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**EMERGING EVIDENCE  
FOR A PROTUMORIGENIC ROLE OF THE  
ENDOTHELIN AXIS  
UNCOVERS NEW THERAPEUTIC TARGETS IN  
MULTIPLE MYELOMA**

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# *ABSTRACT*

Multiple Myeloma (MM) is a monoclonal tumor of bone marrow (BM) plasma cell (PC), usually associated with a number of disease manifestations, including skeletal damage, anemia and immunosuppression. Despite recent therapeutic advances and an increased patient life expectancy, MM still remains an incurable malignancy.

MM PC is characterized by a strong BM niche dependence, acquiring an autonomous capacity of growth only in the latter stages of disease. The endothelin (ET) axis is mainly composed of Endothelin-1 (ET-1), and 2 G-protein coupled receptors, the ET receptor A (ET<sub>A</sub>R) and ET receptor B (ET<sub>B</sub>R). In the last decades it has been demonstrated that the ET axis is able to promote the development and progression of tumor cells in an autocrine and/or paracrine fashion. Accordingly, recent experimental and clinical data obtained in solid tumors have evidenced the possibility of interfering with ET receptors (ETRs) - *via* specific antagonists - for therapeutic purposes. On the basis of these premises, primary aim of this study was to assess whether the ET axis may have a protumorigenic role in MM, therefore becoming a therapeutic target. The experimental evidences we obtained demonstrated that MM cell lines, primary MM PCs and BM microenvironment cells express ET-1 transcript and release the corresponding protein. Interestingly, while ET<sub>A</sub>R was constitutively expressed by PCs from healthy donors (HDs), primary malignant PCs and MM cell lines, ET<sub>B</sub>R was detected only on malignant PCs of 54 of the 100 patients enrolled in the study and in 3/5 MM cell lines. Interestingly, B lymphocytes isolated from HDs BM or peripheral blood (PB) or from MM patients PB, did express ET<sub>A</sub>R but not ET<sub>B</sub>R. Based on the evidence that the expression of ET<sub>B</sub>R was strictly connected to the neoplastic transformation, we next demonstrated an altered methylation status of the ET<sub>B</sub>R promoter gene in malignant PCs from ET<sub>B</sub>R-expressing MM patients and MM cell lines. The possibility of interfering with ETRs in MM was investigated *in vitro* using RPMI-8226 and U266 MM cell lines through the use of ET<sub>A</sub>R and ET<sub>B</sub>R selective antagonists. Both ET<sub>A</sub>R and ET<sub>B</sub>R antagonists, used alone and/or in combination, decreased RPMI-8226 and U266 cell lines viability. Based on these data we next evaluated the potential therapeutic significance in MM of Bosentan, a dual ETRs antagonist already used in clinical practice for pulmonary arterial hypertension. In agreement with our *in vitro* experiments, Bosentan appeared to be effective in inhibiting the viability of RPMI-8226 and U266 cell lines by reducing p-p44/42 MAPK. Furthermore, this action appeared to be synergic to that of bortezomib, a proteasome inhibitor drug already used in first-line treatment of MM.

Overall our data demonstrate that the ET axis is a potential therapeutic target in MM. Further data are awaited in order to establish the prognostic significance of the aberrant expression of ET<sub>B</sub>R in MM.

Il Mieloma Multiplo (MM) è una neoplasia ematologica dovuta alla proliferazione e all'accumulo nel midollo osseo (MO) di plasmacellule (PCs) monoclonali. Il MM è una neoplasia dell'adulto che si presenta con una serie di manifestazioni cliniche tra cui danno scheletrico, anemia e immunosoppressione. Nonostante i recenti progressi compiuti in campo terapeutico abbiano aumentato la sopravvivenza dei pazienti che ne sono affetti, il MM rimane ad oggi una patologia incurabile. Negli ultimi decenni è stato dimostrato come nei tumori solidi l'endotelina 1 (ET-1) agisca come fattore di crescita autocrino e/o paracrino per le cellule neoplastiche, partecipando allo sviluppo e alla progressione del tumore stesso. Inoltre, dati clinici e sperimentali hanno evidenziato come l'inibizione dei recettori endotelinici possa garantire un'opportunità per lo sviluppo di approcci terapeutici che prevedano l'uso combinato di antagonisti recettoriali e chemioterapici. Sulla base di queste considerazioni, lo scopo del presente studio è stato quello di valutare un possibile coinvolgimento dell'asse endotelinico nella patogenesi del MM. I dati ottenuti hanno consentito di stabilire che linee cellulari di MM, cellule primarie di MM e i componenti del microambiente midollare stromale esprimono il trascritto di ET-1 e rilascino la corrispondente proteina; inoltre, contrariamente a quanto osservato in PCs isolate dal MO di donatori sani ed esprimenti solo ET<sub>A</sub>R, ET<sub>B</sub>R è stato evidenziato sulle PCs maligne di 54 dei 100 pazienti arruolati nello studio e in 3/5 linee cellulari di MM. Anche linfociti B immaturi isolati dal MO e maturi isolati dal sangue periferico di donatori sani, o di pazienti affetti da MM esprimono ET<sub>A</sub>R ma non ET<sub>B</sub>R. Tali evidenze hanno consentito di ipotizzare che la presenza di ET<sub>B</sub>R rappresenti un evento strettamente correlato al clone neoplastico e a dimostrare successivamente come essa derivi da un'alterata metilazione genica del promotore di ET<sub>B</sub>R. La possibilità di interferire con l'asse ET nel MM è stata indagata *in vitro* sulle linee di MM RPMI-8226 e U266 mediante l'utilizzo di antagonisti selettivi per ET<sub>A</sub>R e ET<sub>B</sub>R. Poiché l'azione dei 2 antagonisti è risultata sinergica nel ridurre la proliferazione cellulare, abbiamo valutato il possibile significato terapeutico nel MM di Bosentan, un duplice antagonista di ET<sub>A</sub>R e ET<sub>B</sub>R già utilizzato nella pratica clinica per la terapia dell'ipertensione arteriosa polmonare. I nostri esperimenti hanno dimostrato come Bosentan sia in grado di inibire *in vitro* la proliferazione delle linee RPMI-8226 e U266 riducendo l'attivazione delle p-p44/42 MAPK. Tale azione appare inoltre sinergica rispetto a

quella esercitata da bortezomib, un farmaco, inibitore del proteasoma, già utilizzato nel trattamento di prima linea dei pazienti con MM.

I dati ottenuti questo studio evidenziano un ruolo protumorigenico dell'asse endotelinico nel MM e ne indicano il potenziale ruolo di bersaglio terapeutico. Studi futuri stabiliranno se l'aberrante espressione di ET<sub>B</sub>R possa avere un significato prognostico negativo.

# *INTRODUCTION*

## ***1 Multiple Myeloma***

Multiple Myeloma (MM) is a plasma cell (PC) disorder that accounts for 1% of all malignant diseases in white population and 13% of all hematological cancers. The median age at diagnosis is 66 and men are more frequently affected than women. The neoplastic PCs, generally derived from one clone producing monoclonal immunoglobulin, proliferate and accumulate in the bone marrow (BM) and cause skeletal damage, the hallmark of MM. But, a part from osteolytic lesions due to uncomplete bone metabolism, the malignancy is associated with a number of disease manifestations, including anemia and immunosuppression due to loss of normal hematopoietic stem cell function and end-organ damage due to immunoglobulin secretion [1, 2]. The aetiology of this cancer remains poorly understood, however several case-control studies have reported association between family history of MM and increased MM risk [3] suggesting that the aetiology of MM, as well as that of many other cancers, involves a genetic component. Increased radiation exposure also may play a role [4]; furthermore, multiple case control studies have suggested an elevated risk of MM among agricultural workers: insecticides, herbicides and fungicides have all been hypothesized as the basis for this increased risk [5].

### ***1.1 Diagnosis and Stages of Multiple Myeloma***

The diagnosis of MM is based on the presence of at least 10% clonal BMPCs and monoclonal (M) protein in serum or urine. The criteria that define MM are summarized by the acronym CRAB and include hypercalcemia ( $>11,5\text{mg/dL}$ ), renal insufficiency (creatinine  $>2\text{mg/dL}$ ), anemia (hemoglobin  $<10\text{g/dL}$  or  $2\text{g/dL}<\text{normal}$ ) and presence of bone lesions. The International Myeloma Working Group (IMWG) recently updated the definition of MM to include biomarkers in addition to existing requirements of CRAB features, that is: 60% or more clonal PCs in the BM, involved/uninvolved serum free light chain (FLC) ratio of 100 or more and/or MRI with more than one focal lesion (involving bone or BM). Additionally, the presence of one or more osteolytic lesions seen on skeletal radiography, whole body MRI or PET/CT fulfills the criteria for bone (Table 1). After diagnosis, patients with active myeloma are categorized according to stage based on either the Durie-Salomon staging system or the International Staging System (ISS) or better, on the revised International Staging System (R-ISS). The Durie-Salomon staging system is based on tumor cell density in the BM and measures

end-organ damage (renal insufficiency, anemia, hypercalcemia, lytic bone lesions) and immunoglobulin (Ig) levels. The ISS is based on levels of serum  $\beta$ -2 microglobulin and serum albumin to divide the disease into 3 stages with different prognostic significance. Compared with the Durie-Salomon staging system, the ISS is able to predict prognosis and overall survival (OS) and is easier to use for patients with previously untreated MM. Finally, the R-ISS incorporates factors included in the ISS ( $\beta$ -2 microglobulin and serum albumin), but also serum lactate dehydrogenase (LDH) and high risk chromosomal abnormalities detected by interphase fluorescence in situ hybridization (FISH) (Table 2) [6].

MM is usually, but perhaps not always, preceded by an age dependent pre-malignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which is present in 1% of adults. MGUS cells secrete monoclonal Ig and progress to malignant MM, expressing the same Ig, at a rate of 1% per year. Despite MM, patients with MGUS present a serum M protein less than 3.0 g/dL, less than 10% clonal PCs in the BM, little or no M protein in the urine and no lytic bone lesions, renal insufficiency, hypercalcemia, or anemia related to the PC proliferative process (Table 1) [7]. The cause of malignant progression of MGUS is not well understood; however, genetic changes, BM angiogenesis and cytokines related to myeloma bone disease may all play a role in the progression of MGUS to MM. Furthermore, a number of parameters are helpful in predicting the likelihood of progression of MGUS to MM. The concentration of the M protein at diagnosis represents the most important predictor of progression to a PC disorder. If the initial M protein value is less than 0.5 g/dL, the risk of progression at 20 years is 14%, whereas patients with an M protein of 2.5 g/dL have a 49% risk of progression. Moreover, patients with an IgM or an IgA monoclonal protein have an increased risk of progression, compared with patients who have an IgG protein [8]. Also the percentage of BMPCs at diagnosis and the abnormal serum FLC ratio can indicate the risk of progression: several studies [9, 10] have reported that having more than 5% BMPCs is an independent risk factor for progression. Furthermore, the risk of progression is higher in patients with an abnormal FLC ratio than in patients with a normal ratio (hazard ratio (HR)=3.5) and this is independent of the concentration and type of serum M protein [11]. Management of patients with MGUS should be tested again in 4 to 6 months to exclude an evolving MM. Patients with low-risk MGUS may be reevaluated every 2 years, whereas those with high-risk MGUS should be followed annually for life or until they develop an unrelated condition that severely limits life expectancy. It is important to detect a serious PC disorder before complications such as

renal failure or pathologic fractures occur. At the time of the follow-up examination, a careful history and physical examination should be performed, looking for symptoms or signs of one of the malignant disorders known to evolve from MGUS. The serum and urine M protein values should be measured, as well as the complete blood count, calcium, and creatinine [7].

Smouldering Myeloma (SMM) is an asymptomatic PC proliferative disorder characterized by the presence of a serum M protein level 3 g/dL or greater, 10% or more clonal PCs in the BM, or both (Table 1). It represents a more advanced premalignant stage than MGUS. Small amounts of M protein in the urine and a reduction of uninvolved Ig in the serum are not uncommon in SMM. Most importantly, as in MGUS, there is no evidence of end-organ damage, no hypercalcemia, renal insufficiency, anemia, or bone lesions. In fact, SMM patients have MGUS from the biologic standpoint, but the diagnostic criteria differ from the MGUS criteria to highlight the higher risk of progression in SMM compared with MGUS. It is also important to recognize this entity because patients with SMM should not be treated until symptomatic disease occurs. SMM accounts for approximately 10% to 15% of all cases of newly diagnosed MM. Many patients with SMM progress to symptomatic disease and the risk of progression is higher than in patients presenting with MGUS. It has been shown that the risk of progression is 10% per year for the first 5 years, 3% per year for the next 5 years, and then 1% to 2% per year for the next 10 years [12]. The median duration of progression to symptomatic disease ranges from 1 to 5 years. Two subsets of SMM have been described: evolving SMM, in which there is a progressive increase of the serum M protein level and patients progress to MM within the first 5 years, probably having an early MM without organ damage; and non-evolving SMM, in which patients with newly diagnosed SMM will not progress in the first 10 years after diagnosis, but probably have a premalignant state (biological MGUS), even though the clonal BMPCs percentage or M protein level is higher than that specified in the clinical definition of MGUS and M protein value abruptly increases when symptomatic MM develops [13]. An increase in the number or proliferation rate of circulating PCs determined by immunofluorescence is an important risk factor for progression [14] but the three most important prognostic factors for progression of SMM are a serum M protein level greater than 3.0 g/dL, an IgA immunoglobulin, and urine M protein excretion of more than 50 mg per day. Patients with two or three of these risk factors have a median time to progression of 17 months, whereas those with one risk factor have a median time to progression of 40 months and those with none of the factors have a progression time of 95 months [15]. As with MGUS, also for SMM have been developed a risk-

stratification model to predict risk of progression. Three risk factors have significant prognostic value: (1) BMPCs greater than 10%; (2) serum M protein greater than 3.0 g/dL; and (3) abnormal FLC ratio less than 0.125 or greater than 8. The probability of progression at 5 years is 25% for those with one risk factor, 51% in the presence of two risk factors, and 76% if all three risk factors were present. If there are doubts about the differentiation of SMM from MM and whether to begin chemotherapy immediately, one should withhold treatment and reevaluate in 2 or 3 months. One must keep in mind that patients with SMM may remain stable for a long time. Patients should be reevaluated 3 to 6 months after the recognition of SMM; if they are stable, they can be seen at 6-month intervals. There is no evidence that OS is lengthened by giving chemotherapy to asymptomatic patients [7, 16].

For most myeloma patients, the PC proliferation is restricted to the BM. However, when tumor mass expands and severe organ impairment or symptoms increases, symptomatic MM can progress into a more aggressive form, the Extramedullary Myeloma (EMM). EMM is defined by the presence of PCs outside the BM (mostly liver, skin, central nervous system, pleural effusion, kidneys, lymph nodes, and pancreas) and may be found in up to 30% of MM patients across the overall disease course. The molecular mechanisms underlying the hematogenous spread of PCs outside the BM are only partially known and involve hypoxia and an altered expression of adhesion molecules. The prognosis of EMM is poor and the median OS of patients who experience an extramedullary disease is < 6 months. EMM patients should be considered as having high-risk myeloma and treated accordingly [17]. Sometimes EMM is associated with plasma cell leukemia (PCL) which is a rare and aggressive variant of myeloma characterized by the presence of circulating PCs. PCL can be classified into primary and secondary forms: primary PCL occurs at diagnosis and does not arise from pre-existing MM; secondary PCL is a leukemic transformation of end-stage MM and occurs in patients with relapsed/refractory MM. The diagnosis of PCL is based upon the presence of >20% of PCs in whole leucocytes with an absolute count of  $2 \times 10^9/L$  PCs in the peripheral blood (PB). The prognosis of PCL is very poor, with a median OS of only 7 months with standard chemotherapy, therefore it requires innovative treatment approaches incorporating various modalities to improve outcome [18].

**Table 1: International Myeloma Working Group (IMWG) Diagnostic Criteria for MGUS, SMM and MM.**

Reproduced from [6].

<b>Diagnosis</b>	<b>Disease Definition</b>
<b>MGUS</b>	<p>All 3 criteria must be met:</p> <p>Serum M protein (IgG or IgA or IgM) &lt; 3g/dL</p> <p>Clonal BMPCs &lt;10%</p> <p>Absence of myeloma-defining events (MDE) or amyloidosis</p>
<b>SMM</b>	<p>Both criteria must be met:</p> <p>Serum M protein (IgG or IgA) <math>\geq</math> 3g/dL or urinary M protein (Bence Jones Protein) <math>\geq</math> 500mg/24h and/or Clonal BMPCs 10-60%</p> <p>Absence of MDE or amyloidosis</p>
<b>Active (Symptomatic) Myeloma</b>	<p>Both criteria must be met:</p> <p>Clonal BM PCs <math>\geq</math>10% or biopsy proven bone or extramedullary Myeloma</p> <p>And</p> <p>Anyone or more of the following MDE:</p> <ul style="list-style-type: none"> <li>. calcium &gt; 0.25 mmol/L (&gt;1mg/dL) higher than the upper limit of normal or &gt;2.75mmol/L (&gt;11mg/dL)</li> <li>.Renal insufficiency (creatinine &gt;2mg/dL (&gt;177<math>\mu</math>mol/L) or creatinine clearance &lt;40ml/min</li> <li>. Anemia (hemoglobin &lt;10g/dL or hemoglobin &gt;2g/dL below the lower limit of normal)</li> <li>. one or more osteolytic bone lesions on skeletal radiography, CT or PET-CT</li> <li>. Clonal BM PCs <math>\geq</math>60%</li> <li>. Abnormal serum FCL ratio <math>\geq</math> 100 (involved kappa) or &lt;0.01 (involved lambda)</li> <li>. &gt;1 focal lesions on MRI studies &gt;5mm in size</li> </ul>

**Table 2: Staging System for Multiple Myeloma.** Reproduced from [6].

<i>Staging</i>	<i>ISS</i>	<i>R-ISS</i>	<i>Durie-Salomon</i>
<b><i>I</i></b>	Serum $\beta$ -2 microglobulin <3.5 mg/L, serum albumin $\geq$ 3.5g/dL	ISS stage I and standard-risk* chromosomal abnormalities by FISH and serum LDH < the upper limit of normal	Low cell mass
<b><i>II</i></b>	Not ISS stage I or III	Not R-ISS stage I or III	Intermediate cell mass
<b><i>III</i></b>	Serum $\beta$ -2 microglobulin > 5.5 mg/L	ISS stage III and either high-risk** chromosomal abnormalities by FISH or serum LDH > the upper limit of normal	High cell mass
		<p>*standard risk: t(11;14);</p> <p>**High risk: presence of del(17p) and/or traslocation t(4;14) and/or traslocation t(14;16)</p>	<p><b>Subclassification (either A or B)</b></p> <p><b>A:</b> relatively normal renal function (serum creatinine value &lt;2mg/dL)</p> <p><b>B:</b> abnormal renal function (serum creatinine value &gt;2mg/dL)</p>

## ***1.2 Antigenic profile of malignant plasma cells in MGUS and Multiple Myeloma***

MM PCs can be distinguished from normal PCs based on the expression of several cell surface markers. Important phenotypic characteristics of both normal and neoplastic PCs are the expression of CD138 (syndecan-1) and CD38, while CD45 appeared weakly expressed by healthy PCs (CD45 expression has been reported to decline in vivo during normal PCs differentiation) and variable expressed by neoplastic PCs: its intensity varied from negative to bright. In fact, it has been reported that different PCs populations can be identified based on CD45 expression in myeloma patients characterized by dim to negative expression of CD45 and intermediate to bright expression of the antigen [19]. MM PCs can also be distinguished from normal PCs based on the expression of CD56 and the lack of CD19 expression. CD56 is a neural adhesion molecule expressed in 70-80% cases of MM. Lack of CD56 expression has been shown to be associated with poor prognosis in MM patients treated with conventional chemotherapy. In contrast, CD56 negativity was not found to confer a poor prognosis in patients treated with high-dose chemotherapy and autologous stem cell transplantation (ASCT), suggesting that intensive treatment might overcome the adverse influence of CD56 negative MM [20]. CD19 is a hallmark differentiation antigen of the B-cell lineage and positively regulates antigen receptor signal transduction in mature B-cells. Malignant PCs isolated from MM patients lack CD19, while non-malignant PCs isolated from healthy donors express the antigen. An intriguing observation is the existence of both CD19<sup>-</sup> and CD19<sup>+</sup> PCs in some cases of MGUS [21]. Compared to normal PCs, MM neoplastic clones show the overexpression of the CD44v9 variant isoform that mediates the binding to the BMSCs and is involved in the induction of IL-6 production by these latter. The presence of this variant isoform is associated with poor prognosis [22]. Approximately 20% of MM patients and 50% of PCL patients express the mature B-cell marker CD20. It has been suggested that CD20 expression on MM PCs reflects a more aggressive subtype. This is based on the fact that CD20 expressing MM cases have a shorter survival compared to those whose PCs do not express CD20. Another important MM marker is CD28, which is expressed in the majority of the MM cases but is absent on normal PCs from tonsil and BM. It has been demonstrated that CD28 is expressed in 19% of the MGUS patients, 41% of the MM patients and on human MM cell lines [23]. Recently, it has been demonstrated that MM PCs lack CD27 expression compared to normal BM derived

PCs. Moreover, the antigen reappears in patients who achieved a complete clinical remission compared to newly diagnosed and relapsed MM patients, whereas PCs from MGUS patients displays a high CD27 expression. These data suggest that loss of CD27 is associated with progression of disease. The differential expression of CD27 on MM plasma cells was confirmed by cDNA microarray analysis. Comparing the mRNA expression of more than 5,000 genes, CD27 was the second most downregulated gene in MM PCs compared to normal donor BM PCs [24]. Based on CD33 expression, MM patients can be divided into two groups, the first expressing CD33 and showing a lower survival and the second lacking the antigen [25]. Finally, normal PCs do not express CD117 (also known as c-kit) but the latter was reported on malignant PCs of some myeloma patients that show a better outcome compared to the CD117<sup>-</sup> patients. Therefore, it is possible that CD117 could be one of the markers for transition from MGUS to MM [26]. The table below summarizes the common phenotypic markers used in flow cytometry analysis of MGUS and MM.

**Table 3: Expression of various antigens in PCs of MGUS and MM.** Reproduced from [27].

<i>CLUSTER DESIGNATION</i>	<i>NORMAL DISTRIBUTION AND FUNCTIONS</i>	<i>EXPRESSION IN MGUS AND MM PCS</i>
<b>CD19</b>	All B cells, including lymphoblasts, mature B-lymphoid cells and most of PCs	MGUS – normal PCs express CD19, rather malignant PCs do not MM – only negative or dim CD19 expression on PCs
<b>CD20</b>	During maturation process of pre-B cells and negative expression on PCs	Some subsets of myeloma patients express CD20 on PCs and the expression correlates with a more aggressive phenotype
<b>CD27</b>	Expressed on B cell subsets and on the majority of peripheral T cells, aids in differentiation of mature B cells into PCs	MGUS – consistent high expression on PCs MM – expression is heterogeneous but MM PCs show low intensity compared to MGUS
<b>CD28</b>	The antigen is present on T lymphocytes and serves for T cell activation	MGUS – only very few cases express CD28 MM – expression is heterogeneous but CD28 expressing PCs represented an aggressive phenotype. This antigen is associated always with tumor expansion
<b>CD33</b>	Myeloid and monocytic cells	Only few myeloma patients express CD33 on PCs and the presence of the antigen correlates with lower survival
<b>CD38</b>	It appears on BM precursor cells but is lost on mature B lymphocytes and is one of the few antigens present on the surface of terminally differentiated PCs	Both MGUS and MM PCs express CD38
<b>CD45</b>	B and T cells, dim expression in precursor cells and on PCs	MGUS – equal distribution of CD45 <sup>+</sup> and CD45 <sup>-</sup> PCs MM - variable expressed by neoplastic PCs. Based on CD45 expression, different PCs populations can be identified characterized by dim to negative expression of CD45 and intermediate to bright expression of the antigen. Survival rate is higher for CD45 <sup>+</sup> patient group
<b>CD56</b>	NK and NK-T cells	Most of the myeloma cases express CD56. However, circulating PCs and extramedullary myeloma patients lack CD56 expression
<b>CD117</b>	Progenitors of myeloid, erythroid and megakaryocytic lineage	MGUS – 50% of cases express CD117 MM – one third of myeloma patients express CD117 and have better prognosis comparing to their counterpart
<b>CD138</b>	PCs	MGUS and MM cases express CD138 on PCs

### ***1.3 Role of BM microenvironment in Multiple Myeloma***

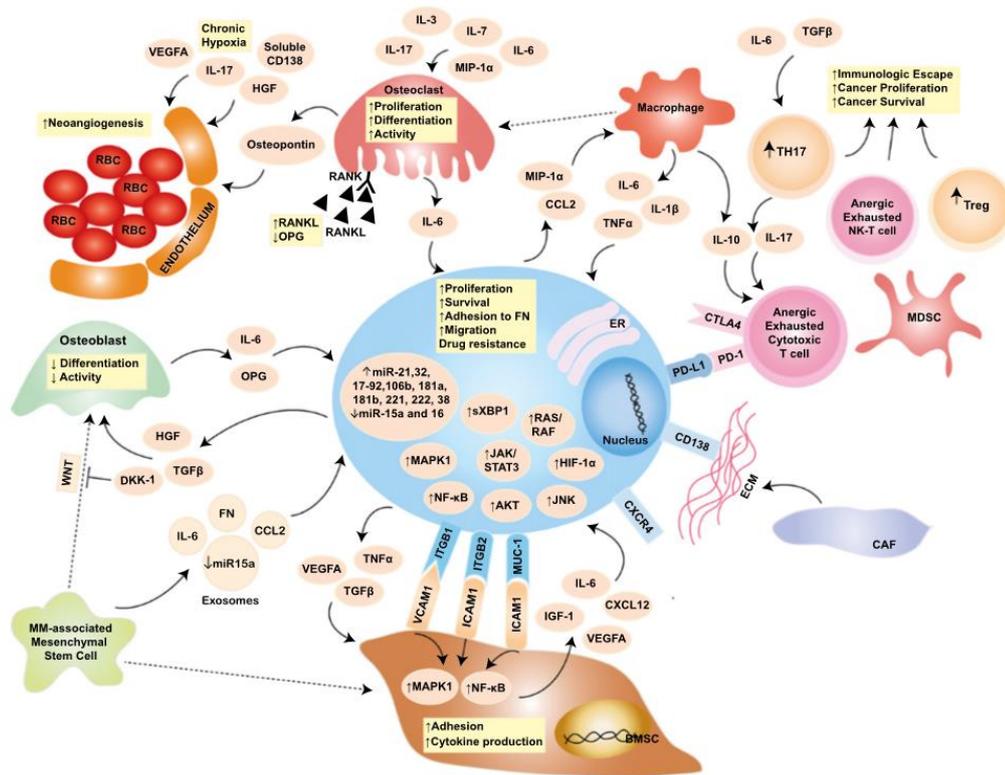
The pathogenesis of MM involves complex interactions between the tumor cells and the BM microenvironment. In the BM long-lived PCs are in close contact both with cellular and structural elements and are highly dependent on the microenvironment for survival, growth and differentiation (Figure 1). The niche aids the growth and spreading of tumor cells by a complex interplay of cytokines, chemokines, proteolytic enzymes and adhesion molecules and confers chemoresistance of MM cells to current therapies. However, as the disease becomes more aggressive, tumor cells become stromal independent. The microenvironment includes the extracellular matrix (ECM) and several types of BMSCs: fibroblastic stromal cells, mesenchymal stromal cells (MSCs), osteoblasts (OBs), osteoclasts (OCs), adipocytes, vascular endothelial cells (ECs), macrophages, dendritic cells (DCs) and lymphocytes. The ECM is the most abundant acellular component of the BM niche and consists mainly in fibronectin (FN), collagen types I and IV, laminin and the glycosaminoglycans heparin sulfate (HS), chondroitin sulfate and hyaluronan (HA). BMSCs are the major constituent of the BM space and the main producers of structural elements of the BM microenvironment such as ECM proteins and basement membrane proteins. Similar to other cellular components of the BM, malignant and normal BMSCs are functionally different and BMSCs derived from MM patients support MM proliferation, resistance to apoptosis, drug resistance, migration and invasion via both direct cell-to-cell contact and through secretion of cytokines and chemokines. Adhesion of myeloma cells to fibronectin confers protection from apoptosis, whereas binding of myeloma cells to BMSCs induces transcription and secretion of several cytokines including interleukin-6 (IL-6), insulin-like growth factor-1 (IGF-1), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), vascular endothelial growth factor (VEGF), interleukin-1 $\beta$  (IL-1 $\beta$ ) and stroma-derived factor-1 (SDF-1) that enhances survival and growth of the neoplastic clone. IL-6 is the major growth and survival factor for myeloma cells, usually involved in the early pathogenesis of myeloma, although IL-6 independence can occur during progression. It triggers proliferation via the Ras, Raf, MEK, mitogen-activated protein kinase (MAPK) cascade and promotes myeloma cell survival via phosphorylation of signal transducer and activator of transcription 3 (STAT-3) and up-regulation of anti-apoptotic molecules such as Mcl-1, Bcl-x<sub>L</sub> and c-Myc, conferring chemoresistance to the neoplastic clone. IL-6 also induces VEGF expression and secretion in myeloma cells and inhibits antigen-presenting function of DCs, hence contributing to the

immunocompromised status characteristic of myeloma. In turn, VEGF interacts with receptors on ECs and enhances migration and proliferation of myeloma cells, augmenting IL-6 production in BMSCs and stimulating marrow angiogenesis, which is inversely correlated with the length of patient survival and correlates with disease activity. IGF-1 is another paracrine factor that enhances survival, growth and drug resistance in myeloma cells by activating Ras-MAPK and PI3K-Akt pathways. Moreover, the cytokine inhibits apoptosis by leading to the phosphorylation of pro-apoptotic proteins (e.g. BAD, a Bcl family member). TNF- $\alpha$  is produced by myeloma cells and BMSCs and up-regulates expression of adhesion molecules on myeloma cells and their ligands on BMSCs, increasing the binding of myeloma cells to the latter, hence promoting myeloma cell survival and protection against apoptotic stimuli. SDF-1 promotes proliferation and induces migration in myeloma cells. Moreover, it increases the secretion of IL-6 and VEGF in BMSCs and functions as a chemoattractant, which localises myeloma cells in BM microenvironment. IL-1 $\beta$  is produced mainly by BMSCs and induces IL-6 production in myeloma cells and activates osteoclasts and bone resorption [1]. Several other osteoclast-activating factors such as parathyroid-hormone-related protein (PTHrP), hepatocytes growth factor (HGF) and TNF- $\alpha$  are generated by the mutual interaction of tumor and BMSCs. Two mechanisms are particularly notable: the first involves macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) which is secreted by MM cells and functions as an osteoclast chemotactic and maturation factors [28]; the second involves a receptor called receptor activator of nuclear factor-kB (RANK). This pathway seems to be the key regulator of osteoclastogenesis. In a physiological healthy state, equilibrium between OBs and OCs function exists, thus guaranteeing adequate bone mass. In MM patients, the osteoblastic niche is depleted in favour of an overabundance of OCs and the increased osteoclastogenesis is determined by the aberrant composition of the soluble BM milieu. The normal balance between the pro-osteoclastogenic RANK (which normally is expressed as a membrane-bound ligand on OBs and T cells and interacts with RANK-L on OC precursors to generate active OCs) and the anti-osteoclastogenic RANK-decoy receptor osteoprotegerin (OPG), that is produced by stromal cells and that protects the skeleton from excessive bone resorption by competing with RANK for RANKL binding, is lost in favour of the former. The active interaction of  $\alpha$ 4 $\beta$ 1 integrin on myeloma cells with vascular cell adhesion molecule-1 (VCAM-1) on BMSCs, causes a marked decrease in OPG secretion and increased the production of RANKL by stromal cells and perhaps also by tumor cells, enhancing the generation of OCs which, in turn, sustain MM proliferation and

angiogenesis by secreting IL-6 and osteopontin (OPN). Moreover, other pro-OCs cytokines such as MIP-1 $\alpha$ , IL-6, IL-7 and IL-3 are also detected at higher concentration in the serum of MM patients compared with healthy donors and contribute to osteoclastogenesis and MM-related diseases [29, 30].

As discussed above, BM microenvironment is crowded by different cellular elements: MSCs are precursor cells retaining the capacity to self-renew and differentiate into a variety of cell types, including fibroblasts, adipocytes, chondrocytes and OBs/OCs. MM-MSCs produce more VEGF-A, IL-6, IL-1 $\beta$  and TNF compared to their normal counterparts, thus favoring MM cells proliferation and inhibiting OBs function and differentiation. Furthermore, it has been shown that MM-MSCs enhance tumorigenesis by transferring oncogenic factors, including IL-6 chemokine (C-C motif) ligand 2 (CCL2) and fibronectin to MM cells via exosomes [31]. Exosomes released by MM cells were also noted to contain a different pattern of miRs; in particular, a lower level of the oncosuppressor miR-15a and upregulated levels of miR-135b, resulting in decreased SMAD family member 5 (SMAD5) expression and impaired capacity of OBs differentiation [32]. Moreover, MM-MSCs carry genomic abnormalities that are not present in healthy MSCs and when co-cultured in vitro with MM cells, acquire a phenotype similar to MM-MSCs in a matter of hours. Exosomes have been also shown to mediate chemoresistance in MM. This observation further supports the hypothesis that cancer microenvironment is not just an innocent bystander in the oncogenic process but contributes actively to cancer pathogenesis. In fact, it has been postulated that exosomes may transfer genetic material (in particular oncogenes) into tumor cells, suggesting a completely novel mechanism for oncogenesis [33]. Also fibroblasts infiltrating the cancer microenvironment differ from their normal counterparts. In particular, cancer associated fibroblasts (CAFs) play a pivotal role in inducing epithelial-mesenchymal transition and in promoting metastasis in solid malignancies, producing a surplus of ECM components that differ from those of healthy individuals [34]. Adipocytes, which are abundant in the BM of elderly individuals, were also shown to support MM pathogenesis [35]. Similar to other cancers, MM cells are capable to escaping immunologic surveillance by inducing immune tolerance and T cell anergy. The composition of lymphocytes present in the MM microenvironment substantially differs from that in a healthy subject. Under the priming of elevated concentration of IL-6 and TGF- $\beta$ , T<sub>H17</sub> cells, a distinct subset of CD4<sup>+</sup> T helper lymphocytes characterized by a peculiar pattern of cytokines production, are abundant in the BM of MM patients. T<sub>H17</sub> cells suppress cancer

immune surveillance by secreting IL-17 and IL-10. IL-17 also serves as a pro-osteoclastogenesis factor, providing an indirect mechanism of MM cells support and contributing to MM-related bone disease [36]. Cytotoxic CD8<sup>+</sup> T cells and natural killer (NK) cells in the BM microenvironment of MM patients differ from their healthy counterpart in the repertoire of T-cell receptor (TCR) co-receptor molecules. Increased expression of PD-1, a TCR co-receptor with inhibitory function, was shown on BM-resident cytotoxic T cells of MM patients. Coupled with over-expression of PD-L1 on the surface of MM cells, the increased expression of PD-1 account as one of the major mechanisms of immunologic tolerance in MM [37]. In MM, the abnormal BM cytokine milieu in states of chronic inflammation causes the accumulation of the heterogeneous population of immature myeloid precursors with immunosuppressive functions called myeloid derived suppressor cells (MDSCs). It has been shown that MM cells are capable to expand MDSCs and that these latter contribute to myeloma pathogenesis at least in 2 ways: first, by exerting an anti-proliferative effect against lymphocytes via increased nitric oxide (NO) production, L-arginine depletion and IL-10 secretion; and second, by differentiating into OCs in the context of a RANKL-rich milieu [38]. Within the tumor microenvironment, MDSCs can also differentiate into tumor-associated macrophages (TAMs), which are phenotypically and functionally distinct from their precursor cells. TAMs can also be the product of differentiation of circulating monocytes, which are attracted to cancer cells via chemoattractants that are secreted by MM cells themselves (CCL-2 and MIP-1 $\alpha$ ). TAMs contribute to MM pathogenesis in 3 different ways: first, they are a major source of IL-6 as well as other pro-MM cytokines such as TNF- $\alpha$  and IL-1 $\beta$ ; second, they produce IL-10, a major mediator of cancer immune tolerance by suppressing the function of T cells; lastly, macrophages contribute substantially to angiogenesis by releasing VEGF-A and NO and by acquiring the capability of differentiating into EC-like cells under the autocrine/paracrine effect of VEGF-A [39]. The BM microenvironment is hypoxic and neoangiogenesis and increased vessel density are hallmarks of MM. Indeed, the elevated expression of HIF-1 $\alpha$ , HIF-2, VEGF-A, HGF, and SDF-1 have all been shown to promote angiogenesis in MM. Hypoxia drives epithelial to mesenchymal transition in MM, thus promoting tumor trafficking and metastasis and appear to induce a stem-like phenotype in vitro. However, increased tumor burden and loss of an inhibitor of angiogenesis during evolution from MGUS to MM, have proved to be the major determinants of increased microvessel density in MM patients [37].



**Figure 1: Role of BM niche in MM pathogenesis.** The blue oval in the center is MM cell which is in close interplay with cellular and acellular components of the BM. The pale orange ovals represent relevant cytokines/chemokines in the BM milieu. Dotted arrows indicate differentiation, whereas solid arrows indicate secretion and/or effect on a target cell. Yellow squares contain a synopsis of the overall effect of cytokines and cell-to-cell contact on the target cell. Key signaling cascades, transmembrane proteins and intracellular organelles, which are of interest for molecular therapies, are represented. Reproduced from [37].

## 1.4 Biology and molecular profiling of Multiple Myeloma

MM is a B-cell neoplasm and, even if the precise nature of the cell of origin in myeloma is not established, current evidence suggests that the malignant clone is likely to be a long-lived PC which derived from B cells that have been exposed to antigen and undergone the B cell maturation process of VDJ recombination, somatic hypermutation and isotype recombination in the germinal center (GC). After stimulation by specific antigens, GC B lymphocytes differentiate into long-lived memory B cells on one hand and PCs on the other hand and these latter, subsequently, home to the BM that is the major site of disease where PCs reside as terminally differentiated and non-proliferating [40]. The natural course of the disease is the result of a multistep process [41] that may progress from MGUS and SMM to symptomatic

myeloma and EMM/PCL. This process is characterized by a series of genetic events that impact in different signaling pathways changing the biologic characteristics of the myeloma cells and determining proliferative and selective advantages (Figure 2). Multiple prognostic factors that reflect host factors, tumor biology, tumor burden and response to treatment have been described; among them, the presence of genetic alterations in PCs has been considered an important prognostic factor, capable to identify patients with different clinical features and response to therapy. Although many genetic alterations are amenable to detection by conventional cytogenetics, their detection is hampered by the low proliferative index of PCs and, consequently, the limited number of metaphases. In addition, some aberrations are cryptic and cannot be detected by standard cytogenetics, thus an apparently normal karyotype is observed in 70-80% of MM patients. However, the introduction of interphase fluorescence in situ hybridization (FISH) studies showed genetic alterations in more than 80% of cases.

Primary alterations can be subdivided in two cytogenetic categories: hyperdiploid and nonhyperdiploid. The hyperdiploid group (45% of cases) is characterized by the presence of trisomies of odd-numbered chromosomes (3,5,7,9,11,15,19 and 21) with patients showing a total of 48-75 chromosomes and a low prevalence of immunoglobulin heavy chain (IgH) translocations involving 14q32 locus. In contrast, the non hyperdiploid group (40% of cases) encompassing hypodiploid, pseudodiploid and near tetraploid MM, is characterized by a high frequency of IgH translocations, indicating that two fundamentally different pathogenic pathways exist for MM development [42]. The ploidy categories are stable over time and rarely change at disease progression. In a small subset of patients (10%), both IgH translocations and hyperdiploid can be observed. This association is considered to have an unfavourable outcome. In addition, there is a subgroup of cases (15%) that present other type of alterations and variable clinical course. Overall, hyperdiploidy showed a more favourable clinical evolution, regarding often elderly individuals which have a higher incidence of MM bone disease. This group is heterogeneous and is characterized by overexpression of largely non-overlapping gene sets associated to different pathways relevant to myeloma biology and with different clinical outcome, such as, cancer testis antigen and mitosis/proliferation-related genes, hepatocyte growth factor (HGF)/IL-6 genes, NF-kB/anti-apoptosis genes and with low expression of HGF [43]. Nonhyperdiploid MM cases are also a heterogeneous group that consists of several molecular subtypes based on the specific partner chromosome involved in the IgH translocation

and mediated primarily by errors during IgH switch recombination process (Table 4). The subgroup includes: Cyclin D translocations, t(4;14) (p16.3;q32) and MAF translocations.

#### ***1.4.1 Cyclin D translocations***

Translocation t (11;14) (q13; q32), with the consequent up-regulation of Cyclin D1 (CCD1) gene, is the most frequent translocation, occurring in approximately 15-20% of newly diagnosed MM patients and in one half of cases of light chain amyloidosis and in MGUS. Other alterations are t (6;14) (p21; q32) (2%) and t (12;14) (p13; q32) (1%), involving Cyclin D3 gene (CCND3) and Cyclin D 2 gene (CCND2), respectively. These translocations are associated with nonsecretory or hyposecretory disease, lymphoplasmacytic or small mature PCs morphology, lambda light chain usage and CD20 expression. In particular, t (11;14) (q13; q32) has been associated with favourable clinical evolution, even if recently the International Myeloma Workshop Consensus has established that t (11;14) does not predict a superior outcome: a recent evaluation of its impact on the outcome of autologous hematopoietic cell transplantation concludes that t(11;14) has a worse outcome than patients with a normal phenotype but better than patients with high risk markers [44, 45].

#### ***1.4.2 t (4;14) (p16.3; q32) translocation***

The second most frequent reciprocal translocation in MM patients is t (4;14) (p16.3; q32), which occurs in about 15% of patients. This is a cryptic translocation that must be evaluated by FISH or reverse transcriptase PCR (RT-PCR) and involves two protein coding genes: MM SET domain (MMSET), a protein with homology to histone methyltransferases, and fibroblast growth factor receptor 3 (FGFR3), an oncogenic receptor tyrosine kinase, both mapped at 4p16.3, which appear upregulated. However, the translocation can be imbalanced with up to 25% losing the derivative chromosome 14 associated with the loss of FGFR3 expression. T (4;14) (p16.3; q32) is associated with the use of IgA heavy chain, lambda light chain and very high prevalence of chromosome 13 deletion/monosomy and identify a subset of MM patients with short survival, even in the context of autologous transplantation [46]. This translocation is also observed in cases with MGUS and frequently in patients with SMM.

### ***1.4.3 MAF (Musculoaponeurotic fibrosarcoma) gene translocations***

MAF (Musculoaponeurotic fibrosarcoma) gene translocations are less frequent, being observed in 5-7% of MM patients. They include: t (14; 16) (q32; q23) (5%), t (14;20) (q32; q11) (2%) and t (8;14) (q24.3; q32) (<1%), involving c-MAF, MAFB and MAFA genes, respectively. These translocations have been associated with IgA isotype, higher frequency of chromosome 13 deletion and a more aggressive clinical outcome. The mechanism of this poor outcome is thought to involve the consequence of MAF upregulation, which include upregulation of CCND2, effects on cell interaction and upregulation of apoptosis resistance. Particularly, the t (14;16) (q32; q23) juxtapose the c-MAF gene (16q13) and the IgH locus at 14q32. The breakpoint occurs in the introns of a very large gene named WWOX (WW domain oxidoreductase), which spans a fragile site (FRA16D) [47]. Finally, t (14;20) (q32; q11) involving MAFB gene, is associated with short survival in MM patients. The same translocation shows a long-term stable disease in MGUS and SMM patients, suggesting that the translocation alone cannot be responsible for adverse clinical behaviour and additional events must be required for disease progression [48].

It is presumed that the translocations cited above usually represent primary or perhaps initiating oncogenic events as normal B cell pass through GC. The observation that patients with MGUS and/or SMM also carry some initial mutations, suggests that they are necessary, but not sufficient, in MM pathogenesis. Late oncogenic events are thought to occur in the BM, after the founder cancer clone is completely differentiated into a long-lived PC (Figure 2). In fact, in MM several recurrent secondary alterations have been described. The most frequent are: deletion/monosomy of chromosome 13, deletion of chromosome 17p13, chromosome 1 abnormalities (1p deletions and 1p gains/amplifications), C-MYC translocations and activating mutations of RAS and BRAF family genes and of Nf-KB pathway.

### ***1.4.4 Chromosome 13 alterations***

Chromosome 13 alterations are detected in 50% of cases and consist in 85% monosomies while the remaining 15% are interstitial deletions. These alterations were first associated with an unfavorable prognosis and short survival, but there is now increasing evidence that their prognostic relevance may be related to their association with high risk IGH translocations, particularly t (4;14) (90% of cases), or t (11;14), being considered as a marker of

nonhyperdiploid MM [49]. The pathologic effect of this chromosome deletion is unknown but it's possible that it may lead to progression due to haploinsufficiency of the retinoblastoma (RB1) tumor suppressor gene [50].

#### ***1.4.5 Deletion 17p13***

This alteration, in which the tumor suppressor gene TP53 maps, is considered the most important molecular cytogenetic prognostic factor in MM patients. TP53 has an important role in promoting apoptosis, senescence or cell cycle arrest in response to DNA damage, while TP53 deletion or mutations may either predispose cells to DNA damage or allow cell survival. It is a late event in MM, being reported in about 10% of cases by FISH studies. Its presence predicts for shorter survival, more aggressive disease, higher prevalence of extramedullary disease, hypercalcemia, short duration of response post-high dose therapy and central nervous system involvement. It is observed in most cases of PCL both primary and secondary and is very uncommon in MGUS [45, 51].

#### ***1.4.6 Chromosome 1 alterations***

Structural aberrations of chromosome 1 are the most frequent additional changes in PC disorders, being found in 45% of MM and in almost all PCL patients. Among them, 1q21 gains/amplifications are highly prevalent in MM and its frequency rises during the course of the disease. This alteration introduces an increased level of genetic instability in myeloma cells. Significantly short survival is observed in patients with 1q21 gain/amplification compared to those lacking this alteration. In addition, higher frequency of 1q21 gain was found in relapsed patients (72% of cases), probably associated with drug resistance. One of the key genes mapped on chromosome 1q21 is CKS1B (CDC28 protein kinase regulatory subunit 1B) that encodes for a positive cell cycle regulator that activates cyclin-dependent kinases to promote proliferation and cell cycle progression. CKS1B is essential for the ubiquitination of the inhibitor of the cell cycle CKDN1B (p27<sup>KIP1</sup>) whose degradation is required for the cellular transition from quiescence to proliferative state. Its overexpression was associated with a high rate of proliferation and poor prognosis in MM patients and a positive correlation between CKS1B expression and 1q21 gain was observed. Moreover, patients with 1q21 gain/amplification show a higher prevalence of adverse IGH translocations as well as other secondary alterations like deletion 13q and 17p [45, 52]. Deletion of 1p have been identified in

approximately 7-40% of myeloma cases using cytogenetics, FISH and comparative genomic hybridization (CGH) and a current region of losses at 1p32.3 affecting CDKN2C (cyclin-dependent kinase inhibitor 2C) (p18<sup>INK4C</sup>) locus was defined. CDKN2C belongs to the INK4 family of cyclin-dependent kinases (cdk) inhibitors which interacts preferentially with the cdk4/6 preventing G1 progression. Chromosome 1p deletion has also been associated to adverse clinical outcome in MM patients and association between 1p deletions and 1q21 gains is frequently observed [45, 53].

#### ***1.4.7 MYC alterations***

In MM rearrangements of the proto-oncogene MYC (8q24) are often complex and involve non reciprocal alterations, duplications and amplifications that can be mediated by secondary events that do not involve B-cell specific recombination mechanisms and sometimes do not involve immunoglobulin loci. These alterations occur in 15% of newly diagnosed MM patients but are also observed in 45% of cases with advanced disease and in nearly 90% of human myeloma cell lines. MYC alterations were also detected in MGUS patients, suggesting it could be an early genetic event in the pathogenesis of MM responsible for progression from MGUS to MM. On the contrary, translocations of MYC and IGH loci are frequently observed as a late event during tumor progression, when the disease becomes more proliferative and less stromal dependent [45, 54].

#### ***1.4.8 Activating mutations of RAS and BRAF***

The members of the Ras GTPase family (H-RAS, N-RAS and K-RAS) are crucial players in many signaling networks connecting a great variety of upstream signals to an even wider set of downstream effector pathways linked to the functional control of a great assortment of cellular outcomes including cell cycle progression, growth, migration, cytoskeletal changes, apoptosis, and senescence. Mutations in RAS genes lead to the production of permanently activated Ras proteins that cause unintended and overactive signaling inside the cell, even in the absence of incoming signals that ultimately lead to cancer development. In MM, this results in the neoplastic PCs independence from the stimuli released by BM microenvironment, including IL-6 and insulin growth factor (IGF). The prevalence of activating NRAS or KRAS mutations in newly diagnosed and relapsed MM tumors is about 15%-18% each, but is substantially higher in tumors that express CCND1 compared with tumors that express CCND2. For MGUS, the

prevalence of NRAS mutations is 7%, but KRAS mutations have not been described. This is consistent with increasing evidence that NRAS and KRAS mutations have overlapping but non-identical effects and with the hypothesis that KRAS mutations provide a molecular mark of the transition of MGUS to MM. Recently, mutations in the proto-oncogene BRAF, that is part of the RAS/MAPK signaling pathway, were also described in 4% of MM tumors, suggesting a possible role for BRAF inhibitors in these cases of MM [50].

#### ***1.4.9 Mutations that activate the Nf-KB pathway***

BMSCs produce a lot of extrinsic ligands of the TNF superfamily including a proliferation-inducing ligand (APRIL) and a B cell activating factor (BAFF) that provide critical survival signals to long-lived PCs by stimulating B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI) and B cell activating factor receptor (BAFF-R) to activate the Nf-KB pathways. Most MGUS and MM tumors highly express Nf-KB target genes, suggesting a continued role of extrinsic signaling in PC tumors. Additionally, activating mutations in positive regulators and inactivating mutations in negative regulators of the Nf-KB pathway have been identified in at least 20% of untreated MM tumors and 50% of MM cell lines, rendering the cell less dependent on ligand-mediated Nf-KB activation. However, small molecules that inhibit extrinsic signaling have been developed as potential therapeutic agents [50].

#### ***1.4.10 Other alterations***

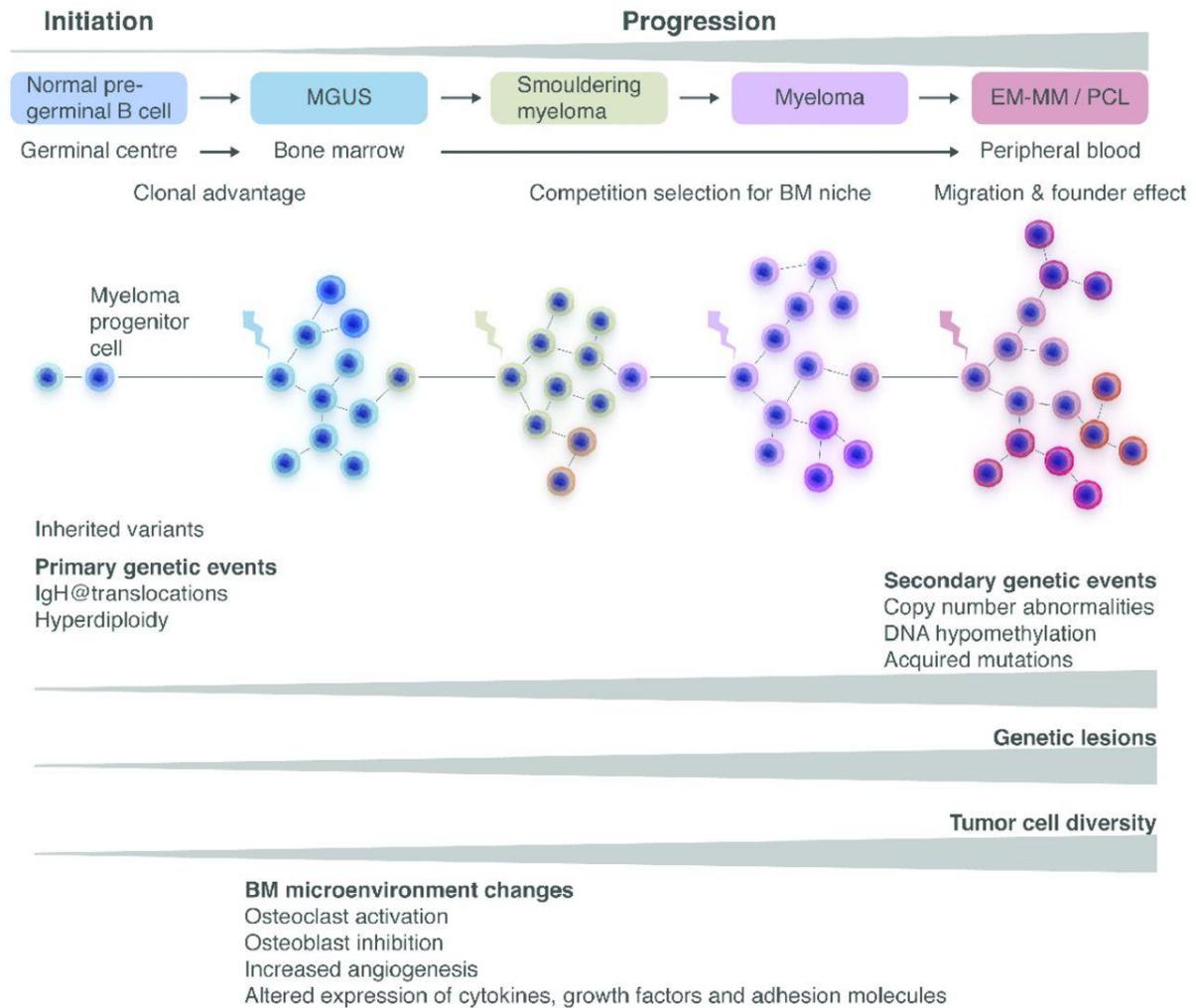
15% of MM patients have other alterations, showing a heterogeneous clinical course. Among them, deletions of the short arm of chromosome 12 occur in about 8% of MM patients and in 24% of cases with PCL. The size of the deletion is variable and it tends to appear in advanced disease, representing a secondary change associated with disease progression [45]. Finally, patients with MM present complex karyotypes which are the consequence of the accumulation of sequential genetic changes that appear during tumor clone development and are associated with disease progression. Taking into account the clinical outcome and the genetic abnormalities, MM can be classified in standard, intermediate and high risk (Table 5).

**Table 4: Distribution of cytogenetic abnormalities in patients with multiple myeloma.** Reproduced from [45].

Group (% of cases)	Genes	Clinical Characteristics
<b>Hyperdiploid (45)</b>		More favorable, IgG-k Older patients
<b>Nonhyperdiploid (40)</b>		Aggressive disease, IgA-λ Younger patients
<b>Cyclin dysregulations</b>		
<b>t (11;14) (q13; q32) (16)</b>	CCND1/IGH	More favorable
<b>t (6;14) (p21; q32) (2)</b>	CCND3/IGH	
<b>t (12;14) (p13; q32) (&lt;1%)</b>	CCND2/IGH	
<b>t (4;14) (p16;q32) (15)</b>	FGFR3-MMSET/IGH	Intermediate risk Less frequent bone lesions
<b>MAF rearrangements</b>		
<b>t (14;16) (q32; q23) (5)</b>	IGH/c-MAF	Aggressive disease
<b>t (14;20) (q32; q11) (2%)</b>	IGH/MAFB	
<b>t (8;14) (q24.3; q32) (&lt;1)</b>	MAFA/IGH	
<b>t (6;14) (p25;q32) (2)</b>	IRF4/IGH	Poor information
<b>Other alterations (15)</b>		Variable

**Table 5: Cytogenetic risk stratification.** Reproduced from [45].

High Risk (20% of patients)	Intermediate Risk (20% of patients)	Standard Risk (60% of patients)
t (14;16) (q32; q23)	t (4;14) (p16; q32) (15)	Hyperdiploid
t (14;20) (q32; q11)	del (13) (q14)	t (11;14) (q13; q32) (16)
del (17) (p13)		t (6;14) (p21;q32) (2)
chromosome 1 alterations		
Complex karyotype		



**Figure 2: Pathogenesis of MM.** MM is a result of a multistep process that may progress from MGUS and SMM to symptomatic myeloma and EMM/PCL. It is thought that transition through these different states requires the acquisition of genetic abnormalities that lead to the development of the biological hallmarks of myeloma. The initial genetic event is thought to occur in the GC, facilitated by the processes of somatic hypermutation and isotype switching and characterizes the founder clone. Later genetic mutations occur at the time of transformation to MM, with de novo mutations acquired during disease evolution and heterogeneously present in different subclones. In fact, later in disease progression, MM PCs developed sufficient genetic abnormalities that let them to acquire a clonal advantage, expand and evolves: they are no longer restrained to growth within the BM and can be found at extramedullary sites and as circulating leukaemic cells. Reproduced from [55].

## ***1.5 Treatment of Multiple Myeloma***

Over the years MM treatment has undergone a strong evolution. For decades, the treatment of MM patients was based on the combination of alkylating agents (melphalan in particular) and steroids (MP- Melphalan Prednisone - therapy). However, with the arrival of the new millennium and the introduction of thalidomide, an immunomodulatory drug (IMiD), and bortezomib, a proteasome inhibitor (PI), a new era for MM treatment has began, that of the so-called “new agents”. Thus, the main protagonists of this revolution were two classes of drugs: that of IMiDs, whose mechanism of action is not entirely clear but is known that they inhibit the production of TNF, IL-6 and stimulate cytotoxic T lymphocytes and NK cell activity against the myeloma cells, also acting as anti-angiogenics, and that of PIs that block the proteasome, producing conflicting regulatory signals and interfering with critical cellular functions by down regulating Nf-KB and other anti-apoptotic proteins, by activating the tumor suppressor protein p53 and by modulating cell cycle proteins and other pro-apoptotic factors. Thalidomide was the first novel agent that demonstrated a significant activity in the treatment of both transplant eligible and not-eligible patients, by reporting superior response rates and overall survival (OS) compared to standard regimen. Analogs of thalidomide, the so called next-generation IMiDs (lenalidomide, pomalidomide), were subsequently tested, both for newly diagnosed and relapsed MM patients [56]. As reported above, bortezomib was the first PI used in the treatment of MM, at diagnosis and relapse of both transplant eligible and not-eligible patients [57]. However, despite the effectiveness, the parenteral administration and the toxicity profile of bortezomib (neurotoxicity of both motor and sensory type) [58], have prompt the investigation of new PI molecules (carfilzomib, oprozomib, ixazomib, marizomib) [59]. There has been a significant improvement in the survival rate of MM patients in the last 10 years; despite this progress, the median OS of patients refractory to both IMiDs and PIs is only 9 months. For this reason, new molecules with different mechanisms of action are appearing in the MM world, sometimes with very promising results. Among them, monoclonal antibodies (MoAbs), whose targets are mostly surface molecules such as CD38 (daratumumab and SAR650984) or CS1 (elotuzumab), and the histone-deacetylase inhibitors (panobinostat and vorinostat), which were tested either as single agents and, especially, in combination with drugs already approved, in order to determine a synergistic action, can be considered the new and more promising drugs for MM therapeutics [59].

Nowadays, the approach to treatment of newly diagnosed MM patients includes an initial therapy, stem cell transplant (if eligible), consolidation/maintenance therapy and treatment of relapse. Transplant eligible patients typically receive approximately 4 cycles of initial therapy followed by stem cell collection and autologous stem cell transplantation (ASCT). Selected patients with standard risk MM who respond well to induction can opt for delay ASCT: in this strategy, stem cells are collected after 4 cycles of initial therapy and cryopreserved for future use. Transplant-ineligible patients are usually treated for 12-18 months.

### ***1.5.1 Initial Therapy***

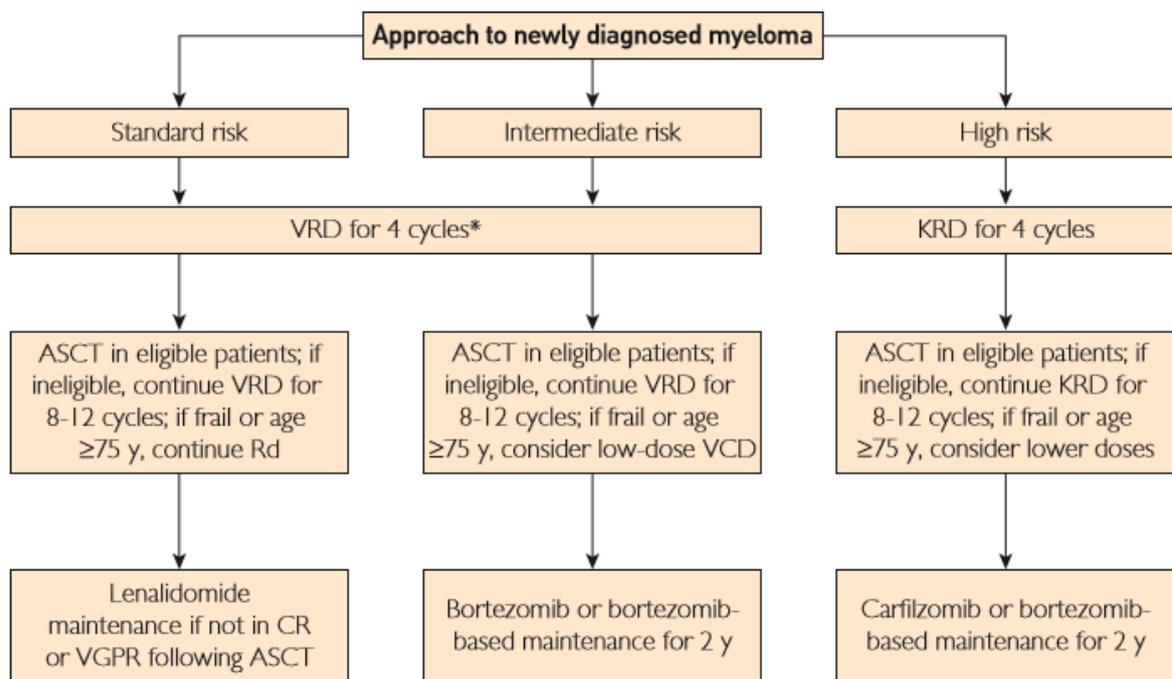
Initial therapy for MM varies across countries depending on drug availability. The most common regimens used in the treatment of newly diagnosed MM are lenalidomide plus dexamethasone (Rd), bortezomib, lenalidomide and dexamethasone (VRD), bortezomib, thalidomide and dexamethasone (VTD) and bortezomib, cyclophosphamide and dexamethasone (VCD). In a recent randomized trial conducted by the Southwest Oncology Group, progression free survival (PFS) OS were significantly superior with VRD compared with Rd [60]. Other studies have reported superior response rates and PFS with VTD compared with other regimens [61, 62]. On the basis of this data, VRD or VTD are the preferred regimens for initial therapy in transplant-eligible patients and in fit transplant-ineligible patients. The low dose dexamethasone regimen (40mg once a week) is preferred in all regimen (Rd, VRD, VTD, VCD, ecc) to minimize toxicity. Similarly, the once-weekly subcutaneous schedule of bortezomib is preferred in all regimen in order to diminish the neurotoxicity, the main adverse effect of the drug. Higher doses of dexamethasone and twice/weekly bortezomib can be considered if a rapid response is desired and in the case of patients with acute renal failure due to cast nephropathy, extensive EMD, PLC or impending cord compression [63]. Patients who are 75 years of age or older or are frail may not tolerate a triplet regimen. In these patients, Rd is a reasonable choice for initial therapy, especially for standard-risk patients [64, 65]. For patients with high risk myeloma, the triplet regimen of carfilzomib, lenalidomide and dexamethasone (KDR) has had high activity in phase 2 trials with stringent complete response rates and minimal residual disease (MRD)-negative rates that appear superior to historical result with VRD [66]. However, because of concerns about cardiac toxicity in a small proportion of patient with carfilzomib, the use of KDR is destined only to patients with high-risk MM in

whom it may be reasonable to administer a regimen with the highest possible complete response rates [66, 67] (Table 6).

### 1.5.2 Autologous Stem Cell Transplant (ASCT)

ASCT improves complete response rates and prolong OS in MM by approximately 12 months. The treatment-related mortality (TRM) rate is 1-2%. Eligibility for ASCT is based on age, performance status and comorbidities. A series of randomized trials showed that survival is similar whether ASCT is done early (immediately following 4 cycles of induction therapy) or delayed (at the time of relapse as salvage therapy). Furthermore, patients with MM can receive a second planned ASCT (tandem transplant) after recovery from the first procedure, especially if they not achieve a complete response or very good partial response (VGPR) with the first transplant. On the contrary, the high TRM and morbidity related to graft-vs-host disease has made conventional allogenic transplants unacceptable for most patients with MM. It is recommended primarily in young patients with high-risk MM in first or second relapse who are willing to accept a high TRM and graft-vs-host disease-related morbidity in return for a small chance at long-term OS [64].

**Table 6: Approach to the treatment of newly diagnosed Multiple Myeloma.** Reproduced from [64].



ASCT= autologous stem cell transplant; CR=complete response; KRD= carfilzomib, lenalidomide and dexamethasone; Rd=lenalidomide plus dexamethasone; VCD=bortezomib, cyclophosphamide and dexamethasone; VGPR=very good partial response; VRD=bortezomib, lenalidomide and dexamethasone; \*=Rd if frail or age  $\geq 75$ .

### ***1.5.3 Consolidation/Maintenance Therapy***

Numerous trials have been conducted over the years testing maintenance therapy in MM, either after ASCT or after 12 to 18 months of standard-dose therapy. However, the agents used were either ineffective, toxic or both and none of these approaches gained ground in clinical practice. Thalidomide had modest PFS and OS benefit as maintenance therapy in 2 randomized trials but has drawbacks of significant non hematologic toxicity [68, 69].

In the post-ASCT setting, maintenance therapy with lenalidomide and bortezomib has shown promise. The OS benefit was primarily in patients who received lenalidomide as part of the initial therapy before ASCT, even if it has been shown that there was also a clear increased risk of second cancers with lenalidomide maintenance therapy. For this reason the pros and cons of lenalidomide maintenance therapy should be considered carefully. It is recommended lenalidomide maintenance therapy in standard-risk patients who do well with lenalidomide-containing initial therapy and do not achieve a VGPR following ASCT [70]. In patients with intermediate- and high-risk MM, it is preferred bortezomib-based maintenance therapy. Furthermore, randomized trials with the new proteasome inhibitor ixazomib, which is administered orally one weekly, are ongoing.

The role of maintenance therapy after an initial 12 to 18 months of treatment for newly diagnosed MM in patients not receiving an ASCT is evolving. Some data indicate that continuous therapy with Rd is superior in term of PFS to Rd given for 18 months [71], but whether this benefit will be seen after 18 months of a triplet therapy such as VRD is unclear. Generally, if Rd is used as initial therapy, it is recommended to continuing it until progression; if a triplet regimen is used, it is recommended to stop therapy after 12 to 18 months in patients with standard-risk disease and continuing with bortezomib maintaining therapy in those with intermediate- and high-risk disease. Randomized trials with the new oral proteasome inhibitor ixazomib are ongoing in this setting as well.

### ***1.5.4 Treatment of relapsed Multiple Myeloma***

The approach to treatment of relapsed MM is complicated. Numerous effective regimens are available and the choice of treatment depends on numerous factors such as drug availability, response to previous therapy, aggressiveness of the relapse, eligibility for ASCT and whether the relapse occurred while the patient was receiving therapy. In eligible patients, ASCT should

be included in the consideration if the patient has never had an ASCT or if the remission duration with a previous ASCT exceed 18 months (no maintenance therapy) or 36 months (with maintenance therapy). Recent data support the use of triplet therapy for relapsed MM, but selected patients with indolent relapse can often be treated with a double regimen such as Rd or pomalidomide plus low-dose dexamethasone (PD). However, the most common regimens and new drugs used in the treatment of relapsed/refractory MM includes:

**Bortezomib- and Lenalidomide-Based Regimens;**

**Carfilzomib- and Pomalidomide-Based Regimen:** Carfilzomib is a keto-epoxide tetrapeptide proteasome inhibitor approved for the treatment of relapsed refractory MM in patients who have been treated previously with lenalidomide and bortezomib. It is a more PI than bortezomib and has a lower risk of neurotoxicity than the latter but a small proportion of patients (5%) may experience serious cardiac adverse experience. Moreover, drug resistance has been observed with carfilzomib treatment [72] and, as bortezomib, the drug presents the important limitation of the intravenously (iv) injection, that makes patients to necessitate the administration of this drug in a clinical setting, which may hamper their quality of life and increase the cost of treatment.

Pomalidomide is an analogue of lenalidomide and thalidomide approved for the treatment of relapsed/refractory MM [73]. It has significantly activity in relapsed/refractory MM, even in patients in whom lenalidomide or lenalidomide plus bortezomib has been ineffective. For this reason, as with Rd, the doublet regimen of PD is a reasonable option for patients with indolent relapse. More often, however, pomalidomide must be administrated in combinations such as pomalidomide, cyclophosphamide, bortezomib and dexamethasone; or carfilzomib, pomalidomide and dexamethasone.

**Panobinostat:** panobinostat is a pan-deacetylase inhibitor approved in 2015 for the treatment of relapsed /refractory MM. It is the first agent from a new class of drugs with meaningful clinical activity in MM in nearly 15 years. Its putative mechanism of action is to block the aggresome pathway, an alternative route for cells to bypass the lethal effect of proteasome inhibitor. By combining bortezomib and panobinostat, there is a simultaneous blockade of both proteasome and aggresome pathways [74]. In a randomized trial of 768 patients, bortezomib-dexamethasone plus panobinostat was associated with superior PFS compared with

bortezomib-dexamethasone plus placebo [75]. However, panobinostat therapy was associated with grade 3 diarrhea in approximately 25% of patients and care should be exercised when using this drug. It is recommended to use lower dose than approved starting dose and to use bortezomib in the once-weekly subcutaneous schedule.

**Liposomal Doxorubicin:** Anthracyclines have marginal single-agent activity in MM. A phase 3 randomized trial found that median time to progression was superior with bortezomib plus pegylated liposomal doxorubicin compared with bortezomib alone. OS at 15 months was also superior [76]. Despite these results, liposomal doxorubicin is infrequently used in the treatment of relapsed MM given the availability of other active agents.

**Monoclonal antibodies:** two monoclonal antibodies targeting CD38 (daratumumab and SAR650984) have shown promise in relapsed/refractory MM. Antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), apoptosis induction via FcR-mediated crosslinking or caspase-dependent MM cell death, modulation of enzymatic activity and immunomodulatory activity, are the various mechanisms of action for daratumumab [77]. In a phase 2 trial, daratumumab (Darzalex) as a single agent produced a response rate of approximately 30% in heavily pretreated patients [78]. On the basis of these data, daratumumab has been recently approved in the United States for use in the relapsed/refractory MM.

Elotuzumab (Empliciti<sup>TM</sup>), a monoclonal antibody targeting the signaling lymphocytic activation molecule F7 (SLAMF7) also known as cell-surface glycoprotein CD2 subset 1 (CS1), has also activity in relapsed MM. SLAMF7/CS1 expression is high in myeloma cells and NK cells, but not on normal tissues, making CS1 a potential target for MM immunotherapies [79]. NK cell-mediated ADCC through the CD1 pathway is the predominant elotuzumab mechanism of action that leads to the release of cytotoxic granules that eventually leads to MM cell death [80] and it has been demonstrated that it also impairs the adhesion of MM cells to BMSCs [81]. Unlike anti-CD38 antibodies, elotuzumab does not appear to have any single agent activity. However, it seems to have synergistic activity when combined with Rd. In a phase 3 trial of 646 patients, elotuzumab plus Rd was superior to Rd in terms of PFS. Based on these data, this drug has been approved in the United States for the treatment of relapsed MM [82]. Furthermore, two randomized studies of lenalidomide and dexamethasone combined with elotuzumab versus lenalidomide and dexamethasone without elotuzumab [79] and

Elotuzumab plus bortezomib and dexamethasone versus bortezomib and dexamethasone [83] in patients with relapsed/refractory MM showed promising results as well.

**Ixazomib:** ixazomib is an oral PI that is active in both the relapsed/refractory setting and in newly diagnosed MM. In a randomized controlled trial in relapsed MM, ixazomib, lenalidomide and dexamethasone were reported to improve PFS compared with Rd [84]. Ixazomib has been approved in the United States for the treatment of relapsed MM even if, currently, it is only approved for use along with lenalidomide and dexamethasone. It has the advantage of once-weekly oral administration but compared with bortezomib, has more gastrointestinal adverse events but lower risk of neurotoxicity. Moreover, ixazomib is under testing for use as single agent maintenance therapy in patients after ASCT and in those who have not received ASCT [85].

**Other Emerging Options:** Other promising agents include marizomib (a new proteasome inhibitor), oprozomib (an oral proteasome inhibitor related to carfilzomib), filanesib (a kinesin spindle protein inhibitor), ABT-199 (a selective BCL-2 inhibitor), dinaciclib (a cyclin dependent kinase inhibitor), and LGH-447 (pan-PIM kinase inhibitor). Each of these agents has single-agent activity in relapsed MM [64].

### ***1.5.5 Supportive Care***

**Hypercalcemia and Skeletal Lesions:** the mainstay of therapy for hypercalcemia is hydration, corticosteroids and bisphosphonates (pamidronate or zoledronic acid). Bisphosphonates are also important to prevent or reduce the number of skeletal lesions. In patients with osteolytic bone disease, the use of local radiation should be limited to those with spinal cord compression from extramedullary tumor extension and to patients with bone pain refractory to analgesics and systemic therapy. Vertebroplasty (injection of methylmethacrylate into a collapsed vertebral body) or kyphoplasty (introduction of an inflatable bone tamp into the vertebral body and after inflation the injection of methylmethacrylate into the cavity) can be used to decreased pain from vertebral fractures.

**Prevention of infections:** patients with MM should receive pneumococcal and influenza vaccination. Intravenously administered gamma globulin every 3 to 4 weeks is indicated if patients have recurrent serious infections associated with severe hypogammaglobulinemia. It is

recommended to do acyclovir for all patients receiving bortezomib or carfilzomib to prevent herpes zoster activation. Prophylaxis against *Pneumocystis jiroveci* should be considered in all patients receiving long-term corticosteroids. However, there is a risk of serious skin toxicity in patients receiving an immunomodulatory agent (thalidomide, lenalidomide) and trimethoprim-sulfamethoxazole. In such patients, alternative antibiotics (such as levofloxacin) and alternative agents for *Pneumocystis* prophylaxis should be considered.

Hyperviscosity syndrome: a small proportion of patients with MM, especially of the IgA subtype, have development of hyperviscosity syndrome. Plasmapheresis promptly relieves the symptoms and should be performed regardless of the viscosity level if the patient has signs or symptoms of hyperviscosity [64].

## ***2. The Endothelin System***

### ***2.1 The discovery of endothelins and endothelin receptors***

The endothelin (ET) system consists of three small peptide ligands, Endothelin 1, 2, 3 (ET-1, ET-2, ET-3), two distinct rhodopsin-like G-Protein Coupled Receptors (GPCRs) subtypes, the Endothelin A and B Receptor (ET<sub>A</sub>R and ET<sub>B</sub>R, respectively) and the Endothelin Converting Enzymes (ECEs), which catalyze the generation of biologically active ETs. ET-1 is the predominant form of the ETs and probably the most biologically relevant isoform. It was originally isolated and sequenced from conditioned media of porcine aortic endothelial cells in 1988, thanks to the collaboration between several groups in Japan [86- 88]. This peptide, 21 amino-acid long, was shown to be an extremely potent vasoconstrictor produced by the endothelial cells and was also found in medium of cultured bovine endothelial cells and in several other mammalian species including rat, mouse and humans [89]. In the same year, the sarafotoxins (SRTXs), a family of peptides with high degree of sequence similarity to ET-1, which act on the same endothelin receptors, were identified in the cardiotoxic venom of the asp *Atractaspis engaddensis*. The venoms evolved to immobilize mammalian prey and can be fatal in humans leading in a rapid rise in blood pressure consistent with coronary vasoconstriction accompanied by changes in the electrocardiogram associated with atrioventricular block and cardiac arrest. The most widely studied are S6b and S6c, the first showing similar affinities for both endothelin receptors (ETRs) with a profile similar to ET-1/ET-2, although less potent, whereas S6c is used as a moderately selective ET<sub>B</sub>R agonist [88, 90, 91]. Later analysis revealed the existence of two other ET-like peptides in humans and these additional isoforms were named ET-2 and ET-3, respectively [88, 92]. Furthermore, another isoform of the ET family was found, the vasoactive intestinal contractor (VIC), so named because of its contractile activity in the ileum. The peptide, which has also been named ET-4 in the literature, is thought to be the mouse and rat orthologous of human ET-2 showing the same synthetic pathway of this latter and having the same affinity for both receptor subtypes [93, 94]. Another important discovery during the early stage of ET research was the detection of ET receptors (ET<sub>A</sub>R and ET<sub>B</sub>R). Although different from each other, both shown to belong to the family of heptahelical G-Protein Coupled Receptors [95, 96]. These two receptors are distinct in their ligand binding affinity and are both distributed in various tissues and cells but with different levels of

expression, suggesting the presence of a multifunctional ET system [91]. In addition, several radioligand binding and pharmacological experiments revealed the existence of another type of Endothelin Receptor in nonmammalian cells: the ETC [91, 97]. Key milestones in the development of pharmacological agents were the first ET<sub>A</sub>R-selective peptide antagonists, BQ123 and FR139317, whereas the first selective peptide ET<sub>B</sub>R antagonist was BQ788. It was immediately clear that the clinical goal was the discovery of orally active, small molecule ET receptor antagonists, that would block the potent and long lasting vasoconstrictor actions of ET in human cardiovascular system. Within 5 years from the discovery of ET-1, the first ET<sub>A</sub>R/ET<sub>B</sub>R antagonist with oral bioavailability was identified and its successor bosentan was the first in class to be introduced into the clinic, initially for the treatment of pulmonary arterial hypertension (PAH). Successively, the ET<sub>A</sub>R-selective antagonist ambrisentan, was approved in clinical use in PAH, followed by the more ET<sub>A</sub>R-selective antagonist sitaxentan which was in few years voluntarily withdrawn owing to cases of idiosyncratic hepatitis, resulting in mortality from acute liver failure. On the contrary, bosentan was the structural basis for the development of macitentan, representing the next generation of antagonists, being more potent, with longer receptor occupancy as well as undergoing conversion to an active metabolite, properties contributing to greater pharmacodynamics and pharmacokinetic efficacy [98].

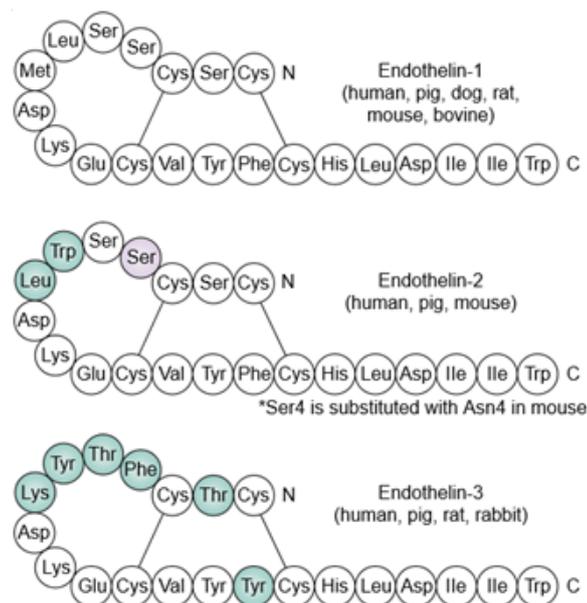
## ***2.2 Endothelins: structure and tissue distribution***

ET-1, ET-2 and ET-3 are characterized by a single  $\alpha$ -helix and two disulfide bridges joining the cysteine residues at position 1-15 and 3-11. The C-terminal end contains amino acids that appear to mediate receptor binding, while the N-terminal residues determine the peptide's binding affinity to the receptor. ET-1 and ET-2 have similar structure, they differ in only two amino acids (Trp and Leu), whereas ET-3 differs in structure at 6 of 21 amino acids [98] (Figure 3).

ETs are ubiquitously expressed (Table 7) acting in an autocrine/paracrine manner and are therefore thought to have a variety of physiologic and pathophysiological functions. ET-1 is the most abundant isoform in the human cardiovascular system and the primary source is thought to be vascular endothelial cells, although the peptide is produced by other cell types, including smooth muscle cells, epithelial cells, macrophages and monocytes, fibroblasts, cardiomyocytes, enteric glia cells in the periphery, as well as choroid plexus and certain neurons and reactive glial cells in the central nervous system. Intense research over the last 10 years has changed the

original view of ET-1 as mainly a vasoconstrictor regulating blood pressure into a biological factor regulating processes such as vascular remodeling, angiogenesis or extracellular matrix synthesis. Furthermore, ETs exerts a number of physiological functions in the lung, kidney and brain, which results in biological responses like normal bronchial tone, modulation of hormones and neurotransmitters release [89, 99] (Figure 4).

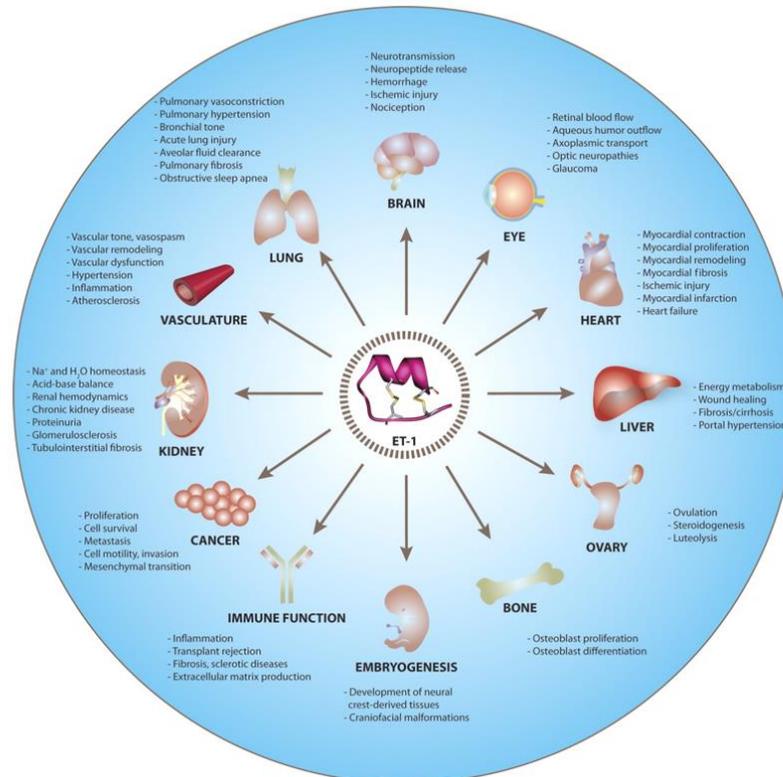
The cellular and organ distribution of ET-2 has not been extensively studied as ET-1 but it seems that it has a more restricted organ distribution. The most likely source of ET-2 in the plasma overspill from endothelial cells and ET-2 mRNA and/or peptide has been detected in a number of human tissues including the vasculature, the heart, the lung, the kidney, the intestine and the ovaries. As a matter of fact, most recent studies suggest the involvement of this endothelin family sibling in the regulation of lung alveolarization, thermoregulation, ovulation and intestinal epithelial cell homeostasis [100]. ET-3 is the only endogenous isoform that, at physiologic concentrations, can distinguish between the two receptor subtypes, because it has the same affinity at ET<sub>B</sub>Rs as ET-1 and ET-2 but has much lower or little affinity for ET<sub>A</sub>R than the other isoforms. ET-3 was the most abundant ET peptide in rat brain, leading to the proposal that this molecule was the “brain” endothelin peptide, particularly because the ET<sub>B</sub>R subtype comprises ~ 90% of the ET receptors in human normal cerebral cortex. However, ET-3 is also found in endothelial cells, renal tubular epithelial cells and intestinal epithelial cells mediating the release of vasodilators including nitric oxide (NO) and prostacyclin [98, 101].



**Figure 3: The structure of the three isoforms of Endothelin:** Green residues indicate those that are different from ET-1. Reproduced from [91].

**Table 7: Cellular source of ET-1, ET-2 and ET-3.** Reproduced from [101].

Endothelin-1	Endothelin-2	Endothelin-3
Endothelial cells	Kidney epithelial cells	Neuronal stromal cells
Cardiomyocytes	Gastrointestinal stromal cells	Glial cells
Aortic vascular Smooth muscle cells	Cardiomyocytes	Adrenal cells
Kidney epithelial cells	Trophoblastic cells	Lung epithelial cells
Neuronal stromal cells	Uterine glandular epithelial cells	Intestinal epithelial cells
Astrocytes		Kidney epithelial cells
Neurosecretory nerve endings in neurohypophysis		
Kidney mesangial cells		
Sertoli cells		
Endometrial cells		
Breast epithelial cells		
Leukocytes		
Myofibroblasts		
Bile duct epithelial cells		

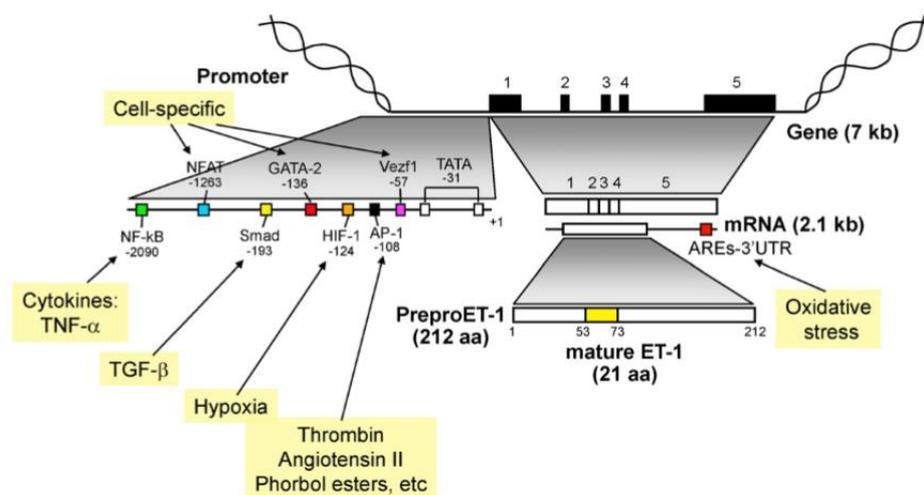


**Figure 4: Overview of ET-1 physiology.** Reproduced from [103]

### ***2.3 Regulation of endothelins expression***

ET-1, ET-2 and ET-3 are encoded by different genes and are regulated at the level of mRNA transcription. ET-1 gene (EDN1) is located on chromosome 6 (at 6p23-p24.1), ET-2 gene (EDN2) on chromosome 1 (at 1p34) and ET-3 (EDN3) on chromosome 20 (at 20q13.2-q13.3). EDN1 and EDN3 contain 5 exons which encode for the precursor preproendothelin-1 (PPET-1) and preproendothelin-3 (PPET-3), respectively, while EDN2 contains only 4 exons and encodes for the preproendothelin-2 (PPET-2) [88, 92, 102]. In addition to the wild-type transcript, 3 alternatively spliced isoforms of ET-1 have also been reported in humans. Each transcript retains the coding sequence for the mature ET-1 peptide but encodes for predicted PPET-1 isoforms that are 211, 178, and 78 amino acids (aa) in length [103]. ET-1 is synthesized and released continuously from endothelial cells and levels of PPET-1 mRNA are modulated predominantly at the level of transcription. EDN1 is under the transcriptional control of a TATA-box-containing promoter highly conserved across all mammals. A functional activator protein-1 (AP-1) site at position -108-102 which is bound by c-fos/c-jun and that mediates the transcriptional response to thrombin, bradykinin, angiotensin II or phorbol esters has been identified in endothelial as well in non-endothelial cells [104-107]. Additional studies have shown the existence of a GATA motif at positions -136-131 which is bound by the transcription factor GATA-2 in endothelial cells [105]. Furthermore, a hypoxia-inducible factor-1 (HIF-1) binding site at position -124-119 in the ET-1 gene is crucial for the hypoxic-stimulated response. Hypoxia induces the expression of ET-1 in endothelial cells and may be the primary cause of vasoconstriction and vessel wall thickening, often seen in progression of pulmonary hypertension. Moreover, also in tumors, hypoxia has an important role in increasing expression of endothelial genes, contributing to disease progression [109- 111]. Additionally, tumor growth factor- $\beta$  (TGF- $\beta$ ) is one of the potent regulators of ET-1 levels both in endothelial and non- endothelial cells and a specific binding site for Smad proteins, the family of TGF- $\beta$  activated transcription factors, has been identified at position -193-171 within the human ET-1 gene promoter. It has been shown that TGF- $\beta$ , acting via the ALK/Smad 3 pathway, induces an increase in PPET-1 via AP-1 site and/or Smad binding element [112, 113]. Finally, in the distal EDN1 promoter, several regulatory elements have been identified. These include hormone-response elements, which are activated by steroid hormones and mineralocorticoids, a nuclear factor-kappa b (NF-kB) binding site, which is activated by glucose and by inflammatory

mediators such as TNF- $\alpha$ , interferon- $\gamma$ , interleukin-1 $\beta$  (IL-1 $\beta$ ) and other cytokines, an E box motif and two consensus nuclear factors of activated T cell (NFAT) binding sites at the position -1263 and -1563 base pairs upstream of the transcription start site [114, 115] (Figure 5). Furthermore, emerging evidence indicates that ET-1 expression is regulated by epigenetic mechanisms, such as DNA methylation and histone modification, as well as by post-transcriptional modifications that affect mRNA stability and microRNA (miRNA)-mediated regulation [103]. Therefore, the production of ET-1 is tightly controlled by transcriptional and post-transcriptional mechanisms and alteration of ET-1 expression are associated with pathogenesis and progression of numerous diseases.



**Figure 5: ET-1 gene regulation.** NF-kB, Nuclear factor kappa B; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; NFAT, nuclear factor of activated T-cells; TGF- $\beta$ , transforming growth factor- $\beta$ ; HIF-1, hypoxia-inducible factor; AP-1, activator protein-1; Vezf1, vascular endothelial zinc finger-1; AREs, adenine- and -uridine rich regions. Nucleotide positions are numbered relative to the transcriptional start site and correspond to the human ET-1 gene (NCBI accession n<sup>o</sup> NC\_000006). Reproduced from [116].

## 2.4 Biosynthesis of endothelins

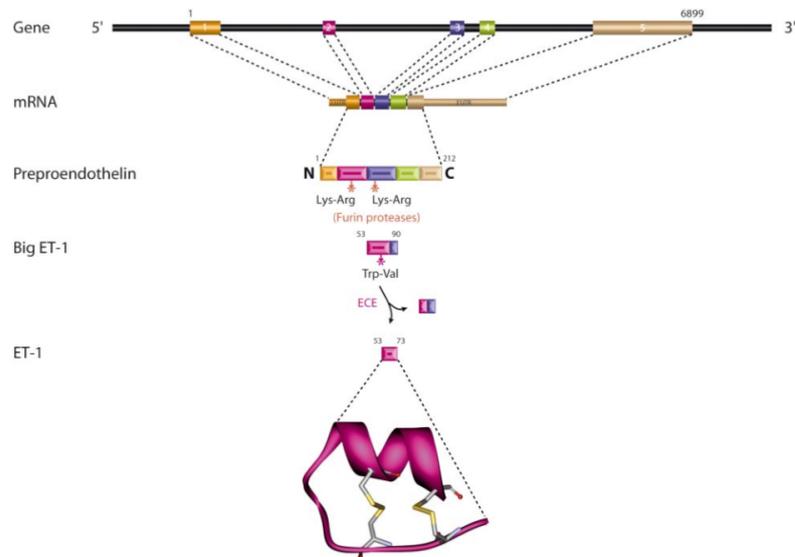
The endothelin peptides are produced through a set of complex molecular processes. The primary translation product of the ET-1 gene is the 212-aa-PPET-1, which is cleaved at Trp21-Val22 by a family of endothelin converting enzymes (ECEs) to form the 38-aa-big-ET-1, and then the biologically active peptide ET-1 (Figure 6). ET-2 and ET-3 synthesis is essentially similar to that of ET-1. Radioligand binding studies using (125)-Big ET-1 demonstrated that at

physiological concentrations Big-ET-1 is inactive and does not bind to either receptor subtype because the accessibility for binding aa 1-5 is greatly reduced in Big-ET-1 compared with ET-1 and the residues 16-22 are also affected, as a result of the folding back of the C-terminus of Big-ET-1. In addition, Big-ET-1 is resistant to proteolytic cleavage by enzymes that are able to metabolize the mature peptide.

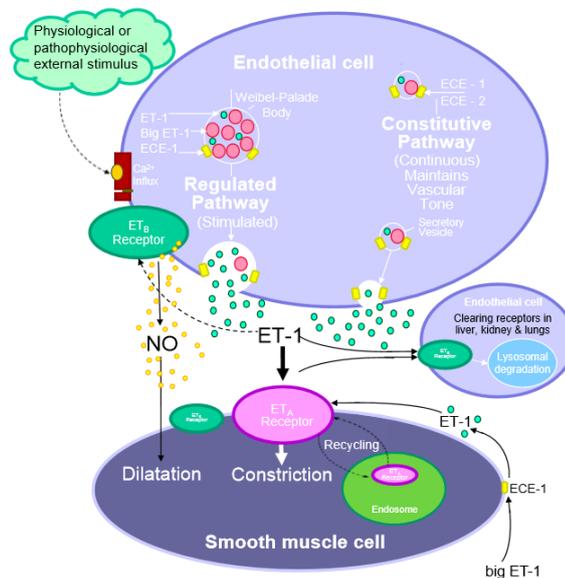
ECEs belong to the M13 group of proteins, a family that includes neutral endopeptidase, Kell blood group antigens, a peptide from phosphate regulating gene (PEX), X-converting enzymes (XCE) and secreted endopeptidases. M13 family members contain type 2 integral membrane proteins with zinc metalloprotease activity. They consist of a short N-terminal cytosolic domain, a single transmembrane segment and a large ectodomain, which contain the catalytic zinc binding motif [117]. Three isoforms of ECEs have been reported, namely ECE-1, ECE-2 and ECE-3; ECE-1 and ECE-2 are the most prominent. ECE-1 is localized in the plasma cell membrane of smooth muscle cells predominantly and its optimal activity is at pH 7. In humans were reported four variants of ECE-1 derived from a single gene namely ECE-1a, ECE-1b, ECE-1c and ECE-1d, which are the result of alternate splicing of ECE-1 mRNA. They differ only in their N-terminal cytosolic sequence but show comparable efficiency of conversion of Big ET-1. Furthermore, they display different patterns of subcellular localization: ECE-1a and ECE-1c were localized at the cell surface; ECE-1b was intracellular and show significant sublocalization in the trans-Golgi network; ECE-1d is expressed at the cell surface, although less strongly than ECE-1a. The existence of these isoforms lets ECE-1 capable of processing Big-ETs both intracellularly and on the cell surface. ECE-2 shares 59% sequence similarity to ECE-1 and also consists in four isoforms possessing a conserved catalytic unit and differing only in their N terminal tail. The isoforms differ in their localization: ECE-2b was found to be highly expressed in neuroendocrine tissues (brain, pituitary and adrenal medulla), whereas the other isoforms were in peripheral tissues. In contrast to ECE-1, the optimum pH for ECE-2 activity is acidic (pH 5.5), which would favor an intracellular localization and a potential role under low pH conditions, for example in ischemia. This difference provides an experimental method for distinguishing between the activities of the two enzymes. In agreement, ECE-2 was found to be localized to the acidic environment of vesicles of the secretory pathway in human endothelial cells but was not detected in Weibel-Palade storage granules. These enzymes are also capable to process other biologically active peptides including bradykinin, substance P and insulin [118, 119]. Both ECE-1 and ECE-2 show preference for Big-ET-1 over Big-ET-2 and

Big-ET-3. However, the absence of both ECE-1 and ECE-2 in mice, point to the existence of ECE isoforms or proteases other than the latter. Data suggest the presence of an ECE-1 independent pathway that processes big-endothelin, possibly involving tissue chymases and non-ECE metalloproteinases [101, 120].

There are two major pathways for endothelin secretion: the constitutive pathway, that involves the continuous release of the protein from the trans-Golgi network to the cell surface in secretory vesicles and the regulated one that involves the stimulated release (Figure 7). The constitutive secretory pathway is modulated at the level of mRNA transcription and has been proposed as the mechanism for the ET release from porcine endothelial cells [86] as well as from most endothelin producing cell types. The constitutive pathway is the primary secretory mechanism, it causes a greater release of ET-1 than the regulated one and is most important in the maintenance of the vascular tone in humans [101, 121]. The other pathway involves the secretion of endothelin in secretory granules, also known as Weibel-Palade bodies, which appear to serve as a storage compartment for ET-1 as well as for other vasoactive compounds including histamine, von Willebrand factor, P-selectin and calcitonin gene-related peptide [122-124] Upon activation of endothelial cells, Weibel-Palade bodies relocate, fuse the plasma membrane and release their contents by exocytosis. The mechanism of exocytosis is typically regulated, occurring after specific types of regulation, such as hypoxia, hypothermia, mechanical stretch, shear stress, agonists like histamine and thrombin and by a variety of stimuli including interleukin (IL)-1 $\beta$ , TNF- $\alpha$ , TGF- $\beta$ , platelet-derived growth factor (PDGF) and vasopressin. Conversely, inhibitory factors include nitric oxide (NO), prostacyclin and atrial natriuretic peptide [121, 125].



**Figure 6: Overview of ET-1 synthesis.** Intron-exon structure and RNA processing pathway are indicated for the EDN1 gene. Translation yields preproET-1 (PPET-1) that is processed in sequential proteolytic steps to generate ET-1. Structure of ET-1 contains 2 disulfide bridges and was rendered from the RCSB Protein Data Bank (PDB 1T7H). Reproduced from [103].



**Figure 7: The dual secretory pathway of ET-1.** In human endothelial cells, ET-1 is synthesized by a dual secretory pathway. ET-1 is continuously released from the small vesicles of the constitutive pathway to interact with ET receptors to contribute to vasomotor tone but is also released from the Weibel-Palade bodies of the regulated pathway in response to external stimuli. Some ET-1 may also interact with endothelial ET<sub>B</sub>Rs in an autocrine manner and limit the constrictor response by the release of vasodilators such as nitric oxide. The ET-1/ET<sub>A</sub>R complex undergoes internalization to the endosome before recycling of the receptor to the cell surface whereas ET-1/ET<sub>B</sub>R complex is internalized and degraded to the lysosome. Reproduced from [98].

ET-1, synthesized by vascular endothelial cells, is released toward the basolateral side of the cells, where it usually acts on smooth muscle cells contributing to the maintenance of vascular tone. Tissue levels of ET-1 are likely to be higher than plasma levels which are in the picomolar range (1-10 pmol/L). In addition, ET-1 has a short half life in blood (about 7 minutes) that could be due to its continuous metabolism and clearance. ET clearance from the circulation is achieved by endothelial ET<sub>B</sub>Rs, also termed “clearance receptors” [126, 127] as evidenced by the fact that selective ET<sub>B</sub>R blockade inhibits the accumulation of intravenously administered radiolabeled ET-1 in tissue [99, 128]. This ET<sub>B</sub>Rs-mediated clearance mechanism, followed by lysosomal degradation, is particularly important in the lung, which clears about 80% of circulating ET-1 [128]. ET-1 clearance is also carry out by the extracellular neutral endopeptidase (NEP), also known as endopeptidase 24.11, through hydrolytic inactivation. In renal tissues, neutral endopeptidase controls the turnover of ET-1; the inhibitors of this endopeptidase increase urinary endothelin levels several fold [129].

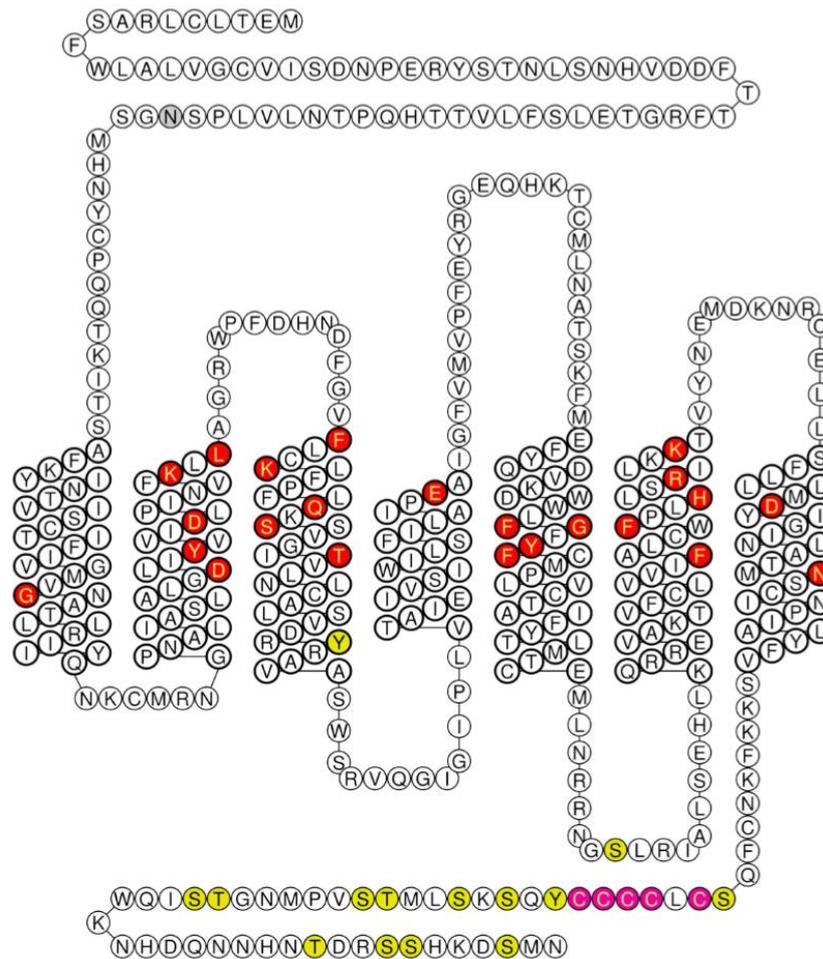
## ***2.5 Endothelin Receptors structure, distribution and function***

ETs interact with two different receptors in humans, the ET<sub>A</sub>R and the ET<sub>B</sub>R, both belonging to the rhodopsin-type superfamily of G-protein coupled receptors (GPCRs) consisting of a long extracellular N-terminus sequence, seven helical transmembrane domains (TMDs), 3 extracellular and 3 intracellular loops, and a cytoplasmic C-terminus tail. TMD IV-VI and the adjacent extracellular loops are involved in receptor binding selectivity, while the carboxyl terminal tail constitutes the signaling domain. The endothelin receptors are distinct in their ligand binding affinity and distribution in tissues and cells. ET<sub>A</sub>R has a high affinity to ET-1 and ET-2, but a low affinity to ET-3. ET<sub>B</sub>R has equally potent affinities to all three endogenous ETs and additionally binds sarafotoxin (S6c).

### ***2.5.1 Endothelin A Receptor***

The human ET<sub>A</sub>R (EDNRA) gene is located on chromosome 4, spans more than 40 kilobases (kb) and consists of 8 exons and 7 introns encoding for a protein 427 aa long. Human EDNRA has 63% sequence similarity with the human EDNRB and displays a high degree of conservation between human and animal species, including pig, dog, sheep (95%), rat (93%) and mouse (92%). The main differences are in the N terminus, rather than within the

transmembrane domains that contribute to binding, and reflect that ET-1 structure is conserved across mammalian species (Figure 8). Posttranslational modifications, phosphorylation, palmitoylation and glycosylation can modulate the function of the receptor. For example, mutation of a cluster of five Cys residues present in the cytoplasmic tail did not alter binding but prevents palmitoylation of the receptor, resulting in a failure of ET-1 to stimulate the transient increase in the cytoplasmic calcium. Truncation of the entire N terminus that included a possible glycosylation site, abolished binding activity, but it is unclear whether this is the result of a structural change or whether glycosylation is important. Phosphorylation is crucial for ligand induced desensitization in ET<sub>B</sub>Rs, with rapid deactivation after ET-1 stimulation and mostly activity lost within 5 minutes. In contrast, ET<sub>A</sub>R did not undergo ligand-induced phosphorylation and continued to signal with little reduction in activity after 5 minutes, consistent with long-lasting constrictor activity. At least three alternatively spliced ET<sub>A</sub>R transcripts have been reported in human tissues, including aorta, heart atria and lung. Deletion of exon 3 (producing a protein with two membrane-spanning domains), exon 4 (producing a protein with three membrane domains), and exon 3 plus exon 4 (producing a protein lacking the third and fourth domain) have been identified, but these did not bind ET-1 when expressed in cells. The significance of alternative splicing of ET<sub>A</sub>R mRNA is unclear but could reduce the abundance of active receptor and reduce functions such as contractility [95, 98, 130, 131].



**Figure 8: Schematic diagram of the human ET<sub>A</sub>R.** Experimental mutations altering receptor function are shown in red. Potential sites for translational modifications are shown as phosphorylation (yellow), glycosylation (grey), and palmitoylation (purple). Reproduced from [98].

### 2.5.2 Endothelin B Receptor

The human ET<sub>B</sub>R (EDNRB) gene is located on chromosome 13, has 7 exons and 6 introns and encodes for a protein 442 aa long with a predicted molecular mass of about 50kDa [132]. The ET<sub>B</sub>R has an unusually long N terminus (Figure 9) that can be cleaved by a metalloprotease to remove the first 64 aa while still retaining ET-1 binding capacity. The carboxyl terminus, as with other GPCRs, is important for intracellular signaling. It is thought that aa associated with the first, second, third and seventh transmembrane spanning domains and associated extracellular loops are mainly involved in ligand binding where most of the naturally occurring or experimental mutations that affect ET ligand binding and ligand receptor selectivity coincide.

Like ET<sub>A</sub>Rs, ET<sub>B</sub>Rs have a surprising number of potential post-translational modifications: there are 13 phosphorylation sites mainly located in the C terminus, a potential glycosylation site in the N terminus (even if there are no evidences that this is important for binding, although in other GPCRs at least one glycosylation site is often critical for receptor expression at cell membrane and function) and four sites for possible palmitoylation. The clustering of Cys residues at the N terminus is conserved across many GPCRs and the degree of C-terminus palmitoylation may act as a selector for coupling with different G proteins leading to different pathways of intracellular signaling [98]. Canonical ET<sub>B</sub>R was cloned from human liver and placental cDNA libraries. Several alternative splice variants of human ET<sub>B</sub>R have been identified. The first splice variant has an additional 10 aa at the second cytoplasmic loop of ET<sub>B</sub>R, exhibited limited distribution in heart, lung, brain and placenta, but has similar binding and functional characteristics as wild-type ET<sub>B</sub>R [133]. A second splice variant from human placental libraries has 436 aa residues and differs from wild-type ET<sub>B</sub>R in the last 52 aa of the carboxyl terminal. Although this splice variant has the same binding properties as wildtype ET<sub>B</sub>R, it lacks any potential palmitoylation sites. mRNA encoding this variant was not particularly abundant, except in skeletal muscle where it represented more than 40% of the total mRNA and the splice variant shares 91% identity to human ET<sub>B</sub>R [134]. A third splice variant in human melanoma cell line has its transcription initiation 939bp upstream from wild-type ET<sub>B</sub>R, an additional 90 aa at the N-terminus and a shorter 3' untranslated region, but exhibits similar binding and functional characteristics as wild-type ET<sub>B</sub>R [135]. Moreover, it was described a mutant ET<sub>B</sub>R associated with Hirshprung disease in which the lacking of 134 base pairs corresponding to exon 5, leads to the untraslation or to the rapidly degradation of the protein, presumably because of its instability. The preferential production of this null function ET<sub>B</sub>R by RNA editing/splicing could be involved in the etiology of some cases of Hirschsprung disease and could be considered possible cause of disease [136]. Based on its in vivo pharmacology, ET<sub>B</sub>R has been further classified into two types, ET<sub>B1</sub>R and ET<sub>B2</sub>R, present on endothelial and on smooth muscle cells, respectively. Nevertheless, the molecular basis for the existence of these subtypes is still lacking and also in competition binding assays, it has been seen that the receptors could not be distinguished by a range of ET ligands [137]. For these reasons current evidence only support the existence of two human subtypes, the ET<sub>A</sub>R and the ET<sub>B</sub>R, according to NC-IUPHAR nomenclature [121]. Finally, the ET<sub>C</sub>R, a third type of ET



osteoblasts, melanocytes and keratinocytes, adipocytes, and various cells in the reproductive tract. ET<sub>B</sub>R is abundantly expressed on vessel endothelial cells but exists also on smooth muscle cells, liver hepatocytes and Ito cells, renal-collecting-duct epithelial cells, airway smooth muscle cells, osteoblasts, neurons of the central and peripheral nervous system, and various cells of the reproductive tract. In particular, highest relative expression of ET<sub>A</sub>R mRNA is associated with heart and lungs, with relative low expression in central nervous system. On the contrary brain regions show high expression of ET<sub>B</sub>Rs and also the lung is a particularly ET<sub>B</sub>Rs rich tissue. In many tissues, therefore, the same cells express both receptors. The relative ratio of the two subtypes, measured by competition binding in human tissue, is shown in figure 10 [98, 99, 101]. Under normal physiologic conditions, the two receptor subtypes exhibit contrasting actions: ET<sub>A</sub>Rs is involved in vasoconstriction, whereas ET<sub>B</sub>Rs in vasodilatation and, in addition, function as clearing or scavenger receptors to internalize the ligand-receptor complex and remove ET-1 from the circulation (Figure 7) [139]. In humans, the lungs and the kidney are important sites for removal of ET-1. This reflects that these organs are ET<sub>B</sub>Rs rich (Figure 10). In fact, 70% of the endothelin receptors in both the cortex and the medulla of the kidney are ET<sub>B</sub>Rs, which localize to endothelial cells throughout the renal vasculature and the same happens for the 50% of the receptors in the lung which are present on the vascular endothelium, consistent with clearing ET-1 [98]. Moreover, several studies have demonstrated that systemic and selective blockade of ET<sub>B</sub>Rs, results in a significant rise in circulating ET-1 [140] and increase in plasma ET-1 have also been reported for mixed antagonists that block both receptors, such as bosentan [141]. These results emphasize the importance of ET<sub>B</sub>Rs in maintaining low circulating plasma levels and the potential for ET-1 levels to rise under pathophysiological conditions where clearance is reduced.

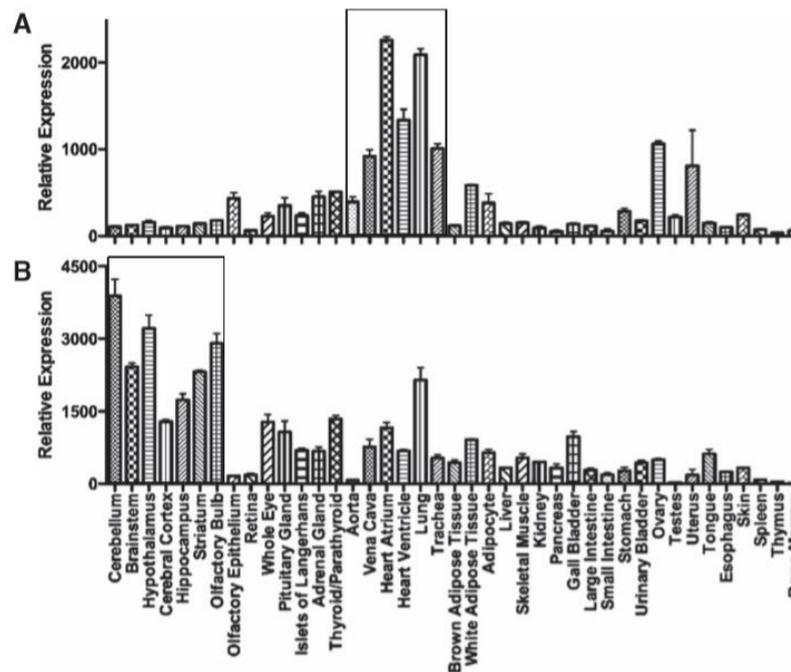


Figure 10: Relative expression of mRNA encoding ET<sub>A</sub>R (A) or ET<sub>B</sub>R (B) in 41 adult tissues. Reproduced from [98].

## 2.6 The Endothelin Signaling Network

ET-1 signaling is extremely complicated and ET receptor activation leads to diverse cellular responses (Figure 11). Owing to their differences in C-terminus sequences, which are pivotal for coupling of G-proteins, the receptors induce divergent intracellular effects. As reported above, ET<sub>A</sub>R primarily mediates vasoconstrictive and proliferative response to ET-1 but is also traditionally accepted that ET<sub>A</sub>R activation leads to hypertension, diabetes and other cardiovascular diseases, such as to the development and progression of cancer, therefore being considered the “bad” ET receptor. On the other hand, ligand activation of ET<sub>B</sub>R leads to induction of intracellular pathways being counter-regulatory to ET<sub>A</sub>R signaling. Indeed, ET<sub>B</sub>R is referred as the “good” one, as it seems to be responsible for the clearance of circulating ET-1 and for its capacity to promote endothelial synthesis of vasodilators NO and prostacyclin [142]. However, it has been shown that also ET<sub>B</sub>R is involved in the development and progression of such types of tumors [143, 144]. In particular, ET<sub>B</sub>R has been reported to behave as either a tumor suppressor [145, 146] or a protumorigenic [147, 148] gene, depending on its actual function in the corresponding normal tissues. Accordingly, ET<sub>B</sub>R was found to be down-regulated or absent in tumors of epithelial origin, whose normal counterparts express ET<sub>B</sub>R with anti-proliferative and regulatory functions [149], whereas it appears overexpressed in

malignancies (e.g. melanoma) whose normal counterparts express ET<sub>B</sub>R as mediator of pro-survival signals [150]. In tumors, this up-regulation of ET<sub>B</sub>R surface expression is due to an altered methylation status of the promoter gene [151]. Table 8 shows the expression of endothelin receptors in human malignancies. Regarding ET-1, elevated plasma levels have been detected in patients with various solid tumors including hepatocellular, gastric and prostatic cancer [152- 154] where levels are greatest in patients with metastatic, hormone refractory diseases. Furthermore, changes in the expression of ET system components have also been demonstrated in premalignant tissues, suggesting that modulation of this system may be an early phenomenon in tumorigenesis.

ET receptors interact with heterodimeric G proteins composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. The  $\alpha$  subunit of G proteins is divided into 4 subfamilies: G $\alpha$ s, G $\alpha$ i, G $\alpha$ q and G $\alpha$ <sub>12</sub> and each G protein activates several downstream effectors. These receptors can be activated either through autocrine production of ligand [155] or through production of the latter from stromal cells [156], that may be expressed physiologically or in response to cancer cells in a paracrine loop. Both activate a similar signaling cascade, resulting in a range of pleiotropic responses (Figure 12).

**Table 8: The endothelin System in Cancer**

	<b>Endothelin Receptor</b>	<b>References</b>
<b>Ovarian Cancer</b>	ET <sub>A</sub> R mRNA is present in primary and metastatic cancer, correlating with tumor grade. ET <sub>A</sub> R mediates all ET-1-induced tumor-promoting effects	[157]
<b>Renal Cancer</b>	ET <sub>A</sub> R is expressed in renal cancer cell lines	[158]
<b>Endometrial Cancer</b>	Both ET <sub>A</sub> R and ET <sub>B</sub> R mRNA levels are lower in endometrial cancer tissue than in non cancerous endometrium	[159]
<b>Prostate Cancer</b>	ET <sub>A</sub> R expression increases with tumor stage and grade and is associated with decreased ET <sub>B</sub> R expression	[160]
<b>Glioblastoma</b>	ET <sub>B</sub> R mediates the ET-1 effects in glioblastoma cell lines	[161]
<b>Bladder Cancer</b>	Both ET <sub>A</sub> R and ET <sub>B</sub> R are expressed in bladder tumors	[162]
<b>Melanoma</b>	ET <sub>B</sub> R is overexpressed and correlates with tumor progression. ET <sub>B</sub> R mediates all ET-1-induced tumor promoting effects	[163]
<b>Bone Malignancies</b>	ET <sub>A</sub> R is expressed in osteoblasts	[164]
<b>Breast Cancer</b>	ET <sub>A</sub> R expression correlates with clinicopathologic	[165]

	parameters of aggressive carcinoma	
<b>Nasopharyngeal Carcinoma</b>	ET <sub>A</sub> R is over-expressed in 74% of tumors	[166]
<b>Colon Cancer</b>	ET <sub>A</sub> R and ET <sub>B</sub> R are over-expressed	[167]
<b>Cervical Cancer</b>	HPV-positive cervical carcinoma cells express functional ET <sub>A</sub> R	[168]
<b>Kaposi's Sarcoma</b>	Both ET <sub>A</sub> R and ET <sub>B</sub> R are expressed	[169]
<b>Lung Cancer</b>	ET <sub>A</sub> R is over-expressed in tumor	[170]
<b>Osteosarcoma</b>	ET <sub>A</sub> R is expressed in tumor cell lines	[171]
<b>Acute Myeloid Leukemia (AML)</b>	ET-1 and ET <sub>A</sub> R are involved in the proliferation of AML cells and favour angiogenesis and chemioresistance of the leukemic cells	[172]
<b>Chronic Lymphocytic Leukemia (CLL)</b>	ET-1 promotes survival and chemioresistance in chronic lymphocytic leukemia B cells through ET <sub>A</sub> R	[173]
<b>Multiple Myeloma (MM)</b>	ET-1 induces resistance to bortezomib via ET <sub>B</sub> R	[174]

### ***2.6.1 ET-1 and cell proliferation***

In cancer, as in physiological conditions, ET-1 acting through its cognate receptor, entails the activation of a network of a multiple signaling pathway, including PLC activity, calcium mobilization and activation of PKC and downstream members of the MAPK family, including ERK signaling, that act in a synergistic and combinatorial fashion to relay the mitogenic signal to the nucleus and promote cell proliferation (Figure 11). In various epithelial tumor cells, including prostate, cervical and ovarian cancers, ET-1 stimulates DNA synthesis and cell proliferation and spontaneous growth is inhibited in presence of ET<sub>A</sub>R but not ET<sub>B</sub>R antagonists, demonstrating that endogenous ET-1 acts as an autocrine modulator of cell proliferation only through ET<sub>A</sub>R. Moreover, the mitogenic activity of ET-1 can be amplified by synergistic interactions with other growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin, insulin growth factor (IGF), platelet-derived growth factor (PDGF), TGF- $\beta$  and IL-6 [176, 177]. Cross-talk between cell surface receptors represents the main mechanism to expand the cellular communication signaling network. A major pathway utilized by many GPCR agonists is the transactivation of epidermal growth factor receptor (EGFR) that leads to RAS-dependent MAPK activation. GPCR-induced EGFR transactivation is mediated by the release of precursors form of EGFR ligands by the activation of matrix-metalloproteinases (MMPs) but EGFR can be also phosphorylate directly by c-Src. For example, in colon cancer cells, EGFR transactivation, appears to proceed through an intracellular pathway involving GPCR,  $\beta$ -arrestin and c-Src [178]. Also in ovarian cancer cells ET-1 causes EGFR activation that is in part responsible for MAPK activation by c-Src and as well as for AKT activation [157, 178]. The cross-signaling between the EGFR/ETAR pathways provides a rationale to combine EGFR inhibitors with ET<sub>A</sub>R antagonists [179].

### ***2.6.2 ET-1 and cell survival***

ET-1 is an anti-apoptotic factor in different cell types and acts by modulating cell survival pathways, such as PI3K-dependent AKT activation of Nf-kB signaling. In particular, the activation of PI3K/AKT pathway leads to the inhibition of Nf-kB Inhibitor (IkB), resulting in dissociation and subsequent nuclear localization of active Nf-kB (Figure 11). In prostate cancer cells, ET<sub>A</sub>R blockade by antrasentan, induces the inactivation of Nf-kB DNA binding activity and a subsequent decrease in the levels of anti-apoptotic cell death [180]. In ovarian cancer

cells, ET-1 markedly inhibited paclitaxel-induced apoptosis, as a result of phosphorylation of the anti-apoptotic protein BCL-2 and the apoptotic response could be restored by treatment with the specific ET<sub>A</sub>R antagonist BQ123 [181]. Thus, ET<sub>A</sub>R blockade may result in antitumor activity by growth inhibition and by inducing apoptosis. When combined with conventional chemotherapy, ET<sub>A</sub>R selective antagonists or dual ET<sub>A</sub> and ET<sub>B</sub>R antagonist (such as macicentan) can more effectively induce apoptosis in vitro and in vivo, as observed in ovarian, prostate, colon and cervical cancer cells [168, 182- 184].

### ***2.6.3 ET-1 and tumor invasion***

In addition to modulating tumorigenesis, ET-1 seems to have a role in tumor progression leading to changes in tumor microenvironment and supporting migration, invasion, epithelial-to-mesenchymal transition (EMT), metastatic growth, angiogenesis and lymphangiogenesis. In tumor progression, changes in cadherin, connexin (Cx), MMPs and integrin expression, have emerged as key factors [163, 185-187]. ET-1, acting through ET<sub>A</sub>R, induces the activity of two families of metastasis-related proteinases: the MMPs and the urokinase-type plasminogen activator system. The regulation occurs at several levels, including gene transcription and secretion and activation of the resultant proenzymes. ET-1 signaling also contributes to increased tumor cell motility and actin fibers disorganization by increasing the activation of p125 focal adhesion kinase (FAK), paxillin and RHO (Figure 11), which have been shown to transduce signals that are involved in tumor cell invasion and metastasis. Moreover, EGFR transactivation by ET<sub>A</sub>R is in part responsible for MMPs activity and tumor cell invasion [157]. The peptide is also capable to induce a transient and dose-dependent reduction of gap junction intercellular communication (GJIC) and Cx and in this context enhances the expression of  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$ , as well as  $\alpha 2\beta 1$  and  $\alpha v\beta 3$  integrins. It has been documented that antibodies directed to  $\alpha 2\beta 1$  and  $\alpha v\beta 3$  integrins strongly reduced the adhesion to type I collagen as well as cell migration induced by ET-1. One hallmark of epithelial cancer progression is epithelial-to-mesenchymal transition (EMT), in which tumor cells undergo loss of polarity and cell-cell junction, acquire a mesenchymal phenotype, the ability to invade the extracellular matrix and to migrate to distant sites. In cancer cells, such as melanoma or ovarian carcinoma cells, activation of ET-1 axis contributes to disruption of normal host-tumor interaction by down-regulating the expression of epithelial cadherin (E-cadherin) and associated  $\beta$ -catenin and concomitant up-regulation of N-cadherin [157]. Moreover, in both normal and malignant

prostate cancer cells,  $\beta$ -catenin transcriptionally activates ET-1 expression. Meanwhile, ET-1 stimulates  $\beta$ -catenin signaling via a PI3K-dependent pathway. The positive inter-regulation between  $\beta$ -catenin and ET-1 signaling plays an important role in promoting proliferation and survival of cancer cells [188].

#### ***2.6.4 ET-1 angiogenesis and lymphangiogenesis***

ET-1 is mitogenic for blood and lymphatic endothelial cells, vascular smooth muscle cells, fibroblasts and pericytes. During the formation of new blood or lymphatic vessels, endothelial cells are stimulated by ET-1 to release proteases (such as MMP-2), which allow the endothelial cell to migrate, proliferate and invade the surrounding tissues to form capillaries. These various stages of vascularization are induced by ET<sub>B</sub>R, which displays a potent additive effect with the VEGF family members in the angiogenic and lymphangiogenic processes [189, 190]. In different tumor types, increased expression of ET-1 and its cognate receptors is significantly associated with the expression of VEGF and its receptors, as well as with microvessel density, indicating that ET-1 and VEGF might have complementary and coordinated roles during neovascularization [176]. Various studies have also demonstrated that there is an important link between ET-1 and HIF-1 $\alpha$ . Under normoxic conditions HIF-1 $\alpha$  is hydroxylated and submitted to a proteasomal degradation. On the contrary, the absent hydroxylation during hypoxia, stabilizes HIF-1 $\alpha$ , enabling it to form a dimeric HIF transcription factor complex that binds to hypoxia-response element (HRE)-binding sites to induce the transcription of pro-angiogenic genes, including those of VEGF family members. ET-1 potentiates the response to hypoxia by amplifying HIF-1 $\alpha$  stability and VEGF production and, at the same time, HIF-1 $\alpha$  also activates the transcription of ET-1 in different cell types. Therefore, an autocrine ET-1 circuit is controlled by the tumor microenvironment and ET-1 itself modifies the environment through HIF-1 $\alpha$  stabilization [191]. Additionally, in hypoxic conditions, ET-1 increases the promoter activity of cyclooxygenase 1 (COX-1) and COX-2 and the production of prostaglandin E<sub>2</sub> (PGE-2) (Figure 11). Silencing of HIF-1 $\alpha$  by small interfering RNA abrogates ET-1-induced COX-2 transcriptional activity, PGE-2 and VEGF production and MMPs activation, thus implicating that both HIF-1 $\alpha$  and COX genes are downstream targets of ET-1 signaling [176]. More recently, studies evaluating the functional contribution of mesenchymal stem cells (MSCs) to tumor angiogenesis demonstrated that IL-6 that is secreted by tumor MSCs may enhance the secretion of ET-1 by cancer cells to increase angiogenesis [192]. Finally, EDNRB

has been identified as one of the gene that has the capacity to organize vasculogenic mimicry, that is the novo generation of capillary like-vessels by deregulated and aggressive tumor cells [193].

### ***2.6.5 ET-1 and immunomodulation***

ET-1 modulates trafficking, differentiation and activation of tumor-infiltrating immune cells (Figure 4). It acts on cancer associated-fibroblasts, tumor associated macrophages (TAMs) and tumor cells promoting the formation of a suppurative tumor stroma, inducing the formation of a specialized niche that sustain tumor maintenance and progression. Acting through its receptors expressed on TAMs, ET-1 activates pro-inflammatory transcription factors and stimulates the release of inflammatory cytokines, triggering macrophage infiltration. In turn, tumor cells, in response to chemokine stimulation, migrate towards the chemoattractant gradient until finding the site for secondary colonization. ETRs activation also causes increased release of VEGF, which is important for tumor cell extravasation and angiogenesis. Concomitantly, during tumor progression, ET-1 through ET<sub>B</sub>R overexpressed in tumor endothelium, controls the endothelial barrier to T cell homing to tumor, by inducing the suppression of intracellular adhesion molecule 1 (ICAM1) expression on endothelial cells, which mediates T cell transendothelial migration and homing to tumors. These observations strong underline that dual antagonists might offer the advantage of simultaneously targeting the tumor cells (through ET<sub>A</sub>R) and enhancing antitumor immune mechanisms (through inhibiting vascular ET<sub>B</sub>R) by augmenting T cell infiltration, as well as by increasing the homing of T cells to tumors [176].

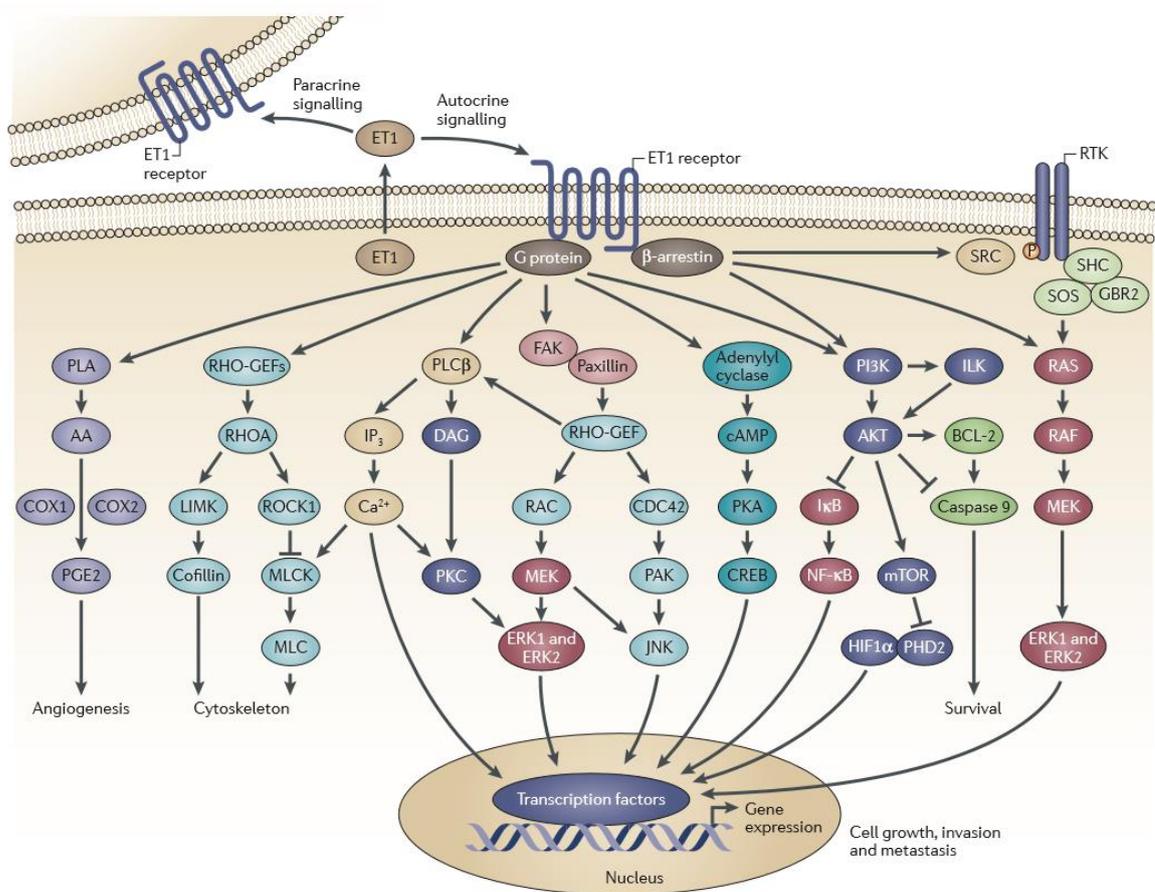
### ***2.6.6 ET-1 and metastasis***

Several studies in model systems have described a broad range of potential ET-1 effects in metastatic colonization. In a mouse orthotopic model of metastatic human ovarian cancer, silencing of  $\beta$ -arrestin or treatment with zibotentan inhibited metastasis by preventing nuclear  $\beta$ -arrestin/-catenin association and by inhibiting the transcription of EMT and metastasis-related genes. This indicates that the metastatic phenotype is linked to the ET<sub>A</sub>R-  $\beta$ -arrestin - mediated  $\beta$ -catenin pathway [194]. Furthermore, in a similar model, the administration of the dual antagonist macitentan in combination with paclitaxel, prevented the progression of ovarian cancer by inhibiting survival pathways both in tumor cells which express ET<sub>A</sub>R and in tumor-

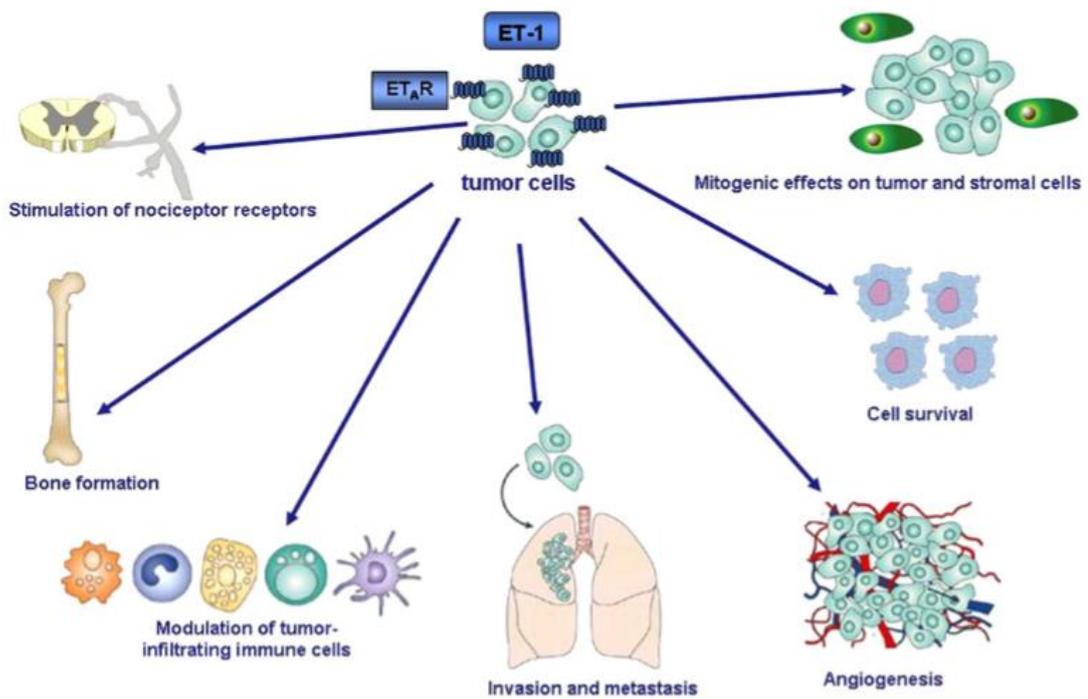
associated endothelial cells that express ET<sub>B</sub>R [182]. It has also been shown that overexpression of ET<sub>B</sub>R increased the incidence of brain metastasis in a preclinical model of spontaneous metastasis of melanomas to the central nervous system (CNS) and that intracranial melanoma growth is inhibited by the ET<sub>B</sub>R antagonist A192621 [195]. Recent studies demonstrated that ET-1, also in the bladder cancer microenvironment, primes cancer cells for pulmonary metastasis and recognized ET-1 as a biomarker for lung metastases. The activation of the ET-1/ET<sub>A</sub>R axis enhanced the migration of both tumor cells and macrophages and induced the expression of inflammatory proteases and cytokines, whose expression was significantly reduced by zibotentan, that was also capable to reduce the development of lung metastasis and decrease the number of TAMs [196]. Moreover, ET-1 has been shown to have a prominent role also in the formation of osteoblastic lesions in patients with metastatic prostate cancer and in those with metastatic breast cancer by up-regulating multiple osteoclast-stimulating factors, such as IL-6, Wnt5a, connective tissue growth factor and RANKL and by downregulating the WNT signalling pathway inhibitor Dickkopf-related-1 (DKK-1) [197]. Evidence is also accumulating to suggest that ET-1 contributes to the pain that is associated with metastatic disease. Local cutaneous injection of ET-1 causes pain and excitation of nociceptors through ET<sub>A</sub>R but this also concurrently produces analgesia through ET<sub>B</sub>R-mediated  $\beta$ -endorphin release and opioid receptor activation [198]. Overall, therefore, ET<sub>A</sub>R blockade could represent a therapeutic option to reduce bone metastasis and associated pain.

### ***2.6.7 ET-1, stemness and chemoresistance***

Interaction between tumor cells and the microenvironmental niche can trigger not only EMT programmes but also the maintenance of stem-cell like characteristics that fuels chemoresistance [199]. Several studies have demonstrated that in ovarian cancer cells ET<sub>A</sub>R is overexpressed, as well as mesenchymal markers such as SNAIL, SLUG, TWIST, vimentin and neural cadherin. On the contrary, there is a decrease in E-cadherin expression. These cells showed phenotypic changes that were consistent with EMT and stemness and the blockade of ET<sub>A</sub>R with selective or mixed antagonists, reversed EMT, inhibited invasiveness and restored chemotherapy sensitivity. These results indicate that the overexpression of ET<sub>A</sub>R can be considered a predictor of chemoresistance and that targeting of ET<sub>A</sub>R could increase the sensitivity of tumors to therapeutic agents [200].



**Figure 11: The Endothelin Signaling Network:** ET receptors (usually ET<sub>A</sub>R but sometimes also ET<sub>B</sub>R) couples to one or more families of GPCRs, as well as to scaffold protein such as β-arrestins. This activates diverse signal-transduction pathways including phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphonate (PIP<sub>2</sub>) into diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>), leading to calcium mobilization and protein kinase C activation and activation of downstream members of the MAPK family, including ERK signaling. ET-1 receptor signaling also control the crosstalk between ET-1 receptors and receptor tyrosine kinases (RTKs) through the recruitment and activation of Src, resulting in downstream pathway activation. Moreover, ET-1 receptor stimulation activate phospholipase A (PLA) and arachidonic acid (AA) and downstream cyclooxygenase 1 (COX1) and COX2, leading to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production. ET-1 receptor signaling also activates focal adhesion kinase (FAK)-paxillin, resulting in the activation of RHO-guanine nucleotide exchange factor (RHO-GEF) and PI3K, leading to hypoxia-inducible factor 1α (HIF-1α) activity. ET-1 activates nuclear factor κB (Nf-κB) signaling via inhibition of Nf-κB inhibitor (IκB), resulting in the dissociation and subsequent nuclear localization of active Nf-κB. ET receptor activation stimulates RHO-GEF and a small GTPase protein, RAS homology A (RHOA), initiating RHO-dependent signaling events through RHO-associated coiled-coil containing protein kinase 1 (ROCK1), causing cytoskeletal remodeling. Reproduced from [176].



**Figure 12: ET-1 involvement in cancer.** The endothelin axis is involved in facilitating several aspects of cancer growth and progression, including cell proliferation escape from cell death, new vessel formation, invasion and metastasis. Moreover, in some tumor types, ET-1 stimulates osteoblast proliferation, alteration of nociceptive stimuli and malignant bone pain. ETs modulate also trafficking, differentiation and activation of tumor-infiltrating immune cells. Reproduced from [201].

## ***2.7 Classification of selective agonists and antagonists of the endothelin system***

Various findings have provided a rationale for the therapeutic targeting of ET-1 signaling and until now several have been the strategies used for this purpose. First, the selective inhibition of ET-1 synthesis by ECE-1 inhibitors or by natural products such as green tea [202] and red wine biologically active polyphenols [203], as well as the modulation of ET-1 degradation by regulating NEP activity [204] but most of all, inhibition of the ET axis using specific, selective and dual competitive ET receptor antagonists, represent an attractive target approach for the treatment of cancer. Over the last 20 years the development of agonists and antagonists of varying degree of specificity for both receptors has been extensive, not only as pharmacological probes but also for candidate drugs. As a matter of fact, a range of specific and selective ET<sub>A</sub>R antagonists and dual ET receptors antagonists, as well as agonism of ET<sub>B</sub>R, have undergone clinical trials and have shown variable efficacy in cancer (Table 9).

### ***2.7.1 Agonists***

Several studies have demonstrated that the activation of ET<sub>A</sub>Rs by ET-1 stimulates cell proliferation, migration, invasion, osteogenesis and angiogenesis in cancers. Conversely, ET<sub>B</sub>Rs may oppose tumor progression by promoting apoptosis and clearing ET-1 [205]. Until now, no ET<sub>A</sub>R-selective agonists (either peptide or nonpeptide) have been discovered. On the contrary, several agonists have been developed for ET<sub>B</sub>Rs. In particular, four synthetic peptides have become established as the most selective agonists at ET<sub>B</sub>Rs, with greater selectivity than ET-3: Sarafotoxin 6c (S6C), one of the four sarafotoxin isoforms originally identified from the snake venom [90]; [Ala<sup>1,3,11,15</sup>]-ET-1, a linear analog of ET-1 where substitution of Ala for Cys residues removes the disulfide bridges, converting the ET-1 structure to an ET<sub>B</sub>R agonist [206]; IRL1620 (Suc- [Glu<sup>9</sup>, Ala<sup>11,15</sup>]-ET-1<sub>8,21</sub>) [207], which consists in a truncated linear analog where the N-terminus has an N-succinyl modification that reduces metabolism by non-specific peptidases; BQ3020 ([Ala<sup>11,15</sup>] Ac-ET-1<sub>6,21</sub>), a linear peptide fragment of ET-1 with an N-acetyl modification [208]. Among these agonists, IRL-1620 (known as SPI-1620 licensed by Spectrum Pharmaceuticals, Technology Drive Irvine, CA, USA) has been tested for clinical application in cancer and a phase 1 clinical trial has been successfully completed showing to selectively and transiently increase tumor blood flow. Since tumor hypoxia leads to therapeutic

resistance while oxygenation of the tumor improves the effectiveness of radiotherapy, the rationale for the study was that stimulating ET<sub>B</sub>Rs would cause vasodilatation to increase the penetration of cytotoxic anti-tumor agents into tumors but reduces the concentration in healthy tissues. In fact, in rat models of prostate and breast cancer, IRL1620 increased the effectiveness of antitumor agents by significantly increasing the amount of drug in tumors [209]. In addition, the agonist improved the efficacy of radiotherapy in treatment of solid tumors enhancing the radiation-induced reduction in tumor volume [210]. As the phase I trial determined the safety, tolerability, pharmacokinetics and pharmacodynamics of IRL1620, a phase II trial was initiated in 2013 to determine the effectiveness of IRL1620 in combination with docetaxel in patients with advanced biliary cancer (<http://clinicaltrials.gov/ct2/show/NCT01773785>) and in combination with docetaxel compared with docetaxel alone for patients with nonsmall-cell lung cancer after failure of platinum-based chemotherapy (<http://clinicaltrials.gov/show/NCT01741155>). Moreover, considering that the human brain contains the highest density of ET receptors, with the ET<sub>B</sub>R subtype comprising about 90% in areas such as cerebral cortex, a new emerging strategy is to use IRL1620 to provide vasodilatation and neuroprotection in rat model of focal ischemic stroke where it has been already shown that the peptide reduced the neurological damage [211].

### ***2.7.2 Antagonists***

There are currently over 15 ET<sub>A</sub>R and/or ET<sub>B</sub>R antagonists being evaluated in clinical trials for a variety of indications, including cardiovascular disease and cancer. The selective ET<sub>A</sub>R and ET<sub>B</sub>R antagonists BQ123 and BQ788 have been valuable tools for the assessment of ET receptor antagonism and have been used extensively in preclinical models. However, as these agents are both peptides, their utility in clinical setting has been limited. Although no data in humans with BQ788 have been published, a number of studies have been performed using BQ123, generally in a setting in which access to small molecule non peptide agents has not been possible. To date, the dual ET<sub>A</sub>R/ET<sub>B</sub>R antagonist bosentan, the selective ET<sub>A</sub>R antagonists antrasentan and YM-598, and the specific ET<sub>A</sub>R antagonist zibotentan are the only ET receptor antagonists that have been evaluated in both preclinical and clinical oncology settings (Table 9) [205].

### 2.7.2.1 ET<sub>A</sub>R-selective antagonists

BQ123 originally isolated from fermentation products of the microorganism *Streptomyces misakiensis*, that has low nanomolar affinity for the receptor [212] and the linear peptide FR139317 [213] are the most widely cited ET<sub>A</sub>R-selective peptide antagonists used in vitro and in vivo. These antagonists are commercially available and are recommended for their very high selectivity for ET<sub>A</sub>R, their solubility and lack of plasma binding but, owing to their peptidic structure, they do not have orally bioavailability, so they are administered intra-arterially and metabolized or excreted over comparatively short period of time. BQ123 and FR139317 are therefore used for short-term, acute investigations in both animal models and in experimental medicine studies that have demonstrated a role for the ET<sub>A</sub>R in cancer cell growth, proliferation, survival, migration invasion and pain [205].

TAK-044, a cyclic hexapeptide also isolated from *Streptomyces misakiensis* with a more modest degree of ET<sub>A</sub>R selectivity, has also been used in experimental and clinical studies, including renal failure and in a multicenter, randomized, double-blind, placebo-controlled trial in patients with subarachnoid hemorrhage [98, 214]. However, the majority of experimental studies have used a number of commercially available experimental ET<sub>A</sub>R antagonists such as PD156707 [215] and A127722 (ABT627, antrasentan) [216].

A range of preclinical investigations of antrasentan in cancer setting have demonstrated its potential anticancer activity (Table 9). Antrasentan dose dependently inhibited ET-1 driven prostate cancer cell line proliferation, inhibited neoangiogenesis in a cervical cancer xenograft model and reduced osteoblastic bone metastases in mice inoculated with a breast cancer cell line. Moreover, when combined with paclitaxel and docetaxel, additive antitumor, pro-apoptotic and antiangiogenic effects were observed in ovarian cancer cell and prostate cancer cells, respectively. A number of Phase II and III clinical trials of antrasentan have been completed, several of these were performed in men with prostate cancer, but the lack of reduction in disease progression and the absence of additive or synergistic effects of combination therapy, did not support its use as first-line monotherapy (Table 9) [205]. On the contrary, the compound has shown promise in patients with type 2 diabetic nephropathy, reducing albuminuria, blood pressure, cholesterol and triglyceride levels [217] and this has led to the initiation of a Phase III multicenter trial (Sonar).

Ambrisentan (Leitairis, Volibris, LU208075, BSF208075) was approved in 2007 by the Food and Drug Administration and in 2008 by the European Medicines Authority as an orally active, once daily drug for treatment of pulmonary arterial hypertension (PAH). The compound is a competitive ET<sub>A</sub>R antagonist with a good bioavailability, long half-life with a low incidence of acute hepatic toxicity and does not induce or inhibit P450 enzymes, so less likely to affect the pharmacokinetics of P450-metabolized drugs. Ambrisentan has fewer side effects, such as an increase in liver enzymes that require liver function tests that is associated with bosentan but nasal congestion and peripheral edema are more prevalent. In 2015, clinical trials with ambrisentan included the treatment of portopulmonary hypertension, hypoplastic left heart syndrome, inoperable chronic thromboembolic pulmonary hypertension, PAH associated with systemic sclerosis and exercise-induced PAH [218, 219].

Zibotentan is the most ET<sub>A</sub>R specific antagonist of the small molecule compounds which, unlike other agents that have activity at both ET<sub>A</sub>R and ET<sub>B</sub>R, showed any effect at ET<sub>B</sub>R. A range of preclinical studies have been undertaken with zibotentan, the majority of which were in models of prostate and ovarian cancer. These studies have demonstrated that complete blockade of ET<sub>A</sub>R with zibotentan reversed ET-1 induced inhibition of apoptosis while allowing pro-apoptotic signaling via ET<sub>B</sub>R. Moreover, in combination with paclitaxel or docetaxel, enhances chemotherapy-induced apoptosis compared with either agent alone, as well as a role of this compound in inhibiting cell proliferation and invasion has been shown [220]. These and other preclinical findings, provided a rationale for the use of zibotentan in clinical studies but, owing to its failure in showing any improvement in survival in phase III trial in patients with resistant prostatic cancer metastatic to bone, zibotentan was discontinued from development by Astra-Zeneca (Cambridge, United Kingdom) [221]. However, the antagonist was made available to other groups and trials included patients with intermittent claudication and renal scleroderma [205].

Sitaxentan was introduced in 2008 as a potent, competitive, long-acting, orally active ET<sub>A</sub>R antagonist, the most highly selective to be approved for treatment of PAH. The compound was withdrawn in 2010 owing to cases of idiosyncratic hepatitis resulting in acute liver failure [222].

YM-589 [Astellas Pharma (formerly Yamanouchi)] is a potent selective ET<sub>A</sub>R antagonist developed through the modification of bosentan. ET<sub>A</sub>R inhibition with this antagonist

significantly reduced tumor growth and liver metastasis in an in vivo model of gastric cancer [223] and significantly inhibited ET-1 induced potentiation of nociception in murine models of cancer pain [224]. These beneficial effects in preclinical models led to the initiation of two randomized Phase II clinical trials to investigate the impact of this agent on pain in patients with prostate cancer (Table 9) but both trials were terminated due to a lack of pain reduction [205].

#### ***2.7.2.2 ET<sub>B</sub>R-selective antagonists***

Few ET<sub>B</sub>R antagonists have been developed, reflecting the potential danger of blocking the beneficial vasodilatation and internalization of ET-1 mediated by this subtype. The most extensively used and highly cited is the peptide BQ788 [225], although a small number of papers have reported on other compounds such as the first small molecule, nonpeptide RO468443 that displayed 2000-fold ET<sub>B</sub>R selectivity [226] and A192621, that has the advantage of having good oral bioavailability in a number of species [216]. Preclinical studies with BQ788 have reported a role for ET<sub>B</sub>R in the survival, growth and metastasis of melanoma and glioma cells [227]. Furthermore, BQ788 inhibited several pathways mediated by ET-1, including bronchoconstriction and cell proliferation and was shown to inhibit clearance of perfused ET-1 [228]. BQ788 has also been used to identify the role of ET<sub>B</sub>R in control of vascular tone in vessels that supply tumor and in growth and invasion of lymphatic endothelial cells and vessels [189] as well as in the mediation of endothelial barrier in T cell homing tumors [229]. However, as BQ123, the use of this antagonist in clinical trial has been limited because of the cost of its development and the need for parenteral administration (the peptide is hydrolyzed by peptidases in the systemic circulation and gastrointestinal tract).

#### ***2.7.2.3 Mixed antagonists in the clinic***

Until now two mixed antagonists have been used in clinical studies, in particular for the treatment of PAH.

*Bosentan* (Tracleer, Ro47-0203) is a sulfonamide and was the first ET orally active antagonist to be approved for clinical use in the treatment of PAH [230]. It has a short half-life but a good bioavailability and, as a competitive mixed antagonist, it blocks both the detrimental effects of ET-1 mediated via ET<sub>A</sub>R, as well as the beneficial actions mediated by ET<sub>B</sub>R. The drug tends

to have lower rates of fluid retention and edema than observed with ET<sub>A</sub>R antagonists. However, liver toxicity is a significant side effect. This is not thought to be as a consequence of blocking ET receptors but by inhibiting the bile salt export pump leading to accumulation of cytotoxic bile salt, resulting in hepatocellular damage. In preclinical studies of human melanoma cell lines, bosentan was observed to inhibit proliferation, decrease cell viability and DNA synthesis and induce apoptosis [231]. These preclinical studies provided a rationale to investigate this agent in the clinical cancer setting but a Phase II clinical study accomplished on patients with metastatic melanoma, reported no difference in time to tumor progression (Table 9) [232]. In 2015, bosentan clinical trials included scleroderma renal crisis, ischemic optic neuropathy, stable severe chronic obstructive pulmonary disease and combined with iloprost in PAH [98].

*Macitentan* (Opsumit, ACT-064992; Actelion) represent the next generation of orally active ET receptor antagonists and was developed by modifying the structure of bosentan to improve efficacy and tolerability [233]. The antagonist is an order of magnitude more potent as bosentan, as a result of longer receptor occupancy (17 minutes versus 70 seconds for bosentan measured by in vitro assays), probably as a result of interaction with different amino-acid residues in the ETRs. Actelion describes the compound as a dual antagonist but on the basis of their own data measuring inhibition of [<sup>125</sup>I]-ET-1 binding to human expressed receptors, it displays ~ 800 fold selectivity for the ET<sub>A</sub>R subtype. A phase III clinical trial was successfully completed in 2012 [234] and the compound gained approval from the US FDA in the 2013 for the treatment of PAH. In 2015, clinical trials with macitentan includes thromboembolic pulmonary hypertension, portopulmonary hypertension and Eisenmenger Syndrome. Perhaps, the most intriguing new target is glioblastoma, where the ET pathway is upregulated, in combination with existing therapy temozolomide (Table 9) [98, 235].

**Table 9: Endothelin receptor antagonists in cancer therapy.** Reproduced from [176].

Compound	Company	ET1 receptor antagonist	Tumour type	Clinical development	Outcome
Zibotentan	AstraZeneca	Specific for ETAR	Non-metastatic CRPC <sup>106</sup>	Phase III (monotherapy)	<ul style="list-style-type: none"> <li>OS HR = 1.13; (95% CI = 0.73–1.76)</li> <li>PFS HR = 0.89; (95% CI = 0.71–1.12)</li> </ul>
			HRPC with bone metastases <sup>144</sup>	Phase III (monotherapy)	<ul style="list-style-type: none"> <li>OS = 24.5 versus 22.5 months</li> <li>PFS = 6.2 versus 6.5 months</li> </ul>
			Metastatic HRPC*	Phase III (zibotentan plus docetaxel)	<ul style="list-style-type: none"> <li>OS = 20.0 versus 19.2 months</li> <li>PFS = 7.0 versus 7.9 months</li> </ul>
Zibotentan	AstraZeneca	Specific for ETAR	NSCLC <sup>145</sup>	Phase II (zibotentan plus pemetrexed)	<ul style="list-style-type: none"> <li>OS = 146 versus 193 days</li> <li>PFS = 110 versus 87 days</li> </ul>
Atrasentan	Abbott	Selective for ETAR	Non-metastatic HRPC <sup>146</sup>	Phase III (monotherapy)	<ul style="list-style-type: none"> <li>TTP = 764 versus 671 days</li> <li>OS = 1,477 versus 1,403 days</li> </ul>
			Metastatic HRPC <sup>147</sup>	Phase III (monotherapy)	<ul style="list-style-type: none"> <li>TTP HR = 0.89 (95% CI = 0.76–1.04)</li> <li>OS HR = 0.97 (95% CI = 0.81–1.17)</li> </ul>
			Metastatic HRPC <sup>†</sup>	Phase III (atrasentan plus docetaxel plus prednisone)	NR
YM-598	Astellas Pharma	Selective for ETAR	Prostate cancer <sup>§</sup>	Phase II (monotherapy)	NR
			Metastatic prostate cancer <sup>  </sup>	Phase II (YM598 plus mitoxantrone plus prednisone)	NR
Bosentan	Actelion	Dual ETAR and ETBR	Metastatic melanoma <sup>148</sup>	Phase II (monotherapy)	SD = 34%
			Metastatic melanoma <sup>149</sup>	Phase II (bosentan plus dacarbazine)	<ul style="list-style-type: none"> <li>OS = 13.0 versus 10.6 months</li> <li>PFS = 1.6 versus 2.8 months</li> </ul>
Macitentan	Actelion	Dual ETAR and ETBR	Recurrent glioblastoma <sup>¶</sup>	Phase I (macitentan plus temozolomide)	NR

CI, confidence interval; CRPC, castration-resistant prostate cancer; ET1, endothelin 1; ETAR, endothelin A receptor; ETBR, endothelin B receptor; HR, hazard ratio; HRPC, hormone-refractory prostate cancer; mCRPC, metastatic CRPC; NR, not reported; OS, overall survival; PFS, progression-free survival; SD, stable disease; TTP, time to disease progression.\*ClinicalTrials.gov identifier: [NCT00617669](https://clinicaltrials.gov/ct2/show/study/NCT00617669). †ClinicalTrials.gov identifier: [NCT00134056](https://clinicaltrials.gov/ct2/show/study/NCT00134056). ‡ClinicalTrials.gov identifier: [NCT00050297](https://clinicaltrials.gov/ct2/show/study/NCT00050297). §ClinicalTrials.gov identifier: [NCT00048659](https://clinicaltrials.gov/ct2/show/study/NCT00048659). ¶ClinicalTrials.gov identifier: [NCT01499251](https://clinicaltrials.gov/ct2/show/study/NCT01499251).

# *AIM OF THE STUDY*

MM is an age-dependent monoclonal tumor of BMPCs, characterized by a strong BM stromal niche dependence and by a significant morbidity due to organ dysfunction [29]. Despite recent developments in novel therapies, such as immunomodulator drugs and proteasome inhibitors, it remains an incurable disease [64]. Although various genomic aberrations have been shown to provide PCs with the ability to proliferate in an uncontrolled manner, increasing evidences suggest critical roles for surface receptors with restricted expression in malignant PCs and for BM microenvironment in mediating MM survival, proliferation and resistance to therapy [37]. Therefore, receptors with a restricted expression on PCs represent attractive new therapeutic targets. In the last decades it has been demonstrated that the ET axis is able to promote the development and progression of tumor cells in an autocrine and/or paracrine fashion. Accordingly, recent experimental and clinical data obtained in solid tumors have evidenced the possibility of interfering with ET receptors (ETRs) - *via* specific antagonists - for therapeutic purposes [236]. Although recent evidence suggests a role for ET-1 axis in MM [174], a complete understanding of the underlying mechanisms activated by ET-1 axis in MM remains to be elucidated. Therefore, our study was aimed at:

- analyzing the expression of ET<sub>A</sub>R and ET<sub>B</sub>R by MM cell lines (U266, RPMI-8226, OPM-2, LP-1, KMS-12-PE), as well as by primary malignant and normal, long lived and/or reactive PCs and PCs precursors (BM immature and mature B lymphocytes and PB naïve and memory B lymphocytes);
- assessing whether malignant PCs and cells from the BM microenvironment express and release ET-1, in order to understand the mechanism (autocrine/paracrine) through which ET-1 acts on the neoplastic clone;
- testing the possibility of targeting the ET-1 receptors in MM for therapeutic purposes.

*MATERIALS AND  
METHODS*

### *Patients, Samples and Cell lines*

BM samples were collected from patients (n=100) with newly diagnosed MM and from volunteer healthy donors (HDs) (n=15) during routine diagnostic assessments and at the time of BM harvest, respectively. Peripheral blood (PB) samples from MM patients (n=8) and HDs (n=15) were obtained in the same settings. Overall, BM and PB samples were collected from March 2010 to July 2016 at the Hematology and Bone-Marrow Transplant Unit of the Verona University Hospital and at the Hematology Unit of the Treviso Hospital. Both patients and HDs provided written informed consent for the collection of samples and subsequent analysis, as approved by our institutions' Ethics Board. Patient demographics, clinical characteristics and treatment are reported in Table 10. Human MM cell lines U266, OPM-2, LP-1, KMS-12-PE, RPMI-8226 and the human breast cancer cell line MCF-7 were purchased from DSMZ, Braunschweig, Germany, and cultured in RPMI 1640 and in Eagle's MEM respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) in 5% CO<sub>2</sub> at 37°C. Mycoplasma contaminations were excluded by the Mycoplasma Species kit (EuroClone, Milan, Italy). Human BM fibroblasts and mesenchymal stromal cells (MSCs), provided by Dr. G. Bassi (Department of Medicine, Verona University, Italy), were cultured in DMEM supplemented with 10% FBS and 1% P/S. Adipocytes, osteocytes, chondrocytes and myocytes were obtained by differentiating MSCs as previously described [237]. Human umbilical vein endothelial cells (HUVEC), a gift of Prof. C. Lunardi (Department of Medicine, Verona University), were cultured in F-12K Medium (Gibco, ThermoFisher Scientific), 0.1 mg/ml heparin, 0.03-0.05 mg/ml EC growth supplement (ECGS) (Sigma-Aldrich, Milan, Italy) and 20% FBS. Cell suspensions from tonsils were provided by Prof. MA Cassatella (Department of Medicine, Verona University). The mononuclear cell fraction was isolated from each BM or PB sample by Ficoll-Paque isolation solution and freshly examined. In 10 cases, CD38<sup>+</sup> PCs were purified from the mononuclear fraction of MM BM samples using a positive immune-magnetic cell selection kit (MACS, Miltenyi Biotec, Bologna, Italy) according to the manufacturers' instructions. The percentage of CD138<sup>+</sup> PCs obtained ranged from 95 to 99%. B lymphocytes were purified from buffy coat using B cell isolation kit II human (MACS, Miltenyi Biotec, Bologna, Italy), according to the manufacturers' instructions.

### ***RNA isolation and reverse transcription quantitative real-time PCR (RT-qPCR)***

Total RNA was isolated from MM cell lines, primary MM CD138<sup>+</sup> PCs, HUVEC, BM fibroblasts, MSCs, adipocytes, osteocytes, chondrocytes and myocytes using the SV Total RNA Isolation System (Promega, Milan, Italy). Reverse transcription (RT) was performed using the SuperScript III Reverse Transcriptase (Life Technologies, Monza MB, Italy) according to the manufacturers' instructions. Briefly, 1 µg of total RNA was reverse transcribed using the random hexamer primers, the obtained cDNA was diluted (2ng/µl) and then amplified by Real Time PCR using the Fast SybrGreen MasterMix (Life Technologies, Monza MB, Italy) and intron-spanning primers for ET<sub>A</sub>R, ET<sub>B</sub>R and ET-1, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Gene expression was quantified by the comparative cycle threshold (C<sub>t</sub>) method, normalizing C<sub>t</sub> values to the housekeeping gene GAPDH and calculating relative expression values. The primer sets used were: 5'-TAAGCAGCGTCGAGAAGTGG-3' and 3'-CGGTTCTTGTCATCTCGTTAT-5' (ET<sub>A</sub>R); 5'-AGACAGGACGGCAGGATCT-3' and 3'-GAACACAAGGCAGGACACAA-5' (ET<sub>B</sub>R); 5'-CACTCCCGAGCACGTTGTTC-3' and 3'-GGATGATGTTCTGGAGAGCC-5' (GAPDH).

### ***Western blotting***

Following cells lysis in 200 µl of lysis buffer (50mM TrisHCl pH 7.5, 150mM NaCl, 1mM Na<sub>3</sub>VO<sub>4</sub>, 0,5% IGEPAL CA630, 1% Triton X100, 1mM PMSF and proteases inhibitors), protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay (Sigma, Milan, Italy). Protein cell extracts (40 µg) were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris Buffer Saline (TBS, with 0,1% Tween 20) overnight at 4°C in and then immunoblotted for 2 h at room temperature with the following primary antibodies: anti-ET<sub>A</sub>R rabbit pAb (ab117521, 1:5000; Abcam, Cambridge, UK), anti-ET<sub>B</sub>R rabbit mAb (EPR7013, 1:1000; Abcam, Cambridge, UK), anti-ET-1 Rabbit pAb (C1C3) (GTX116033, 1:1000; GeneTex Irvine, Usa), anti-phospho-p44/42 MAPK rabbit 1:500, anti-p44/42 MAPK rabbit 1:500, anti-Bcl-xL rabbit 1:1000, anti-β actin mouse mAb (#3700, 1:20000), and anti-GAPDH rabbit (#2118, 1:1000) (all from Cell Signaling Technology, Danvers, USA), and anti-tubulin mouse mAb (1:500; Developmental Studies Hybridoma Bank, Iowa, USA). After washing, membranes were incubated with species-specific horseradish

peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1,5 h (anti-rabbit NA934, 1:10000; anti-mouse NA931, 1:10000; Amersham, GE Healthcare Life Science, Milan, Italy). Blots were then developed using ECL (Amersham) and images were acquired by Image Quant Las 4000mini (GE Healthcare Life Sciences).

### ***Flow cytometric analysis***

Cells were incubated with rabbit polyclonal anti-ET<sub>A</sub>R (NBP2-30068, Novus Biologicals, Littleton CO, USA) for 1 h at room temperature, or with rabbit monoclonal anti-ET<sub>B</sub>R antibody (EPR7013, Abcam, Cambridge, UK) for 30 minutes in ice and, after washing, with PE conjugated secondary antibody Donkey pAb to rabbit IgG (AB98441, Abcam, Cambridge, UK) for 30 minutes. In order to increase the specificity of the staining, 20µl of FcR Blocking Reagent (MACS, Miltenyi Biotec, Bologna, Italy) were added to each sample. BM samples from MM patients, HDs, and the mononuclear cell fraction from tonsils were also stained with 5 µl of the following antibodies: PerCP anti-CD38, APC anti-CD138, APC-Cy7 anti-CD45, PE-Cy7 anti-CD56, FITC anti-CD19 (Biolegend, London, UK). In order to distinguish different steps of B lymphocytes maturation, BM cells from HDs were also stained with APC-Cy7 anti-CD45, FITC anti-CD19, APC anti-CD10 and PE-CY7 anti-CD20, while HDs and MM patients PBMCs were stained with APC-Cy7 anti-CD45, FITC anti-CD19, PE-CY7 anti-CD20, APC anti-CD27. Quantitative fluorescence analysis was performed by the FacsCanto flow cytometer (Becton-Dickinson, New Jersey) using FlowJo software (Tree Star, Inc. Ashland, OR) to collect and analyse data.

### ***Immunohistochemistry***

Immunohistochemistry analysis of ET<sub>B</sub>R expression in BM biopsies from MM patients was performed as previously described [238], with minor modifications. Trophoblast specimens were used as positive controls. Briefly, histological specimens were fixed in neutral, phosphate-buffered 10% formaldehyde solution for 24 h. Subsequently, samples were decalcified with a decalcifying solution of EDTA (DDK Italy, Vigevano, Italy) for 60 minutes and then dehydrated in graded series of ethanol and embedded in paraffin (Merk, Germany) using xylene as intermedium. Sections of 3 µm were cut and mounted on glass slides then, using xylene and graded series of ethanol, samples were deparaffined and antigen-retrieval was performed by the use of a series of buffered solution at different pH and temperatures. Sections were subsequently

incubated with 3% H<sub>2</sub>O<sub>2</sub> and then stained with the primary antibody. Immunohistochemical staining for ET<sub>B</sub>R was performed with the anti-ET<sub>B</sub>R sheep polyclonal antibody (ALX-210-506A-C250, Alexis Biochemicals Corporation, Lausen, Switzerland) at 1:400 dilution and the biotinylated rabbit anti-sheep IgG secondary antibody (BA-6000, Vector; Burlingame, USA) at 1:500 dilution. Omission of primary antibody served as negative control. The reaction was visualized by the binding of a streptavidin-horseradish peroxidase complex to biotin and a colour reaction with 3,3'-Diaminobenzidine (DAB).

### ***Methylated DNA Immunoprecipitation (MeDIP) analysis***

MeDIP was performed as previously described [239], with minor modifications. Briefly, genomic DNA was obtained from primary CD138<sup>+</sup> PCs of 10 MM patients, MM cell lines (U266, OPM-2, LP-1, KMS-12-PE, RPMI-8226) and B lymphocytes by phenol-chloroform extraction, ethanol precipitation and RNase digestion. Subsequently, the purified DNA was sheared by sonication by an ultrasonic homogenizer (Bandelin Sonopuls HD 2070, Bandelin, Berlin, Germany), to produce random fragments ranging in size from 300 to 1000 bp. Four µg of sonicated DNA was then diluted in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), denatured for 10 min at 95°C and immediately placed on ice. The diluted samples were subsequently incubated overnight with 51 µl of 10x IP buffer (10mM Na-phosphate, 1.40 M NaCl, 0,5% Triton X-100) and 5µg of 5-methylcytosine (5mC) monoclonal antibody (clone 33D3, Merck Millipore, Milan, Italy) for 2 h at 4°C in a final volume of 500 ul 1x IP buffer and then immunoprecipitated with 18 µl of Dynabeads Protein G (10003D, ThermoFisher, Waltham, MA USA) for 1h at 4°C in a rotating tube holder; a portion of the sonicated DNA was left untreated to serve as an input control (IN DNA). To purify the immunoprecipitated (IP) DNA and allow the digestion of the antibody and the release of the methylated DNA, each sample was treated with 70µg of proteinase K and incubated overnight at 50°C. The IP DNA was then recovered using the Pure Link PCR Purification Kit (K310002, Thermo Fisher, Waltham, MA USA) and a qPCR was carried out on both IP DNA and IN DNA by using primers specific for control genes (H19 ICR, positive control, always methylated; and HIST 1H3B, negative control, always un-methylated) and for ET<sub>B</sub>R promoter region. Primer sequences for control genes were: H19 ICR, For-5'-CGAGTGTGCGTGAGTGTGAG; Rev-5'-GGCGTAATGGAATGCTTGAA. HIST 1H3B, For-5'-GAGAGCATTAGGGCAGACAAA; Rev-5'-GTTCCCTCAGACAGCCACATTT. Primer

sequences for ET<sub>B</sub>R gene were: set A, For-5'-TAGAGACAAGCAGAGGAAGGAAG; Rev-5'-AGGGCATCAGGAAGGAGTTTC (distance from transcription start site (TSS) +215 +15); set B, For-5'-CGCAGGGGAACCTCTATAACC; Rev-5'-GTGTTTGAAGCCGTTTGGAG (distance from TSS -216 -412). The relative enrichment of target sequences after MeDIP, was then expressed as percentage over IN DNA.

### ***Enzyme-linked immunosorbent assay (ELISA)***

ET-1 levels were measured in conditioned media of HUVEC, BM MSCs, BM fibroblasts and MM PCs after 48 h of culture by using commercially available ET-1 Quantikine ELISA kit (R&D System, Minneapolis, USA, Canada). Briefly, 200 µl of supernatants were incubated in duplicated for 2 h at room temperature in 96-well microplates coated with specific antibody. After washing, samples were treated with substrate solution for 2 h. The enzymatic reaction was stopped and read within 30 minutes at 450 nm. Concentrations were reported pg/mL.

### ***Cell viability assay***

U266 and RPMI-8226 cell lines were plated in 96-well plates (Corning LifeSciences, NY, USA) and treated for 48 h with 10 µM BQ123 or BQ788 (Bachem, Bubendorf, Switzerland), alone or in combination. U266 and RPMI-8226 were also cultured for 48 h in the presence of bosentan (SML1265, Sigma Aldrich, Milan, Italy) at the concentration of 50-100 µM and /or bortezomib (Janssen-Cilag, Beerse, Belgium; discarded patient drug) 1-5 nM. Cell viability rates were assessed by MTT assay (Sigma Aldrich, Milan, Italy) according to the manufacturer's instruction. The effects of interaction of the two drug were analysed according to the median- effect method of Chou [240] using the CalcuSyn Software (Biosoft, Cambridge, UK). The mean combination index (CI) values were evaluated and combination data were plotted as CI vs fraction affected (Fa), defining the CI variability by the Sequential Deletion Analysis method.

### ***Statistical analysis***

Expression of plasma membrane ET receptors with respect to controls was analyzed using 1-way-ANOVA, while differences among groups were tested by Pearson's chi square and Fisher exact test. Correlation between ET<sub>B</sub>R plasma membrane expression and DNA methylation in

CD138<sup>+</sup> PCs was evaluated by the nonparametric Spearman correlation test. Analysis of drug-treated vs vehicle-treated cells was determined using Student's t test. Differences were statistically significant for P values < 0.05. Patients were staged according to Durie and Salomon [241], response criteria were established according to Rajkumar [242]. Time-to-treatment (TTT) was defined as the time from diagnosis to first treatment or last follow-up in case of untreated patients, overall survival (OS) as the time from diagnosis to death or last follow-up. Survival curves were calculated according to Kaplan and Meier, and differences were tested using the log-rank test. Statistical analysis were performed by GraphPad PRISM® version 5-0c, and Stata SE v.14.2 by StataCorp (College Station, TX).

**Table 10: Demographic and clinical characteristics of the 100 MM patients enrolled in the study.**

	<b>number (%)</b>
<b>Median age</b>	66 (41-90) years
<b>M/F</b>	55/45
<b>Stage</b>	
I	23 (23%)
II	22 (22%)
III	53 (53%)
Unclassified	2 (2%)
<b>Acute renal failure</b>	10 (10%)
<b>Classification</b>	
Ig A	17 (17%)
Ig G	62 (62%)
Light chain	16 (16%)
Non secretory	5 (5%)
<b>First-line therapy</b>	84 (84%)
VD/VTD/VMP	64 (76.2%)
MP/MPT	13 (15.4%)
VAD	3 (3.6%)
TD	3 (3.6%)
RT	1 (1.2%)
<b>No therapy</b>	13 (13%)
<b>Lost to follow-up</b>	3 (3%)

VD: Bortezomib-Dexamethasone; VTD: Bortezomib-Thalidomide-Dexamethasone; VMP: Bortezomib-Melphalan-Prednisone. MP: Melphalan-Prednisone; MPT: Melphalan-Prednisone-Thalidomide. VAD: Vincristine-Doxorubicin-Dexamethasone. TD: Thalidomide-Dexamethasone. RT: Radiotherapy on bone lesion.

# *RESULTS*

### ***Expression of ET-1 receptors by MM PCs and normal, long-lived and/or reactive PCs***

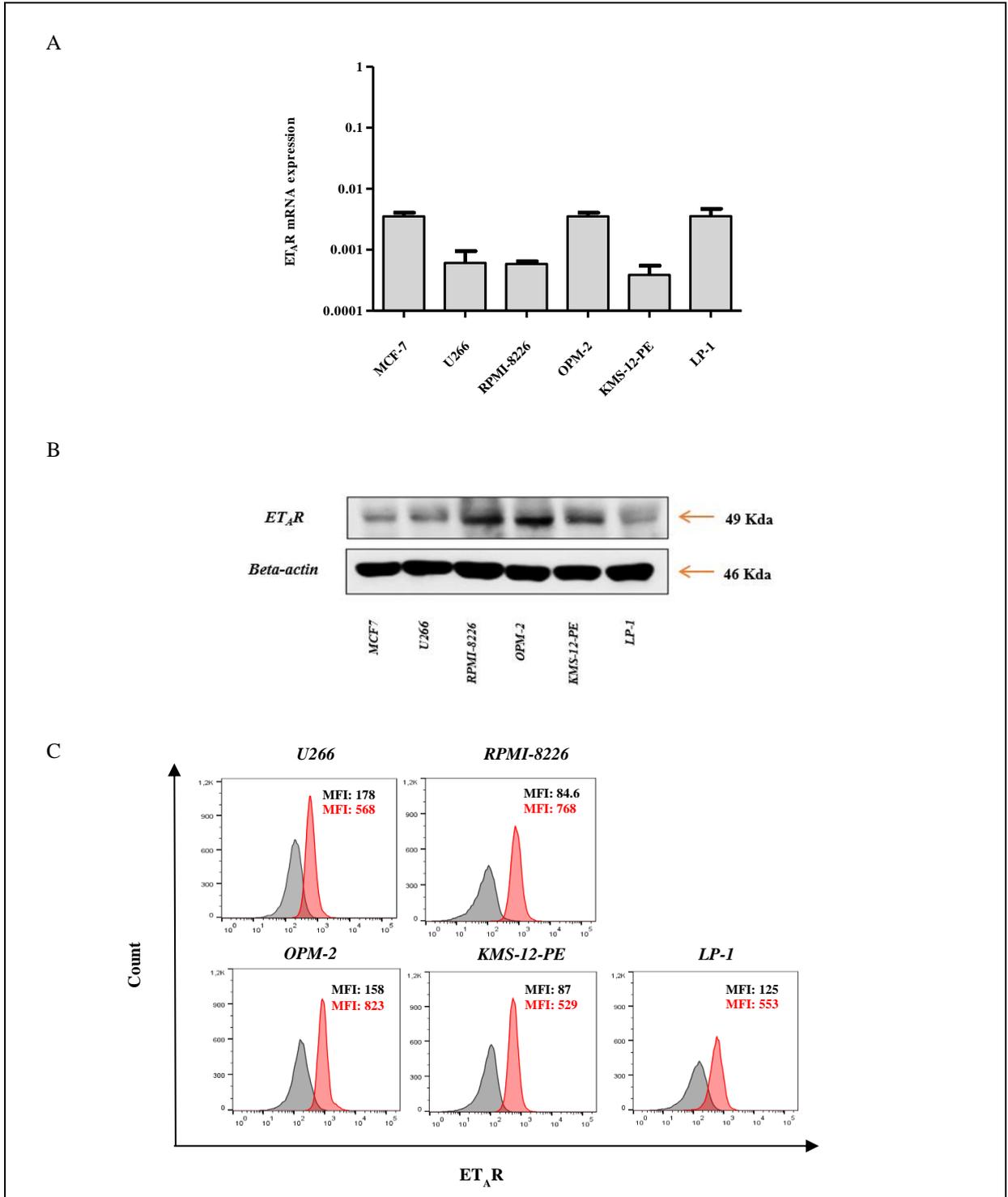
To establish the existence of an ET-1 axis in MM, we first evaluated the expression of ET<sub>A</sub>R and ET<sub>B</sub>R in a panel of MM cell lines, including U266, RPMI-8226, OPM-2, KMS-12-PE and LP-1. Although both ET<sub>A</sub>R and ET<sub>B</sub>R were detected by RT-qPCR and Western blot (Figure 13 A, B and Figure 14 A, B), flow cytometry revealed that only ET<sub>A</sub>R was expressed on the plasma membrane in all cell lines (Figure 13 C), while ET<sub>B</sub>R could be detected only on the plasma membrane of U266, RPMI-8226 and LP-1, but not on OPM-2 and KMS-12-PE (Figure 14 C). For primary MM PCs, we evaluated by flow cytometry 100 BM samples obtained at first diagnosis through BM aspirate. Malignant and normal BM PCs were identified on the basis of the following flow cytometry immunophenotyping panel: CD38/CD138/CD45/CD56/CD19 [243]. Both normal and neoplastic PCs presented a strong expression of CD38 and CD138, while CD45 appeared weakly expressed by normal PCs (CD45 expression has been reported to decline in vivo during normal PCs differentiation) and variable expressed by neoplastic PCs: its intensity ranged from negative to bright. In fact, it has been reported that different PC populations can be identified based on CD45 expression in myeloma patients characterized by dim to negative expression of CD45 and intermediate to bright expression of the antigen [19]. In addition, normal PCs always appeared CD56<sup>-</sup> and CD19<sup>+</sup> whereas neoplastic PCs express CD56 in the majority of cases and lack CD19 [20]. In our study malignant PCs were distinguished from normal, long-lived PCs in BM samples based on their expression of CD56 but not of CD19 (Figure 15). We found that ET<sub>A</sub>R was constitutively expressed by all primary MM PCs (Figure 16 A and C), while ET<sub>B</sub>R was detected in 54 out of the 100 cases under examination (Figure 16 B and D). To ascertain whether ET<sub>A</sub>R and, particularly, ET<sub>B</sub>R are constitutively expressed by normal, long-lived PCs, or, alternatively, they are abnormally up-regulated during neoplastic transformation, we next evaluated PCs in 15 BM samples from HDs, as well as in 5 inflamed tonsils, by flow cytometry. While ET<sub>A</sub>R was constitutively expressed by normal, long-lived and reactive PCs (Figure 16 A and 17 A), ET<sub>B</sub>R was instead always absent (Figure 16 B and 17 A). In our series, ET<sub>B</sub>R<sup>+</sup> and ET<sub>B</sub>R<sup>-</sup> patients did not differ with regard to age, sex, disease classification, stage, presence of bone lytic lesions, or renal failure (Table 11). Moreover, the expression of ET<sub>B</sub>R in terms of median mean fluorescence intensity (MFI), did not differ among patients in different stages of disease [stage I: 2.75 (1.59-

19.38); stage II: 3.45 (1.54-7.64); stage III: 3.51 (1.44-81.22); P=0.81]. BM biopsies obtained from 10 MM patients, were also tested in immunohistochemistry (IHC) for ET<sub>B</sub>R expression and trophoblast specimens were used as positive controls. Figure 17 B displays a representative ET<sub>B</sub>R<sup>+</sup> and ET<sub>B</sub>R<sup>-</sup> patient out of the 10 examined.

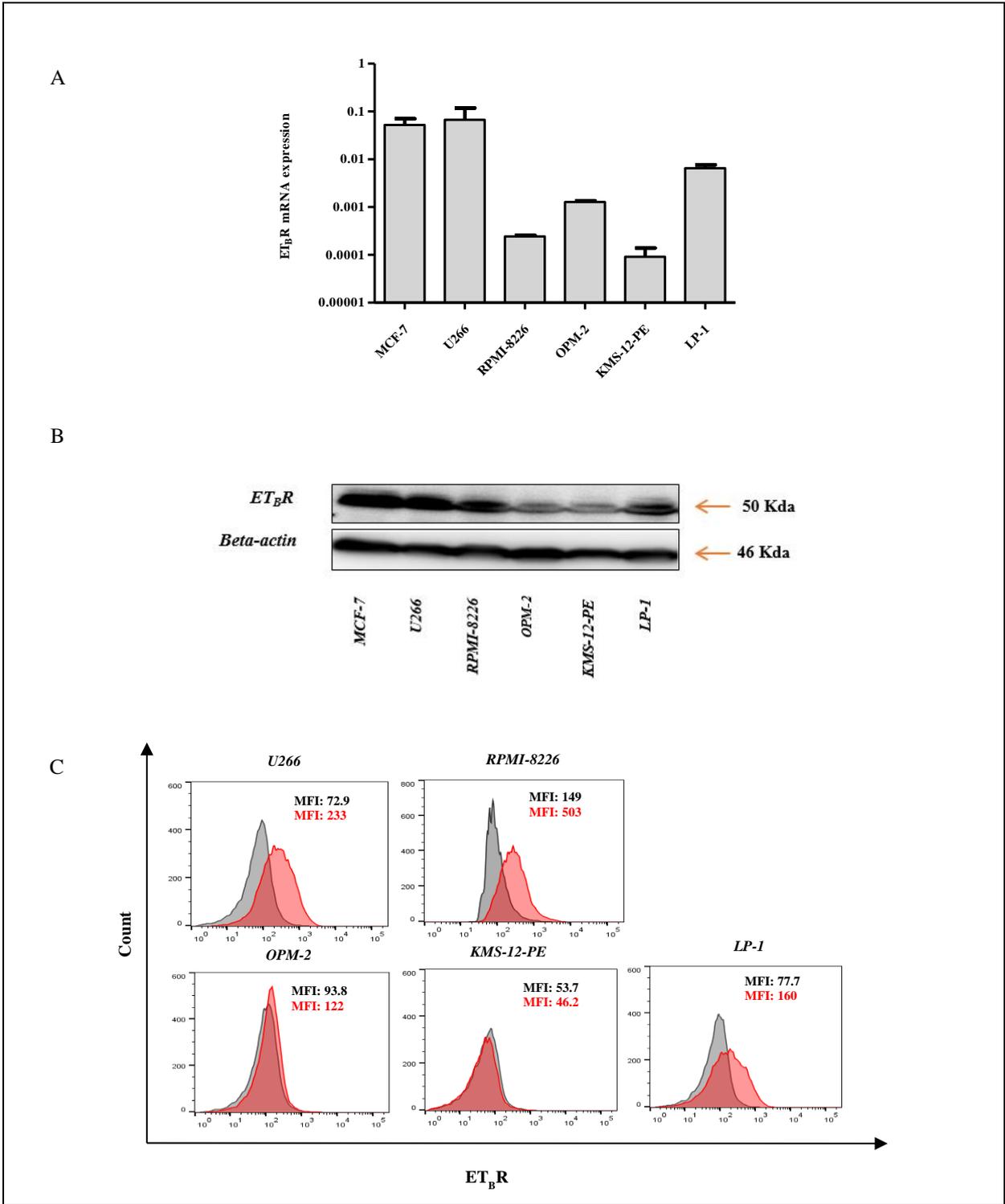
***ET<sub>B</sub>R is aberrantly up-regulated during neoplastic transformation of PCs since the receptor is always absent on PCs precursors***

We evaluated the expression of ET-1 receptors also on healthy PCs precursors, i.e. BM immature and mature B lymphocytes and PB naïve and memory B cells identified by the following immunophenotyping panels: CD45/CD19/CD10/CD20 for immature and mature BM B lymphocytes and CD45/CD19/CD20/CD27 for PB naïve and memory B cells (Figure 18). Noteworthy, also BM immature (CD45<sup>+</sup>/CD19<sup>+</sup>/CD10<sup>+</sup>/CD20<sup>-</sup>) and mature (CD45<sup>+</sup>/CD19<sup>+</sup>/CD10<sup>-</sup>/CD20<sup>+</sup>) B lymphocytes and PB naïve (CD45<sup>+</sup>/CD19<sup>+</sup>/CD20<sup>+</sup>/CD27<sup>-</sup>) and memory (CD45<sup>+</sup>/CD19<sup>+</sup>/CD20<sup>+</sup>/CD27<sup>+</sup>) B cells from 15 HDs, were found to express ET<sub>A</sub>R, but not ET<sub>B</sub>R (Figure 18). Similarly, PB B lymphocytes from 8 ET<sub>B</sub>R-expressing MM patients displayed ET<sub>A</sub>R, but not ET<sub>B</sub>R (Figure 19).

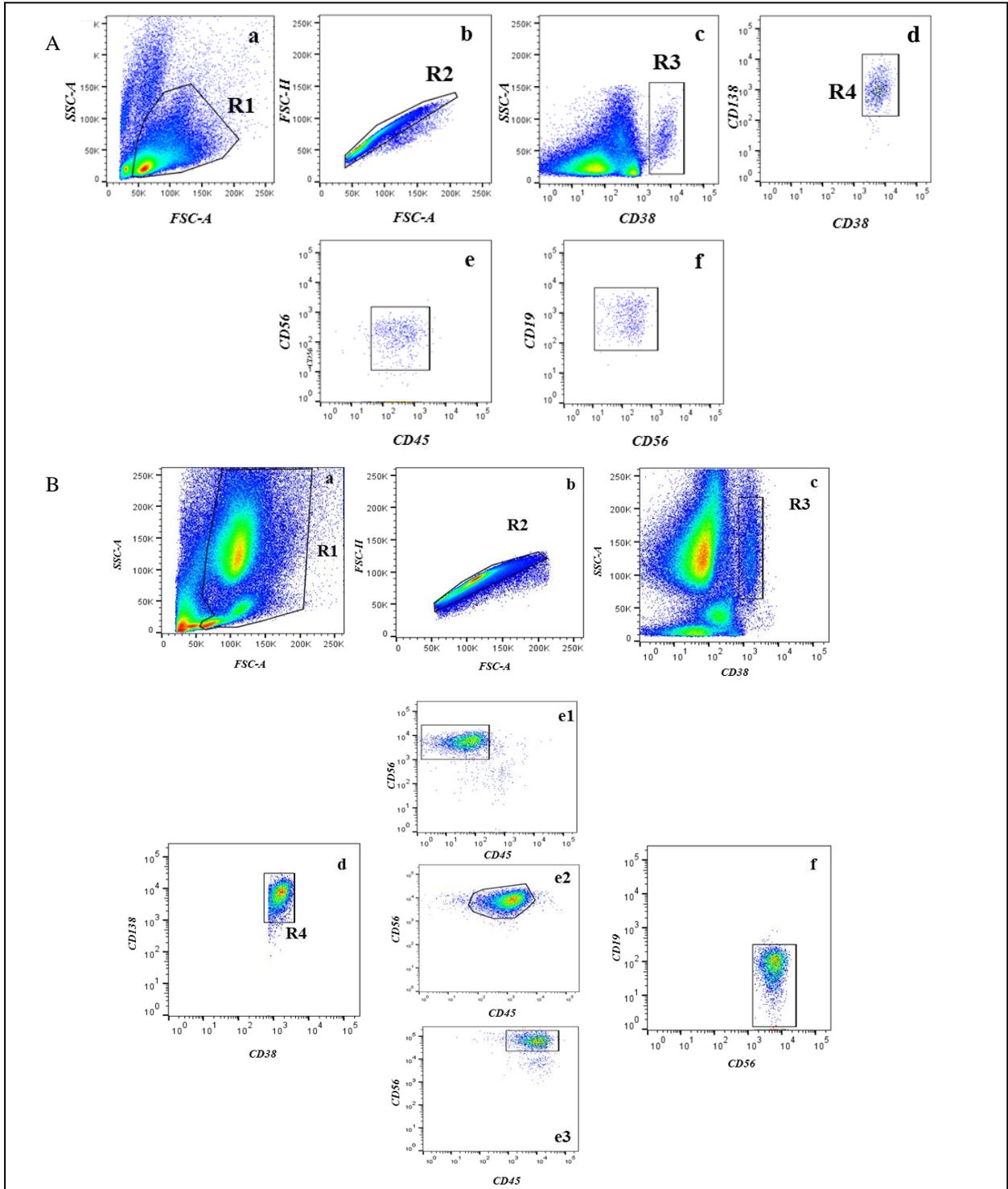
Collectively, our data indicate that normal or reactive PCs constitutively express ET<sub>A</sub>R, but not ET<sub>B</sub>R. On the contrary, ET<sub>B</sub>R is aberrantly up-regulated during neoplastic transformation, as revealed by its expression in malignant PCs (Figure 16 B and D).



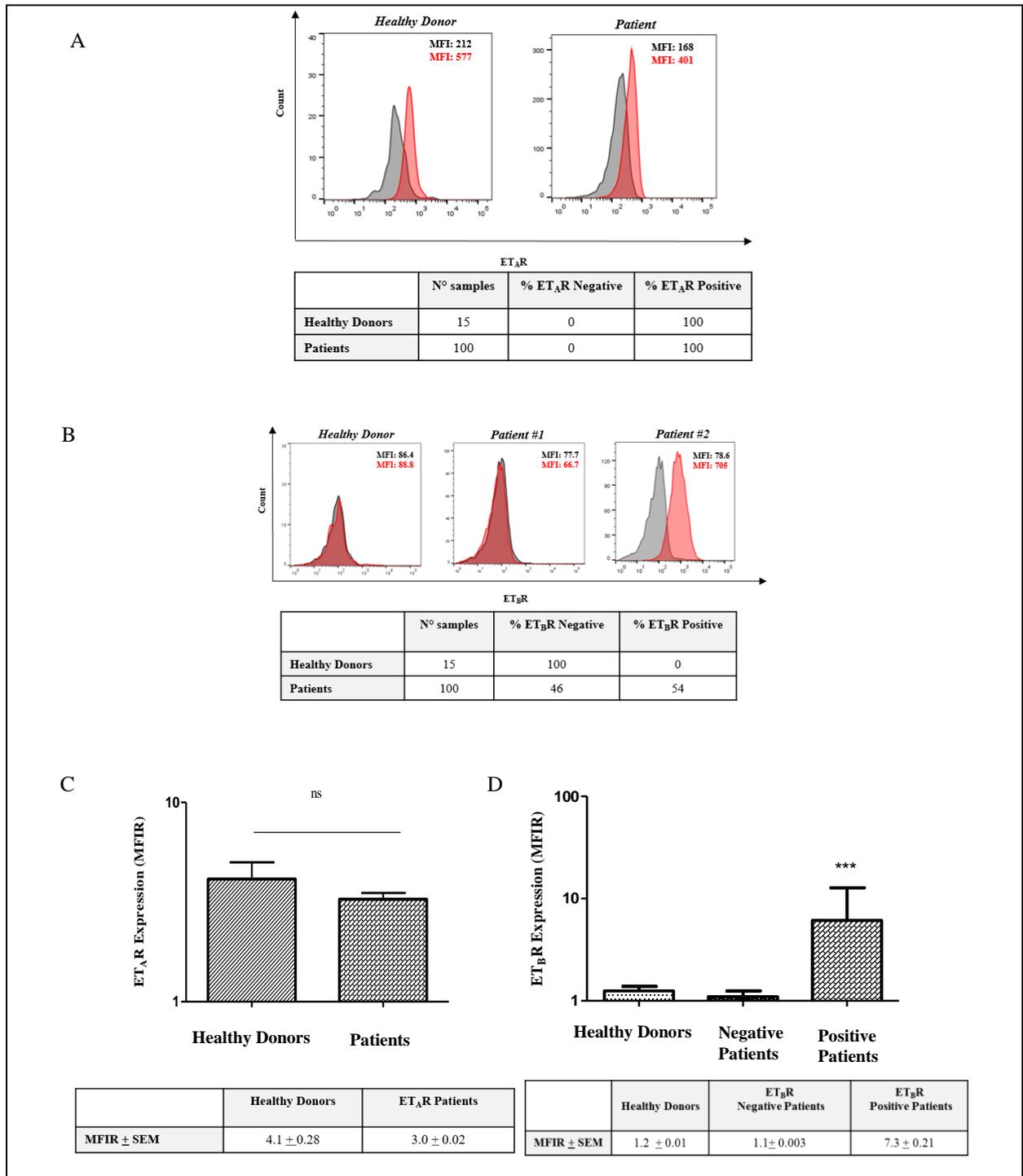
**Figure 13: Expression of ET<sub>A</sub>R by MM cell lines.** Analysis of ET<sub>A</sub>R mRNA and protein expression in U266, RPMI-8226, OPM-2, KMS-12-PE and LP-1 cell lines. (A): RT-qPCR. Data represent the mean value of ± SD of 3 independent experiments. (B): ET<sub>A</sub>R protein expression by Western blot analysis. MCF-7: breast cancer cell line used as positive control. (C): Flow cytometry analysis of plasma membrane ET<sub>A</sub>R. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining.



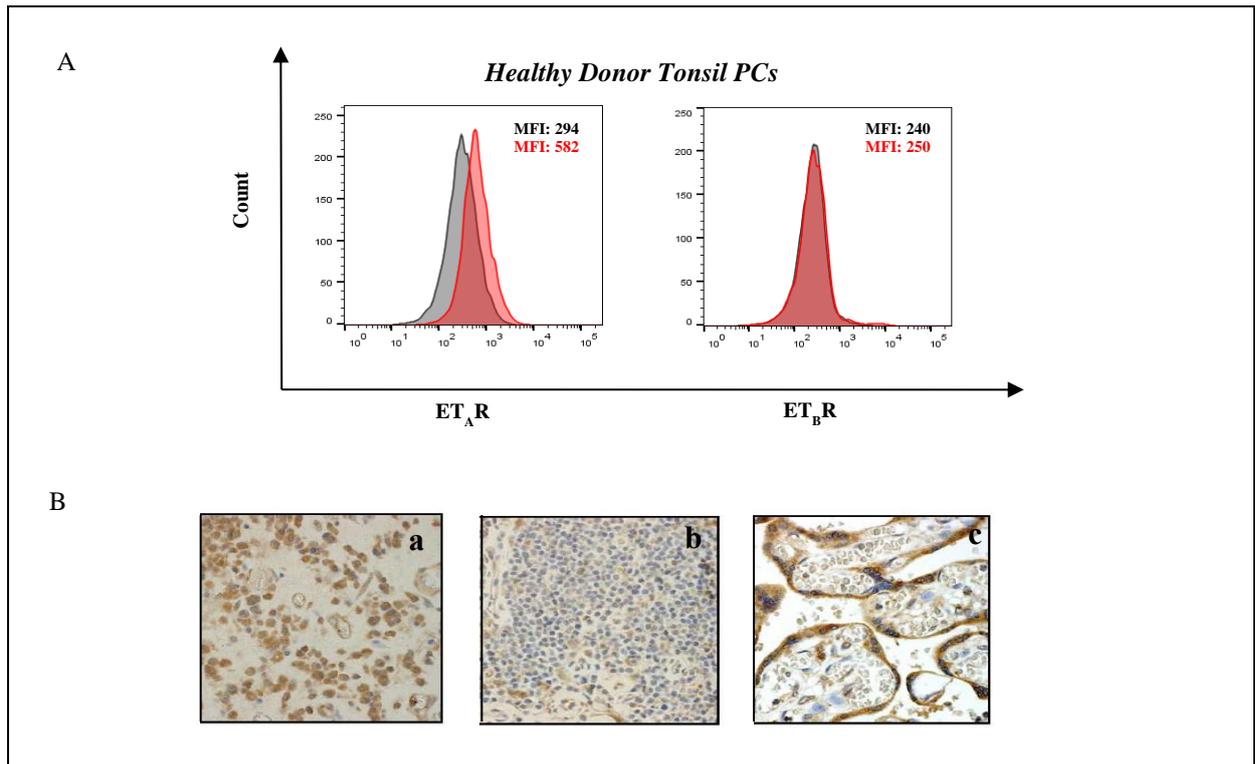
**Figure 14: Expression of ET<sub>B</sub>R by MM cell lines.** Analysis of ET<sub>B</sub>R mRNA and protein expression in U266, RPMI-8226, OPM-2, KMS-12-PE and LP-1 cell lines. (A): RT-qPCR. Data represent the mean value of  $\pm$  SD of 3 independent experiments. (B): ET<sub>B</sub>R protein expression by Western blot analysis. MCF-7: breast cancer cell line used as positive control. (C): Flow cytometry analysis of plasma membrane ET<sub>B</sub>R. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining.



**Figure 15: Five-color flow cytometry analysis of PCs from an HD and a MM patient: sequential gating strategy.** Panel A: representative HD; Panel B: representative MM patient. (a) Poligonal region 1 (R1) includes live cells on the basis of morphological parameter (FSC/SSC); (b) R2 excludes doublets from analysis; (c and d) R3 includes all CD38<sup>+</sup> cells, which are also CD138<sup>+</sup> (R4); (e) CD45 and CD56 in normal vs neoplastic PCs. CD45 is weak in normal PCs, but variably expressed (dim to bright: e1, e2, e3) by MM PCs. PCs from HDs are usually CD56<sup>-</sup>, while MM PCs express CD56 in the majority of cases. (f) PCs from HDs are usually CD19<sup>+</sup>, while malignant PCs are CD19<sup>-</sup>.



**Figure 16: Expression of ET-receptors by primary normal and neoplastic PCs.** Displayed are flow cytometric histograms depicting the relative fluorescence intensity of one representative case of 15 HDs and 100 MM patients stained with anti-ET<sub>A</sub>R (A) and anti-ET<sub>B</sub>R (B) Abs. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining. PCs were identified on the basis of the gating strategy reported in figure 15. Histograms (C and D) depict the ratio of the mean fluorescence intensity (MFIR) obtained between MFI of each sample and its control on PCs for ET<sub>A</sub>R (C) and ET<sub>B</sub>R (D). The cut off positivity for ET<sub>A</sub>R and ET<sub>B</sub>R (i.e.  $\geq 1.5$ ) was established based on the MFIR. Data reported in the tables below the histograms (C and D) are presented as the mean value of MFIR  $\pm$  SEM. \*\*\*  $P < 0.0001$ .

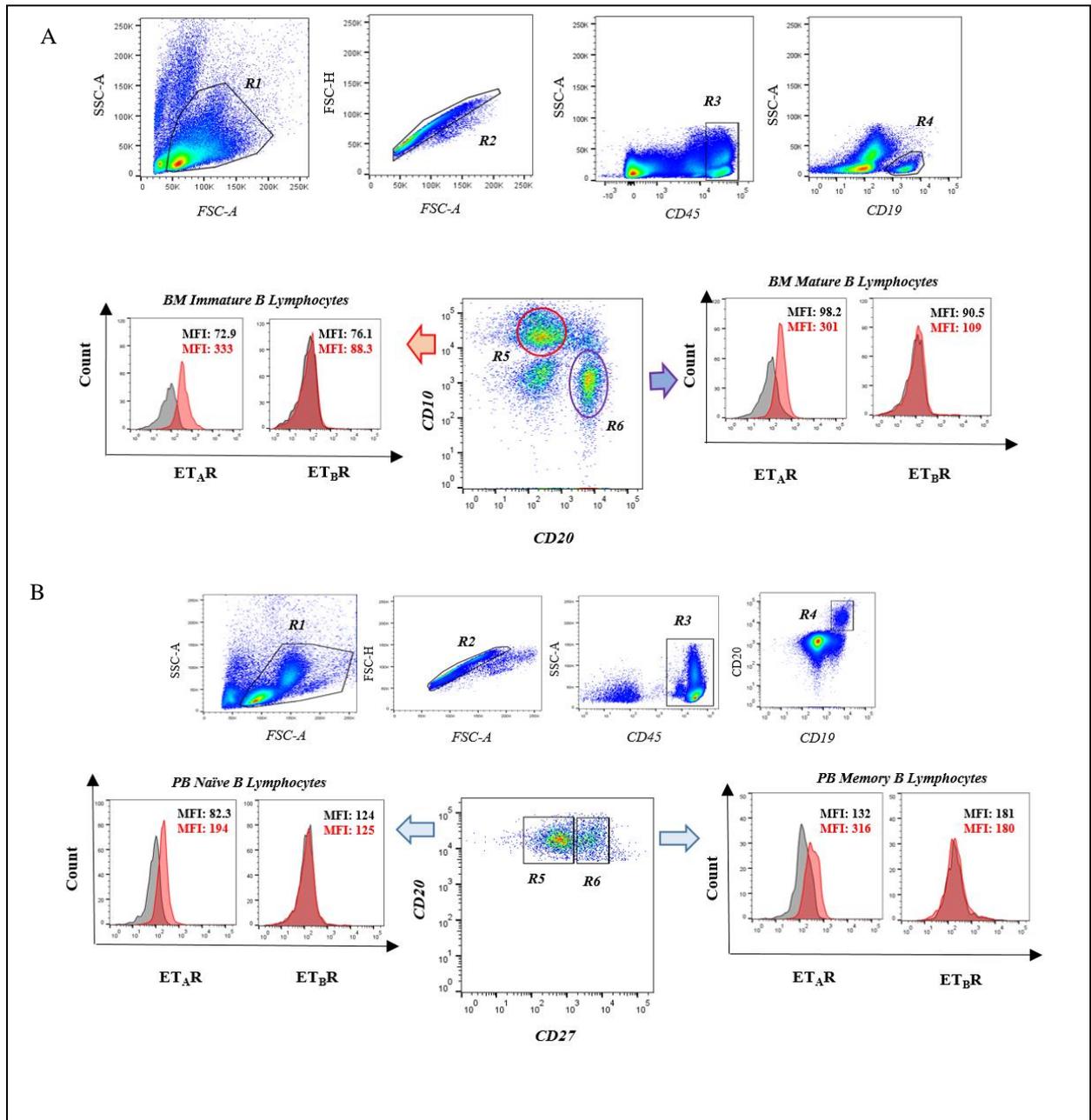


**Figure 17: Expression of ET-receptors by reactive PCs and immunohistochemistry of ET<sub>B</sub>R in MM biopsies.** (A) Displayed are flow cytometric histograms depicting the relative fluorescence intensity of one representative case out of 5 inflamed tonsils stained with anti-ET<sub>A</sub>R and anti-ET<sub>B</sub>R Abs. PCs were identified on the basis of the gating strategy reported in figure 15 A. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining. (B) ET<sub>B</sub>R immunohistochemical staining pattern in MM biopsies: a) representative positive patient; b) representative negative patient; c) positive control. a) and b) 200X and c) 400X magnification.

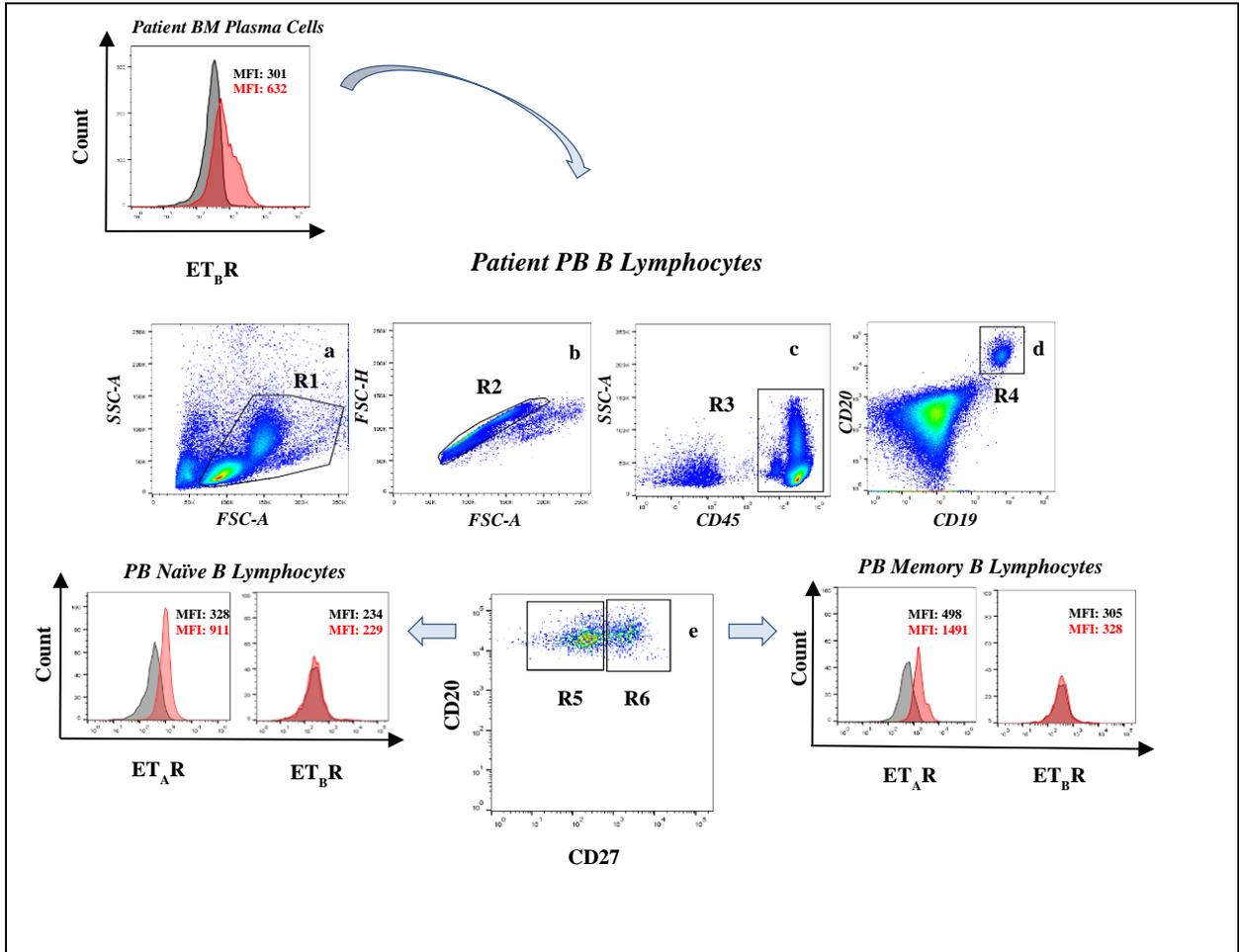
**Table 11: Demographic and clinical characteristics of ET<sub>B</sub>R<sup>-</sup> (n=46) vs ET<sub>B</sub>R<sup>+</sup> (n=54) patients.**

	ET <sub>B</sub> R <sup>-</sup>	ET <sub>B</sub> R <sup>+</sup>	P
<b>Age ≥65 Years</b>	30 (65%)	26 (48%)	0.087
<b>Male Gender</b>	23 (50%)	31 (57%)	0.457
<b>Stage*</b>			
<b>I</b>	12 (26%)	11 (20%)	0.711
<b>II</b>	9 (19.5%)	13 (24%)	
<b>III</b>	23 (50%)	30 (56%)	
<b>Classification</b>			
<b>Ig A</b>	7 (15%)	10 (18.5%)	0.502
<b>Ig G</b>	31 (67%)	31 (57%)	
<b>Light Chain</b>	5 (11%)	11 (20%)	
<b>Non Secretory</b>	3 (6.5%)	2 (3.7%)	
<b>Acute Renal Failure</b>	3 (6.5%)	7 (13%)	0.318
<b>Osteolytic Lesions</b>	32 (69.5%)	40 (74.1%)	0.881

\* 2 patients were unclassified



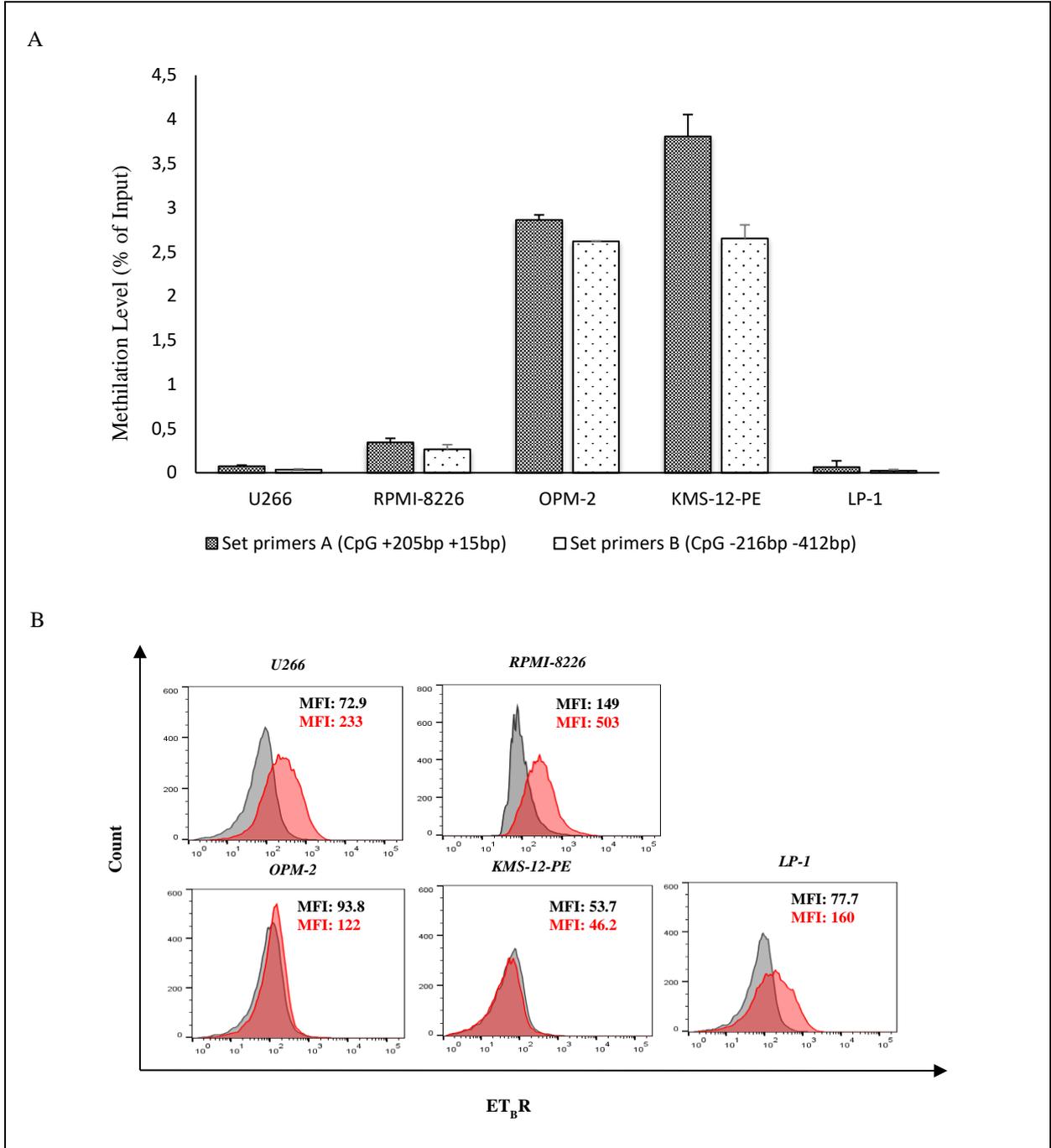
**Figure 18: ET<sub>A</sub>R and ET<sub>B</sub>R expression on HD PCs precursor.** Flow cytometry analysis of: (A) BM immature and mature B lymphocytes; (B) PB naïve and memory B lymphocytes, from a representative HD. In panels (A) and (B) polygonal region 1 (R1), includes live cells on the basis of morphological parameters (FSC/SSC), R2 excludes doublets from analysis and R3 includes CD45<sup>+</sup> cells; R4 includes CD19<sup>+</sup> B lymphocytes in (A) which are also CD20<sup>+</sup> in (B); circular regions 5 and 6 (R5 and R6) in (A) include BM immature (CD10<sup>+</sup> and CD20<sup>-</sup>) and mature (CD10<sup>-</sup> and CD20<sup>+</sup>) B lymphocytes, respectively; R5 and R6 in (B) include PB naïve (CD20<sup>+</sup> and CD27<sup>-</sup>) and memory (CD20<sup>+</sup> and CD27<sup>+</sup>) B cells, respectively. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining.



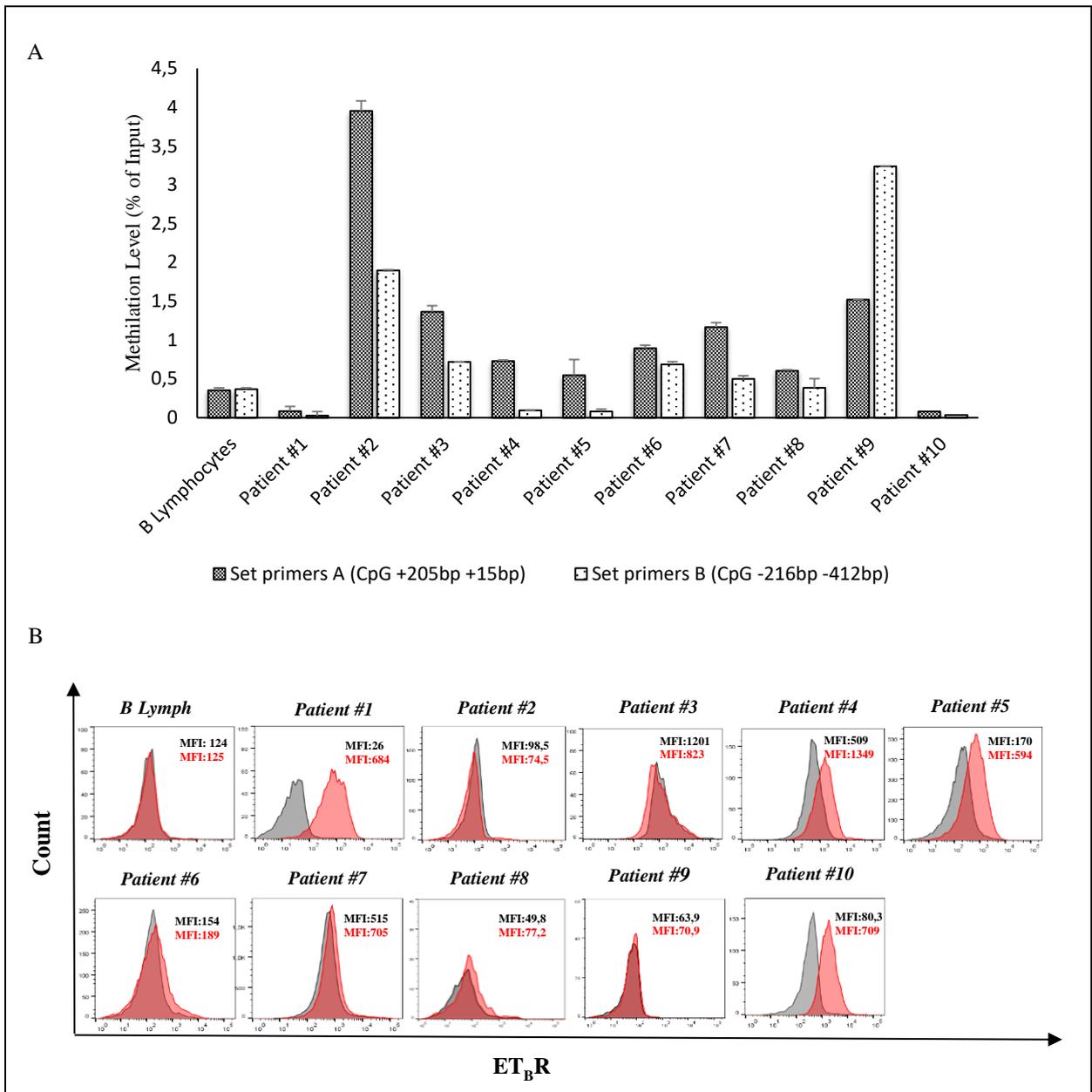
**Figure 19: ET<sub>A</sub>R and ET<sub>B</sub>R expression on PB B lymphocytes from ET<sub>B</sub>R-expressing MM patients.** Flow cytometry analysis of PB naïve and memory B lymphocytes from a representative ET<sub>B</sub>R-expressing MM patient. Polygonal region 1 (R1) in (a) includes live cells on the basis of morphological parameters (FSC/SSC); R2 in (b) excludes doublets from analysis; R3 in (c) includes CD45<sup>+</sup> cells; R4 in (d) includes CD19<sup>+</sup> and CD20<sup>+</sup> B lymphocytes; R5 and R6 in (e) include naïve (CD20<sup>+</sup> and CD27<sup>-</sup>) and memory (CD20<sup>+</sup> and CD27<sup>+</sup>) B cells, respectively. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining.

***Hypomethylated ET<sub>B</sub>R gene promoter locus is associated with aberrant expression of ET<sub>B</sub>R in malignant PCs***

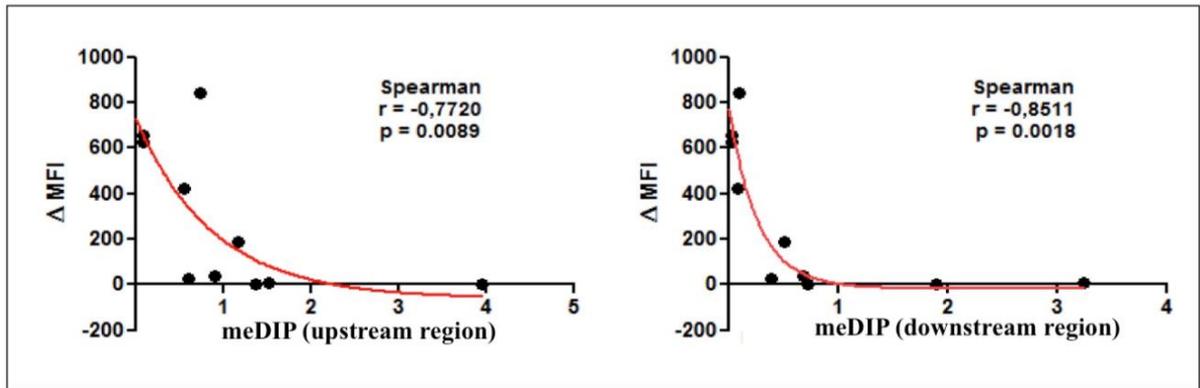
Previous studies have uncovered that the aberrantly increased expression of ET<sub>B</sub>R is associated with low methylation levels of CpG islands located at the ET<sub>B</sub>R gene promoter in melanoma and bladder cancer cells [151]. Therefore, we investigated whether ET<sub>B</sub>R expression in MM is regulated by epigenetic deregulation mechanisms. We measured the extent of methylation in a CpG island located in a highly CG-rich region of the ET<sub>B</sub>R locus, spanning from position -792 to +451 with respect to the classically defined ET<sub>B</sub>R TSS [244, 245]. Such CpG island was analyzed by using 2 different primer sets (i.e., A and B) amplifying a region upstream (from +205 bp to +15 bp) and downstream (from -216 bp to -412 bp) of TSS, respectively [245, 246]. Interestingly, we found that the two ET<sub>B</sub>R<sup>-</sup> MM cell lines (i.e., OPM-2 and KMS-12-PE) were highly methylated at the ET<sub>B</sub>R gene promoter regions, while MM cell lines displaying ET<sub>B</sub>R on the surface (i.e. U266, RPMI-8226 and LP-1) showed lower DNA methylation levels (Figure 20 A and B). To ascertain whether the same correspondence between ET<sub>B</sub>R surface expression and gene promoter methylation levels also occurs in primary MM PCs, we next analyzed CD138<sup>+</sup> PCs isolated from highly infiltrated (i.e. ≥60%) BM samples obtained from ET<sub>B</sub>R<sup>+</sup> (n=4), and ET<sub>B</sub>R<sup>-</sup> (n=6) MM patients. B lymphocytes obtained from PB samples of HDs (n=3) were also evaluated. As shown in figure 21, CD138<sup>+</sup> PCs with the highest expression of ET<sub>B</sub>R (patients #1, #4, #5 and #10) displayed the lowest methylation levels at the CpG island of the ET<sub>B</sub>R gene promoter. Conversely, CD138<sup>+</sup> PCs with no, or very low expression of ET<sub>B</sub>R (patients #2, #3, #6, #7, #8 and #9) displayed elevated DNA methylation levels. The inverse correlation between ET<sub>B</sub>R expression and DNA methylation in CD138<sup>+</sup> PCs is statistically significant and is more stringent for the downstream region (Spearman  $r = -0,8511$ ,  $p = 0.0018$ ) than for the upstream one of the CpG island (Spearman  $r = -0,7720$ ,  $p = 0.0089$ ) (Figure 22). Overall, data appear in agreement with an inhibitory role of DNA methylation in the control of ET<sub>B</sub>R expression. Data also indicate that, in a proportion of MM cases, malignant PCs express ET<sub>B</sub>R as a consequence of an epigenetic dysregulation occurring at the corresponding promoter region.



**Figure 20: DNA Methylation levels of the ET<sub>B</sub>R gene and surface ET<sub>B</sub>R expression in MM cell lines.** Methylation levels expressed as percent of methylation of ET<sub>B</sub>R promoter (A) and flow cytometry analysis of the corresponding surface ET<sub>B</sub>R expression (B) in MM cell lines. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining. Data in (A) represent the mean value  $\pm$  SD of 3 independent experiments, each performed in triplicate compared to the control.



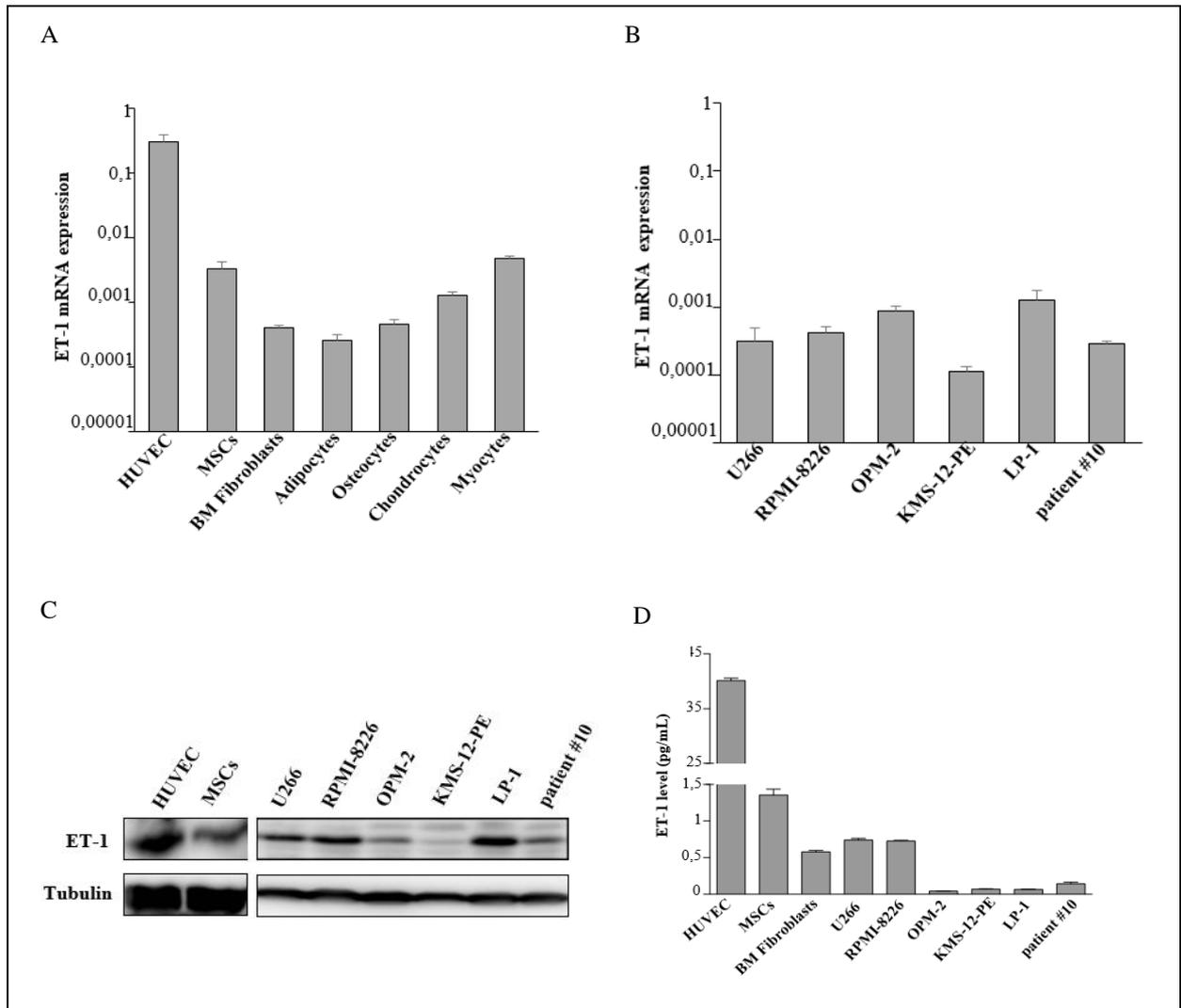
**Figure 21: DNA Methylation levels of the ET<sub>B</sub>R gene and surface ET<sub>B</sub>R expression in primary MM PCs.** Methylation levels expressed as percent of methylation of ET<sub>B</sub>R promoter (A) and flow cytometry analysis of the corresponding surface ET<sub>B</sub>R expression (B) in primary MM PCs. B lymphocytes obtained from PB samples of HDs (n=3) were also evaluated. Filled grey histogram: control (secondary antibody only); filled red histogram: specific staining. In (A) error bars represent standard errors calculated from triplicate qPCR reactions.



**Figure 22: Correlation between ET<sub>B</sub>R surface expression and methylation levels at the ET<sub>B</sub>R promoter.** Non parametric Spearman correlation between specific ET<sub>B</sub>R staining with respect to control (ΔMFI), and MeDIP levels at the ET<sub>B</sub>R gene, as measured in 2 regions amplified upstream (left) and downstream (right) with respect to the TSS. The Spearman correlation coefficient (r) and p-value are indicated.

### ***BM microenvironment cells and malignant PCs express and release ET-1***

BM microenvironment is normally composed of vascular ECs and stromal cells [30]. To establish whether the BM microenvironment may be a source of ET-1, we initially analyzed the expression of ET-1 mRNA, either in HUVEC (used as a surrogate of BM vascular ECs, i.e., positive control), or in BM stromal cells (i.e., MSCs, fibroblasts, and MSCs-derived adipocytes, osteocytes, chondrocytes, and myocytes), by RT-qPCR. Figure 23 A shows that all BM stromal cells express ET-1 mRNA, albeit at lower levels than HUVEC. Moreover, since the ET-1 axis acts also in an autocrine fashion in different cancer cells [176], we evaluated whether malignant PCs from MM patients or MM cell lines express ET-1 mRNA. As shown in Figure 23 B, primary MM PCs and MM cell lines were found to express ET-1 mRNA at levels comparable to BM stromal cells. Significantly, RT-qPCR data were also confirmed at the protein level, as shown by the Western blot and ELISA assay in Figure 23 C and D, demonstrating that both BM microenvironment cells (i.e., HUVEC, MSCs) and malignant PCs (primary and cell lines) produce and release ET-1. Collectively, these data indicate that both BM microenvironment cells and malignant PCs may represent ET-1 source, and that, in MM, the ET-1 axis may act in a paracrine and/or autocrine fashion.



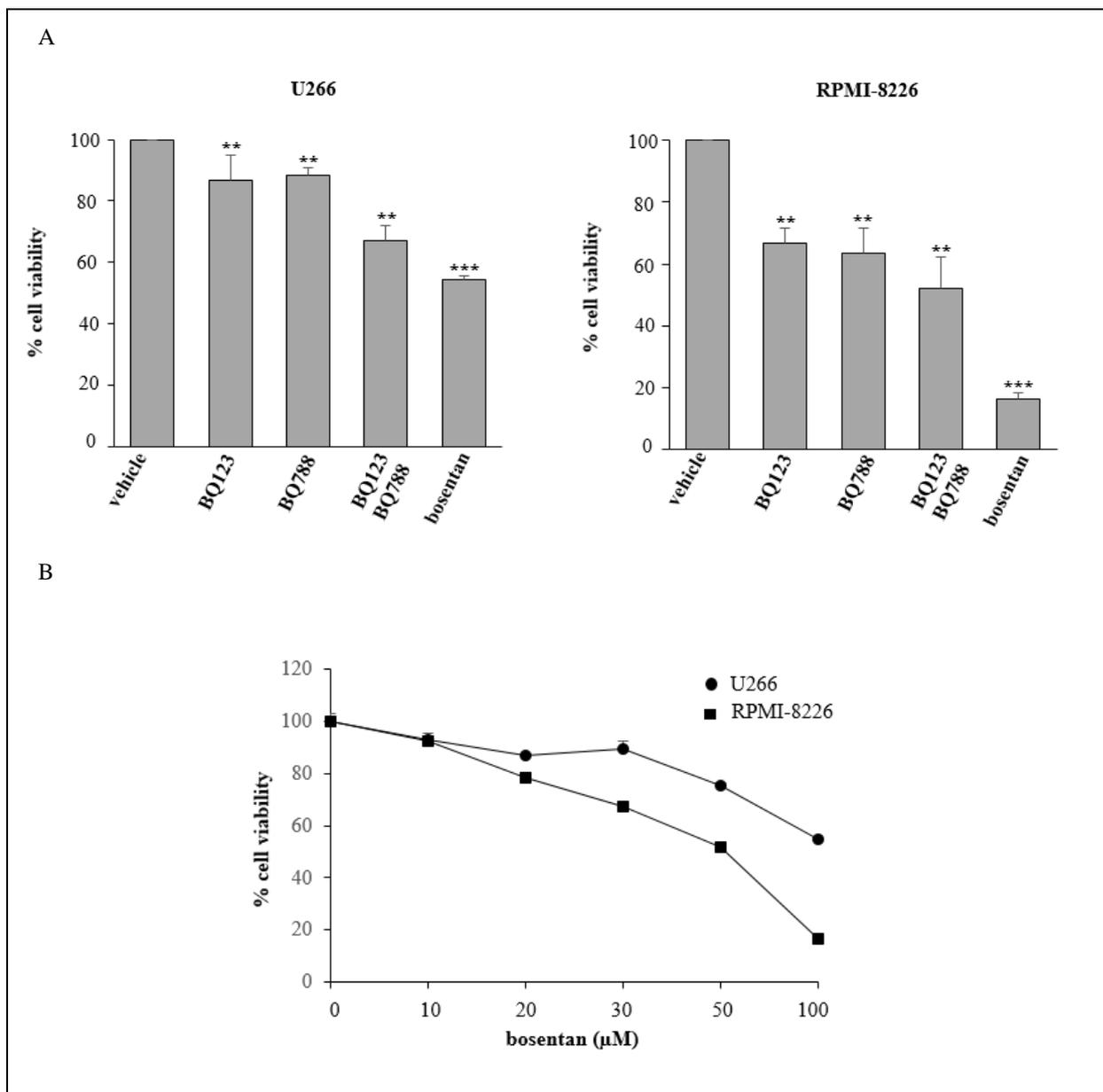
**Figure 23: BM stromal cells, MM cell lines and primary MM PCs express and produce ET-1.** RT-qPCR analysis of ET-1 mRNA expression normalized on GAPDH mRNA in: (A) BM stromal cells and (B) MM cell lines and primary MM PCs (representative case). Data represent the mean value  $\pm$  SD of 3 independent experiments. (C) Western blot analysis of ET-1 in BM stromal cells, MM cell lines and primary MM PCs (representative case). (D) ET-1 levels measured by ELISA in 48 h-supernatants from HUVEC, BM stromal cells, MM cell lines and primary MM PCs (representative case). Data represent mean value  $\pm$  SD of two independent experiments each performed in duplicate.

### ***ET-1 affects viability of MM cells in an autocrine fashion***

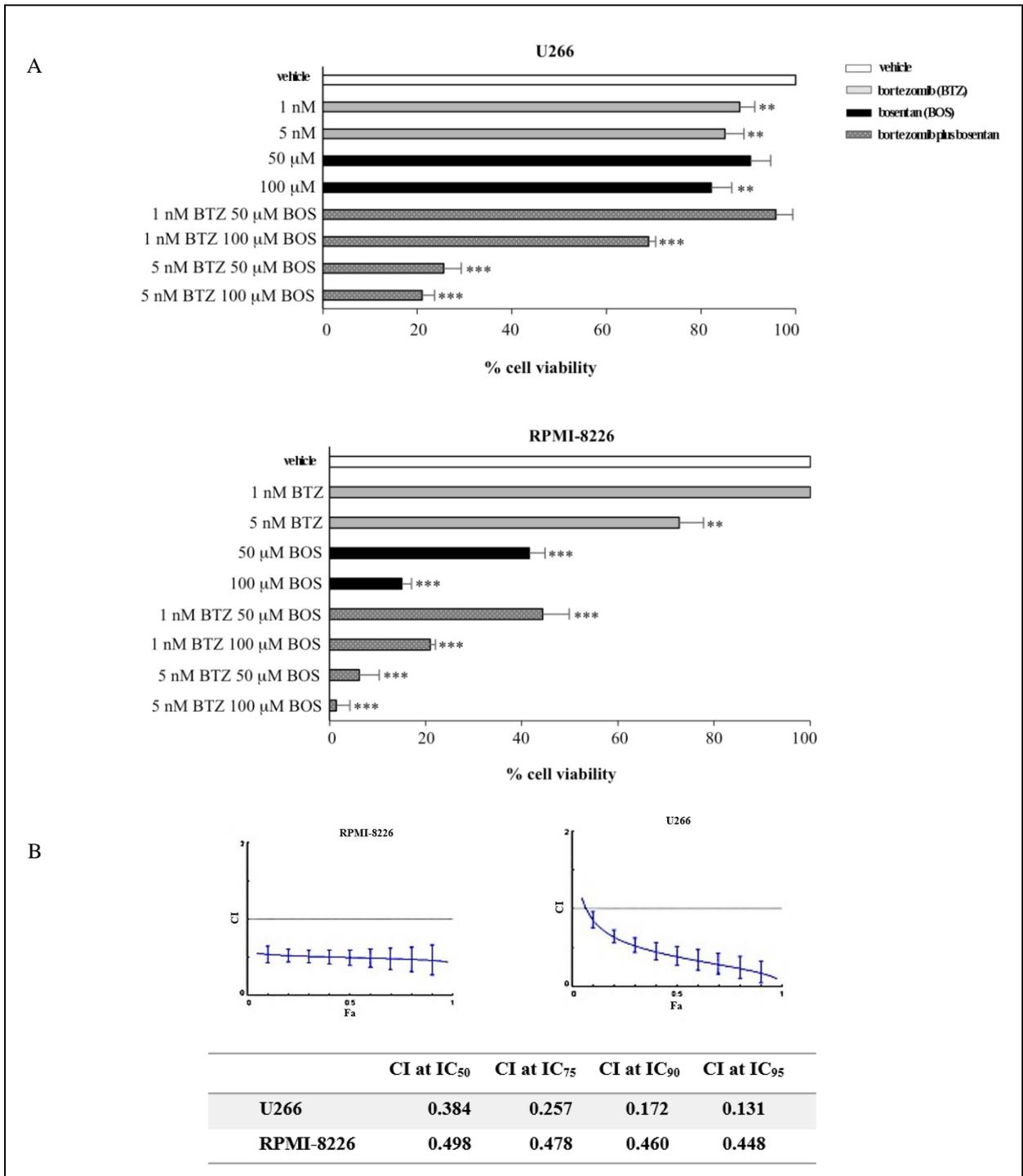
Next, we evaluated whether ET-1 could act as an autocrine growth factor in malignant PCs, using U266 and RPMI-8226 cell lines, which express both ET<sub>B</sub>R and ET<sub>A</sub>R. Given that these cells produce considerable levels of ET-1 (Figure 23 B and C), we hypothesized an autocrine role of the ET-1 axis in the same cells. To test such an hypothesis, we cultured cells in the presence of specific ET<sub>A</sub>R and ET<sub>B</sub>R antagonists, namely BQ123 and BQ788, respectively, alone and/or in combination. Consistent with our assumption, both BQ123 and BQ788, used alone and/or in combination, reduced significantly the viability of U266 and RPMI-8226 cell lines (Figure 24 A), demonstrating an autocrine role for the ET-1 axis in MM setting. To further demonstrate the autocrine role of ET-1 we tested bosentan, an orally active dual ET-receptor antagonist, which is currently licensed in Europe and USA for the treatment of pulmonary arterial hypertension [98], in MM cell lines, U266 and RPMI-8226. Bosentan reduced U266 and RPMI-8226 cell viability over a 48 h culture (Figure 24 A), being significantly more effective than BQ123 and BQ788 used in association (Figure 24 A). Notably, bosentan displayed a dose-dependent effect in both cell lines, and it exerted a stronger anti-proliferative effect in RPMI-8226 (50 mM IC<sub>50</sub>) than in U266 (50 mM IC<sub>75</sub>) cells (Figure 24 B).

### ***The dual ET-1 receptor antagonist, bosentan, synergizes with bortezomib to inhibit MM cells viability***

Given that ET-1 axis may act as an escape mechanism to treatment with the proteasome inhibitor bortezomib [174], currently approved as upfront treatment in MM [59], we next sought to verify whether bosentan could potentiate the effect of bortezomib. Although both cell lines were sensitive to 5 nM bortezomib, the combined exposition to 100 mM bosentan and 5 nM bortezomib, further impaired viability of U266 and, at higher levels, RPMI-8226 cell lines, exerting stronger effects than bosentan or bortezomib, used alone at the same concentration (Figure 25 A). Moreover, bosentan and bortezomib were found to have synergistic effects on cell viability based on CalcuSyn Software (Figure 25 B). Taken together, our results show that the ET-1 axis might represent a new therapeutic target in MM and that bosentan in combination with bortezomib-based therapy could be effective in enhancing cytotoxic effects in MM.



**Figure 24: BQ123, BQ788 and Bosentan affect viability of MM cell lines.** (A) U266 and RPMI-8226 cells were incubated in the presence of 10  $\mu$ M BQ123 and/or BQ788, and 100  $\mu$ M bosentan for 48 h. Cell viability was determined by the MTT assay. Each point represents the mean  $\pm$  SD of 5 independent experiments, each one performed in triplicate. \*\* $P < 0.005$  and \*\*\*  $P < 0.001$  vs control. (B) U266 and RPMI-8226 cell viability after a 48 h treatment with increasing concentrations of bosentan. Each point represents the mean  $\pm$  SD of 5 independent experiments, each one performed in triplicate.



**Figure 25: Bosentan and Bortezomib synergistically decrease viability of MM cell lines.** (A) U266 and RPMI-8226 cells were treated for 24 h with increasing concentrations of bosentan and bortezomib, used alone or in combination. Cell viability was determined by the MTT assay. Each point represents the mean  $\pm$  SD of 5 independent experiments, each one performed in triplicate. \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$  vs control. (B) Fa-CI plots displaying the interaction between bosentan and bortezomib in U266 and RPMI-8226 cells. Each point represents the mean  $\pm$  SD of 5 independent experiments, each one performed in triplicate. Table below the Fa-CI plots displays the combination indices (CI) in synergism experiments using bosentan and bortezomib in constant ratio.  $< 0.1$  = very strong synergism,  $0.1-0.3$  = strong synergism,  $0.3-0.7$  = synergism,  $0.7-0.85$  = moderate synergism,  $0.85-0.9$  = slight synergism.

***Bosentan exerts anti-proliferative effects in MM cells by inhibiting the MAPK/ERK and pro-survival pathways***

To evaluate the mechanisms underlying the anti-proliferative effects exerted by BQ123, BQ788 and bosentan, we examined the MAPK/ERK signaling pathway involved in ET-1-mediated protumorigenic functions [176]. We showed variations of p-p44/42 MAPK in U266 and especially in RPMI-8226 cells treated with 10  $\mu$ M BQ123 and BQ788, or 100  $\mu$ M bosentan for 48 h. We also evaluated the combinatory effects of bosentan and 5 nM bortezomib under the same experimental conditions. As shown by Western blotting in Figure 26 A, elevated basal levels of p-p44/42 MAPK were found in both cell lines under resting conditions. Bosentan reduced p-p44/42 MAPK levels in a substantial fashion in both cell lines, while BQ123 and BQ788 used in combination were more effective in RPMI-8226 but not in U266 cell line (Figure 26 A). Bosentan was also found to potentiate the bortezomib-induced reduction of p-p44/42 MAPK levels in U266 and RPMI-8226 cell lines (Figure 26 A). Finally, bosentan alone down-modulated Bcl-xL expression in RPMI-8226 cells, inducing in both cell lines a fully reduction of Bcl-xL when used in association with bortezomib (Figure 26 B). Taken together, our results confirm the potential role of bosentan alone and/or in combination with bortezomib, in the treatment of MM, further indicating its capacity to interfere with major signaling and anti-apoptotic pathways.



# *DISCUSSION*

Although a protumorigenic role of ET-1 was previously described in a number of solid tumors [205], as well as in B-cell chronic lymphocytic leukemia [173], little is known on an eventual existence of the ET-axis in MM [174].

In this study, we report that primary MM PCs express ET-1 receptors, as well as that MM PCs themselves and BM microenvironment cells produce ET-1. Interestingly, we found that ET-1 is released in significant amounts not only by ECs which are usually overrepresented in MM, but also by other cellular components of BM microenvironment, including MSCs. With regard to ET-1 receptors, surface ET<sub>B</sub>R was detectable only in a proportion of cases, being evidenced in 54% of MM BM samples and in 60% of MM cell lines examined, while ET<sub>A</sub>R was constitutively expressed by all malignant PCs. Based on these data, and on the lack of ET<sub>B</sub>R, but not ET<sub>A</sub>R, surface receptor expression in normal long-lived BM or tonsil PCs and immature and mature B lymphocytes, we interpreted as aberrant the expression of ET<sub>B</sub>R at least in a number of MM cases. Interestingly, previous studies had demonstrated that epigenetic dysregulation is an important contributor to MM pathogenesis, with global DNA methylation changing significantly during disease progression [247], as a consequence of lesions in gene encoding histone methyltransferases and DNA methylation modifiers [248]. Given that up-regulation of ET<sub>B</sub>R surface expression is due to an altered methylation status of the promoter gene [151], we hypothesized that the expression of ET<sub>B</sub>R could be regulated by epigenetic mechanisms also in MM. Consistent with this hypothesis, we demonstrated that the ET<sub>B</sub>R promoter locus of ET<sub>B</sub>R<sup>+</sup> MM samples or cell lines display a lower methylation rate than ET<sub>B</sub>R<sup>-</sup> primary MM samples, MM cell lines and B lymphocytes, suggesting a negative prognostic role of ET<sub>B</sub>R as MM biomarker.

Data concerning the function of ET<sub>B</sub>R seem relatively controversial. Indeed, ET<sub>B</sub>R has been reported to behave as either a tumor suppressor [145, 146] or a protumorigenic [147, 148] gene, depending on its actual function in the corresponding normal tissues. Accordingly, ET<sub>B</sub>R was found to be down-regulated or absent in tumors of epithelial origin, whose normal counterparts express ET<sub>B</sub>R with anti-proliferative and regulatory functions [149], whereas it appears overexpressed in malignancies (e.g. melanoma) whose normal counterparts express ET<sub>B</sub>R as a mediator of pro-survival signals [150]. Our *in vitro* findings demonstrate that ET<sub>B</sub>R may exert a protumorigenic function in MM, as it delivers survival signals and synergizes with ET<sub>A</sub>R. In fact, MM cell lines showed a decreased viability when exposed to the ET<sub>B</sub>R antagonist BQ788, concordantly with data previously obtained in melanoma [150, 249], glioma [227] and

glioblastoma [161]. A recent study reports the role of ET<sub>B</sub>R in two MM cell lines expressing ET-1 [174]. Interestingly, those authors also found that ET<sub>B</sub>R mediates pro-survival signals since the exposition of MM cell lines to the ET<sub>B</sub>R antagonist BQ788 decreased cells viability and reduced the ET-1 mediated resistance to bortezomib [174]. Based on these findings, and on our current data regarding the expression of ET<sub>B</sub>R by PCs from a proportion of patient BM samples, it would be conceivable to anticipate a negative prognostic significance for ET<sub>B</sub>R in MM. Unfortunately we couldn't observe a significant difference in terms of TTT or OS between ET<sub>B</sub>R<sup>+</sup> and ET<sub>B</sub>R<sup>-</sup> patients (not shown). However the cohort that we analyzed was therapeutically treated in a quite heterogeneously manner (Table 10), thus making it difficult to establish whether ET<sub>B</sub>R<sup>+</sup> patients had a worse outcome (eventually due to an increased resistance of their disease to bortezomib) than those expressing ET<sub>A</sub>R only. Therefore, the prognostic role of ET<sub>B</sub>R will be clarified only by prospective studies analyzing larger cohorts of homogeneously treated patients. Interestingly, while in melanoma the extent of ET<sub>B</sub>R expression was shown to parallel with disease aggressiveness [250], we observed an elevated ET<sub>B</sub>R expression even in initial stages of MM, therefore allowing us to speculate that a very early epigenetic event may occur in malignant PCs.

The expression of either ET<sub>A</sub>R only, or ET<sub>A</sub>R and ET<sub>B</sub>R, by MM PCs might have immediate therapeutic implications, given that ET-1 receptor antagonists are currently used in clinics, even though under non-hematological settings [235]. In fact, we report that the dual ET- receptor antagonist, bosentan, was effective in reducing viability of MM cell lines *in vitro*, even showing synergistic effects with bortezomib. As a consequence, besides being highly effective, the combination of the two drugs might reduce the toxic effects of bortezomib that usually occur *in vivo* [58]. Interestingly, similarly to other drugs administered in MM, bosentan is also expected to act on BM microenvironment, mostly by targeting ET<sub>B</sub>R expressing ECs [251], and reducing angiogenesis.

Our data also indirectly indicate that in U266 and RPMI-8226 cell lines the MAPK/ERK pathway is strongly involved in mediating the autocrine pro-survival signals exerted by ET-1 through ET<sub>A</sub>R and ET<sub>B</sub>R. Accordingly, BQ123 and BQ788 in combination and, mostly, bosentan, were found to effectively down modulate p-p44/42 MAPK. Besides the autocrine function of ET-1 in MM, observations made in ECs and BM stromal cells also suggest an *in vivo* paracrine role for the ET-1 axis, especially in the MM stroma-dependent phase [41]. Further information on the paracrine role of ET-1 will derive from *in vitro* experiments targeted

at evaluating the ET-1-mediated relationship between BM microenvironment cells and primary MM PCs.

In summary, our study uncovers an autocrine and paracrine ET-1-mediated mechanism of MM PCs survival together with the potential role of the ET-1 axis as therapeutic target. Further pre-clinical and clinical studies are awaited in order to definitively establish the effectiveness and safety profile of ET-receptor antagonists in MM.

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