as shown below (immature to mature forms):

Early precursor B (early pre-B) ALL (also called pro-B ALL)

Common ALL

Pre-B ALL

Mature B-cell ALL (also called Burkitt leukaemia). This type is rare, accounting for only about 2% to 3% of childhood ALL. It is essentially the same as Burkitt lymphoma and is treated differently from most leukaemias.

Infant leukaemia" generally refers to acute lymphoblastic leukaemia (ALL) or acute myeloid leukaemia (AML) diagnosed in a child before 1 year of age. A high proportion of acute leukaemias occurring in infants are characterized cytogenetically by balanced chromosomal translocations involving the mixed lineage leukaemia (MLL) gene at chromosome 11q23, known as t(4:11).

1.2. Clinical Presentations

The typical and atypical symptoms and clinical findings of childhood ALL have been published (Onciu *et al.,* 2014). Of relevance skeletal abnormalities are commonly seen in children and adolescent with leukaemia. Orthopedic surgeons frequently misdiagnose pediatric leukaemia with musculoskeletal condition and patients receive a significant delay in diagnosis of acute leukaemia. Blood examinations show: no blasts, low platelets, low hemoglobin, and high C- reactive protein. Low bone turnover and osteopenia are the major manifestation of bone metabolism involvement (Kobayashi *et al.,* 2005). These abnormalities can mimic several orthopedic pathologies at presentation, with a variable delay in the correct diagnosis. These manifestations occur before diagnosis. At presentation and prior initiating

chemotherapy serum specific bone formation markers are low. These include osteocalcin, type I collagen carboxy terminal propetide, and bone specific alkaline phosphatrase are low. Similarly parameters measuring bone degradation are low, as urinary telopeptide and type I carboxy terminal telopetptide. Thus both bone formation and bone resorption markers seems to be altered suggesting an overall low bone turnover state (Halton *et al.,* 1995; Rogalsky *et al.,* 1986; Sorva *et al.,* 1997; Moab and Halton, 2014).

1.3. Muscolo-skeletal system alterations in acute lymphocytic leukaemia

The leukemogenic characteristics of blasts are explained by their intrinsic properties and by extrinsic factors due to the altered cross talk with their microenvironment. Normal haematopoiesis requires complex bidirectional interactions between the bone marrow microenvironment (niche) and hematopoietic stem cells (HSCs). A spectrum of signaling pathways can regulate the interactions of HSCs with the niche (Lane *et al.,* 2009; Lane *et al.,* 2011). Bone marrow components of the normal haematopoietic stem progenitor cells (HSPCs) niche may play an essential role in the disease progression and deregulation of normal haematopoiesis occurring during the leukemogenic process (Sipkins *et al.,* 2005; Burger *et al.,* 2007). Leukemic cells alter osteoblastic and osteoclastic cell function resulting in measurable skeletal changes, which may impair normal hematopoiesis (Frish *et al.,* 2011). However, the cellular and molecular players, which contribute to a favorable leukaemia microenvironment, remain poorly understood. HSPCs are thought to reside in specific niches; cavities within the BM formed by specialized bone resorbing cells, the osteoclast (OCs) derived from monocytes/macrophage lineage. Osteoblasts (OBs) bone generating cells, arise from mesenchimal stem cells and together with other cells types like vascular endothelial cells or reticular cells they provide to HSPCs a nurturing microenvironment to grow (Calvi *et al.,* 2003; Zhang *et al.,* 2003; Arai *et al.,* 2004; Stier *et al.,* 2005; Adams and Scadden, 2006; Lymperi *et al.,* 2011). The ability of leukaemia cells to alter the niche components in the bone marrow is well documented and the effects are well described in acute myelocytic leukemia (AML) models: in mouse primary stromal dysfunction of osteoprogenitors can cause secondary neoplastic disease as myelodisplasia and secondary leukaemia (Raaijmakers *et al.,* 2010), mouse leukaemia stem cells (LSC) engraft into the hematopoietic microenvironment (Lane *et al.,* 2011), similarly human LSC home to and engraft within the osteoblast-rich area

of the bone marrow, both acquiring chemotherapy resistance (Ishikawa *et al.,* 2007). Analogous results were observed in an *in vitro* model of acute lymphoblastic leukaemia where mesenchimal secretion of asparagine synthetase conferred resistance to asparaginase drug (Iwamoto *et al.,* 2007). Furthermore leukaemia cells have direct effects on bone metabolism altering host formation and turnover in AML mouse (Frish *et al.,* 2011). Imbalance of bone remodeling factors has been suggested to be involved in HSPC mobilization in human (Li *et al.,* 2013). In steady state conditions, only low levels of HSPCs egress to the circulation. Treating with granulocytes colony stimulating factor (G-CSF) breaks the balance between HSPCs and bone lining osteoblasts causing HSPCs mobilization (Li *et al.,* 2013). In concomitance with the G-CSF treatment it is possible to observe a clinical phenomenon known as bone remodeling process. Accompanied by bone pain (Vial *et al.,* 1995) this reflects the dramatic reduction in bone turnover. Continuous G-CFS treatment to prevent neutropenia causes osteopenia, a decrease of bone mineral density and vertebral compression fractures (Bishop *et al.,* 1995; Dale *et al.,* 2003).

1.4. The Receptor Activator Nuclear Factor NF-kB Ligand (RANKL) pathway in bone metabolism

One of the most relevant pathways involved in bone homeostasis is the axis mediated by Receptor Activator Nuclear Factor NF-kB Ligand or RANKL (also known as TNSF11, TRANCE, OPGL, CD254) and its receptor RANK. RANKL is a member of the TNF super family and it is synthesized and expressed on cell membrane of bone marrow stromal cells and osteoblast. However RANKL expression can be detected in other tissues as spleen, placenta stomach, thyroid lung, brain, thymus, lymph nodes, and finally on osteoclasts and peripheral blood lymphocytes (Kartsogiannis *et al.,* 1999; Anderson *et al.,* 1997). RANK is the receptor for RANKL and it is known to be essential for osteoclasts as it is required for their differentiation, maturation and survival (Kong *et al.,* 1999). Osteoprotegerin (OPG) is a decoy receptor for RANKL, thus inhibiting binding to osteoclasts and preventing osteoclastogenesis differentiation. RANK is expressed by dendritic cells, fibroblast, B and T cells, osteoclasts (reviewed in Theoleyre *et al.,* 2004). Co stimulatory signal to the RANKL/RANK interaction are represented by immune receptor tyrosine-based activation motif (ITAM) bearing adapter proteins, as DAP12 or Fcε receptor I γ chain (FcRγ). Both are required for osteoclast maturation and activation (Mocsai *et al.,* 2004; Humphrey *et al.,*

2005). Upon activation a tyrosine phosphorylation on ITAM domain recruits SYK kinase, which in turn initiate a transduction cascade including several pathways as phospholypase C γ (PLCγ), calcium mobilisation, phosphatidylinositol-3 kinase (PI3K), RAS, nuclear factor kB (NFkB) and nuclear factor for activation of T cells (NFAT) (Shinohara *et al.,* 2008). DAP12 associates to multiple cell surfaces activating receptor depending on cell type like CD300d (Lanier *et al.,* 2009). Myeloid cells and above all natural killer NK highly express DAP12. Among lymphocytes, B cells can also express it. DAP12 is involved in innate immunity driven by NK and myeloid cells. Furthermore is involved in B cell dependent adaptive immune response. DAP12 deficient mice B cells and B cells from patients with a recessive genetic disorder named Nasu Hakola resulting fron loss of DAP12 are both characterized by abnormal increased responses through Toll like receptor TLR9. Thus in physiological conditions DAP12 act as negative regulator of B cell proliferation upon induction of adaptive immunity (Nakano-Yomizo *et al.,* 2011). Dap12-/- mice show a mild osteopetrotic phenotype whereas double mutant Dap12-/- FcRγ -/- are severely osteopetrotic. Integrin αvβ3 is in the same signalling complex in a linear sequence. In absence of these three players the osteopetrotic phenotype is very strong, with a defect in the osteoclasts ability to resorb bone because lacking of cytoskeleton organization (Zou *et al.,* 2014). A role of RANKL/RANK pathway in disease patho-physiology of chronic leukaemia and multiple myeloma is already known, as well as for metastatic spread of solid tumors (Jones *et al.,* 2006; Palfox *et al.,* 2012; Tan *et al.,* 2011). RANKL/RANK have been reported to regulate immune response NK mediated in acute myeloid leukaemia (Schmiedel *et al.,* 2013). In pathological conditions RANKL is over expressed in primary malignant bone tumours, as osteosarcoma, chondrosarcoma, giant cell tumour (Grimaud *et al.,* 2003) and multiple myeloma (Giuliani *et al.,* 2001).

1.5. Defective osteoclasts and osteopetrosis in human

Osteoclasts, the only bone-resorbing cell, arise from the myeloid lineage, including macrophages and monocytes. Upon activation, the osteoclast's plasma membrane attach to the underlying bone forming the sealing zones, belts of specialized adhesion structures called podosomes. Attachment to the bone matrix is allowed by integrin receptors, such as αvβ3. The osteoclast releases hydrogen ions through the carbonic anhydrase into the resorptive cavity, acidifying and aiding dissolution of the mineralized bone matrix into Ca_2+, H_3PO_4,

H_2CO_3, water and other substances. The mobilization of the mineral phase opens the way to hydrolytic enzymes, such as members of the cathepsin and matrix metallo-protease (MMP) groups, which are released to digest the organic components of the matrix. These enzymes are released into the compartment by lysosomes. Among hydrolytic enzymes, cathepsin K is of most importance. Cathepsin K is secreted from the ruffled border into the resorptive pit. Cathepsin K transmigrates across the ruffled border by intercellular vesicles and is then released by the functional secretory domain. Within these intercellular vesicles, cathepsin K, along with reactive oxygen species generated by TRAP, further degrades the bone extracellular matrix (Cappariello *et al.,* 2014). RANKL and macrophage colony simulating factor M-CSF are required during osteoclast differentiation. In human osteoclast defects represent a rare and hereditable bone disorder characterized by high bone mass due to insufficient activity of osteoclasts. Two major clinical osteopetrosis forms have been identified; the autosomal recessive osteopetrosis (ARO) often lethal in early childhood, and the second form autosomal dominant osteopetrosis ADO II, known as Albers-Schonberg disease or marble bones, seen in adults. The first form is caused by homozygous mutation of *TCIRG1* gene that impairs osteoclast acid secretion. The latter can be due to a homozygous mutation to *CLCN7* and *OSTM1*, which form a molecular complex. ADO II is associated with serious clinic complications, such as fractures, delayed fracture healing and osteomyelitis (Del Fattore *et al.,* 2008; de Vernejoul *et al.,* 2010). Coudert (Coudert *et al.,* 2014) performed a transcriptomic analysis by comparing gene expression profile of ADO II with healthy donors. They found among other genes that *SERPINE2* and *WARS* were differentially expressed and suggested those to be part of the ADO II phenotype, together with the already characterised function of *CLCN7* and its partner *OSTM1*. *SERPINE2* is involved in the removal of non-collagenous proteins that are present in the non-mineralized bone matrix: it was also significantly down regulated in ADO II patients. Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA by their cognate amino acid. Tryptophanyl-tRNA synthetase (WARS) catalyzes the aminoacylation of tRNA(trp) with tryptophan and is induced by interferon. Tryptophanyl-tRNA WARS was significantly down regulated in ADO II (Coudert *et al.,* 2014).

The functional activity of the bone system are ensured not only by the major players as OCs and OBs, but several other accessory cells, not directly involved in the formation or degradation of the skeleton support and cooperate to ensure the physiological bone homeostasis.

1.6. Bone homeostasis maintenance: old and new players

Bone homeostasis relays on the functional activity of OBs and OCs. They work in concert to ensure the correct balance between bone formation and degradation. In the niche context the role of stromal and mesenchimal stem cells is well known. However among these a rare population exist which is represented by Megakaryocytes (MKs), responsible for platelets release, and also involved in the maintenance of the bone marrow niche through the expression of components of the basement membrane, necessary components of the extra cellular matrix microenvironment (Malara *et al.,* 2014). MK can produce RANKL and OPG, and their expression is modulated by oestrogen (Bord *et al.,* 2004; Bord *et al.,* 2005). Megakaryocytes grown in co-colture with osteoblasts were able to increases osteoblastic OPG synthesis while suppressing RANKL expression, demonstrating that in vitro MK have a direct effect on bone forming cells (Bord *et al.,* 2005). Mice with deletion of p45 NF-E2 results in the arrest of megakaryocytes differentiation and are characterized by high mass phenotype. In these mice both OCs and OBs number were increased and they were functional. Furthermore the increased bone mass was observed only in bony sites of hematopoiesis. This impairment was due to a missed cross talk with OBs rather than OCs (Kacena *et al.,* 2004). An *in vitro* study showed that MK could also influence OCs precursor. Co-colture of mature MKs with PBMC CD14+ cells inhibited the formation of OCs reducing their number and, affecting their functionality (Beeton *et al.,* 2006). Mice in which OBs function is ablated or overexpressed both resulted in early blocks of B cell development (Wu *et al.,* 2008). Furthermore haematopioetic cells are involved in the regulation of the bone homeostasis, directly and indirectly influencing bone cells, specifically the osteoclasts digesting cells.

1.7. B-lymphocytes and the bone players: a tight relationship

B-lymphocytes are in close proximity with the bone cells, osteoblasts and osteoclasts. Perturbation of B lymphopoiesis affects the bone mass, and on the other side alteration of bone homeostasis affects B lymphopoiesis. B cell development occurs within the vascular microenvironment of the BM. Stromal cells, and OBs produce factors like chemokine C-X-X

motif chemokine 12 (CLCX12), stem cell factors (SCF), IL7, RANKL and OPG, which are critical for early B cell development (Manilay and Zouali 2014; Toraldo *et al.,* 2014). RANKL and osteoprotegerin produced by stromal cells are also important for B cells development specifically at the pre-B to immature transition. Mesenchimal and OBs cells contributes to support all developmental transition in B lymphopoiesis through local secretion of B lymphocytes survival factors as vascular cell adhesion molecule-1 (VCAM-1)-mediated adhesion and *in situ* secreted IL7 and CXCL12 (Egawa *et al.,* 2001; Miller *et al.,* 2002).

B lymphopoiesis is a highly regulated process which starts from the progenitors in the fetal liver, move to the BM, to end the maturation in the secondary lymphoid organs (SLOs), such as the spleen and lymphonodes. After B cells mature in the bone marrow, they migrate through the blood to SLOs, where receive a constant supply of antigen through circulating lymph. At the SLOs, B cell activation begins when the B cell binds to an antigen via its BCR. Only after the mature B cell meet the T cell derived helper can terminally differentiate into immunoglobulin (Ig)- secreting plasma cell (Ghia *et al., 2010*). The B cell development is organized around the assembly of a functional B-cell receptor (BCR) through a process of gene rearrangement called V(D)J recombination. The molecular players responsible for the correct rearrangements to have a functional BCR are different transcription factors, required at specific time of the different developmental transitions. Several genes are involved in B cell maturation (Figure 1). Of this Pax5 is specifically required for B cell development at the pro B to pre B transition. Pax5 codes for the transcription factor B cell lineage specific activator protein (BSAP). It is expressed exclusively in the B-lymphocytes lineage extending from pro-B cells to mature but not to the terminally differentiated plasma cells. Pax5 deletion causes among various deleterious effects the arrest of all B cell development beyond the early pro B stage.

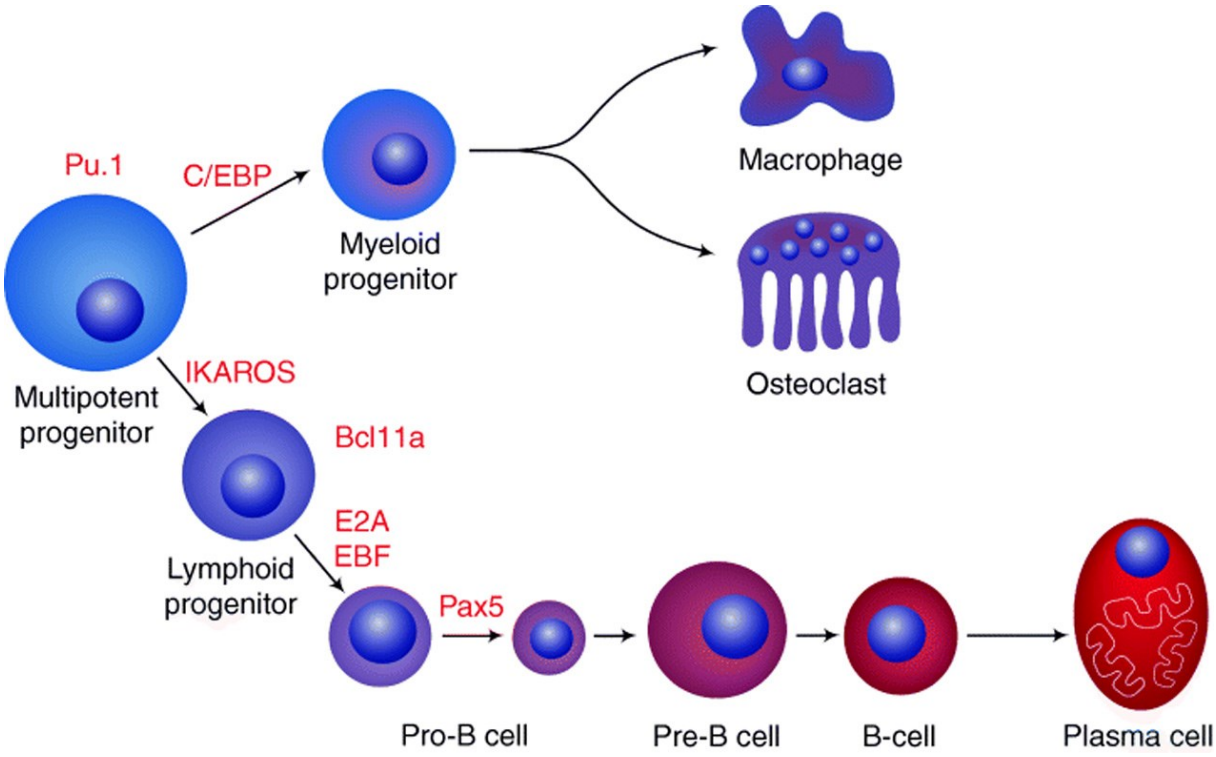

Figure 1: B cell differentiation and its transcription factors. (From Horowitz *et al.,* 2008).

These pro B cells have the ability to be maintained in an undifferentiated phase if grown in presence of IL7, the removal of which cause these cells to differentiate into multiple hematopoietic phenotypes including osteoclasts. Furthermore in Pax5-/- pro B cells, treatment with MCS-F and RANKL can induce the formation of osteoclasts (Yoshida *et al.,* 1990).

Mice with deletion of Pax5 are osteopenic due to an increase of osteoclast number, and not to the lack of B cells (Horowitz *et al.,* 2004). In Pax5 deficient mice the alteration are specific for osteoclast and B cells, and not other haematopoietic compartments are involved (Yoshida *et al.,* 1990). Pax 5 gene is responsible for the correct activation and suppression respectively of B cell lineage specific genes and myeloid precursors. Uncommitted haematopoietic progenitors including Pax5-/- pro B cells express genes of different lineages (Nutt *et al.,* 1999; Miyamoto *et al.,* 2002). At commitment Pax5 activates target genes as components of the pre BCR. Pax5 depressed genes include among others RANKL, MCSF-R and Notch1 with the result that B cells are not anymore responsive to myeloid signals (Nutt *et al.,* 1999). During B cell differentiation the transcriptional activity responsible for activation and repression of specific genes is accompanied by cytokines produced by stromal cells, which became essentials in the transition phase. Interleukin 7 has a pivotal role in B lymphopoiesis at the transition from pro B cells to pre B cells. IL-7 overexpressing mice have expanded number of pre B cells, and increased bone resorption, probably because IL-7 stimulated B

cells expresses RANKL and can thus activate osteoclast-resorbing activity (Myamura *et al.,* 1997). Another mice model that highlight the importance of the cross talk between OCs and B cells is represented by *oc/oc* mice, which are osteopetrotic due to the absence of resorbing osteoclasts caused by a mutation in *tcgr1* gene. Those mice behave similarly to IL7-/- models, presenting the arrest at the transition between pro B and pre B. In these mice IL-7 injection caused the partial rescue of B lymphpoiesis by engaging a population of bone marrow cells (described by Blin Wakkach *et al.,* 2004) B220+ CD11b+ into B lymphocytes through the induction of Pax5 and the inhibition of the myeloid lineage specific genes (Blin Wakkach *et al.,* 2006). This model and the phenotype observed showed that a mixed lineage population exist in the BM and that factors as IL7 can revert the myeloid lineage phenotype towards the B lymphoid one. IL-7R deficient mice have suppressed B cell development and increased bone mineral density (Peschon *et al.,* 1994). B cell autonomous RANKL expression is also important for B cells development as shown in RANKL knock out (KO) mice. Osteopetrotic RANKL KO mice have a clear block at the haematopoiesis between the pro and pre B cells. RANKL deficient mice show severe osteopetrosis, because no osteoclasts are present and in turn no bone marrow spaces can be formed. B cell differentiation is arrested at the pro B pre B stage. Number of cells is reduced and an intrinsic defect exists in these pre B cells (Kong *et al.,* 1999; Kim *et al.,* 2000). Another informative model is represented by an established pre B cell line named preBR. This cell line was able to differentiate into immature B cells *in vitro* after removal of IL7 from the colture medium (Kato *et al.,* 2003). RANKL expression was dependent upon IL7 and transfectant overexpressing RANKL were induced to proliferate through inhibition of the G1 arrest. RANKL also increased the percentage of cells S/G2/M phase. Physiologically RANKL expression was thus shown to be restricted to the pre B cell stage (Kato *et al.,* 2003). Oestrogen deficiency mimics the IL7 overexpression, causing an increased B lymphopoiesis. Ovariectomy mice and subsequent oestrogen withdrawal, resulted in marked accumulation of B220+ pre B cells, which activity was linked to the up regulated bone resorbing activity of osteoclasts (Miyamura *et al.,* 1997). Figure 2 summarize the cytokines requirements for B cell development in mice as reviewed by Manilay (Manilay and Zouali, 2014). Injection of IL7 partially restores B lymphopoiesis but not completely, indicating that not only the niche but also cell intrinsic factors are involved in interaction with OCs.

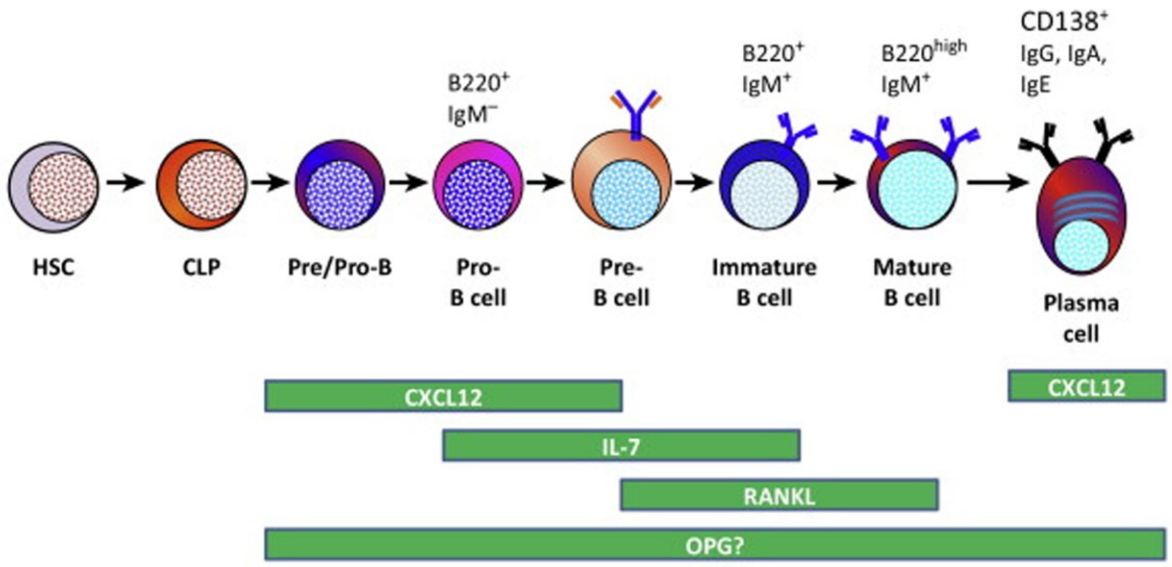

Figure 2: Cytokine requirements during B cell differentiation in mice. Adapted from Maniklay and Zouali, 2014.

Treatment with zoledronic acid (ZA) in wild type mice could induce mild osteopetrosis affecting B cell development. The effect of ZA seemed to be linked to the microenvironment modification rather then a B specific phenotype. B cells progenitors were less retained in the bone marrow and were homed instead in other organs as the spleen. Histological analysis of ZA mice femora confirmed trabecular region with mineralised bone matrix, and disorganised. Tartarate resistant acid phosphatase (TRACP) showed a large number of OCs not attached to the bone surface. ZA effect was thus decreasing OCs resorbing activity because they loss their adhesion ability, rather than to a decrease in OC number (Mansour*et al.,* 2011). ZA treated mice showed that B cell compartment was affected and no others. Mansour (Mansour *et al.,* 2011) suggested that the decreased cell number of mature B cells could be due to an impaired retention and mobilisation of B cell precursors outside the bone marrow. In this frame osteoclasts are arising as bone cells strongly connected to the B cells and indeed they share transcription factors involved in their maturation and differentiation. As described above the B cells and osteoclasts cross talk is made of diverse modes of communication. A novel mechanisms of cell-cell communication relays on extracellular vesicles as it will be described in the next section.

1.8. Cell to cell communication: extracellular vesicles as carriers of information

Haematopoietic precursors from which osteoclasts and B cells originate resides in the bone marrow niche. The niche has a physical meaning but it is also made of a complex net of interactions. Different cell populations contribute to maintain suitable condition for HSPCs to grow and develop. In this frame intracellular communication between cells and their microenvironment is essential for its functionality and integrity. Beyond cell-cell interactions cross talk between niche components is traditionally viewed as mediated by soluble factors; however new contribution of shed membrane vesicles has been recently appreciated (Ratajczak 2006; Mause and Weber, 2010).

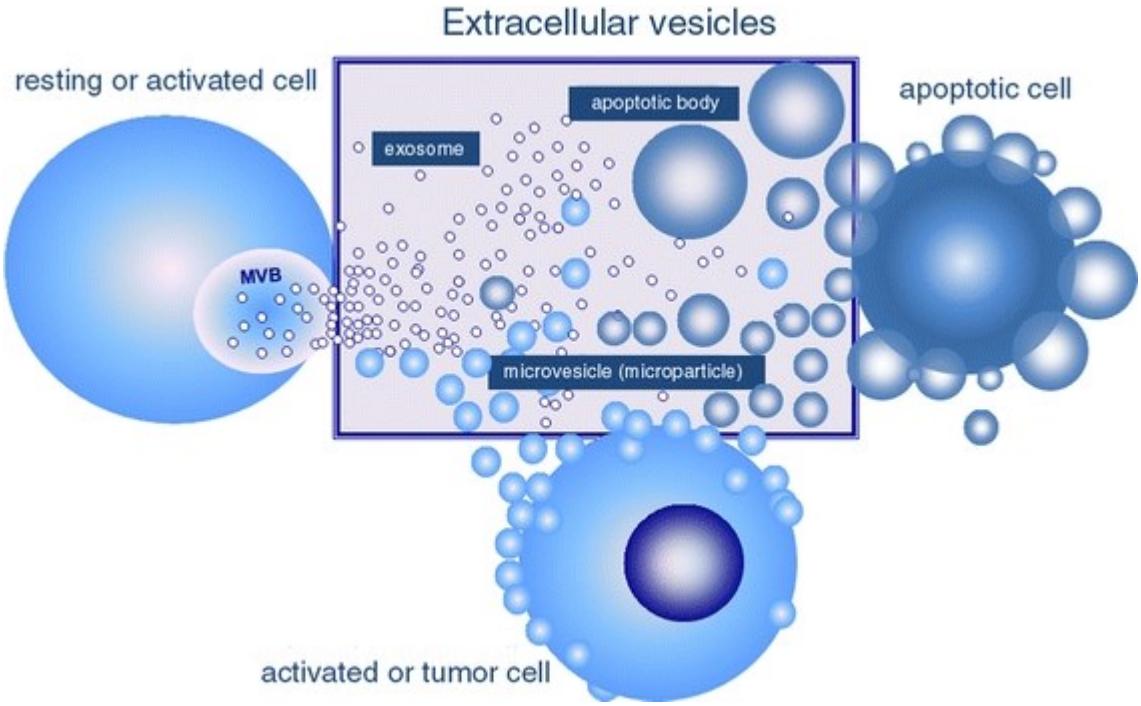

Figure 3: Schematic representation of the extracellular vesicles. Major populations include exosomes, microvesicles and apoptotic bodies (From Gyorgy *et al.,* 2011).

Secreted membrane-enclosed vesicles, collectively called extracellular vesicles (EVs), which include exosomes, ectosomes, microvesicles, microparticles, apoptotic bodies and other EV subsets, encompass a very rapidly growing scientific field in biology and medicine. They differ for their biogenesis and for their size. Exosomes are budded out after plasma membrane fuses with multivesicular bodies (MVBs), which are endosomal structures carrying multiple vesicles in the cytosol (Raposo *et al.,* 2013). The released exosomes are homogenous vesicles,

of around 40-100 nm in diameter (Simons *et al.,* 2008). While it is known that exosomes contain endosomal proteins such as tetraspanins (CD9, CD63), Alix, and TSG101, and are used as exosomal markers (Simons *et al.,* 2009; Simpson *et al.,* 2008), for microvesicles, there are not yet identified common markers. Microvesicles are also known as ectosomes, shedding vesicles, microparticles, and plasma membrane-derived vesicles, and originate from the plasma membrane by outward budding and fission (Murilidharan-Chari *et al.,* 2010). Microvesicles are about 50-1.000 nm in diameter, but their density is undefined (Murilidharan-Chari *et al.,* 2010). The most important difference between exosomes and microvesicles is in their biogenesis. Microvesicles are generated by budding from the plasma membrane and thus resemble the plasma membrane composition of the parent cells. On the other hand, exosomes generated by inward budding and fission with MVBs, are composed of various cytosolic proteins related to endolysosomal pathways (Figure 3). Exosomes and microvesicles carry specific markers, but those markers are not completely unique to distinguish them from each other. EVs interact with recipient cells by ligand-receptor interactions, fusion, and internalization via receptor-mediated endocytosis or macropinocytosis (reviwed by Yoon *et al.,* 2014). In this study we will focus only on a group of membrane bodies with diameter comprised between 100 and 1000 nm, and we will refer to them simply as extracellular vesicles (EVs).

Extra-vesicular bodies are surrounded by double layer cell membrane, which are released by cells, both *in vivo* and *in vitro*, in physiological and pathological conditions. In physiological conditions the shedding of EVs accompanies cell activation and growth. In pathological conditions such as solid tumours their content promotes progression and metastasis of the disease (Ratajczak *et al.,* 2006; Peinado *et al.,* 2011; Ghasemi *et al.,* 2013). EVs are shed from a variety of cell types. They are present in body fluids such as blood, urine, ascites (Graves *et al.,* 2004; Piccin *et al.,* 2007; Smalley *et al.,* 2008; Taylor and Gercel-Taylor, 2008). Recently it has been recognized their potential as indicators in the diagnosis, prognosis and follow up of different diseases and in compromised health conditions where the level of EVs bodies is generally increased (van Doormal *et al.,* 2009; Fleissner *et al.,* 2012).

Fast growing cells tend to release more EVs, than slow growing cells. The increase in EVs release can be induced by several factors, as cell activation, hypoxia and radiation, oxidative injury, complement cascade, upon cytoplasmic calcium released and by degradation of the membrane skeleton (reviwed by Ratajczak *et al.,* 2006).

EVs packaged with bioactive molecules as miRNAs, mRNA, proteins have been reported to be secreted by differentiated cells and tumor cells (Cocucci *et al.,* 2009; Ratajczak *et al.,*

2006; Ghosh *et al.,* 2010). It has also been suggested that extra-vesicular bodies are involved in stem cell plasticity, being able to modulate specific cells at different cycling phases (Alliota *et al.,* 2010).

In lymphocytic leukaemia EVs were suggested to modulate the hematopoietic niche, and found at increased levels in plasma of B-cell chronic lymphocytic leukaemia (CLL) patients. Early-stage disease is characterized by predominantly platelet derived extra-vesicular bodies. With the progression of the disease the number of B-lymphocyte–derived EVs increases compared to platelet ones. These extra-vesicular bodies are able to modulate stromal cells. In CLL the EVs level was increased compared to control patients. The secreted EVs were able to modulate the *in vitro* the bone marrow stromal cells, causing the activation of the AKT/mammalian target of rapamycin/p70S6K/hypoxia-inducible factor 1α axis, with the production of a pro-leukemic factor, the vascular endothelial growth factor. In addition EVs were responsible of the activation of the β catenin pathway. EVs from CLL cells were thus able to transfer phospho receptor tyrosine kinase Axl directly to the stromal cell (Ghosh *et al.,* 2010).

These data highlight the importance of EVs in the tumor progression and their ability to modulate the microenvironment. The world of extracellular vesicles is a fast growing field with multiple applications. Today we still lack standardization of the isolation and absolute quantification procedures. Notably their availability in diverse body fluids might provide an easy accessible source of biomarkers to use in clinic, making them very interesting.

2. Aim of the study

Leukaemia disease and the skeleton are physically connected within the bone marrow. Bone involvement and its alterations in leukaemia are well documented. However the mechanism responsible of this phenomenon is not well known. The low bone turn over is the major manifestation at diagnosis, even before blast crisis. Acute lymphocytic leukaemia mimics several orthopaedic pathologies, causing a delay in the correct diagnosis. Furthermore the therapy aggravates the bony phenotype with consequence in the long-term survival of young patients. Beyond the protection that the skeleton represent for the hematopoietic compartment there is a net of interactions that in both directions ensures the physiological grow of lymphocytes and bone cells, and alteration of this homeostasis causes at the same time the bone or the lymphopoiesis imbalance. RANKL pathway is a shared key involved in the regulation of both compartments. We asked if RANKL is altered in paediatric B ALL diagnosis, and if RANKL expression on blasts cells had a functional meaning. Specifically if B ALL blasts were able to influence the osteoclast cells, which carries the RANK receptor for RANKL. Furthermore we characterised peripheral blood and bone marrow extracellular vesicles in controls patients and compared them to the peripheral blood population at diagnosis analysing leukaemia and bone specific marker RANKL. This study wanted to address the possibility that blasts actively influence the bone marrow environment, specifically bone resorbing cells, and possibly beyond known interaction modes trough the production of extra-vesicular bodies.

Our study underlines the importance of cross talk between blasts cells and bone cells, and the knowledge of the mechanism behind their interaction might help to anticipate the diagnosis including acute lymphocytic leukaemia in the differential diagnosis of musculoskeletal manifestations. Furthermore could be important to tailor the therapy in order to reduce the long-term squeal of the therapy side effects.

3. Materials and methods

RANKL expression on leukaemia Bone Marrow blast cells

Patients

For B cell specific membrane RANKL expression patients PBMC and BM cells were obtained at the time of the diagnosis before therapy (68 B Common, 4 pre B, 7 pro B) and from healthy patients (11 stop therapy, and 4 maintenance) by density gradient centrifugation (Ficoll, Histopaque 1077, Sigma-Aldrich St. Louis, MO, USA). The local medical ethics committee approved this study.

Flow cytometry analysis

Flow cytometry analysis (FACS) was performed using specific antibodies (table 1), according to manufacturer's instruction.

Table 1: antibodies used in flow cytometry analysis.

Cluster of differentiation	Company	Fluorophore	Target cell
CD7	Becton Dickinson	FITC	T cells
CD15	Becton Dickinson	FITC	Monocytes precursor
CD41	Becton Dickinson	FITC	Megakaryocytes, platelets
CD14	Becton Dickinson	PE	Monocytes
CD254 clone MIH24	eBioscience	PE	B cells, blasts, others
CD45	Beckam coulter	ECD	Pan leucocytes
CD33	Beckam coulter	PC5	Myeloid cells
CD3	Beckam coulter	PC7	T cells
CD19	Beckam coulter	PC7	B cells, blasts
CD61	Beckam coulter	PC7	Megakaryocytes, platelets
CD19	Becton Dickinson	APC	B cells, blasts

A simplified antigen panel for leukaemia diagnosis includes the antigen CD19+, a leukemic blast marker, a pan leukocyte marker CD45, T specific antigen CD7 or CD3, a myeloid specific marker CD33, that allows for detection of granulocytes, monocytes and neutrophils

(see tables) and CD254. Fluorescence values for CD254 are given in median fluorescence intensity (MFI).

Primary human osteoclast precursors isolation

Mononuclear cells were isolated from human peripheral blood buffy coats from healthy donors by gradient centrifugation (Ficoll, Histopaque 1077, Sigma-Aldrich St. Louis, MO, USA). Subsequently, CD14+ monocytes were sorted using anti-CD14 coated magnetic beads Miltenyi Biotec GmbH Germany), according to manufacturer's instruction. Purity of isolated population was assessed by FC (CD14 for monocytes, CD7 for T lymphocytes, CD19 for B lymphocytes, CD45 pan leucocyte, CD33 for myeloid lineage)

Cells were re-suspended in α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), and subsequently used in co-colture experiments as described below.

Co-culture experiment

Co-colture into chamber slides

3 x 10^5 PBMC CD14+ cells/ml were seeded in each 4-Well Culture Slides (Falcon).

The negative control were 3 x 10^5 PBMC CD14 grown in maintenance medium supplemented with MCS-F (20ng/ml); the positive control were 3 x 10^5 PBMC CD14 grown in osteoclastogenic medium (M-CSF 20 ng/ml, soluble recombinant RANKL 50 ng/ml).

3 x 10^5 PBMC CD14+ cells were grown in co-colture with 1 x 10^5 B cell precursor leukaemia cell line (SEM ACC 546 from DSMZ) in maintenance medium.

3 x 10^5 PBMC CD14+ cells were grown in co-colture with 5 x 10^5 CD19+ cells from BM of diagnosis or whole BM from healthy patients in maintenance medium.

To assess RANKL specificity to induce multinucleation recombinant OPG was used to inhibit RANKL activity. The OPG was added both to the maintenance medium and to the osteoclastogenic medium, in controls and in wells with primary leukaemia cells and SEM cells.

The culture medium was supplemented with MCS-F (25 ng/ml, Cell guidance system) and recombinant receptor activator nuclear factor ligand RANKL (50 ng/mL, Cell guidance system), OPG (50 ng/mL, Cell guidance system). SCF (Miltenyi), FLT3 (Miltenyi) for

primary leukemic cells (see table 2 for list of cytokine) and refreshed every 4 days, for a period of 12 days.

Table 2: Cytokines used in co-colture experiments.

Cytokines	Company	Working concentration
MCS-F	Cell guidance system	25 ng/mL
RANKL	Cell guidance system	50 ng/mL
OPG	Cell guidance system	25 ng/mL
FLT3	Miltenyi	20 ng/mL
SCF	Miltenyi	5 ng/mL

Multinucleation and TRAP assay

Differentiated osteoclats were identified as tartarate–resistant acid phosphatase (TRAP-positive) cells with three or more nuclei using Acid Phosphatase Leukocyte (TRAP) Kit (SIGMA), according to manufacturer's instruction. Multinucleated cells were visualized and counted by light microscopy at Zeiss imager M1 equipped with Axiocam MRc5, Axiovision. A minimum of 200 cells was counted per each chamber and results are given as percentage.

Bone plate Co-coltures

Primary human osteoclast precursors were seeded in each well of the bone plate (OsteoAssay™ Plate, Lonza) at 30,000 precursors

The negative control were 3 x 10^4 PBMC CD14 grown in maintenance medium supplemented with MCS-F (20 ng/ml); the positive control were 3 x 10^4 PBMC CD14 grown in osteoclastogenic medium (M-CSF 20 ng/ml, soluble recombinant RANKL 50 ng/ml).

3 x 10^4 PBMC CD14+ cells were grown in co-colture with 1 x 10^4 B cell precursor leukaemia cell line in maintenance medium.

3 x 10^4 PBMC CD14+ cells were grown in co-colture with 3 x 10^4 CD19+ cells from BM of diagnosis or whole BM from healthy patients in maintenance medium.

To assess RANKL specificity to induce multinucleation recombinant OPG was used to inhibit RANKL activity. The OPG was added both to the maintenance medium and to the osteoclastogenic medium, in controls and in wells with primary leukaemia cells and SEM cells.

The culture medium was supplemented with MCS-F (25 ng/ml, Cell guidance system) and recombinant receptor activator nuclear factor ligand RANKL (50 ng/mL, Cell guidance system), OPG (50 ng/mL, Cell guidance system). SCF (Miltenyi), FLT3 (Miltenyi) for primary leukemic cells (see table for list of cytokine) for a period of 9 days.

Resorption pits staining and enumeration

CD14+ cells were plated onto bone plates (Lonza Osteo-assay) and treated with 20 ng/ml M-CSF and 50 ng/ml RANKL for 7 days. The cells were completely removed with 10% hypochlorite and the bone plate was stained with toluidine blue 1%. Photographs of the resorption pits were obtained under a light microscope at 40× magnifications, with Opetech microscope equipped with DP200 camera and Deltapix software. Resorption pits number per each well was evaluated using ImageJ software.

Gene expression profile

RNA was extracted from bone marrow of patients, as well as from a series of healthy pediatric BM (HBM). RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Gene expression profile were obtained using GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CS, USA) and normalized using RMA package from Bioconductor (www-r-project.org).(Bresolin *et al.,* 2010).

To study the involvement of B ALL in bone metabolism we make a list of genes involved in osteolysis pathway derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Unsupervised hierarchical clustering analysis using Euclidian distance and Ward methods were used to assess the different expression profiling of these genes in B-ALL patients and healthy donors.

The Student t test was used for evaluate the differences between patients and HBM samples for selected genes. The Search Tool for the Retrieval of Interacting Genes (STRING) tool was utilized to build a protein-protein interaction (PPI) network and (DAVID Functional Annotation Bioinformatics Microarray Analysis was used to identify molecular networks) among genes resulted to be differently expressed. Table 3 shows the leukaemia subpopulation that has been used in this gene profile analysis.

Table 3: Gene expression profile ALL B subtype.

Class	SubType
1	ALL – mature B cells with t(8;14)
2	ALL – prepreB, CALL with rearranged 11q23 (*MLL*)
3	ALL – CALL, preB with t(9;22)
4	ALL – all T subtypes
5	ALL – CALL with t(12;21)
6	ALL – t(1;19)
7	ALL - hyperdiploid
8	ALL – CALL, preB without t(9;22)
18	Non-Leukaemia or normal BM

Data analysis

Statistical analysis was performed using Prism 4.02 (Graph Pad Software, San Diego, CA). Experiments were performed in duplicate or triplicate, and results were presented as mean of replicate experiments. Statistical significance was evaluated by the Mann Withney or unpaired Student *t* test where the latter was not possible. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

Extracellular vesicles characterization

Patients

Peripheral Blood (PB) and Bone Marrow (BM) were collected after informed consent from healthy donors (Stop Therapy, Maintenance) (6 PBs, 5 BMs) and patients at diagnosis of ALL B (20 B common, 3 pre B, 2 pro B, 1 relapse).

Plasma Preparation for Extracellular vesicles analysis

Cells, apoptotic bodies and eventually immune-complexes are removed from plasma by a combination of steps at the refrigerated centrifuge (Thermo scientific MicroCL21R) and filtration. Whole plasma is obtained by centrifuging at 1,500 g for 15'. Subsequent centrifugation for 15 minutes at 2,500 g (twice) depletes platelets in the plasma sample. The resulting supernatant is designated plasma platelet free (PFP). Plasma is finally filtered with 1,2 μm (FP 30/1 CA-S Whatman), to remove remaining debris.

Extracellular vesicles isolation

EVs were isolated previously described (Ghosh *et al.,* 2010) with minor modifications. PFP is centrifuged at 20,000 x g 60' to obtain the pellet of extra cellular vesicles. The pellet is re-suspended in a protein-free buffer phosphate-buffered saline (PBS) and stored at 4°C for further analysis.

Extracellular vesicles Labelling

For extra cellular vesicles labeling PFP is incubated with CellTrace™ CFSE (Carboxyfluorescein Succinimidyl ester) Cell Proliferation Kit Molecular probe (working concentration 3 µM) and stained for 20 minutes at room temperature with gentle agitation. After filtration PFP was centrifuged at 20,000 x g 60' to obtain the pellet of extra cellular vesicles. The pellet is re-suspended in 20 µl phosphate-buffered saline (PBS) and labeled with 1 µl of the following antibodies for flow cytometry analysis: CD254 PE, CD61 PC7, CD19 APC (see table 1). Samples are incubated for 30' at room temperature. After incubation PBS cold is added up to 450-µl final volume ready for analysis.

Extracellular vesicles Flow cytometry analysis

Prior FC analysis 50 µl of 3 µm polystyrene beads (Polyscience.Inc Polybead® polystirene) were added to each sample and in the control tube at a final concentration of 200 beads /µl. These beads were used both as a relative size reference and as internal standard for measuring the acquired samples beads number.

FS settings were adjusted using MegaMIx-Plus FSC beads (Biocytex) according to the manufacturer procedures.

Samples were analyzed using MoFlow Beckman Coulter flow cytometer.

The number of EVs was calculate by bead count assay as described before (Shet *et al.,* 2003; Jayachandran *et al.,* 2008; Jayachandran *et al.,*2013).

Atomic Force Microscopy analysis of Extracellular vesicles

A standard coverslip was cut into pieces 1"x0.5" (ca. 2.5 cm x 1.25 cm) using a diamond tip to fit into the AFM sample holder. The surface was cleaned first with pure deionized water (diH2O) and soap (rinse in diH2O) and then with acetone (rinse in EtOH). Both cleaning steps were ultrasound-assisted (80 kHz for 60 s) and the samples were dried in a N_2 flow. Just before sample deposition, the samples were exposed to UV light for 60 s to further clean and

activate the surface. We used a CPII AFM from Digital Instruments (now Bruker) equipped with a V-shaped Si_3N_4 cantilever 0.6 μm thick of the sharp MicroLever (ML) series by Park Instruments, featuring a curvature radius of 2 nm. We chose in particular a ML06E cantilever (force constant 0.10 N/m, resonant frequency 38 kHz). Non-contact mode was also used to better visualize the morphology of isolated membrane bodies. AFM does not suffer from tip or sample degradation effects and is the eligible method for studying biological samples.

4. Results

CD254 BM membrane expression in B ALL patients at diagnosis.

As reported in literature leukaemia bone involvement is a clinical feature of patients before, during and after diagnosis of B ALL. RANKL (CD254) is the major and best-characterized player of the bone metabolism. It has been previously shown that it can be expressed in cells of B lineage and its expression is associated to bone erosion in several disease included rheumatoid arthritis or periodontitis (Kawai *et al.,* 2006). For this reason we wanted to study if CD254 expression was a feature of leukemia patients' blasts or B cells from healthy controls. In the attempt to address this point a flow cytometry approach was chosen. Membrane RANKL expression was analysed in the context of B ALL antigen panel.

A typical analysis of leukaemia at diagnosis includes the antigen CD19+, a leukemic blast marker, a pan leucocyte marker CD45, T specific antigen CD7 or CD3, a myeloid specific marker CD33 (see table 1). We analyzed a total of 67 patients at diagnosis (56 common, 7 pro B, 4 pre B) and 22 controls (18 Stop therapy, 4 maintenance). Data are given as mean fluorescence intensity of CD254 gated on CD19+ cells. CD19/CD254 double positive cells of control patients where compared to CD19/CD254 double positive cells from patients at diagnosis (Figure 4 A).

CD19+ cell of patients at diagnosis showed a heterogeneous but significantly higher level of membrane RANKL expression compared to control CD19+ bone marrow cells. The highest RANKL expression was associated to the Common B ALL types rather than to the other leukaemia subpopulations (pro and pre B diagnosis), in which RANKL expression was not significantly higher if compared to controls (data not shown).

CD19+ PB and BM membrane RANKL expression was not different, showing a similar pattern of expression between the two compartments (7 Common diagnosis were analyzed) (Figure 4 B).

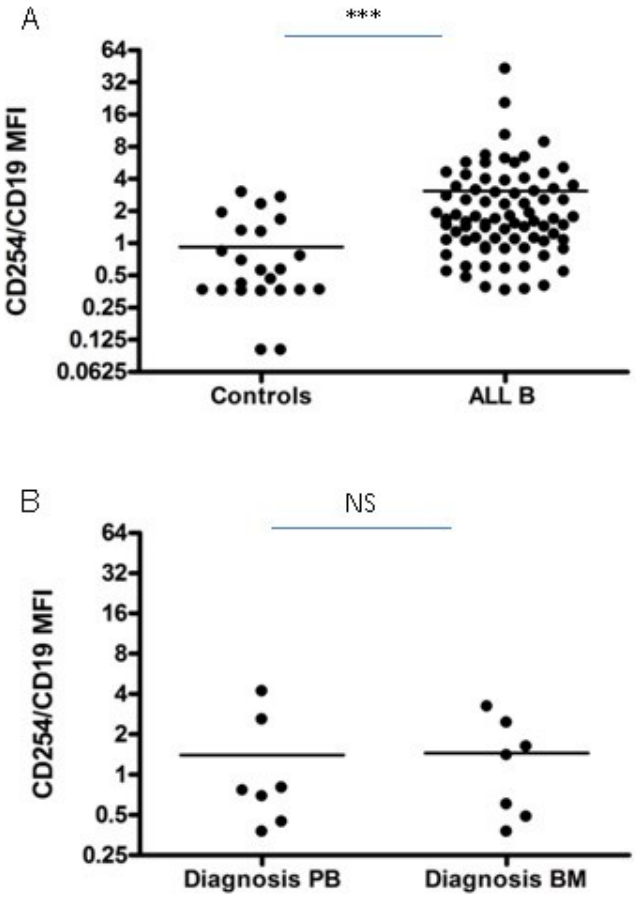

Figure 4 A and B: **RANKL surface expression on BM cells from patients at diagnosis.** 67 Diagnosis (56 B ALL Common, 7 B ALL pro B, 4 pre B ALL) and 22 controls (18 Stop therapy, 4 maintenance) were investigated by flow cytometry using the RANKL monoclonal antibody (mAb) MIH24. Malignant and healthy B-lymphocytes cells were gated based on CD19. (A) Controls versus B ALL patients at diagnosis, including common, pre and pro B subtypes. (B) PB and BM CD254 expression of patients at diagnosis (7 Common). Statistical analysis was performed using the Mann–Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

RANKL expressing B leukaemia cell line SEM could induce differentiation of PBMC CD14+ into resorbing osteoclasts.

Our previous data showed that RANKL membrane expression was significantly up regulated in patients at diagnosis compared to healthy controls.

We thus ask if the expression of membrane RANKL was able to influence the cells involved in the bone metabolism, specifically the osteoclasts which carries on their membrane RANK, the receptor for the ligand CD254.

In order to assess the ability of leukemic cells to influence osteoclasts precursor and their ability to differentiate into multinucleated cells and digest bones, we used SEM leukaemia cells line expressing RANKL, and we co cultured them with osteoclast precursor PBMC CD14+ cells on human bone slices for 7 days. After this period all the cells were removed and the bone slices were stained. In each co-colture experiment with SEM cell line we established as a negative control a well containing CD14+ cells only grown in maintenance media (MCS-F). The positive control was represented by the CD14+ cells grown in presence of soluble recombinant RANKL as inducer of the full differentiation of the precursor into resorbing osteoclasts. SEM cell line expressing membrane RANKL were co cultured with CD14+ cells in maintenance medium (MCS-F only), and their ability to influence CD14+ towards differentiation into mature OCs was evaluated by number of resorption pits in each well. The RANKL specificity in inducing resorption by OCs was evaluated by using the soluble RANKL inhibitor, OPG. Pits were analyzed by light microscopy and the number of pits in each well was counted using Image J. Experiment were performed in duplicate. Figure 5 is a representative image of the resorption pits as visualized at the microscope with the 40X magnification.

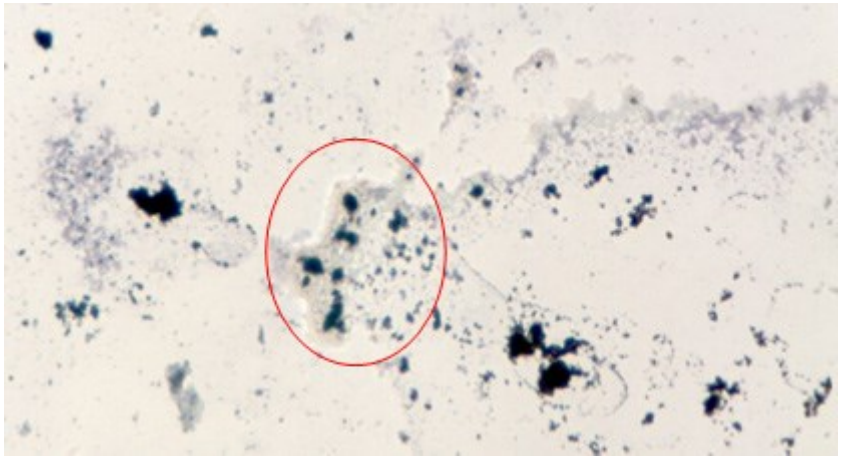

Figure 5: Representative image of resorption assay. Resorption pits and lacunae formation on a human bone surfaces (96 well, Lonza Osteoassay).

PBMC CD14+ co-colture with leukemic cell line SEM on bone plates.

PBMC CD14+ monocytes were incubated in the presence of RANKL and MCS-F (as described in materials and methods) on a human bone surfaces for 7 days, or in presence of leukaemia cell line SEM expressing membrane RANKL (MFI 6). We observed pits formation with light microscopy (magnification 40×). Resorption pits/well were analyzed and counted using Image J software.

PBMC CD14+ cells grown in presence of soluble recombinant RANKL showed a significant increase of resorption pits if compared to the untreated condition (Figure 6 A). The OPG treatment however was not significantly able to reduce the number of pits formed by CD14+ cells (Figure 6 B). CD14+ cells in co-colture with SEM cell line showed a higher number of resorption pits when compared to our untreated sample (Figure 6 C). CD14+ cells in co-colture with SEM and treated with OPG showed a reduction of pits number in a significant manner. Figure 6 shows a single experiment performed in duplicate and showed that this leukemia cell line expressing RANKL (MFI 6), could indeed induce the resorption of the bone substrate, a resorption that seemed to be dependent on RANKL, as OPG significantly reduced the pits number (Figure 6 D).

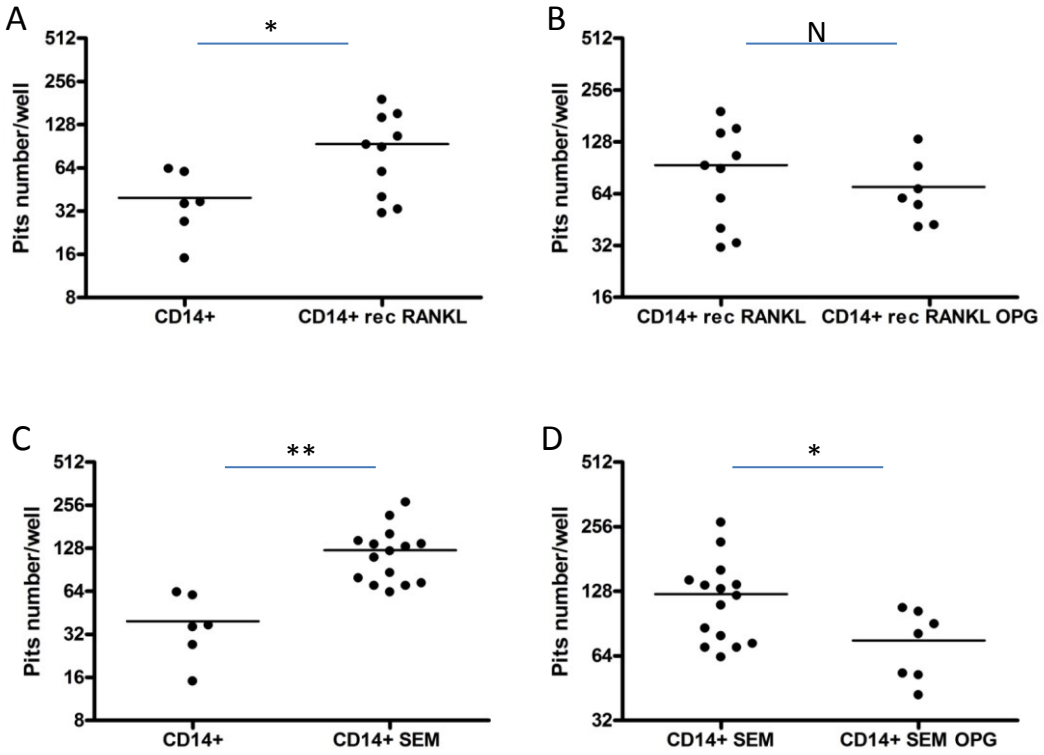

Figure 6 A-D: PBMC CD14+ and SEM leukaemia cell line co-colture on Osteo-assay human bone plate (single experiment). After cells removal resorption pits were stained with toluidine blue and counted using Image J. (A) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml, considered as negative control). These were compared to PBMC CD14+ cells grown in osteoclastogenic medium (MCS-F 20 ng/ml, soluble recombinant RANKL 50 ng/ml, considered as positive control), in order to assess the ability of PBMC CD14+ to differentiate in resorbing osteoclasts. (B) PBMC CD14+ cells grown in osteoclastogenic medium (MCS-F 20 ng/ml, soluble recombinant RANKL 50 ng/ml) were compared to PBMC CD14+ cells grown in osteoclastogenic medium (MCS-F 20 ng/ml, soluble recombinant RANKL 50 ng/ml) supplemented with recombinant OPG, to evaluate the osteoclastogenic dependence on RANKL cytokine. (C) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml) were compared to PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line, expressing membrane RANKL at high level (MFI: 6). (D) PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line were compared to PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line supplemented with recombinant OPG, to assess the RANKL dependent ability to induce osteoclasts activity by resorption pits formation. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

RANKL expressing SEM cells could induce differentiation of PBMC CD14+ into resorbing osteoclast.

The first co-colture experiment with leukemia cell line showed the ability of these cells to induce bone resorption. We then performed two more experiments the results of which are summarized in figure 7.

Figure 7 A-B shows the results of three different experiment performed co culturing SEM with CD14+ cells. SEM cell lines co-cultured with osteoclast precursor were able to increase in a significant manner the number of resorption pits (Figure 7 A), however the treatment with the RANKL inhibitor OPG did not significantly to reduce the number of resorption pits (Figure 6 B). The Figure 7 is the result of three different experiments, which were performed using SEM expressing different level of membrane RANKL. We can speculate that when the OPG is more abundant than the available RANKL, the soluble decoy receptor binds to other ligands expleting an anti apoptotic effect on mature OCs. Alternatively other pathways, different from RANKL, are involved.

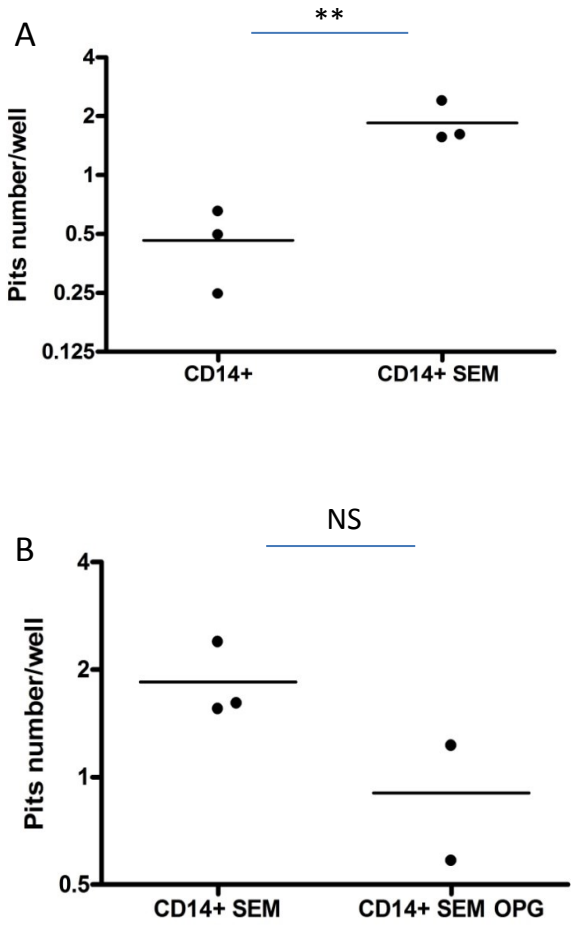

Figure 7 A and B: PBMC CD14+ cells in co-colture with SEM leukaemia cell line in three different experiments performed on Osteo-assay human bone plate and pits number evaluation. (A) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml) were compared to PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line, expressing membrane RANKL. (B) PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line and PBMC CD14+ cells grown in co-colture with SEM leukaemia cell line supplemented with recombinant OPG, were compared to assess the RANKL dependent ability to induce resorption pits formation. Values were normalized to the positive control (resorption pits number in osteoclastogenic media). Statistical analysis was performed using the Unpaired T test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

RANKL induced multinucleation of PBMC CD14+ cells in chamber slide colture system.
We next wanted to test the ability of primary bone marrow leukemic cell to influence the osteoclast precursor PBMC CD14+ cells. In order to do that we performed co-colture experiment in chamber slides with the aim to understand if leukemic cells expressing membrane RANKL can induce fusion of single PBMC CD14+ cells into giant multinucleated cells.
The figure 8 shows the detail of a mature multinucleate osteoclast after TRAP staining (more than 3 nuclei can be visualized).

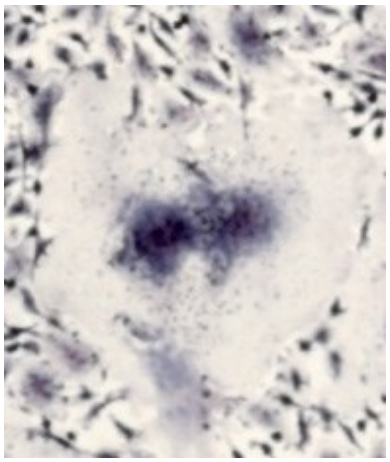

Figure 8: representative image of a multinucleated cell derived from PBMC CD14+ cells treated with recombinant RANKL. Detail adapted from figure 9 B.

Figure 9 A-D is an example of TRAP staining of PBMC CD14+ osteoclast precursors grown in chamber slides. Recombinant human soluble RANKL in the colture the media induced multinucleation of PBMC CD14+ cells (Figure 9 C). OPG treatment completely abolished that fusion in to polykaryon of PBMC CD14+ cells (Figure 9 B and D).
Figure 10 A-D shows an example BM cells from patients at diagnosis and control patient in cocolture with PBMC CD14+ cells. TRAP staining was used to evaluate the ability to induce multinucleation of PBMC CD14+ cells. The OPG treatment reduced the multinucleation both in the diagnosis and controls.

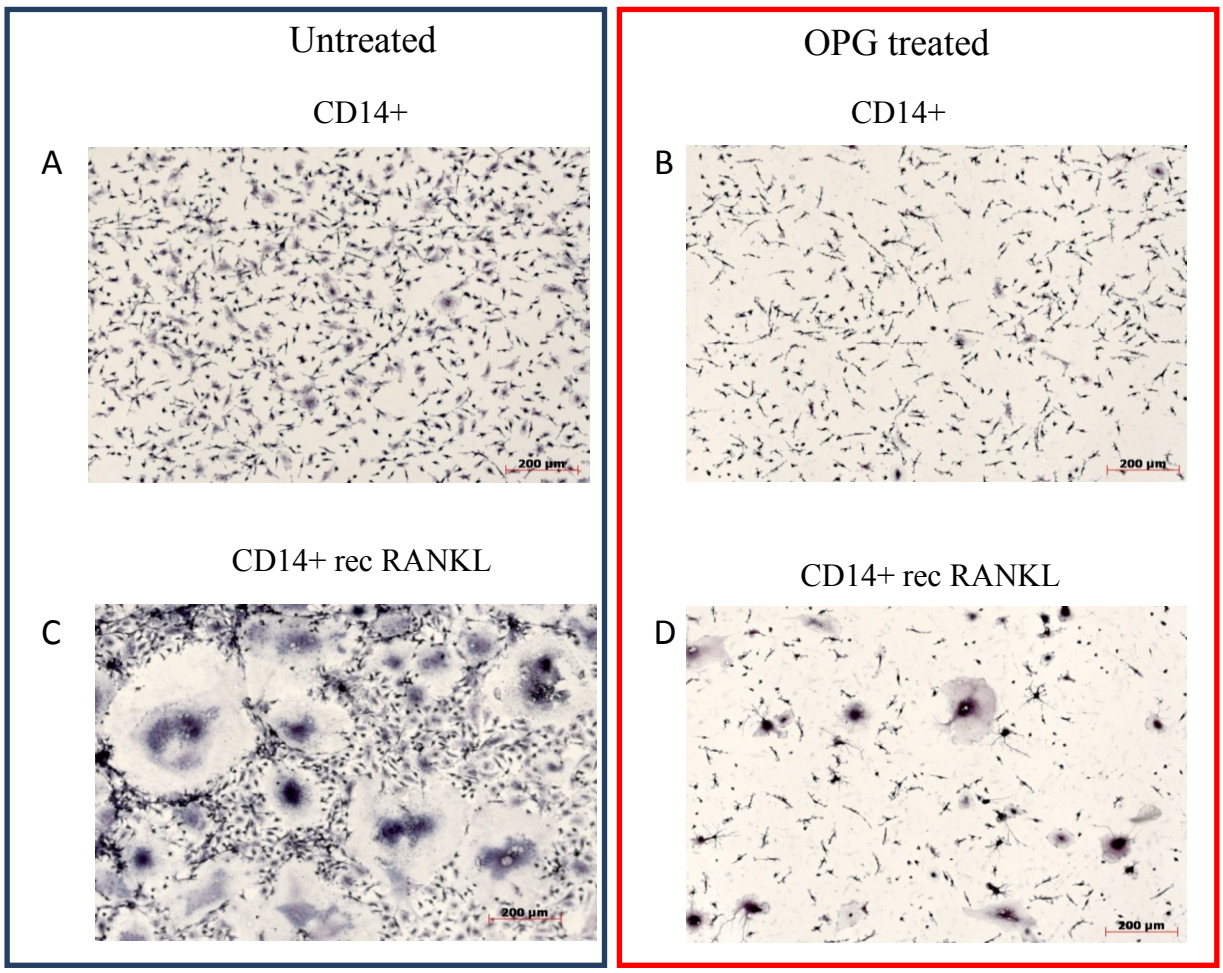

Figure 9 A-D: 20 x light microscopy representative images of PBMC CD14+ cultured in chamber slides. Tartarate-Resistant Acid Phosphatase (TRAP) was used to stain osteoclasts cells, which are TRAP resistant and multinucleated (with three or more nuclei). (A) Only PBMC CD14+ mononuclear cells were observed in unstimulated PBMC cultures (negative control). (B) Only mononuclear cells were observed in unstimulated PBMC cultures treated with OPG. (C) Multinucleated cells from PBMC stimulated with MCS-F and RANKL. (C) No multinucleated cells from PBMC stimulated with MCS-F and RANKL were upon OPG treatment.

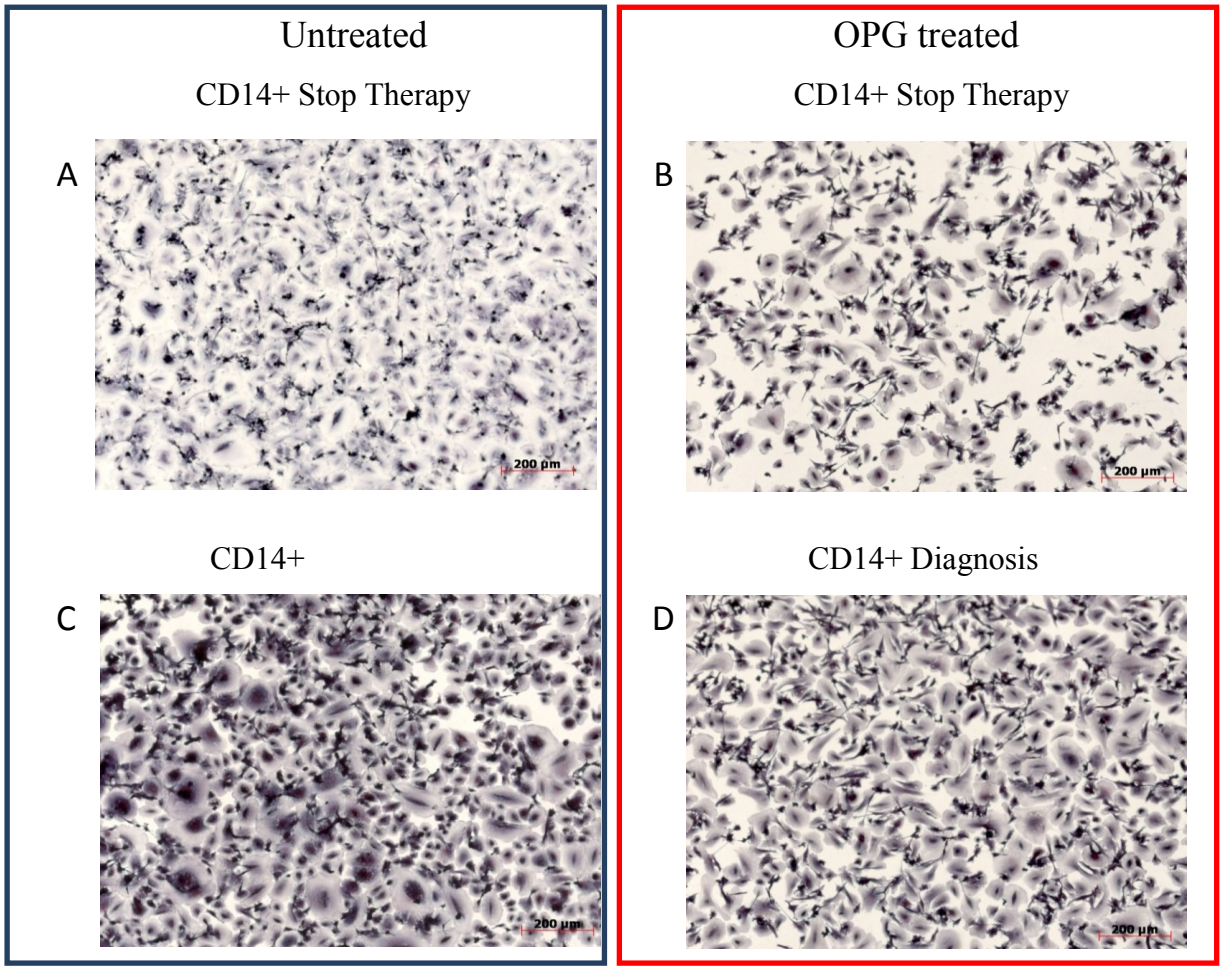

Figure 10 A-D: 20 x light microscopy representative images of PBMC CD14+ co-cultured with whole BM from control patients and diagnosis patients in chamber slides. Tartarate-Resistant Acid Phosphatase (TRAP) was used to stain osteoclasts cells, which are TRAP resistant and multinucleated (with three or more nuclei). (A) Only few multinucleated cells were observed in PBMC CD14+ co cultures with whole BM from a control patient. (B) Only mononuclear cells were observed in PBMC CD14+ co cultures with whole BM from a control patient treated with OPG. (C) Multinucleated cells were observed in PBMC CD14+ co cultures with whole BM from a diagnosis patient. (D) Reduced number of multinucleated cells from PBMC CD14+ co cultures with whole BM from a diagnosis patient treated with OPG.

Chamber slide PBMC CD14+ co-colture with diagnosis and control patients.

Figure 11 (A-H) represents the data from five different experiments performed using primary bone marrow cells from patients at diagnosis and controls. The membrane RANKL expression of diagnosis was variable (data not shown). For these experiments we considered all patients independently from their RANKL expression. In summary there were no significantly differences between our positive and negative controls (Figure 11 A). No differences between our negative control and our diagnosis (Figure 11 B). Similarly there were no differences between our diagnosis and controls (Figure 11 C). However the number of multinucleate cells in our positive control and in our diagnosis was increased compared to the negative. Furthermore the percentage of multinucleate cells in CD14+ co-colture with diagnosis was higher than the one with control patients (Figure 11 D). OPG treated co-colture percentage of multinucleate cells of diagnosis was significantly reduced compared to diagnosis not treated with the inhibitor (Figure 11 G).

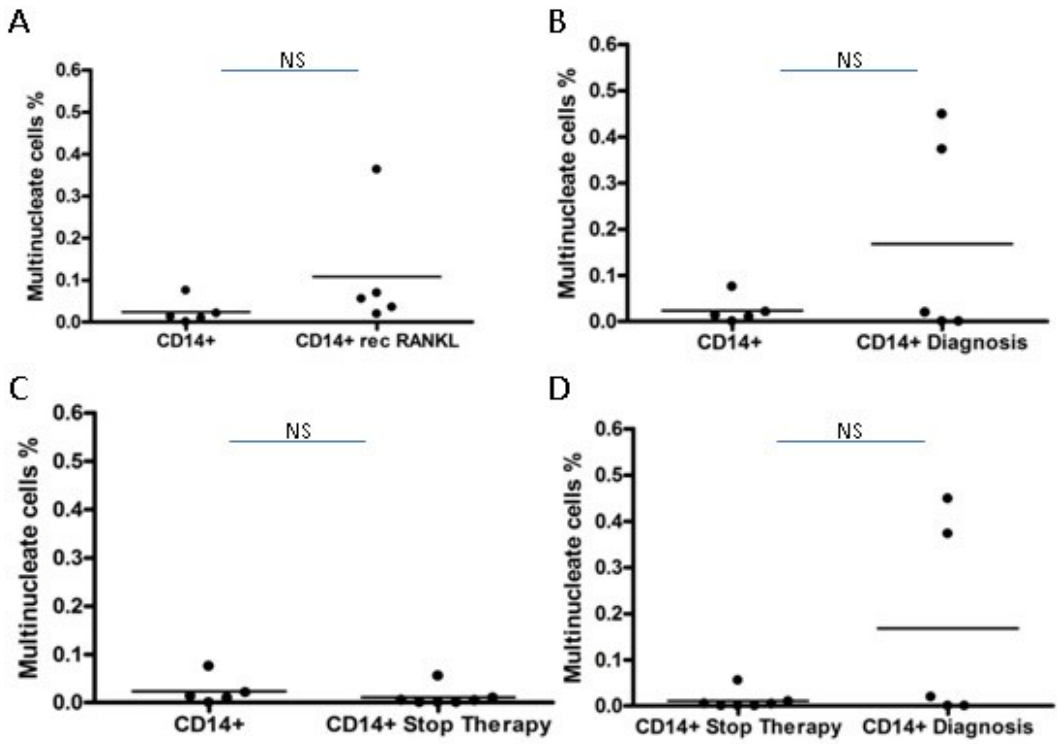

Figure 11 (A-D): 5 different experiment of PBMC CD14+ co cultured with whole BM from control patients and diagnosis patients in chamber slides and showing different membrane RANKL expression. (A) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml, considered as negative control). These were compared to PBMC CD14+ cells grown in osteoclastogenic medium (M-CSF 20 ng/ml, soluble recombinant RANKL 50 ng/ml, considered as positive control). (B) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml) were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis. (C) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml) were compared to PBMC CD14+ cells grown in co-colture with controls patients. (D) PBMC CD14+ cells grown in co-colture control patients were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis. A minimum of 200 cells was counted per each well. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

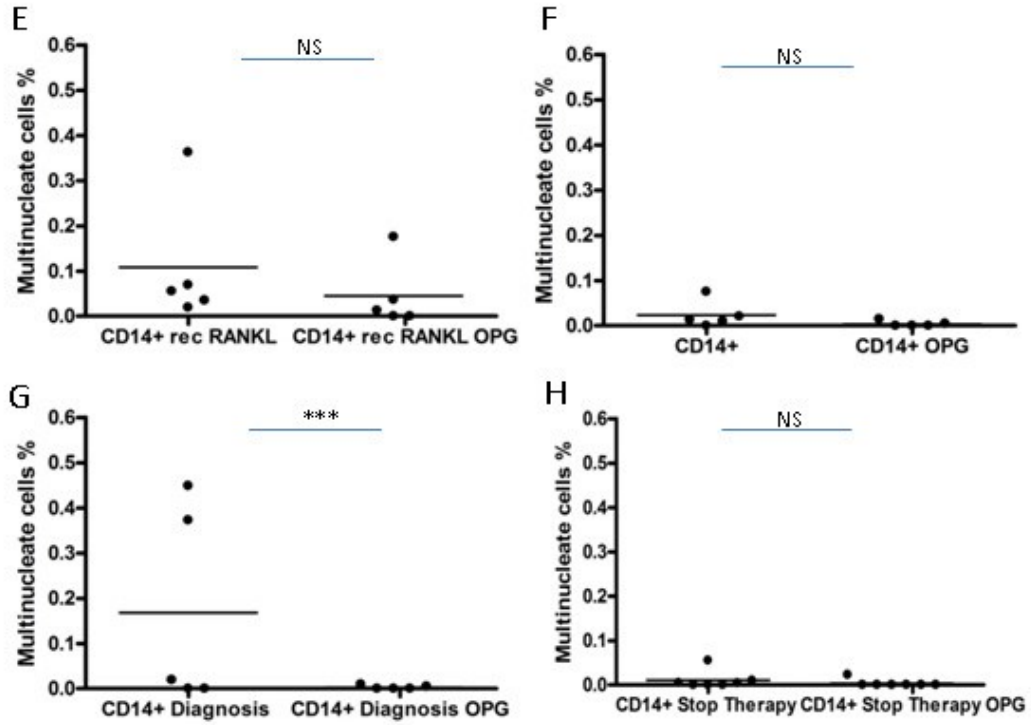

Figure 11 (E-H): 5 different experiment of PBMC CD14+ co cultured with whole BM from control patients and diagnosis patients in chamber slides and showing different membrane RANKL expression. (E) PBMC CD14+ cells grown in osteoclastogenic medium were compared to PBMC CD14+ cells grown in osteoclastogenic medium in presence of OPG. (F) PBMC CD14+ cells grown in maintenance medium were compared to PBMC CD14+ cells grown in maintenance medium in presence of OPG. (G) PBMC CD14+ cells grown in co-colture with patients at diagnosis were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis in presence of OPG. (H) PBMC CD14+ cells grown in co-colture with control patients were compared to PBMC CD14+ cells grown in co-colture with control patients in presence of OPG. A minimum of 200 cells was counted per each well. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

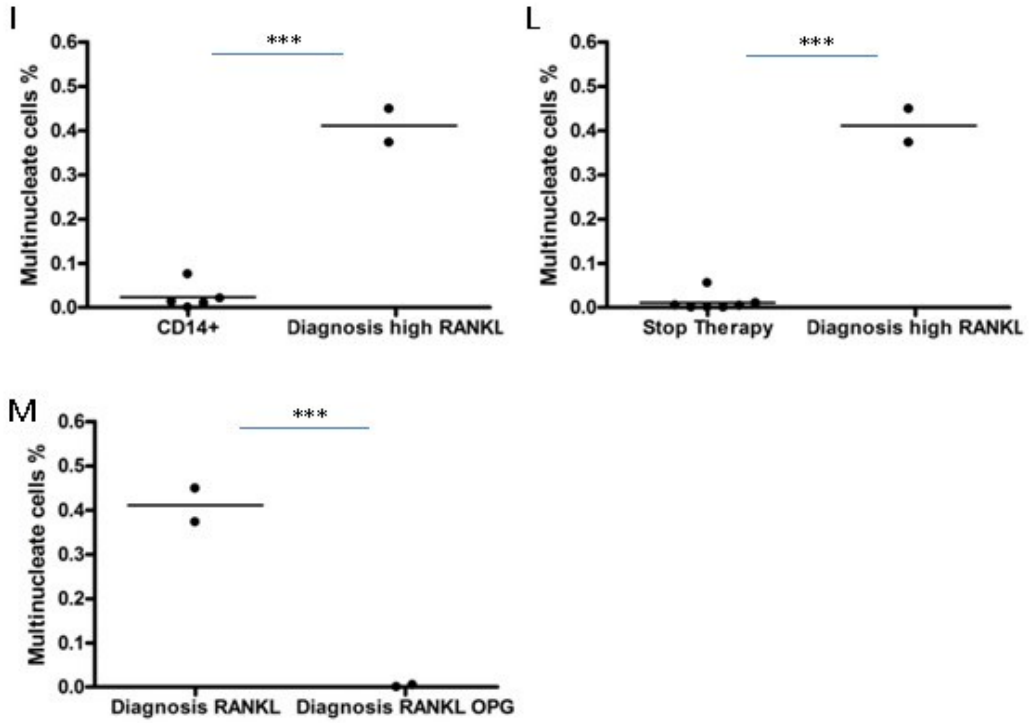

Figure 11 (I-M): PBMCs CD14+ co cultured in chamber slides with whole BM from control patients and diagnosis patients showing high membrane RANKL expression (2 different experiments). (I) PBMC CD14+ cells grown in maintenance medium were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis expressing high membrane RANKL. (L) PBMC CD14+ cells grown in co-colture with control patients were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis expressing high membrane RANKL. (M) PBMC CD14+ cells grown in in co-colture with patients at diagnosis expressing high membrane RANKL were compared to in co-colture with patients at diagnosis expressing high membrane RANKL in presence of OPG. A minimum of 200 cells was counted per each well. Statistical analysis was performed using the Unpaired T test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

High RANKL membrane expression diagnosis blast cells induced multinucleation of PBMC CD14+ cells in chamber slides co-colture system.

The positive correlation between higher membrane RANKL expression and the percentage of multinucleate cells is shown in figure 12 A, which includes all patients involved in this experiment, both controls and diagnosis.

High and low RANKL expressing BM cells from diagnosis were compared. We found there was a significant increase in the percentage of multinucleate cells in high RANKL expressing patients (Figure 12 B).

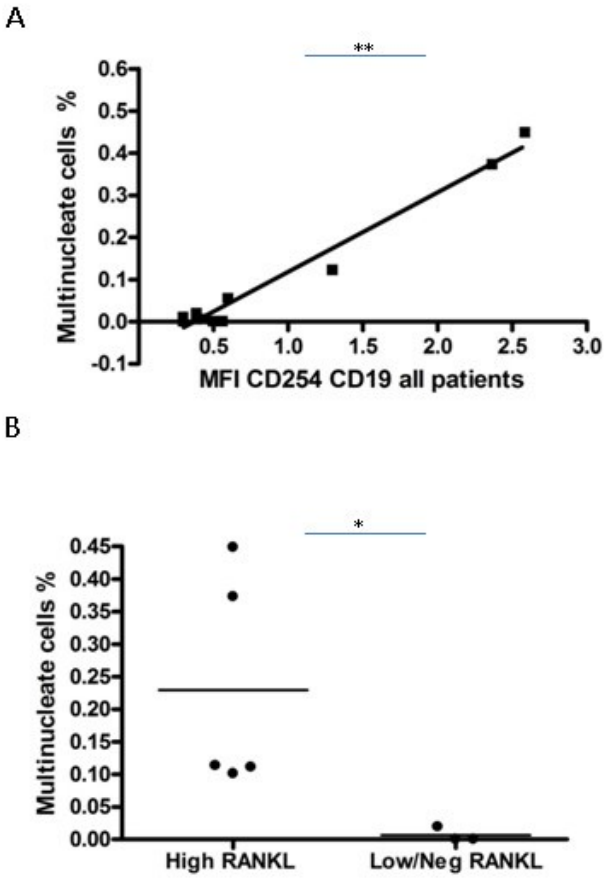

Figure 12 A and B: (A) Correlation MFI CD25/CD19 and multinucleation in patients grown in chamber slide co-coltures (7 Stop therapies, 5 diagnoses). The MFI of CD254 gated on CD19+ cells significantly correlates with the number of multinucleate cells (Spearman positive correlation). (B) Multinucleate cells generated from PBMC CD14+ co-colture with BM of patients at diagnosis (chambers co-colture) and grouped according to their RANKL expression. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

RANKL expressing blasts cells can induced multinucleation of CD14+ precursor in bone plates.

In order to confirm our analysis of B ALL cells ability to influence PBMC CD14+ cell towards osteoclast differentiation we performed co-colture experiment using bone slices substrates.

For this purpose we used bone marrow from patients high membrane RANKL expression (MFI between 2,5 and 7) and from a control patient (3 different experiments made in triplicate or duplicate).

Figure 13 A-H summaries the results. The percentage of multinucleate cells in the untreated co-colture was significantly lower compared to RANKL stimulated cells. The untreated PBMC CD14+ cells showed a significant lower percentage of multinucleate cells compared to diagnosis patients. Co-colture with diagnosis showed significantly higher number of multinucleate cells compared to controls patients. The OPG treated co-colture showed a reduction of multinucleate cells in all cases analyzed but none of them were significantly affected by the OPG treatment. Once again we saw that the OPG inhibitor of RANKL does not always work on arresting PBMC CD14+ differentiation towards osteoclast lineage. Further analyses are required.

Of note, for these experiment we used 2 BMs from patients with known bone involvement, one of them was suffering for extensive bone lesions at the time of the therapy. This patients was showing the highest RANKL membrane expression among the one here analyzed.

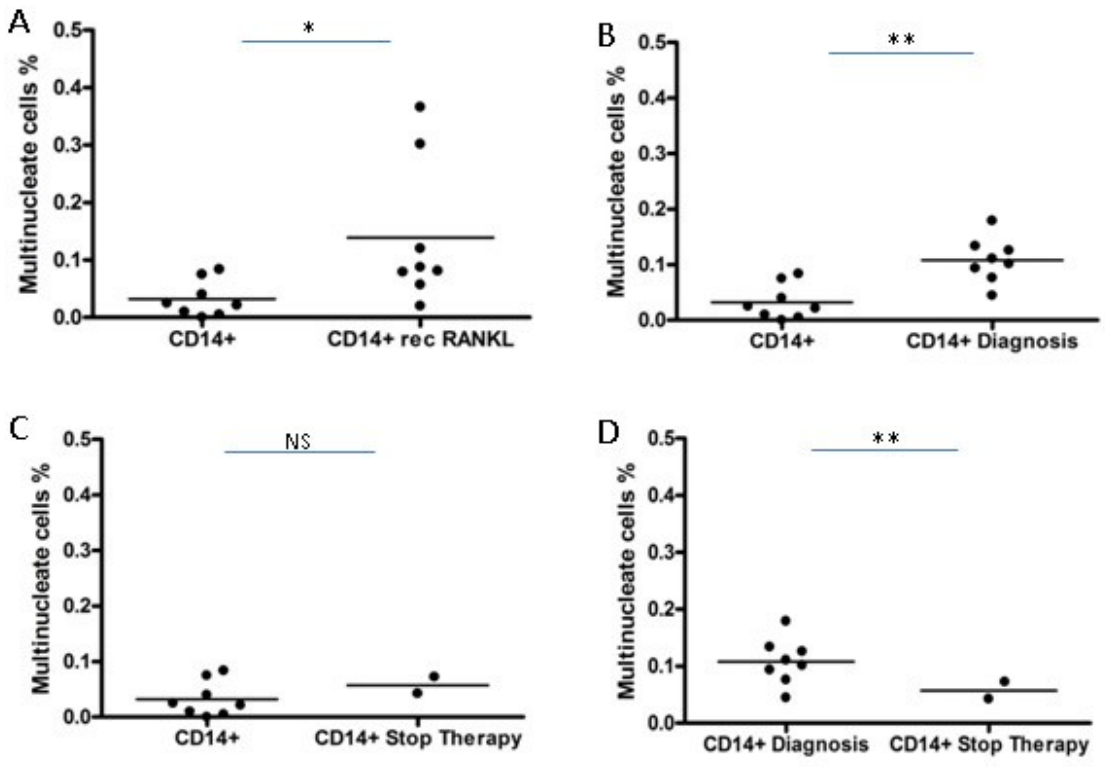

Figure 13 (A-D): PBMC CD14+ co cultured in bone plates with whole BM from control patients and diagnosis patients showing membrane RANKL expression (3 different experiments). (A) PBMC CD14+ cells grown in maintenance medium (MCS-F 20ng/ml, considered as negative control). These were compared to PBMC CD14+ cells grown in osteoclastogenic medium (M-CSF 20 ng/ml, soluble recombinant RANKL 50 ng/ml, considered as positive control). (B) PBMC CD14+ cells grown in maintenance medium were compared to PBMC CD14+ grown in co-colture with patients at diagnosis with different RANKL expression (see table). (C) PBMC CD14+ cells grown in maintenance medium were compared to PBMC CD14+ cells grown in co-colture with control patients. (D) PBMC CD14+ grown in co-colture with patients at diagnosis with different RANKL expression were compared to PBMC CD14+ cells grown in co-colture with control patients. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

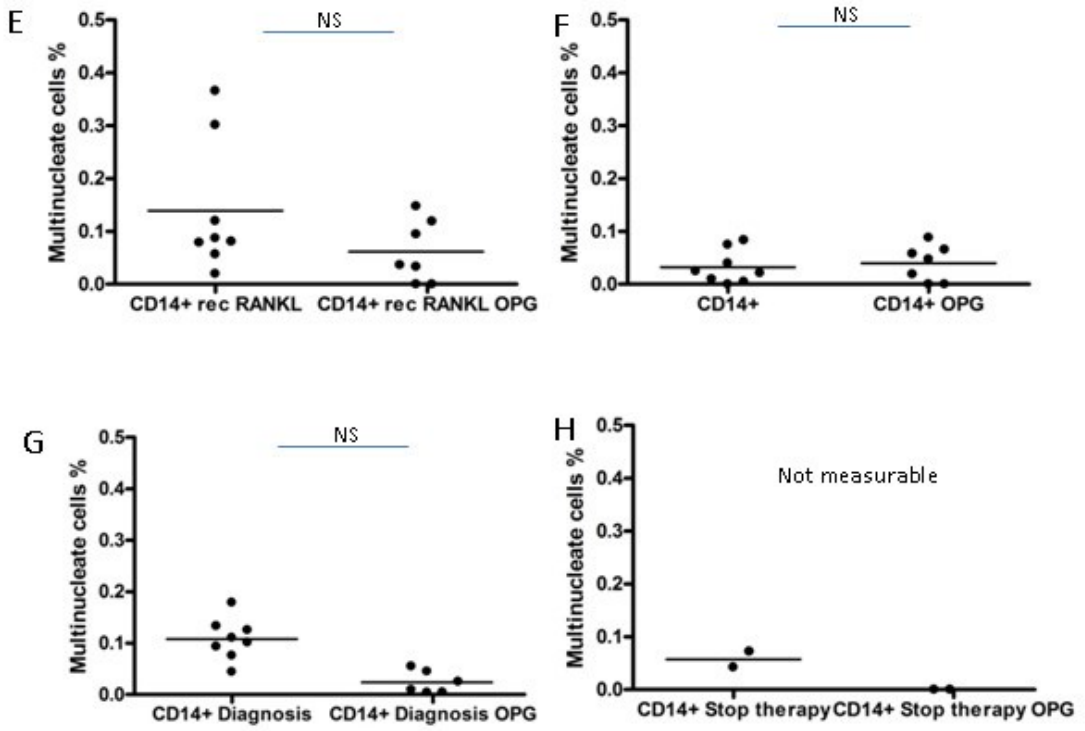

Figure 13 (E-H): PBMC CD14+ co cultured on bone plates with whole BM from control patients and diagnosis patients showing membrane RANKL expression (3 different experiments). (E) PBMC CD14+ cells grown in osteoclastogenic medium were compared to PBMC CD14+ cells grown in osteoclastogenic medium in presence of OPG. (F) PBMC CD14+ grown in maintenance medium were compared to PBMC CD14+ grown in maintenance medium in presence of OPG. (F) PBMC CD14+ cells grown in osteoclastogenic medium (M-CSF 20 ng/ml, soluble recombinant RANKL 50 ng/ml were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis expressing RANKL. (G) PBMC CD14+ grown in co-colture with control patients were compared to PBMC CD14+ cells grown in co-colture with patients at diagnosis expressing RANKL. (H) PBMC CD14+ grown in co-colture with control patients were compared to PBMC CD14+ grown in co-colture with control patients and treated with OPG. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

Gene expression profile of B ALL patients.

Down regulation of genes involved in osteoclastogenesis.

With the attempt to understand if blast cells behave as stromal like cells, supporting OCs as seen from the above results, or as OCs precursor themselves, we performed a gene profile analysis. RANKL can be involved in three different pathways, as a ligand in the in B cell lymphoiesis, and in osteobalstogenesis pathway or can be involved through RANK receptor in the osteocalstogeneisis.

We analyzed the osteoclastogenic and osteoblastogenic and the B cell differentiation pathway pathways derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Surprisingly we found that there were no differences between BM of control patient and diagnosis of B ALL concerning the osteogenic and B lymphopoiesis pathways (data not shown), however there were for the osteoclastogenic one. We found that control patients and diagnosis clustered and that in the B ALL cohort here analyzed the osteoclastogenic pathway was down regulated. Figure 14 A shows the heat map and in the red boxes are genes differentially expressed. As shown by the Search Tool for the Retrieval of Interacting Genes **(**STRING) analysis those genes are interacting partner of this pathway (Figure 14 B).

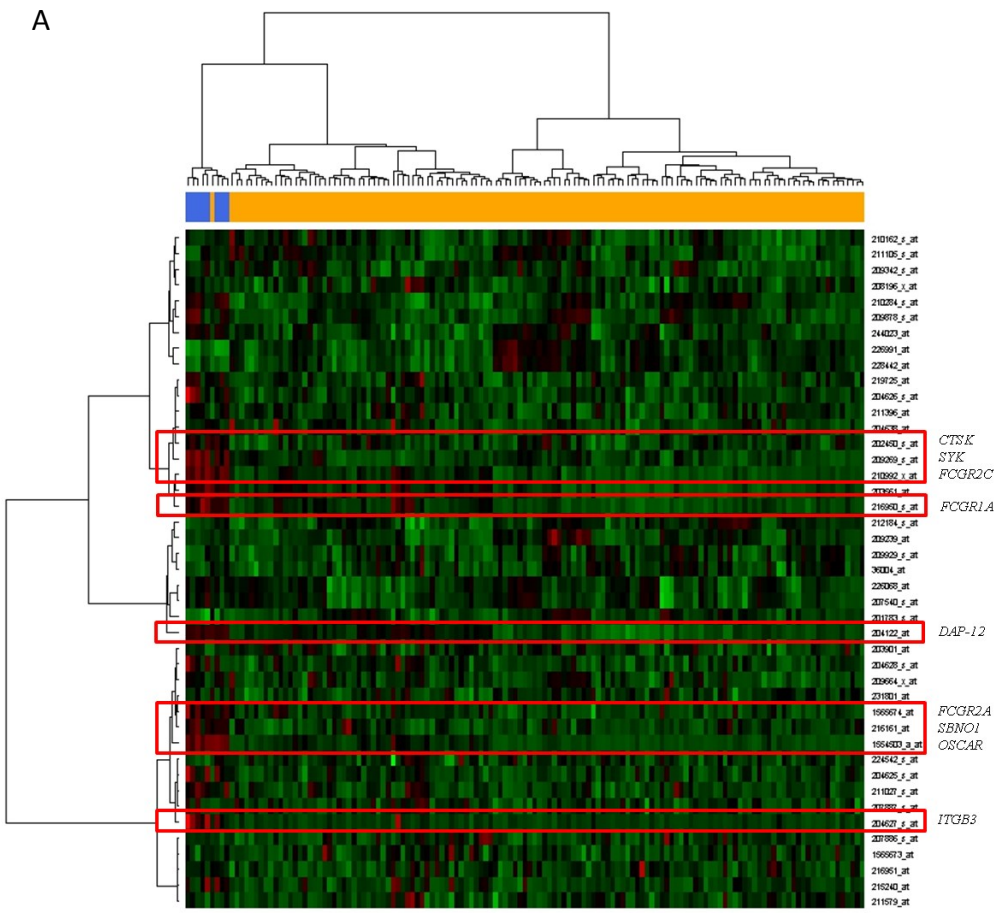

Figure 14 A and B: Osteoclastogenesis KEGG derived pathway and interacting proteins. (A) Heat map of genes involved in osteolysis pathway derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Unsupervised hierarchical clustering analysis using Euclidian distance and Ward methods were used to assess the different expression profiling of these genes in B-ALL patients and healthy donors. Genes differentially expressed are highlighted in red. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

B

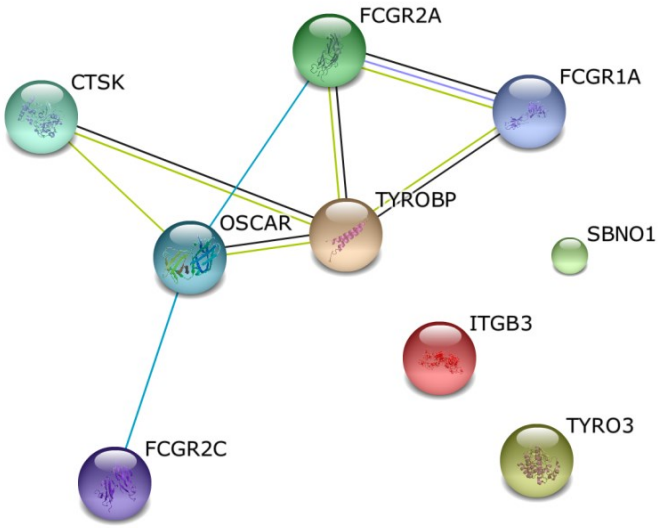

Figure 14 (B): Search Tool for the Retrieval of Interacting Genes (STRING). Protein-protein interaction of genes differentially expressed according to gene expression profile and built using the STRING.

Genes involved in osteoclasts specific metabolism and in osteopetrosis disease.

In order to understand if the down regulation was associated to a defect of osteoclast like genes and not to genes that could also be involved in B cell differentiation (as DAP12 and SYK), we performed GEP analysis on a group of genes that are involved in osteoclasts maturation differentiation and eventually in bone disease associated to not functional osteoclasts (Coudert *et al.,* 2014), Figure 15.

Interestingly we found a strong separation in clusters of control patients versus diagnosis. This suggests that the alteration of osteoclast specific genes have a role in B ALL etiology that requires further analysis, as it will be discussed in the next sections.

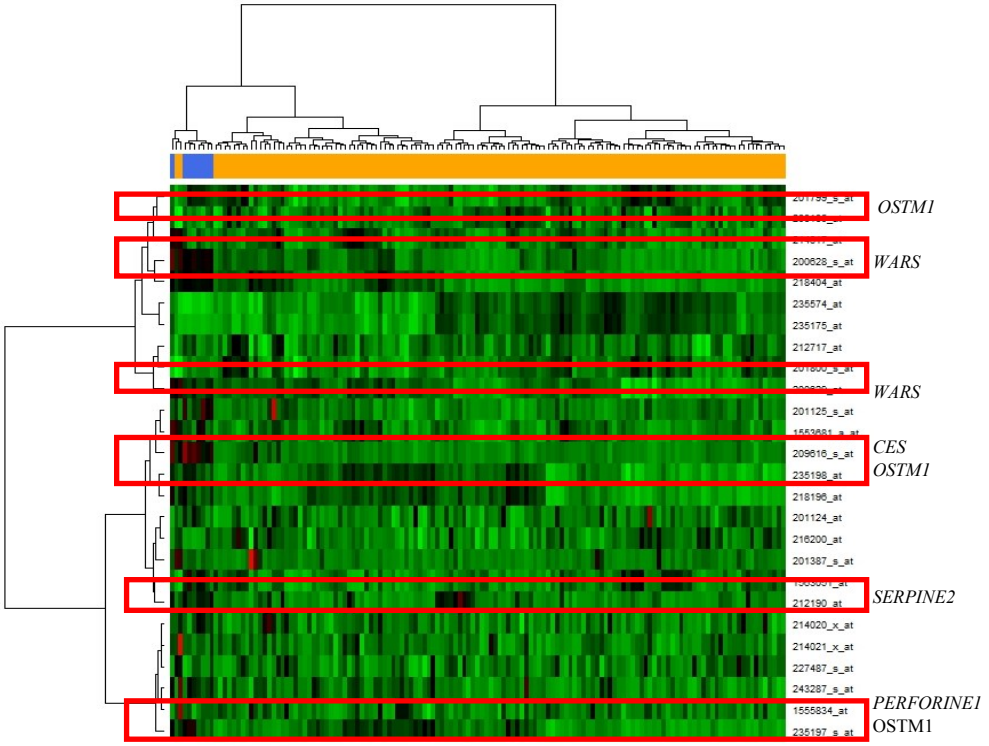

Figure 15: Heat map of genes with known osteoclastsic functions and involved in osteopetrosis. Unsupervised hierarchical clustering analysis using Euclidian distance and Ward methods were used to assess the different expression profiling of these genes in B-ALL patients and healthy donors. Genes differentially expressed are highlighted in red. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

Plasma EVs from B ALL patients at diagnosis and controls.

Flow cytometry characterization of Extracelular Vescicles membrane antigens.

Our co-colture experiment results showed that B ALL blats cells with high membrane RANKL expression could influence the OCs precursor through a pro osteoclastic phenotype. We could not address if the effects mediated by RANKL was direct, in contact – contact mode, or indirect by release of soluble factors.

Here we studied the cell-cell communication through EVs, and we characterized EVs from peripheral blood plasma of patients at diagnosis, and from peripheral blood and bone marrow plasma of control patients.

We isolated EVs from the peripheral blood (PB) plasma of untreated B ALL patients at diagnosis as well as healthy subjects (Stop therapy and maintenance). The isolation protocol was adapted from a previous work on chronic lymphocytic leukaemia (CLL) (Ghosh *et al.,* 2010), with minor modifications. In order to analyze EVs at the flow cytometry, we used the cytoplasmic dye CFSE that allows the selection of intact membrane bodies containing cytoplasm. Our EVs gate was also defined on the basis of forward/side scatter with the use of 3 μm beads and were considered positive events the ones falling in the in the range of 300-800 nm. This allowed us to exclude the eventual residual cells or platelets that could have left behind the plasma platelet free preparation (Figure 16 A and B).

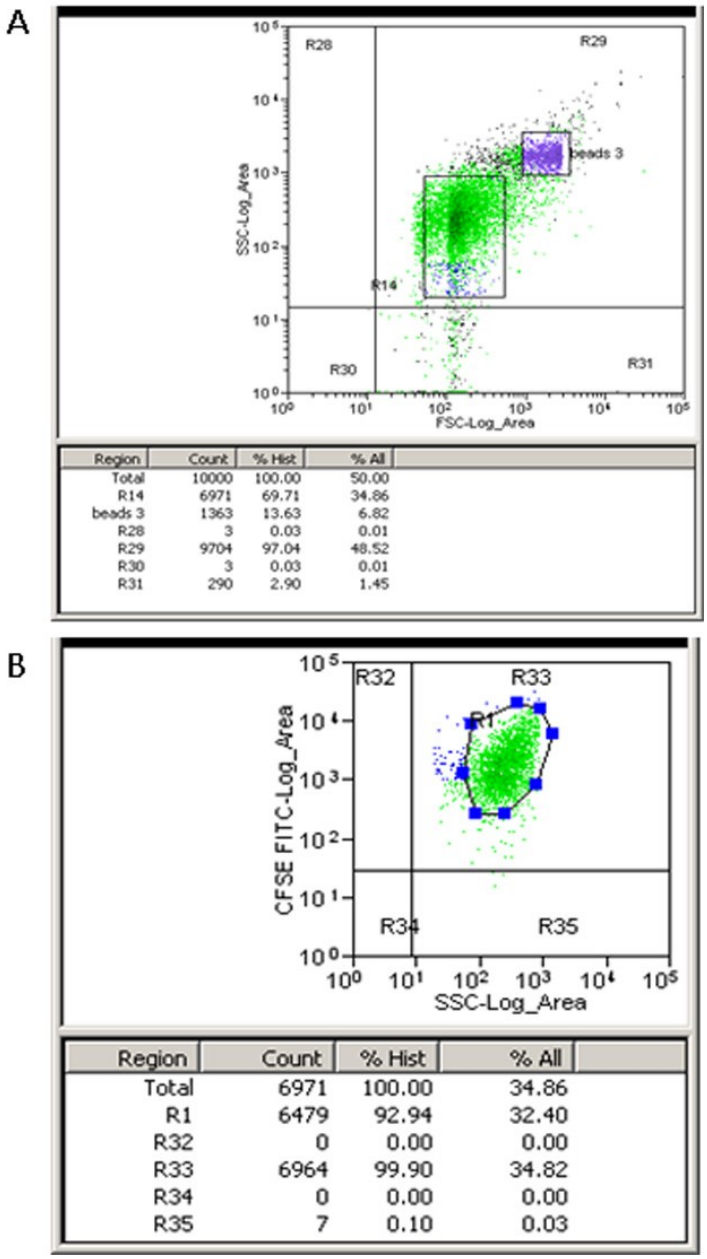

Figure 16 A and B: (A) Representative flow cytometry dot plots of EVs physical properties. (A) EVs isolated from PB of a patient at diagnosis and characterized morphologically. EVs gate according to Side Scatter (SSC) and Forward Scatter (FSC), and polystyrene beads used for size definition (3 μm). The scattergram shows the events detected in the gate from a sample re-suspended in PBS and containing beads. Beads number is standard and the same per each test tube and serve to number EVs using the bead count assay. (B) Identification of CFSE labeled EVs only, in the FITC- fluorescence channel.

Atomic Force Microscope: size overview

Atomic force microscopy showed that the EVs isolated are heterogeneous population with the majority ranging in size from 300 nm to 500 nm.

Figure 17 (A-C) are representative images taken with the AFM in both contact (A-B) and non-contact mode (C). Analysis were performed on EVs form PB and BM of controls and patients at diagnosis. The first two figures were taken on a BM plasma sample of EVs from diagnosis patients in contact mode. Figure A shows a typical population as seen in AFM. Figure B shows the measure of the isolated EVs. Figure 17 C representative image in contact mode of the EVs shape. AFM microscopy was performed to confirm that we are isolating membrane vesicles rather than cell debris or immune-complexes. The size and the shape matched with our expectations.

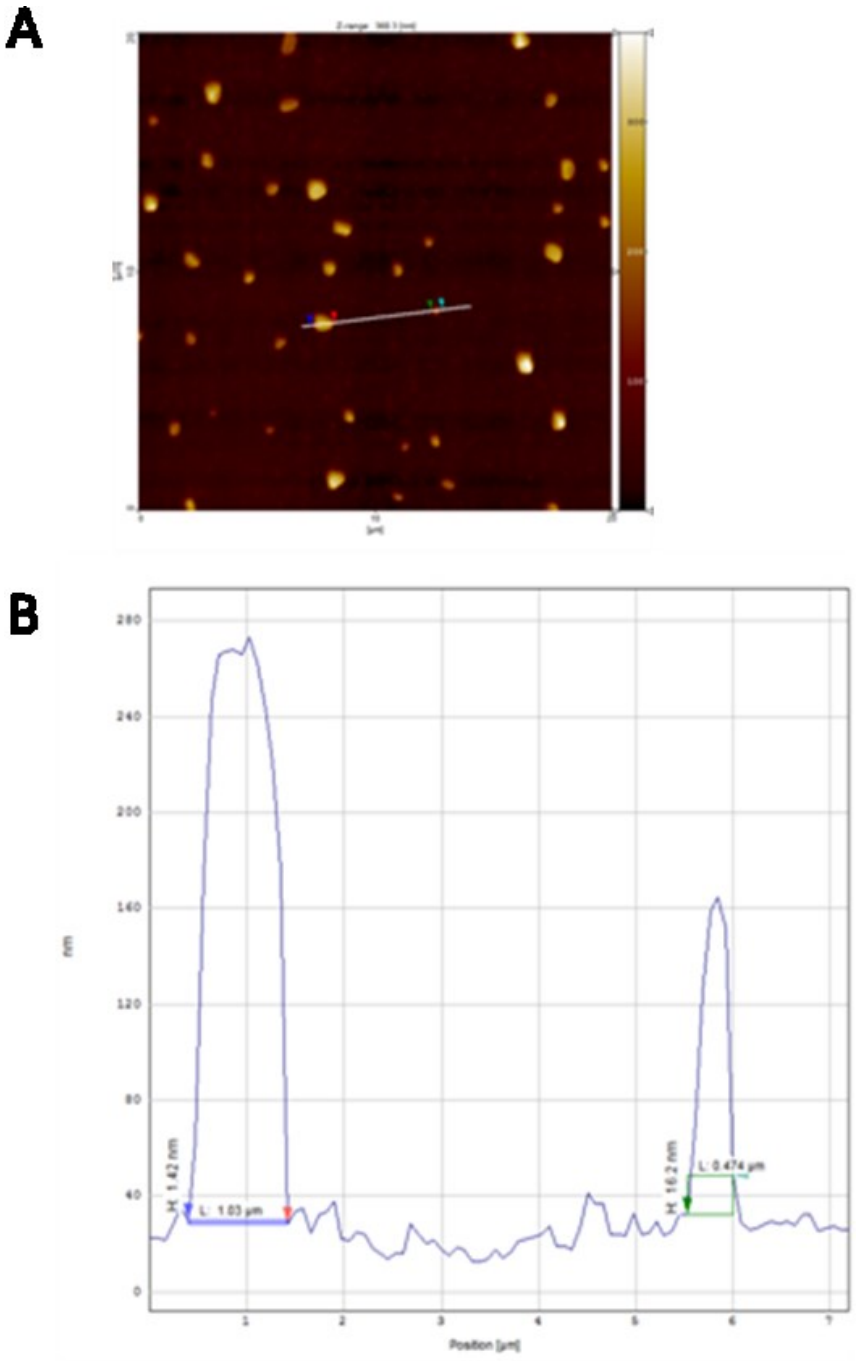

Figure 17 A - C: AFM representative imaging of isolated EVs. (A) AFM imaging in 20 µm x 20 µm scan. All measurements were performed in air and in contact mode with constant force. Image resolution was 256 pixels x 256 pixels, the set point force was 5 pN and the scan rate was 1 Hz.
(B) AFM imaging measured profiles taken on 20µmx20µm scans. Profiles are indicative of the EVs height and width. The representative range of our isolated population is between 100 nm and 300 nm.

C

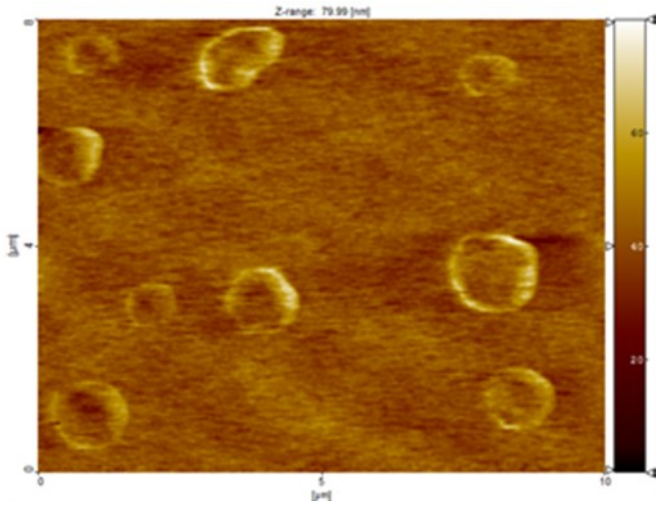

Figure 17: (C) AFM imaging in non-contact mode: representative image of 10umx8um scan on EVs, taken in. non-contact mode. Only shape was considered in this picture.

EVs surface antigen characterization by flow cytometry.

In the normal plasma the majority of EVs originates from platelets (80%), and only about 10% are represented by leukocytes EVs (George *et al.,* 1982; Caby *et al.,* 2005). In chronic lymphocytic leukaemia most the EVs are produced by CD19+ blasts (Ghosh *et al.,* 2010).

We used CD19 as marker for EVs derived from blasts and normal B cell, CD61 marker for platelet/megakaryocyte and CD254 as bone specific marker (Figure 18).

We considered the CFSE positive events calculated as described in material and methods, as the total population irrespectively of the surface markers. We found that significant greater levels of EVs were present in the plasma of our control patients compared to leukemic patients ($P<0.001$). The analysis of the CDs markers was performed gating on CFSE+ EVs.

CD61 was highly expressed in control patients, and was significantly lowered in leukemic patients at diagnosis ($P<0001$), as described before. Interestingly CD254 EVs were present in the control plasma and significantly increased compared to diagnosis. In the controls the numbers suggest that most of the CD61 are also CD254 positive. Other cell populations can produce CD254+ EVs, but our antigen panel does not allow knowing which ones. Due to the heterogeneous CD19 expression in our controls, there was no difference with the diagnosis but the trend is negative, with low EVs CD19+ at diagnosis compared to healthy subjects. In control patients' most part of CD19+ EVs population is also CD254 positive. At diagnosis there is a minor population CD19+ CD254 double positive. This confirmed that blasts cells might not be the major source of EVs in the plasma of patients at diagnosis, and it is consistent with the observation of the BM cells, as it is described in the next section.

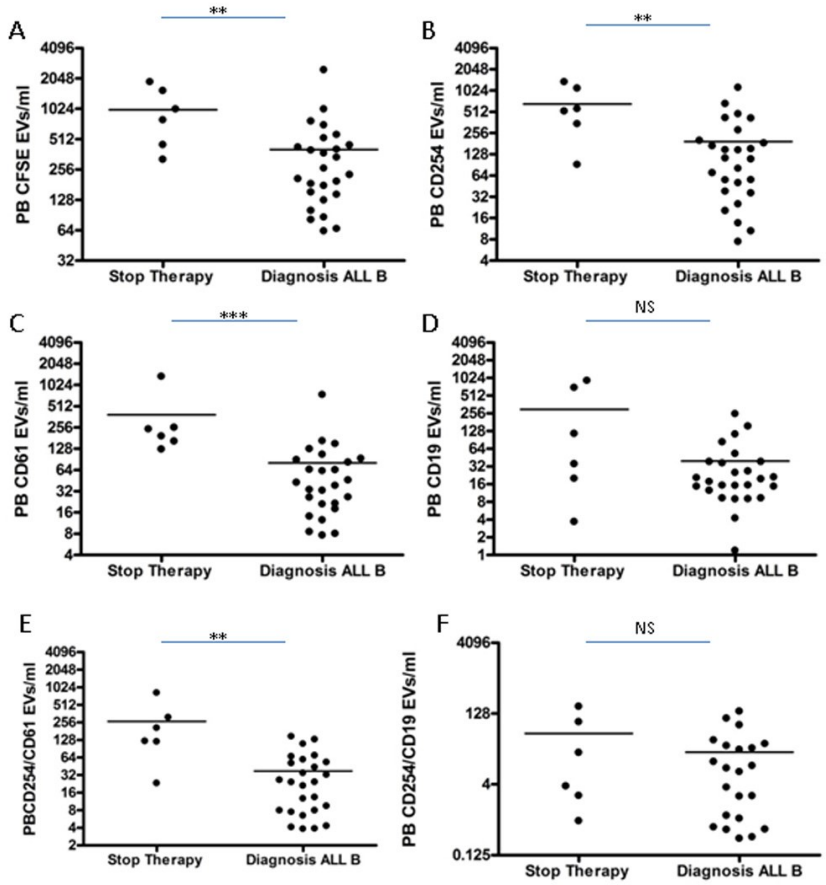

Figure 18 A-F: Characterization of CDs on PB plasma EVs. (A) Cytoplasmic dye CFSE was used to characterize the overall number of EVs in control patients (6 stop therapy) versus diagnosis patients (20 B ALL Common, 3 pre B ALL, 2 pro B ALL, 1 relapse). (B) CD254 bone marker expression was evaluated in EVs CFSE+ from control patients and compared to diagnosis patients. (C) CD61 megakaryocytes/platelets marker expression was evaluated in EVs CFSE+ from control patients and compared to diagnosis patients. (D) CD19 leukaemia blasts and B-lymphocytes expression was evaluated in EVs CFSE+ from control patients and compared to diagnosis patients. (E-F) Double positive CD254/CD61 and CD254/CD19 EVs population analysis: controls versus diagnosis patients. Statistical analysis was performed using the Mann–Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

Bone Marrow and peripheral blood compartments: EVs in control patients.

The same analysis of the PB markers described before was performed on plasma from bone marrow (BM) in control patients only (Figure 19). Similarly to the PB, the EVs from BM shows that the most represented populations are CD254+ and CD61+ positives. Moreover the overall number of EVs in BM is significantly higher compared to PB. The numbers suggests that the EVs observed in the PB derive from bone marrow parental cells.

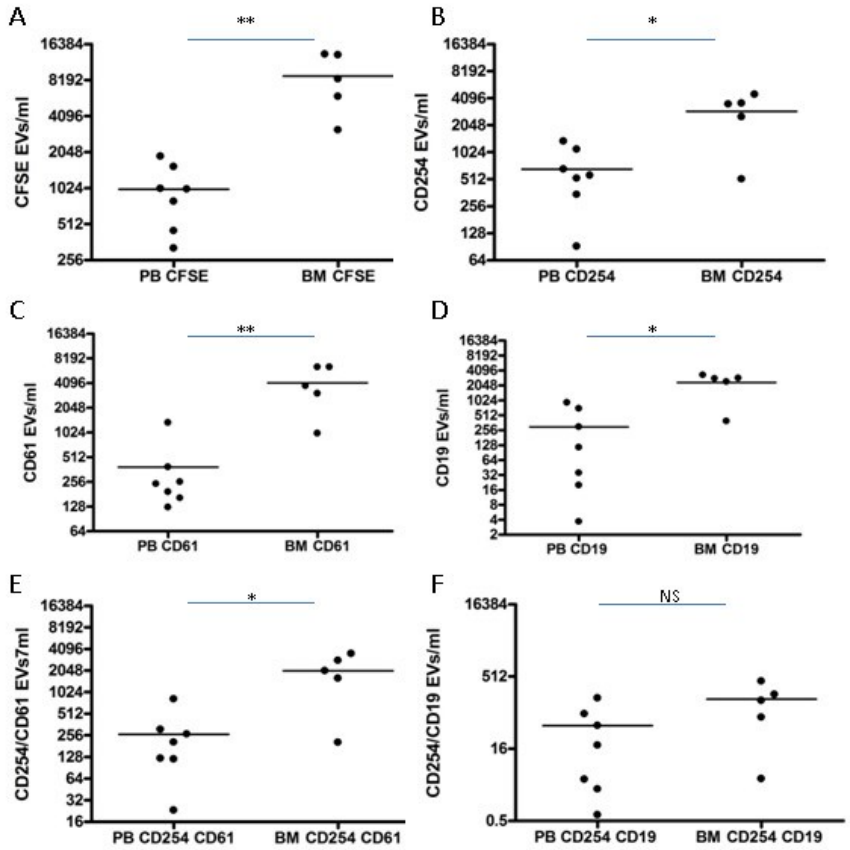

Figure 19 (A-F): Characterization of CDs on PB and BM plasma EVs from control patients. (A) Cytoplasmic dye CFSE was used to characterize the overall number of EVs in control patients (6 PBs, 5 BMs). EVs number/ml were calculated as described before. CFSE + EVs were then characterized for different CDs. (B) CD254 bone marker expression was evaluated in EVs CFSE+. (C) CD61 megakaryocytes/platelets marker expression was evaluated in EVs CFSE+ from control patients. (D) CD19 leukaemia blasts and B-lymphocytes expression was evaluated in EVs CFSE+ from control patients. (E-F) Double positive CD254/CD61 and CD254/CD19 EVs population analysis in controls PB versus BM. Statistical analysis was performed using the Mann–Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

Bone Marrow compartment: CD254 and CD61 cell membrane expression in control and patients at diagnosis.

PB EVs analysis of control and diagnosis patients showed the presence of a double positive CD254/CD61 population and that in control patients the overall production of the membrane bodies is greater compared to patients at diagnosis.

We wanted to understand if the situation described in the PB through EVs was representative of the bone marrow. We asked how CD254 marker is associated to CD61 antigens in healthy patients. For this purpose we performed flow cytometry analysis on bone marrow cells of controls and diagnosis (Figure 20). We found that CD61+ cells also express CD254 in the BM of the control patients. The number of CD61/CD254 in patients at diagnosis is lowered compared to controls because of the overwhelming CD19+ blast population. The presence of CD61/CD254 double positive cells suggests that those could be the source of EVs present in the PB and at major extent in BM.

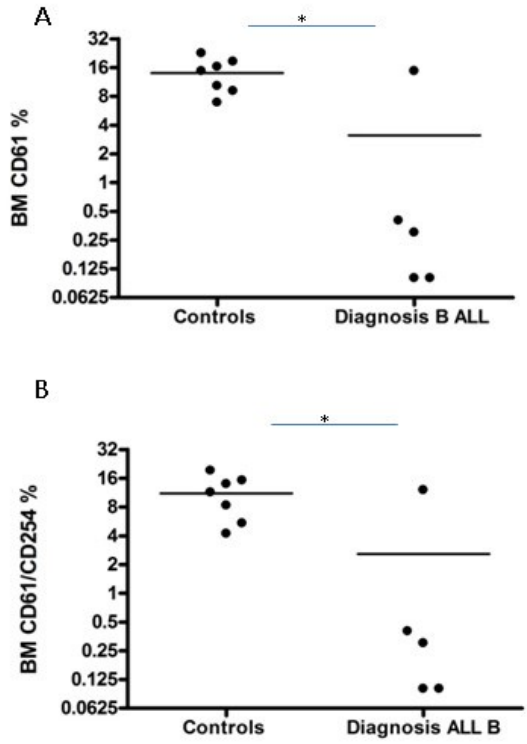

Figure 20 A and B: Megakaryocytes/plateles markers in BM cells from control patients and B ALL diagnosis patients. (A) Flow cytometry analysis of CD61 and (B) CD61/CD254 expression on freshly isolated BMs cells from controls (5 stop therapy, 1 maintenance) and diagnosis (6 ALL B). Results are given as percentage. Statistical analysis was performed using the Mann–Whitney U test. Differences were considered to be statistically significant at *P* values <. 05 and were indicated with asterisks (<. 05 *, < .001 **, < .0001 ***).

5. Discussions

RANKL membrane expression in B ALL blast cells.

The importance of CD254 in leukaemia is represented by its pivotal role in bone metabolism. Leukaemia disease is often associated with musculoskeletal involvement, a condition that accompanies the patients from the diagnosis to the follow up. CD254 can be expressed by the lymphoid lineage by activated T cells and this is linked to their ability to positively stimulate the resorption activity of osteoclasts. Furthermore it has been shown that activated CD19+ B cells, similarly to activate T cells are involved in osteoclast activation and bone degradation in periodontal disease, rheumatoid arthritis and in B cell lymphoma neoplasm (Kong *et al.,* 1999; Kawai *et al.,* 2006; Horowitz, 2010). Thus in literature it is well described that deregulation in the RANKL expression and pathways activated by this ligand can have a pro osteoclastic effect.

Initially our flow cytometry analysis reveled that blasts cells from B ALL patients at diagnosis had a significantly increased RANKL membrane expression compared to control patients. Membrane RANKL expression in patients at diagnosis was heterogeneous (Figure 4). Pre B and pro B cells did not showed the same RANKL expression as common B ALL (data not shown). RANKL membrane expression in leukemic cells has been reported on AML primary blasts (Schmiedel *et al.,* 2012) but B ALL expression is a novelty. This first data suggest that RANKL expressed on blasts cells could also have a functional role in acute lymphocytic leukaemia.

We thus decided to study how CD19+ blast cells can influence the bone environments, and specifically the bone digesting osteoclasts. To do this we performed 2D co-colture experiment on bone plates using a leukaemia cell line SEM, expressing membrane RANKL. We tested the ability of leukaemia cell line to induce differentiation of PBMC CD14+ osteoclasts precursor into resorbing cells, by analyzing number of pits on the bone substrate (Figures 6 and 7). SEM cells were able to induce differentiation of the PBMC CD14+ in bone degrading osteoclast (see Figures 6 and 7). However the only known RANKL inhibitor OPG was not able to significantly reduce the bone digestion by OCs, both in SEM co-colture and in OPG treated positive control (Figure 7).

We then analyzed the ability of primary BM cells from diagnosis and from control patients to influence the PBMC CD14+ differentiation towards the mature osteoclast. We choose the

TRAP staining assay and we studied the first step of osteoclasts differentiation, the ability to fuse to generate multinucleate cells (with more then 3 nuclei), see Figure 10 and 11.

To investigate this aspect we performed co-colture in chamber slides using patients at diagnosis showing different RANKL expression and controls (Figure 11 A-H).

Only patients at diagnosis with high RANKL expression were able to induce multi-nucleation of PBMC CD14+ in a significant manner (Figure 11 I-M). Moreover the treatment with OPG significantly reduced the ability of RANKL expressing cells to induce fusion into giant OCs (Figure 11 E-H).

Same experiment was performed on bone plates, which seemed a more physiological substrate for OC precursors adhesion and differentiation. This set of experiments showed that BMs from patients at diagnosis expressing RANKL could induce osteoclasts precursor to fuse to form multinucleate cells (Figure 13 A-D). However the OPG treatment was not efficient blocking the RANKL activity (Figure 13 E-H).

The OPG is the only known inhibitor for RANKL. The fact that the OPG is not always efficient in blocking multinucleation might also suggest that other mechanisms other then RANKL are involved, and this aspect need to be further investigated. However OPG can bind to another receptor on OCs, which is the TNF-related apoptosis inducing ligand (TRAIL). This is an apoptotic receptor, which may have the opposite effect in condition where RANKL is not sufficient to interact with the OPG and be sequestered it. OPG concentration in the co-colture system is probably more than the RANKL produced by blasts. Nonetheless there is a positive correlation between the RANKL membrane expression and the number of multinucleate cells, as shown in figure 14 A. Patients at diagnosis showing high RANKL expression (MFI above 2,5 to 7) had an increased number of multinucleate cells when compared with controls and diagnosis patients with low RANKL expression (MFI below 2) (Figure 14 B). Whit these data we cannot exclude that only specific CD14+ are responsive to CD254, and further analysis are required. The OPG anti-apoptotic effect has been shown for committed OCs (Chamoux *et al.,* 2008), thus it is possible that CD254+ blasts cells exert their osteoclastogenic effects on more immature osteoclasts. Other molecules, already in use in clinic, could offer an interesting alternative to recombinant OPG, (Denosumab). It could be tested in this system to assess the ability to inhibit high bone degradation in patients with high RANKL expression.

These finding support the concept that there is an important cross talk between B cells and the microenvironment. Until now the attention has been mainly focused on stromal osteoblastic cells, rather then on bone resorbing cells. However the importance of osteoclasts in the

hematopoietic compartment it is well known, above all from osteopetrotic mice models. Osteoclasts are physically responsible for the niche formation, and hematopoietic cells egression. Furthermore feedback signals from OCs are necessary for osteoblastic cells activity, stimulating their activity. An example is the osteoporosis treatment where anti-resorptive drugs used to reduce the bone erosion leads to a concomitant reduced bone formation (Charles and Aliprantis 2014). In leukaemia we see that a plethora of different bone alterations come at presentation, during the therapy and persist as a long-term sequel after treatment. In this study we analyzed RANKL membrane expression on patients with known bone involvement. Two of them were also used in co-colture experiment, and we clearly saw that in those cases the multinucleation was associated with high RANKL MFI. Our data support the idea that blasts cells can expresses RANKL on their membrane and that this can influence osteoclast precursors to multinucleate. The immune system is functionally and physically related to bone. Members if the TNF ligand/receptor superfamily and several cytokines are essentials for the function and normal development of both systems (Manabe 2001; Monsour *et al.,* 2011; Horowitz *et al.,* 2005). Moreover immune and bone cells have common progenitors. Commitment to the B cell lineage depends on Pax5, and Pax5 deficient mice pro B cells can differentiate in to osteoclasts as well in absence of IL7 and estrogen (Blin Wakkach *et al.,* 2006; Miyaura *et al.,* 1997).

RANKL deficient mice showed a severe osteopetrosis, because of the complete lack of osteoclasts. These mice also showed a disruption of the B cells differentiation, were transition from pro B to pre B stage is compromised and this defect is a B cell intrinsic phenomenon and not to due to the microenvironment. B cell differentiation from hematopoietic stem cells to immature B cells in the bone marrow is achieved after successive rearrangement of the gene segments of the heavy IgH and light IgL chain loci (Tonegawa *et al.,* 1983). When a functional rearrangement occurs at the pro B to pre B stage the cells will express the pre B cell receptor (pre BCR) (Karasuyama *et al.,* 1990; Melchers *et al.,* 1993). When pre BCR is expressed on pro B cells surface those can expand in the presence of low IL7 concentration. IL7 is produced by stromal cells and is required both for lymphopoiesis and osteoclastogenesis, providing a nurturing microenvironment for their progenitors cells. Also IL7 deficient mice show osteopetrosis and suppression of early B lymphopoiesis. In contrast IL7 overexpressing mice or IL7 treated mice shows and increase in B lymphopoiesis and bone loss (Stoddart *et al.,* 2000; Miyaura *et al.,* 1997).

Kato showed that RANKL/TRANCE membrane expression in mice is stage specific regulated Kato *et al.,* 2003). Pre B cells, which are proliferative, express RANKL and can differentiate

further to the immature cells with removal of IL7. Thus RANKL transcription depends on IL7, and it is limited to proliferating cells, such as Abelson virus transformed pre B cell lines or IL7 dependent pre B cells. RANKL induce cells to proliferate after an inhibition of G1 arrest. The physical proliferation of pre B cells increases the differentiation rate.

We did not see clear association between high RANKL expression and the cell cycle, even though we suspect that RANKL expression on blasts cells might be associated to a more differentiated blats sub population. When dividing diagnosis and controls by their CD254 MFI into quartiles (data not shown), we saw that the patients with known bone lesions are in the III and IV quartile. The third and fourth quartiles were also associated to the highest number of aneuploidy cell cycle, even though this did not correlate with DNA index, or S phase. Cell cycle analysis was performed on the whole bone marrow blast population, but the patients we analyzed showed heterogeneity for the RANKL expression as described before. During our flow cytometry analysis we often observed that CD254/CD19 double positive cells were not always representative of the whole blasts population, as if within the CD19+ gate there were two different sub groups. Similarly to the results described by Kato, among blast population double positive CD19+CD254+ cells could represent a more differentiated population of leukemic cells.

Further cell cycle analysis are required in order to understand RANKL membrane expression role in cell cycle; DNA index or S phase length might not be fully informative of other phases as the spindle formation and checkpoint activity. Our patients' data need to be implemented and the RANKL expression needs to be studied for correlation with possible prognostic factors. The fact that blasts can express at high level RANKL and the possibility that this can induce bone resorption should be taken into account. Recently a Danish study has focused on the delay of the diagnosis of B ALL due to atypical presentations. They reported a study of 100 children which symptoms, signs and laboratory findings at the time of the presentation. They found that among children with pre B ALL, musculoskeletal pain is reported by more than half, and that 10% manifest joint or bone lesion resembling arthritis and osteomyelitis. Children with skeletal lesions have a longer diagnostic interval with slower disease progression and associated with good prognosis, unless diagnosis is much delayed (Heinrich *et al.,* 1994; Muller *et al.,* 1998). They suggested the blast crisis when the full-blown bone marrow occurs is a late occurrence of disease progression. Today the efficacy of treatment has greatly improved and knowing more about RANKL function might help to anticipate and tailor therapy, or could be used for the stratification of the patients. Whether the multinucleation induced on osteoclasts precursor by RANKL expressing blasts can end with

full osteoclast activation has to yet to be addressed, but our results are in agreement with work made by other groups and with clinical data.
Many other points need to be addressed in order to consider this bone marker in leukaemia and RANKL expression need to be studied in a more complex of interaction as it will be described in the next section.

Gene Expression Profile analysis in B ALL patients at diagnosis and controls.

Our results strongly suggested that high membrane RANKL expression in B ALL blasts could induce multinucleation of osteoclasts precursor (Figure 11 and 13). The 2D co-colture system was informative of the effects that leukaemia cells had on CD14+ OCs precursor. However we do not know from these data if leukemic cells do serve as osteoclasts precursor itself.
We performed gene profile analysis in order to gain information about the possibility that leukemic blats and RANKL expression could influence the bone metabolism through the three major pathway in which RANKL is involved: osteoclastogenic, osteoblastogenic and B cell differentiation as derived from KEGG. Our cohort includes all the B ALL classes as shown in table 3 in material and methods.
Manabe (Manabe *et al.,* 2001) reported that in mice B lymphoid lineage cells expressed RANKL and had the ability to support osteoclast differentiation. Furthermore a subset of normal bone marrow cells that expresses markers of B lymphoid lineage cells in early developmental stages can serve as osteoclast progenitors and are able to differentiate in to resorbing osteoclasts *in vitro* (Manabe *et al.,* 2001; Blin-Wakkach *et al.,* 2006).
With this in mind we performed GEP analysis and found that among the pathways analyzed only the osteoclastogenic pathway was altered in our B ALL cohort (Figure 14 A).
Among genes differentially expressed there are interacting partner as shown in figure 14 B. Of these genes DAP12 and CD300d have been directly implicated in B cell differentiation.
DAP12 is the product of the gene *TYROBP*. Is an immune receptor tyrosine-based activation motif-bearing adapter protein. It represents a co-stimulatory pathway in the RANKL cascade. Mutations of this gene are responsible of a recessive genetic disorder named Nasu-Hakola. Upon ligand binding DAP12 trigger intracellular activation via the ITAM motif, reaching the SYK family kinases and inducing their phosphorylation.
Yokomizo (Yokomizo *et al.,* 2011) described DAP12 and its role in human B cells. They showed that DAP12 together with CD300d negatively regulates B cell mediated adaptive immune responses and suppress their B cell receptor (BCR) mediated proliferation. Absence

of DAP12 in human B cells made them hyper responsive to BCR mediates stimulation also increasing their proliferation rate.

SYK regulates several signaling events in lymphoid cells (Uckun *et al.,* 2010). After its activation SYK phosphorylates several signaling molecules triggering a cascade of signal transduction that affect activation, proliferation ad survival (Uckun *et al.,* 2014). SYK it is a part of the pre BCR signaling in B cell precursors as well as BCR signaling in mature B-lymphocytes and it plays an important role in early specification and maturation events during B cell ontogeny (Uckun *et al.,* 2014). SYK is also involved in normal osteoclast function *in vivo* and *in vitro*. Osteocalsts defective for SYK showed no defect in their differentiation but were unable to re organize their cytoskeleton and as such their ability to resorb bone (Zou *et al.,* 2007). DAP12 and mature B-lymphocytes act synergistically to maintain bone mass under physiological conditions. When osteoclasts function is compromised as in *Dap12* mutant mice (KΔ75, osteopetrotics) they can function as accessory cells to regulate osteoclasts function (Anginot *et al.,* 2007). On the other side mice overexpressing DAP12 have an osteopenic bone phenotype due to an increased number of osteoclasts on the surface of trabecular and cortical bone. Again the increased osteoclastogenesis results in an impaired hematopoiesis with concomitant arrest of B cell differentiation arrest at the pre-proB/pre B stage (Despars *et al.,* 2013).

Thus some of the genes are directly involved in B cell maturation whereas others have a more important role in the functional differentiation of osteoclasts, mainly in their cytoskeleton reorganization rather than in their differentiation.

With the attempt to better understand if the osteoclastogenic involvement of B ALL leukaemia cells was due to an impaired B cell differentiation or rather to the possibility that blasts cells could be a defective or dormant osteoclast precursor we analyzed some of the gens that are specifically involved in human osteopetrosis (Lange *et al.,* 2006; Coudert *et al.,* 2014) (Figure 15).

Of the gene analyzed we found that were differentially expressed genes that are known or for their role in osteoclasts functionality as *OSTM1*, *SERPINE2,* and *WARS*.

OSTM1 and CLC-7 both co localize in the late endosomes and lysosomes of various tissues included the ruffled border of resorbing osteoclasts. OSTM1 and CLC-7 form a molecular complex. Together they are required for acidification of the resorption lacunae (Lange *et al.,* 2006).

SERPINE2 it is involved in the removal of non-collagenous proteins that are present in the non-mineralized bone matrix, but it is not required for their differentiation or for the

resorption on bone matrix (Daci *et al.,* 1999). WARS catalyze the aminoacylation of tRNA (Trp) with tryptophan for protein synthesis, and is involved in osteoclasts differentiation (Chabbi Achengli *et al.,* 2012).

To data this is the first study performed on B ALL leukaemia blast cells that focus on the interaction with bone degrading cells. Importantly here we highlighted the possibility that leukemia B cells and osteoclast share more than common transcription factors at early differentiation stages, but their respective development is closely linked.

We need to understand if RANKL expressing primary blasts population can induce bone resorption and lacunae formation. If blast cells, which do not express RANKL, can be differentiated into osteoclasts upon RANKL and MCS-F stimulation.

The GEP analysis unveiled the possibility that beyond a direct effect on osteoclast mediated by RANKL expressing blast, there are shared pathways between leukemia and osteoclasts alteration of which might contribute to the disease progression. Our next focus will be on the RANKL expression on pro B and pre B cells and the effects the down regulation or overexpression can induce in these cells. The description of RANKL membrane expression on blasts represents a small piece of a puzzle of interactions within the bone marrow.

Notably in this context of cell-to-cell communication the shedding of extra cellular vesicles could be relevant and could work cooperatively to transfer signals through cells within the bone marrow. In the next section we will focus on the surface antigen characterization of EVs in the peripheral blood of B ALL patients at diagnosis and controls.

Flow cytometry characterization of extracellular vesicles in B ALL.

The importance of communication between tumor cells and their microenvironment through the shedding of Extracellular Vesicles (EVs) has been well documented. In leukaemia B-lymphocytes secrete antigene-presenting vesicles (Raposo *et al.,* in 1996). They found that human and murine B-lymphocytes can release exosomes and those had a role in inducing antigen specific response. Extra cellular vesicles can be released by CLL blast cells and influence with their cargo stromal cells in favor of the leukemogenic process (Ghosh *et al.,* 2010). The second part of this study was thus focused on the characterization of the EVs populations in the PB plasma of B ALL leukemic patients at diagnosis and in control patients.

For this purpose we choose the flow cytometry approach, which is actually the election methodology and allows a rapid identification of membrane antigen and semi quantitate definition of EVs populations (Figure 16 A).

We were interested in the analysis of EVs CD19 expression, a specific B cell and leukaemia marker, in CD61 markers of platels/megakaryocytes and finally in the CD254, which represent, the major marker of bone metabolism.

As reported before in solid tumors and in CLL, the disease progression is associated with an increase in the number of EVs in the PB of patients. Ghosh work showed that CLL is characterized by an increasing number of EVs CD19+ the far we go with the disease progression, whereas the platelets/megakaryocyte CD61 was reduced compared to the healthy controls (Figure 18 A-F).

We used cytoplasmic CFSE dye to mark the total EV populations in PB from patients. We then analyzed single populations for their binding ability to selected markers. Finally we analyzed and compared double positive populations, in order to understand if bone marker CD254 was associated to one of the two populations investigated.

We expected to have the same conditions described by Ghosh (Ghosh *et al.,* 2010) in our pediatric B ALL cohort. However the first observation came from the CFSE population, which was overall significantly decreased in patients at diagnosis. CD19+ the most represented EVs population in CLL was not the most represented population B ALL, suggesting that blasts cells do not produce the large amount of EVs expected. There was instead a significant decrease of CD61 population in diagnosis compared to controls as well as for CD254 population. The reduced number of CD61 EVs observed at diagnosis, was in agreement with Ghosh work. Moreover bone marrow and peripheral blood of leukaemia patients at presentation is characterized by low platelets count. If we assume that the CD61+ EVs are of platelets origin, this is consistent with the clinical features of leukaemia patients. Taken together the data showed that there was a suppressed release of EVs, not restricted to CD61 population, but which also involves the CD254 and CD19 groups. The difference with Ghosh could be due to the fact that we analyzed an acute phase, rather than a chronic condition, in which blasts cells are present for a long period of time. Consistently with the fact that CD19 blasts cells do not produce a significant amount of CD19+ EVs we found there were no association between EVs production and the blast number at diagnosis. It is clear that in this process other cells are involved and we have to keep in to account a possible involvement of lymph node and spleen populations. Furthermore we do not exclude the possibility that blasts cells can release exosomes, smaller in size and not detectable by flow cytometry, which were not studied in this work.

CD254 bone marker together with CD61 was the most represented population among the ones we studied. CD254 is a marker of bone metabolism but it also involved in the B cell

development and is expressed by different cell type in specific conditions, as activated T and B cells. Megakaryocytes (MKs) also express CD254, and have been shown to be important in the bone homeostasis (Beeton *et al.,* 2008; Bord *et al.,* 2010).

In order to understand if CD254 was specifically associated to one or the other population in patients at diagnosis and controls we analyzed double positive populations CD254/CD61 and CD254/CD19. Control patients showed that most of the CD61 are also positive to CD254, whereas in patients at diagnosis the double positive population was significantly less represented. This scenario was similar for CD19/CD254 double positive population. On the other side in patients at diagnosis the majority of the CD254 was not associated either to CD19 or CD61, suggesting the presence of other parental cells involved in the release of CD254+ EVs in the PB. We know that mature megakaryocytes (CD61+ cells) are involved in bone remodeling through the RANKL pathway (Bord *et al.,* 2010). Moreover increasing evidence suggests that circulating microparticles expressing CD61 originate predominantly from megakaryocytes rather than from platelets (Rank *et al.,* 2011; Flaumenhaft *et al.,* 2009; RANK *et al.,* 2010). We suspect that in control patients the majority of CD254/CD61 double positive EVs are of MKs origin, and that those might have a role in the bone homeostasis.

Peripheral blood and bone marrow EVs population in controls patients were also analysed. We found that the overall number BM EVs is significantly higher compared to PB. The results shows a similar pattern of antigen expression as seen in PB with the difference that all the BM EVs populations are much more numerous (Figure 19 A and B).

With the attempt to gain information about the origin of the CD254/CD61 double positive population we assessed if the BM cells of control patients present double positive CD254/CD61 cells. Flow cytometry analysis showed that the BM of controls patients was characterized by the presence of these cells, potentially involved in the production of EVs that we observed in plasma (Figure 20 A-B).

In conclusion we saw that the PB EVs from patients at diagnosis and controls reflect the bone marrow situation. CD254 bone marker was present both in controls and diagnosis. The abundance of total EVs population in controls was significantly increased compared to leukemic patients at diagnosis. Importantly CD254 was mainly associated to CD61 in controls rather than in diagnosis. Unless we expected a predominant CD19+ population at diagnosis we found that the EVs blast production was very limited.

With this study we were able to show that it is possible to isolate EVs from PB of patients at diagnosis and to characterize them for their surface antigen markers by flow cytometry.

Unexpectedly we found that the EVs production seen in physiological condition is suppressed in leukaemia.

CD254 and CD61 were the most represented populations both in controls and diagnosis.

Further analyses are required to understand if CD254 EVs population has a physiological meaning and thus can have importance in diagnosis or follow up. In view of the data described in the previous sections we need to address if CD254 EVs can be produced by membrane RANKL expressing blasts. Morover we should investigate which is the functional role of these EVs in the bone marrow, if they carry specific cargo and if they have a specific tropism. It is known that CD254 EVs can be released by mesenchimal cells and have a specific tropism (Cappariello *et al.,* 2015).

These findings highlight the importance of EVs in the microenvironment and the importance of these membrane bodies as carriers of information to distal sites from the parental cells.

The field of EVs is developing faster. Their role in the pathophysiology of several diseases is well described as reviewed by Yoon and collaborators (Yoon *et al.,* 2014). Despite the numerous reports on EVs analytical isolation issues, at date standardized precedures for the isolation and quantification of EVs are not available. Nonetheless several groups are working in this direction and preliminary results for the use of plasma and blood in diagnostic device are described, with the final aim to use it directly in lab on chip diagnostic tools.

6. Conclusions

The purpose of this study was to understand if leukaemia cells could influence the bone marrow microenvironment, acting on osteoclast bone resorbing cells and through the major bone metabolism player RANKL. We observed a significantly higher expression of membrane RANKL in patients at diagnosis if compared with controls. We found that blasts cells from B ALL bone marrow could induce the fusion of PBMC CD14+ osteoclast precursor promoting their association in to multinucleate cells. The ability to induce the multinucleation was linked to high RANKL expression, and we showed that two patients known for their bone lesion at diagnosis expressed high RANKL membrane and were able to induce multinucleation.

Beside this ability to induce multinucleation the GEP analysis revealed that leukemic bone marrow is characterized by a significant down regulation of genes that are involved in osteoclast differentiation maturation and activation if compared to healthy subjects. The gene profile analysis of leukaemia bone marrow resembles that of a hybrid phenotype between a defective osteoclast and not fully committed B cell. Leukemic blasts are well described for their inability to differentiate into normal B cells, but no information specifically links these blast cells to osteoclasts or defective osteoclasts. Cell line FLG29.1 is an example of myeloid leukaemia cells that under certain colture conditions can differentiate into fully resorbing osteoclasts (Gattei *et al.,* 1992). However data are not available for B cell committed leukaemia precursors. FLG29.1 is a myeloid cell line, and the gap between a myeloid cell and mature osteoclasts is close, as both come from a common committed progenitor. Less obvious is the link between an osteoclast and a B lymphoid committed cell.

Here we are observing two different aspects of the same disease. On one side the RANKL expression on blast cells might represent a more differentiated blast sub population, maybe a fraction that is going thorough differentiation. As described by Kato (Kato *et al.,* 2000) RANKL induced pre B cells to proliferate after an inhibition at the G1 phase, thus moving on through their differentiation.

On the other side we have the osteoclastic like “suppressed” background, highlighted by the GEP that could represent a step before, when the switching from myeloid lineage and B compartment is altered.

Literature is a source of precious information of this phenomenon. The transcription factor Pax5 is a common regulator of B lymphoid differentiation and osteoclasts precursor as well.

At B cell commitment Pax5 represses diverse biological activities including receptor signaling, cell adhesion, migration and transcriptional control and cellular metabolism.
Pax5-/- pro B cells and common lymphoid progenitors display lymphoid and myeloid lineage promiscuity of gene expression. Among gene repressed by Pax5 at the pro B cells there are RANKL and MCS-F R. In Pax5-/- cells factors as RANKL, which continue to be expressed, may support deregulated osteolysis as RANKL expression can stimulate OCs activity.
We think that at early stage of leukaemia the mechanism involved in switching off myeloid specific genes and in switching on B cell specific patterns could be compromised.
In osteopetrotic mice as in IL7-/- mice the transition between pro and pre B is blocked, leading to a reduced B cell numbers, as it is described for *oc/oc* mice. Moreover there is a concomitant increase in myelopoiesis with a high number of inactive OCs (Schlissel MS, Corcoran LM *et al.,* 1991; Gilliland DG, Griffin JD., 2002).
Although the differentiation pathways for lymphoid and myeloid lineage are mutually exclusive, there is a certain degree of plasticity, and B220+ cells isolated from murine bone marrow are able to differentiate in vitro into osteoclasts (Manabe *et al.,* 2001).
Osteopetrotic models showed accumulation of a bone marrow population that is B220+ CD11b+. In vitro the treatment of IL7 induced the differentiation of B220+ CD11b+ cells into B lymphocytes through the induction of Pax5 and the inhibition of myeloid markers with partial restoration of the B cell population and thus of the B lymphopoiesis (Blin-Wakkach *et al.,* 2006). A model of mild inducible osteopetrosis is recreated by Zoledronic Acid (ZA) treatment as described by Monsour (Monsour *et al.,* 2011). Using this system they were able to study the effects of the inducible bone marble phenotype on stromal cells included osteoblasts. They showed an indirect effect that altered the expression of CXCL12 and IL-7 decrease by stromal cells and associated with reduced osteoblastic engagement. ZA induced disadvantageous condition for B lymphopoiesis leading to the retention of B cell progenitors outside of their bone marrow niches. These effects were not directly (Mansour *et al.,* 2001).
Figure 21 summarizes the possible scenarios that RANKL membrane expression could cause in different B ALL leukemia subtypes.
In conclusion the growing skeleton in leukaemia is vulnerable and it is more sensitive to osteotoxic therapy. Early recognition and intervention strategies are essential, and in this context the deep knowledge of B lymphopoiesis and osteoclastogenesis shared pathways are important to anticipate diagnosis and to prevent long-term sequelae from altered bone metabolism.

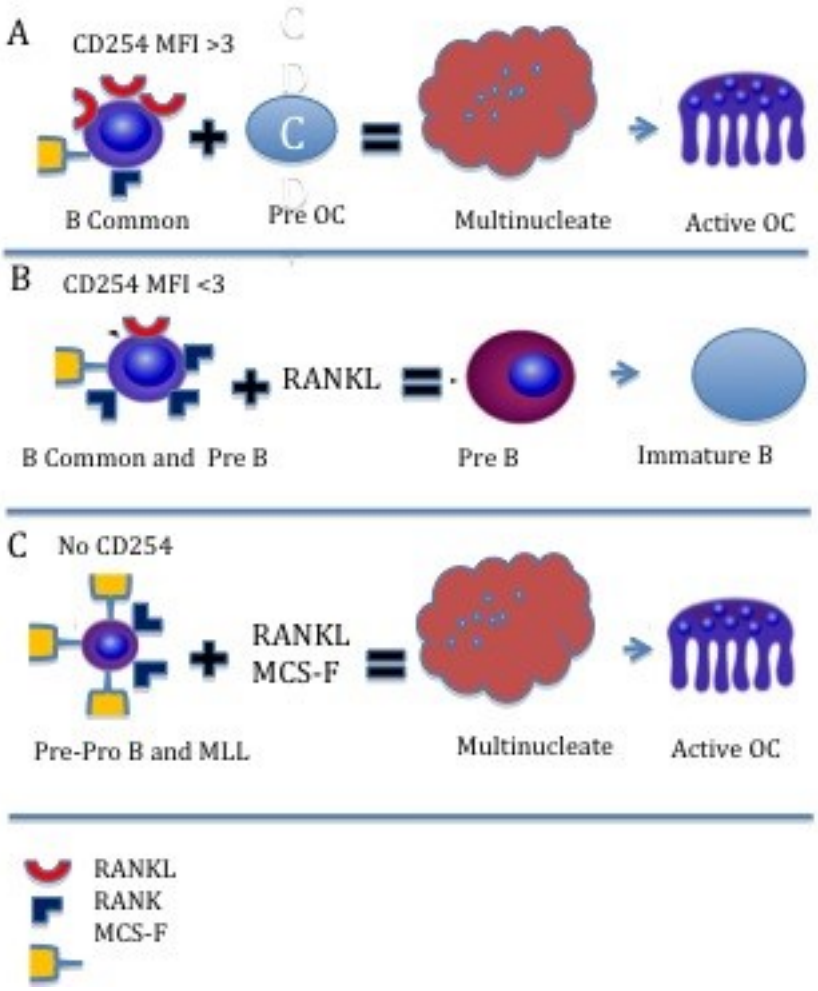

Figure 21: Model and hypothesis of RANKL expression in B ALL subtypes. (A) RANKL membrane expression on B ALL common could induce pre OCs to multinucleate and activate their resorption activity. (B) B ALL and Pre B with low RANKL expression might be forced to move on through proliferation and differentiation to pre B and to immature B stage by inducing RANKL expression. (C) More immature B ALL forms or MLL could be pushed through osteoclastic like differentiation by exogenous RANKL and MCS-F stimulation.

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