Alma Mater Studiorum-Università di Bologna

Dottorato di Ricerca in:

Biotecnologie, Farmacologia e Tossicologia

Progetto formativo n.2 "*Farmacologia e Tossicologia*"

Ciclo XXIV

Settore Concorsuale di afferenza: **05/G1**

Settore Scientifico disciplinare: **BIO 14**

The impact of polymorphisms in P-gp, DNA repair and folic acid metabolism genes in newly diagnosed multiple myeloma patients treated with thalidomide plus dexamethasone, with or without bortezomib

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Esame finale anno 2012

General background

1. Multiple myeloma

Multiple myeloma (MM) is a progressive clonal B-cell disorder characterized by proliferation and accumulation of malignant plasma cells in the bone marrow and, less frequently, at extra-medullary sites (Figure 1) [1,2]. This malignant cells are phenotypically similar to long-lived plasma cells, including a strong dependence on the bone marrow microenvironment for survival and growth [3]. They typically secrete a single electrophoretically homogenous immunoglobulin (Ig) product, known as the monoclonal (M) protein (Figure 2), whereas the normal Ig levels are decreased [4,5]. In most of the cases the serum M-protein is of the IgG class, the IgA class is frequently involve as well, whereas IgM, IgE and IgD are rarely found.

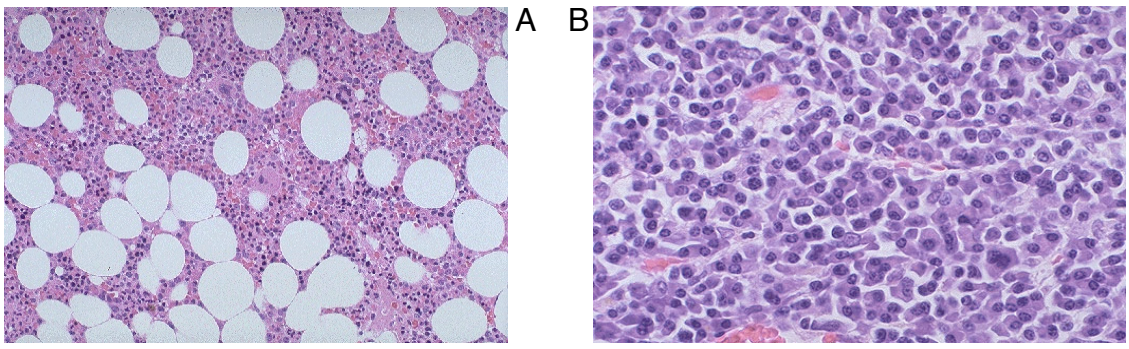

Figure 1. Smear of normal bone marrow (**A**) and in a patient affected by MM (**B**), with extensive infiltration by malignant plasma cells.

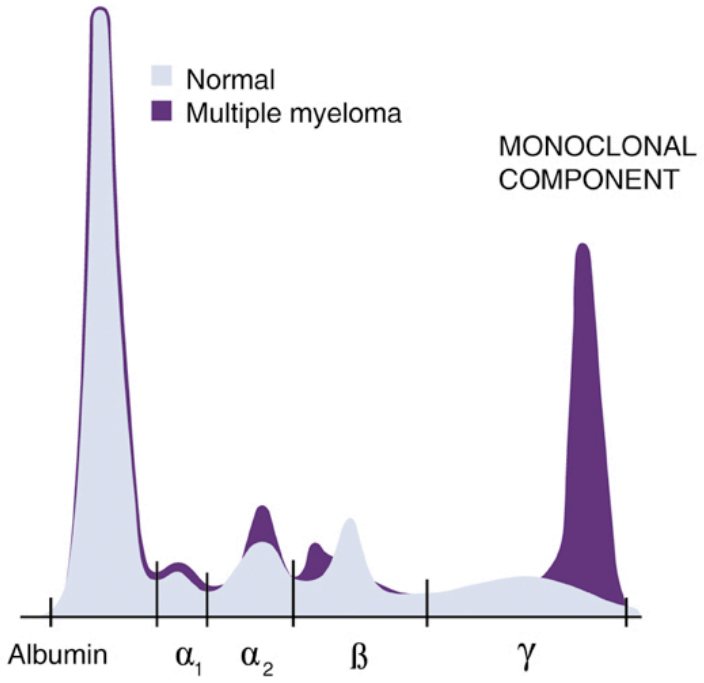

Figure 2. Electrophoretic pattern of a normal person (blue) and of a MM patient (violet).

2. Epidemiology and etiology of multiple myeloma

MM is a devastating, incurable malignancy which constitutes 1% of al cancers. It represents the second most frequent malignancy of the blood after lymphomas, accounting for 10% of all haematological malignancies [6]. MM is a disease of the elderly, the median age at onset is approximately 70 years. Only 15% of patients are aged less than 60 years, and rarely - less than 2-3% of patients - are diagnosed before the age of 40 [7]. On a worldwide scale, it is estimated that about 86 000 incident cases occur annually, accounting for about 0.8% of all new cancer cases. About 63 000 subjects are reported to die from the disease each year (~1% of all cancer deaths) [8]. MM incidence rate is significantly affected by race and gender. It is more common in the black race, followed by Maoris, Hawaiians, Israeli Jews, northern Europeans, US and Canadian whites, respectively [5,9,10]. The lowest rates occur in the Middle East, Japan, and China [9]. MM is also significantly higher in males than females among both, black and white population.

The cause of MM is still uncertain. The strongest environmental factor associated with an increased risk of developing MM is ionizing radiation [11]. However further studies on nuclear bomb survivors in Japan found no such relation [12]. Other factors associated with increased risk of MM are smoking, exposure to metals, agricultural chemicals, benzene and other petroleum products [11,13]. A direct genetic linkage to the etiology of MM has not yet been established. However, the remarkable difference in the incidence rate between different races, and the preservation of these incidence patterns regardless migration, suggest that susceptibility to MM may be determined by hereditary and genetic rather than environmental factors.

3. Clinical features of multiple myeloma

The clinical signs and symptoms in MM may vary greatly. Skeletal destructions or osteolytic lesions (Figure 3) are a characteristic feature of MM, being found in 70% of all cases. The lower back, ribs, and spine are the most commonly affected areas. The lesions are due to an unbalanced process between the cells reabsorbing bone (osteoclasts) and the cells producing bone (osteoblasts). The skeletal lesions and their accompanying hypercalcemia give rise to asthenia, cachexia, bone pain, fractures, compression of the spinal cord, and renal insufficiency, and are major causes of morbidity [3]. As the malignant cells grow they displace red blood cells and excrete inhibitory factors that prevent erythropoiesis, leading to anemia. MM patients are also more susceptible to bacterial infections due to deficiencies in both the humoral and cellular immunity. Renal failure is one of the most serious adverse complications of MM, and is caused by accumulation of Ig as well as deposition of calcium in the kidneys, leading to obstruction and inflammation. Neurological symptoms are most commonly related to the effect of the tumor mass, e.g. compression of the spinal cord or the nerves, but can also be due to hypercalcemia, hyperviscosity, or depositions of amyloids [3].

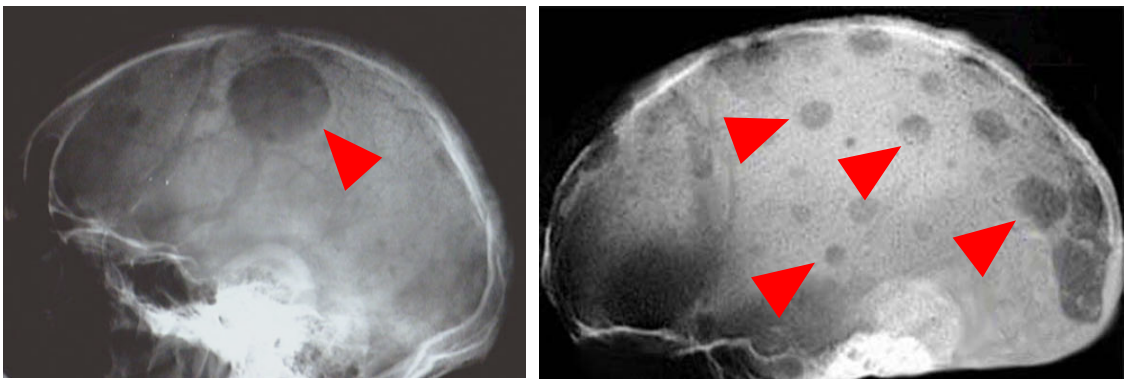

Figure 3. Typical bone lesion induced by MM: the skull χ-ray shows rounded "punched out lesion" (arrowhead)

3.1. Diagnosis and course of the disease

The diagnosis of MM is based on the presence of M-protein, bone marrow plasmacytosis and evidence of organ or tissue-related damage (i.e. bone lesions, kidney failure) to the body as a result of myeloma, and not other cause. Recently, the International Myeloma Working Group agreed on new consensus criteria for the classification of multiple myeloma and other gammopathy [4]. In this classification, the concept of end-organ damage was introduced to distinguish between monoclonal gammopathy of undetermined significance (MGUS), asymptomatic myeloma (smouldering MM -SMM) and symptomatic myeloma.

MGUS and SMM are asymptomatic, pre-malignant disorders, characterized by clonal expansion of plasma cells within the bone marrow, which is responsible for the presence of an M-protein in the serum, but with no evidence of end-organ impairment [4,14]. Patients with MGUS and SMM are often diagnosed by chance, as M-proteins are frequently identified during investigation of unrelated symptoms or during health screening. These patients are associated with an increased risk of developing and require lifelong observation in order to detect signs of transformation. The purpose of monitoring is to try to identify transformation to a malignant disorder at an early stage, when there is no significant irreversible lytic bone disease, renal failure or other disabling symptoms and at a stage when the patient is fit enough to benefit from increasingly effective treatments.

MGUS or SMM patients are not treated unless progression occurs. However, SMM needs to be differentiated from MGUS in the clinical setting, as its rate of transformation is markedly higher (Figure 4; [15]). The rate of progression of MGUS is ~1% per year *vs* 10% per year for SMM. By virtue of this different probability of progression between SMM and MGUS, SMM patients should be managed differently in terms of frequency of follow-up [14,16].

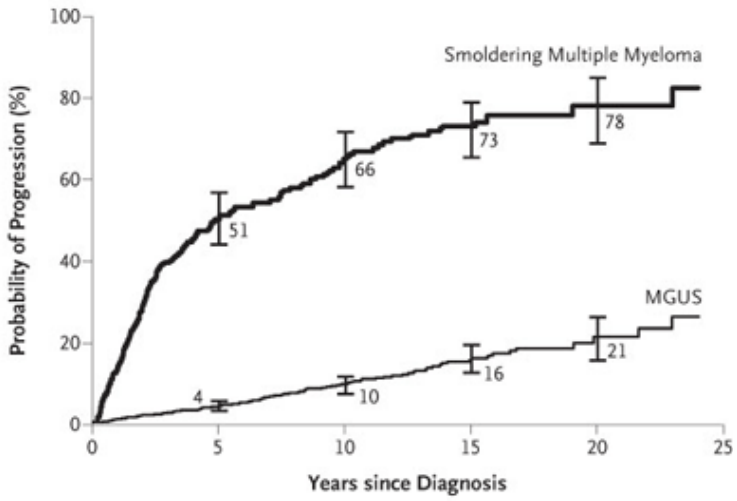

Figure 4. Probability of progression to active MM in patients with SMM or MGUS (vertical bar represents 95% confidence intervals).

At present no methods are available to distinguish those who will later develop MM from those who do not. Until recently it was not clear whether all MM were preceded by an MGUS phase. A study by Landgren *et al.*, and another by Weiss *et al.*, offered important clues about MGUS and its relationship to MM [17,18]. These two studies indicated that virtually all MM cases were preceded by an MGUS phase. This is a key finding that helps to fill a gap in our understanding of myelomagenesis. However, the events that trigger progression of MGUS to MM is are currently still unknown. These with other studies led to the generation of a disease model based on the multistep progression of normal to MGUS through to myelomatous plasma cells. In this model the initiating event is thought to be an immortalisation episode in plasma cell, which initiates the formation of a clone. It

has been suggested that such clone may remain quiescent and non-accumulating without producing end organ damage (MGUS/SMM stage). If transformation occurs, plasma cells accumulate within the bone marrow leading to organ and tissue impairment. This disease usually enters a quiescent phase of variable duration, followed by a late stage of drug resistance with resistance to apoptosis and independence from the bone marrow microenvironment.

The multi-step model of the molecular pathogenesis of MM as proposed is summarized in Figure 5 [3,19].

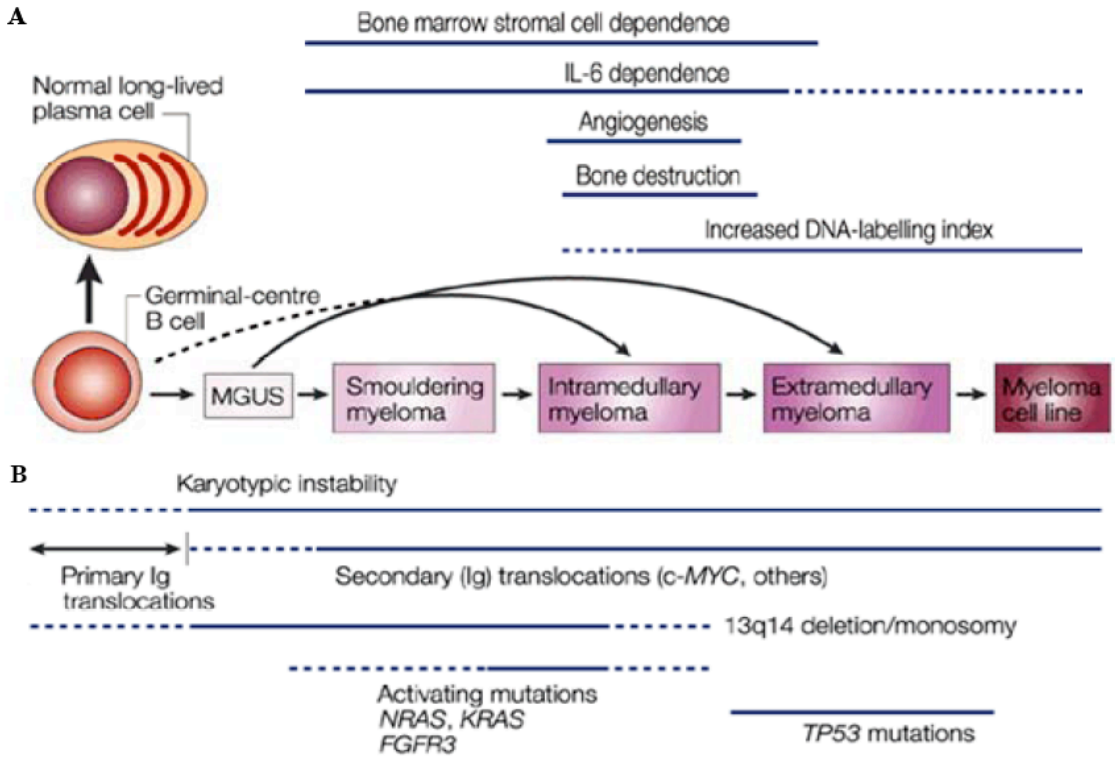

Figure 5. Development and molecular pathogenesis of MM. (**A**) Developing MM occurs either from a MGUS, or arises directly from a normal germinal-centre B cell. Plasma cells accumulate within the bone marrow (intramedullary MM), leading to manifestation of clinical features. Thus, intramedullary myeloma is associated with severe secondary features (lytic bone lesions, anaemia, immunodeficiency and renal impairment) and, in some patients with tumours occurring in extramedullary sites (blood, pleural fluid and skin). With progression to malignant myeloma, complex changes occur in the bone marrow microenvironment, i.e. induction of angiogenesis, suppression of cell-mediated immunity, and development of paracrine signalling loops (involving cytokines such as IL-6, IGF-1, and VEGF). These changes lead to interactions of myeloma cells, bone marrow stromal cells, and microvessels which, taken together contribute to persistence of the tumour and its resistance to drugs. (**B**) Oncogenic events occur in MGUS and throughout the course of MM, such as karyotypic instability; primary and secondary immunoglobulin (Ig) translocations, chromosome deletion, and gene mutations.

4. Genetic abnormalities in multiple myeloma

The acquisition of recurrent chromosomal abnormalities is an early event in MM development, as many of the genetic changes identified in the PC of MM patients have also been found in MGUS and SMM. Although, the mechanisms responsible for the acquisition of these changes is not well understood, current evidence suggests that in many cases an abnormal response to antigenic stimulation may be a key factor [20-22].

Conventional cytogenetics and fluorescent in-situ hybridization (FISH) have shown that numeric abnormalities occur in the genes of MM cells in both a non-hyperdiploid and a hyperdiploid pattern (Figure 6; [23]). Non-hyperdiploid abnormalities (black triangle in Figure 8) usually includes one of the seven recurrent IgH translocations as an early event, hyperdiploid (white triangle in figure 6) is associated with multiple trisomies. Monosomy/deletion of chromosome 13 (Δ13; grey triangle in figure) has also been suggested to be an early abnormality shared by MGUS and MM tumours. IgH translocations, hyperdiploid, and Δ13 are all early and partially overlapping events; however, the relative timing of their occurrence is not yet completely understood. Secondary chromosomal rearrangements and other abnormalities, implicated in disease progression, can occur at any time during tumourigenesis. These includes MYC rearrangement, activation of N or K-RAS mutations, FGFR3 mutations, inactivation or mutation of TP53, RB1 and PTEN; and inactivation of cyclin-dependent kinase inhibitors *CDKN2A* and *CDKN2C*.

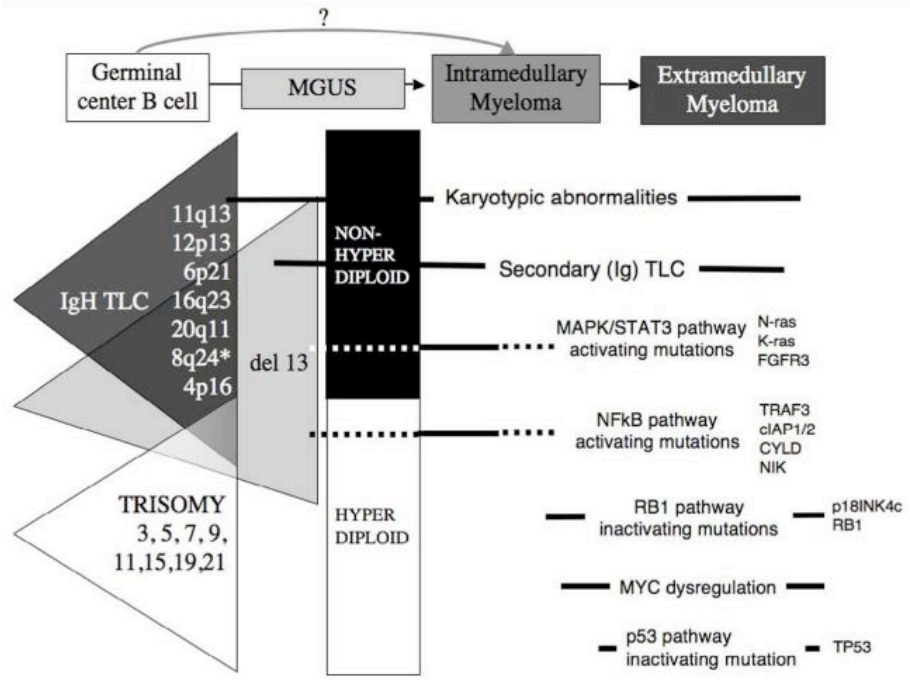

Figure 6. Disease stages and timing of oncogenic events.

Translocations involving the Ig heavy chain locus (14q32) are present in approximately 75% of the newly diagnosed. The translocation partners of 14q32 are quite heterogeneous with 4p16.3, 11q13 and 16q23 being most frequently involved. The t(4;14)(p16.3;q32) and t(14;16)(q32;q23) are associated with poor prognosis after high-dose chemotherapy [24].

The t(4;14)(p16.3;q32) is present in approximately 20% of the patients. The translocation results in expression of multiple myeloma SET domain (mmset) and/or fibroblast growth factor receptor 3 (FGFR3), which promotes myeloma cell proliferation and prevents apoptosis [25]. The t(14;16)(q32;q23) is present in 10% of the patients and results in expression of c-maf [26]. Other chromosomal abnormalities associated with poor prognosis are 17p13 deletion (p53), and translocations involving c-myc (8q24) [27,28].

The t(11:14)(q13;q32) and hyperdiploid karyotype are chromosomal abnormalities associated with a favorable prognosis. The t(11:14)(q13;q32) is detected in 20% of the patients and results in expression of cyclin D1 [29,30]. Hyperdiploid karyotype is observed in 40-50% of the patients with multiple myeloma. The

majority of these patients have a chromosome pattern, which consists of the combination trisomies of chromosomes 5, 7, 9, 11, 15, 19 and 21, and low prevalence of chromosome 13 deletions. Several studies have shown that hyperdiploid-myeloma patients have a better prognosis than non-hyperdiploid-myeloma patients [31,32].

5. Prognostic factors associated with tumor burden

The tumor burden can be assessed by means of the Durie and Salmon staging system, which was specifically obtained from mathematical models for evaluation of tumor mass. Multiple myeloma was divided in three tumor burden groups, which correlated with survival [33]. Recently, a new staging system has been proposed. The International Staging System (ISS) was obtained from statistical analysis of potential prognostic factors in a large international data set of symptomatic myeloma patients.

The individual most powerful prognostic marker is the serum β2-microglobulin level, which is a single variable that measures a combination of indices: cell proliferation, cell mass, and renal function. Genetic factors are also important prognostic markers. Favourable prognostic marker include a β2-microglobulin level < 2.5 mg/L, absence of deletion or monosomy of chromosome 13, and t(11;14). Prognostic markers related to an adverse outcome include increase in plasma cell labelling index, increased levels of serum β2-microglobulin, and circulating myeloma cells. Complete deletion of chromosome 13 or its long arm, t(4,14) as well as increased density of bone marrow microvessels are also adverse prognostic factors [1].

Based on the results of two widely available laboratory tests, serum β2-microglobulin and albumin concentration, multiple myeloma is divided in three stages in which the median survival ranging from 29 to 62 months (Table 1) [34]

Table 1. International Staging System for multiple myeloma [34]

Stage	Criteria	Survival (months)
I	Serum β2-microglobulin < 3.5 mg/l and serum albumin ≥ 35 g/l	62
II	Serum β2-microglobulin < 3.5 mg/l and serum albumin < 35 g/l or serum β2-microglobulin > 3.5 to < 5.5 mg/l	45
III	Serum β2-microglobulin ≥ 5.5 mg/l	29

6. Treatment of multiple myeloma

To date MM remains an incurable disease. However, treatment improves the clinical situation in 75% of patients and multiple periods of remission and relapse can occur. In particular, high-dose chemotherapy supported by haematopoietic stem cell transplantation (HSCT), and the latest integration of several novel agents (Figure 7; [2]), into each step of therapeutics have substantially improved the outcome of patients with MM [35].

MM treatment can be divided into three phases: induction, consolidation and maintenance. Currently the treatment of choice for symptomatic MM is high-dose chemotherapy with haematopoietic stem cell transplantation (HSCT). Autologous HSCT uses the patient's own stem cells, whereas allogeneic/syngeneic HSCT employs MHC (i.e. HLA) identical or twin donor bone marrow. If HSCT is not an option (depending on the individual situation of the patient i.e., age, state of the disease, physical fitness) a simple induction regime with conventional chemotherapy, single agent treatment (e.g., dexamethasone) or new treatments (thalidomide, bortezomib) possibly in combination with other drugs are applied.

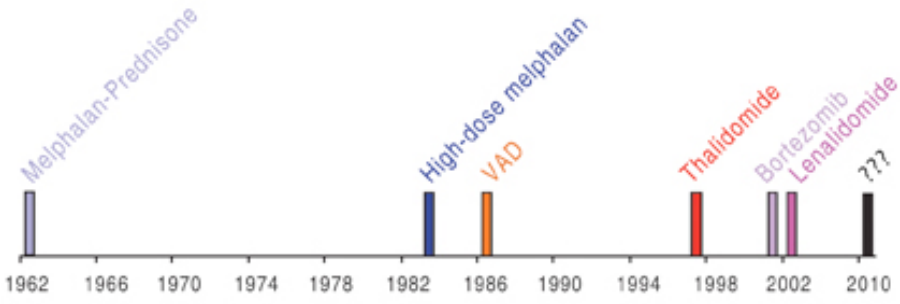

Figure 7. Timeline of treatment evolution in MM.

6.1. Conventional agents

The first successful myeloma treatment - a combination of melphalan and prednisone - was introduced in the late 1960s, and was further improved by high-dose drug regimens with autologous stem-cell transplantation in the 1980s. Median survival after conventional treatments was only 3 to 4 years, but high-dose treatment followed by autologous stem-cell transplantation extended median survival to 5 to 7 years [36]. This was the treatment for MM until the 1980s when vincristine, doxorubicin, and dexamehasone (VAD) followed by autologous transplant became the standard of care for eligible patients [37,38].

Most newly diagnosed MM patients <65 years of age (or older if fit) are candidates for autologous transplant. Therefore initial therapy must avoid agents with cumulative myelosuppression in order to permit collection of an adequate number of stem cells. Common pre-autologous transplant induction regimens have included the VAD regimen. This produces partial remission (PR) in about 50% of patients and complete remissions (CR) (no evidence of monoclonal protein and <5% marrow plasma cells) in 5 to 10% of patients

6.2. Novel agents

The new era of treatment for multiple myeloma was not initiated until the late 1990s with the introduction of thalidomide, its analogue lenalidomide, and bortezomib.

6.2.1. Thalidomide

In 1999, thalidomide was introduced as a new therapeutic agent in the treatment of multiple myeloma. The rationale for the use of thalidomide was based on studies showing increased bone marrow microvascularity in multiple myeloma [39] and the observation that thalidomide had anti-angiogenic activity in animal models [40]. The first clinical trial with thalidomide was conducted by Singhal *et al*. [41] in patients with relapsed or refractory multiple myeloma. The response rate was 30%. The event-free and overall survivals at 2 years were 20% and 48%, respectively. When Thalidomide showed promising activity in relapsed myeloma, it was quickly combined with Dexamethasone (TD regimen) in an attempt to develop an oral alternative to the cumbersome VAD regimen. Dexamethasone and the other steroids are useful in myeloma treatment because they can stop white blood cells from travelling to areas where cancerous myeloma cells are causing damage. This decreases the amount of swelling or inflammation in those areas and relieves associated pain and pressure. Several studies have confirmed the efficacy of thalidomide alone and in combination with dexamethasone or chemotherapy for the treatment of myeloma patients with relapsed and refractory disease [42-45].

Subsequently, thalidomide was extensively investigated in newly diagnosed patients. Three studies reported on the combination TD [46-48]. Objective responses were observed in 63% to 72% of patients, with a complete response rate of approximately 10%. Even there are no randomized studies comparing TD, and VAD like regiments, a matched case-control study by Cavo *et al*., [49] reported a significantly higher response rate with TD as compared to VAD (76% vs. 52%); the complete response rate was 10% and 8%, respectively. Overall,

these studies indicate that the TD is a relatively safe and effective induction regimen that does not impair stem cell collection. Thalidomide is being investigated also in the maintenance setting for its effect on the duration of response after high-dose chemotherapy and HSCT [50]. In this study – – IFM 99 2 – patients were randomly assigned to no maintenance treatment, maintenance with pamidronate alone or maintenance with thalidomide and pamidronate. Thalidomide increased the overall survival compared with the other two groups. The 4-years probability of survival was 77% in the no maintenance group, 74% in the pamidronate group and 87% in the thalidomide and pamidronate group.

Disadvantages of thalidomide include a variety of side effects such as deep vein thrombosis, constipation, peripheral neuropathy, and fatigue, which often restrict dose and treatment duration thus reducing drug effectiveness [51].

6.2.2. Lenalidomide

Lenalidomide is an immunomodulatory drug, analogue of thalidomide, that has demonstrated significantly more potent preclinical activity compared with thalidomide, and without sedative and neurotoxic adverse effects [52]. A multicenter phase II study [53] reported a response rate of 24%. Approximately, one third of the patients who did not respond to lenalidomide alone, had an additional responses when dexamethasone was added to the regimen. Two other randomized phase III studies have compared lenalidomide and dexamethasone to dexamethasone alone in patients with relapsed or refractory disease. Interim analyses of both studies showed a higher response rate and improved time to progression in favour of the lenalidomide and dexamethasone group [54]. Recently, Rajkumar et al [55] investigated lenalidomide in combination with dexamethasone in newly diagnosed multiple myeloma. The response rate was 91% with a (near) complete response rate of 38%, and an adequate number of stem cell was obtained in all patients.

6.2.3. Bortezomib

Bortezomib is a novel proteasome inhibitor, which is highly active in patients with multiple myeloma. The proteasome-ubiquitin pathway is a ubiquitous and essential intracellular system that degrades many labile proteins regulating cell cycle, apoptosis, transcription, cell adhesion, angiogenesis, and antigen presentation [56,57]. Bortezomib, is a small molecule that is a potent and selective inhibitor of the 26S proteasome which is the primary component of the protein degradation pathway of the cell (Figure 8; [57,58]). Given the broad array of substrates, the 26S proteasome has been shown to be involved in cell cycle control, cell differentiation, transcription, DNA repair, and immune response, [59-61]. The antimyeloma mechanism of bortezomib is still subject of intense study.

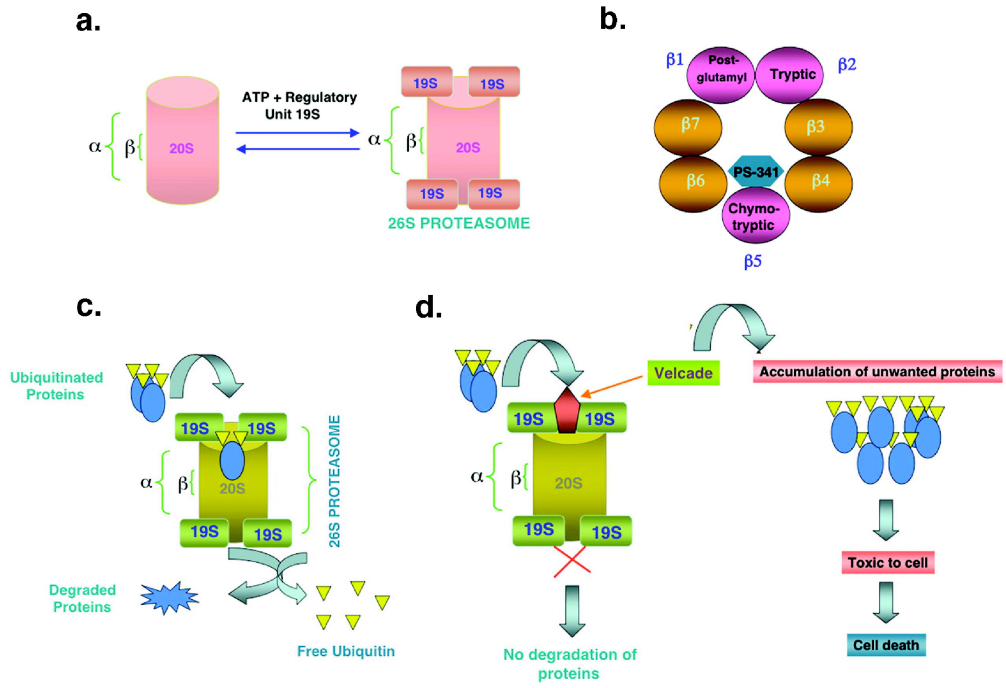

Figure 8. (**A**) Structure and function of proteasomes; (**B**) cross-sectional view of 26S proteasome complex; (**C**) process of degradation of ubiquitinated proteins by proteasome complex; and (**D**) bortezomib/velcade blocks the proteasomal protein degradation resulting in accumulation of cytotoxic proteins.

Bortezomib is currently believed to exert its effects through multiple pathways that target both the tumor cell and its microenvironment [62]. A phase II study in patients with relapsed or refractory multiple myeloma treated with bortezomib

demonstrated a response rate of 35% [63]. The median overall survival was 16 months, with a median duration of response of 12 months. A smaller, randomized study confirmed the activity of bortezomib [64]. In both studies some responses occurred after addition of dexamethasone in patients with no or a suboptimal response to bortezomib alone. Chromosome 13 deletion and elevated β2-microglobulin, generally considered as poor prognostic factors were not predictive of poor outcome in patients treated with bortezomib [65]. A subsequent international, multicenter phase III study in 669 patients, who had a relapse prior therapies were randomized to receive bortezomib or high-dose dexamethasone [66]. Bortezomib demonstrated to be superior to high-dose dexamethasone in terms of response rate (38% *vs* 18%), time to progression (6.2 months *vs* 3.5 months) and 1-year survival (80% *vs* 66%).

Based on preclinical findings of synergistic anti-myeloma activity with other agents, bortezomib-based combination regimens are under clinical investigation. Preliminary data from studies of bortezomib alone [67] or in combination with dexamethasone [64], liposomal-doxorubicin [68], melphalan and prednisone [69-70], TD [71] or cyclophosphamide and prednisone [72] indicate encouraging activity with manageable toxicities in advanced and newly diagnosed myeloma patients. Several studies have also assessed bortezomib-based regimens as pre-transplantation induction treatment. Bortezomib and dexamethasone [73] or bortezomib, adriamycin and dexamethasone [74] showed to be promising regimens with high complete response rates (25%) and no stem cell toxicity.

6.2.3.1. Bortezomib resistance

Although bortezomib revolutionized treatment of MM, prolonging survival in relapsed myeloma as well as newly diagnosed disease, resistance to therapy develops inevitably. Furthermore, nearly a third of the patients with multiple myeloma never respond to treatment with bortezomib. There are several ways to escape the effects of proteasome inhibition by malignant cells (Figure 9, [62]).

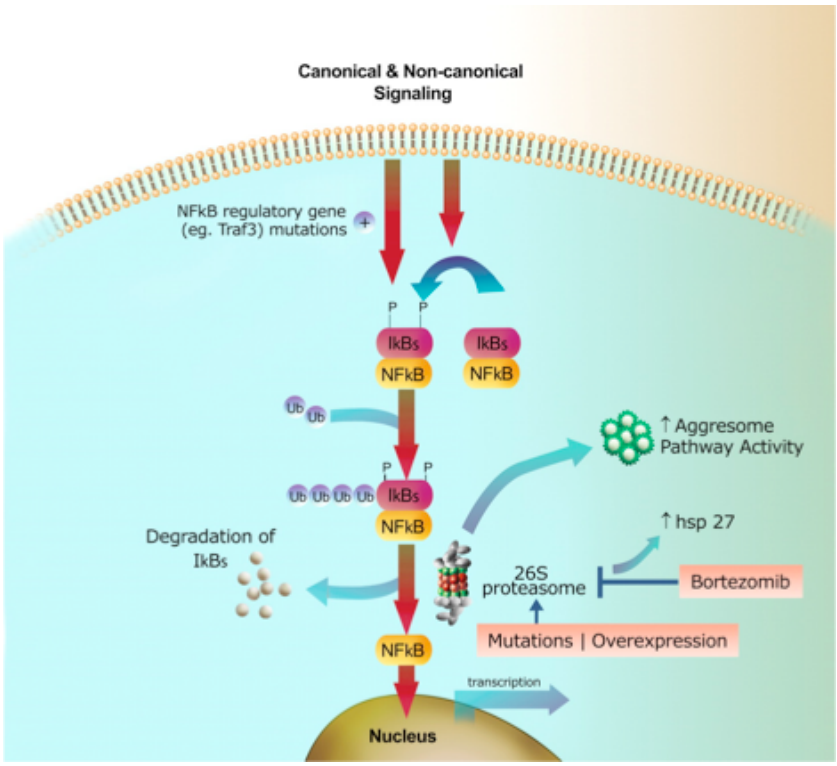

Figure 9. Mechanisms of resistance and susceptibility to proteasome inhibition

6.2.3.1.1. Cell intrinsic resistance

Drug can be effluxed from cells by transporters expressed on the external cell membrane after up-regulation of efflux transporters like glicoprotein-P (P-gp). Once inside cell, alterations in binding site for bortezomib in the proteasome complex can prevent drug to bind to it. As a third resistance mechanism we could point the increasing efficiency of alternate mechanisms of protein degradation (the aggresome pathway). Modulation of cell signaling pathways that are affected by proteasome inhibition, like DNA repair pathway, may be another mechanism of resistance.

P-gp - The mechanism of resistance to bortezomib is multifactorial and while little is known about the interaction of bortezomib with P-gp, there are indications that overexpression of this pump may contribute to resistance to this agent. Rumpold *et al* .[75] showed that knockdown of P-gp resensitises P-gp-expressing cells to proteasome inhibitors.

Proteasome β5 unit - The activity of Bortezomib is directed mainly against the β5 unit of the proteasome. It has been reported that some mutations in this catalytic unit may impair binding of the drug and thus decrease proteasome inhibition and consequently bortezomib efficacy [76]. In addition, significant up-regulation of the PSMB5 subunit following exposure to bortezomib has been noted.

Aggresome pathway - Recent studies have revealed an alternative system to the proteasome for degradation of polyubiquitinated misfolded/unfolded proteins, termed the aggresome [77-78]. The aggresome pathway therefore likely provides a novel system for delivery of aggregated proteins from cytoplasm to lysosomes for degradation [79]. In view of this consideration, aggresome pathway potentially may compensate for proteasome inhibition and contribute to drug resistance. It has been hypothesized that inhibition of both proteasomal and aggresomal protein degradation systems could induce accumulation of polyubiquitinated proteins and significant cell stress, followed by activation of apoptotic cascades [79].

Heat shock protein Hsp27 – Upregulation of the heat shock protein Hsp27 confers resistance to bortezomib-induced cell death through a mechanism still undefined. Recently in a study by Chauhan *et al*., [80] blocking Hsp27 using an antisense to Hsp27 (AS-Hsp27) restores sensitivity to PS-341 in PS-341-resistant DHL4 lymphoma cells. These findings provide the first evidence of potential mechanisms of PS-341 resistance and suggest a therapeutic advantage of using an AS-Hsp27 to overcome PS-341 resistance.

NFkB pathways - Activation of the non-canonical NF-kB pathway has been recognized in patients with multiple myeloma, and attributed to interactions of the myeloma cell with the bone marrow microenvironment [81]. TRAF3 is a recognized regulator of the non-canonical NF-kB pathway and bortezomib has found to have a remarkable activity in patients with inactivation of TRAF3. This finding suggests that one of its most important mechanisms of action in MM is the inhibition of the non-canonical NF-kB. pathway. Therefore, it is possible that the specific targeting of the direct NF-kB regulators NIK and IKKα may be particularly effective in MM treatment, a finding that could allow tailoring the use of this class of drugs in the future [81].

6.2.3.1.2. Host-mediated resistance

Interactions of MM cells with the bone marrow stromal cells (BMSCs) microenvironment play a critical role in the development of drug resistance. The crucial role in this mechanism, play cytokines and/or adhesion molecules. The most important cytokine in MM biology is interleukin-6 (IL-6). Under normal conditions, IL-6 drives B-cell differentiation, but in MM it causes proliferation, and inhibits apoptosis of myeloma cells. Nuclear factor-κB (NF-κB), a transcription factor member of the Rel family, is constitutively activated in MM and its activation in both MM and BMSCs mediates further IL-6 secretion, resulting in increased MM and BMSCs interaction [79]. IL-6 may promote resistance to bortezomib through different mechanisms [82]:

- Prevention of apoptosis via the PI3K-AKT pathway;
- Impaired immune functions by blocking differentiation of monocytes to dendritic cells;
- Induction of VEGF - *vascular endothelial growth factor* - secretion, which promotes angiogenesis, stimulates growth and migration of MM cells;
- Promoting cell proliferation via the RAS-MAPK pathway.

6.2.3.2. Overcoming bortezomib resistance: Emerging therapies

New therapies such as heat shock protein (HSP) 90 inhibitors, Akt inhibitors, histone deacytelase (HDAC) inhibitors, BCL2 inhibitors, pro-apoptotic peptides and other proteasome inhibitors are in preclinical studies to provide the framework for phase I and II clinical trials [83]. These new agents are tested singly or more commonly, in combination with other MM therapies.

Microarray profiling showed that bortezomib induces HSP90 gene transcripts in MM cells [84]. The combination of Hsp90 inhibitor, 17AAG and bortezomib can block this stress response and increase cytotoxicity [85]. A clinical trial combining bortezomib and 17AAG is currently ongoing to see if the combination can overcome bortezomib resistance.

Bortezomib down-regulates ERK, Jak/STAT and PKC signaling pathways but activates the Akt survival pathway *in vitro* [86]. Hideshima *et al*. demonstrated that perifosine, an Akt inhibitor, when combined with bortezomib, is able to abrogate this response and induce synergistic MM cytotoxicity *in vitro* [83]. A phase II clinical trial is currently evaluating this combination.

Other important new combinations are the HDAC inhibitors SAHA or LBH589 with bortezomib. The HDAC inhibitors are able to block protein degradation through the aggresome autophagy pathway and upregulate proteasomal degradation; conversely, blockade of the proteasome with bortezomib upregulates aggresome activity [84-85]. Preclinical studies have shown that combinations that block both pathways of protein degradation induce synergistic cytotoxicity in MM. A phase II trial of LBH589 is now ongoing in MM, with a combination LBH589 and bortezomib trial to follow. Richardson *et al.* [89] had reported modest single agent activity of SAHA in a phase I trial in 11 advanced MM patients and we are currently awaiting results of a combination trial involving SAHA and bortezomib.

Specific background

7. Personalized therapy

Personalized therapy, contrary to popular belief, it is not a new and modern concept. Personalized medicine has always been present in medicine, and observations of individual differences in response to food and drugs date back to as early as the 6th century BC. Pythagoras first made the observation that some individuals fall ill after ingesting uncooked fava beans, and disallowed his followers to eat them. It was not until the 1950s that the link between glucose-6-phosphate dehydrogenase deficiency, haemolytic anemia, and fava beans was established [90].

We have long recognized that each patient is unique in clinical presentation, prognosis, treatment tolerance, supportive care needs, and outcomes. We now recognize that, just as each patient is different in how he or she is affected by cancer, each cancer has a distinctive biology and natural history, and every disease called “cancer” comprises smaller subsets with distinctive features and differing outcomes that require personalized treatment plans [91].

8. Pharmacogenetics and pharmacogenomics

Pharmacogenetics and pharmacogenomics deal with the role of genetic factors in drug effectiveness and adverse drug reactions. These disciplines have their origin in the 1950s with the emergence of human biochemical genetics. The role of genetics, as a potential cause of adverse drug reactions was set out in a first review by Motulsky in 1957 with the programmatic title “*Drug reaction, Enzymes and biochemical Genetics*”. The term, pharmacogenetics was coined by Friedrich Vogel from Heidelberg in 1959. In the late 1960s, Vessel showed remarkable similarity of disposal for several drugs in identical twins who shared 100% of their genes, opposed fraternal twins who only share 50% [92]. The development of pharmacogenetics over the years remained slow since relatively

few drug responses or adverse drug reactions were under control of a single gene. Family studies were difficult and a direct DNA study of drug response was not yet possible. There was little or no impact on clinical pharmacology, drug development and clinical medicine. The increasing availability of DNA technology and *in vitro* molecular tests advanced the field. Pharmacogenetic research has seen an explosion of interest in the last decades by physicians, geneticists and the pharmaceutical industry, as reflected by the rapid increase in the number of publications in the medical literature [93].

The term pharmacogenomics was introduced in 1990s with the emergence of the Human Genome project and the development of the genome sciences [94-95]. New technology such as microarrays allowed search for multiple genes affecting drug responses. The analysis of DNA abnormalities that characterize a disease is now leading to therapeutic drugs acting on disease-specific DNA mutations [96]. After the completion of the Human Genome Project in 2003, genomics has become a mainstay of biomedical research and pharmacogenetics has been forecasted to be one of the first clinical applications arising from the new knowledge [97]. Indeed, the research efforts in the field of pharmacogenetics expressed as the number of publications listed on PubMed have steadily increased until levelling out in 2009 at 1100-1200 publication per year [98]. By contrast, the clinical use of pharmacogenetic testing did not meet the initial high expectations and has lagged considerably behind, despite the significant body of evidence supporting its usefulness. As a result of the unmet promises many clinicians have become somewhat disillusioned regarding pharmacogenetics in recent years. Indeed, expectations of the effect of a single polymorphism on drug response were unrealistically high [99]. Still, pharmacogenetics holds the promise of advancing drug therapy. The ultimate goal of pharmacogenetics is personalized medicine, rather than the established "*one size fits all*" approach to drugs and dosages, according to the specific genetic make-up of a given patient. Environment, diet, age, lifestyle and state of health can all influence the individual response to the pharmacological treatment, but understanding the influence of genetics could be the key to create personalized medicine: selecting the right drug

and administering it at the dose that have the maximum efficacy and the least toxicity.

Pharmacogenetics and pharmacogenomics combines traditional pharmaceutical sciences such as biochemistry with modern genetics. In particular, biotechnology and molecular biology of our days let us look inside our genetic code. Today, physicians can perform some impressive feats that were just exciting new ideas in research 10 years ago. A clinician can cure a woman with human epidermal growth factor receptor 2 gene-amplified breast cancer, by adding trastuzumab to standard adjuvant chemotherapy. Just after lunchtime, the same clinician can review a report of the results of polymerase chain reaction tests on DNA in the peripheral blood of an asymptomatic patient with chronic myelogenous leukemia (CML) and if necessary, change the patient's prescription from imatinib to dasatinib or nilotinib to maintain the patient's clinical remission. At the end of the day, a patient with KRAS–mutated metastatic colorectal cancer can be saved from unnecessary toxicity and cost by discussing whether to proceed with cetuximab or panitumumab treatment [100]. This is just few examples of how understanding SNPs could greatly benefit the therapeutic index of a patient. Currently The American Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved more than 77 drugs with pharmacogenomic information into drug label inserts [100,101].

In Table 2 are reported some significant examples of pharmacogenomics biomarkers, describing prevalence, and authority guidelines [101,102].

Table 2. Example of pharmacogenomic biomarkers in the context of disease, and authority guidelines

Drug	Indication	Causative genotype and its effects	Clinical Directive on Label
Abacavir (Ziagen, GlaxoSmithKline)	HIV-1	HLAB*5701 Hypersensitivity	Black-box warning: "Prior to initiating therapy with abacavir, screening for the HLAB*5701 allele is recommended."Your doctor can determine with a blood test if you have this gene variation.

Azathioprine (Imuran, Prometheus)	Renal allograft transplantation, rheumatoid arthritis	TPMT*2, TPMT*3A, and TPMT*3C Severe myelotoxicity	"TPMT genotyping or phenotyping can help identify patients who are at an increased risk for developing Imuran toxicity.""Phenotyping and genotyping methods are commercially available."
Carbamazepine (Tegretol, Novartis)	Epilepsy, trigeminal neuralgia	HLAB*1502 Stevens-Johnson syndrome or toxic epidermal necrolysis	Black-box warning: "Patients with ancestry in genetically at-risk populations should be screened for the presence of HLAB*1502 prior to initiating treatment with Tegretol.Patients testing positive for the allele should not be treated with Tegretol." "For genetically at-risk patients, high-resolution HLAB*1502 typing is recommended."
Cetuximab (Erbitux, Imclone)	Metastatic colorectal cancer	KRAS mutations Efficacy	"Retrospective subset analyses of metastatic or advanced colorectal cancer trials have not shown a treatment benefit for Erbitux in patients whose tumors had KRAS mutations in codon 12 or 13. Use of Erbitux is not recommended for the treatment of colorectal cancer with these mutations."
Clopidogrel (Plavix, Bristol-Myers Squibb)	Anticoagulation	CYP2C19*2*3 Efficacy	"Tests are available to identify a patient's CYP2C19 genotype; these tests can be used as an aid in determining therapeutic strategy. Consider alternative treatment or treatment strategies in patients identified as CYP2C19 poor metabolizers."
Irinotecan (Camptosar, Pfizer)	Metastatic colorectal cancer	UGT1A1*28 Diarrhea, neutropenia	"A reduction in the starting dose by at least one level of Camptosar should be con- sidered for patients known to be homozygous for the UGT1A1*28 allele." "A laboratory test is available to determine the UGT1A1 status of patients."
Panitumumab (Vectibix, Amgen)	Metastatic colorectal cancer	KRAS mutations Efficacy	"Retrospective subset analyses of metastatic colorectal cancer trials have not shown a treatment benefit for Vectibix in patients whose tumors had KRAS mutations in codon 12 or 13. Use of Vectibix is not recommended for the treatment of colorectal cancer with these mutations."
Trastuzumab (Herceptin, Genentech)	HER2-positive breast cancer	HER2 expression Efficacy	"Detection of HER2 protein overexpression is necessary for selection of patients appropriate for Herceptin therapy because these are the only patients studied and for whom benefit has been shown." "Several FDA-approved commercial assays are available to aid in the selection of breast cancer and metastatic gastric cancer patients for Herceptin therapy."

Warfarin (Coumadin, Bristol-Myers Squibb)	Venous thrombosis	CYP2C9*2*3 and VKORC1 Bleeding complications	Includes the following table: Range of Expected Therapeutic Warfarin Doses Based on CYP2C9 and VKORC1 Genotypes.

8.1. Basic principles of pharmacogenetics

By definition, pharmacogenetics refers to the study of inherited differences (variation) in drug metabolism and response. On the other hand pharmacogenomics refers to the general study of all of the many different genes that determine drug behavior. The distinction between the two terms is considered arbitrary, however, now the two terms are very often used interchangeably [103].

A gene is a part of the DNA that codes for a type of protein or for a RNA chain that has a specific function in the organism. There are two alleles per autosomal gene (one paternal and one maternal). Together the two alleles form the genotype. Heterozygotes have two different alleles, and homozygotes have two of the same alleles. Genetic variation can consist of deletions, insertions, inversions, and copy number variation [104]. Most sequence variations are single nucleotide polymorphisms (SNPs), a single DNA base pair substitution that may result in a different gene product. As a result of this genetic variation many genes have multiple variants. The most common allele in a population is referred to as the wild type. Some of the variant alleles code for non-functional or decreased functional proteins. Allele frequencies can vary greatly in different ethnic populations. Phenotype refers to the trait resulting from the protein product encoded by the gene.

8.2. Single nucleotide polymorphisms (SNPs)

Polymorphisms are genetic variations that occur with different frequency in different populations. These variations could be represented by insertion or deletion, but the most common variations are SNPs. A SNP is a DNA sequence

variation occurring when a single nucleotide in the genome differs among members of a species or between chromosomes in an individual. In the human genome the number of SNPs is estimated around 3.2 millions and they are responsible for the 90% of genetic human variability (105). SNPs are classified in three groups depending on where they are located in the genome: (i) c-SNP, variations located in coding region , exons, whose presence could modify or not the amminoacidic sequence of the protein (non synonymous and synonymous, respectively), (ii) p-SNP, located in perigenic region and (iii) r-SNPs, random SNPs that are located in the intragenic region (Figure 10).

Polymorphisms in key genes encoding drug transporters and metabolizing enzymes influence intracellular drug delivery. Pharmacogenetics has indeed proven to be a potential source of biomarkers able to predict drug response and adverse drug reactions.

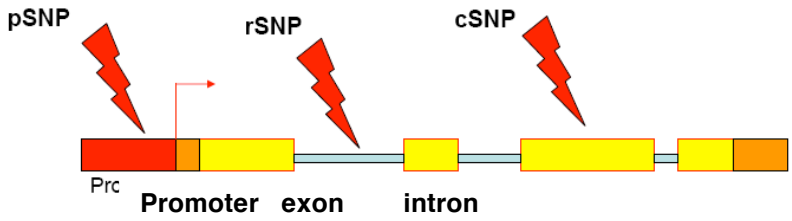

Figure 10. Single nucleotide polymorphisms classification depending on their position in the genome.

Currently, pharmacogenetic information is accumulating rapidly and it was reported that based on the available pharmacogenetic information it is possible to generate advice for nearly 100 drugs for a patient with a completely sequenced genome [106]. To date, many efforts within the field of pharmacogenetics have been aimed at the improvement of drug therapy with “high risk” medications such as within the field of oncology. Yet the clinical use of genotyping prior to drug prescription and dispensing is not widely practiced, largely by a lack of scientific evidence for improved patient care by pharmacogenetic testing [107].

several “players” can be identified [108], including the biotechnology and analytical industry, the pharmaceutical industry, research institutions, funding agencies, regulatory agencies, clinicians, and patients. These players each have substantial roles, both individually and in collaboration, in developing and implementing clinical applications of pharmacogenetics. Initiatives have been proposed such as a pharmacogenetic research network that includes a series of integrate groups with expertise in pharmacology, genomic science, bioinformatics and clinical science. The group located at Stanford University is responsible for the development of a public database that focuses on genotype and phenotype data relevant to pharmacogenomics. The Royal Dutch Association for the Advancement of Pharmacy established the Pharmacogenetics Working Group in 2005, a 15-member multidisciplinary working group, comprising clinical pharmacists, physicians, clinical pharmacologists, clinical chemists, epidemiologists, and toxicologists are represented. This ore only two example of what it is moving on this panorama.

The pharmacogenomics studies require a large number of subjects and multi-disciplinary teams with complementary expertise, as well as the ability to genotype a very large number of polymorphisms and haplotypes, frontier joined with Genome Wide Associations Studies (GWAS) [109]. The main current problems related to pharmacogenomics are: poorly defined phenotypes; studies of non-functional mutations; ethical aspects such as the use of genomic information; unclear sources for covering diagnostic costs and lack of founding for large prospective randomized studies and not only retrospective studies.

Aim of the study

With the advent of novel therapies, patients with MM are enjoying a longer survival. Bortezomib as a single agent, has been shown to be an highly effective treatment against cancer cells, and unlike most anticancer agents, bortezomib is effective against MM even when MM cells have already showed multi drug resistance. Bortezomib is unique because it enhances the sensitivity of cancer cells to other treatment drugs. Therefore, the trend is to combine bortezomib with other conventional agents, such as dexamethasone, thalidomide, doxorubicin and melphalan and novel agents, such as lenalidomide. In addition, there are other clinical trials looking at the combination of bortezomib with other investigational agents. The reason that bortezomib is used in so many different combinations is that it is a very effective agent in MM. Despite many phase II and III studies show favorable results in treating MM, the treatment outcome for patients are often varied and difficult to predict, and more and more patients are relapsing to the treatment.

The general aim of my three-years research period was to investigate resistant mechanisms in MM with regard to bortezomib treatment. In particular, I have been focusing on the relationship between pharmacogenetics biomarkers and drug response, on the basis of an *ex vivo* approach.

The main hypothesis of the work presented in this dissertation is that DNA damage repair pathways mediate resistance to agents used to treat MM - and other cancers in general - and that one of the proposed mechanisms of action for proteasome inhibitors, as bortezomib, is inhibition of DNA damage repair.

Following the above mentioned hypothesis, the **first objective** was to conduct a retrospective study in a subset of 454, previously untreated, MM patients enrolled in a randomized phase III open-label study, and to investigate a panel o SNPs in DNA repair and Folate pathway genes, as candidate for bortezomib responsiveness.

There are indications that overexpression of P-gp may contribute to resistance to bortezomib. For example it has been shown that knockdown of P-gp resensitises leukaemia cells to proteasome inhibitors. Despite these important mechanisms of resistance little is known about the interaction of bortezomib with P-gp. For this reason, the **second objective** was to demonstrate that bortezomib is a P-gp substarte in an *in vitro* model and subsequently we investigated if SNPs in ABCB1, coding for P-gp, may contribute to the phenomenon of bortezomib resistance.

Materials and methods

9. Materials and methods - first objective

SNPs in DNA repair and Folate pathway genes, as candidate for bortezomib responsiveness in newly diagnosed MM patients

9.1. Study population

A total of 454 MM patients were retrospectively enrolled in this pharmacogenetic study. These patients represented a subset of a randomized phase III trial, on V-TD vs TD in newly diagnosed and previously untreated MM patients [110]. Their selection was based exclusively on availability of a written informed consent for correlative sub-studies, according to the Helsinki Declaration and later Amendments. The study was approved by all local ethical committees of the 73 hospitals of the GIMEMA (Gruppo Italiano Malattie EMatologiche dell'Adulto) Myeloma Network in Italy involved in the study. In Figure 11 (modified from Cavo et al., [110]) is summarized the treatment protocol.

9.2. Evaluation of bortezomib response

Laboratory and clinical investigations, done to assess response in the population of interest, are described in a comprehensive manner by Cavo *et al*., [110]. Bone marrow biopsy and aspirate samples were obtained at baseline, at pre-specified time-points, and as needed to confirm complete response. Response and progression were reported by investigators according to criteria of the European Group for Blood and Marrow Transplantation [111], with the addition of categories for near complete response (100% reduction in M protein according to electrophoresis, but immunofixation positive) [63] and very good partial response (≥90% reduction in serum M protein, and less than 100 mg urine M protein per day) [112]. Patients with complete response who lacked confirmation from bone marrow biopsy samples were centrally downgraded to very good partial response.

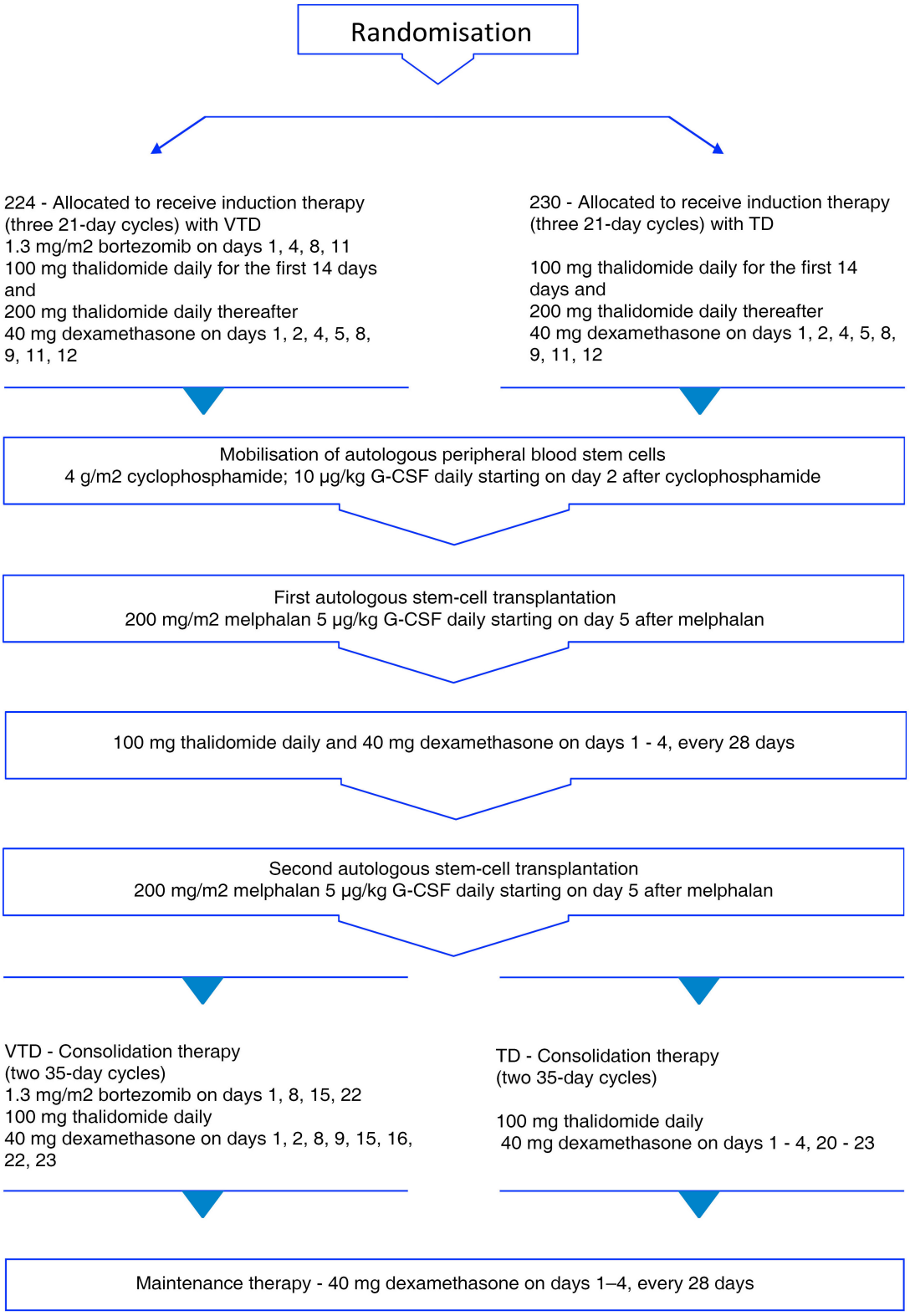

Figure 11. Trial design [G-CSF: granulocyte colony-stimulating factor]

9.3. DNA extraction

DNA was extracted from a fresh fraction of plasma cells using QIAamp® DNA Mini Kit (Quiagen, Hilden, Germany). For several samples DNA extraction was also carried, following the same procedure, from peripheral blood. The kit provides a fast and easy method for DNA purification from whole human blood, lymphocytes, plasma, serum, and body fluid and sample may be either fresh or frozen. After treatment with lysis buffer, the kit provides proteinase K, commonly used to digest proteins, to remove contamination and inhibits nucleases that might degrade DNA. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the silica membrane of the QIAamp spin column. To improve the purity of the eluted DNA membranes were washed two times with two different washing buffers (Buffer AW1 and Buffer AW2). Purified DNA was finally eluted from the membrane in a concentrated form in Buffer AE. All the passages for DNA isolation were performed following the protocol provided with the kit. The material collected was stored at -20°C.

9.4. DNA quantification

The DNA quantification was performed using NanoVue™, an innovative spectrophotometer able to measure small volumes of very concentrated or highly absorbing samples. Nucleic acids were quantified at 260 nm. DNA quality was also evaluated quantifying the absorbance (A) at 260 and 280 nm. All samples gave a A_{260}/A_{280} ratio greater than 1.8, indicating that the DNA is reasonably clean of proteins that could intefere with downstream applications.

9.5. Genotyping analysis

Genotypes were determined by PCR following the restriction fragment length polymorphism (RFLP) and/or the real-time PCR approach according to published methods or as recommended by manufacturer. The genotype results were regularly confirmed by repetition of 100% of the samples. Positive and negative

controls were included in each reaction as quality control. In addition, accuracy of genotyping was confirmed by repetition of 100% of the samples. The replicates were 100% concordant.

9.6. PCR RFLP

The principle components (Table 3) to perform a PCR are primers, containing sequences complementary to the target region, DNA polymerase, necessary to enzymatically assemble a new strand of DNA, deoxynucleoside triphosphates (dNTPs), from which the DNA polymerases synthesize a new DNA strand, buffer solution and usually divalent cations that help the reaction (usually $MgCl_2$).

Table 3. Principle components to perform PCR

Components	Final concentration
Water	0.52X
10X Buffer	1X
MgCl2 solution	2.00 mM
dNTPs	0.15 mM
Forward primer	0.13 μM
Reverse primer	0.13 μM
Taq polymerase	0.03 U/μL

DNA was added at this mixture and the thermal cycling program was performed. The DNA is replicated in every cycles and the amount increased exponentially. After the amplification of the sequence of interest, the appropriate restriction enzyme was used to recognize, if present, the restriction site creating DNA fragments. The fragments obtained after incubation with the restriction enzyme

(usually 37°C, overnight) were separated through electrophoresis at 200 V for 30 minutes, on pre-cast polyacrilamid gels 10% TBE (BIO RAD, Hercules, CA, USA). The DNA was then analyzed using ethidium bromide, an intercalating agent commonly used as a fluorescent tag. When exposed to UV ray, its fluorescence is 20-fold higher after binding DNA. The image of the gel was acquired using a digital photo camera connected to VERSADOC-4000 (BIO RAD) and visualized with QUANTITY ONE software. Three were the possible situation that can merge from the analysis: homozygosis for the *wild type* (*wt*) allele, homozygosis for the SNP or hetherozigosis, in which only one of the two alleles presents the SNP and the other one is *wt*.

9.7. Real Time PCR

Real time PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or mRNA in a sample. Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles. More specifically for our use, an allelic discrimination assay was used to detect variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants of a polymorphism site in a target template sequence. One fluorescent dye detector is a perfect match to the *wt* (allele 1) and the other fluorescent dye detector is a perfect match with the SNP allele (allele 2). The allelic discrimination assay classifies unknown samples as: i) homozygotes (samples having only allele 1 or allele 2) and ii) heterozygotes (samples having both allele 1 and 2). The allelic discrimination assay measures the change in the fluorescence of the dyes associated with the Taqman probes VIC® and FAM™ (Applied Biosystems, Foster City, CA), that selectively bind wt or SNP allele. The reaction was prepared using 10 ng DNA, 20X or 40X Taqman genoyping assay mix, 2X Taqman®Universal Master Mix, No AmpErase®UNG and water RNAse free, for a total volume of 25 μL. Each reaction was run in presence of a negative

control, no template control (NTC). The analysis was performed using Real Time PCR System 7300 (Applied Biosystems). Three were the steps performed for the allelic discrimination with Taqman probes: i) a pre-read run, using an allelic discrimination plate that measures the fluorescence at the baseline; ii) an amplification run, using a standard curve plate document to generate real-time PCR data that consist in 95°C for 10 min, 40 cycles at 92°C for 15 sec and 60°C for 1 min and, finally, iii) an allelic discrimination run (post-read run), using the allelic discrimination plate to analyze the data and discriminate the genotype, after removing the baseline fluorescence. Allelic discrimination assays use the fluorogenic 5' nuclease chemistry: fluorigenic probes, reporter (R), are used to detect specific PCR product as it accumulates during the PCR cycles. Additionally, the two probes at the 3'end bind the quencher (Q) and a minor groove binder (MGB) to improve the probe affinity for the target DNA. The process could be described in 4 passages: i) polymerization: R and Q are attached to the 5' and 3' end of a probe; ii) strand displacement, when both dyes are attached to the probe, R dye emission is quenched; iii) cleavage, during each extension cycle: the DNA polymerase system cleaves the R dye from the probe, iv) completion of polymerization: the R dye emits its characteristic fluorescence (Fig. 12). The signal is extremely specific because the probes do not interfere and the fluorescent signal is present only when the binding between probes and target DNA is correct.

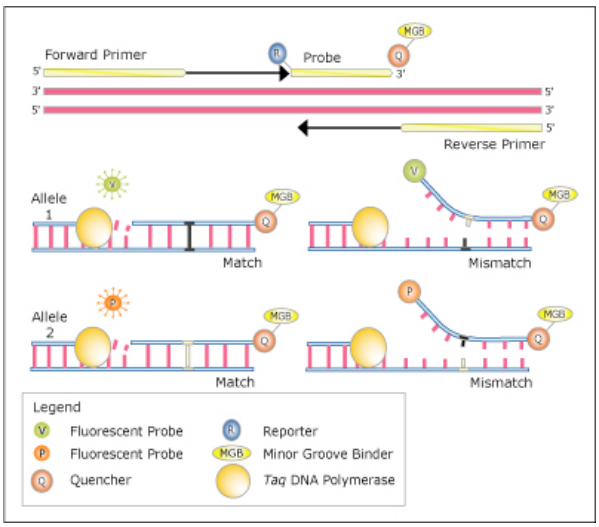

Figure 12. Molecular basis for TaqMan-based allelic discrimination.

The tables below shows the polymorphisms analyzed for each gene and the methods used for the analysis (Table 4-6).

Table 4. Details on investigated SNPs in DNA repair genes.

Gene rs unique code [amino acid change or non-coding region]	Method*
APEX1	
rs1130409 [Asp148Glu]	RFLP [113] RT TaqMan assay C_8921503_10
hOGG1	
rs1052133 [Ser326Cys]	RFLP [114] RT TaqMan assay C_3095552_1
NBS1	
rs1805794 [Gln185Glu]	RFLP [115]
XPD	
rs1799793 [Asp312Asn]	RT TaqMan assay C_3145050_10
rs13181 [Lys751Gln]	RFLP [116] RT TaqMan assay C_3145033_10
XRCC1	
rs861539 [Gln399Arg]	RFLP [116] RT TaqMan assay C_622564_10
XRCC3	
rs861539 [Thr241Met]	RFLP [116] RT TaqMan assay C_3145033_10
XPA	
rs1800975 [5' UTR region]	RT TaqMan assay C_482935_1_
rs2808668 [Intronic region]	RT TaqMan assay C_9312100_20

XPC	
rs2228000 [Ala499Val]	RT TaqMan assay C_16018061_10
rs2228001 [Gln902Lys]	RT TaqMan assay C_234284_1_

*RFLP = PCR-RFLP analysis carried out according to published methods (reference parenthetically); RT = Real-Time PCR with TaqMan allelic discrimination assay (Applera, Foster City, USA);

Table 5. Details on investigated SNPs in folate metabolic pathway genes.

Gene rs unique code [amino acid change or non-coding region]	Method*
DHFR	
rs70991108 [Intron 1]	PCR/electrophoresis [117]
FOLR1	
rs2071010 [5' UTR region]	RT TaqMan assay C_15861044_10
MTHFR	
rs1801133 [Ala222Val]	RFLP [118] RT TaqMan assay C_8714009_10
rs1801131 [Glu429Ala]	RFLP [118] RT TaqMan assay C_850486_20
MTR	
rs1805087 [Asp919Gly]	RFLP [119] RT TaqMan assay C_12005959_10
MTRR	
rs1801394 [Ile49Met]	RFLP [120] RT TaqMan assay C_3068176_10
rs1532268 [Ser175Leu]	RT TaqMan assay C_3068164_10
rs162036 [Lys350Arg]	RT TaqMan assay C_3068152_10

MTRR	
rs10380 [His595Tyr]	RT TaqMan assay C_7580070_1
RFC	
rs1051266 [Arg27His]	RFLP [121]
SHMT	
rs1979277 [Leu474Phe]	RFLP [122]
	RT TaqMan assay C_3063127_10
TYMS	
rs45445694 [enhancer region]	PCR/electrophoresis [123]

* PCR/electrophoresis and RFLP = PCR-RFLP analysis carried out according to published methods (reference parenthetically); RT = Real-Time PCR with TaqMan allelic discrimination assay (Applera, Foster City, USA);

Table 6. Details on investigated SNPs in ABCB1 gene.

Gene rs unique code [amino acid change or non-coding region]	Method*
ABCB1	
rs10245483 [Promoter region]	RT TaqMan assay C_2573447_20
rs3213619 [Promoter region]	RT TaqMan assay C_27487486_10
rs1128501 [Gly185Val]	RT TaqMan assay C_7586664_10
rs1128503 [Gly412Gly]	RT TaqMan assay C_7586662_10
	RFLP [Goreva *et al.*, 2004]
rs60023214 [Ile1145Ile]	RFLP [124]
rs2032582 [Ser893Thr or Ser893Thr]	RFLP [125]

*RFLP = PCR-RFLP analysis carried out according to published methods (reference parenthetically); RT = Real-Time PCR with TaqMan allelic discrimination assay (Applera, Foster City, USA);

9.8. Statistical analysis

Genotypes distribution, in the overall population was tested for Hardy-Weinberg equilibrium (HWE) with exact test. Additive models, with SNPs represented as number of minor allele (0, 1 or 2), were used to assess association between individual SNPs and treatment outcome after 4 months of induction therapy. Relative risk (RR) and 95% CI were estimated with Cox proportional hazard models and evaluated with likelihood ratio tests. All statistical tests were two-sided, with statistical significance defined as $P = 0.05$. Statistical analyses were conducted using SAS version 9.1 (SAS Institute Inc., Cary, NC).

10. Materials and methods - second objective

Bortezomib as P-gp substrate and ABCB1 variants candidate for bortezomib responsiveness in newly diagnosed MM patients

10.1. Bi-directional transport studies

10.1.2. Materials

MDCK–MDR1, MDCK Caco-2 cell lines and all components necessary for proper cell culture growth conditions (described below) were purchased from the University of California, San Francisco Cell Culture Facility (San Francisco, CA).

Bortezomib was obtained from LC Laboratories. GG918 (GF 120918: N-[4-[2-(3,4-Dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)ethyl]phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridinecarboxamide) was a kind gift from GlaxoSmithKline, Research Triangle Park, NC). Falcon PET cell culture inserts (pore size 0.4 μm, diameter 4.2 cm2) and Costar six-well plates were obtained from Fisher Scientific (Santa Clara, CA). All solvents were HPLC grade and were obtained from Fisher Scientific.

10.1.3. Cell Cultures

Caco-2 cells were grown in a humidified 5% CO2 atmosphere at 37ºC using minimal essential medium (MEM) Eagle's with Earle's balances salt solution (BSS) containing: 2 mM L-glutamine, 5.5 mM glucose, and 2.2 g/L sodium bicarbonate (NaHCO3), which was supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% of heat-inactivated FBS (custom made from the UCSF Cell Culture Facility), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were grown to 90-100% confluence and harvested using 0.05% trypsin EDTA. Monolayers were prepared by seeding harvested cells onto inserts at a density of 250,000 cells/insert. Growth medium for the Caco-2 was refreshed 48

hours post-seeding and then twice weekly, including one day prior to the experiment. Caco-2 cell monolayers were used for bidirectional transport experiments 21-28 days post-seeding.

MDCK cells were grown in a humidified 5% CO2 atmosphere at 37ºC using Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (custom made from the UCSF Cell Culture Facility), 100 U/mL penicillin and 100 U/mL streptomycin; 1% of nonessential amino acid. MDCK–MDR1 cell culture medium was as described above with addition of 80 ng/mL colchicine as a selective supplement. Cells were grown to 90-100% confluence and harvested using 0.25% trypsin EDTA. Monolayers were prepared by seeding harvested cells onto inserts at a density of 250,000 cells/insert, and maintained for 7 days before the experiment. Fresh media was added to the cells 3 days after seeding and 24 h before the transport study.

10.1.4. Bidiretional Transport Experiments

The transport assays were performed following a modified protocol previously described. [126]

Cell monolayers were preincubated in transport buffer (Hank's buffered salt solution containing 25 nM HEPES and 1% FBS, pH 7.4) at 37ºC for 20 min. Transepithelial electrical resistance (TEER) values were measured across the monolayer's using a Millicell (Millipore Corporation, Bedford, MA) equipped with chopstick electrodes (Figure 13). Approximate MDCK TEER values were 200 to 250 Ω cm2, MDCK–MDR1 values ranged from 900 to 1300 Ω cm^2, and Caco-2 values ranged from 240 to 320 Ω cm^2.

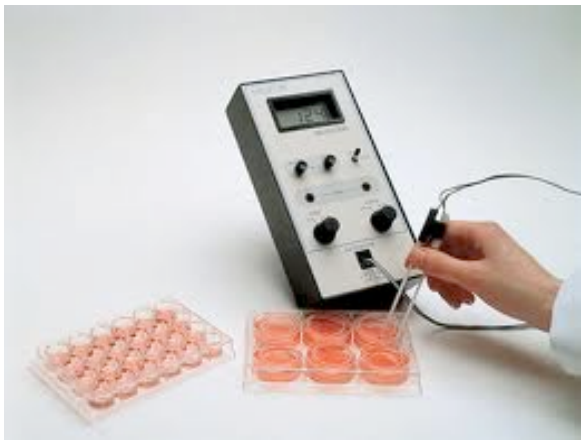

Figure 13. Millicell (Millipore Corporation, Bedford, MA) equipped with chopstick electrodes for TEER measurement

All experiments were performed in triplicate. In the case of the MDCK-MDR1 and parental MDCK cells, both these transfected and control cell lines were run on the same day to account for potential between-day variability of LC-MS/MS measurement and TEER values.

To measure P-gp effect in mediated transport, solutions were prepared containing 1µM drug in Hank's buffered salt solution modified and compared further to bidirectional transport with and without GG918, a P-gp inhibitor. To measure A→B transport, 1.5 mL of drug solution was added to the apical side (A) and 2.5 mL of Hank's buffered salt solution to the basolateral side (B). B→A transport was evaluated adding 2.5 mL of drug in B side and 1.5 mL of Hank's buffered salt solution in A side. To evaluate P-gp effect, the same procedure above was used adding 500 nM of GG918 in both sides. All experiments were run at 37ºC with a shaking speed of 25 strokes/minute in the Boekel Shake 'N' Bake Incubator Shaker II (Boekel Scientific, Feasterville, PA).

Cells were incubated in a 37,5 ºC shaker, and 200 µL aliquots were taken from the receiver side at 1, 2, and 3 h time intervals. At the first two time points, 200 µL was replaced with fresh receiver solution to maintain the original starting volumes. After the last time point, the apical solution was removed by suction, and each filter was dipped in three different beakers containing large volumes of ice-cold phosphate-buffered saline. The inserts were inverted to remove residual liquid.

When dried, membranes were removed from the inserts and sonicated (in an ultrasonic bath) in 1mL of 25% ACN for 15 min. This volume was than centrifuged for 10 min at 10,900g, and the resulting supernatant was analyzed by LC/ MS/MS as well as all 200 µL aliquots.

10.1.5. Measurement of bortezomib by LC-MS/MS

An API 4000 using Electrospray/positive ionization was employed. Multiple Reaction Monitor (MRM) was set at 367.008-226.129 m/z for bortezomib and 237.01-194.02 m/z for carbamazepine, CBZ, (internal standard). The column was Phenomenex, Gemini, C18 (100 mm x 2 mm, 5 µm particle size) with a mobile phase consisting of A, 99.9% H_2O containing 0.1% formic acid and B, 99.9% ACN containing 0.1% formic acid.

The gradient program was as follows: 0–6 min, B, 25–90%; 6.01–8 min, B, 25%. The flow rate was 0.5 mL/min. Running time for each sample was 8 min.

10.1.6. Data analysis

The apparent permeability (P_{app}) values (cm/s) were calculated as follows, where the rate of transport was measured from the flux of drug across the cells (ng/h).

P_{app} = Rate of transport/(surface area x initial donor concentration)

Efflux ratio was defined as the ratio of basolateral to apical (B→A) over apical to basolateral (A→B) transport.

10.1.7. Statistical analysis

Experimentally derived in vitro data are shown as mean ± SD (n=3). Student's t-test was used to analyze differences between two groups. The p-value for statistical significance was set at <0.05.

10.2. Identification of P-gp in MM cells

The cell line used was U-266, cells derived from human MM. They were cultured in RPMI 1640 medium with L-glutamine, sodium pyruvate, and Hepes buffer (ATCC), supplemented with 10% fetal bovine serum (FBS, Lonza, inactivated for 30 minutes at 57°C), 5% CO_2-95% air at 37°C in cell incubator. The medium was supplemented with 1% penicillin (10.000 Units/mL)/streptomycin (10 mg/mL) and replaced every 2 or 3 days to maintain a cells density of about 5*10^5 cells/mL.

Cells were exposed to increasing concentration of bortezomib (Aurogene,). The parental, sensitive cell line was maintained in parallel cultures without bortezomib to be used as control. Powder was stored at room temperature and a stock solution (10 mM) was prepared dissolving the drug in DMSO (1:6000). The starting treatment concentration of cells was 0.0125 μM. The medium was replaced every 2 or 3 days with the necessary bortezomib concentration. The resistance was observed when the cells acquired the ability to grow in the presence of a specific concentration of the drug. U266 resistant cells were collected at the following bortezomib concentrations: 0.0125 μM and 0.025 μM. The process required about 2 months. Proteins were collected from cells treated at each concentrations following the protocol described in *Protein isolation* (10.2.1.).

10.2.1. Protein isolation

To isolate proteins Qproteome Mammalian protein Prep kit (QIAGEN, Hilden, Germany) was used. The lysis buffer was added with 10μL/mL of Protease inhibitor. Cells were washed with cold DPBS and treated with lysis buffer. The supernatant containing protein was stored at -80°C. Bradford solution was prepared using Protein Assay (Bio-Rad) and a standard curve was built using increasing concentration of BSA (Bovin Serum Albumin). Probes were measured at 595 nm in Photometer (Eppendorf) and the concentration obtained using the standard curve.

10.2.2. Electrophoresis

Gel electrophoresis was necessary to separate native or denatured protein by the length of the polypeptide or by the 3-D structure of the proteins. Protein probes were prepared at the concentration of 20 μg in 6.5 μL of Lysis Buffer. The marker used to identify protein was Spectra^{TM} Multicolor Broad Range Protein Ladder (Fermentas) and the gels used were pre-cast 10% Bis-Tris Gel (Biorad) in 20x buffer (Biorad) running at 150 V.

10.2.3. Western Blot

Proteins were than transferred to a PVDF (Polyvinildifluoridon) membrane (0.45 μM, Millipore) where they were probed and than detected using antibodies specific to the target protein.

The transfer from the gel to the membrane was done preparing the Blot-Sandwich. After the transfer, membranes were washed 5-10 minutes in TBS-T buffer on the shaker and then incubated 1 hour at room temperature with 5% milk, which blocks all remaining hydrophobic binding sites to prevent unspecific probing of the antibody. The membranes were then incubated with primary antibody, diluted in 5% milk, 4°C overnight on the shaker.

Antibody	Characterization	Dilution
B-actine	Mouse monoclonal IgG-1 antibody (Anti-β-actin, Clone AC-15 Sigma)	1:10 000
P-gP	Mouse monoclonal IgG-2a antibody (Clone C219 Enzo, Lörrach, Germany)	1:250

The membranes were washed three times, then incubated with secondary antibody diluted in 5% milk on the shaker.

Antibody	Characterization	Dilution
Anti-mouse	Rabbit peroxidase conjugate IgG-A antibody. Rabbit-anti-mouse (Sigma)	1:2000

After washing the detection of proteins was done using ECL™ Western Blot Detection Reagent (GE Healthcare).

10.3. Genotyping analysis

Genotypes were determined by PCR following the restriction fragment length polymorphism (RFLP) and/or the real-time PCR following the methods described in chapter 9.1.

Results

Demographic and disease characteristic at diagnosis of the overall population, and stratified by treatment are reported in Table 7. The Mean age at diagnosis is 56.2 ± 7.2 year, confirming MM is principally a disease of the elderly [7]. 57% are male and 43% female; this is in accordance with the literature on MM in which is reported an higher frequency of the disease in males than females, independently from the race. Regarding myeloma subtype, 63.4% were IgG, 19.8% were IgA and 15.8 harboured the light chain (BJ). The randomisation sequence was computer generated and was stratified by international staging system (ISS) disease stage [34]. 45.6% of the patients were in stage I, 37.7% in stage II, and 16.7% in stage III. Data on cytogenetic abnormalities, del(13q), t(4;14), and del(17p), detected by fluorescence in-situ hybridisation on highly purified bone marrow plasma cells, were available in more than 90% of patients. Cytogenetic abnormalities were not present in 203 patients (44.7%).

Demographic and disease characteristics were well balanced between treatment groups at baseline (Table 7).

Table 7. Demographic and disease characteristic at diagnosis in the overall population

	Overall (*n* 454)	**TD (*n* 230)**	**V-TD (*n* 224)**
Age (years)	56.2 ± 7.2	55.9 ± 7.4	56.3 ± 6.9
Mean ± SD (range)	(23.1 - 66.3)	(23.1 - 65.9)	(34.5 - 66.3)
Sex			
Male *n* (%)	259 (57.0%)	129 (56.1%)	130 (58.0%)
Female *n* (%)	195 (43.0%)	101 (43.9%)	94 (42.0%)
Myeloma subtype			
IgG *n* (%)	288 (63.4%)	144 (62.6%)	144 (64.3%)
IgA *n* (%)	90 (19.8%)	50 (21.7%)	40 (17.6%)
Light chain *n* (%)	72 (15.8%)	33 (14.3%)	39 (17.4%)
Other *n* (%)	4 (1%)	3 (1.3%)	1 (< 1%)

ISS disease stage			
I	207 (45.6%)	104 (45.2%)	103 (46%)
II	171 (37.7%)	87 (37.8%)	84 (37.5%)
III	76 (16.7%)	39 (17.0%)	37 (16.5%)
β2-microglobulin (mg/L)			
Mean ± SD (range)	3.4 ± 2.3 (0.2 - 15.7)	3.8 ± 2.2 (1.2 - 12.8)	3.8 ± 2.5 (0.2 - 15.7)
Albumin (g/L)			
Mean ± SD (range)	3.8 ± 0.63 (1.8 - 5.4)	3.8 ± 0.62 (1.8 - 5.2)	3.8 ± 0.63 (2.1 - 5.4)
Haemoglobin (g/L)			
Mean ± SD (range)	11.2 ± 1.9 (5.4 - 16.0)	11.3 ± 2.0 (6.0 - 16.0)	11.1 ± 1.9 (5.4 - 15.1)
Platelets ($\times 10^9$ per L)			
Mean ± SD (range)	242.1 ± 83.6 (72 - 903)	239.6 ± 75.6 (74 - 644)	244.7 ± 91.1 (72 - 903)
Bone marrow plasma cells			
Mean ± SD (range)	52.7 ± 23.7 (2 - 100)	52.9 ± 24.3 (2 - 100)	52.5 ± 23.1 (2 -100)
Presence of del(13q)	193/230 *	97/119	96/111
Presence of t(4;14)	83/339 *	47/182	39/182
Presence of del(17p)	30/393 **	17/199	13/194
None	203	106	97

* Missing data for 31 subjects; ** Missing data for 4 subjects

Table 8 shows that rates of complete, near complete response and very good partial response were significantly higher after induction therapy with V-TD than with TD ($P < 0.0001$, RR 0.34, 95% CI 0.2 - 0.57). Also the overall response rate (ORR: Complete plus near complete response) was significantly higher after induction therapy with V-TD than with TD ($P < 0.0001$, RR 0.78, 95% CI 0.7 - 0.86). Interestingly none of the patients assigned to the V-TD regimen had disease progression during induction therapy. On the contrary of the 49 patients belonging to the stable/progressive disease group in the TD regimen, 11 had disease progression during induction therapy.

Table 8. Treatment response in the overall population and according to treatment

	Overall	TD	V-TD
Complete response	52 (11.4%)	11 (4.8%)	41 (18.3%)
Near complete	45 (10.0%)	16 (6.9%)	29 (12.9%)
Very good partial response	103 (22.7%)	36 (15.6%)	67 (29.9%)
Partial response	189 (41.6%)	118 (51.3%)	71 (31.7%)
Stable/Progressive disease	65 (14.3%)	49 (21.3%)	16 (7.1%)
ORR	97 (21.4%)	27 (11.7%)	70 (31.2%)

ORR: Complete plus near complete response.

11. Results - first objective

SNPs in DNA repair and Folate pathway genes, as candidate for bortezomib responsiveness in newly diagnosed MM patients

11.1. Treatment response according to polymorphisms in genes of the folic acid and DNA repair pathways.

All the studied SNPs in both pathways (folic acid metabolism and DNA repair) were in Hardy-Weinberg equilibrium as demonstrated from the reported p value below.

Table 9 shows distribution of polymorphisms in genes involved in the folic acid metabolism pathway in the overall population and in poor responder only. In the overall population frequency of the variant allele were as follow: RFC: 0.410 (Hardy-Weinberg P = 0.648); FOLR: 0.053 (Hardy-Weinberg P = 0.918); MTHFR: 0.425 (Hardy-Weinberg P = 0.656) and 0.329 (Hardy-Weinberg P = 0.615) for rs1801133 and rs1801131 respectively; MTR: 0.189 (Hardy-Weinberg P = 0.952); MTRR: 0.459 (Hardy-Weinberg P = 0.926) for rs1801394, 0.333 (Hardy-Weinberg P = 0.730) for rs1532268, 0.151 (Hardy-Weinberg P = 0.881) for rs162036 and 0.129 (Hardy-Weinberg P = 0.813) for rs10380; SHMT1: 0.236 (Hardy-Weinberg P = 0.861); DHFR: 0.404 (Hardy-Weinberg P = 0.772); TS: 0.563 (Hardy-Weinberg P = 0.885) and 0.382 (Hardy-Weinberg P = 0.945) for rs45445694 and rs34489327 respectively.

When stratifying the population according to poor response (very good partial response, partial response and stable/progressive disease; Table 9), we observed a significant association between poor responder after four months of induction therapy and MTFHR rs1801131 genotype (p = 0.0428), also after adjustment for treatment response (p = 0.0388). This finding, ascribe to MTHFR the role of prognostic factor for therapeutic outcome independently from the regimen.

Table 9. Treatment response in the overall population according to polymorphisms in genes of the folic acid pathway

Genes		Overall Population	Poor Responders*	p value	RR (95% CI)	p value	RR (95% CI) Treatment adjusted
RFC	wt	152	116	0.696		0.778	
	He	232	185		1.04 (0.94-1.17)		1.02 (0.96-1.08)
	SNP	70	56		1.05 (0.91-1.21)		1 (0.92-1.09)
FOLR	wt	406	316	0.322		0.644	
	He	44	37		1.08 (0.94-1.24)		0.98 (0.90-1.07)
	SNP	2	2		-		-
MTHFR rs1801133	wt	144	112	0.7668		0.8007	
	He	234	187		1.03 (0.92-1.15)		1.01 (0.96-1.07)
	SNP	76	58		0.98 (0.84-1.14)		1.04 (0.9- 1.2)
MTHFR rs1801131	wt	211	166	**0.0428**		**0.0388**	
	He	187	154		1.05 (0.95-1.15)		1 (0.96-1.04)
	SNP	56	37		0.84 (0.69-1.03)		0.86 (0.73-1.01)
MTR	wt	299	239	0.5219		0.99	
	He	138	104		0.94 (0.84-1.05)		0.98 (0.91-1.06)
	SNP	17	14		1.03 (0.82-1.29)		1.03 (0.8-1.33)
MTRR rs1801394	wt	134	100	0.1825		0.3524	
	He	223	175		1.05 (0.93-1.19)		1.05 (0.97-1.13)
	SNP	97	82		1.13 (0.99-1.29)		1.03 (0.95-1.12)
MTRR rs1532268	wt	206	166	0.1989		0.4339	
	He	192	152		0.98 (0.89-1.08)		0.97 (0.93-1.02)
	SNP	55	38		0.86 (0.71-1.04)		0.98 (0.83-1.15)
MTRR rs162036	wt	328	256	0.8595		0.99	
	He	113	90		1.02 (0.91-1.14)		1.01 (0.96-1.07)
	SNP	12	10		1.07 (0.82-1.38)		1.04 (0.42-2.57)

MTRR rs10380	wt	346	271	0.6264		0.99	
	He	97	76		1 (0.89-1.13)		1.01 (0.95-1.07)
	SNP	10	9		1.15 (0.93-1.42)		1.03 (0.29-3.67)
SHMT1	wt	263	201	0.3962		0.5701	
	He	168	137		1.07 (0.97-1.18)		1.02 (0.97-1.07)
	SNP	23	19		1.08 (0.89-1.32)		0.97 (0.86- 1.1)
TS rs45445694	wt	85	65	0.7899		0.3704	
	He	227	178		1.03 (0.89-1.18)		1.02 (0.96-1.09)
	SNP	142	114		1.05 (0.91-1.21)		0.98 (0.91-1.07)
TS rs34489327	wt	174	135	0.7326		0.4806	
	He	212	166		1.01 (0.91-1.12)	.	1.03 (0.98-1.09)
	SNP	67	55		1.06 (0.92-1.21)	.	1.02 (0.95- 1.1)
DHFR	wt	157	128	0.1564		0.5257	
	He	226	179		0.97 (0.88-1.07)		0.98 (0.93-1.02)
	SNP	70	49		0.86 (0.72-1.02)		0.96 (0.88-1.06)

* Poor responder: includes very good partial response, partial response and stable/progressive disease.

When considering patients with stable/progressive disease as poor responder only, MTR genotype was associated with treatment response (Table 10). In particular the variant allele was associated with favourable response (p = 0.0281). However this association disappears after adjustment for treatment regimen (P = 0.0505).

Table 10. Treatment response in the overall population according to polymorphisms in genes of DNA repair pathways

Genes		Overall Population	Poor Responders*	p value	RR (95% CI)
MTR	wt	299	49	0.0281	
	He	138	16	.	0.71 (0.42-1.2)
	SNP	17	0	.	0 (0 – 0)

* Poor responder: stable/progressive disease.

Table 11 shows distribution of polymorphisms in genes involved in the DNA repair pathway in the overall population and in poor responder only. In the overall population frequency of the variant allele were as follow: XPD: 0.416 (Hardy-Weinberg P = 0.676) and 0.423 (Hardy-Weinberg P = 0.871) for rs1799793 and rs13181 respectively; XRCC1: 0.345 (Hardy-Weinberg P = 0.464); XRCC3: 0.412 (Hardy-Weinberg P = 0.658); NBS1: 0.309 (Hardy-Weinberg P = 0.851); APEX1: 0.384 (Hardy-Weinberg P = 0.604); hOGG1: 0.203 (Hardy-Weinberg P = 0.782); XPA: 0.326 (Hardy-Weinberg P = 0.698) for both rs2808668 and rs1800975 that are in complete linkage, as reported in the literature; XPC: 0.240 (Hardy-Weinberg P = 0.691) and 0.468 (Hardy-Weinberg P = 0.972) for rs222800 and rs2228001 respectively.

None of the other investigated SNPs in folic acid pathway or DNA repair pathway (Table 11) resulted associated with treatment outcome.

Table 11. Treatment response in the overall population according to polymorphisms in genes of DNA repair pathways.

Genes		Overall Population	Poor Responders*	p value	RR (95% CI)	p value	RR (95% CI) Treatment adjusted
xpd ex10	wt	149	119	0.904		0.99	
	He	232	181		0.98 (0.88-1.09)		0.96 (0.85-1.09)
	SNP	73	57		0.98 (0.84-1.13)		0.96 (0.84- 1.1)
xpd_ ex23	wt	149	114	0.2168		0.4851	
	He	226	185		1.07 (0.96-1.19)		1.02 (0.97-1.09)
	SNP	79	58		0.96 (0.82-1.13)		0.98 (0.88-1.09)
xrcc1	wt	185	147	0.7501		0.99	
	He	225	174		0.97 (0.88-1.08)		0.97 (0.92-1.02)
	SNP	44	36		1.03 (0.88-1.21)		1.02 (0.73-1.43)
xrcc3	wt	151	120	0.4969		0.6811	
	He	232	185		1 (0.9-1.11)		1.02 (0.97-1.07)
	SNP	71	52		0.92 (0.78-1.08)		1.04 (0.88-1.23)
nbs1	wt	219	177	0.5442		0.99	
	He	189	145		0.95 (0.86-1.05)		0.97 (0.91-1.02)
	SNP	46	35		0.94 (0.79-1.12)		1.02 (0.83-1.24)
apex	wt	165	126	0.2543		0.1395	
	He	229	187		1.07 (0.96-1.19)		0.99 (0.95-1.04)
	SNP	60	44		0.96 (0.81-1.14)		0.91 (0.8-1.03)
hOGG1	wt	292	229	0.7424		0.99	
	He	140	112		1.02 (0.92-1.13)		1.03 (0.91-1.17)
	SNP	22	16		0.93 (0.71-1.21)		0.92 (0.76-1.11)
XPA rs2808668	wt	201	154	0.3827		0.2797	
	He	210	171		1.06 (0.96-1.17)		1.04 (0.99-1.09)
	SNP	43	32		0.97 (0.8-1.18)		1.04 (0.88-1.22)

XPA rs1800975	wt	201	154	0.3827		0.2694	
	He	210	171		1.06 (0.96-1.17)		1.04 (0.99-1.09)
	SNP	43	32		0.97 (0.8-1.18)		1.04 (0.88-1.22)
XPC rs222800	wt	256	201	0.7153		0.7641	
	He	175	139		1.01 (0.92-1.12)		0.98 (0.94-1.03)
	SNP	21	15		0.91 (0.69-1.2)		1.02 (0.8-1.3)
XPC rs2228001	wt	127	105	0.2498		0.8382	
	He	225	177		0.95 (0.86-1.06)		1 (0.95-1.05)
	SNP	98	72		0.89 (0.77-1.03)		0.98 (0.9-1.07)

* Poor responder: includes very good partial response, partial response and stable/progressive disease.

Regarding the relationship between treatment response, we considered the outcome in the overall population and adjusted data for treatment regimen.

12. Results - second objective

Bortezomib as P-gp substrate and ABCB1 variants candidate for bortezomib responsiveness in newly diagnosed MM patients

12.1. Bidirectional transport studies

12.1.1. Bidirectional transport in MDCK cells

Transport profiles of 1µM bortezomib across MDCK cells in control and inhibitory conditions are depicted in Table 12 and Figure 14.

Table 12. Bortezomib transport and inhibition across MDCK cell monolayer

Cell line	Condition	P_{app}, cm/sec x 10^{-6}		Efflux ratio
		AtoB	BtoA	
MDCK	control	1.65±0.12****	8.79±0.77**	5.33
	w/ 0.5 μM GG 918	3.43±0.05****	5.48±0.24**	1.60

P_{app}, permeability; A, apical side; B, basolateral side.

****$p < 0.0001$, statistical difference observed, using paired, two-tailed t-test

**$p < 0.01$, statistical difference observed, using paired, two-tailed t-test

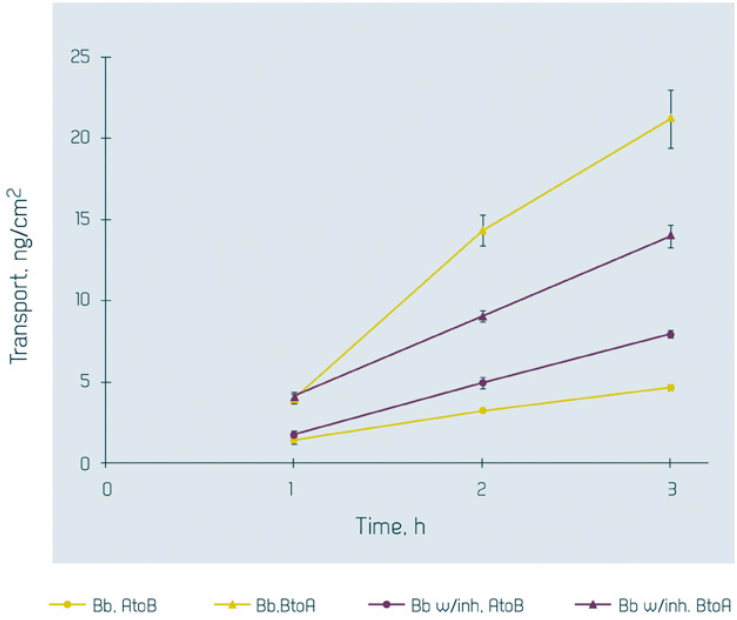

Figure 14. Bortezomib transport and inhibition across MDCK cell at 1, 2, 3 hours time points.

In both, control and inhibitory conditions, apparent permeability in direction B→A was greater than A→B. To determine whether bortezomib's transport was polarized, transepithelial fluxes were measured in both directions. Efflux ratio (B→A/ A→B) in control transport buffer was 5.33 and reduced to 1.60 in presence of GG918 (0.5 μM) (Table 12). According to the results shown in Table 12 after inhibition of P-gp by GG918 there was a significant increase of P_{app} in the A→B direction and decrease in B→A.

The intracellular accumulation increased in A→C direction after addition of GG918 from 4.92 ng to 7.01 ng (Table 13). In B→C direction the intracellular accumulation increased after addition of GG918 but this change wasn't statistically significant.

Table 13. Bortezomib intracellular accumulation in MDCK cells

Cell line	Condition	Drug accumulation, ng	
		AtoC	BtoC
MDCK	control	4.92±0.64*	2.92±1.57
	w/ 0.5 μM GG918	7.01±0.81*	4.64±0.81

A, apical side; B, basolateral side; C, intracellular accumulation.

*p < 0.05, statistical difference observed, using paired, two-tailed t-test.

12.1.2. Bidirectional transport in MDCK–MDR1 cells

In MDCK-MDR1, in control and inhibitory conditions, apparent permeability in direction B→A was greater than A→B (Table 14) (Figure 15). Addition of P-gp inhibitor decreased efflux ratio from 82.0 in control to 4.92 in inhibitory conditions. GG918 provoked 18-fold increase in apparent permeability in direction A→B.

Table 14. Bortezomib transport and inhibition across MDCK-MDR1 cell monolayer

Cell line	Condition	P_{app}, cm/sec x 10^{-6}		Efflux ratio
		AtoB	BtoA	
MDCK-MDR1	control	0.19±0.02**	15.6±1.4	82.0
	w/ 0.5 μM GG918	3.46±0.69**	17.0±2.3	4.92

Papp, permeability; A, apical side; B, basolateral side.

**p < 0.01, statistical difference observed, using paired, two-tailed t-test.

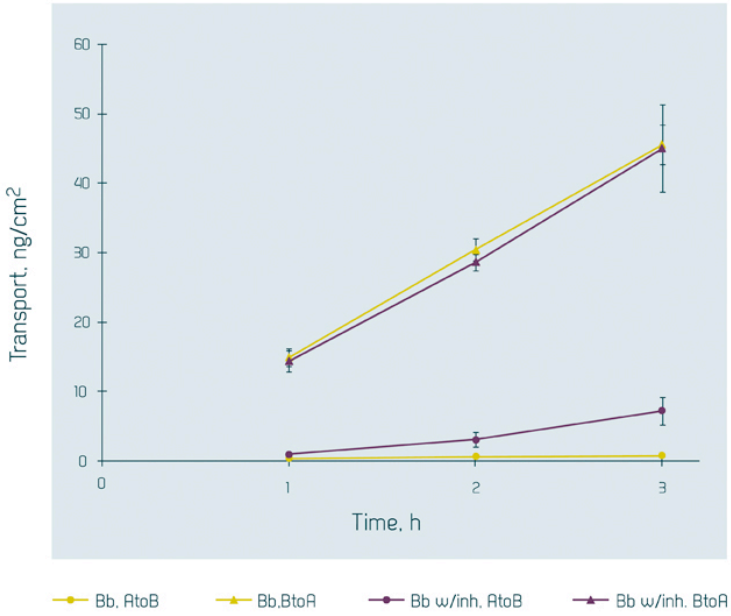

Figure 15. Bortezomib transport and inhibition across MDCK-MDR1 cell at 1, 2, 3 hours time points.

The intracellular accumulation increased in the A→C direction after addition of GG918 from 3.01 ng to 6.87 ng but didn't change in B→C direction (Table 15).

Table 15. Bortezomib intracellular accumulation

Cell line	Condition	Drug accumulation, ng	
		AtoC	BtoC
MDCK-MDR1	control	3.01±0.28**	5.42±0.31
	w/ 0.5 μM GG918	6.87±1.12**	5.83±0.90

A, apical side; B, basolateral side; C, intracellular accumulation.

**p < 0.01, statistical difference observed, using paired, two-tailed t-test

12.1.3. Bidirectional transport in Caco-2 cells

As shown in Table 16 and Figure 16 in Caco-2 cells, apparent permeability in direction B→A was greater than A→B in both, control and inhibitory condition. Efflux ratio (B→A/ A→B) in control transport buffer was 3.35 and reduced to 1.13 in presence of GG918 (0.5 μM) (Table 16). According to the results shown in Table 5 after inhibition of P-gp there was a significant increase of P_{app} in the A→B direction and decrease in B→A.

Table 16. Bortezomib transport and inhibition across Caco-2 cell monolayer

Cell line	Condition	P_{app}, cm/sec x 10^{-6}		Efflux ratio
		AtoB	BtoA	
Caco-2	control	3.1±0.1****	10.4±1.0***	3.35
	w/ 0.5 μM GG918	4.61±0.04****	5.19 ±0.43***	1.13

Papp, permeability; A, apical side; B, basolateral side,

***p < 0.001, statistical difference observed, using paired, two-tailed t-test

****p < 0.0001, statistical difference observed, using paired, two-tailed t-test

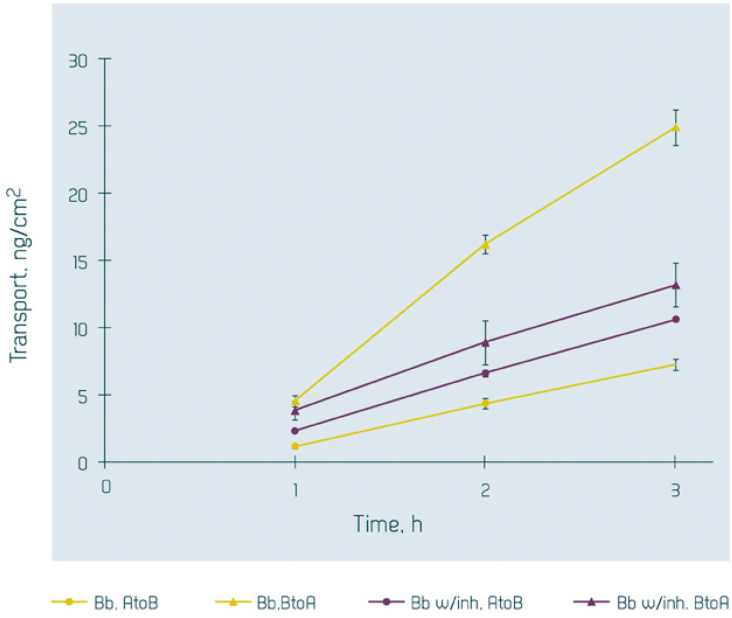

Figure 16. Bortezomib transport and inhibition across Caco-2 cell at 1, 2, 3 hours time points.

The intracellular accumulation decreased in A→C direction after addition of GG 918 but didn't change in B→C (Table 17).

Table 17. Bortezomib intracellular accumulation

Cell line	Condition	Drug accumulation, ng	
		AtoC	BtoC
Caco-2	control	10.58±0.21**	3.81±0.45
	w/ 0.5 μM GG918	5.47±1.73**	3.11±1.31

A, apical side; B, basolateral side; C, intracellular accumulation

**$p < 0.01$, statistical difference observed, using paired, two-tailed t-test

12.2. Expression of P-gp in multiple myeloma cells after treatment with bortezomib

The viability of U266 cells was significantly affected already at concentrations 0.0125 μM of bortezomib, leading to an inhibition of 50% after 48 h treatment.

Protein expression of ABCB1 - In order to determine the effects of long-term bortezomib treatment, bortezomib-resistant MM cell line we generated through incubation of U266 cells with increasing drug concentrations over a time period of four months. Due to the key role of ABCB1 transporter for intracellular bortezomib concentrations, we determined ABCB1 protein levels. There was ABCB1 protein expression in the U266 cells, under chronic conditions; Caco2 cells, a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells, were used as positive control, because they express ABCB1 (Figure 17).

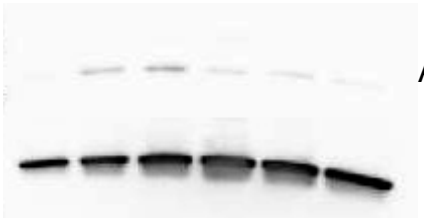

Figure 17. ABCB1 protein expression in U266 during long-term treatment (Caco2 cells were loaded as positive control. The lower bands represent β-actin)

The bidirectional study highlighted the importance of P-gp as bortezomib transporter. The western blot study confirm high expression of this efflux transporter in multiple myeloma cell line. Additionally, expression of P-gp was induced after treatment with bortezomib. The successive pass was to valuate the influence of SNP's in the gene codifying for P-gp in response to therapy.

12.3. Treatment response according to polymorphisms in ABCB1 gene.

In the overall population frequency of the variant ABCB1 allele were as follow: 0.492 (Hardy-Weinberg P = 0.449) for rs60023214; 0.443 (Hardy-Weinberg P = 0.642) for rs1128503; 0.422 (Hardy-Weinberg P = 0.631) for rs2038502; 0.423 (Hardy-Weinberg P = 0.814) for rs10245483; 0.384 (Hardy-Weinberg P = 0.604); 0.022 (Hardy-Weinberg P = 0.962) for rs3213619. All the studied SNPs were in Hardy-Weinberg equilibrium as demonstrated from the reported P value.

Table 18 shows that there was no correlation between ABCB1 SNPs analysed and treatment response.

Interestingly we found a significant association between ABCB1 variants and cytogenetic abnormalities (Table 19). In particular presence of the variants ABCB1 rs60023214 and rs2038502 are associated with the presence of del(17) (P = 0.0147 and P = 0.099 respectively) whereas ABCB1 rs2038502 is associated with presence of t(4;14) (P = 0.0217). These findings are very interesting. To the best of my knowledge is the first time that a genetic SNP is associated with presence of genetic abnormalities in MM patients. However, the mechanistic reason for this is unknown.

Table 18. Treatment response in the overall population according to polymorphisms in ABCB1 gene

Genes		Overall Population	Poor Responders*	p value	RR (95% CI)	p value	RR (95% CI) Treatment adjusted
ABCB1 rs60023214	wt	119	58	0.6876		0.9814	
	He	190	86		1 (0.9-1.12)		1.01 (0.95-1.06)
	SNP	114	49		0.95 (0.83-1.09)		1 (0.94-1.07)
ABCB1 rs1128503	wt	140	62	0.2537		0.441	
	He	193	90		1.09 (0.97-1.21)		1.04 (0.97-1.12)
	SNP	89	40		1 (0.87-1.16)		1.03 (0.94-1.12)

ABCB1 rs2038502	wt	138	57	0.5765		0.9183	
	He	215	105		1.03 (0.92-1.16)		1.01 (0.96-1.07)
	SNP	68	30		1.08 (0.94-1.24)		1.01 (0.94-1.08)
ABCB1 rs10245483	wt	139	61	0.8858		0.99	
	He	214	99		0.98 (0.89-1.09)		0.95 (0.84-1.09)
	SNP	70	33		0.96 (0.83-1.12)		0.98 (0.83-1.15)
ABCB1 rs3213619	wt	405	184	0.1688		0.1628	
	He	18	9		1.15 (0.99-1.34)		1.16 (0.99-1.35)

Table 19. Association between ABCB1 variants and cytogenetic abnormalities.

Genes		Overall pop.*	Del(17) Yes	P value	RR (95% CI)	Overall pop.*	T(4;14) Yes	P value	RR (95% CI)
ABCB1 rs60023214	wt	119	4	**0.0147**		126	31	0.1805	
	He	190	21		3.29 (1.16-9.34)	205	36		0.71 (0.47-1.09)
	SNP	114	5		1.3 (0.36-4.74)	119	19		0.65 (0.39-1.08)
ABCB1 rs1128503	wt	140	6	0.2448		146	33	0.4359	
	He	193	17		2.06 (0.83-5.08)	209	36		0.76 (0.5-1.16)
	SNP	89	7		1.84 (0.64-5.28)	94	17		0.8 (0.47-1.35)
ABCB1 rs2038502	wt	138	4	**0.0099**		144	38	**0.0217**	
	He	215	23		3.69 (1.3-10.4)	230	39		0.64 (0.43-0.95)
	SNP	68	3		1.52 (0.35-6.61)	73	9		0.47 (0.24-0.91)
ABCB1 rs10245483	wt	139	8	0.7302		147	25	0.5409	
	He	214	17		1.38 (0.61-3.11)	227	48		1.24 (0.8-1.92)
	SNP	70	5		1.24 (0.42-3.65)	76	13		1.01 (0.55-1.85)
ABCB1 rs3213619	wt	405	29	0.7882		430	82	0.9181	
	He	18	1		0.78 (0.11-5.38)	20	4		1.05 (0.43-2.57)

* Overall pop. = Overal population

Conclusions

The principle aim of this study was to investigate some of the possible biological predictors of response and resistance to multiple myeloma treatment with bortezomib as well as prognostic factors for therapeutic outcome independently from the regimen. Inter-individual variability is one of the cause of differences in response to bortezomib therefore the study had mainly the pharmacogenetic approach.

At first, the resistance to MM treatment with bortezomib was approached from a strictly pharmacodynamic point of view. One of the proposed mechanisms of action of bortezomib as well as common resistance mechanism to cancer treatment is directly depending on DNA repair system. This linkage was a trigger to hypotheses that poor response to MM treatment may be correlated with inter-individual differences in genes coding for main proteins in DNA repair system and folic acid metabolism pathway which directly supports DNA repair system [126].

Our analysis identified two SNPs in folic acid metabolism pathway, which might be very important for the future understanding of multiple myeloma disease as well as its treatment. MTR gene was associated with favourable response in the overall population of MM patients. However, this relation, disappear after adjustment for treatment response.

MTFHR rs1801131 genotype was associated with poor response to therapy induction in newly diagnosed MM patients. This relation - unlike in MTR – was still significant after adjustment for treatment response. Identification of this genetic variant in MM patients could be used as an independent prognostic factor for therapeutic outcome in the clinical practice.

Secondly, P-gp was hypnotized as a factor of resistance on the pharmacokinetic and pharmacodynamic level. P-gp is well known efflux transporter affecting disposition of small molecules in the whole body as well as their concentration in the site of action. It’s capacity to eliminate xenobiotics from cancer cells and from eliminating organs, as liver, is well known cause of therapy

failure. Bi-directional transport study clearly demonstrated high efflux activity of P-gp on bortezomib what directed investigation to pharmacogenetics of this transporter.

Permeability rate values measured for bortezomib in bi-directional transport study together with solubility values found in the literature let possible classification of bortezomib in Biopharmaceutics Classification System as a Class III drug [127]. This discover can have a crucial implication in better understanding of bortezomib's pharmacokinetics with respect to the importance of membrane transporters as well as in the future preparation of oral formulation of this drug.

To investigate pharmacodynamic implications of bortezomib interaction with P-gp, significant up-regulation of this glicoproteine after bortezomib treatment was confirmed by western blot analysis. Successively most frequent SNPs in ABCB1 (gene coding for P-gp) were correlated with MM treatment response as well as cytogenetic abnormalities.

None of the Five SNPs in ABCB1 correlated with treatment responsiveness, although we found a significant association between these variants and cytogenetic abnormalities. In particular, deletion of chromosome 17 and t(4;14) translocation were significantly present in harboring the rs60023214 and rs2038502 variants respectively. This new linkage between genetic make-up in the healthy somatic cells with chromosome abnormality in MM cancer cells may open an interesting discussion about hereditary characteristic of this disease.

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