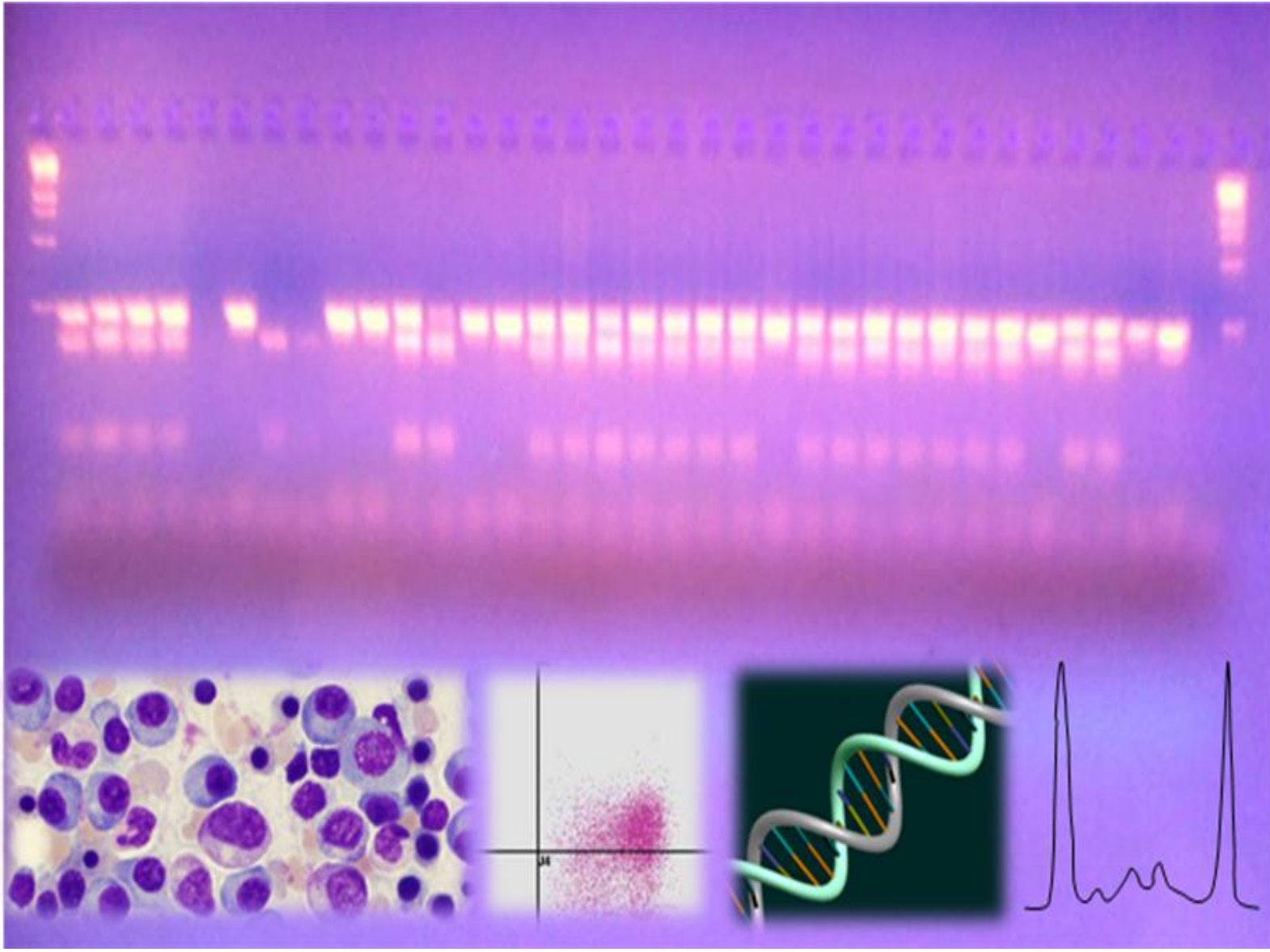

Maria João Nunes Vicente Mexia Leitão

Identification of predictive molecular markers for disease progression and response to therapy in Monoclonal Gammopathies

Mestrado em Investigação Biomédica

Coimbra

2011

· U C ·

UNIVERSIDADE DE COIMBRA

Maria João Nunes Vicente Mexia Leitão

Identification of predictive molecular markers for disease progression and response to therapy in Monoclonal Gammopathies

Dissertation presented to obtain the Masters Degree in Biomedical Research at Faculty of Medicine in University of Coimbra, Portugal
Work performed under supervision of Dr. Ana Bela Sarmento Ribeiro, and co-supervision of Dr. Ana Cristina Gonçalves,

UNIVERSIDADE DE COIMBRA

Agradecimentos

À Professora Doutora Ana Bela Sarmento quero expressar os meus sinceros agradecimentos pela orientação científica deste trabalho e pela disponibilidade com que me recebeu.

À Drª Cristina Gonçalves pela orientação e ajuda ao longo do trabalho em laboratório, pela disponibilidade e pelos conhecimentos transmitidos.

À Sílvia Neves pelo apoio incondicional, pela partilha de conhecimentos, pela amizade e companhia fundamentais.

Às colegas Raquel Alves e Ana Raquel Fonseca pela ajuda no trabalho prático durante o ano.

À Drª Vera Alves pelo auxílio na elaboração da técnica de citometria de fluxo.

À minha colega e camarada Ana Raposo pelo companheirismo no 1º ano de Mestrado, que não esqueço; à Cátia Costa, Maria Madeira, Daniela Silva e Deolinda Santinha pela amizade e companhia nestes últimos dois anos, pelos cafés sempre a correr e pela partilha de experiências.

Às minhas colegas de curso e de Mestrado e grandes amigas de tantos anos, Ana Margarida Soares e Alexandra Martins, pela ajuda e companhia em dias e noites de trabalho e não só, pela partilha de conhecimentos mas sobretudo pela camaradagem em caminharmos sempre juntas nos últimos seis anos. Que nos mantenhamos sempre unidas. Obrigada por me aturarem, eu até conseguiria sem vocês mas não era a mesma coisa!

À Isabel Ferreira, a quem admiro e agradeço profundamente o apoio e a amizade incondicional ao longo deste ano. Muito obrigada por tudo, Belita.

Ao restante grupo do CNC em especial à Ana Rufino, Joana Liberal, Vera, Mónica, Bruno e João pelas pequenas ajudas aqui e ali, pela companhia e pelas brincadeiras.

Ao Professor Paulo Pereira, ao Doutor Henrique Girão pelo apoio e interesse, ao Steve e à Doutora Carla Marques pelo interesse e disponibilidade neste ano.

Ao Professor Doutor Francisco Ambrósio, à Doutora Raquel Santiago e à Filipa Baptista pela simpatia e gentileza que tiveram comigo.

To Professor Sukalyan Chatterjee , for his friendship and support in difficult times and for sharing his beliefs and some of his great knowledge not only in Science but also in Art, Literature and Life, in such a generous way I could never forget. Thank you so much.

À minha querida amiga Mariana Ribeiro pelo seu incentivo e amizade de tantos anos, pelo brilhante exemplo que é para mim e por nunca me perder de vista mesmo quando estamos longe e o tempo não chega.

Ao Pedro Gaspar, pela ajuda técnica, paciência e amizade.

A todos os meus restantes Amigos e Amigas, por tudo. Um agradecimento especial ao João Casalta Lopes (Jedu) pelo carinho e pelo conforto de o saber sempre por perto.

À minha amiga Fernanda, que me acompanha desde que me lembro de existir, e que tão bem me conhece. Pela paciência, pelas conversas, desabafos, comidinhas deliciosas e pela sua dedicação e amizade que têm para mim um valor inestimável.

Ao meu Pai e ao meu irmão, obrigada.

Aos 'Nunes', aos 'Vicentes' e aos 'Filipes', pelo conforto e pelo apoio. Aos meus primos e primas, Tios e Tias em especial à Marta, à Bia, à Joaninha e à Inês, Tio Pedro e Tia São, obrigada pelo carinho e pelo interesse. Aos meus primos-sobrinhos pequeninos, que me ajudam sempre a rir à gargalhada, a brincar como gente pequena e a não esquecer o que realmente importa.

À minha Mãe, Joana, a quem vezes sem conta calham a má-disposição dos piores dias, a teimosia e os problemas. Obrigada pelo amor e apoio sem limites que sempre me dedicaste. Pela força, pelos valores e pelo alento que me transmitiste durante todos estes anos, apesar das adversidades. Por estares sempre comigo e por seres a melhor amiga que se pode desejar. Sem ti teria sido impossível atingir este e tantos outros objectivos. Obrigada.

Index

Abbreviations

ABC- ATP-binding cassette

ATP- adenosine Triphosphate

BM- bone marrow

CD- cluster of differentiation

CFU- colony forming unit

CSC- cancer stem cell

CSF- colony stimulating factor

CYP- cytochrome P450

DMSO- dimethyl sulfoxide

DNA- deoxyribonucleic acid

DNTPs- deoxyribonucleotide triphosphate

FBS- fetal bovine serum

FC- flow cytometry

FDA- Food and Drug Administration

FISH- fluorescence in situ hybridization

HCSC- hematopoietic cancer stem cell

HMCL- human MM cell lines

HRD- hyperdyploid

HSC- hematopoietic stem cell

IC50- half maximal inhibitory concentration

Ig- immunoglobulin

IGF- insulin like growth factor

IkB- inhibitor kappa B

IL- interleukin

IMM- indolent (asymptomatic) Multiple Myeloma

IMWG- International Myeloma Working Group

MAPK- mitogen-activated protein kinases

MDR- multiple drug resistance protein (Pgp)

MG- Monoclonal Gammopathy

MGUS- Monoclonal Gammopathy of Undetermined Significance

MM- Multiple Myeloma

MRP- multidrug resistance-related protein

NF-kB- nuclear factor kappa B

NHRD- non-hyperdyploid

PB- peripheral blood

PBs- plasmablasts

PCL- plasma cell leukemia

PCR- Polymerase Chain Reaction

PCs- plasma cells

P-gp- glycoprotein P

RBC- red blood cells

RFLP- Restriction Fragment Length Polymorphism

RNA- Ribonucleic acid

sFLC- serum free light chain

SMM- Smoldering Multiple Myeloma

SNP- single nucleotide polymorphism

TGF- tumor growth factor

TNF- tumor necrosis factor

Ub- Ubiquitin

UC- ubiquitin conjugates

UPS- Ubiquitin Proteasome System

VEGF- vascular endothelial growth factor

Resumo

O Mieloma Múltiplo (MM) é uma neoplasia hematológica incurável caracterizada por uma proliferação clonal de plasmócitos (PCs) no microambiente da medula óssea (MO). A doença evolui frequentemente a partir de uma Gamapatia Monoclonal de Significado Indeterminado (MGUS) que progride para Mieloma Múltiplo Assintomático/Indolente (MMI) e finalmente para Mieloma Múltiplo sintomático. Alterações genéticas/epigenéticas que ocorrem nos plasmócitos desempenham um papel importante na patogenia do Mieloma e influenciam a resposta à terapia. Não existem marcadores biológicos fiáveis que permitam diferenciar plasmócitos e prever a progressão dentro dos vários estádios da doença (MGUS, MMI e MM). Apesar de alguns agentes terapêuticos como a Talidomida, a Lenalidomida e o Bortezomib aumentarem a taxa de sobrevivência no MM, um terço dos doentes desenvolve resistência à terapêutica.

Com este estudo pretendeu-se avaliar alguns mecanismos moleculares envolvidos na predisposição de doentes com Gamapatia Monoclonal e na transição de MGUS para MM que possam contribuir para a sensibilidade ou resistência à terapia, nomeadamente com inibidores do proteossoma. Em particular, pretendeu-se compreender melhor o papel dos polimorfismos *C3435T* e *T673C* dos genes MDR1 e CYP3A4 respectivamente assim como dos níveis de expressão das proteínas transportadoras MDR1 e MRP, dos conjugados de ubiquitina e do NF-kB. Utilizaram-se amostras de sangue e MO de 51 doentes com Gamapatia Monoclonal (24 doentes com MGUS, 4 com Mieloma Indolente e 23 com Mieloma Múltiplo Sintomático). Como controlos, foram utilizados 51 indivíduos saudáveis e 9 controlos não-neoplásicos. Procedeu-se à análise genotípica através da técnica de PCR-RFLP utilizando as enzimas de restrição *MBOI* (MDR1) e *Alw26I* (CYP3A4). Para além disso, realizou-se o estudo de citometria de fluxo nas amostras de MO dos doentes e avaliou-se a percentagem de células que expressam as proteínas Pgp/MDR1, MRP, UC e NF-kB,

assim como os seus níveis de expressão. Os plasmócitos malignos foram diferenciados dos normais através da expressão de CD138 e CD19.

Na análise genotípica, observou-se que o genótipo CC poderá predispor ao desenvolvimento da doença já que se revelou muito mais frequente nos doentes do que nos controlos saudáveis (57% *vs* 18%, respectivamente), apresentando um risco cerca de três vezes superior, face aos genótipos CT e TT (odds ratio = 2,756; CI: 1,070-7,145; p= 0,0378). Nos estudos de citometria de fluxo, observou-se um aumento de plasmócitos totais nos doentes relativamente aos controlos não-neoplásicos e a percentagem de plasmócitos malignos mais elevada nos doentes com MM em relação aos restantes sub-grupos. Em geral, os plasmócitos normais dos doentes apresentavam maiores níveis de expressão em todas as proteínas em relação aos controlos mas também em relação aos plasmócitos neoplásicos dos doentes, excepto para a MDR1. Nos doentes com MM verificou-se uma diminuição na percentagem de células a expressar e uma expressão mais fraca de transportadores, comparando com os doentes com MGUS. No entanto, nos doentes a nível geral os plasmócitos normais apresentaram níveis mais altos de expressão de Pgp e MRP em relação aos malignos. Finalmente, tendo em conta o *follow-up* dos doentes, verificou-se que um dos doentes com melhor resposta à terapia apresentava o genótipo TT, apesar de ter níveis de expressão de MDR1 elevados.

Os resultados confirmam o aumento de plasmócitos malignos no MM e sugerem que durante a progressão de MGUS para MM ocorrem transformações nos transportadores da família ABC (proteínas MDR1 e MRP), NF-kB e conjugados de ubiquitina que poderão influenciar o comportamento dos plasmócitos neoplásicos iniciais. Por outro lado, os polimorfismos no gene MDR1 podem ser determinantes no risco para desenvolver a neoplasia e influenciar a sua evolução, constituindo importantes marcadores no *follow-up*, avaliação de prognóstico e na previsão de resposta à terapia.

Abstract

Multiple myeloma (MM) is an incurable neoplastic plasma-cell disorder that is characterized by clonal proliferation of malignant plasma cells (PCs) in the bone marrow microenvironment. It is thought to evolve most commonly from a monoclonal gammopathy of undetermined significance (usually known as MGUS) that progress to smoldering (asymptomatic) myeloma (SMM) and finally to symptomatic myeloma (MM). Several genetic/epigenetic abnormalities occurring in tumor plasma cells play a major role in the pathogenesis of myeloma and may influence the response to therapy. Reliable biological markers to differentiate plasma cells of MGUS, SMM or MM, that could also predict the progression are currently lacking. Despite the availability of new therapeutic agents such as thalidomide, lenalidomide and bortezomib, that have changed the management of MM and extended overall survival, nearly a third of the patients still develop resistance to chemotherapy.

With this study we wanted to evaluate some molecular mechanisms involved in predisposition of MG and in the transition from MGUS to Multiple Myeloma that could also contribute to sensitivity and/or resistance to therapy, namely to proteasome inhibitors. In particular we tried to better understand the role of MDR1 *(C3435T)* and CYP3A4 *(T673C)* polymorphisms, and the expression levels of ABC proteins (MDR1 and MRP), ubiquitin conjugates and of the transcription factor NF-kB.

To attain these objectives we have used 51 patient samples, 24 diagnosed with MGUS, 4 with IMM (indolent/asymptomatic) and 23 with MM. As controls we have studied 51 healthy individuals and 9 non-neoplastic controls. Genotypic analysis was carried out by PCR-RFLP using the restriction enzymes *MBOI* (MDR1) and *Alw26I* (CYP3A4). Furthermore, we carried out a flow cytometry study on bone marrow plasma cells (PCs) to evaluate cell percentage and membrane levels of Pgp/MDR1, MRP1; ubiquitin conjugates (UC) and NF-kB. Malignant plasma cells were distinguished from normal ones based on CD19 and CD138 expression.

In genotyping analysis, we observed that CC genotype might predispose to malignancy since it is much more frequent in patients comparing with healthy controls (57% *vs* 18%, respectively) which may confer an increased risk of Monoclonal Gammopathy (MG) facing the other genotypes (CT and TT) (odds ratio = 2,756; CI: 1,070-7,145; p= 0,0378).

In flow cytometry studies, we observed that MG patients had increased number of total PCs compared to non-neoplastic controls and within sub-groups of disease, MM patients presented the higher percentage of neoplastic PCs. In general, normal population of PCs in MG patients had higher expression of all proteins (MDR1, MRP, UC and NF-kB) compared to non-neoplastic controls but, taking into account normal and neoplastic PCs from patients, the latter had lower expression of proteins, except for MDR1. Concerning neoplastic PCs, we observed in MM a decrease in the percentage of cells and weaker general expression of efflux transporters comparing with MGUS but in all MG patients benign PC population showed higher Pgp and MRP expression levels in relation with neoplastic PCs.

Finally, we observed that concerning patients' follow-up, a patient presenting TT genotype had a better response to therapy comparing to other patients' with different genotypes, even though it showed higher levels of MDR1 expression.

Our data confirm the highest content of malignant PCs in MM and suggest that during progression from MGUS to MM, changes in ATP-binding cassette transporters, NF-kB and UC occurs which may influence initial neoplastic PCs behavior. On the other hand, MDR1 polymorphisms may determine the risk to develop these plasma neoplasias and influence disease evolution, being valuable markers in patients' follow-up, evaluation of prognostic and prediction of response to therapy.

Part I: Introduction

1. Hematopoiesis

In hematopoiesis, the various types of blood cells are all generated from a single type hematopoietic pluripotent stem cell (HSC) in bone marrow (BM), which gives rise to separate cell lineages, the more-restricted myeloid and lymphoid progenitor cells (Figure 1) (Lodish, 2003).The hematopoietic system, therefore, works as a hierarchy of cells. Pluripotent stem cells are rare, perhaps 1 in every 20 million nucleated cells in BM. In general, HSCs is $CD34^{+}/CD38^{-}$ on immunological testing and morphologically resembles a small or medium-sized lymphocyte, although the exact phenotype of pluripotent HSC is unknown.

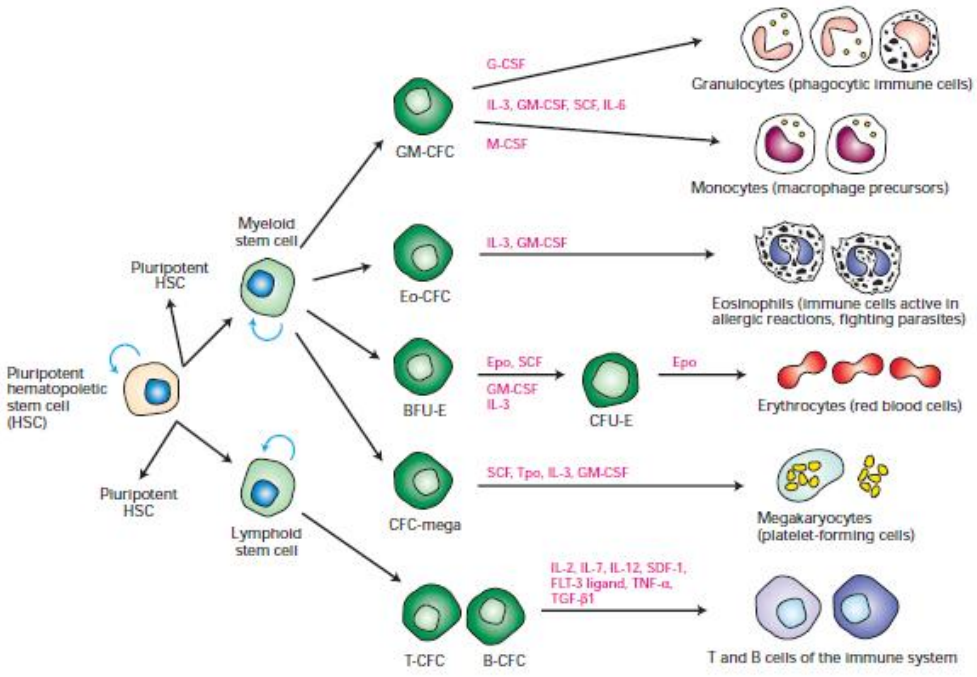

Figure 1. Growth factors/cytokine-induced proliferation, commitment and differentiation of blood cells. Cytokines/growth factors that support the process are indicated (red labels). GM = granulocyte macrophage; Eo = eosinophil; E = erythrocyte; mega = megakaryocyte; T = T-cell; B = B-cell; CFU = colony-forming unit; CSF = colony-stimulating factor; IL= interleukin; SCF = stem cell factor; Epo = erythropoietin; Tpo= thrombopoietin; TNF = tumor necrosis factor; TGF = transforming growth factor; SDF= stromal cell–derived factor; FLT-3 ligand = ligand for fms-like tyrosine kinase receptor 3 [*Adapted from M. Socolovsky et al.,1998*].

Committed progenitor cells they give rise to are irreversibly ancestors of only one or a few blood cell types and divide rapidly but only a limited number of times. There is considerable amplification in the system: one stem cell is capable of producing about 10^6 mature blood cells after 20 cell divisions. At the end of it, they develop into terminally differentiated cells, which usually divide no further and die after several days or weeks (Lodish, 2003).

BM is also the primary site of origin of lymphocytes (which differentiate from a common lymphoid precursor). The stem cell has the capability for self-renewal so that marrow cellularity remains constant in a normal healthy steady state (Hoffbrand *et al*., 2006).

BM microenvironment is crucial for the maintenance of HSC as well as for growth and differentiation of blood components. Adipocytes, fibroblasts, endothelial cells and macrophages are the major contributors for BM microenvironment balance, as they regulate growth factors secretion, later reaching the blood stream through micro vessels. These factors are proteins usually called colony-stimulating factors (CSFs). Some of them circulate in the blood and act as hormones, while others act in the BM either as secreted local mediators (cytokines) or as membrane-bound signals that act through cell-cell contact (Alberts *et al.*, 2008).

The precursor cells are capable of responding to these CSFs which regulate proliferation and differentiation of the precursor cells for various blood cell lineages (lineage-specific growth factors), leading to increased production of one or other cell line depending on organism needs (Lodish, 2003). This property of self-renewal proliferation inherently makes these cells more valuable to picking mutations and encouraging tumorigenesis. On the other hand, tumorigenesis can also be due to single nucleotide polymorphisms (SNPs) carried by the genome depending on the change introduced by the SNP.

2. Tumorigenesis

It has been recognized for many years that tumorigenesis is a multistep process comprising several steps such as initiation, promotion and progression (which can be triggered by some irreversible alteration in several genes). For that reason, cancer is nowadays considered a genetic disease involving multiple genetic or epigenetic alterations that contribute to the progressive transformation of normal cells towards a malignant phenotype with biological advantages either for its abnormal accelerated proliferation and/or resistance to apoptosis (Hanahan and Weinberg, 2000). Molecular alterations can be potentiated by several external carcinogenic agents, such as chemicals and radiation, endogenous factors related to inflammation or compromised immune system. At genetic level, this alterations comprise irreversible changes in the DNA sequence, such as mutations, deletions, translocations or amplifications that may result in activation of oncogenes, inactivation of tumor suppressor genes or formation of novel chimeric proteins (Galm *et al.*, 2006).

Increasing data from several human cancers suggest that neoplastic cells within individual tumors are functionally heterogeneous cells despite their clonal origins. In particular, the potential for long-term proliferation appears to be restricted to subpopulations of cancer stem cells (CSC) (Ghosh and Matsui, 2009).

Stem cells occur in many different somatic tissues and are important participants in their physiology. The attribute of CSC self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype (Figure 2) *(Jordan et al.,* 2006). Cancer can be generated from either differentiated cells or CSC, which can suffer genetic and epigenetic alterations that way modifying genetic expression patterns involved in division, proliferation and cell death processes.

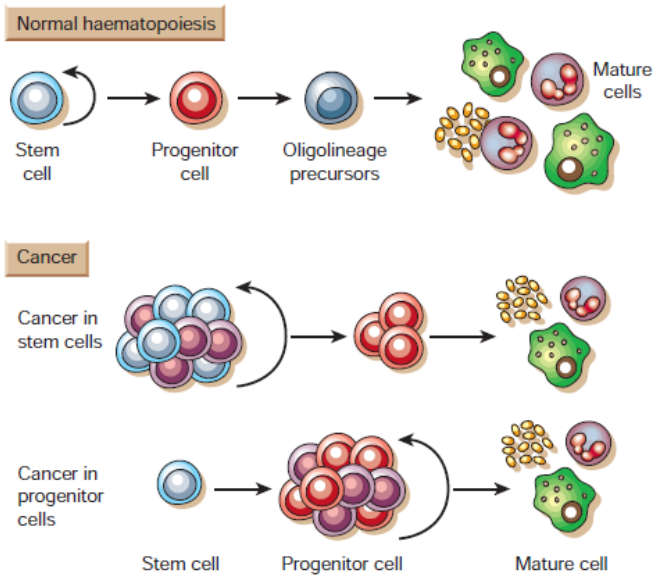

Figure 2. Comparison of stem cell self-renewal during normal haematopoiesis and in cancer (leukaemic transformation). In hematopoiesis, stem cell self-renewal are tightly regulated (top), while during CSC transformation of the same mechanisms may be deregulated to allow uncontrolled self-renewal (middle) [*Reya T. et al., 2001*].

3. Hematological neoplasias

The hematopoietic system is the best characterized somatic tissue with respect to stem-cell biology. Hematopoietic-cell cancers such as leukemia are clearly different from solid tumors, but certain aspects of hematopoietic stem-cell biology are relevant to our understanding of the broad principles of CSC biology. In various types of leukemia, CSCs have been unequivocally identified, and several biologic properties of these stem cells have been found to have direct implications for therapy (Jordan et al., 2006).

In multiple myeloma (MM), the existence of CSCs has long been proposed since early experiments examining the growth potential of mouse plasma cell tumors were carried out over four decades ago. However, the exact nature of the myeloma stem cell and its

relationship to normal plasma cell and B cell development is unclear (Ghosh and Matsui, 2009). Multiple myeloma cells are reminiscent of hematopoietic stem cells in their strict dependence upon the bone marrow microenvironment. However, from all other points of view, multiple myeloma cells differ markedly from stem cells (Zipori, 2010).

4. Multiple Myeloma

4.1. Epidemiology

Multiple myeloma is an incurable neoplastic plasma-cell disorder that is characterized by clonal proliferation of malignant plasma cells (PCs) in the bone marrow microenvironment, monoclonal protein in the blood or urine, and associated organ dysfunction (Kyle and Rajkumar, 2004). It accounts for approximately 1% of neoplastic diseases and 15% of hematologic cancers. In Western countries, the annual age adjusted incidence is 5.6 cases per 100,000 persons. Age-adjusted rates of the disease are 6.9 per 100,000 men compared with 4.5 per 100,000 women; the rate is nearly twice as high in black persons than in Caucasians (Nau and Lewis, 2008).

It is the second most common hematological malignancy and presents primarily in elderly patients, with a median age at diagnosis of approximately 72 years in Europe (De La Rubia and Sanz, 2011). The number of older patients with this disease is expected to rise over time as a consequence of the increased life expectancy of the normal population. Exposure to ionizing radiation, farming pesticides or possibly petrochemicals also increases the risk. There is an increased incidence of MM in patients with rheumatoid arthritis or obesity. However, no clear risk factor can be identified in most patients with MM (Sirohi and Powles, 2006). Median overall survival can be from a few months to a couple of years. Despite the development of novel drugs, such as

proteasome inhibitors and derivatives of thalidomide, MM remains incurable and the majority of patients eventually succumb to cancer (Bommert *et al*., 2006).

4.2. Pathogenesis and clinical features

Immunoglobulin (Ig) molecules contain two linked heavy chains, with one light chain attached to each (Figure 3). Generally, PCs produce immunoglobulins to fight infection. However, monoclonal MM PCs proliferate and overproduce M protein (abnormal IgG, IgM, or IgA, or rarely IgE or IgD). MM cells also produce abnormal light chain proteins (κ or λ), cytokines that stimulate osteoclasts and suppress osteoblasts, and angiogenic factors that promote new blood vessel formation (Nau and Lewis, 2008).

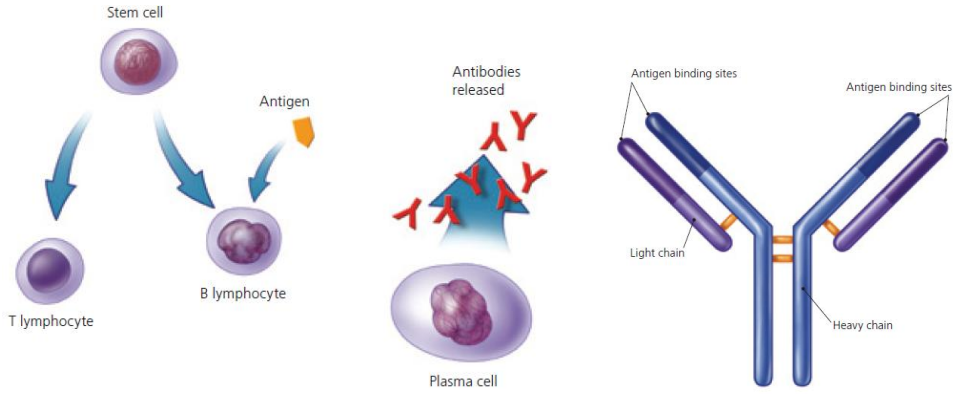

Figure 3. **PCs development from stem cells in the BM**. Stem cells can develop into B cells (B lymphocytes). When foreign substances (antigens) enter the body, B cells develop into PCs; Normal immunoglobulin molecule containing paired heavy chains with one smaller light chain attached to each that produce Igs (antibodies) to help fighting disease [*C. Nau et al., 2008*].

Clonal expansion of the tumor cells results in excessive production of monoclonal immunoglobulin and its presence in serum or urine is the main diagnostic feature of the disease. MM PCs produce IgG in 2/3 of the cases, IgA in 1/3 and rarely IgM or IgD. A typical serum protein electrophoresis in Multiple Myeloma shows a monoclonal peak in the γ-globulin region with decreased levels of β and α-globulins (Figure 4) (Hoffbrand *et*

al., 2006). Urine often contains Bence-Jones protein (2/3 of the cases) consisting of κ or λ light free chains of the same type of protein seen in serum. It is important to note that 34% of patients are asymptomatic at presentation with incidental abnormalities on total protein, creatinine, calcium, or hemoglobin laboratory levels (Kyle *et al*., 2003b).

Clinical manifestations of symptomatic MM include: extensive osteolitic lesions, fractures and bone pain (due to hyper function of osteoclasts stimulated by interleukin IL-6 and others cytokines); hyper viscosity syndrome, cryoglobulinemia and amyloidosis (resulting of the presence of monoclonal protein); kidney insufficiency (characterized by excretion of monoclonal light chains of Bence-Jones protein) and hypercalcaemia: serum calcium level greater than 11 mg/dL (2.75 mmol/L); serum creatinine level greater than 2 mg/dL (180 µmol/L); impaired hematopoiesis with anemia [hemoglobin level less than 10 g/dL (100 g/L)], cytopenia and *rouleaux* (in late stages of disease) (Nau and Lewis, 2008).

Figure 4. **A typical serum protein electrophoretic profile in MM**. The figure shows a monoclonal peak in the γ-globulin region with decreased levels of β e α-globulins [Adapted from *Kyle RA, et al. 2004*].

Median overall survival can be from a few months to a couple of years. Serum β2-microglobulin is one of the useful prognostic indicators, reflecting the kidney function overall state. Levels under 4mg/L correspond to a better prognosis (median overall survival of 48 months). These myeloma-related impairments are best remembered using

the terminology CRAB (hypercalcemia, renal insuficiency, anemia, bone lesions) (Kyle et al., 2003a).

4.3. Staging and Molecular events

Multiple Myeloma is often preceded by a premalignant plasma cell disorder such as smoldering (asymptomatic/indolent) Multiple Myeloma (SMM/IMM) or by a monoclonal gammopathy of undetermined significance (MGUS), which occurs in at least one third of patients (Lynch *et al*., 2005).

Robert Kyle coined the term "monoclonal gammopathy of undetermined significance" (MGUS) in 1978 (Kyle, 1978). Long-term follow-up studies of MGUS patients show an excess risk of developing MM (Kyle *et al*., 2002). However, a key gap in our understanding is whether MM is always preceded by MGUS or if MM typically arises *de novo* (Landgren *et al.,* 2009). The risk of progression from these premalignant conditions to MM is affected by the level of monoclonal immunoglobulin, the presence of non-IgG gammopathy, an abnormal serum free light-chain (sFLC) ratio, the fraction of bone marrow plasma cells bearing an aberrant phenotype, increased bone marrow plasma cells, decreased levels of polyclonal immunoglobulin and aneuploidy (Dispenzieri *et al*., 2008; Kyle *et al.*, 2007). However, the proportion of MM that develops from MGUS or SMM is unknown and remains an important unresolved issue in the understanding of the pathogenesis of myeloma (Kuehl and Bergsagel, 2002; Landgren et al., 2009; Palumbo and Anderson, 2011). It has also been postulated that MM that arises from a preexisting plasma cell disorder with distinct genomic features, a unique pattern of response to therapy and a more favorable outcome (Fonseca *et al*., 2002; Fonseca et al., 2009; Zhan et al., 2007).

Based on the above, there are still currently lacking reliable biological markers to either differentiate plasma cells of MGUS, IMM, or MM or to predict progression from MGUS/IMM to MM (Landgren, 2010). With progression of MGUS to myeloma, complex

genetic and epigenetic events occur in the neoplastic plasma cell and in the bone marrow microenvironment (Figure 5) (Bergsagel and Kuehl, 2005).

A complex signaling network sustains malignant cells survival and mediates tumor progression and drug resistance. Major signaling pathways involved are the IL-6R/STAT3, RAS/MAPK, PI3K/AKT, NOTCH, WNT and NF-kB pathways. On the other hand, MM cells induce microenvironment cell signaling which results in osteolytic bone destruction, neoangiogenesis and indirect tumor growth support. In contrast to oncogenic pathways, which are regularly affected by mutations, tumor suppressor genes are rarely mutated in MM and predominantly associated with advanced disease and extramedullary manifestations.

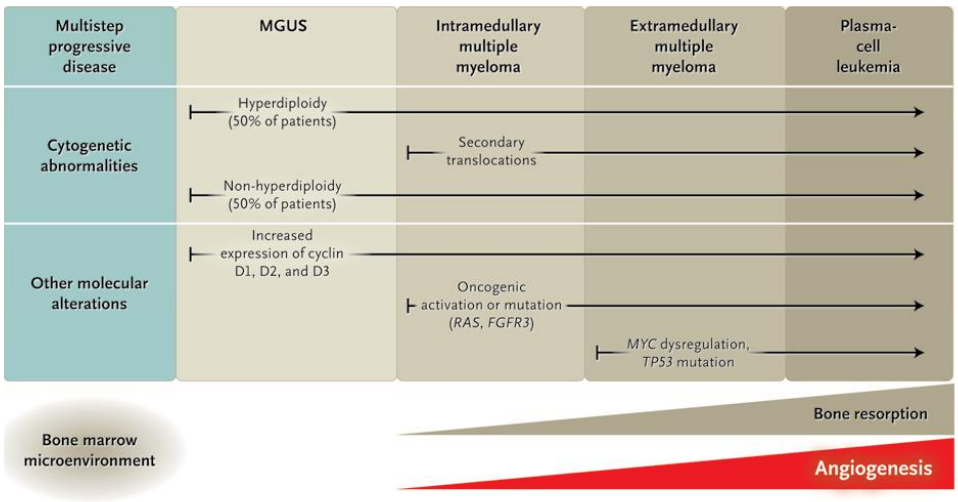

Figure 5. Multistep Pathogenesis of Multiple Myeloma. Early chromosomal abnormalities (IgH translocations or trisomies) are shared by PCs in MM and in MGUS. Secondary translocations involving *MYC* (8q24), *MAFB* (20q12), and *IRF4* (6p25) genes are common in multiple myeloma but quite rare in MGUS. Mutations of *RAS* or *FGFR3, MYC* dysregulation, deletion in p18 or loss of expression or mutation in *TP53* are found only in MM and play a key role in determining tumor progression and drug resistance. Also, changes in gene expression, in particular the up-regulation of transcription factors (NF-kB), have been reported in PCs from patients with MGUS but not in those from patients with MM [*Palumbo et al. 2011].*

Selective activation of the p53-dependent pathway by pharmacologic antagonists of the p53-inhibitor MDM2 induces apoptosis of primary MM cells (Bommert *et al*., 2006). IgH translocations involving the IgH locus (14q32) are early and fundamental events in the pathogenesis of PCs neoplasias as well as structural chromosome abnormalities (Bergsagel and Kuehl, 2005; Braggio and Renault, 2007; Chng et al., 2007).

Secondary late-onset translocations and gene mutations that are implicated in disease progression include complex karyotypic abnormalities in c-*MYC* (8q24) gene that are common in multiple myeloma but quite rare in MGUS; the activation of *N-RAS* and *K-RAS*, mutations in *FGFR3* and *TP53* are found only in multiple myeloma and play a key role in determining tumor progression and drug resistance (Palumbo and Anderson, 2011).

4.4. Diagnosis

According to diagnostic criteria provided by the International Myeloma Working Group (IMWG) in 2010, MGUS is defined as follows (all three must be met): i) serum monoclonal protein under 3 g/dL; ii) clonal BM PC's under 10% and iii) absence of end-organ damage (hypercalcemia, renal insufficiency, anemia, and bone lesions that can be attributed to the plasma cell proliferative disorder) (Kyle *et al*., 2010).

The diagnosis of myeloma is based on the presence of at least 10% clonal bone marrow plasma cells and monoclonal protein in serum or urine. In patients with true non secretory myeloma, the diagnosis is based on the presence of 30% monoclonal bone marrow plasma cells or a biopsy proven plasmacytoma (Roodman, 2009).

Myeloma is classified as asymptomatic or symptomatic, depending on the absence or presence of myeloma-related organ or tissue dysfunction (CRAB criteria described before) (Palumbo and Anderson, 2011). The diagnostic criteria for smoldering (asymptomatic) MM is also serum M protein level of 3 g/dL (30 g/L) or more, 10% or

more bone marrow PCs, and no related organ or tissue impairment (i.e., no end-organ damage, including bone lesions) or symptoms (Table I). The diagnostic criteria for symptomatic MM is the presence of a M protein (serum or urine), bone marrow clonal PCs or plasmacytoma and myeloma-related organ or tissue impairment (Nau and Lewis, 2008). In the current literature, asymptomatic MM is now synonymous with SMM (Kyle et al., 2003a).

Table I. Differential diagnosis of Monoclonal Gammopathies

Monoclonal gammopathy	*Incidence*	*Serum findings*	*Bone marrow findings*	*Clinical clues*
MGUS[3]	1 to 2 per 100 adults older than 50 years	M protein level of less than 3 g per dL (30 g per L)	Less than 10 percent plasma cells	Absence of myeloma-related organ and tissue impairment
Smoldering (asymptomatic) multiple myeloma[5,10]	5 to 7 per 1,000,000	M protein level of 3 g per dL or greater (IgG, IgA, IgM, IgD, or free light chains)	10 percent or more plasma cells	Absence of myeloma-related organ and tissue impairment
Symptomatic multiple myeloma[6,10]	5 to 7 per 100,000	M proteins (40 percent of patients with multiple myeloma have a level less than 3 g per dL)	Plasma cells (5 percent of patients with multiple myeloma have fewer than 10 percent plasma cells)	Presence of at least one myeloma-related organ and tissue impairment

[*Nau C. K. et al, 2008*]

The recommended tests for the diagnosis of myeloma include the taking of a detailed medical history and physical examination, routine laboratory testing: complete blood count, chemical analysis, serum and urine protein electrophoresis with immunofixation, and quantification of monoclonal protein (a clonal PC neoplasm must expand to ~10^9 cells before it produces enough Ig to be recognized as monoclonal IgM by serum electrophoresis) and BM examination [trephine biopsy plus aspirate for cytogenetic analysis or fluorescence in situ hybridization (FISH)] (Mahindra et al., 2010).

Interleukin-6, C-reactive protein and plasma cell antigen (e.g., CD38, CD138) measurements, with DNA ploidy and cell cycle analysis, are increasingly being used to predict response to therapy (Nau and Lewis, 2008).

4.5. Prognosis and Follow-up in MM

Any chromosomal abnormality (in this case specific translocations in the Ig heavy chain region detected by FISH) is associated with a worse outcome when compared to normal karyotype (Avet-Loiseau et al., 2007). High risk disease and poor prognosis are defined by the presence of one of the following parameters in International Staging System (Table II) (Fonseca et al., 2009; Greipp et al., 2005; Kyle and Rajkumar, 2009). Flow cytometric immunophenotyping is a follow-up tool as sensitive as other advanced molecular techniques to set prognostic markers, predict progression risk from MGUS to MM and detect minimal residual disease, during and after therapy (Davis *et al*., 1997; Gupta et al., 2009).

Table II. International Staging System for Multiple Myeloma

Stage	*Criteria*	*Percentage of patients in this stage*	*Median survival (months)*
I	Serum β_2-microglobulin level less than 3.5 mg per L (297 nmol per L) Serum albumin level of 3.5 g per dL (35 g per L) or greater	28	62
II	Not stage I or III	33	45
III	Serum β_2-microglobulin level of 5.5 mg per L (466 nmol per L) or greater	39	29

This staging defines three risk groups based on serum β2-microglobulin and albumin levels *[Greipp PR et al., 2005]*

In monoclonal gammopathies, two different PC populations are usually observed: one is normal, the other is malignant, and both of these populations have distinct phenotypes (Olteanu et al., 2008). The frequency of malignant PCs to total BM PCs is associated with the progression risk of MGUS and SMM to MM (Perez-Persona et al., 2007). FC can assess BM aspirates suspensions for a range of the most comprehensive antigens that play a significant role in the characterization of normal and malignant plasma cells.

According to European Myeloma Network (EMN) recommendation for the diagnosis of MM, MGUS and reactive conditions by flow cytometry antigens, such as CD19/CD56/CD117/CD20/CD38/CD27/CD81/CD200, must be used. In addition, antigens, such as CD45/CD56/CD117/CD38, have been identified as prognostic markers for myeloma. Characteristic changes in aberrant plasma cells include absence of CD19, lack of CD45, decreased CD38 and/or overexpression of CD56 (Rawstron et al., 2008). CD138 is a differentiating antigen appearing only after the plasmablastic stage (PC progenitors). PC precursors ($CD138^{+}$) retain the ability to divide while fully matured PC ($CD138^{++}$) lack this ability (Gupta et al., 2009; Jego et al., 1999). Soluble CD138 molecules in plasma work as a prognostic factor as it reflects tumor mass and apoptotic index (Lovell et al., 2005; Seidel et al., 2000). Studies have proved that adhesion molecule CD138 can be used as a strong therapeutic target because of its unique expression (Craig and Foon, 2008). CD38 also can be used as target but it lacks specificity (due to its broad expression); its expression in malignant PCs is lower compared to normal PCs. MGUS and MM cases express CD138 on PCs, but its expression is dim on malignant PCs (Bataille et al., 2006). CD19 is a marker of B cells, including most of the PCs. In MGUS, normal PCs express CD19, rather malignant PCs do not. In MM, there is only negative or dim CD19 expression on PCs. Indeed, loss of CD19 is found to be associated with tumor progression in MGUS and MM patients (Raja et al., 2010). CD45 is present in both B and T cells, with dim expression in precursor and some PCs. In MGUS there is equal distribution of $CD45^{+}$ PCs and $CD45^{-}$ PCs whereas in MM its expression is not well characterized but survival rates is higher in $CD45^{+}$ cases and most reports agree that $CD45^{-}$ phenotype represents the malignant PCs in MM (Moreau et al., 2004). CD56 is a NK and NK-T antigen. Its expression strongly correlates with $CD45^{-}$ PCs. It is a valuable marker in diagnosis- most of MM cases express CD56. However, circulating PCs and extramedullary MM patients lack its expression (Bataille et al., 2006).

Several groups described two distinct populations of normal and malignant PCs in the BM of MGUS and MM. Normal PCs are characterized by low forward/side scatter (FSC/SSC) and high CD38 expression with $CD19^{+}/CD56^{-}$ phenotype. Malignant PCs are $CD19^{-}/CD56^{+}$ or $CD19^{+}/CD56^{+}$) with high FSC/SSC and low CD38 expression. It was also found that almost all cases of MM have 95% abnormal plasma cells, compared with 60% and 18% of SMM and MGUS cases, respectively (Figures 6 and 7) (Kovarova et al., 2009; Sezer et al., 2001).

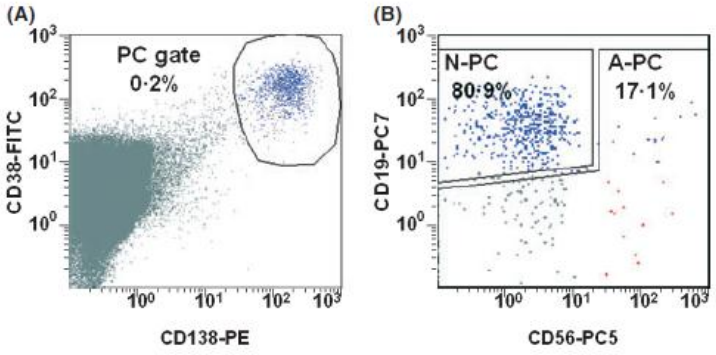

Figure 6. Flow Cytometry Analysis in MGUS and MM PCs. (A) Identification of PCs in MGUS by using the markers CD138 and CD38. (B) Showing the high frequency of normal PCs (N-PC) in MGUS comparing to the frequency of malignant /abnormal PCs (A-PC) (Raja et al., 2010).

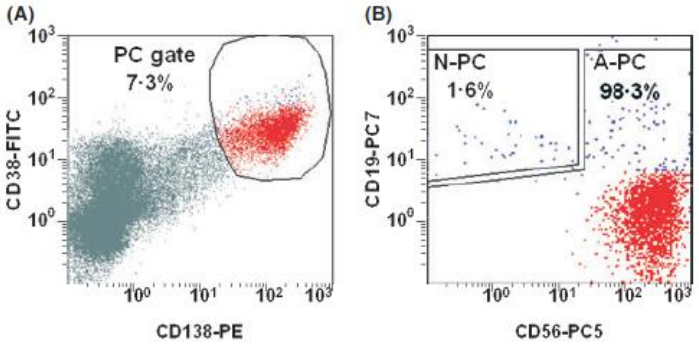

Figure 7. (A) PC infiltration in the bone marrow of MM patient. (B) Existence of high frequency of malignant/abnormal PCs (A-PC) in MM comparing to normal PCs (N-PC) (Raja et al., 2010).

5. MM Treatment

5.1. The role of Proteasome inhibition in MM

The 26S proteasome is a large protein complex, present in both the cytoplasm and the nucleus of all eukaryotic cells. It is necessary for the degradation of intracellular proteins in eukaryotic cells whereas extracellular/ transmembrane proteins are typically degraded by the aggresome/lysosomal pathway (Adams, 2004). It consists of a 20S catalytic core and two 19S regulatory complexes that cap the 20S core particle at both ends, forming 2 outer and 2 inner rings that are stacked to form a cylindrical structure (Chari *et al.*, 2010). The lysine residues of those proteins targeted for degradation are covalently modified with a polyubquitin protein chain, with each ubiquitin tag consisting of a 76 amino acid polypeptide. The ubiquitin chain is recognized by the lid-like structure of the19S subunit and then removed. The target protein is then denatured in an energy dependent manner by the 6 ATPases at the base of the 19S subunit and threaded into the center of the 20S subunit (Orlowski *et al*., 2002). Selected ubiquitinated proteins by the 19S complex are then directed for catalytic degradation by the 20S complex, comprising two α-subunits and two proteolytic β-subunits, arranged in $\alpha_7\beta_7\beta_7\alpha_7$ fashion, surrounding a central cavity where the catalytic sites are found (Groll *et al.*, 2000). Three of the seven h subunits, β1, β2, and β5, are proteolytically active with different substrate specificities. β1 subunit catalyzes a postglutamyl peptidyl hydrolytic-like activity; β2 subunit catalyzes a trypic-like activity; and β5 subunit catalyzes a chymotryptic-like activity (Figure 8) (Wang *et al*., 2008).

The UPS plays an essential role in regulating the intracellular concentration of specific proteins, thereby maintaining homeostasis within cells. Inhibition of the 26S proteasome prevents this targeted proteolysis, which can affect multiple signaling cascades within the cell related to regulation of cell growth and proliferation (e.g cyclins, p53, NF-kB) (Adams, 2003). On the other hand, disruption of normal homeostatic mechanisms can

lead to cell death. This critical cellular function of the UPS has been successfully targeted for cancer therapy, as highlighted by the efficacy of proteasome inhibitor bortezomib in a wide spectrum of hematological, namely MM, and solid tumors (Sanchez-Serrano, 2006). Cancer cells are more sensitive to proteasome inhibition than normal cells. It is clear, that there must be some feature(s) of cancer cells, lacking in normal cells, that sensitizes them to proteasome inhibition.

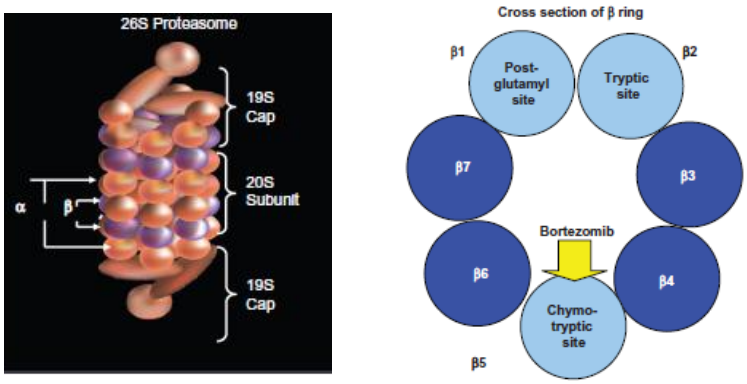

Figure 8. Structure of 26S proteasome. The 26S proteasome is formed when the 20S catalytic core is capped by 19S regulatory subunits at both ends in an ATP dependent fashion; Cross section of the β ring of the 20S subunit of the proteasome: chymotrypsin (β5), trypsin (β2), and post-glutamyl peptide hydrolase (β1). Bortezomib inhibits the chymotryptic site [*Adapted from Chari et al 2010*].

Nuclear factor kappa B (NF-kB), for instance, is often suggested to be a reason for such disparity in cell responsiveness because of its role in tumor promotion, survival and metastasis (Cvek and Dvorak, 2008). Several studies proposed that this factor may be the answer to the link between chronic inflammation and tumorigenesis (Karin et al., 2006). In fact, NF-kB constitutive activation (because of genetic abnormalities) is characteristic of many malignant tumors, for example, MM (Annunziata et al., 2007; Keats et al., 2007). NF-kB pathway requires the proteasome to cleave inhibitor-kB (IkB) and releases the heterodimer p50:p65 for nuclear translocation, leading to gene transcription. Such NF-kB activation promotes cancer development through the

expression of cell cycle genes, apoptosis inhibitors, invasive proteases and so on (Karin, 2006). Hence, if NF-kB requires proteasome activity, proteasome inhibition leads to the blockage of NF-kB and cancer cell death. Moreover, this factor, which can be activated by anticancer drugs, offers a good explanation for the known ability of proteasome inhibitors to sensitize malignant cells to standard therapeutics (Nakanishi and Toi, 2005; Pham et al., 2007).

5.2. Pharmacogenomics of Bortezomib

Bortezomib (VELCADE®), a modified dipeptidyl boronic acid (approved by the FDA on May 13, 2003), is a reversible inhibitor of the chymotrypsin-like proteolytic activity of the 26S proteasome in mammalian cells. This drug exhibits cytotoxic, growth-inhibitory, and antitumor activities in several *in vitro* and *in vivo* assay systems and binds to the proteasome at lower concentrations than it does to other tested proteases (Bross et al., 2004). Bortezomib is currently believed to exert its effects through multiple pathways that target both the tumor cell and its microenvironment, leading to decreased cell proliferation and induction of apoptosis (Uttamsingh *et al.*, 2005).

One central mechanism by which bortezomib functions in MM is via the inhibition of the breakdown of IκB and consequently, stabilization of the NF-κB complex (Adams, 2003). This prevents NF-κB translocation to the nucleus with consequent inactivation of multiple downstream pathways known to be important in MM cell signaling (Karin *et al.*, 2002). It also decreases the adhesion of the myeloma PC's to stromal cells which increases sensitivity to apoptosis, as well as interrupting prosurvival paracrine and autocrine cytokine loops in the bone marrow microenvironment mediated by IL-6, insulin like growth factor (IGF-1), vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF-α) (Hideshima *et al.*, 2003; Hideshima *et al*., 2001).

Other effects in MM include inhibition of angiogenesis, inhibition of DNA repair (blocks the degradation of tumor suppressor p53) and impairment of osteoclast activity (Rajkumar et al., 2005). Tumor cells appear to be more sensitive to the effects of proteasome inhibition than normal cells due to a loss of checkpoint mechanisms occurring during tumorgenesis; this means that normal cells can usually recover as the inhibition is transient and reversible (Field-Smith *et al.*, 2006).

Bortezomib is 83% bound to human plasma proteins at therapeutic concentrations. *In vitro* studies with human liver microsomes and human cDNA-expressed cytochrome P450 isozymes indicate that Bortezomib is primarily oxidatively metabolized via the cytochrome P450 enzymes, CYP3A4, CYP2C19, CYP1A2, being CYP3A4 and CYP2C19 the major contributors to bortezomib metabolism (Uttamsingh *et al.*, 2005).

Bortezomib is approved for intravenous administration of 1.3 mg/m^2 and has a maximum plasma concentration (C_{max}) of ~150nM. The boronic acid group was found to be essential for activity, and the metabolites are not pharmacologically active. Like many other cancer drugs, bortezomib is a cytotoxic agent with a narrow therapeutic index. Thus, it is important to understand bortezomib metabolism and the consequences of inhibition or induction of that metabolism, to predict possible drug-drug interactions (Lu *et al.*, 2006). Hence, limited evidence indicates that advanced cancer patients may have decreased capacity to metabolize certain chemotherapeutic agents due to decreased CYP activity (Helsby *et al.*, 2008).

5.3. Frontline Treatment Strategies

The treatment strategy is mainly related to age (Anderson *et al*., 2009). Combination chemotherapy with melphalan and prednisone (MP) has been used since the 1960s and, until recently, has remained the most widely accepted treatment option for elderly patients with MM, and more complex combinations have shown higher toxicity and no

survival advantage over MP (Facon *et al*., 2006). However, in an attempt to change this scenario, new drugs such as thalidomide, bortezomib or lenalidomide have been incorporated in the frontline therapy of elderly patients with MM (Figure 9) (De La Rubia and Sanz, 2011).

Symptomatic (active) disease should be treated immediately whereas patients with smoldering (asymptomatic) multiple myeloma should not undergo treatment (Kyle *et al*., 2007). It requires only clinical observation, since early treatment with conventional chemotherapy has shown no benefit (Kyle *et al*., 2010; Kyle and Rajkumar, 2004; Kyle *et al*., 2007).

Biological age and the presence of organ dysfunction determine treatment choice and drug dose. The level of response is associated with an improved long-term outcome (Palumbo and Anderson, 2011). A complete response is defined as the elimination of detectable disease on routine testing (Kyle and Rajkumar, 2009; Ladetto *et al*., 2010). Combined therapy with dexamethasone plus bortezomib, lenalidomide or thalidomide is the treatment of choice for patients with relapsed or refractory myeloma (Richardson *et al*., 2005). In a randomized study, combination therapy with bortezomib, thalidomide and dexamethasone was superior to therapy with thalidomide plus dexamethasone with respect to both response rate and progression-free survival (Palumbo and Anderson, 2011).

Recent therapeutic trends favor adapting the treatment for a specific patient according to those patients' risk factors. Although such risk-adapted strategies have not been prospectively validated, it has been recommended the use of bortezomib-containing regimens for high-risk disease and lenalidomide or thalidomide-containing regimens for standard-risk disease (Avet-Loiseau *et al.*, 2010).

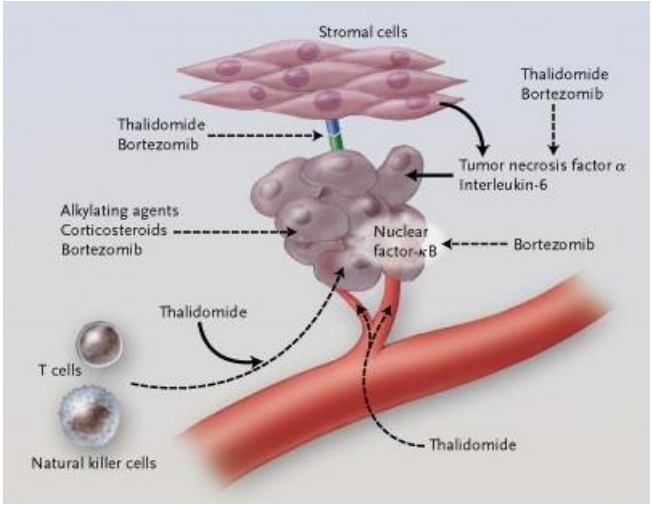

Figure 9. Proposed mechanism of action for Multiple Myeloma therapies Conventional therapy combined with thalidomide, lenalidomide, or bortezomib is administered in patients older than 65 years of age. Thalidomide plus Bortezomib target TNFα and IL-6 and Bortezomib single therapy targets NF-kB. [*Kyle et al., 2004*].

6. Drug resistance and associated polymorphisms

Resistance to chemotherapy develops inevitably and limits the effectiveness of anti-cancer drug treatment. Tumors may be intrinsically drug-resistant or develop resistance to chemotherapy during treatment. Acquired resistance is a particular problem, as tumors not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action (Longley and Johnston, 2005). Drug resistance can occur at many levels, including increased drug efflux, drug inactivation, alterations in drug target, processing of drug-induced damage, and evasion of apoptosis. The ability to predict response to chemotherapy and to modulate this response with targeted therapies allows for selection of the best treatment for individual patients (Ross *et al*., 2005).

Although several prognostic factors have been identified for newly diagnosed myeloma and for patients with relapsed and/or refractory disease, genetic factors that may influence responses to therapy, especially in the latter setting, have been less well defined. One possible factor is variant multiple drug resistance protein 1 (MDR1) genotypes (over than 50 SNPs described so far), which have been associated with differential expression of the MDR1 gene and its 170 kDa protein product, P-glycoprotein, also known as the ATP binding cassette Transporter B1 (ABCB1) (Jamroziak et al., 2009). MDR1 is a transmembrane protein that acts as an energy dependent drug efflux pump for chemotherapeutic drugs commonly used against hematologic malignancies. Indeed, the three most common MDR1 SNPs, C3435T, C1236T, and G2677T/A, had been found to influence the outcome of myeloma treated patients. Polymorphism C3435T (exon 26) originates a silencing mutation which, as far as some authors are concerned, can compromise gene expression levels and lead to drug resistance. In this particular case, homozygous patients for allele T have a better response to therapy due to lower expression levels of MDR1, when compared to heterozygous and homozygous patients for allele C (Drain et al., 2009; Krishna and Mayer, 2000).

Moreover, overexpression of MDR1 in myeloma cells might contribute to treatment failure in patients receiving proteasome inhibitors. Therapy resistance may also be mediated by another multidrug resistance associated protein, also known as ATP binding cassette, subfamily C, member 1 (MRP1, or ABCC1). Like MDR1, it confers resistance to anthracyclines and because the MRP1 mutation Arg723Gln has an effect on MRP1 expression and trafficking, it significantly reduced MRP1 mediated resistance to a wide spectrum of drugs (Buda et al., 2010; Cavaco et al., 2003; Jamroziak et al., 2009).

The codifying gene for sub family of CYP3A (previously referred on Bortezomib pharmacogenomics) is located in chromossome 7 (7q21.1) and is responsible for metabolizing about 50% of prescript drugs. The isoenzyme CYP3A4 is the major

element of this family mainly because of the several substrates it can react with but also for its liver protein amounts. There are already described 20 polymorphisms for this enzyme, being the most common allelic form the CYP3A4*1B (392 A>G) and some authors suggest that it is associated with increased protein activity (van Schaik, 2008). In Portuguese population the most important allelic form is CYP3A4*2 which frequency in Caucasians varies from 0 to 5% and which incidence is closer to the maximum, 4,5%. Hence, this can be a determinant factor in metabolizing drugs in Portuguese population, as this allelic form is associated with decreased activity of the enzyme (Cavaco et al., 2003).

Polymorphism studies mediating drug metabolism aim to identify markers which can possibly allow predicting response to therapy in patients in terms of sensitivity and drug levels. Therefore, the major candidates are the allelic forms which present higher frequencies in a population and influence enzyme activity. Ultimate goal focus its application on therapeutic approaches, allowing drug adjustment for each patient, based on polymorphism profile (Longley and Johnston, 2005).

Malignant cells may develop several mechanisms to escape the effects of proteasome inhibition, including alterations in the proteasome complex itself, leading to decreased function, increasing the efficiency of alternate mechanisms of protein degradation or modulation cell signaling pathways that are affected by proteasome inhibition. Despite promising clinical activity, some patients with multiple myeloma failed to respond to bortezomib therapy (nearly a third of the patients). Moreover, the efficacy for bortezomib may differ between tumor types (Oerlemans et al., 2008; Ross et al., 2005).

A mutation involving the β5 unit of the proteasome catalytic unit (Ala49Thr) leads to impaired binding of bortezomib and thus decreased proteasome inhibition. Investigators also noted a significant up regulation of the *PSMB5* subunit following exposure to bortezomib and other proteasome inhibitors. Furthermore, while mutations such as this

may explain development of resistance, baseline differences in susceptibility may be due to polymorphisms involving the *PSMB5* locus (Oerlemans et al., 2008).

Recent studies have identified mutations involving genes associated with regulation of NF-kB pathways that result in constitutive activation of the NF-kB pathway. Cells that carry these mutations appear to be particularly sensitive to the effects of proteasome inhibition, a finding that could allow us to tailor the use of this class of drugs in the future (Figure 10) (Vangsted et al., 2009).

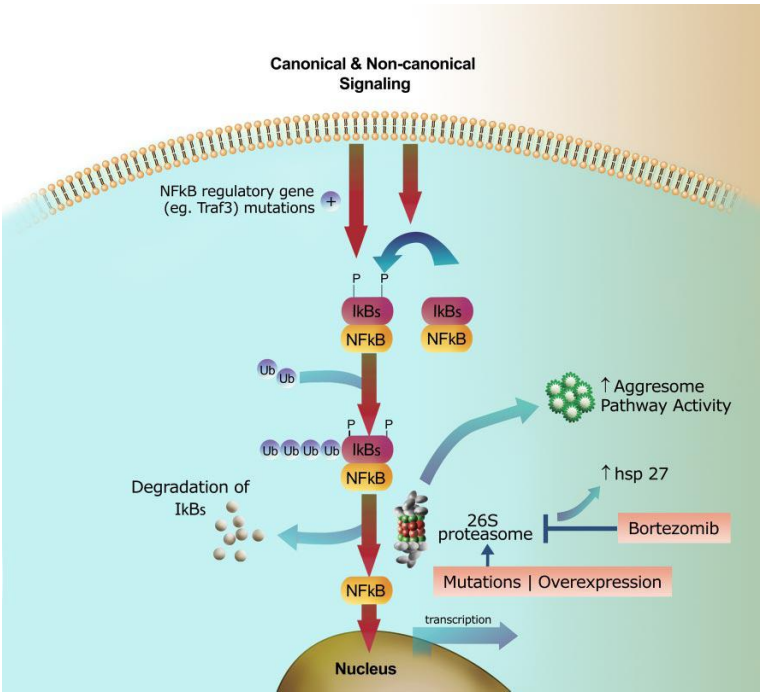

Figure 10. Mechanisms of resistance to Bortezomib. Constitutive NF-kB signaling can result from mutations in regulatory genes, such as *TRAF3*, leading to increased cell sensitivity to proteasome inhibition. Mechanisms of resistance include: (1) the 26S proteasome acquiring resistance due to mutations or overexpression of the PSMB5 subunit; (2) proteasome inhibitors being antagonized by upregulation of heat shock proteins, such as Hsp27; and (3) increased activity of the aggresome pathway (Kumar and Rajkumar, 2008).

However, the mechanism of bortezomib resistance in myeloma remains elusive. (Kumar and Rajkumar, 2008). Understanding the mechanisms of resistance to proteasome

inhibition would not only allow for better use of proteasome inhibitors such as bortezomib, but should also allow for the rational design of synergistic drug combinations.

Part II: Goals

This study aimed to:

- Evaluate some molecular mechanisms involved in predisposition to monoclonal gammopathies, and in transition from MGUS to Multiple Myeloma;

- To clarify possible molecular mechanisms that could influence sensitivity and/or resistance to therapy in these patients, in particular we tried to better understand the role of *MDR1* (C3435T) and CYP3A4 (T673C) polymorphisms, and the expression levels of ABC proteins (MDR1 and MRP), ubiquitin conjugates and the transcription factor NF-kB.

  We hope that this work could contribute to the identification of molecular markers that could predict disease progression and response to therapy.

Part III: Materials & Methods

1. Materials

- **Isolation and cryopreservation of leukocytes**

Sterile Phosphate Buffered Saline (PBS) (Sigma-Aldrich); ph 7.4, Dimethylsulfoxide (DMSO) (Sigma-Aldrich), FBS (GIBCO), culture medium Roswell Park Memorial Institute (RPMI 1640) (GIBCO), RBC Lysis Buffer: (NH_4Cl; $KHCO_3$; Na_2EDTA).

- **DNA extraction and quantification**

QIAamp ® DNA Blood Mini Kit (QIAGEN); ethanol 100% (AppliChem); nuclease free water (Ambion); NanoDrop (THERMOscientific).

- **PCR-RFLP**

For PCR: Agarose (Lonza, USA), Nuclease free water (Ambion), TAE buffer (BioRad), DNA ladder (Promega), Buffer (Qiagen), Q Solution (Qiagen), DNTPs (Qiagen), primers for MDR1 (Thermo Scientific) and CYP3A4 (Thermo Scientific), HotStart polymerase (Qiagen), Thermocycler BioRad iQ5 (BioRad, Hercules, USA), ethidium bromide (BioRad), BioRad GelDoc 2000 (BioRad, Hercules, USA). PCR primers used for PCR-RFLP were as follows: *MDR1 (3435T)*, Sense 5'-(TGC TGG TCC TGA AGT TGA TCT GTA AC)-3', Antisense 5'-(ACA TTA GGC AGT GAC TCG ATG AAG GCA)-3'; *CYP3A4 A-392-G (*1B)*, Sense: 5'-(AAT GAG GACAGC CAT AGA GAC AAG GcC)-3', Antisense: 5'-(CAA TCA ATG TTA CTG GGG AGT CCA AGG G)-3' (lower case is indicative of mismatched nucleotides incorporated for the generation of restriction enzyme recognition sequences).

PCR products were restricted with the following enzymes: *Alw26I* for *CYP3A4, T673C* SNP detection (Promega, Madison, USA) with Tango Buffer (Fermentas) and *MBOI* for *MDR1, C3435T* SNP detection (Promega, Madison, USA) with Buffer NEB 4 (New

Biolabs, England). All digestions were performed in accordance with recommendation of commercial suppliers.

- **Flow Cytometry**

Flow Cytometer FACSCalibur (Becton Dickinson); fixation and permeabilization kit for flow cytometry (Intra Cell Kit, Immunostep); Phosphate Buffered Saline (PBS) (Sigma-Aldrich); Lysis Buffer (BD BioSystems); anti-CD138-APC (BD Biosystems); anti-CD19-PerCP-Cy5.5 (BD Biosystems); antibody anti-ubiquitin conjugates-FITC (Santa Cruz Antibodies); antibody anti-NF-kB-PE(BD Biosystems).

2. Methods

To achieve the proposed goals we used patients and controls samples obtained from PB and/or BM aspirates collected for routine diagnostic procedures in individuals over 18 years old. Patients have clinical and laboratorial criteria of monoclonal gammopathies (MG), namely Monoclonal Gammopathy of Undetermined Significance (MGUS), Indolent/Smoldering Multiple Myeloma (IMM/SMM) and Multiple Myeloma (MM). As controls we used non-neoplastic patients and healthy individuals.

The patient and controls material was collected according to the principles in the Helsinki II Declaration, approved by the Local Ethical Committee and have an informed consent.

2.1. Leucocyte Isolation - Erythrocyte Lysate Technique

For each patient sample (PB and BM), three volumes of RBC Lysis Buffer (NH_4Cl, 155mM; $KHCO_3$, 10mM; Na_2EDTA, 1mM) were added to the whole blood sample collected with EDTA, in a falcon and incubated in ice for 5 minutes while mixing several times. PBS was added to perform a certain volume and the sample was then centrifuged at 500 *xg* for 10 minutes. The supernatant was decanted and 5 mL additional volumes of the RBC Lysis Buffer were then added to the pelleted white blood cells and incubated for 5 minutes in ice as well as mixed for several times. PBS were again added to the tubes that are centrifuged at 250 *xg* for 10 minutes and the supernatant decanted. To the obtained pellet of white blood cells, it was added equal volumes of medium both RPMI 1640 supplemented with 40% of FBS and RPMI 1640 supplemented with 20 % of DMSO and frozen in vials at -80 °C, for further use in Flow Cytometry analysis.

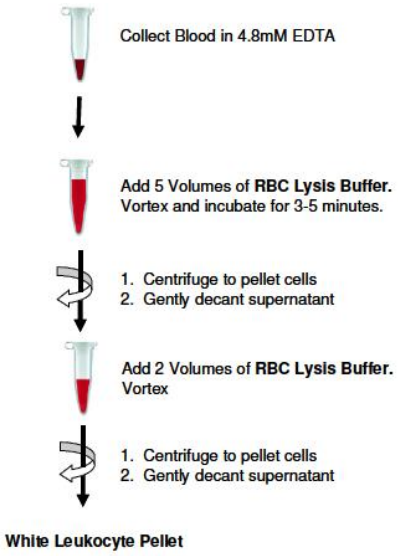

Figure 11. Procedure for Differential Red Blood Cell Lysis. White blood cells (buffy coat) are obtained when RBC and platelets get pelleted and then removed after several lysing and washing steps (*www.norgenbiotek.com).*

2.2. DNA Extraction and quantification

Fresh or cryopreserved blood samples from patients and controls were handled in a sterilized environment. Qiagen DNA extraction protocol is silica based. The protocol involves the incubation of the cellular material in a lyses buffer that contains a detergent along with proteinase K. The lyses' buffer destabilizes cell membranes, leading to the breakdown of cellular structure. The addiction of a chaotropic salt after cell lyses disrupts the protein structure by interfering with hydrogen bonding, Van der Waals and hydrophobic Interactions. Cellular proteins are largely insoluble in chaotropic salt and can be removed by centrifugation. This DNA extraction method is based on the binding properties of silica support (glass particles). DNA binds to the particles with high affinity in the presence of the chaotropic salt. After the other cellular components have been removed, the DNA can be released from the silica support by

suspending them in water. Without the chaotropic salt, DNA no longer binds to the glass particles and is released into solution.

The isolated DNA needs to be studied for its quality and quantity by using a Spectrophotometric Method. It is an analytical method used for determining the purity (quality) and quantity of the isolated DNA. The absorbance is measured at 260nm, and at this wavelength an absorbance of 1.0 corresponds to 50 µg/ml of double stranded DNA.

The equipment in use concerns a Spectrophotometer connected with specific Software. Besides this, DNA samples, the buffer in which samples were diluted previously and sterile distilled water are required. The procedure starts by setting a standard curve by analyzing 2 µl of buffer, in the wavelength of 260-280nm and is followed by the addition of 2µl of each isolated DNA sample to the detector. The absorbance is measured at 260nm and 280nm in the spectrophotometer to determine the quality (purity) and quantity of DNA (µg/ml). DNA concentration in the sample is calculated by the following formula:

Quantity of DNA (µg/ml) = Dilution Factor x Standard (50µg/ml) x O.D at 260nm

If DNA is pure the ratio between the concentration at 260 and 280 nm (260/280) will be 1.8-2.0; if DNA is contaminated with proteins, the ratio will be <1.8; if DNA is contaminated with RNA, the ratio will be >2.0.

2.3. PCR-RFLP (Restriction Fragment Length Polymorphism)

RFLP is a widely used technique to detect known mutations and variations using specific restriction endonucleases (Figure 16). These enzymes recognize specific sequences and cut double strand DNA within their recognition sequence to produce

fragments. Fragments thus produced get separated on agarose gel electrophoresis and are visualized after staining with ethidium bromide which enables analysis of sequence variations of discrete region. Normal and defective genes give rise to different restriction patterns, if change is in the recognition site of restriction enzyme.

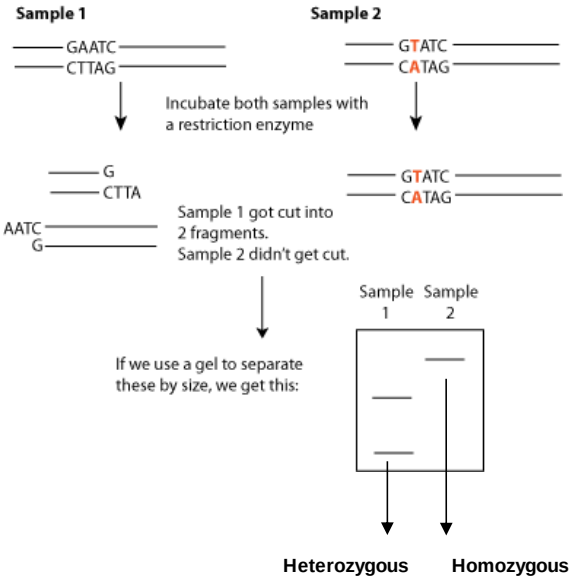

Figure 12. Digestion of heterozygous and homozygous samples by Restriction enzymes (RFLP). In electrophoresis of heterozygous sample (sample 1), digestion with the enzyme produces two bands (more than one cut site) and in homozygous sample (sample 2) there is only one site to cut, producing only one band.

After DNA extraction and quantification, 5µL of each sample was added to the PCR mix (20µL) for either *MDR-1* and *CYP3A4* testing, contents being to the former (1x): Buffer (Tris·Cl, KCl, $(NH_4)_2SO_4$,15 mM $MgCl_2$; pH 8.7; 10x), Q solution (5x), DNTPs (0,2mM), primers forward and reverse (0,5µM), Hot start *Taq Polymerase* (5U/µL), nuclease free water; and to the latter: primers (0,25µM), remaining equal all the other contents. The PCR was performed to get final products for digestion (35 cycles of: denaturation for 1 minute at 95°C; annealing for 45 seconds at 61°C (MDR1 primers) or 55°C (CYP3A4

primers); extension for 30 seconds at 72°C; holding end at 4°C). After that, an electrophoresis was carried out in 4% agarose gel (with ethidium bromide staining) to guarantee that all samples would amplify the required gene and be ready for the following step - digestion with restriction enzymes. PRC products (15µL) were added to digestion mix (5µL; 1x): *MBOI* enzyme solution (25U/µL), NEB4 Buffer (10x) and water; *Alw26I* solution (10U/µL), Tango Buffer (10x) and water. Digestion was carried out overnight at 37°C in thermocycler. Restriction fragments were analysed in 4% agarose gel and visualized by UV transillumination in a BioRad GelDoc 2000.

2.4. Flow Cytometry studies

Cells from MG patients and non-neoplastic controls were initially stained with the antibodies anti-CD138-APC (BD Biosystems) and anti-CD19-PerCP-Cy5.5 (BD Biosystems) in order to identify both normal ($CD138^+/CD19^+$) and neoplastic ($CD138^+/CD19^-$) plasma cell populations. Approximately 1 million of bone marrow (BM) cells from patients and controls were then incubated with 1 µg of monoclonal antibody anti-CD138-APC, anti-CD19-PerCP-Cy5.5 and monoclonal antibody anti-MDR1-FITC or anti-MRP1-FITC for 15 minutes at room temperature, in absence of light. Next, cells were incubated with 2 mL of Lysis Buffer (BD BioSystems) for 10 minutes at room temperature, in absence of light. After that, cells were washed with Phosphate Buffer Saline (PBS) by centrifugation for 5 minutes at 300 xg, ressuspended in 400 µL of PBS and analyzed in a Flow Cytometer FACSCalibur (Becton Dickinson).

In order to evaluate cytoplasmic expression of poli-ubiquitinated proteins and NF-kB transcription factor, approximately 10^6 cells from GM patients and non-neoplastic controls were initially stained with the antibodies anti-CD138-APC and anti-CD19-PerCP-Cy5.5 for 15 minutes at room temperature, in absence of light. After that, cells were washed with PBS by centrifugation for 5 minutes at 300 xg. Then, cells were

ressuspended in 100 µL of Solution A (fixation solution) – IntraCell Kit (ImmunoStep), and incubated for 15 minutes at room temperature, in absence of light. After that, cells were washed with PBS by centrifugation for 5 minutes at 300 xg. In the following step, cells were ressuspended in Solution B (permeabilization solution) – IntraCell Kit (ImmunoStep), and incubated with 1 µg of monoclonal antibody anti-ubiquitin conjugates-FITC and 1 µg of monoclonal antibody anti-NF-kB-PE, for 15 minutes at room temperature, in absence of light. Finally, cells were washed with PBS by centrifugation for 5 minutes at 300 g, ressuspended in 400 µL of PBS and analyzed in the Flow Cytometer FACSCalibur.

A total of 50.000 cells were acquired using the CellQuest™ program and results were analyzed and quantified using the Paint-a-Gate 3.02 program. Results are expressed in terms of percentage of positive cells expressing each protein and mean intensity of fluorescence (MIF) and represent mean membrane expression of MDR1 and MRP and cytoplasmic expression of ubiquitin conjugates and NF-kB, in MG patients and non-neoplastic controls.

2.5. Statistical Analysis

All the data obtained was evaluated by ANOVA, T-student and Multivariate analysis (logistic regression) in GraphPad Program Prism 5. For each experimental condition, the results are presented as the mean value ± standard deviation (SD). Adjusted odds ratios (ORs) were calculated and reported at 95% confidence intervals (CI). Significance is considered when $p<0.05$ (*), $p<0,01$ (**) and $p<0,001$ (***).

Part IV: Results

1. Characterization of samples from both patients with Monoclonal Gammopathy and non-neoplastic controls

For this study, we have used 51 patient samples, 24 diagnosed with MGUS, 4 with IMM (indolent/asymptomatic) and 23 with MM (Figure 13). As controls we have studied 9 non-neoplastic patients and 51 healthy individuals. Patients and controls were acquired in collaboration with Hematology service in Coimbra's University Hospital (HUC). Patient samples were obtained from peripheral blood (PB) and Bone Marrow (BM) aspirates (EDTA or heparin tubes) during routine diagnostic procedures in patients over 18 years old that have clinical and laboratorial criteria, cytogenetic study and cytometry analysis of MM. The patients group included 30 women and 21 men (age range: 41-89 years; median= 71 years). Non-neoplastic controls included 6 women and 3 men (age range: 59-86; median= 66 years). Patient material was collected according to the principles in the Helsinki II Declaration, approved by the Local Ethical Committee and has a patient's informed consent. As we can observe in Figure 13, 47% of the patients (24 patients) were diagnosed with Monoclonal Gammopathy of Undetermined Significance (MGUS); 45% (23 patients) with Multiple Myeloma (MM) and just a small percentage (8%) (4 patients) was classified as Indolent Multiple Myeloma (IMM).

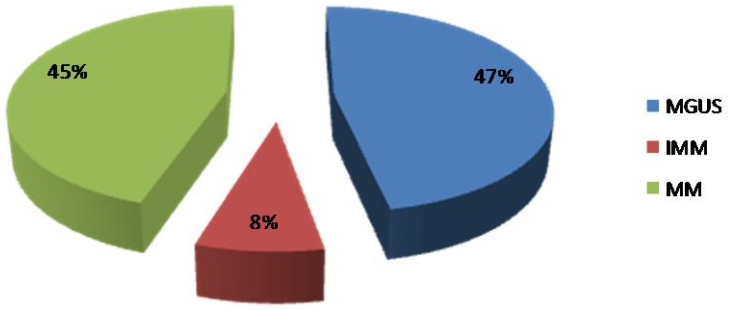

Figure 13. Distribution of MG patients. MGUS-Monoclonal Gammopathy of Undetermined Significance (blue, 47%); MM-Multiple Myeloma (green, 45%); IMM-Indolent Multiple Myeloma (red, 8%).

2. Genotypic characterization of the MDR-1 polymorphisms (SNP C3435T) and CYP3A4 (SNP T673C) in MG patients and healthy controls by Restriction Fragment Length Polymorphism (RFLP)

In order to determine the role of the polymorphic variants of MDR1 (3435T) and CYP3A4 (673C) genes in MG disease, namely in MGUS, IMM and MM, we performed genotypic characterization of 51 patients (24 MGUS, 4 IMM and 23 MM) and 51 healthy controls. Genotypic analysis was carried out by PCR-RFLP using the restriction enzymes *MBOI* (MDR1) and *Alw26I* (CYP3A4), as described in material and methods, to obtain a differentiated band pattern. Restriction fragments were analysed in 4% agarose gel (with ethidium bromide staining) and visualized by UV transillumination in a BioRad GelDoc 2000 (Figure 14).

Figure 14 represents the RFLP agarose gel from the MDR1 gene sequence, obtained from 43 patients and 43 controls (13 patients and 6 controls represented in figure 14). Hence, after digestion of the MDR1 PCR product (248pb), we could observe that: alelle T would produce one band of 232b and allele C would reflect two bands in the gel (170 and 60pb) ; for genotype CC (homozygous for C allele) there was a pattern of 2 bands in the gel; for TT genotype (homozygous for allele T) there was a single band in the gel with 232 pb and for genotype CT (heterozygous) we obtained a pattern of 3 bands in the gel (232pb, 170pb, and 60pb) (Figure 14).

Figure 15 represents the RFLP agarose gel from the CYP3A4 gene sequence [CYP3A4 T673C (Ser222Pro)], obtained from 43 patients and 43 controls (12 patients and 4 controls represented in figure 15). After PCR product (309 pb) digestion with *Alw26I* restriction enzyme we could observe that all samples obtained from either patients or controls exhibited the same restriction pattern of 2 bands in the gel, reflecting the genotype TT (256 pb and 53 bp). RFLP was performed for the second time to check if the undifferentiated band pattern remained the same and we could observe that again all patients and controls showed the same genotype (Figure 15).

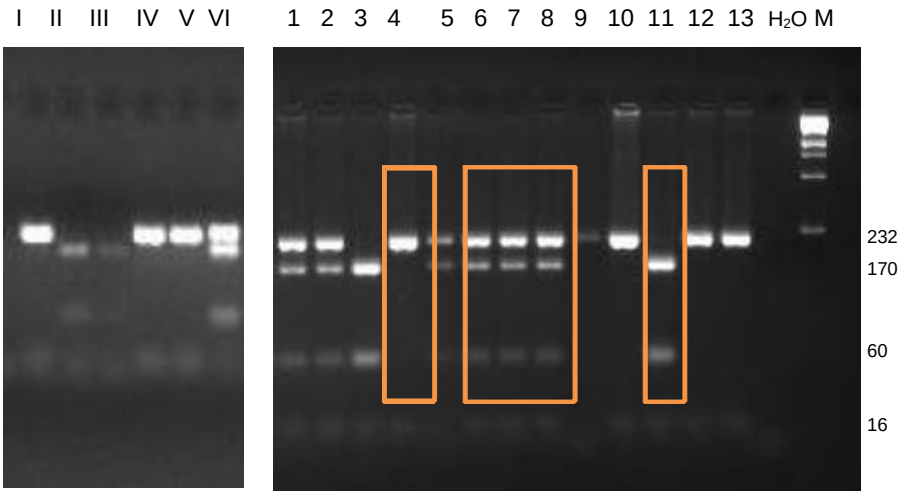

Figure 14. Analysis of the polymorphic MDR1 gene variants (C3435T) by PCR-RFLP. Genomic DNA from both group of patients and healthy controls, was extracted from whole PB and amplified by PCR. After PCR-RFLP, restriction fragments were analysed in 4% agarose gel (with ethidium bromide staining) and visualized by UV transillumination in a BioRad GelDoc 2000. In the presence of the MDR polymorphism (C*3435T*), digestion of the 248pb PCR products produced 3 fragments (232, 170 and 60 bp). Columns 1-13 show the results obtained in 13 patient's genotyping; and columns I-VI show healthy controls' genotyping. Colum 4 exhibits one fragment of 232 pb, reflecting the genotype TT; columns 6, 7 and 8 show 3 fragments, reflecting CT genotype and column 11 shows 2 bands, representing the CC genotype. Colums I, IV and V show three healthy controls exhibiting TT genotype, II and III have CC genotype and control VI has the CT genotype. M= molecular weight marker.

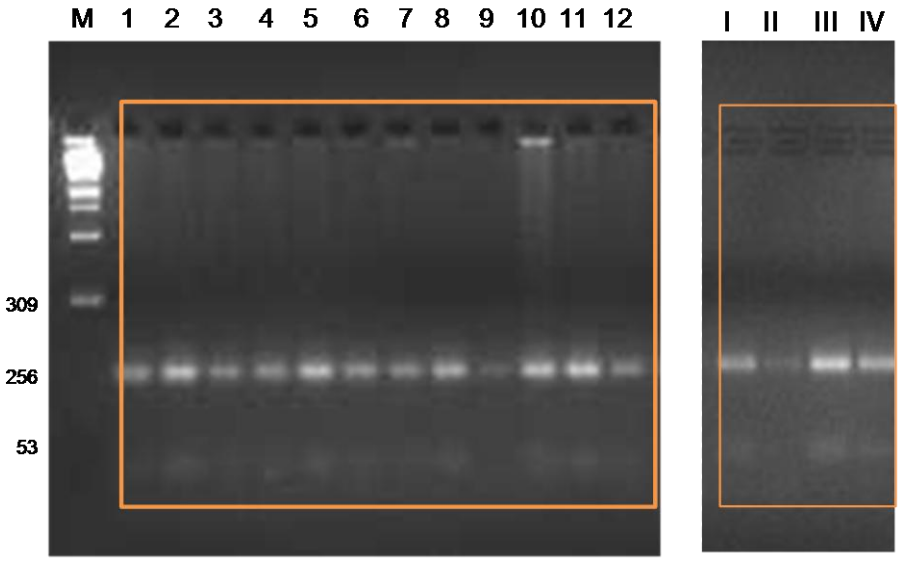

Figure 15. Analysis of the polymorphic CYP3A4 gene variants (673T) by PCR-RFLP. When CY3A4 polymorphism was present (*T673C*), digestion of the 309pb PCR products produced 256 and 53bp fragments. Columns show both patients (1-12) and controls (I-IV) genotyping. All patients and controls seemed to present the same digestion pattern in the gel, which corresponds to two bands (256 and 53pb) and therefore to genotype TT. M= molecular weight marker.

In order to verify the frequency of C and T alleles in general population of MG patients compared to healthy controls, we evaluated allelic and genotypic frequencies from both populations. Table IV represents allelic and genotypic frequencies of the polymorphism *C3435T* of the MDR1 gene in studied MG patients and healthy individuals and Table V represents risk associated of the polymorphism *C3435T* of the MDR1 gene in both patients and controls (Tables IV and V).

From genotyping analysis, we observe that CC genotype is much more frequent in patients comparing with healthy controls (57% *vs* 18%, respectively). CT genotype revealed to be the most common genotype for both patients and healthy controls (51% *vs* 55%, respectively) and TT the most rare genotype (12% vs 27%, respectively).

On the other hand, allele C was more frequent than allele T in patients (63% *vs* 37%, respectively) and the opposite was true for healthy controls (allele T more frequent than allele C- 55% *vs* 45% respectively) (Table IV).

Table III: Allelic and genotypic distribution of the MDR1 gene *C3435T* polymorphism in MG patients and controls

	Alleles		Genotypes		
	C n (%)	**T n (%)**	**CC n (%)**	**CT n (%)**	**TT n (%)**
Patients	54 (63%)	32 (37%)	16 (37%)	22 (51%)	5 (12%)
Controls	46 (45%)	56 (55%)	9 (18%)	28 (55%)	14 (27%)

To analyze if these *MDR* gene polymorphic variations are associated with the risk of MG development we evaluated the Odds ratio, using the Fisher test. As we can observe in Table V, the genotype CC may confer an almost threefold increased risk of MG facing the other genotypes (CT and TT), since it is much more frequent in patients compared to healthy controls (odds ratio = 2,756; CI: 1,070-7,145; p= 0,0378).

Table IV: Evaluation of the risk associated with the polymorphism *C3435T* of MDR1 gene in MG patients and controls

Genotype	Patients n(%)	Controls	*Odd's ratio (CI 95%)*	*p*
CC	16 (37%)	9 (18%)	2.756 (1.070- 7.145)	0.0378
CT	22 (51%)	28 (55%)	0.8605 (0.3814-1.942)	0.8360
TT	5 (12%)	14 (27%)	0.3477 (0.1138- 1.063)	0.0729

We also evaluated genotype frequency within the sub-groups of MG patients (MGUS, MM and IMM). We observed that the highest frequency of *MDR1 (C3435T)* variant genotype CC was observed in MGUS patients (47% when compared to 35% in MM patients), while in MM patients, the most common genotype was CT (Figure 16).

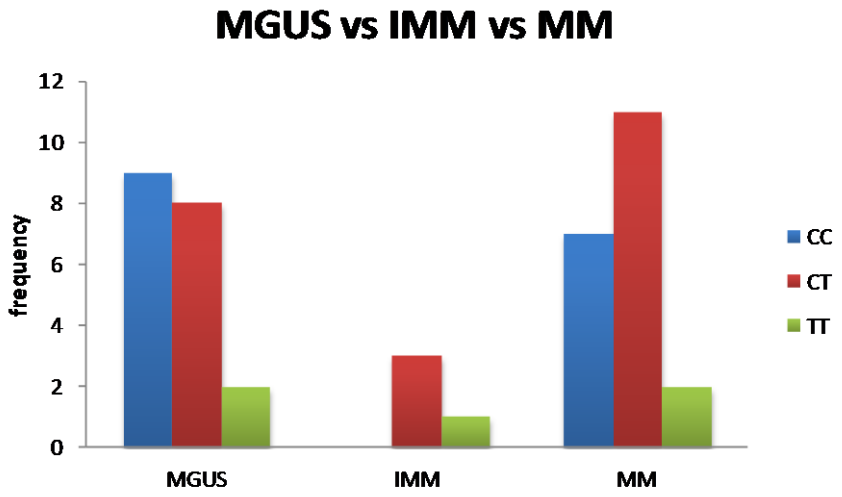

Figure 16. MDR genotypic frequencies in patients in different stages of MM disease. MGUS patients present the higher frequency of CC genotype (47% *vs* 35% in MM); CT genotype is the most frequent in MM patients and TT the less represented genotype in all stages of disease.

3. Evaluation of membrane MDR1 and MRP proteins expression and intracellular UC and NF-kB expression in MG patients and non-neoplastic controls by flow cytometry

To study the distribution of MDR1 protein (Pgp) among the plasma cell subpopulations ($CD138^+$) in bone marrow of Monoclonal Gammopathy patients and non-neoplastic controls, a 4-color labeling procedure was performed and the expression levels and percentage of positive cells for each protein were analyzed on gated $CD19^+$ and $CD19^-$ plasma cells (Figure 17).

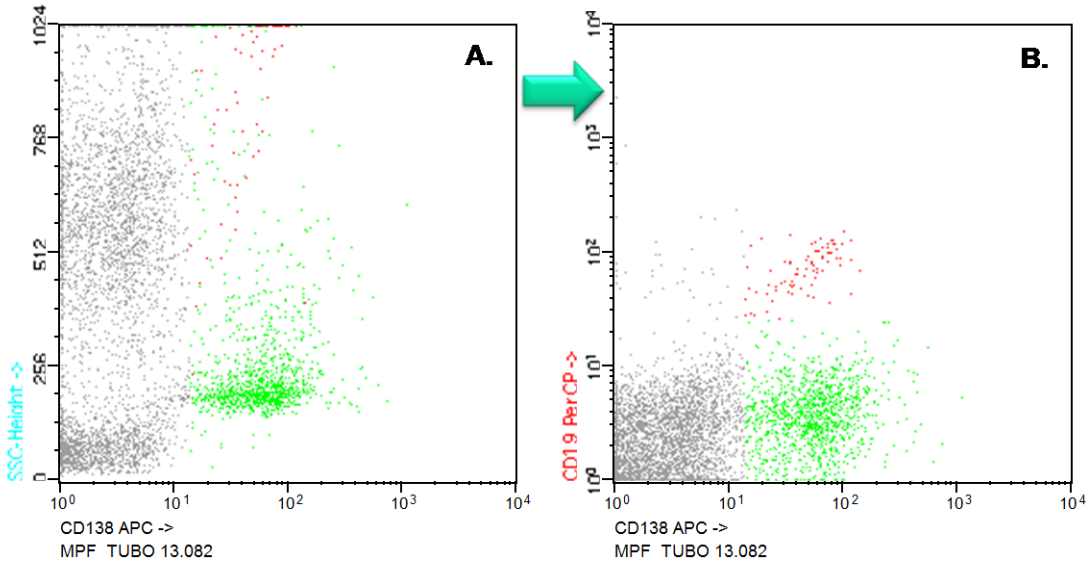

Figure 17. Identification of plasma cell subpopulations from bone marrow of a patient with Monoclonal Gammopathy by flow cytometry. After gating plasma cell ($CD138^+$ cells), the normal ($CD138^+/CD19^+$) and malignant plasma cells ($CD138^+/CD19^-$) are discriminated based on CD19 expression. **(A)** Dot Plot representing the identification of bone marrow plasma cells from a MG patient bases on membrane antigen CD138 expression. **(B)** Dot Plot representing the identification of normal and neoplastic bone marrow plasma cells from a MG patient based on the expression of CD19 membrane antigen of (green= neoplastic, PCs $CD138+/CD19^-$; red= normal, PCs $CD138+/CD19^+$). (Cells were labelled with monoclonal antibodies anti-CD138 and anti-CD19 as previously described in material and methods section).

After the identification of plasma cells subpopulations, we analyzed the percentages of total, normal and neoplastic plasma cells in bone marrow of MG patients and controls (Figure 18). As shown in Figure 18-A, we observed a significantly increase in percentage of plasma cells in MG patients, when compared to non-neoplastic controls (Non-neo Ctls). We also observed that neoplastic plasma cells (Neo PCs) were predominant in MG patients (Figure 18-B) and particularly in MM patients (Figure 18-C). However, we did not find significant differences on the percentage of neoplastic plasma cells between the different subtypes of plasma cell disorders (Figure 18-D).

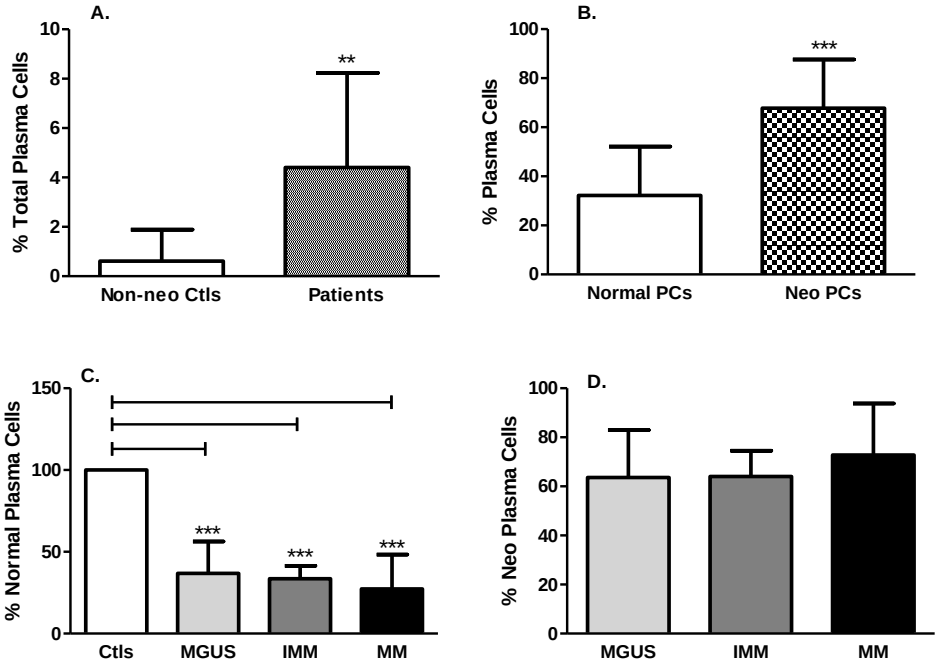

Figure 18. Representation of plasma cell populations in Monoclonal Gamopathies patients and non-neoplastic controls. Plasma cells were gated from the total cell population as described in Figure 17. **(A)** Percentage of total plasma cells (Controls *vs* MG patients); **(B)** Percentage of Plasma cells (PCs) in MG patients (normal PCs *vs* neoplastic PCs); **(C)** Percentage of normal population of PCs in MGUS vs IMM vs MM); **(D)** Percentage of neoplastic PCs (MGUS vs IMM vs MM). Results are expressed in percentage (%) (A and C) or in Mean Fluorescence Intensity (MIF) (B and D) and represents the mean ±SD of each represented sample population (** p<0,01; *** p<0,001).

Next, we analyzed the expression levels of MDR1, MRP, ubiquitin conjugates (UC) and NF-kB proteins, in normal plasma cells ($CD138^+/CD19^+$) of both Monoclonal Gammopathy patients and non-neoplastic controls. Then we performed the same analysis only in MG patients, regarding the two plasma cell (PCs) populations: normal and neoplastic (Figures 19-22). In figure 19, we observe that normal and neoplastic PCs in MG patients, tend to present increased percentage of cells and higher levels of membrane MDR1/P-glycoprotein (PgP) expression (MIF) when compared with PCs in non-neoplastic controls (Figure 19-A and B, respectively) . On the other hand, when we compared normal and neoplastic PCs in MG patients, we observed increased levels of PgP expression in neo PCs (statistically significant, p<0,001), (Figure 19-C and D, respectively).

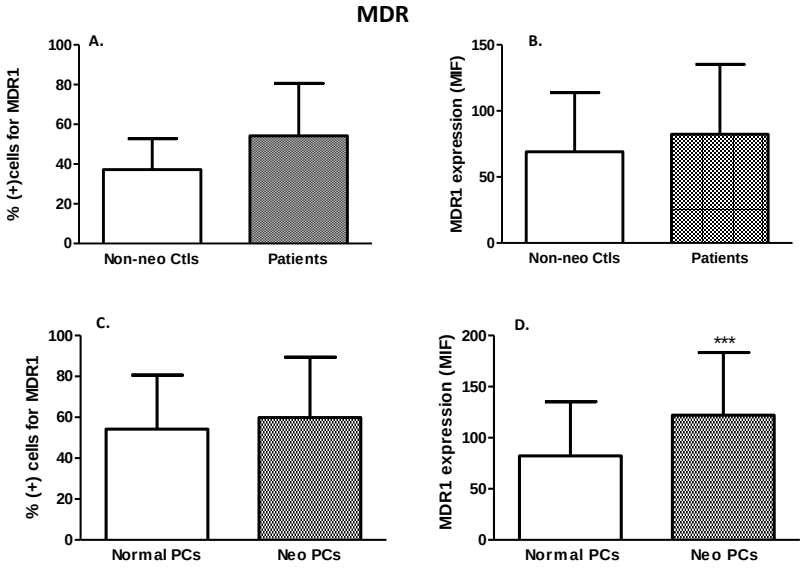

Figure 19. Expressing plasma cells and expression levels of MDR1 protein. MDR1, MRP1, UC and NF-KB expression were detected by specific antibodies staining and flow cytometry analysis. Plasma cells were gated for the intact cell population as shown in Figure 17, and 50 000 events were acquired per sample. **(A)** Percentage of PCs expressing MDR1 protein in non-neoplastic controls (non-neo Ctls) *vs* MG patients; **(B)** MDR1 expression levels in non-neo Ctls *vs* MG patients; **(C)** Percentage of PCs expressing MDR1 protein in normal PCs *vs* neoplastic PCs in MG patients; **(D)** MDR1 protein expression levels in normal PCs *vs* neoplastic PCs of MG patients. Results are expressed in percentage (%) (A and C) or in Mean Fluorescence Intensity (MIF) (B and D) and represents the mean ±SD of each represented sample population (*** p<0,05).

Concerning levels of MRP expression, we could observe an increased percentage of normal PCs expressing this protein in MG patients (statistically different from non-neoplastic controls, p<0,001), along with higher levels of membrane expression (p<0,05) (Figure 20-A and B, respectively). However, it is also noticed that there is a significant impairment of MRP membrane expression levels and percentage of positive cells in neoplastic PCs when we compare them with normal ones in MG patients (p<0,001) (Figure 20-D and C, respectively).

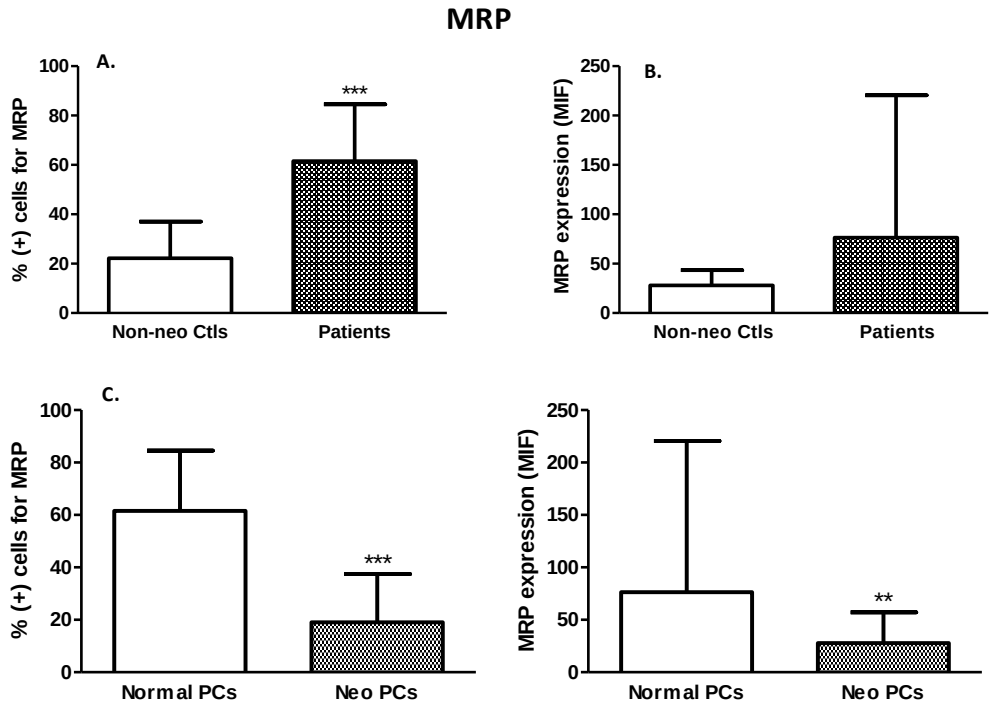

Figure 20. Expressing plasma cells and expression levels of MRP protein. **(A)** Percentage of plasma cells (PCs) expressing MRP protein in non-neoplastic controls (non-neo Ctls) *vs* MG patients; **(B)** MRP expression levels in non-neoplastic controls *vs* MG patients; **(C)** Percentage of PCs expressing MRP protein in normal PCs *vs* neoplastic PCs in MG patients; **(D)** MRP protein expression levels in normal PCs *vs* neoplastic PCs in MG patients. Results are expressed in percentage (%) (A and C) or in Mean Fluorescence Intensity (MIF) (B and D) and represents the mean ±SD of each represented sample population (**p<0,05, ***p<0,001).

Besides MDR and MRP expression, we also assessed expression levels of Ubiquitin Conjugates (UC) and NF-kB (Figures 21 and 22, respectively). As we can observe in these figures, there is an increased number of PCs expressing these markers in normal PCs in MG patients when compared with those cells in non-neoplastic controls. Figures 21-A and 22-A, respectively, show that UC and NF-kB expressing cells in MG patients, are significantly higher than in controls (UC, p<0,01; NF-kB, p<0,001). However, we observed a slightly decrease in intracellular UC and NF-kB expression levels in neoplastic PCs compared with those detected in normal PCs (Figure 21-B and 22- B, respectively).

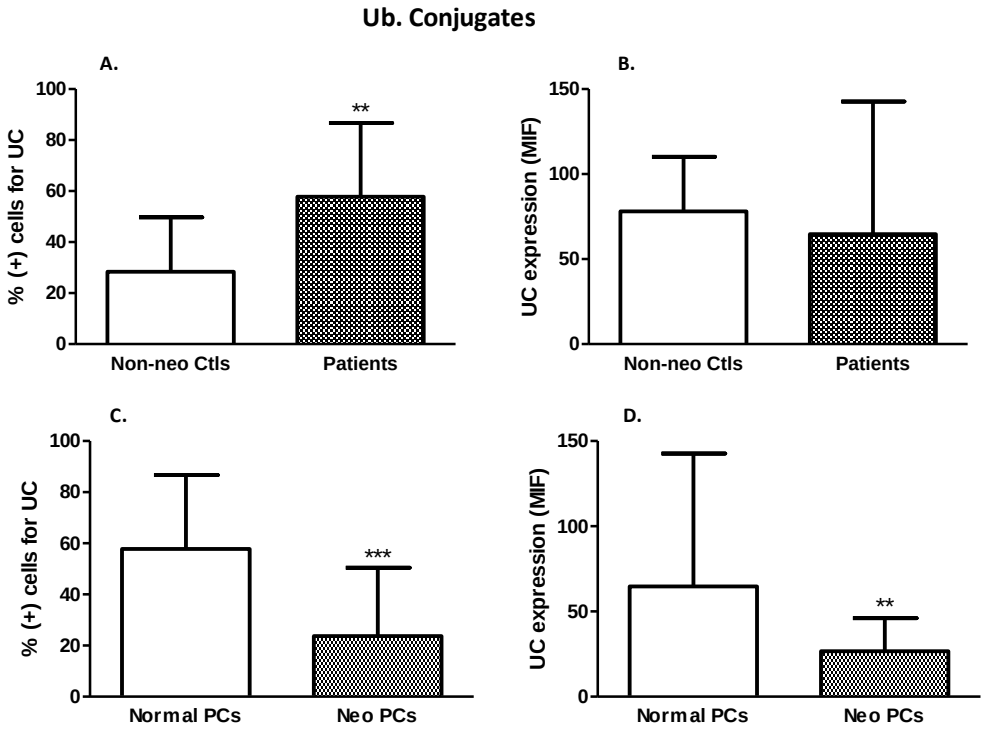

Figure 21. Expressing plasma cells and expression levels of UC proteins. **(A)** Percentage of plasma cells (PCs) expressing ubiquitin conjugates protein (UC) in non-neoplastic controls (non-neo Ctls) *vs* MG patients; **(B)** UC expression levels in non-neoplastic controls *vs* MG patients; **(C)** Percentage of PCs expressing UC protein in normal PCs *vs* neoplastic PCs in MG patients; **(D)** UC expression levels of in normal PCs *vs* neoplastic PCs in MG patients. Results are expressed in percentage (%) (A and C) or in Mean Fluorescence Intensity (MIF) (B and D) and represents the mean ±SD of each represented sample population (**p<0,01, ***p<0,001).

Comparing patients' normal PCs with neoplastic PCs, we observe a decrease in UC expression levels in the latter (statistically different from normal PCs, p<0,01) and in the percentage of cells expressing these conjugates compared with normal PCs (p<0,001) (Figure 21-C and D, respectively). Taking into account PCs expressing NF-kB and its expression levels, we observed increased percentage of positive cells (p<0,01) along with decreasing levels of intracellular expression of this transcription factor, in patients, when compared to controls (Figure 22-C and D, respectively).

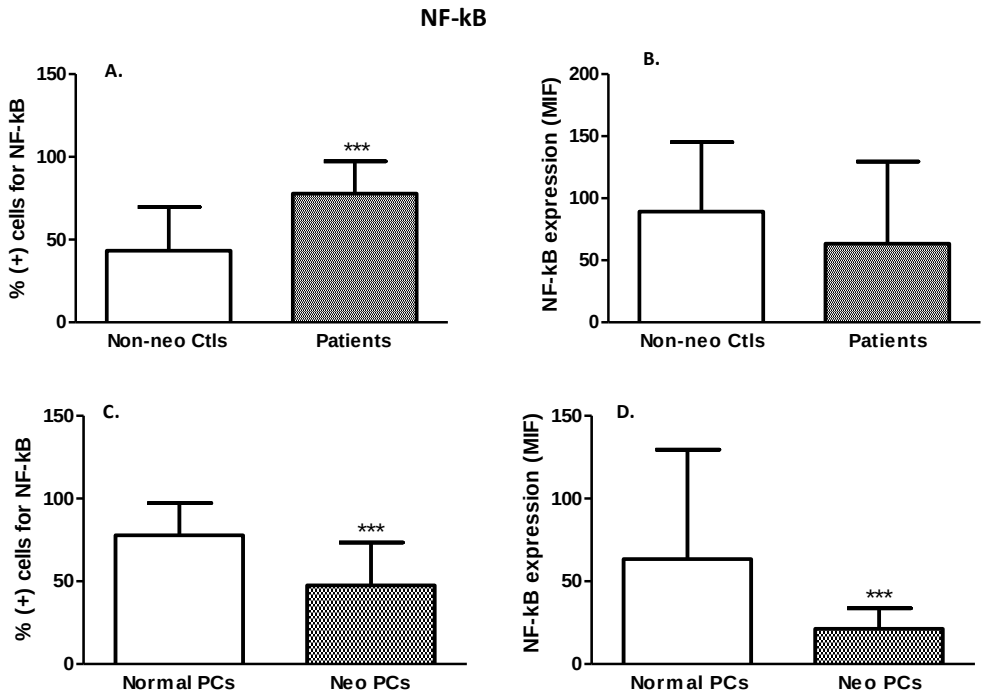

Figure 22. Expressing plasma cells and expression levels of NF-kB. **(A)** Percentage of plasma cells (PCs) expressing NF-kB in non-neoplastic controls (non-neo Ctls) *vs* MG patients; **(B)** NF-kB expression levels in non-neoplastic controls *vs* MG patients; **(C)** Percentage of PCs expressing NF-kB proteins in normal PCs vs neoplastic PCs of MG patients; **(D)** NF-kB expression levels in normal PCs *vs* neoplastic PCs in MG patients. Results are expressed in percentage (%) (A and C) or in Mean Fluorescence Intensity (MIF) (B and D) and represents the mean ±SD of each represented sample population (**p<0,01, ***p<0,001).

Thereafter, we analyzed the expression levels of MDR1, MRP1 (Figure 23), ubiquitin conjugates and NF-kB (Figure 24) in normal plasma cells ($CD138^+/CD19^+$) (Figures 23

and 24) and neoplastic plasma cells ($CD138^+/CD19^-$) (Figure 25), according to the different MG subgroups (MGUS, IMM and MM).

We observed a significantly increased number of cells expressing MRP1 (p<0,001), Ubiquitin conjugates (p<0,01) and NF-kB (p< 0,001) in MM when comparing with non-neoplastic controls (Figure 23-C and D and Figure 24 respectively). MDR and MRP expression levels were higher in MM but both UC and NF-kB expression levels are higher in controls (Figure 23 and 24). Neoplastic PCs have higher expression levels of MDR, but lower expression of the other proteins (Figure 25).

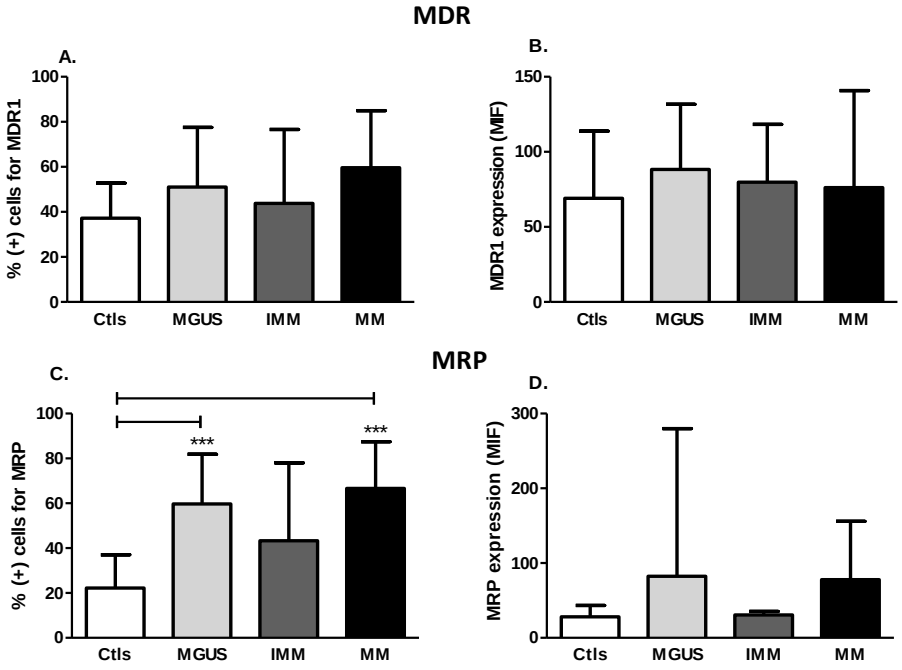

Figure 23. Expressing PCs and expression levels of MDR1 and MRP1 proteins in sub-groups of MG patients (MGUS, IMM and MM) and non-neoplastic controls. MDR1, MRP1, UC and NF-KB expression were detected by specific antibodies staining and flow cytometry analysis. Plasma cells were gated for the intact cell population as shown in Figure 17, and 50 000 events were acquired per sample. Results are expressed as a percentage of untreated controls. **(A)** Percentage of PCs expressing MDR1 protein; **(B)** Expression levels of MDR1; **(C)** Percentage of PCs expressing MRP protein; **(D)** Expression levels of MRP protein (***p<0,001).

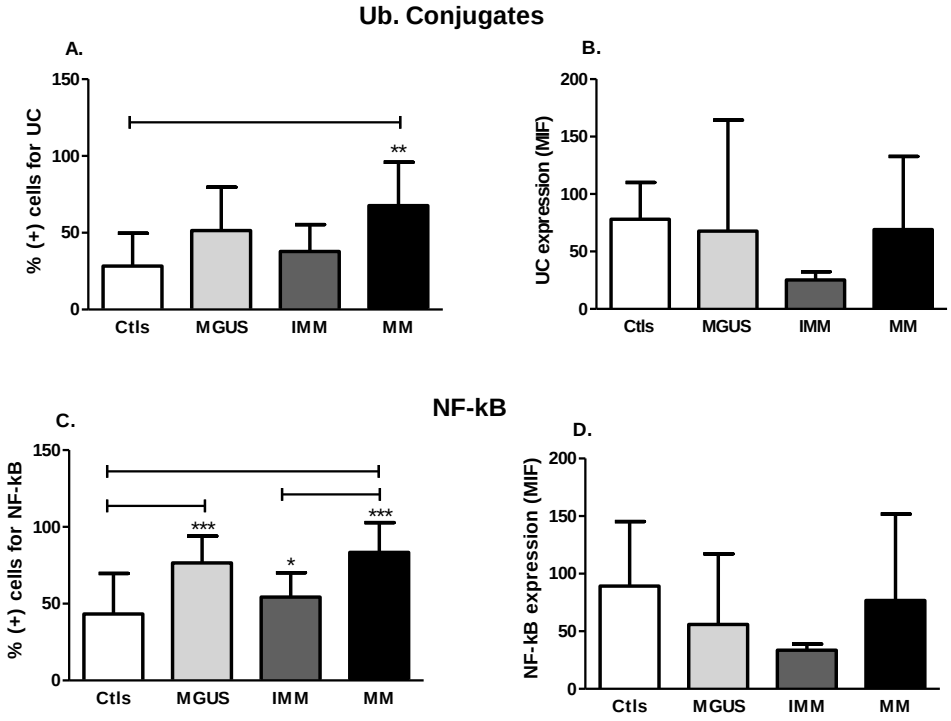

Figure 24. Expressing PCs and expression levels of MDR1 and MRP1 proteins in sub-groups of MG patients (MGUS, IMM and MM) and non-neoplastic controls. MDR1, MRP1, UC and NF-KB expression were detected by specific antibodies staining and flow cytometry analysis. Plasma cells were gated for the intact cell population as shown in Figure 17, and 50 000 events were acquired per sample. **(A)** Percentage of PCs expressing MDR1 protein; **(B)** Expression levels of MDR1; **(C)** Percentage of PCs expressing MRP protein; **(D)** Expression levels of MRP protein ($p<0,01$; ***$p<0,001$).

Next we analyzed the same parameters but only regarding neoplastic PCs population in all three sub-group stages (MM, MGUS and IMM) (Figure 25). Although, none of the following results are statistically significant, when concerning neoplastic population of PCs. However, in MGUS patients the number of cells expressing the MDR protein and the expression levels tend to be higher compared with the observed in IMM and MM patients. Regarding MRP, UC and NF-kB, there are more PCs cells expressing all of the proteins in MM than in MGUS patients, but the expression levels tend to be lower.

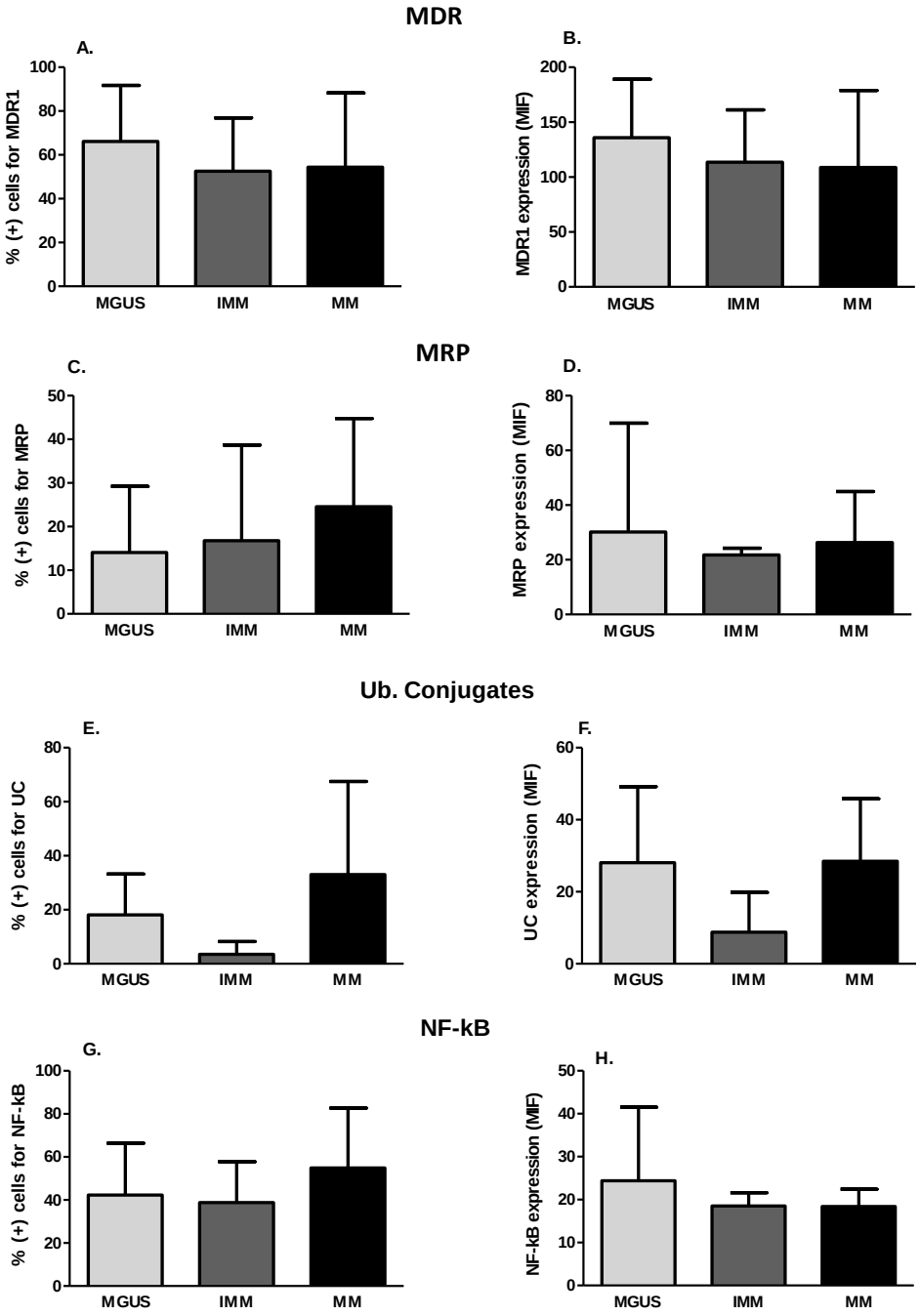

Figure 25. Expressing PCs and expression levels of MDR1, MRP, UC and NF-kB proteins (MGUS vs IMM vs MM). **(A)** Percentage of PCs expressing MDR1 protein; **(B)** Expression levels of MDR1; **(C)** Percentage of PCs expressing MRP protein; **(D)** Expression levels of MRP protein; **(E)** Percentage of PCs expressing UC proteins; **(F)** Expression levels of UC proteins; **(G)** Percentage of PCs expressing NF-kB protein; **(H)** Expression levels of NF-kB.

Finally, we analyzed MDR1, MRP, UC and NF-kB proteins in normal and neoplastic PCs in each sub group of patients. In the general context (data not shown), neoplastic populations in each group of patients always presented significantly lower expression levels and number of cells expressing the proteins, except for MDR1. Concerning MDR1 (Figure 26), MGUS patients were the only group in which we could observe significantly increased number of neoplastic PCs expressing this protein than normal PCs (*p<0,05) and expression levels also increased (**p<0,01) (Figures 26-C and D).

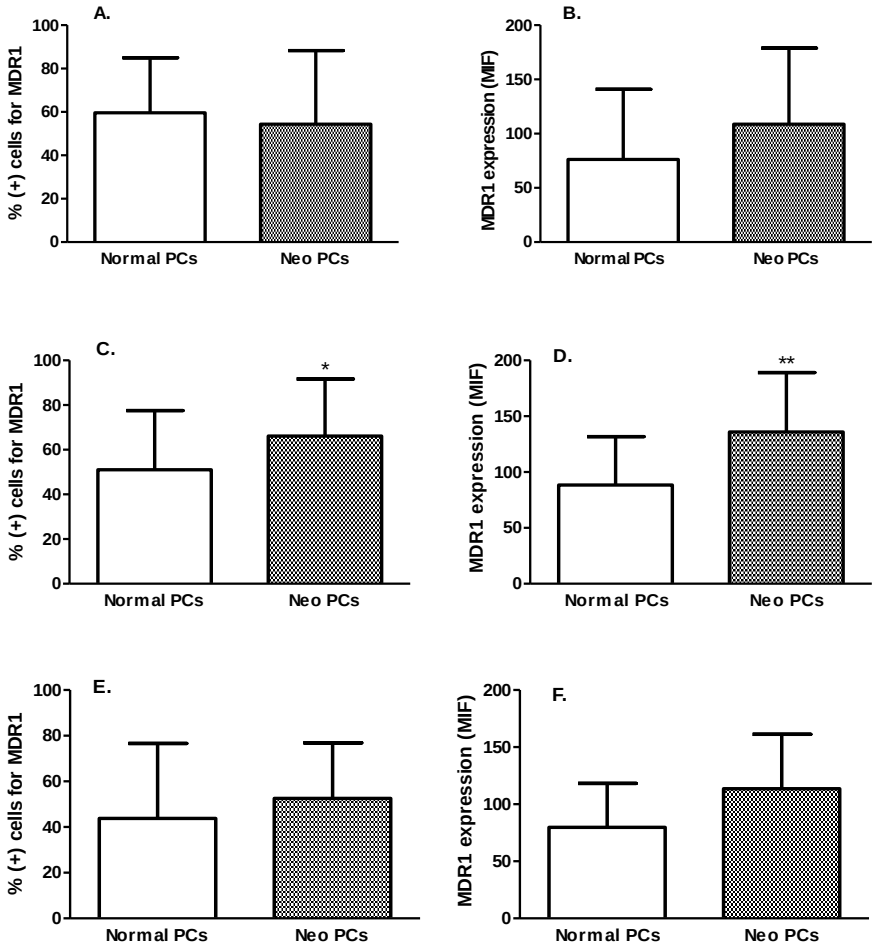

Figure 26. Comparative evaluation between the MDR1 expressing cells and expression levels in normal and PCs in MGUS, IMM and MM. **(A)** Percentage of PCs expressing MDR1 protein in MM; **(B)** Expression levels of MDR1 in MM; **(C)** Percentage of PCs expressing MRP protein in MGUS; **(D)** Expression levels of MRP protein in MGUS; **(E)** Percentage of PCs expressing UC proteins in IMM; **(F)** Expression levels of UC proteins in IMM (*p<0,05; **p<0,01).

Having set the results from the assessment of genotypes and protein expression, we ultimately attempted to establish a relation between MDR1 genotypes and its expression in neoplastic population of plasma cells in MG patients. Regarding this, we could observe that between genotypes CC and CT there was no difference neither in percentage of cells expressing the protein nor in expression levels of Pgp. On the other hand, the highest levels of MDR1 expression/percentage of expressing cells were seen in patients presenting TT genotype, although the number of cells expressing the protein was quite similar for all three genotypes (Figure 27).

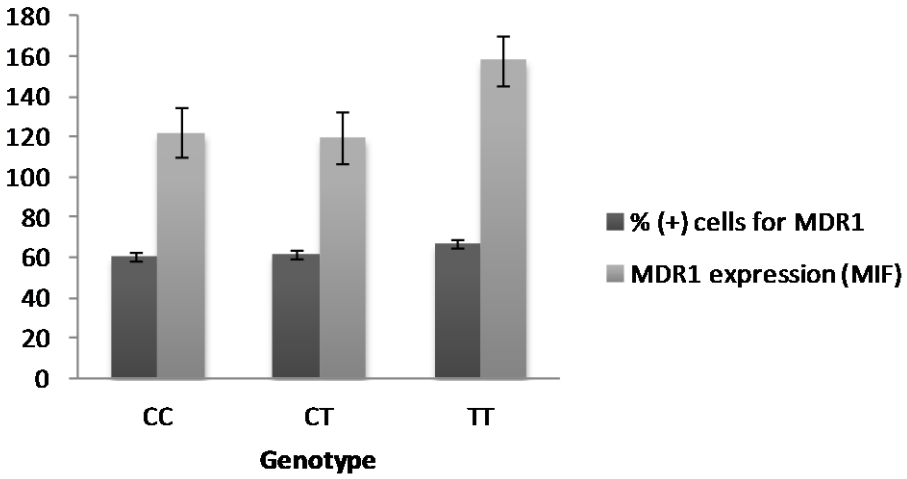

Figure 27. Correlation between MDR1 genotype and MDR1 expression/ percentage of expressing cells in MG patients. After genotyping MG patients and having the expression levels of MDR1 assessed, we compared these two results to evaluate whether or not there was a parallel between them. We could observe than between genotypes CC and CT there was no relevant difference but genotype TT presented increased expression levels of MDR1 protein.

Amongst all MG patients, it was only possible for us to evaluate the follow-up response to therapy in eight of them. Table VI describes which treatment options were taken for each patient according to previous diagnosis as well as the level of response showing the MDR1 expression/percentage of expressing cells in normal and neoplastic plasma cell populations. We could observe that within the two patients with very good partial response (VGPR), presents higher levels of MDR1 in neoplastic PCs when compared to patients with partial response (PR). In the general context, three over the four patients presenting the highest levels of MDR1 expression match the ones treated with bortezomib combined regiments and had partial response to therapy. There was only a single patient in which progression of disease could be attested and corresponds to a CC genotype, which, as seen before, shows an approximately threefold risk for patients to develop malignancy but response to therapy could not be assessed.

However, these results are not significant because they refer to a restricted number of patients by now, as the other patients' follow-up was not yet assessed.

Table VI- Correlation between treatment strategies/response and MDR1 expression, in eight of MG patients

Patient	*Diagnostic*	*Genotype*	*Therapy*	*Response*	*% (+) cells*	*MDR1 MIF (normal PCs)*	*% (+) cells*	*MDR1 MIF (neo PCs)*
1	MM	TT	MPT	VGPR	81	81	78	179
2	MM	Na	Bort-Dex	VGPR	84	65	97	170
3	IMM	CT	Cy-Dex + Bort-Dex	PR	36	39	64	172
4	MM	Na	MPT	PR	62	247	18	15
5	MM	CC	MP	PR	60	24	6	17
6	MM	CT	Bort-Dex	PR	20	67	90	129
7	MM	Na	MP	PR	87	26	13	17
8	IMM	CT	Cy-Dex + MPT	PR	58	123	16	89
9	MM	CC	Len-Dex	Progression	89	55	90	157

Na= non-analyzed (genotype could not be assessed due to lack of sample volume for DNA extraction or non purity of obtained DNA); **MPT** = Melphalan/Prednisolone/Thalidomide; **Bort** = Bortezomib; **Dex** = Dexamethasone; **Cy**= Cyclosporine; Len= Lenalidomide; **VGPR** = very good partial response; **PR** = partial response.

Part V: Discussion

Current models assume that MM evolves through a multistep transformation process being the first pathogenic step a premalignant monoclonal gammopathy of undetermined significance (MGUS). With progression of MGUS to Myeloma, complex genetic and epigenetic events occur in the neoplastic plasma cell and in the BM microenvironment (Bergsagel and Kuehl, 2005).

NF-kB was originally described as a B-cell transcription factor, which is required for proper regulation of normal B cell differentiation. NF-kB was also found constitutively active in primary MM cells, and blockade of this transcription factor leads to apoptosis. The ubiquitin-proteasome pathway plays an essential role in regulating the intracellular concentration of specific proteins, thereby maintaining homeostasis within cells. Inhibition of the 26S proteasome prevents this targeted proteolysis, which can affect multiple signaling cascades within the cell. This critical cellular function has been successfully targeted for cancer therapy, as highlighted by the efficacy of proteasome inhibitor bortezomib in a wide spectrum of hematological neoplasias, namely MM (Bommert *et al*., 2006; Bross *et al*., 2004)

Despite promising clinical activity, some patients with multiple myeloma failed to respond to bortezomib therapy (nearly a third of the patients). Moreover, the efficacy for bortezomib may differ between tumor types. Understanding the mechanisms of resistance to proteasome inhibition will not only allow better use of proteasome inhibitors such as bortezomib, but should also allow the rational design of synergistic drug combinations (Oerlemans *et al.*, 2008; Ross *et al*., 2005).

Malignant cells may develop several mechanisms to escape the effects of proteasome inhibition, including alterations in the proteasome complex itself, leading to decreased function, increasing the efficiency of alternate mechanisms of protein degradation or modulation cell signaling pathways that are affected by proteasome inhibition (Oerlemans *et al.*, 2008). On the other hand, bortezomib is primarily oxidatively metabolized via the cytochrome P450 enzymes, CYP3A4, CYP2C19, CYP1A2, being

CYP3A4 and CYP2C19 (Pekol *et al*., 2005; Uttamsingh *et al*., 2005) those affecting the sensitivity and resistance to this proteasome inhibitor.

Other possible factor is variant multiple drug resistant (MDR) genotypes, which have been associated with differential expression of the MDR1 gene and its 170 kDa protein product, P-glycoprotein, also known as the ATP binding cassette Transporter B1 (ABCB1). Indeed, the three most common MDR1 SNPs, *C3435T*, *C1236T*, and *G2677T/A*, have been found to influence the outcomes of myeloma treated patients. Moreover, overexpression of MDR1 in myeloma cells might contribute to treatment failure in patients receiving proteasome inhibitors, since Pgp conferred bortezomib resistance in some preclinical models (Buda *et al*., 2009). Therapy resistance may also be mediated by another multidrug resistance associated protein, also known as ATP binding cassette, subfamily C, member 1 (MRP1, or ABCC1) (Buda *et al*., 2010).

Recent studies have identified mutations involving genes associated with regulation of NF-kB pathways that result in constitutive activation of the NF-kB pathway. Cells that carry these mutations appear to be particularly sensitive to the effects of proteasome inhibition, a finding that could allow us to tailor the use of this class of drugs in the future (Kumar and Rajkumar, 2008).

Understanding the mechanisms of resistance to proteasome inhibition will not only allow a better use of proteasome inhibitors such as bortezomib, but should also allow the rational design of synergistic drug combinations.

With this study we wanted to evaluate some molecular mechanisms involved in predisposition of MG and in the transition from MGUS to Multiple Myeloma that could also contribute to sensitivity and/or resistance to therapy. In particular we tried to better understand the role of MDR1 (*C3435T*) and CYP3A4 (*T673C*) polymorphisms, and the expression levels of ABC proteins (MDR1 and MRP), ubiquitin conjugates and of the transcription factor NF-kB.

To achieve the first aims of this project we evaluated the role of MDR1 (*C3435T*) and *CYP3A4*2* (*T673C*) polymorphisms in MG and the prognostic value in response to therapy of MGUS/IMM/MM patients. We have analyzed 43 patients simultaneously for the presence of these two SNPs as well as in 43 healthy controls.

Pgp appears to work in concert with CYP3A4 by improving presentation of drugs (specifically xenobiotics) in the intestine (Suzuki and Sugiyama, 2000). Both are promiscuous proteins with a large range of substrates and a marked overlap in substrate specificity (Dussault and Forman, 2002). The transcription of their corresponding genes is induced by xenobiotics through a common nuclear receptor (PXR- pregnane X receptor). *CYP3A4*2* harbour an exonic SNP (*T673C*) that was associated with an altered catalytic activity, with a six-fold increase in K_m for a calcium channel blocker and MDR1 *C3435T* SNP was associated with diminished Pgp function (Sakaeda, 2005). CYP3A4/MDR1 variant associations may exist, due to the metabolic relationship of their products (Cavaco *et al*., 2003).

In our study, we could observe that there was no variance in *CYP3A4*2* polymorphic form in both patients and controls, all of them presenting the same restrictive pattern in PCR-RFLP consisting in revelation of allele T (2 bands in gel). This is described as the most common allelic form for *CYP3A4*2* and one of the most frequent in Portuguese population (Cavaco *et al*., 2003). Hence, these results had nothing to add to our study since it showed no difference in patients and controls and cannot be correlated with disease. However, this analysis should be performed again to a broader range of samples.

It is generally accepted that inherited variation in transport and metabolism of environmental toxins can determine malignant transformation. Due to the wide spectrum of drugs and carcinogens being Pgp (MDR1) substrates, significant literature has been published on the relation of MDR1 SNPs with pharmacokinetics and predisposition to diseases (Sakaeda, 2005). (Jamroziak *et al.*, 2009) and others

showed that MDR1 *C3435T* SNP is associated with susceptibility to cancer, including pediatric acute lymphoblastic leukemia and renal epithelial tumors (Jamroziak *et al.*, 2009).

From genotyping analysis we observed that CC genotype may confer an almost threefold increased risk to developed MG, facing the other genotypes, since it is much more frequent in patients comparing with healthy controls (odds ratio= 2,756; CI: 1,070-7,145; p= 0,0378). Possibly it can predispose individuals to develop these type of malignancies. CT genotype revealed to be the most common genotype for both patients and healthy controls and TT genotype the rarest. This last should confer protection since its frequency is higher in controls when compared to CC genotype (odds ratio = 0,3477; CI: 0,1138-1,063; p=0,0729).

Regarding MDR1 polymorphism *C3435T,* also described as the most frequent in Portuguese population, we could observe that allele frequencies and distributions of this MDR1 SNP were comparable in MG patients and healthy controls. There was just a slightly significant variance of the CC genotype in patients comparing to controls ($p<0,05$). Moreover, Allele C frequencies were also significantly different ($p<0,05$) between patients and controls. The results suggest that allele C could predispose to disease, once it is more frequent in patients, while allele T may have a protective role. The sub group of patients presenting the highest frequency of MDR genotype CC was MGUS (47% when compared to 35% in MM patients), raising the question that CC genotype could be related to cases presenting early detectable Monoclonal Gammopathy rather than development/arising *de novo* symptomatic MM stage of disease.

MM patients differ in type and amount of past carcinogen exposure. A recent *ex vivo* study shows that silent MDR1 polymorphisms (specifically *C3435T*) can modify Pgp conformation and thus polymorphisms' effect can be substrate specific. These results raise the question that functional effect of this polymorphism may be just relevant

among subjects undergone specific carcinogenic exposure. Furthermore, a recent report shows that survival rate of MM patients treated with Pgp substrate drugs is dependent upon *C3435T* and *T2677G* SNPs (Maggini *et al*., 2008).

To characterize the percentage and expression levels of ABC proteins (MDR1 and MRP), NF-kB and UC in plasma cells we had first identified plasma cells and analyzed these proteins in normal and neoplastic plasma cells, by flow cytometry. As expected, the percentage of total plasma cells in controls was lower than in MG patients and within those, neoplastic plasma cells were higher than the amount of normal ones. There was also a significant difference between the percentages of total PCs of controls compared to MM diagnosed patients. Neoplastic PCs in MGUS patients tended to be lower compared to the same population in MM patients.

In general, normal population of PCs in MG patients had higher expression of proteins compared to non-neoplastic controls but, taking into account normal and neoplastic PCs from patients, the latter had lower expression of all proteins, except for MDR1. This suggests that normal PCs in patients may no longer have a real normal phenotype and can represent a transition stage to neoplastic cells, influencing their behavior.

The lifetime risk of progression into symptomatic multiple myeloma lies between 15% and 59% for patients with MGUS or SMM (Hillengass *et al*., 2011). The ratio between normal and neoplastic PCs has been identified to make differential diagnosis between MGUS and MM. A study showed that MGUS group of patients had ≥ 20% of normal PCs to total BM PCs whereas no MM patients had had ≥ 20% of normal PCs (Sezer *et al*., 2001). However, this ratio is hard to assess because MM is a very diffuse and focal disease and the total tumor cell burden may appear spuriously low in a hemodiluted BM aspirate, which means that when a BM aspirate is performed, it may not get representative sample of BM cells, including neoplastic plasma cells.

Concerning neoplastic PCs, we observed in MM a decrease in the percentage of cells and weaker general expression of efflux transporters comparing with MGUS. On the

other hand, in all these patients benign PC population show higher Pgp and MRP expression levels in relation with neoplastic PCs. There were also a higher percentage of cells expressing MRP in controls comparing to MGUS and MM patients ($p<0,001$) but slightly lower percentage in MGUS comparing to MMs. These results suggest that may be occurring changes in ABC proteins expression thus influencing malignant transformation within stages.

Concerning the number of cells expressing UC and NF-kB, an increased number of cells expressed both of them in neoplastic PCs from MM patients compared to other subgroups. Comparing patients and controls this difference is also noticed, patients having increased number of expressing cells. UC should reflect ubiquitination and proteasome function. As referred previously, NF-kB was found constitutively active in primary MM cells and blockade of this transcription factor leads to apoptosis by bortezomib/other proteasome inhibitors. On the other hand, another study shows that primary MM cells frequently harbor proteasome inhibitor-resistant pathway (PIR) activity, further enhanced by the presence of patient-derived BM stromal cells (BMSCs) (Markovina *et al.*, 2008). Our results do not show relevance in expression levels but differences in the number of expressing cells may be likely relevant to drug resistance development in some patients since interaction with BMSCs can potentiate PIR pathway activity, further studies need to elucidate molecular mediators of BMSCs-induced PIR, to try inhibiting this interaction and allow proteasome inhibitors to effectively block NF-kB.

Regarding MDR1 genotypes and its protein expression altogether, the highest levels of MDR1 expression/percentage of expressing cells were seen in patients presenting TT genotype with also a VGPR. However, high levels of MDR1 expression were also seen in patients with CC/CT genotype. As discussed previously, MDR1 *C3435T SNP* is associated with decreased Pgp function, which is related to an impaired metabolism of drugs including proteasome inhibitors (bortezomib) and others.

A previous report suggested that may be a predictive value of MDR1 genotypes in modifying the outcome of advanced myeloma. In particular, in MDR1 *C3435T SNP*, the allele T has been found to be associated with weaker drug efflux activity. It may be explained by a less efficient doxorubicin efflux, resulting in a longer drug exposure than CC or CT genotype carriers and consequently a greater level of cytotoxicity (Buda *et al.*, 2010). Therefore, TT carriers should be expected to show a better response to chemotherapy, which is also seen in our study, although it presents high levels of MDR1 expression.

Our results are suggestive about the potential role for MDR1 SNP in modulating the outcome in MG patients. However, the range of individuals studied and followed in terms of therapy is not very wide which unable direct conclusions at this moment. For that reason, there is a strong need for further enlarge study population to confirm if these results remain true for a wider range of MG patients, with the ultimate goal of using these data to tailor therapy to the genetic makeup of individual patients.

Part VI: Conclusions & Future Perspectives

In Western countries, the frequency of Myeloma is likely to increase in the near future as the population ages. Ongoing studies are incorporating thalidomide, lenalidomide, or bortezomib in treatment approaches to further improve outcomes by defining combinations associated with maximal tumor reduction, evaluating consolidation or maintenance therapies that delay tumor regrowth, and determining which regimens provide a benefit with favorable side-effect profiles in specific subgroups of patients.

Efforts are under way to develop risk-adapted strategies in which therapies may be based on knowledge of genetic polymorphisms or mutations that modulate molecular pathways that underlie disease pathogenesis. New proteasome inhibitors, immunomodulatory drugs and humanized monoclonal antibodies are also being currently investigated in clinical trials.

In conclusion, our data confirm the highest content of malignant PCs in MM and suggest that during progression from MGUS to MM, changes in ATP-binding cassette transporters, NF-kB and UC occurs which may influence the neoplastic PCs behavior. On the other hand, *MDR1* polymorphisms may determine the risk to develop these plasma neoplastic diseases and can influence disease evolution. However, this study will take into account patients' follow-up allowing the evaluation of the prognostic and prediction of sensitivity or resistance to therapy.

If confirmed by prospective studies, these data suggest that such SNP analysis along with assessment of molecular markers could be used to identify patients with an increased likelihood of benefiting from rationally design regiments including proteasome inhibitors such as bortezomib.

Part VII: References

Adams, J. (2003). Potential for proteasome inhibition in the treatment of cancer. Drug Discov Today *8*, 307-315.

Adams, J. (2004). The proteasome: a suitable antineoplastic target. Nat Rev Cancer *4*, 349-360.

Alberts, B., Wilson, J.H., and Hunt, T. (2008). Molecular biology of the cell, 5th edn (New York, Garland Science).

Anderson, K.C., Alsina, M., Bensinger, W., Biermann, J.S., Chanan-Khan, A., Cohen, A.D., Devine, S., Djulbegovic, B., Gasparetto, C., Huff, C.A.*, et al.* (2009). NCCN clinical practice guidelines in oncology: multiple myeloma. J Natl Compr Canc Netw *7*, 908-942.

Annunziata, C.M., Davis, R.E., Demchenko, Y., Bellamy, W., Gabrea, A., Zhan, F., Lenz, G., Hanamura, I., Wright, G., Xiao, W.*, et al.* (2007). Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell *12*, 115-130.

Avet-Loiseau, H., Attal, M., Moreau, P., Charbonnel, C., Garban, F., Hulin, C., Leyvraz, S., Michallet, M., Yakoub-Agha, I., Garderet, L.*, et al.* (2007). Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood *109*, 3489-3495.

Avet-Loiseau, H., Soulier, J., Fermand, J.P., Yakoub-Agha, I., Attal, M., Hulin, C., Garderet, L., Belhadj, K., Dorvaux, V., Minvielle, S.*, et al.* (2010). Impact of high-risk cytogenetics and prior therapy on outcomes in patients with advanced relapsed or refractory multiple myeloma treated with lenalidomide plus dexamethasone. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *24*, 623-628.

Bataille, R., Jego, G., Robillard, N., Barille-Nion, S., Harousseau, J.L., Moreau, P., Amiot, M., and Pellat-Deceunynck, C. (2006). The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy. Haematologica *91*, 1234-1240.

Bergsagel, P.L., and Kuehl, W.M. (2005). Molecular pathogenesis and a consequent classification of multiple myeloma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 6333-6338.

Bommert, K., Bargou, R.C., and Stuhmer, T. (2006). Signalling and survival pathways in multiple myeloma. Eur J Cancer *42*, 1574-1580.

Braggio, E., and Renault, I.Z. (2007). Alterações moleculares no Mieloma Múltiplo. Revista Brasileira de Hematologia e Hemoterapia *29*, 10-16.

Bross, P.F., Kane, R., Farrell, A.T., Abraham, S., Benson, K., Brower, M.E., Bradley, S., Gobburu, J.V., Goheer, A., Lee, S.L.*, et al.* (2004). Approval summary for bortezomib for injection in the treatment of multiple myeloma. Clin Cancer Res *10*, 3954-3964.

Buda, G., Martino, A., Maggini, V., Orciuolo, E., Galimberti, S., Gentile, M., Morabito, F., Antognoni, F., Brunori, M., Barale, R.*, et al.* (2009). MDR1 C3435T polymorphism indicates a different outcome in advanced multiple myeloma. Acta Haematol *122*, 42-45.

Buda, G., Ricci, D., Huang, C.C., Favis, R., Cohen, N., Zhuang, S.H., Harousseau, J.L., Sonneveld, P., Blade, J., and Orlowski, R.Z. (2010). Polymorphisms in the multiple drug resistance protein 1 and in P-glycoprotein 1 are associated with time to event outcomes in patients with advanced multiple myeloma treated with bortezomib and pegylated liposomal doxorubicin. Ann Hematol *89*, 1133-1140.

Cavaco, I., Gil, J.P., Gil-Berglund, E., and Ribeiro, V. (2003). CYP3A4 and MDR1 alleles in a Portuguese population. Clin Chem Lab Med *41*, 1345-1350.

Chari, A., Mazumder, A., and Jagannath, S. (2010). Proteasome inhibition and its therapeutic potential in multiple myeloma. Biologics *4*, 273-287.

Chng, W.J., Glebov, O., Bergsagel, P.L., and Kuehl, W.M. (2007). Genetic events in the pathogenesis of multiple myeloma. Best practice & research Clinical haematology *20*, 571-596.

Craig, F.E., and Foon, K.A. (2008). Flow cytometric immunophenotyping for hematologic neoplasms. Blood *111*, 3941-3967.

Cvek, B., and Dvorak, Z. (2008). The value of proteasome inhibition in cancer. Can the old drug, disulfiram, have a bright new future as a novel proteasome inhibitor? Drug Discov Today *13*, 716-722.

Davis, B.H., Foucar, K., Szczarkowski, W., Ball, E., Witzig, T., Foon, K.A., Wells, D., Kotylo, P., Johnson, R., Hanson, C.*, et al.* (1997). U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: medical indications. Cytometry *30*, 249-263.

De La Rubia, J., and Sanz, M.A. (2011). Treatment of multiple myeloma in the elderly: realities and hopes. Leukemia & Lymphoma *52*, 9-14.

Dispenzieri, A., Kyle, R.A., Katzmann, J.A., Therneau, T.M., Larson, D., Benson, J., Clark, R.J., Melton, L.J., Gertz, M.A., Kumar, S.K.*, et al.* (2008). Immunoglobulin free light chain ratio is an independent risk factor for progression of smoldering (asymptomatic) multiple myeloma. Blood *111*, 785-789.

Drain, S., Catherwood, M.A., Orr, N., Galligan, L., Rea, I.M., Hodkinson, C., Drake, M.B., Kettle, P.J., Morris, T.C., and Alexander, H.D. (2009). ABCB1 (MDR1) rs1045642 is associated with increased overall survival in plasma cell myeloma. Leuk Lymphoma *50*, 566-570.

Dussault, I., and Forman, B.M. (2002). The nuclear receptor PXR: a master regulator of "homeland" defense. Crit Rev Eukaryot Gene Expr *12*, 53-64.

Facon, T., Mary, J.Y., Pegourie, B., Attal, M., Renaud, M., Sadoun, A., Voillat, L., Dorvaux, V., Hulin, C., Lepeu, G.*, et al.* (2006). Dexamethasone-based regimens versus melphalan-prednisone for elderly multiple myeloma patients ineligible for high-dose therapy. Blood *107*, 1292-1298.

Field-Smith, A., Morgan, G.J., and Davies, F.E. (2006). Bortezomib (Velcadetrade mark) in the Treatment of Multiple Myeloma. Ther Clin Risk Manag *2*, 271-279.

Fonseca, R., Bailey, R.J., Ahmann, G.J., Rajkumar, S.V., Hoyer, J.D., Lust, J.A., Kyle, R.A., Gertz, M.A., Greipp, P.R., and Dewald, G.W. (2002). Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood *100*, 1417-1424.

Fonseca, R., Bergsagel, P.L., Drach, J., Shaughnessy, J., Gutierrez, N., Stewart, A.K., Morgan, G., Van Ness, B., Chesi, M., Minvielle, S.*, et al.* (2009). International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *23*, 2210-2221.

Galm, O., Herman, J.G., and Baylin, S.B. (2006). The fundamental role of epigenetics in hematopoietic malignancies. Blood Rev *20*, 1-13.

Ghosh, N., and Matsui, W. (2009). Cancer stem cells in multiple myeloma. Cancer Lett *277*, 1-7.

Greipp, P.R., San Miguel, J., Durie, B.G., Crowley, J.J., Barlogie, B., Blade, J., Boccadoro, M., Child, J.A., Avet-Loiseau, H., Kyle, R.A.*, et al.* (2005). International staging system for multiple myeloma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 3412-3420.

Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D.M., Huber, R., Glickman, M.H., and Finley, D. (2000). A gated channel into the proteasome core particle. Nat Struct Biol *7*, 1062-1067.

Gupta, R., Bhaskar, A., Kumar, L., Sharma, A., and Jain, P. (2009). Flow cytometric immunophenotyping and minimal residual disease analysis in multiple myeloma. Am J Clin Pathol *132*, 728-732.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Helsby, N.A., Lo, W.Y., Sharples, K., Riley, G., Murray, M., Spells, K., Dzhelai, M., Simpson, A., and Findlay, M. (2008). CYP2C19 pharmacogenetics in advanced cancer: compromised function independent of genotype. Br J Cancer *99*, 1251-1255.

Hideshima, T., Mitsiades, C., Akiyama, M., Hayashi, T., Chauhan, D., Richardson, P., Schlossman, R., Podar, K., Munshi, N.C., Mitsiades, N.*, et al.* (2003). Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. Blood *101*, 1530-1534.

Hideshima, T., Richardson, P., Chauhan, D., Palombella, V.J., Elliott, P.J., Adams, J., and Anderson, K.C. (2001). The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res *61*, 3071-3076.

Hillengass, J., Moehler, T., and Hundemer, M. (2011). Monoclonal gammopathy and smoldering multiple myeloma: diagnosis, staging, prognosis, management. Recent Results Cancer Res *183*, 113-131.

Hoffbrand, A.V., Moss, P.A.H., and Pettit, J.E. (2006). Essential haematology, 5th edn (Malden, Mass. ; Oxford, Blackwell Pub.).

Jamroziak, K., Balcerczak, E., Calka, K., Piaskowski, S., Urbanska-Rys, H., Salagacka, A., Mirowski, M., and Robak, T. (2009). Polymorphisms and haplotypes in the multidrug resistance 1 gene (MDR1/ABCB1) and risk of multiple myeloma. Leuk Res *33*, 332-335.

Jego, G., Robillard, N., Puthier, D., Amiot, M., Accard, F., Pineau, D., Harousseau, J.L., Bataille, R., and Pellat-Deceunynck, C. (1999). Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood *94*, 701-712.

Jordan, C.T., Guzman, M.L., and Noble, M. (2006). Cancer stem cells. N Engl J Med *355*, 1253-1261.

Karin, M. (2006). Nuclear factor-kappaB in cancer development and progression. Nature *441*, 431-436.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer *2*, 301-310.

Karin, M., Lawrence, T., and Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell *124*, 823-835.

Keats, J.J., Fonseca, R., Chesi, M., Schop, R., Baker, A., Chng, W.J., Van Wier, S., Tiedemann, R., Shi, C.X., Sebag, M.*, et al.* (2007). Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell *12*, 131-144.

Kovarova, L., Buresova, I., Buchler, T., Suska, R., Pour, L., Zahradova, L., Penka, M., and Hajek, R. (2009). Phenotype of plasma cells in multiple myeloma and monoclonal gammopathy of undetermined significance. Neoplasma *56*, 526-532.

Krishna, R., and Mayer, L.D. (2000). Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci *11*, 265-283.

Kuehl, W.M., and Bergsagel, P.L. (2002). Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer *2*, 175-187.

Kumar, S., and Rajkumar, S.V. (2008). Many facets of bortezomib resistance/susceptibility. Blood *112*, 2177-2178.

Kyle, R.A. (1978). Monoclonal gammopathy of undetermined significance. Natural history in 241 cases. Am J Med *64*, 814-826.

Kyle, R.A., Child, J.A., Anderson, K., Barlogie, B., Bataille, R., Bensinger, W., Blade, J., Boccadoro, M., Dalton, W., Dimopoulos, M.*, et al.* (2003a). Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. In (British Journal of Haematology Blackwell Publishing).

Kyle, R.A., Durie, B.G., Rajkumar, S.V., Landgren, O., Blade, J., Merlini, G., Kroger, N., Einsele, H., Vesole, D.H., Dimopoulos, M.*, et al.* (2010). Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *24*, 1121-1127.

Kyle, R.A., Gertz, M.A., Witzig, T.E., Lust, J.A., Lacy, M.Q., Dispenzieri, A., Fonseca, R., Rajkumar, S.V., Offord, J.R., Larson, D.R.*, et al.* (2003b). Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clinic proceedings Mayo Clinic *78*, 21-33.

Kyle, R.A., and Rajkumar, S.V. (2004). Multiple myeloma. N Engl J Med *351*, 1860-1873.

Kyle, R.A., and Rajkumar, S.V. (2009). Treatment of multiple myeloma: a comprehensive review. Clin Lymphoma Myeloma *9*, 278-288.

Kyle, R.A., Remstein, E.D., Therneau, T.M., Dispenzieri, A., Kurtin, P.J., Hodnefield, J.M., Larson, D.R., Plevak, M.F., Jelinek, D.F., Fonseca, R.*, et al.* (2007). Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. N Engl J Med *356*, 2582-2590.

Kyle, R.A., Therneau, T.M., Rajkumar, S.V., Offord, J.R., Larson, D.R., Plevak, M.F., and Melton, L.J., 3rd (2002). A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N Engl J Med *346*, 564-569.

Ladetto, M., Pagliano, G., Ferrero, S., Cavallo, F., Drandi, D., Santo, L., Crippa, C., De Rosa, L., Pregno, P., Grasso, M.*, et al.* (2010). Major tumor shrinking and persistent molecular remissions after consolidation with bortezomib, thalidomide, and dexamethasone in patients with autografted myeloma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *28*, 2077-2084.

Landgren, O. (2010). Monoclonal gammopathy of undetermined significance and smoldering myeloma: new insights into pathophysiology and epidemiology. Hematology Am Soc Hematol Educ Program *2010*, 295-302.

Landgren, O., Kyle, R.A., Pfeiffer, R.M., Katzmann, J.A., Caporaso, N.E., Hayes, R.B., Dispenzieri, A., Kumar, S., Clark, R.J., Baris, D.*, et al.* (2009). Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood *113*, 5412-5417.

Lodish, H.F. (2003). Molecular cell biology, Vol 22, 5th edn (New York, W.H. Freeman and Company).

Longley, D.B., and Johnston, P.G. (2005). Molecular mechanisms of drug resistance. J Pathol *205*, 275-292.

Lovell, R., Dunn, J.A., Begum, G., Barth, N.J., Plant, T., Moss, P.A., Drayson, M.T., and Pratt, G. (2005). Soluble syndecan-1 level at diagnosis is an independent prognostic factor in multiple myeloma and the extent of fall from diagnosis to plateau predicts for overall survival. Br J Haematol *130*, 542-548.

Lu, C., Gallegos, R., Li, P., Xia, C.Q., Pusalkar, S., Uttamsingh, V., Nix, D., Miwa, G.T., and Gan, L.S. (2006). Investigation of drug-drug interaction potential of bortezomib in vivo in female Sprague-Dawley rats and in vitro in human liver microsomes. Drug Metab Dispos *34*, 702-708.

Lynch, H.T., Watson, P., Tarantolo, S., Wiernik, P.H., Quinn-Laquer, B., Isgur Bergsagel, K., Huiart, L., Olopade, O.I., Sobol, H., Sanger, W.*, et al.* (2005). Phenotypic heterogeneity in multiple myeloma families. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 685-693.

Maggini, V., Buda, G., Martino, A., Presciuttini, S., Galimberti, S., Orciuolo, E., Barale, R., Petrini, M., and Rossi, A.M. (2008). MDR1 diplotypes as prognostic markers in multiple myeloma. Pharmacogenet Genomics *18*, 383-389.

Mahindra, A., Hideshima, T., and Anderson, K.C. (2010). Multiple myeloma: biology of the disease. Blood Rev *24 Suppl 1*, S5-11.

Markovina, S., Callander, N.S., O'Connor, S.L., Kim, J., Werndli, J.E., Raschko, M., Leith, C.P., Kahl, B.S., Kim, K., and Miyamoto, S. (2008). Bortezomib-resistant nuclear factor-kappaB activity in multiple myeloma cells. Mol Cancer Res *6*, 1356-1364.

Moreau, P., Robillard, N., Avet-Loiseau, H., Pineau, D., Morineau, N., Milpied, N., Harousseau, J.L., and Bataille, R. (2004). Patients with CD45 negative multiple myeloma receiving high-dose therapy have a shorter survival than those with CD45 positive multiple myeloma. Haematologica *89*, 547-551.

Nakanishi, C., and Toi, M. (2005). Nuclear factor-[kappa]B inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer *5*, 297-309.

Nau, K.C., and Lewis, W.D. (2008). Multiple myeloma: diagnosis and treatment. Am Fam Physician *78*, 853-859.

Oerlemans, R., Franke, N.E., Assaraf, Y.G., Cloos, J., van Zantwijk, I., Berkers, C.R., Scheffer, G.L., Debipersad, K., Vojtekova, K., Lemos, C.*, et al.* (2008). Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. Blood *112*, 2489-2499.

Olteanu, H., Wang, H.Y., Chen, W., McKenna, R.W., and Karandikar, N.J. (2008). Immunophenotypic studies of monoclonal gammopathy of undetermined significance. BMC Clin Pathol *8*, 13.

Orlowski, R.Z., Stinchcombe, T.E., Mitchell, B.S., Shea, T.C., Baldwin, A.S., Stahl, S., Adams, J., Esseltine, D.L., Elliott, P.J., Pien, C.S.*, et al.* (2002). Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *20*, 4420-4427.

Palumbo, A., and Anderson, K. (2011). Multiple myeloma. N Engl J Med *364*, 1046-1060.

Pekol, T., Daniels, J.S., Labutti, J., Parsons, I., Nix, D., Baronas, E., Hsieh, F., Gan, L.S., and Miwa, G. (2005). Human metabolism of the proteasome inhibitor bortezomib: identification of circulating metabolites. Drug Metab Dispos *33*, 771-777.

Perez-Persona, E., Vidriales, M.B., Mateo, G., Garcia-Sanz, R., Mateos, M.V., de Coca, A.G., Galende, J., Martin-Nunez, G., Alonso, J.M., de Las Heras, N.*, et al.* (2007). New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. Blood *110*, 2586-2592.

Pham, C.G., Bubici, C., Zazzeroni, F., Knabb, J.R., Papa, S., Kuntzen, C., and Franzoso, G. (2007). Upregulation of Twist-1 by NF-kappaB blocks cytotoxicity induced by chemotherapeutic drugs. Mol Cell Biol *27*, 3920-3935.

Raja, K.R., Kovarova, L., and Hajek, R. (2010). Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders. Br J Haematol *149*, 334-351.

Rajkumar, S.V., Richardson, P.G., Hideshima, T., and Anderson, K.C. (2005). Proteasome inhibition as a novel therapeutic target in human cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 630-639.

Rawstron, A.C., Orfao, A., Beksac, M., Bezdickova, L., Brooimans, R.A., Bumbea, H., Dalva, K., Fuhler, G., Gratama, J., Hose, D.*, et al.* (2008). Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica *93*, 431-438.

Richardson, P.G., Sonneveld, P., Schuster, M.W., Irwin, D., Stadtmauer, E.A., Facon, T., Harousseau, J.L., Ben-Yehuda, D., Lonial, S., Goldschmidt, H.*, et al.* (2005). Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med *352*, 2487-2498.

Roodman, G.D. (2009). Pathogenesis of myeloma bone disease. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK *23*, 435-441.

Ross, J.S., Symmans, W.F., Pusztai, L., and Hortobagyi, G.N. (2005). Pharmacogenomics and clinical biomarkers in drug discovery and development. Am J Clin Pathol *124 Suppl*, S29-41.

Sakaeda, T. (2005). MDR1 genotype-related pharmacokinetics: fact or fiction? Drug Metab Pharmacokinet *20*, 391-414.

Sanchez-Serrano, I. (2006). Success in translational research: lessons from the development of bortezomib. Nat Rev Drug Discov *5*, 107-114.

Seidel, C., Sundan, A., Hjorth, M., Turesson, I., Dahl, I.M., Abildgaard, N., Waage, A., and Borset, M. (2000). Serum syndecan-1: a new independent prognostic marker in multiple myeloma. Blood *95*, 388-392.

Sezer, O., Heider, U., Zavrski, I., and Possinger, K. (2001). Differentiation of monoclonal gammopathy of undetermined significance and multiple myeloma using flow cytometric characteristics of plasma cells. Haematologica *86*, 837-843.

Sirohi, B., and Powles, R. (2006). Epidemiology and outcomes research for MGUS, myeloma and amyloidosis. Eur J Cancer *42*, 1671-1683.

Suzuki, H., and Sugiyama, Y. (2000). Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. Eur J Pharm Sci *12*, 3-12.

Uttamsingh, V., Lu, C., Miwa, G., and Gan, L.S. (2005). Relative contributions of the five major human cytochromes P450, 1A2, 2C9, 2C19, 2D6, and 3A4, to the hepatic metabolism of the proteasome inhibitor bortezomib. Drug Metab Dispos *33*, 1723-1728.

van Schaik, R.H. (2008). CYP450 pharmacogenetics for personalizing cancer therapy. Drug Resist Updat *11*, 77-98.

Vangsted, A.J., Klausen, T.W., Gimsing, P., Andersen, N.F., Abildgaard, N., Gregersen, H., and Vogel, U. (2009). A polymorphism in NFKB1 is associated with improved effect of interferon-{alpha} maintenance treatment of patients with multiple myeloma after high-dose treatment with stem cell support. Haematologica *94*, 1274-1281.

Wang, L., Kumar, S., Fridley, B.L., Kalari, K.R., Moon, I., Pelleymounter, L.L., Hildebrandt, M.A., Batzler, A., Eckloff, B.W., Wieben, E.D.*, et al.* (2008). Proteasome beta subunit pharmacogenomics: gene resequencing and functional genomics. Clin Cancer Res *14*, 3503-3513.

Zhan, F., Barlogie, B., Arzoumanian, V., Huang, Y., Williams, D.R., Hollmig, K., Pineda-Roman, M., Tricot, G., van Rhee, F., Zangari, M.*, et al.* (2007). Gene-expression

signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. Blood *109*, 1692-1700.

Zipori, D. (2010). The Hemopoietic Stem Cell Niche Versus the Microenvironment of the Multiple Myeloma-Tumor Initiating Cell. Cancer Microenvironment *3*, 15-28.