MOLECULAR PROFILING IN MULTIPLE MYELOMA

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CHAPTER 1

GENERAL INTRODUCTION

1.1 MULTIPLE MYELOMA

Multiple Myeloma (MM) is a malignant plasma cell disorder accounting for 1% of all malignant diseases and 10% of hematological malignancies. The annual incidence world-wide of MM is approximately 0.4 to 5 per 100.000, with high incidence rates in North America, Australia/New Zealand, Northern Europe, and Western Europe compared with Asian countries. Within the United States, the incidence in African Americans is about double that in Caucasians, whereas persons of Japanese and Chinese origin have lower rates.[1] In the Netherlands the annual incidence of MM is 5 per 100.000 and increases progressively with age, the median age of diagnosis is 70 years.[2]

MM is characterized by clonal expansion of malignant plasma cells in the bone marrow. The myeloma plasma cell is a post-germinal centre plasma cell which has undergone somatic hypermutation and immunoglobulin class switching. MM cells secrete a monoclonal protein (M-protein) which can be detected in serum and/or urine. The M-protein is IgG in 50% of patients, and IgA in 30% of patients or consists of light chain (15%). In rare cases, secretion of IgD (1%–2%), IgM (0.2%), or IgE (even less frequent), or absence of secretion (non-secretory MM) is found.[3,4]

Osteolytic bone lesions are the hallmark of MM. Other characteristic clinical features include renal injury, anemia, hypercalcemia and immunodeficiency with recurrent infections. These features may result directly from mass accumulation of plasma cells in tissues (plasmacytomas) or indirectly from effects of the M-protein and/or cytokines secreted by the plasma cells. Furthermore a high level of M-protein can cause hyperviscosity, renal failure and neuropathy.

1.1.1 Diagnosis

The diagnosis MM is based on M-protein level, bone marrow plasmacytosis and related organ or tissue impairment (ROTI) also often referred to as CRAB (hyper**c**alcemia, **r**enal insufficiency, **a**nemia and **b**one lesions). Table 1 lists the diagnostic criteria as are established by the international myeloma working group in 2003 (IMWG),[5] which was subsequently updated in 2009.[6] Only the criteria for monoclonal gammopathy of undetermined significance, smoldering myeloma, and multiple myeloma are shown here.

1.1.2 Pathophysiology of multiple myeloma

MM is thought to evolve most commonly from a monoclonal gammopathy of undetermined significance (MGUS) that progresses to smoldering myeloma (SMM) and, finally, to symptomatic myeloma (Figure 1).[7,8]

MGUS is defined by the presence of a serum M-protein less than 3 g/ 100 ml, a normal or slightly elevated percentage of plasma cells in the bone marrow, but less than 10%, without clinical symptoms or features characteristic for MM (Table 1). MGUS becomes increasingly

common with age, being present in 1% and 3% of patients older than 50 years and 70 years, respectively. MGUS converts to overt MM with a rate of approximately 1% each year. The initial concentration of serum monoclonal protein was found to be the most important risk factor for progression to MM.[9] However, in other studies plasma cell percentage, monoclonal urinary light chain or a reduction in one or more uninvolved immunoglobulins, as well as erythrocyte sedimentation rates were independent risk factors for malignant transformation.[10]

SMM is characterized by an M-protein in serum more that 3 g/100 ml, and/or an elevated percentage of plasma cells in the bone marrow, more than 10%, however without clinical symptoms or features characteristic for MM (Table 1). Risk of progression was found to decrease with time, being 10% per year in the first 5 years, but only 3% per year in the following 5 years.[11]

Table 1. IMWG diagnostic criteria for plasma cell disorders, updated in 2009.[5,6] Criteria for MGUS, SMM and MM.

Monoclonal gammopathy of undetermined significance (MGUS)

All three criteria must be met:

- Serum monoclonal protein < 3 g/ 100 ml
- Clonal bone marrow plasma cells < 10% and
- Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia and bone lesions (CRAB) that can be attributed to the plasma cell proliferative disorder

Smoldering multiple myeloma (also referred to as asymptomatic multiple myeloma)

Both criteria must be met:

- Serum monoclonal protein (IgG or IgA) ≥ 3 g/ 100 ml and/or clonal bone marrow plasma cells ≥ 10% and
- Absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia or renal failure that can be attributed to a plasma cell proliferative disorder

Multiple myeloma

All three criteria must be met except as noted:

- Clonal bone marrow plasma cells ≥ 10%
- Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) and
- Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically
  - Hypercalcemia: serum calcium ≥ 11.5 mg/ 100 ml or
  - Renal insufficiency: serum creatinine > 1.73 mmol/l)
  - Anemia: normochromic, normocytic with a hemoglobin value of > 2 g/ 100 ml below the lower limit of normal or a hemoglobin value < 10 g/ 100 ml
  - Bone lesions: lytic lesions, severe osteopenia or pathologic fractures

Abbreviations: AL, amyloid light chain; MRI, magnetic resonance imaging.

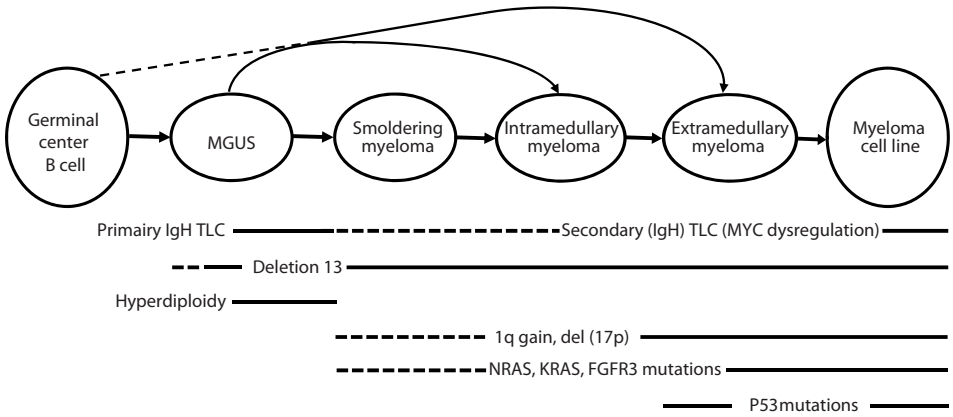

Figure 1. Multistep Pathogenesis of Multiple Myeloma. (Adapted from: Bergsagel and Kuehl.[180,205])
The approximate timing of occurrence of chromosomal changes and mutations are shown by thick lines, with dashed lines reflect some uncertainty as to the precise time at which these changes occur. TLC, translocation.

Cytogenetic aberrations

Non-IgM-MM, -SMM and -MGUS are exclusively post-germinal centre tumors, i.e. all of these tumors have undergone immunoglobulin gene somatic hypermutation, VDJ recombination, antigen selection, and (usually) isotype switch recombination. Myeloma plasma cells have phenotypic features of plasma blasts or long-lived plasma cells, and are usually distributed at multiple sites in the bone marrow.[12]

Although the ultimate genetic event leading to MM is still unknown, it is apparent that translocation of an oncogene to the immunoglobulin heavy chain (IgH) locus at 14q32 is one of the initial immortalizing events in the molecular pathogenesis of MM.[13] The IgH locus is strongly transcriptionally active in B cells, due to the presence of the IgH enhancers, and transfer of an oncogene to this region can result in dysregulation of the gene in question. These translocations are referred to as primary translocations, emphasizing their role in the pathogenesis of MM.[14]

The primary translocations appear to be mediated by errors in one of three B-cell specific DNA modification mechanisms: V(D)J recombination, somatic hypermutation and IgH switch recombination. In MM this predominantly concerns errors in IgH switch recombination, leading to aberrant isotype switching.[15]

The prevalence of IgH translocations varies with the disease stage: nearly 50% in MGUS or SMM, 55% to 73% in intramedullary MM, 85% in primary plasma cell leukemia (PCL), and > 90% in human myeloma cell lines (HMCL).[16-18] There are six recurrent partners (oncogenes) dysregulated by translocations involving the IgH locus at 14q32; *WHSC1* (*MMSET)* and usually *FGFR3* at locus 4p16 in t(4;14), *CCND1* at 11q13 in t(11;14), *c-MAF* at 16q23 in t(14;16),

MAFB at 20q11 in t(14;20) and *CCND3* at 6p21 in t(6;14).[7,19-23] The discovery of recurrent translocations prompted the standard use of cytogenetic analysis in MM.

It has been hypothesized that the pathogenesis of MM involves two distinct pathways, which results in hyperdiploidy (HRD) in 60% and nonhyperdiploidy (NHRD) in 40% of tumors, schematically presented in Figure 1.[24-26] HRD tumors have 48–75 chromosomes, typically with extra copies of odd-numbered chromosomes (3, 5, 7, 9, 11, 15, 19, and 21), or derivatives of these chromosomes. NHRD tumors have < 48 and/or > 75 chromosomes, but only infrequently have extra copies of these same chromosomes. Primary translocations are present in nearly 70% of NHRD tumors, but only in about 10% of HRD tumors.[27]

The most frequent copy number change is deletion of chromosome 13 (del(13)) present from 20% in MGUS, 50% in MM, to nearly 70% in plasma cell leukemia or HMCL.[7] Secondary events involved in the progression of MM include translocation t(8;14) activating the oncogene *c-Myc* at 8q23. This translocation is rare or absent in MGUS, but occurs in 15% of MM tumors, 44% of advanced tumors, and nearly 90% of HMCLs.[7] In addition, presence of amplification of 1q21 (1q gain) was not detected in MGUS, however in 45% in SMM, 43% in newly diagnosed MM, and 72% in relapsed MM.[28] Inactivation of the *p53* tumor suppressor gene at 17p13 by allelic loss (del(17p)) is present in 11% of MM patients, and in approximately 40% of PCL and HMCLs[18,29] Mutations of *TP53* appear to occur mostly in advanced stages of MM.[7] Additional frequently mutated genes include *K-RAS, N-RAS* and *FGFR3*. Other genetic abnormalities involve epigenetic dysregulation, such as alterations in microRNA expression.[30,31]

Bone marrow micro-environment

Another characteristic of (early stage) MM is its presence in and dependence on bone marrow micro-environment. The bone marrow micro-environment consists of extracellular matrix proteins such as fibronectin, collagen, laminin and osteopontin; and cell components including hematopoietic stem cells, progenitor and precursor cells, immune cells, erythrocytes, bone marrow stromal cells, bone marrow endothelial cells, as well as osteoclasts and osteoblasts. The interaction of myeloma cells with the micro-environment regulates processes such as homing of MM cells, MM proliferation, angiogenesis, and bone degradation (Figure 2).

Homing of myeloma plasma cells to the bone marrow is mediated by the chemokine SDF1α, which interacts with its receptor CXCR4 on MM cells. SDF1α induces motility, internalization of CXCR4, and cytoskeletal rearrangement in MM cells. Adhesion of myeloma cells to the bone marrow is regulated by CD44, very late antigen 4 (VLA4), VLA5, leukocyte function-associated antigen 1 (LFA1), neuronal adhesion molecule (NCAM), intercellular adhesion molecule (ICAM1), and syndecan 1(CD138).

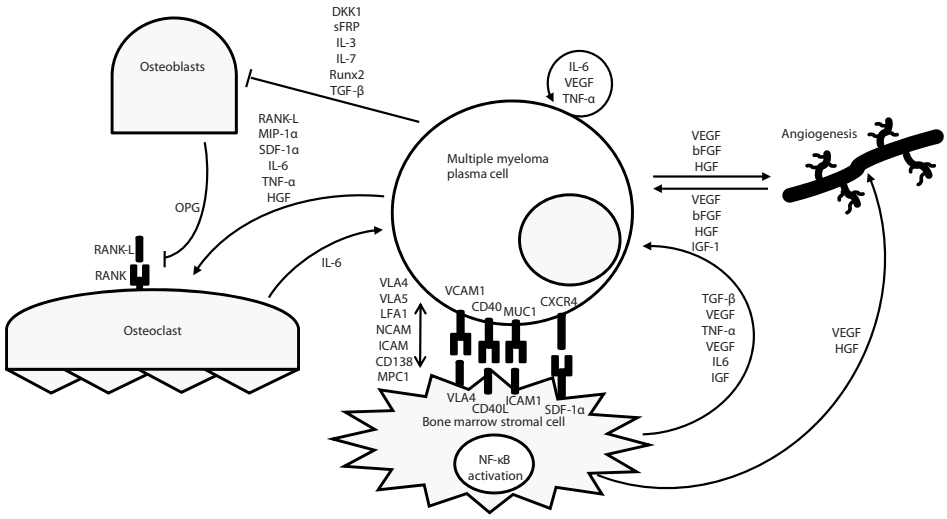

Figure 2. Interaction between myeloma plasma cell and bone marrow microenvironment in multiple myeloma. (Adapted from Palumbo et al.,[8] Lemaire et al.,[206] and Hideshima et al.[32])

MM cell adhesion to the bone marrow stromal cells activates transcription and secretion of cytokines, including interleukin-6 (IL6), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF), involved in multiple myeloma cell growth, survival, drug resistance and migration, whereas factors such as VEGF and basic fibroblast growth factor (bFGF) are involved in angiogenesis. Other important processes regulated by interaction between bone marrow micro-environment and the myeloma plasma cell include osteoclastogenesis and inhibition of osteogenesis, responsible for the osteolytic lesions characteristic of MM, spread of tumor by the bloodstream from one site to another within the bone marrow.[7,32]

NF-κB signaling pathway

In addition to the genetic events described above, the activation of the nuclear factor-kappa B (NF-κB) pathway was more recently shown to be a common event in the biology of MM, attributing to interactions of the myeloma cell with the bone marrow micro-environment.[33] Two pathways are involved in NF-κB signaling, the canonical and noncanonical NF-κB pathway, presented in Figure 3.

In the canonical pathway, IKKβ phosphorylates the inhibitory subunits IkBα, IkBβ, or IkBε, leading to their degradation by the proteasome. As a result, the NF-κB heterodimers p50/p65 and c-rel/p65 accumulate in the nucleus. In the noncanonical pathway, IKKα

homodimers phosphorylate p100/NFKB2, resulting in proteasomal removal of an inhibitory C-terminal domain and generating the NF-κB p52 subunit.[34,35] Consequently, the p52/RelB heterodimers accumulate in the nucleus. These p50/p65 and p52/RelB heterodimers activate transcription of anti-apoptotic factors, cytokines, growth factors, enzymes, cell cycle regulators and cell adhesion molecules important in the growth and survival of myeloma plasma cells.

Both inactivating mutations in NF-κB inhibitors such as *TRAF* and *cIAP1/ BIRC2* and activating mutations in triggers of the NF-κB pathway such as *CD40* and *NIK* are involved. These mutations lead primarily to constitutive activation of the noncanonical NF-κB pathway.[33,36]

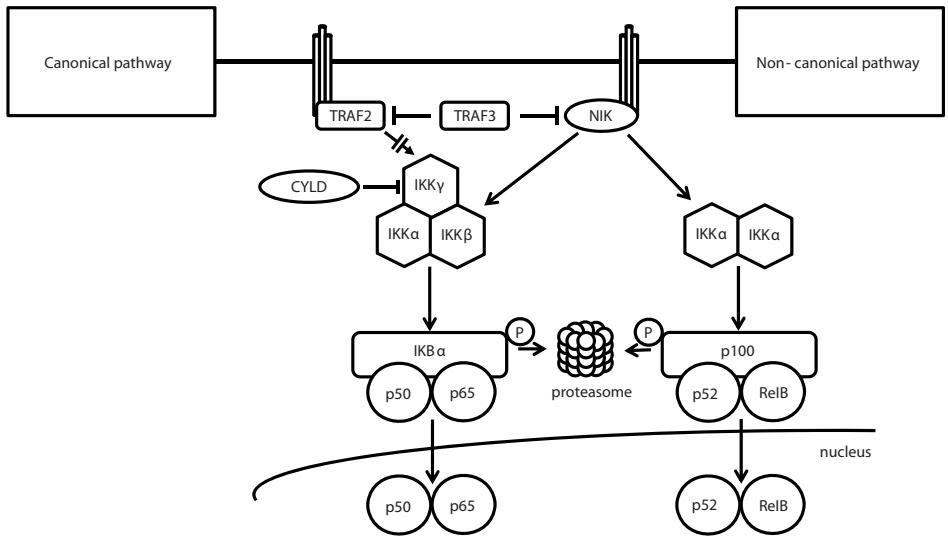

Figure 3. Schematic of Canonical and Non-canonical NF-κB signaling pathway. (Adapted from: Annunziata et al.[36])

1.2 BONE DISEASE IN MULTIPLE MYELOMA

Osteolytic bone lesions represent one of the most frequent and debilitating complications of MM, occurring in approximately 80% of patients,[37] and often resulting in skeletal related events such as pathologic fractures, need for radiation or surgery to bone, spinal cord compression and hypercalcemia. Osteolytic bone lesions are caused by an imbalance in

osteoclast mediated bone resorption and osteoblast mediated bone formation, shifting the balance towards bone resorption.[38]

Different mechanisms contribute to the increased osteoclast mediated bone resorption. Major elements reported to be involved in osteoclast activation are myeloma cell derived macrophage inflammatory protein-1a *(MIP-1a)* and the *RANK/RANK-L/OPG* system. Myeloma cells are able to induce expression of receptor activator of NF-κB ligand *(RANK-L)* in the bone marrow micro-environment of MM patients.[39] *RANK-L* is expressed on myeloma cells, osteoblasts, but can also, via an IL-7 mediated process, be expressed on T-cells.[40] *RANK-L* can induce osteoclast differentiation and activation directly by binding to its receptor *(RANK)* on osteoclastic cells.[39] Decoy receptor osteoprotegrin *(OPG)*, on the other hand, shows decreased expression. *OPG* is a soluble receptor produced by osteoblasts to preserve bone mass. Normally, *OPG* blocks the effects of *RANK-L* by preventing interaction between *RANK-L* and *RANK*.[41] The disturbed *OPG-RANK-L* ratio leads to a increased *RANK-L-RANK* interaction, which results in the differentiation and activation of osteoclasts.[39]

Another important factor responsible for myeloma bone disease is *MIP-1a*.[42] *MIP-1a* activates osteoclast chemokine *CXCR5* plus *RANK-L*, which, in its turn, activate osteoclast *RANK* and competes with *OPG*.[43]

Bone resorption due to osteoclast activity is blocked by bisphosphanates,[44] however the inability to repair lesions indicates an important defect on the side of osteoblast differentiation and osteoblast activity in osteolytic lesions.

Recent studies have emphasized the role of molecular characteristics of the myeloma plasma cell in the development of osteolytic bone lesions. Performing gene expression profiles (GEPs) of CD138-purified bone marrow aspirates of MM patients, Tian et al. associated the overexpression of WNT signaling inhibitors, such as *Dickkopf-1* (*DKK-1)*, with the presence of focal bone lesions.[45] WNT signaling is important for the growth and differentiation of osteoblasts and acts in different developmental processes.[46] *DKK-1*, a WNT signaling inhibitor, inhibits both osteoblast differentiation and function, and increases osteoclast activity.[47]

The elucidation of bone disease development and treatment of this complication in MM remains a challenge.

1.3 PROGNOSTIC FACTORS

The International Staging System (ISS), which is based on serum β_2-microglobulin (B2M) and albumin, is widely used to classify patients with newly diagnosed MM in three prognostic categories.[48] ISS has consistently been confirmed as a solid prognostic factor in clinical trials.[49] Additional clinical factors which can give an indication of prognosis include serum lactate dehydrogenase (LDH), serum monoclonal isotype IgA, presence of extramedullary

disease, presence of renal failure at diagnosis, morphology of plasmablastic disease and plasma cell leukemia.[8,50] In addition to ISS staging, cytogenetic aberrations present in the myeloma cells have an independent impact on prognosis. Chromosome 13 aberrations are frequently found in MM; using conventional cytogenetics, this percentage constitutes approximately 15% of patients. However using interphase fluorescence in situ hybridization (FISH), approximately 50% of patients are found to harbor chromosome 13 abnormalities, with most being complete monosomy 13 (85%), while the remaining 15% constitute deletion 13 (del(13)). Monosomy 13 and del(13) are associated with a poor prognosis when detected by metaphase FISH or conventional karyotyping,[51] however when detected with interphase FISH, the negative impact on survival disappears in the absence of t(4;14).[29,52]

Several groups have demonstrated that translocations t(4;14) and t(14;16) are associated with poor survival.[18,29,52] In contrast, the presence of t(11;14) has either a favorable or a negligible influence on the prognosis.[29,52]

Other relevant adverse cytogenetic features are del(17p) and 1q gain. Approximately 10% of MM patients harbor a del(17p), i.e. a hemizygous deletion, which is associated with an increased occurrence of *p53* mutations. Recent studies demonstrated *p53* mutations in 27–37% of patients with del(17p) compared to < 1% of patients without.[53,54] Whether the patients with del(17p) but without mutations, contain functional p53 has not been demonstrated. In patients with MM treated with conventional chemotherapy or high-dose therapy, del(17p) is associated with aggressive disease and much shorter survival.[18,29]

Recent evidence has suggested that the poor prognosis associated with translocations t(4;14), t(14;16) and del(17p) is lost in cases where these events occur in presence of a trisomy characteristic of hyperdiploidy.[55]

Chromosome 1 aberrations are the most common structural aberrations in MM and mostly involve deletions in 1p and amplifications in 1q. Amplification of 1q21 occurs in approximately 35% of patients, and has been shown to correlate with poor prognosis.[28,56-58] The 1q21 region includes genes such as *CKS1B,* of which overexpression leads to growth and proliferation of myeloma cells, and *PSMD4*, described in two high-risk signatures, extensively outlined in chapter 1.3.2.[59,60] Other frequent abnormalities are deletions of the short arm of chromosome 1 (del(1p)). These concern interstitial deletions spanning the region 1p13–1p31, and are associated with a poor prognosis.[61,62]

1.3.1 Molecular classifications

An important contribution to the elucidation of the pathogenesis of MM and identification of prognostic markers is made by Bergsagel et al.,[27,63] and Zhan et al..[64] Bergsagel et al. developed the translocation/cyclin D (TC) classification, subdividing myeloma patients into 8 subgroups based on the presence of genes (in)directly dysregulated by translocations and cyclin D overexpression: *WHSC1(MMSET)* and *FGFR3* t(4;14) (TC *4p16*), *CCND1* t(11;14)

(TC *11q13*), *MAF* or *ITGB7* and *CX3CR1* t(14;16)/t(14;20) (TC *maf)*, and *CCND3* t(6;21) (TC *6p21)*. The remainder of samples was classified based on expression of *CCND1* (TC *D1*), *CCND1* and *CCND2* (TC *D1_D2*) or only *CCND2* (TC *D2*), of which expression was compared to the expression of these genes in normal bone marrow plasma cells, and a small rest group expressing no D-type cyclins (None).[27]

Concerning the prognostic impact, Bergsagel and Kuehl reported a substantially shortened survival either with standard or high-dose therapy in patients belonging to subgroup TC *4p16* (median overall survival (OS), 26 months and 33 months, respectively). Patients in subgroup TC *maf* showed a similar or even worse prognosis (median OS, 16 months with conventional therapy). A better survival following both conventional chemotherapy and high-dose therapy was observed for the TC *11q13* subgroup and the small subgroup TC *6p21* which was grouped together with the TC *11q13* based on an overlapping gene profile.[63]

The University of Arkansas for Medical Sciences (UAMS) classification by Zhan et al. was generated by performing an unsupervised clustering of purified myeloma plasma cell samples of newly diagnosed MM patients.[64] After removing the cases which showed a high degree of contamination of myeloid and/or plasma cell markers defined by gene expression profiling, a gene set of 1559 highly variable expressed genes clustered the remaining samples into 7 subgroups characterized by distinct GEPs. These included a cluster characterized by overexpression of **M**M**S**ET in all samples and FGFR3 overexpression in 75% of samples, named MS cluster. The samples in the MF cluster showed increased expression of ***M****AF* or *MA**F**B,* however clustered together based on common downstream targets, such as *CCND2, CX3CR1,* and *ITGB7*. *DKK1* was one of the most significant downregulated genes in this cluster. *DKK1,* as mentioned above, inhibits osteoblast differentiation and function.[45,47] The finding of underexpression of this gene was consistent with the relatively low incidence of bone lesions observed in the MF group. The lowest percentage of patients having 3 or more MRI-defined bone lesions, however, was observed in another cluster, one of the clusters characterized by a high frequency of hyperdiploidy (67%). This cluster showed no clear genetic signature, and was designated **l**ow **b**one (LB) cluster. Consistent with this, relative low levels of the WNT signaling antagonists, *FRZB* and *DKK1*, were observed. Overexpressed genes included *IL6LR, PHACTR3, BIK, EDN1,* and *CST6*.

The CD clusters, CD-1 and CD-2, were both characterized by overexpression of *CCND1* and *CCND3*, genes dysregulated by t(11;14) and t(6;14), respectively. The CD-1 cluster showed a distinct gene profile from the CD-2 cluster, the latter distinguished by overexpression of B-cell marker *MS4A1/CD20,* early B-cell marker *VPREB* and B-cell transcription factor *PAX5*.

A hyperdiploid karyotype was found in more than 90% of the cases which clustered together and constituted the HY cluster. *GNG11, TRAIL (TNFSF10),* as well as genes involved in WNT signaling, *FRZB* and *DKK1,* and the MIP–1α chemokine receptor *CCR5* were among the top upregulated genes. This cluster was one of the clusters with approximately 60%

or more patients exhibiting 3 or more MRI-defined bone lesions. Underexpressed genes included genes mapping to chromosome 1q, i.e. *TAGLN2, CKS1B,* and *OPN3*.

The proliferation (PR) cluster was characterized by the overexpression of numerous cell cycle- and proliferation-related genes and cancer-testis antigen (CTA) genes (e.g., *MAGEA6, MAGEA3, GAGE1, GAGE4*). CTAs belong to a family of tumor associated antigens (TAAs) and are protein antigens with normal expression restricted to adult testicular germ cells, however are aberrantly activated and expressed in a proportion of various types of human cancer. The presence calls of some CTA genes have been reported to correlate with significantly shorter event-free survival, such as *CTAG1B*, *CTAG2*, *MAGEA1*, *MAGEA2*, *MAGEA3*, and *MAGEA6*.[65] In addition, this group also had a significantly higher gene expression-defined proliferation index (PI) than the other groups.

Concerning their prognostic value, the clusters showed significant differences in event-free survival (EFS) and OS. Clusters MS, MF and PR, together the high risk group, showed a significant worse EFS and OS in comparison to low risk group, including HY, CD-1, CD-2 and LB. Although excluded from analysis upfront, Zhan et al. also observed that samples showing a myeloid/normal plasma cell signature exhibited more favorable baseline features and a significant better EFS and OS than the remaining samples lacking this signature.

1.3.2 Molecular prediction of survival

The use of GEPs led to more insight in the heterogeneity and molecular differences in MM. Furthermore GEP defined clusters of MM samples showed differences in prognosis. These findings formed the rationale to develop genetic signatures for prediction of prognosis. The 7 gene signatures outlined here are depicted in table 2; the trials generating the datasets used to develop the gene signatures or used for validation purposes are shown in table 4.

The Millennium signature (MILLENIUM-100) was build on relapsed/refractory MM patients included in one of the three trials, APEX,[66] SUMMIT,[67] or CREST,[68] assessing the efficacy of bortezomib, and will be extensively described in chapter 5.[69]

At the same time, Shaughnessy et al. published a gene expression signature to identify high-risk MM.[70] This signature consisted of 70 genes, of which 21 mapped to chromosome 1. Of these 21 genes, up-regulated genes mapped to chromosome 1q, while the downregulated genes mapped to chromosome 1p. The ratio of mean expression levels of up-regulated to down-regulated genes defined a high-risk score. A cut-off for this score was based on K-means clustering, which defined a high-risk proportion of 13%. This 70-gene high-risk score (GEP70/UAMS-70) was an independent predictor of outcome endpoints in multivariate analysis, including the ISS and high-risk translocations. Multivariate discriminate analysis revealed that a smaller subset of 17 genes (GEP17/UAMS-17) could predict outcome as well as the UAMS-70.

Recently, an additional GEP80/UAMS-80 model was developed based on gene expression levels measured in 142 patients before and 48 hours after they received a

bortezomib test dose. Of 1051 genes the expression levels changed significantly relative to baseline expression. Finally, of these 1051 genes, 80 differentially expressed genes were identified whose 48-hour expression levels were highly associated with PFS, and validated in independent datasets. The UAMS-80 model identified 9% of patients with a poor prognosis among those with UAMS-70-defined low-risk disease and 41% of patients with favorable prognosis among those with UAMS-70-defined high-risk disease.[60]

Table 2. Short description of gene signatures, populations of patients in which they were generated, platforms used and datasets in which they were built and/or validated .

Trials	Population	Platform	Validated in independent datasets	Ref.
MILLENIUM-100	Relapse/ refractory	Affymetrix U133 A+B	Built on APEX (substudy)/SUMMIT, tested in APEX	[69]
UAMS-70/ UAMS-17	Newly diagnosed	Affymetrix U133 2.0	Within datasets from TT2 and TT3	[183]
UAMS-80	Newly diagnosed	Affymetrix U133 2.0	Built on TT3A, tested in TT3B and TT2	[60]
IFM-15	Newly diagnosed	Custom designed	APEX, UAMS and Mayo dataset	[182]
MRC-IX-6	Newly diagnosed	Affymetrix U133 2.0	APEX and UAMS dataset	[72]
GPI-50	Newly diagnosed	Affymetrix U133 2.0	UAMS	[73]

Decaux et al. developed a 15-gene model (Intergroupe Francophone du Myélome (IFM)-15) for prediction of high-risk MM. Genes in the IFM-15 were cell-cycle regulated genes involved in cell-cycle control, DNA replication, DNA repair, DNA packaging, mitosis, and spindle-assembly checkpoint.[71]

When comparing the IFM-15 and UAMS-17 signatures, Decaux et al. found that when competing with the UAMS-17, the IFM-15 did not remain a significant independent variable in the UAMS data set of patients treated with thalidomide based therapy (TT2) and did not identify the early disease related deaths in the UAMS data set of patients treated with bortezomib based therapy (TT3).

The Medical Research Council (MRC) gene signature was developed on the MRC-IX trial and consisted of 6 genes (MRC-IX-6). The identification of samples with a poor prognosis was based on the expression ratios of 6 genes: *BUB1B* vs. *HDAC3*, *CDC2* vs. *FIS1*, and *RAD21* vs. *ITM2B*; if any one of these pairs had a ratio of ≥ 1, the test was positive for poor prognosis. Cases tested positive for the 6-gene test showed a median OS of 13 months vs. 45 months when tested negative; they also had a shorter PFS of 11 months vs. 22 months in the negatively tested cases as demonstrated in the MRC-IX data. The MRC-IX-6 remained

a significant independent variable when other prognostic factors, including ISS, del(17p), and poor prognosis IgH translocations, were taken into account.[72]

Table 3. Short description of the trials used in generating the gene signatures and for validation purposes.

Trials	Population	Gene signatures	Treatment	Ref.
APEX (039)	Relapse	MILLENIUM-100	bortezomib vs. dexamethasone, crossover to bortezomib if progressive disease*	66
SUMMIT (025)	Relapse/refractory	MILLENIUM-100	bortezomib	67
CREST (024)	Relapse/refractory	MILLENIUM-100	bortezomib 1.0 or 1.3 mg/ m^2, add dexamethasone if suboptimal response	68
TT2	Newly diagnosed	UAMS-70/ UAMS-17/ UAMS-80	VAD-DCEP-CAD-DCEP, HDM (2x), DCEP-CAD, Interferon maintenance. Randomization for thalidomide or no thalidomide added to all phases in treatment	100
TT3	Newly diagnosed	UAMS-70/ UAMS-17/ UAMS-80	VTD-PACE, HDM (2x), consolidation VTD-PACE. Maintenance with VTD in year 1 and TD in years 2 and 3 (TT3A) or VRD for 3 years (TT3B)	193
MRC-IX	Newly diagnosed, transplant eligible	MRC-IX-6	CTD vs. CVAD, HDM, randomized to thalidomide maintenance therapy or no maintenance	194,195
MRC-IX	Newly diagnosed, transplant ineligible	MRC-IX-6	CTDa vs. MP, randomized to thalidomide maintenance therapy or no maintenance	102

Abbreviations: VAD: Vincristin, adriamycin, dexamethasone; DCEP: dexamethasone, cyclophosphamide, etoposide, and cisplatin; CAD: cyclophosphamide, adriamycin, and dexamethasone; HDM: high-dose melphalan; VTD-PACE: bortezomib, thalidomide, dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide; VTD: bortezomib, thalidomide, dexamethasone; TD: thalidomide, dexamethasone; VRD: bortezomib, lenlidomide, dexamethasone; CTD: cyclophosphamide, thalidomide, dexamethasone; CTDa; as CTD, but with dose adjustment for thalidomide; MP: melphalan, prednisone
*These patients were included in a companion study (040). The 040 study also directly enrolled 263 patients who had more than 3 prior therapies

Recently, a GEP based proliferation index (GPI-50) was reported. The GPI was significantly predictive for EFS and OS and was largely independent of clinical prognostic factors, e.g. serum B2M, ISS, associated high-risk chromosomal aberrations, e.g. translocation t(4;14), and UAMS-17 and IFM-15 high risk signatures.[73]

The UAMS-70 has now been incorporated in a risk stratification system, Mayo Stratification for Myeloma And Risk-adapted Therapy (mSMART). The mSMART additionally includes FISH, metaphase cytogenetics, and plasma cell labeling index and can be used in clinical practice to adjust treatment regimens according to risk status. However, the mSMART has not been validated in prospective clinical trials.[74]

Table 4. Phase 2 and 3 trials of thalidomide-based regimens in preparation for ASCT.

	No. of patients	≥ PR (%)	≥ VGPR(%)	nCR+CR(%)	CR(%)	Median follow-up	PFS (median/ months)	P-value	OS (median/ months)	P-value	Refs
TD vs. VAD	100 vs. 100	76 vs. 52	19 vs. 14	13 vs. 13	10 vs. 8		NR		NR		[95]
TD vs. D	103 vs. 104	63 vs. 41	NR	NR	4 vs. 0		NR		NR		[98]
TD vs. VAD	100 vs. 104	NR	44 vs. 42	NR	NR		NR		NR		[196]
TAD vs. VAD	268 vs. 268	88 vs. 79	66 vs. 54	NR	31 vs. 23	52 months	34 vs. 25	$P < .001$	73 vs. 60	$P = .77$	[99]
CTD vs. CVAD	379 vs. 370	92 vs. 90	74 vs. 62	NR	50 vs. 37	47 months	34 vs. 33	$P = .57$	Not reached vs. not reached	$P = .20$	[194]
TT2 + thal vs. TT2 without thal	323 vs. 345				64 vs. 64[#]	42 months	56% vs. 44%[*]	$P = .01$	65% vs. 65%[**]	$P = .90$	[100]

Abbreviations: TD: Thalidomide, dexamethasone; VAD: Vincristin, adriamycin, dexamethasone; D: Dexamethasone; TAD: Thalidomide, adriamycin, dexamethasone; CTD: Cyclophosphamide, thalidomide, and dexamethasone; CVAD: Cyclophosphamide, vincristin, adriamycin, dexamethasone; TT2: VAD-DCEP-CAD-DCEP, HDM (2x), DCEP-CAD, Interferon maintenance. Randomization for thalidomide or no thalidomide added to all phases in treatment. VAD: Vincristin, adriamycin, dexamethasone; DCEP: dexamethasone, cyclophosphamide, etoposide, and cisplatin; CAD: cyclophosphamide, adriamycin, and dexamethasone; HDM: high-dose melphalan. [#]at 4 years, [*]5 yrs EFS, [**]5 yrs OS.

1.4 TREATMENT

1.4.1 History

Until the discovery of melphalan, no effective treatment was available for MM. Melphalan was introduced in 1958 by Blokhin et al., showing benefit in three patients with MM.[75] This beneficial effect was confirmed in 1962 in 8 of 24 myeloma patients showing significant improvement.[76] Since then, numerous trials have been conducted to find the optimal way to use melphalan in the treatment of MM patients. The introduction of prednisone in 1967, and its efficacy in the treatment of MM, was followed by a trial comparing the combination of melphalan and prednisone to melphalan alone, resulting in a prolonged survival of 6 months in favor of MM patients treated with melphalan in combination with prednisone.[77,78] This resulted in the formation of a classic regimen, MP (melphalan-prednisone). The following 30 years, search for agents and combination of agents with the classic regimen failed to further improve OS.[79] With the introduction of autologous stem cell transplantation (ASCT), allowing higher doses of melphalan to be administered, a further improvement of outcome was achieved.

1.4.2 High dose therapy and autologous stem cell transplantation

In 1983, the first series of MM patients were successfully transplanted with autologous bone marrow after high dose melphalan (HDM).[80] In addition, it was observed that beyond a dose of 100 mg/m2, stem cell rescue is required to keep treatment related mortality below 5%.[81]

In 1996, a randomized trial by IFM showed that ASCT resulted in significantly more disease-free individuals and better OS than did conventional chemotherapy (5-year OS 52% *vs.* 12%; mortality 3% for both treatment arms).[82] Similar results were obtained in the MRC-VII trial.[83] However, a systematic review of nine randomized controlled trials with over 2 years median follow-up in 2411 patients reported that the HR of death with high dose therapy (HDT) was 0.92 (95% CI 0.74–1.13) and a HR of disease progression with HDT of 0.75 (95% CI 0.59–0.96).[84] These results show that early HDT with single ASCT conferred a free survival (PFS) benefit ($P = 0.02$), but no OS benefit ($P = 0.40$) compared with conventional chemotherapy. In addition, they also reported an increased risk of treatment-related mortality associated with HDT (HR 3.01; 95% CI 1.64–5.5).[84]

1.4.3 Novel therapeutic agents

Immunomodulatory drugs (IMiDs)

The development of novel agents changed the landscape of MM treatment. In the end of the last era, this commenced with the introduction of thalidomide (Thalidomide®). The use of thalidomide in the treatment of MM was rationalized by its anti-angiogenic activity in animal models.[85] Thalidomide, α-*N*(phthalimido)glutarimide, is a derivative of glutamic acid. It causes inhibition of TNF-α production following lipopolysaccharide-stimulation of

human peripheral blood mononuclear cells and is therefore pharmacologically classified as an immunomodulatory drug (IMiD).[86] Other immunomodulatory properties include augmentation of natural killer cell activity and stimulation of cytotoxic T-cells.[87] The exact mechanisms by which thalidomide and other IMiDs inhibit MM cell growth remain to be elucidated. IMiDs are hypothesized to act through multiple mechanisms. Evidence suggests that in addition to the anti-angiogenic effects by inhibition of VEGF and bFGF, thalidomide and IMiDs may have antineoplastic effects by blocking signaling through NF-κB,[88] and by inducing apoptosis via the caspase-8/death receptor pathway.[89] In addition, thalidomide and IMiDs inhibit adhesion of MM cells to stromal cells and thereby overcome cell adhesion-mediated drug resistance (CAM-DR). Furthermore, thalidomide and IMiDs inhibit activity and/or secretion of cytokines in MM cells and bone marrow stromal cells. These cytokines include IL-6, IL-1β, IL-10 and TNF-α, responsible for MM cell growth, survival, drug resistance, migration and expression of adhesion molecules.[90]

Thalidomide achieved overall response rates (ORR) of 32% in patients with relapsed and refractory patients and of 36% in patients with previously untreated MM, making it the first agent with single agent activity since the discovery of melphalan.[91,92]

Thalidomide in combination with dexamethasone (TD) resulted in even further improved response rates and proved to be superior to dexamethasone alone both in relapsed /refractory MM patients and in newly diagnosed MM patients.[93-98]

Based on the results of a randomized phase III study by Rajkumar et al. in newly diagnosed MM patients, which showed higher response rates in favor of TD compared with high-dose dexamethasone,[98] the United States Food and Drug Administration granted accelerated approval for TD in patients with newly diagnosed MM.

In newly diagnosed transplant candidates, induction with thalidomide in combination with chemotherapy followed after ASCT with thalidomide maintenance improved response rates and prolonged PFS, however this was not followed by a longer OS.[99,100] One of the hypotheses is emergence of tumor resistant clones in patients with prolonged exposure to thalidomide.

In the MRC-IX trial including newly diagnosed patients, the combination cyclofosfamide, thalidomide, dexamethason (CTD) vs. cyclofosfamide-VAD (CVAD) preceeding HDM/ ASCT, resulted in significant higher ORR (92% vs. 90%), and was non-inferior in terms of PFS and OS.[101] In patients ineligible for ASCT, receiving an attenuated dosing regimen of CTDa, a significant higher ORR was observed in comparison to melphalan, prednisone (MP) (64% vs. 33%); PFS and OS were similar.[102] In another trial in patients ineligible for ASCT, melphalan, prednisone, thalidomide (MPT) vs. MP yielded significant higher ORR, EFS and OS: 66% vs. 45%, 13 vs. 9 months, and 40 vs. 31 months, respectively.[103] Data of a number of trials are shown in table 4.

Concerning adverse events, it became clear that thalidomide was associated with venous thrombo-embolic events (VTE), however mainly when thalidomide was combined

with dexamethasone or other chemotherapeutic agents.[92] VTEs following thalidomide combinations were reported to occur in 15–28% of patients.[92,104] Furthermore, it became evident that the thrombotic activity of thalidomide was not limited to the venules. Arterial thrombotic events following the use of thalidomide occurred in 5.6% of patients.[105] The intervention with thrombocyte aggregate inhibitors, with secondary anti-inflammatory effects and low molecular weight heparins (LMWHs) for patients with increased risk on developing VTE, strongly reduced the incidence of VTE.[106]

Another important side effect concerned the development of polyneuropathy (PN). Thalidomide induced polyneuropathy (TiPN) will be discussed in chapter 1.5.

The search for IMiDs with an even more potent but less toxic profile, led to the development of lenalidomide (Revlimid®) and pomalidomide (Actimid®), both structural analogues of thalidomide. Lenalidomide and pomalidomide are not only up to 50.000-fold more potent at TNF-α inhibition *in vitro* compared with thalidomide, they are also much more potent than thalidomide in their ability to co-stimulate T cells.[107,108] Additional characteristics of these IMiDs include the ability to disrupt tumor-microenvironment interactions and their improved direct anti-proliferative activity compared to thalidomide.[109]

In relapsed/ refractory MM patients, lenalidomide combined with dexamethason induced ORR of 61%, with a median time to progression of 11 months, and a median OS of 30 months.[110] Lenalidomide, dexamethason vs. dexamethason resulted in significant higher ORR, 60% vs. 24%, and a significant improved OS.[111] Lenalidomide plus high-dose dexamethasone (RD) is active in newly diagnosed MM.[112-114] A recent randomized trial found that lenalidomide plus low-dose dexamethasone (Rd), which used a lower dose of dexamethasone (40 mg once weekly), showed less toxicity and better PFS than RD, table 5.[115]

In patients ineligible for ASCT, melphalan, prednisone, lenalidomide, followed by lenalidomide maintenance (MPR-R) was found to be superior to MPR and MP in terms of ORR (77% vs. 68% vs. 50%) and median PFS (31 vs. 15 vs. 14 months). Differences in OS were not significant.[116]

Pomalidomide is the latest IMiD. Data suggest that pomalidomide is the most potent of the IMiDs.[117-120] In two subsequent studies enrolling patients with relapsed/refractory MM after 1 to 4 prior lines of treatment, including refractoriness to lenalidomide, salvage therapy with pomalidomide was combined with low-dose dexamethasone. In one study, the overall rate of at least PR was 63%, including 33% CR or VGPR, and median PFS was 11.6 months. Among patients refractory to prior lenalidomide, PR or better was seen in the 32% to 40% range, suggesting a lack of cross-resistance between lenalidomide and pomalidomide.[121,122]

In contrast to thalidomide, both lenalidomide and pomalidomide had less commonly observed neurosedative toxicity. Dose-limiting neutropenia and thrombocytopenia were the most common toxicities seen with these drugs. As observed with thalidomide, VTEs during treatment with lenalidomide and pomalidomide have been reported in various trials, although rarer when the drug is used as a single drug. The incidence is markedly

reduced when aspirin is given concomitantly; LMWH or Coumadin is needed in patients at high risk of VTE.[123,124]

IMiDs, novel insights in mechanism of action

Recently, *cereblon (CRBN)* was identified as a primary target of thalidomide teratogenicity. In the late 1950s, thalidomide was widely prescribed to pregnant women as a sedative and for treatment of morning sickness, but was found to be teratogenic, causing multiple birth defects, which led to its withdrawal from the market in 1961. Ito et al. showed that thalidomide, by directly binding to CRBN, inhibits the function of the E3 ubiquitin ligase complex (composed by proteins CRBN, DNA binding protein1 (DDB1), and Cullin 4 (Cul4)). In both zebrafish and chicks, the importance of the E3 ubiquitin ligase complex could be demonstrated for limb outgrowth. The inhibition of this E3 ligase complex has been proposed to result in abnormal BMP and Fgf8 signaling, resulting in growth defects.[125] Acknowledging this, the role of *CRBN* in the mechanism of action of thalidomide and IMiDs as antitumor agents was further investigated by Zhu et al.. Several observations were indicative that *CRBN* is involved in antimyeloma activity of IMiDs: first, knockdown of *CRBN* resulted in inhibition of proliferation and induction of apoptosis in HMCL. Secondly, *CRBN* down-regulation was found to result in IMiD resistance in HMCL. Binding to *CRBN* was obligatory to exert IMiD activity, demonstrated by phthalimide, an analog of thalidomide that does not bind to *CRBN*, which did not show activity on any HMCL tested. Thirdly, loss of *CRBN* expression did not affect the HMCL response to other unrelated drugs, such as bortezomib, dexamethasone, and melphalan. Fourthly, preliminary data suggest that a reduction of *CRBN* expression was observed in > 85% of lenalidomide-resistant MM patients. This evidence suggests that *CRBN* is a direct target of IMiDs, required for anti-myeloma activity, while *CRBN* downregulation might be one of the mechanism of IMiD resistance.[126]

Proteasome inhibitors

The proteasome is a multicatalytic protease complex that is responsible for ubiquitin-dependent turnover of cellular proteins.[127-129] Inhibition of the proteasome leads to an accumulation of substrate proteins and cell death.[130] The 26S proteasome complex (2000-kDa), consists of a 20S (700-kDa) part, the catalytic domain, which harbors protease activities, and a 19S component which recognizes polyubiquitinated proteins and subsequently unfolds and removes ubiquitin from substrates. The 20S part contains four stacked rings of seven subunits each. The inner two rings contain β-subunits that encode the three major catalytic activities of the proteasome, chymotrypsin-like (CT-L) (β5), trypsin-like (β2), and caspase-like (β1). Of these, the CT-L activity is the rate-limiting step of proteolysis and has thus become one of the primary targets for the proteasome inhibitor drug class. The ubiquitin-proteasome pathway has become a validated target for cancer therapy with the approval of the proteasome inhibitor Bortezomib.[131]

Bortezomib (Velcade®), introduced by Orlowski et al.,[132] is a dipeptide boronic acid analog, which specifically and reversibly inhibits the CT-L activity of the 20S proteasome. The proteasome degrades the intracellular inhibitor, inhibitory kappa B (IκB), of NF-κB. Bortezomib thus increases the level of IκB thereby blocking the activity of NF-κB, and abrogating the effects of this transcriptional regulator which is involved in cancer cell proliferation, inhibition of apoptosis and cell migration.[133,134] However, recently it was reported by Hideshima et al. that bortezomib did not act through inhibition of the NF-κB pathway. Actually, bortezomib was found to activate 2 upstream NF-κB-activating kinases (RIP2 and IKKβ), promoting downregulation of IκB, and increasing NF-κB activity. Furthermore, it was reported that bortezomib also fails to block NF-κB *in vivo*, determined by measuring nuclear localization of NF-κB's RelA/p65 subunit.[135] It is clear that the relation between bortezomib and NF-κB signaling requires further analysis.

Other mechanisms for the cytotoxic effect of bortezomib involve the effect on the unfolded protein response (UPR). Differentiation of B-lymphocytes to normal plasma cells involves massive expansion of the endoplasmatic reticulum (ER) to enable them to produce large quantities of proteins, the immunoglobulins. The production of high quantities of immunoglobulin in turn activates the UPR which coordinates correct protein folding or regulates proteasomal degradation of misfolded proteins.[136] UPR includes three mechanisms to manage the large increase of unfolded proteins: transcriptional induction of target genes enhancing protein folding, general translational repression, and ER-associated degradation pathway (ERAD) to eliminate misfolded proteins.[137] Two of them involve the activation of transcription factors XBP-1 and ATF6, whereas the third depends on translational repression mediated by PERK/eIF2α. Proteasome inhibition prevents the degradation of misfolded proteins by ERAD, eliciting ER stress response or UPR, which eventually lead to the induction of apoptosis.[138,139] As myeloma cells produce and secrete large amounts of immunoglobulin, their threshold for induction of ER stress and pro-apoptotic UPR following proteasome inhibition may be lower.[140]

Bortezomib has proven to be active in relapsed and/or refractory MM, showing high response rates and a survival advantage.[66-68,132] In newly diagnosed myeloma patients eligible for ASCT, bortezomib in combination with dexamethasone (VD) compared to standard vincristine, adriamycin, dexamethasone (VAD) induction resulted in a significant increase in ORR (79 vs. 63%), and VGPR or better (38 vs. 15%). Responses further improved after one or two ASCTs and a trend towards improvement in median PFS in favor of the VD induction regimen was observed, as shown in table 6.[141] Bortezomib in combination with cytostatic drugs, such as adriamycin (PAD) followed by one or two ASCTs and bortezomib maintenance for 2 years resulted in significantly higher response rates, a prolonged PFS and OS when compared to VAD induction.[142] Furthermore, bortezomib was able to improve response rates, PFS and OS in patients with poor prognostic markers such as t(4;14) and del(17p).[142]

Table 5. Phase 2 and 3 trials of lenalidomide based regimens in preparation for ASCT.

	No. of patients	≥ PR (%)	≥ VGPR (%)	nCR+CR (%)	CR(%)	Median follow-up	PFS (median/ months)	P-value	OS (median/ months)	P-value	Refs
RD vs. Rd	214 vs. 208	81 vs. 70	50 vs. 40	18 vs. 14	13 vs. 10	35.8 months	19 vs. 25	*P* = .02	not reached vs. not reached*	*P* = .40	115
RVD	66	100%	67	39	29	21 months	18 mo, 75%		18 mo, 97%		197
VDCR vs. RVD vs. VCD vs. VCDmod	41 vs. 42 vs. 32 vs. 17	88 vs. 83 vs. 78 vs. 100	59 vs. 50 vs. 41 vs. 59	34 vs. 38 vs. 31 vs. 47	24 vs. 24 vs. 22 vs. 47		NR		NR		198

Abbreviations: RD; Lenalidomide, dexamethasone; Rd: Lenalidomide, low dose dexamethasone; RVD: Lenalidomide, bortezomib, dexamethasone; VDCR: bortezomib, dexamethasone, cyclophosphamide, lenalidomide; VCD: bortezomib, cyclophosphamide, dexamethasone; VCDmod (as for VDC but with an additional dose of C on d 15)
* 1 year OS was 96% in the Rd compared with 87% in the RD (p = 0.0002). As a result, the trial was stopped and patients on high-dose therapy were crossed over to low-dose therapy.

Table 6. Phase 3 trials of bortezomib-based regimens in preparation for ASCT.

	No. of patients	≥ PR (%)	≥ VGPR (%)	nCR+CR (%)	CR (%)	Median follow-up	PFS (median/ months)	P-value	OS (median/ months)	P-value	Refs
V (single agent)	64	41	NR	9	NR	29 months	17		1-yr, 92%		199
V ± D	32	88	NR	25	6		NR		NR		200
PAD2 (1.3mg/ m^2)/ PAD1 (1.0mg/ m^2)	20/21	95 vs. 89	81 vs. 53	57 vs. 42	43 vs. 37	40 months	29 vs. 24	*P* = .19	2-yr, 95% vs. 2-yr, 73%	*P* = .22	201
VDD	30	87	77	57	NR	23.8 months	2-yr, 80%		2-yr, 92%		202
CyBorD	33	88	61	43	3		NR		NR		203
VD vs. VAD	223 vs. 218	NR	68 vs. 47		40 vs. 23	32 months	36 vs. 30	*P* = .06	3-yr, 81% vs. 3-yr, 77%	*P* = .50	141
PAD vs. VAD	413 vs. 414	90 vs. 83	76 vs. 56	49 vs. 34	36 vs. 24	41 months	35 vs. 28	*P* = .00	5-yr, 61% vs. 5-yr, 55%	*P* = .05*	142
VTD vs. TD	236 vs. 238	96 vs. 89	89 vs. 74	71 vs. 54	58 vs. 41	36 months	3-yr, 68% vs. 3-yr, 56%	*P* = .01	3-yr, 86% vs. 3-yr, 84%	*P* = .30	204

Abbreviations: V: Bortezomib; D: Dexamethasone; PAD: Bortezomib, adriamycin, dexamethasone; VDD: Bortezomib, liposomal doxorubicin, dexamethasone; CyBorD Cyclofosfamide, bortezomib, dexamethasone, VD: Bortezomib, dexamethasone; VAD: Vincristin,adriamycin, dexamethasone; VTD: Bortezomib, thalidomide, dexamethasone; TD: Thalidomide, dexamethasone.
* in multivariate analysis

In the VISTA trial for patients ineligible for ASCT, bortezomib, melphalan and prednisone (VMP) showed significantly higher ORR (71% vs. 35%), time to progressive disease (24 vs.17 months), and 3 years OS (69% vs. 54%).[143]

Bortezomib is generally well tolerated in patients of all ages, however one of the key toxicities of bortezomib concerned the development of a painful, sensory polyneuropathy,[66-68,144] which will be more extensively discussed in chapter 1.5.

The most common reported hematological adverse event (AE) was cyclical thrombocytopenia, occurring in 28% of MM patients treated with bortezomib, however predominantly in patients with a low baseline platelet count. This concerned a transient thrombocytopenia, with recovery occurring within the 10-day period during which treatment was suspended, and was not associated with serious bleeding complications.[67,68]

Carfilzomib is a second generation proteasome inhibitor of the epoxyketone class that is structurally and functionally distinct from bortezomib.[145] Carfilzomib is a potent and highly selective inhibitor of the CT-L activity of the 20S proteasome, which results in antiproliferative and proapoptotic effects in cell lines representative of hematologic malignancy, particularly MM.[146] In addition, it provides irreversible proteasome inhibition that leads to a more sustained response in preclinical studies than observed with bortezomib.[145]

In clinical studies carfilzomib has demonstrated substantial antimyeloma activity while exhibiting a well tolerated side-effect profile. In patients with relapsed/ refractory MM, twice-weekly consecutive-day single-agent carfilzomib 20 mg/m^2 for 3 weeks every 28 days, escalating to 27 mg/m^2 in the second cycle was associated with a 54% ORR in bortezomib naive patients and a 26% ORR in bortezomib and IMiD refractory patients. Painful neuropathy was minimally reported, suggesting a possible advantage over other proteasome inhibitors. With single-agent carfilzomib, dose-limiting toxicity was hematologic and included thrombocytopenia and neutropenia.[147-151]

NPI-0052 or salinosporamide A (Marizomib®), is a small molecule derived from fermentation of Salinospora, a new marine Gram-positive actinomycete;[152] like carfilzomib it is also an irreversible inhibitor of CT-L activity, and has a low IC50 for trypsin-like activity.[153]

In vivo, NPI-0052 has shown prolonged survival in animal models of MM.[153] Numerous studies in hematologic malignancies have shown NPI-0052 to have synergistic activity in combination with various agents, including with lenalidomide in a mouse model of MM.[154] Studies have also shown NPI-0052 and bortezomib in combination to result in synergistic effects in MM cells and *in vivo*.[154,155]

Another second-generation, orally bioactive proteasome inhibitor is MLN9708/ MLN2238. In preclinical studies, MLN9708 immediately hydrolyzes to MLN2238, the biologically active form. MLN2238 is an N-capped dipeptidyl leucine boronic acid which predominantly inhibits CT-L activity of the proteasome and induces accumulation of ubiquitinated proteins. MLN2238 inhibits growth and induces apoptosis in MM cells resistant to conventional and bortezomib therapies without affecting the viability of normal

cells. In animal tumor model studies, a head-to-head analysis of MLN2238 vs. bortezomib showed a significantly longer survival time in mice treated with MLN2238 than mice receiving bortezomib.[156,157]

1.4.4 Promising (targeted) therapies

The myeloma treatment paradigm continues to evolve in the era of novel agents. Novel agents have been or are now being evaluated alone or combined with the described novel IMiDs and proteasome inhibitors to further improve outcome, especially in patients who are refractory to mentioned treatments. These include alkylators, such as bendamustine, with structural similarities to both alkylating agents and purine analogs, but no cross-resistance with alkylating agents and other drugs *in vitro*. Furthermore, these are inhibitors of histone deacetylase (HDACis), which are agents that modulate activities of many oncogenes and tumor suppressor genes. The clinical activity of HDACis as single agents appear to be rather modest,[158,159] however phase II trials which assess the efficacy of HDACis in combination with bortezomib show promising results.[160,161] In addition, monoclonal antibodies (mAb) against MM-related target antigens have been, or are currently being explored. Currently, CS1 is one of the most promising targets for mAb-mediated MM therapy. CS1 is a human membrane glycoprotein and member of the immunoglobulin superfamily. Its cell surface expression is found nearly ubiquitous in MM cells, and only limited in normal cells.[162,163] Elotuzumab (HuLuc63) is a fully humanized mAb against CS1. Its principal mechanism is thought to be antibody-dependent cellular cytotoxicity (ADCC).[163] As monotherapy in a dose escalation study, elotuzumab induced no responses, however 26.5% of relapsed/refractory MM patients had stable disease (SD), with acceptable toxicity.[164] The combination of elotuzumab with bortezomib or lenalidomide showed encouraging activity in patients with relapsed/refractory MM.[165,166]

1.5 PERIPHERAL NEUROPATHY (PN)

From the early days of thalidomide and bortezomib use in clinical trials, data on AEs of these agents emerged and it became evident that PN following thalidomide and bortezomib based treatment was one of the main non-hematological dose-limiting side-effects. This treatment related neurotoxicity significantly affected patients' quality of life and myeloma treatment, with requirements of dose reduction, delay, or even premature termination of successful treatment.[167]

Typically, drug-induced PN involves the longest nerves in the extremities, causing a symmetric, length-dependent PN spreading from distal to proximal in a glove-and-stocking distribution.[168] The type of PN most frequently reported concerns sensory PN, including hypoesthesia (numbness), paraesthesia (tingling, pin-prick sensation), and hyperaesthesia

in the toes and fingers. Severe thalidomide-induced and bortezomib-induced sensory PN can result in areflexia and loss of proprioception, placing patients at risk for injury through ataxia and gait disturbance.[169] Motor neuropathy occurs infrequently and if present, it mostly occurs in the context of severe sensory peripheral neuropathy. Symptoms include muscle cramps, muscle atrophy, or loss of strength in distal muscles. Autonomic symptoms, such as orthostatic hypotension, bradycardia and constipation can occasionally occur and are induced by damage to small fibres.[168,170]

In general, severity of neuropathy is graded using the National Cancer Institute's Common Toxicity Criteria for Adverse Events (version 3.0), NCI-CTC version 3 (table 7).[171]

Table 7. National Cancer Institute's Common Toxicity Criteria for Adverse Events criteria, version 3.0.

Grade	0	1	2	3	4
Neuropathy – sensory	Normal	Asymptomatic; loss of deep tendon reflexes or paresthesia (including tingling) but not interfering with function	Sensory alteration or paresthesia (including tingling), interfering with function, but not interfering with activities of daily living	Sensory alteration or paresthesia interfering with activities of daily living	Disabling
Neuropathic pain	None	Mild pain not interfering with function	Moderate pain; pain or analgesics interfering with function but not interfering with activities of daily living	Severe pain; pain or analgesics severely interfering with activities of daily living	Disabling

Thalidomide induced peripheral polyneuropathy (TiPN) CTC grade 1–4 is reported to occur in 27–54% of patients, with 2–23% CTC grade 3-4.[98-100,172,173] Dose and treatment duration of thalidomide are the two most crucial risk factors for development of peripheral neuropathy. Therefore, it has become common practice to avoid daily doses higher than 200 mg and to limit treatment duration.[174]

Bortezomib induced peripheral polyneuropathy (BiPN) typically concerns a painful and predominantly sensory neuropathy resulting from axonal degeneration,[175-177] which occurs within the first courses of bortezomib treatment and does not seem to increase after the fifth cycle of bortezomib.[144] Bortezomib in combination with dexamethasone or other chemotherapeutical agents is associated with development of BIPN, with CTC grade 1-4 occurring in 37–46% in MM patients, regardless relapsed/refractory or newly diagnosed, and 7–26% CTC grade 3–4 BiPN.[66-68,141,143]

In the majority of patients, BiPN was found to be reversible and did not seem to be influenced by number or type of previous treatments, baseline glycosylated hemoglobin level, or diabetes mellitus history.[144] However, another study found that the risk of BiPN was

greater in patients who had PN and diabetes mellitus at baseline,[178] whereas in the VISTA trial, baseline PN was the only consistent risk factor for any BiPN, for ≥ grade 2 BiPN, and for ≥ grade 3 BiPN.[179]

During the years of experience with thalidomide and bortezomib use in clinical trials, specific guidelines, based on NCI-CTC version 3.0, have been developed and recently updated to avoid serious and irreversible neurotoxicity, (table 8).[174] In addition; a newer CTC version is currently available, NCI-CTC version 4.0, which now includes neuropathic pain. This thesis contains a chapter extensively reviewing BiPN, chapter 10.

Table 8. Recently updated dose-modification guidelines for thalidomide-induced and bortezomib-induced neurotoxicity.

Grade	Grade 1	Grade 1 with neuropathic pain or grade 2	Grade 2 with neuropathic pain or grade 3	Grade 4
Thalidomide	No action or 50% dose reduction	Discontinue thalidomide. If the neuropathy resolves to grade I or better, treatment may be restarted at 50% dose reduction, if the benefit/ risk ratio is favorable.	Discontinue treatment	Permanent discontinuation
Bortezomib	If the patient is on a biweekly schedule: reduce current bortezomib dose by one level or prolong dosing interval to once weekly If the patient is already on a weekly schedule: reduce current bortezomib dose by one level	If the patient is on a biweekly schedule: reduce current bortezomib dose by one level or prolong dosing interval to once weekly. If the patient is already on a weekly schedule: reduce current bortezomib dose by one level or consider temporary discontinuation of bortezomib. If the neuropathy resolves to grade 1 or better, once weekly treatment with reduced bortezomib dose may be restarted if the benefit-to-risk ratio is favorable	Discontinue bortezomib, if the neuropathy resolves to grade 1 or better, once weekly treatment with reduced bortezomib dose may be restarted if the benefit-to-risk ratio is favorable	Permanent discontinuation

1.6 NOVEL TECHNOLOGIES FOR MOLECULAR PROFILING

Gene expression profiling (GEP)

Gene expression profiling is the measurement of the expression of thousands of genes at once. As described in this introduction, gene expression profiling enabled subdivision of MM patients into different molecular subgroups, resulting in GEP based molecular classifications with prognostic value.[180,181] Furthermore, gene based high-risk signatures identified MM patients with poor survival,[60,69,72,73,182,183] and GEP associated genetic markers with presence of bone disease.[45] The arrays used in our research are Affymetrix GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix Inc.), comprised of more than 54000 probe sets and 1300000 distinct oligonucleotide features. With these micro-arrays the expression level of over 40000 transcripts and variants can be analyzed, corresponding to over 30000 human genes

Single nucleotide polymorphism (SNP)

A SNP is a DNA sequence variation occurring when a single nucleotide, A, T C, G, in the genome differs between individuals or paired chromosomes in an individual. SNPs are the most common type of genetic variation among people, and have a minor allele frequency (MAF) of at least 1% of the population. The minor allele frequency refers to the allele of the SNP with the lowest frequency in the population. It is estimated that SNPs with a MAF of ≥ 1% occur every 100 to 300 bases along the 3-billion-base human genome, resulting in the presence of 10 million common SNPs, which constitute 90% of the variation in the population.[184,185]

SNPs can occur within coding sequences of genes, non-coding regions of genes, or in intergenic regions. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous; if a different polypeptide sequence is produced they are non-synonymous. SNPs that are not in protein coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA.

SNPs may help to predict an individual's susceptibility to develop particular diseases, treatment outcome, and treatment related toxicity.

The Bank On A Cure (BOAC) SNP chip, used in chapter 9, was a custom SNP chip designed to examine genetic variations and association with disease risk and clinical outcome in multiple myeloma.[186,187] The BOAC SNP panel, based on the Affymetrix/ Gene Chip Targeted Genotyping Platform, contains 3404 SNPs in 983 genes, selected using a candidate gene approach. A candidate gene lists was created based on functionally relevant polymorphisms playing a role in normal and abnormal cellular functions, inflammation and immunity, as well as drug responses.[188] Each gene in the candidate list was systematically investigated with a selection of SNP databases to harvest SNPs that may have a functional effect on gene action. SNPs were selected from the candidate list if they were non-synonymous SNPs with

a MAF ≥ 2%. In addition, SNPs in promoter regions present in homologous regions between human and mouse with a MAF ≥ 2%, in or adjacent to transcription binding sites, were selected. Furthermore, admixture SNPs, which differ in frequency between Asian, African and European groups, were added to allow for corrections for racial specific variations.[189]

Whole genome/exome sequencing (WGS/WES)

A novel, more powerful way to gain insight in factors involved in the development of cancer is to sequence either the whole genome or the protein-coding exome from tumor and normal tissue from the same patient to identify acquired somatic mutations. Recently, a publication came out reporting on the results of whole-genome sequencing (WGS) in 23 samples from MM patients and whole-exome sequencing (WES) in 16 samples from MM patients. 164,687 exons can be sequenced by WES and enables identification of protein coding mutations, but cannot detect non-coding mutations and rearrangements. Tumor-specific mutations were identified by comparing each tumor to its corresponding normal. A number of discoveries were outlined, which include the identification of frequent mutations in genes involved in RNA processing, protein translation and UPR. Apart from previously described mutations, in genes such as *p53*, *NRAS* and *KRAS*, other statistically significant, mutated genes included *DIS3*, *CCND1* and *PNRC1*.[190]

Whole genome/exome sequencing is a powerful, new technology enabling the investigation of novel mutations. The key challenge will be to distinguish driver mutations from passenger mutations. Driver mutations push cells towards cancer, and are causally implicated in oncogenesis, whereas passenger genes represent by-products of cancer cell development, which do not contribute to the development of cancer but have occurred during the growth of the cancer.[191] Combining whole genome/exome sequencing with sequential sampling of myeloma may provide important answers in this context, as was recently reported. Using WGS, Egan et al. demonstrated 15 mutations, present at four time points in a single patient, i.e. at diagnosis, at first relapse, at second relapse and at the plasma cell leukemic phase of disease. Included within these 15 were *LRRC4C, ATXN1* and *LTB*.[192] Future studies, including more sequentially collected samples, will provide insight into novel genes important for myeloma development.

1.7 SCOPE OF THIS THESIS

The work presented in this thesis revolves around molecular profiling of purified myeloma plasma cells. Determining the molecular characteristics of purified myeloma plasma cells at diagnosis, our aim was to try to unravel (to some extent) issues which prevent optimal treatment of multiple myeloma:

- We know that multiple myeloma is a heterogeneous disease, based on detection of translocations involving the IgH locus and their subsequent dysregulated genes, however concerning the heterogeneity of cytogenetically hyperdiploid patients there is still a lot unknown. Furthermore, we know that there are differences in outcome between subgroups of patients. Which subgroups can we distinguish and what is the impact of novel agents on prognosis of these subgroups?
- Furthermore, despite the use of novel agents, there is still a percentage of patients showing a poor PFS and OS. Can we identify this group of patients? Furthermore, can we find genetic markers with predictive potential for specific treatments or agents?
- As described above, peripheral neuropathy often prevents (continuation) of treatment with bortezomib, and has a negative impact on quality of life. Questions raised are: can we detect molecular factors in myeloma plasma cells and polymorphisms in peripheral blood associated with PN, and are we able to identify and predict this group of patients with increased risk of developing PN?

Chapter 2 describes the results of the multi-center, phase III trial, HOVON-65/GMMG-HD4, comparing bortezomib in induction and post-intensification vs. conventional chemotherapy and thalidomide post-intensification in newly diagnosed MM patients. The side studies described in chapters 3, 4, 6, 7, 8 and 9 are based on the clinical and outcome data from this trial.
Chapter 3 presents a classification of MM patients based on translocation status and correlated GEPs and describes 10 distinct clusters, confirming the 7 UAMS clusters and in addition 3 novel clusters. **Chapter 4** is a small chapter, yet unpublished, added to chapter 3 to investigate the effect of bortezomib on outcome of the clusters as defined in chapter 3.

To use GEPs in order to define prognosis, **chapter 5**, a paper by Mulligan and colleagues to which we contributed by performing part of the analyses, describes a pretreatment gene expression pattern and predictive classifier that is significantly associated with the response to bortezomib. In **chapter 6** we describe the development and performance of a gene expression based high-risk signature for outcome of MM patients.
Chapter 7 focuses specifically on cancer testis antigen (CTA) expression in newly diagnosed and relapsed myeloma patients.
Chapter 8 focuses on the role of *CRBN* in IMiD antitumor activity, based on the recent publications described in chapter 1.4.3. Chapter 8 concerns a short report on the role of *CRBN* expression in outcome following thalidomide maintenance treatment in the HOVON-65/GMMG-HD4 trial.

The frequent development of PN following novel agents triggered us to look into the molecular profiles of MM plasma cells at diagnosis. In **chapter 9** the scope was to define molecular characteristics of these myeloma plasma cells of patients who developed PN following treatment with a bortezomib based induction regime. In addition, constitutional

genetic polymorphisms (SNPs) using peripheral blood DNA of MM patients associated with development of BiPN were determined. **Chapter 10** reviews certain aspects of BiPN, including clinical presentation, pathogenesis, risk factors, assessment and management of BiPN.

It is evident that the increase in knowledge on MM biology has resulted in development of important new treatment possibilities for MM. The research conducted and described in this thesis, with use of novel techniques such as gene expression profiling and SNP analysis, contributed considerably to the insight in different aspects of multiple myeloma.

It is unquestionable that multiple myeloma concerns a complex, heterogeneous disease which consists of distinct (cyto)genetic subgroups with prognostic impact. In addition, using gene expression profiling, a genetic signature was developed which enabled identification of high-risk myeloma patients. Furthermore, genetic markers such as *CRBN* and CTAs show prognostic value in myeloma patients treated with novel agents, and could be potential markers in the future to guide treatment choice. Finally, we have shown that molecular factors in myeloma plasma cells as well as polymorphisms in peripheral blood, are associated with development of BiPN, providing a basis for development of predictors for patients at risk for development of PN or other treatment related toxicities.

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CHAPTER 2

BORTEZOMIB INDUCTION AND MAINTENANCE TREATMENT IN PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA: THE OPEN-LABEL, RANDOMIZED PHASE 3 HOVON-65/GMMG-HD4 TRIAL

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ABSTRACT

Background: We investigated if bortezomib during induction and maintenance improves survival in newly diagnosed Multiple Myeloma (MM).

Methods: 827 eligible patients with newly diagnosed symptomatic MM were randomized to receive induction therapy with VAD (vincristine, doxorubicin, dexamethasone) or PAD (bortezomib, doxorubicin, dexamethasone) followed by high-dose melphalan and autologous stem cell transplant. Maintenance consisted of daily thalidomide 50 mg (VAD) or 2-weekly bortezomib 1.3 mg/m^2 (PAD) for 2 years. The primary analysis was progression-free survival (PFS) adjusted for ISS stage.

Results: Complete response (CR) including nearCR was superior after PAD induction (15% vs. 31%, ($p < 0.001$)) and bortezomib maintenance (34% vs. 49% ($p < 0.001$)), respectively. After a median follow-up of 41 months PFS was superior in the PAD arm, i.e. median 28 versus 35 months (HR = 0.75, 95% CI = 0.62–0.90, p = 0.002). In multivariate analysis overall survival (OS) was better in the PAD arm (HR = 0.77, 95% CI = 0.60–1.00, p = 0.049). In high-risk patients presenting with elevated creatinine > 2 mg/dL bortezomib significantly improved PFS from median 13 to 30 months (HR = 0.45, 95% CI = 0.26–0.78, p = 0.004) and OS from median 21 to 54 months (HR = 0.33, 95% CI = 0.16–0.65, $p < 0.001$). A benefit was also observed in patients with deletion 17p13 (median PFS 13 vs. 22 months; HR = 0.47, 95% CI = 0.26–0.86, p = 0.01; median OS 24 months vs. not reached at 54 months; HR = 0.36, 95% CI = 0.18–0.74, p = 0.003)).

Conclusions: We conclude that bortezomib during induction and maintenance improves complete response and achieves superior PFS and OS.

INTRODUCTION

High Dose Melphalan (HDM) followed by autologous stem cell transplantation (ASCT) is considered a standard of care for younger patients with Multiple Myeloma (MM).[1-3] In the setting of ASCT, high complete response (CR) rates have consistently resulted in prolonged progression free survival (PFS) and overall survival (OS).[4] Combined chemotherapy with vincristine, doxorubicin, dexamethasone (VAD) has been a standard induction approach in this setting.[5,6] Proteasome inhibition (bortezomib) and immunomodulatory drugs (thalidomide, lenalidomide) have significant activity in patients with relapsed/refractory MM.[7-10] Bortezomib combined with melphalan/prednisone achieves 30% CR in newly diagnosed non-transplant eligible patients.[11] Likewise, bortezomib combined with dexamethasone (VD) and thalidomide (VTD) results in an improvement of CR and in prolongation of PFS.[12,13] Although CR is considered to be predictive for prolonged survival, these schedules have not resulted in an improved OS.[14,15] In high-risk MM patients presenting with renal failure or with cytogenetic abnormalities such as deletion of chromosome band 17p13 and translocation t(4;14) survival remains especially short.[16-18] Consolidation or maintenance therapy following HDT improves CR rate and PFS.[19] Maintenance with thalidomide prolongs PFS but not OS.[20-22] In addition, lenalidomide maintenance resulted in a better PFS in one transplant study and both PFS and OS in another.[23,24] Bortezomib has been studied as post-ASCT consolidation therapy.[25-27] Use of bortezomib as maintenance therapy has not yet been addressed in younger patients. We report on the results of an open-label randomized phase 3 trial which was designed to evaluate the role of sustained bortezomib treatment during induction and maintenance.

PATIENTS AND METHODS

Eligibility

Patients 18–65 years of age with newly diagnosed MM Salmon Durie stage II/III, WHO performance status 0–2, or WHO 3 when caused by MM, were eligible. Exclusion criteria were systemic AL amyloidosis, non-secretory MM, neuropathy grade ≥ 2, active malignancy during the past 5 years with the exception of basal carcinoma of the skin or stage 0 cervical carcinoma, HIV positivity, or serum bilirubin ≥ 30 mmol/l or transaminases ≥ 2.5 normal level). Prior corticosteroids were allowed for a maximum of 5 days. Patients with renal impairment were not excluded. Local radiotherapy for painful MM lesions was allowed.

Study design

This investigator sponsored open-label randomized phase 3 trial was designed and performed by the Dutch-Belgium Hemato-Oncology Group (HOVON) and the German-

speaking Myeloma Multicenter Group (GMMG) using a joint protocol, data management and analysis. Patients were randomly assigned 1:1 to VAD induction,[5] followed by intensification with HDM and ASCT, followed by maintenance therapy with thalidomide (arm A); or bortezomib, doxorubicin and dexamethasone (PAD)[28] followed by intensification with HDM and ASCT, followed by maintenance with bortezomib for 2 years (arm B). This design was chosen to evaluate sustained bortezomib treatment (64 administrations) as in VISTA.[11] Randomized assignments to VAD or PAD were balanced with the use of a biased-coin minimization procedure, with the bias dependent on the average imbalance between the numbers of patients already assigned to each treatment arm overall and within the stratification factors of the new patient.[29] The minimization was initially based on the factors of hospital, Salmon & Durie stage (2 vs. 3) and LDH level (≤ ULN vs. > LDH). After the 3rd amendment, the minimization was based on both hospital and ISS stage (I vs. II vs. III). VAD was administered as 3 cycles of intravenous (i.v.) vincristine 0.4 mg days 1v4, doxorubicin 9 mg/m^2 days 1–4 and oral dexamethasone 40 mg on days 1–4, 9-12, 17–20, q 28 days. PAD induction included 3 cycles of i.v. bortezomib 1.3 mg/m^2 days 1, 4, 8, 11, doxorubicin 9 mg/m^2 days 1–4 and oral dexamethasone 40 mg on days 1–4, 9–12, 17–20, q 28 days. Stem cells collection was performed 4-6 weeks after induction as described.[22] High-dose melphalan (200 mg/m^2) and ASCT were administered as described.[22] Per protocol one or two cycles of HDM were planned, according to HOVON (one) and GMMG (two) standards, respectively. Patients randomized to VAD received maintenance with thalidomide 50 mg daily for 2 years, starting at 4 weeks after HDM. Patients randomized to PAD received maintenance with i.v. bortezomib 1.3 mg/m^2 two-weekly for 2 years (52 gifts) starting at 4 weeks after HDM. Patients with an HLA-identical sibling could proceed to non-myeloablative allogeneic stem cell transplantation (AlloSCT) after HDM. Maintenance was not given after AlloSCT. Supportive care was given as described in the supplementary file.

This study was approved by the Ethics Committees of the Erasmus University MC, the University of Heidelberg and the participating sites. All patients gave written informed consent and the trial was conducted according to the European Clinical Trial Directive 2005 and the Declaration of Helsinki.

Response assessments and endpoints

Clinical characteristics were registered at diagnosis. Cytogenetic studies were performed as described in the supplementary file.[30] Evaluation of response was performed according to modified European Group for Blood and Marrow Transplantation (EBMT) criteria.[31] NearCR (nCR) and Very Good Partial Response (VGPR) were implemented as in the International Myeloma Working Group (IMWG) uniform response criteria.[32] nCR was defined as CR with positive or missing immunofixation,[8] while VGPR was defined as > 90% reduction of serum M-protein and urine light chain < 100 mg/24h. CR required negative serum/urine immunofixation and bone marrow morphology evaluation. Responses were assessed

after induction, after first and second transplantation and at 2 month intervals during maintenance and in follow up until progression. Progression free survival (PFS) was calculated from randomization until progression, relapse or death, whichever came first. Patients who received a non-myeloablative AlloSCT were however censored at the date of AlloSCT. PFS without censoring of AlloSCT patients has been denoted as PFS_A. Overall survival (OS) was measured from randomization until death from any cause. Patients alive at the date of last contact were censored.

Statistical analysis

The primary objective of the study was to compare PFS between the treatment arms. The expected PFS in the VAD arm was 50% at 3 years. To detect with a power of 80% a hazard ratio (HR) of 0.74, which corresponds to an increase of 3-year PFS from 50% to 60% (2-sided significance level $\alpha = 0.049$, because of one planned interim analysis at a significance level $\alpha = 0.001$), and assuming 3 years accrual, additional follow up time of 2 years and 10% AlloSCT, 800 patients had to be randomized and 356 events had to be observed. All analyses were performed by intention-to-treat, and the primary analysis was done with a multivariate Cox regression including adjustment for ISS stage.

Secondary endpoints included response, PFS_A (without censoring AlloSCT patients), PFS/PFS_A from last HDM, OS, safety and toxicity. The effect of treatment arm and covariates on response was analyzed using logistic regression. Odds ratios (ORs) were calculated with 95% confidence intervals (CI). PFS, PFS_A and OS were estimated by the Kaplan-Meier method and 95% CIs were constructed. Survival endpoints were analyzed with Cox regression. HRs and corresponding 95% CIs were determined. The analyses regarding treatment arm were performed with and without adjustment for covariates. In the statistical analysis plan it was specified that the following variables would be included in the multivariate Cox regression analyses: age, sex, WHO performance (0 vs. 1 vs. 2 vs. 3), Salmon & Durie stage (2 vs. 3), IgA (no vs. yes), IgG (no vs. yes), LDH (normal vs. > upper limit of normal (ULN)), ISS (I vs. II vs. III vs. unknown), FISH del(13q14) and study group (HOVON vs. GMMG). The aim of the multivariate analysis was to evaluate the impact of adjustment for covariates on the HR and 95% CI of treatment arm. Kaplan-Meier curves were generated to illustrate survival.

Analysis of treatment toxicity was done by tabulation of adverse events (AEs) by treatment arm and cycle. AEs were graded using the NCI Common Toxicity Criteria for Adverse Events, CTCAE, version 3.0. The proportion of patients with specific AEs was compared using the chi-squared test or Fisher's exact test, whichever appropriate.

Secondary analyses were planned for prognostic factors, especially b_2-microglobulin, cytogenetic/FISH abnormalities, serum albumin, age, LDH level and Salmon & Durie A/B stage with respect to response rate, PFS, and OS from registration. The heterogeneity of the treatment effect in subgroups was explored post hoc by estimation of the HRs for survival endpoints for each subgroup, together with 95% CIs.

In order to be able to include all patients in the multivariate analyses, the MICE method of multiple imputations was used to cope with missing data on these baseline covariates.[33] Each of those datasets was then analyzed separately, and estimates of the parameters of interest were averaged across the 20 copies to give a single estimate for the ORs and HRs with 95% CIs.[34]

One planned interim analysis was performed after 75 events for PFS and the results were presented confidentially to an independent data and safety monitoring board. After inclusion of the last patient, interim results for PFS based on initial registered 750 patients were presented at the annual meeting of the American Society of Hematology in 2010.[35] Monitoring was based on the reported SAEs. Data cutoff date for this manuscript was April 12, 2011. The median follow up of the 588 patients still alive is 41 months (maximum: 66 months). All reported P values are 2-sided, and have not been adjusted for multiple testing.

RESULTS

Patients

833 patients were recruited from May 2005 until May 2008. Two (VAD) and 4 (PAD) ineligible patients were excluded from analyses for reasons of non-secreting myeloma (n = 4), misdiagnosis or prior treatment (n = 2): 827 patients were randomly assigned to VAD (n = 414) or PAD (n = 413). Patient characteristics at inclusion did not differ between the two groups (Table 1; all P-values > 0.10). Eighty-one patients (10%) with impaired renal function (Salmon-Durie stage B) i.e. serum creatinine > 2 mg/dL were included.

Table 1. Baseline Patient and Disease Characteristics.

Characteristics	VAD N = 414		PAD N = 413	
Study group – no. (%)				
HOVON	213	(51)	219	(53)
GMMG	201	(49)	194	(47)
Median age, years (range)	57 (25–65)		57 (31–65)	
Male sex – no. of patients (%)	247	(60)	253	(61)
WHO performance stage – no. (%)				
0	183	(44)	193	(47)
1	173	(42)	170	(41)
2	47	(11)	31	(8)
3	8	(2)	15	(4)
Unknown	3	(1)	4	(1)

Table 1. *Continued*

Characteristics	VAD N = 414		PAD N = 413	
ISS stage – no. (%)				
I	144	(35)	144	(35)
II	124	(30)	150	(36)
III	107	(26)	81	(20)
Unknown	39	(9)	38	(9)
M-protein isotype – no. (%)				
IgA	97	(23)	92	(22)
IgG	234	(57)	251	(61)
IgD	3	(1)	5	(1)
LCD	78	(19)	63	(15)
Other	2	(0)	2	(0)
M-protein light chain – no. (%)				
Kappa	278	(67)	277	(67)
Lambda	136	(33)	135	(33)
Unknown	0	(0)	1	(0)
Creatinine, mg/dL – no. (%)				
≤ 2 mg/dL	368	(89)	376	(91)
> 2 mg/dL	45	(11)	36	(9)
Unknown	1	(0)	1	(0)
Number of skeletal lesions – no. (%)				
0	96	(23)	102	(25)
1–2	42	(10)	44	(11)
≥ 3	264	(64)	255	(62)
Unknown	12	(3)	12	(3)
Serum LDH – no. (%)				
≤ ULN	331	(80)	329	(80)
> ULN	72	(17)	72	(17)
Unknown	11	(3)	12	(3)
Genetic abnormalities – no. (%)				
del(13q)				
done	372	(90)	361	(88)
positive, % of done	164	(44)	148	(41)
t(4;14)				
done	262	(63)	250	(61)
positive, % of done	35	(13)	35	(14)
del(17p)				
done	313	(76)	289	(70)
positive, % of done	40	(13)	25	(9)
Median β_2-microglobulin – mg/L	3.40		3.40	
Median hemoglobin – mmol/L	6.7		6.6	
Median calcium – mmol/L	2.31		2.34	
Median BM plasma cells – %	45		40	

Abbreviations: VAD, vincristine, doxorubicin, dexamethasone; PAD, bortezomib, doxorubicin, dexamethasone; WHO, World Health Organization; ISS, International Staging System; LCD, light-chain disease; LDH, serum lactate dehydrogenase; BM, Bone Marrow. ULN, upper limit of normal

Adherence to treatment

In Figure 1 the flow of patients through the protocol is shown. Intensification with first HDM was achieved in 84% of patients and 69% of GMMG patients completed a second HDM. Of those patients after HDM, in the VAD arm 77/347 (22%) went off protocol after HDM because of AlloSCT (n = 21, 6%), persisting toxicity (n = 11, 3%) or other reasons (n = 45, 13%), while 270 (78%) patients started maintenance treatment. In the PAD arm 123/352 (35) patients went off protocol because of AlloSCT (n=28, 8%), persisting toxicity (n = 47, 13%, mainly polyneuropathy) or other (n = 48, 14%), while 229 (65%) started maintenance with bortezomib (p = 0.004) (Figure 1). Persisting toxicity was an exclusion criterium for starting maintenance and within the group of patients who went off protocol, excluding AlloSCT, this was observed in 11/77(14%) in the VAD arm versus 47/123 (38%) in the PAD arm (p < 0.001). Progression or relapse were a reason to stop maintenance prematurely in 86/270 (32%) patients during thalidomide vs. 74/ 229 (32%) patients during bortezomib, respectively.

Normal completion of maintenance was achieved in 73/270 (27%) patients in the VAD arm and 109/229 (47%) patients in the PAD arm (Table 2).

Response

Overall response is given in Table 3. The CR rate was 24% in patients who were randomized to VAD and 36% in the PAD arm (p < 0.001). nCR+CR rates were 34% vs. 49%, respectively (p < 0.001). After induction treatment all responses, i.e. ≥ PR, ≥ VGPR and ≥ nCR were superior in the PAD arm. Response rates increased from induction to significantly higher ≥ VGPR and ≥nCR following HDM/ASCT in the PAD arm. nCR+CR after transplantation was 15% (VAD) vs. 31% (PAD), respectively (p < 0.001). During maintenance an upgrade of response (from <PR to PR or < VGPR to VGPR or < nCR to nCR or < CR to CR) was observed in 24% of patients in the VAD arm and 23% in the PAD arm (Table 3). The median time to any response upgrade after start maintenance was 6 months (range, 1–35 months) vs. 7 months (range, 1–57 months).

Progression-free Survival and Overall Survival

The median PFS was 28 months (VAD) and 35 months (PAD) respectively. Patients who were assigned to PAD had a significantly better PFS (HR = 0.75, 95% CI = 0.62-0.90, p = 0.002) when adjusted for ISS (primary analysis) and also in the multivariate analysis (HR = 0.74, 95% CI = 0.62–0.89, p = 0.001) (Table 4 and Figure 2A). The median PFS_A (i.e. without censoring of AlloSCT) was 28 months vs. 34 months, respectively (Figure 2B).

Median OS was not reached at 66 months in both arms, with 5-year OS of 55% (VAD) vs. 61% (PAD) (Figure 2C). When OS was adjusted for ISS, a HR of 0.81, 95% CI = 0.63–1.05, p = 0.11) was noted, but OS difference reached statistical significance in the multivariate analysis (HR 0.77, 95% CI = 0.60–1.00, p = 0.049). However it should be noted that both

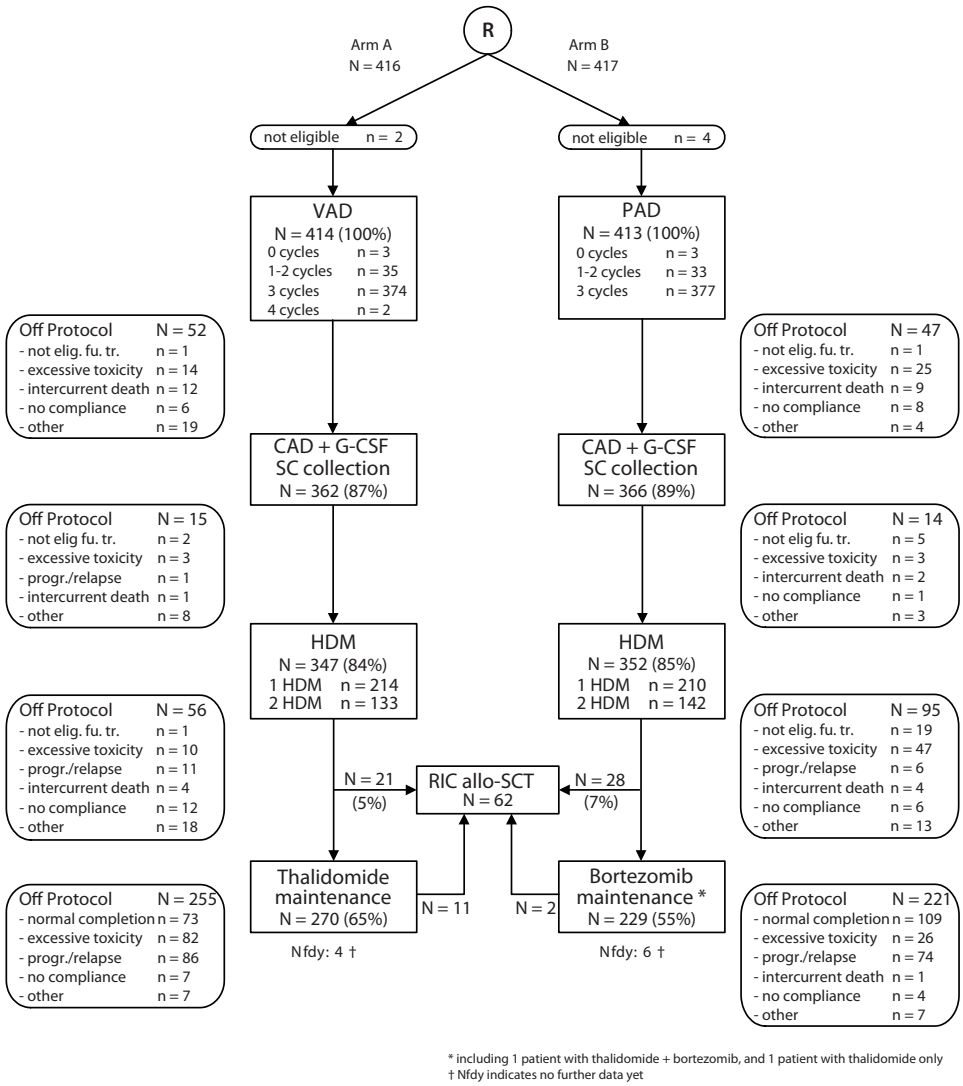

Figure 1. Flow diagram of 827 adult patients with multiple myeloma in the HOVON-65/GMMG-HD4 study by treatment arm.

HRs and 95% CIs are very similar. OS also differed between study groups with the GMMG patients showing a better OS (HR = 0.75, 95% CI = 0.57–0.97, p = 0.03). An analysis of PFS calculated from the time of last HDM (n = 645) showed a significant difference in favor of the PAD arm, median 26 months vs. 31 months (Figure 3A). This indicates that while post-transplant bortezomib and thalidomide both achieved response upgrades, bortezomib

contributed more to improvement of PFS. Moreover, in a Landmark analysis starting at 12 months after randomization in 585 patients (382 VAD, 302 PAD) who had received HDM/ASCT and were still without progression, PFS (p = 0.04; Figure 3B) and OS (p = 0.05; Figure 3C) were improved in the PAD arm.

Table 2. Feasibility of maintenance treatment.

	VAD + thalidomide	PAD + bortezomib
treatment started – #	270	229
Still on treatment after		
6 months, %	78	90
12 months, %	54	76
18 months, %	40	64
24 months, %	27	47

Table 3. Response after Induction, after high-dose melphalan and overall. Response according to Prognostic Factors.

	VAD (N = 414)		PAD (N = 413)		P value
Response after induction – no. of patients (%)					
CR	7	(2)	29	(7)	<0.001
≥ nCR	20	(5)	46	(11)	<0.001
≥ VGPR	59	(14)	174	(42)	<0.001
≥ PR	222	(54)	322	(78)	<0.001
Response after HDM – no. of patients (%)					
CR	37	(9)	85	(21)	<0.001
≥ nCR	62	(15)	127	(31)	<0.001
≥ VGPR	150	(36)	254	(62)	<0.001
≥ PR	312	(75)	363	(88)	<0.001
Response Overall – no. of patients (%)					
CR	99	(24)	147	(36)	<0.001
≥ nCR	140	(34)	201	(49)	<0.001
≥ VGPR	230	(56)	312	(76)	<0.001
≥ PR	343	(83)	373	(90)	0.002
Response upgrade during maintenance – no. of patients (%)					
Any response upgrade	99	(24)	93	(23)	0.64
< CR → CR	45	(11)	48	(12)	0.73
< nCR → nCR	16	(4)	23	(6)	0.25
< VGPR → VGPR	27	(7)	20	(5)	0.30
< PR → PR	11	(3)	2	(0)	0.008

Table 3. *Continued*

	VAD (N = 414)		PAD (N = 413)		P value
ISS stage I – no. of patients (%)	N = 144		N = 144		
CR	37	(26)	59	(41)	0.006
≥ nCR	58	(40)	78	(54)	0.018
≥ VGPR	94	(65)	115	(80)	0.005
≥ PR	130	(90)	132	(92)	0.68
ISS stage II	N = 124		N = 150		
CR	29	(23)	51	(34)	0.05
≥ nCR	35	(28)	70	(47)	0.002
≥ VGPR	59	(48)	109	(73)	<0.001
≥ PR	100	(81)	134	(89)	0.04
ISS stage III	N = 107		N = 81		
CR	24	(22)	26	(32)	0.14
≥ nCR	35	(33)	35	(43)	0.14
≥ VGPR	57	(53)	56	(69)	0.03
≥ PR	82	(77)	70	(86)	0.09
ISS stage unknown	N = 39		N = 38		
CR	9	(23)	11	(29)	0.56
≥ nCR	12	(31)	18	(47)	0.13
≥ VGPR	20	(51)	32	(84)	0.002
≥ PR	31	(79)	37	(97)	0.01
β_2-microglobulin > 3 mg/L – no. of patients (%)	N = 220		N = 223		
CR	50	(23)	77	(35)	0.006
≥ nCR	67	(30)	103	(46)	<0.001
≥ VGPR	114	(52)	163	(73)	<0.001
≥ PR	173	(79)	198	(89)	0.004
Creatinine > 2 mg/dL – no. of patients (%)	N = 45		N = 36		
CR	6	(13)	13	(36)	0.02
≥ nCR	12	(27)	19	(53)	0.02
≥ VGPR	18	(40)	28	(78)	<0.001
≥ PR	29	(64)	31	(86)	0.02
Genetic abnormalities – no. of patients (%)					
del(13/13q)	N = 164		N = 148		
≥ nCR	53	(32)	76	(51)	<0.001
≥ VGPR	88	(54)	124	(84)	<0.001
t(4;14)	N = 35		N = 35		
≥ nCR	11	(31)	20	(57)	0.03
≥ VGPR	20	(57)	30	(86)	0.007
del(17p)	N = 40		N = 25		
≥ nCR	8	(20)	13	(52)	0.008
≥ VGPR	17	(43)	18	(72)	0.02

Abbreviations: VAD, vincristine, doxorubicin, dexamethasone; PAD, bortezomib, doxorubicin, dexamethasone; ISS, International Staging System; BM, Bone Marrow. CR, complete response; nCR, near complete response; VGPR, very good response; PR, partial response.

Table 4. Multivariate analysis of risk factors for PFS and OS.

	PFS			OS		
Risk factor	HR	95%CI	*P-value*	HR	95% CI	*P-value*
Treatment arm	0.74	0.62 – 0.89	0.001	0.77	0.60 – 1.00	0.049
Age	1.00	0.99 – 1.02	0.53	1.00	0.99 – 1.02	0.61
Female sex	0.85	0.70 – 1.02	0.08	0.85	0.65 – 1.11	0.24
ISS stage	1.24	1.09 – 1.41	0.001	1.45	1.21 – 1.74	<0.001
WHO PF	1.25	1.11 – 1.42	<0.001	1.52	1.29 – 1.79	<0.001
IgA*	1.59	1.19 – 2.13	0.002	1.81	1.19 – 2.75	0.006
IgG†	1.33	1.03 – 1.72	0.03	1.54	1.07 – 2.23	0.02
Salmon&Durie stage 3	1.02	0.79 – 1.33	0.86	1.02	0.70 – 1.48	0.93
LDH > ULN	1.28	1.01 – 1.63	0.04	1.68	1.24 – 2.29	0.001
del(13/13q14)	1.39	1.13 – 1.70	0.002	1.70	1.29 – 2.23	<0.001
SG (2 HDM policy)	0.87	0.73 – 1.05	0.15	0.75	0.57 – 0.97	0.03

Abbreviations: ISS, International Staging System; WHO PF, World Health Organization Performance Status; LDH, Serum lactate dehydrogenase; SG, Study Group; PFS, Progression-free Survival; OS, Overall Survival.

* M-protein is IgA, as compared to no IgA (= IgG, IgD, light-chain disease (LCD) and other)

† M-protein is IgG, as compared to no IgG (= IgA, IgD, LCD and other)

Safety

The safety profiles and most common toxicities are listed in Table 5. Peripheral neuropathy (PNP) occurred more often in the PAD arm. Within the first year of treatment, PNP grade 2–4 was reported in 18% (VAD) and 40% of patients (PAD) (HR = 1.50; 95% CI = 1.20–1.88; P < 0.0001). In addition, newly developed grade 3-4 PNP occurred in 8% of patients during thalidomide maintenance and 5% during bortezomib maintenance. In 82/270 (30%) patients on thalidomide maintenance, toxicity was a reason to stop treatment prematurely as compared to 26/229 (11%) patients on bortezomib maintenance (P < 0.001). The most frequent toxicities occurring during maintenance are listed in Table 5.

Secondary analyses

In order to explore a possible differential effect of bortezomib treatment in any of the subgroups, the effect of treatment was estimated separately by HRs for PFS and OS, with associated 95% CIs combined with tests for interaction. The interactions were especially significant for serum creatinine, FISH del(13q) and FISH del(17p). Results of these subgroups will be presented in detail.[36]

In patients with del(17p), both PFS (median PFS:12 vs. 22 months; HR = 0.47, 95% CI = 0.26–0.86, p=0.01) (Figure 5E) and OS (median OS:24 vs. 54 months; HR = 0.36, 95% CI = 0.18–0.74, p = 0.003) (Figure 5F) were significantly better in the PAD arm. In patients

without del(17p) OS was identical in both treatment arms (HR = 0.96, 95% CI = 0.69–1.34, p = 0.81). An extensive analysis of all FISH abnormalities has been published separately.[36]

Because the 2 HDM/ASCT policy was statistically significant in the multivariate analysis for OS, it was decided also to analyze some of the outcomes for HOVON (1 HDM/ASCT) and GMMG (2 HDM/ASCT) separately. In the HOVON patients, the nCR/CR rate after maintenance was 29% in VAD and 47% in PAD (OR = 2.21, 95% CI = 1.49–3.30, P < 0.001). PFS was median 24 vs. 32 months (HR = 0.70, 95% CI = 0.55–0.91, P = 0.006). OS was 55% at 5 years in both arms (HR = 0.87, 95% CI = 0.62–1.20, P = 0.39). In the GMMG patients, the nCR/CR rate after maintenance was 39% in VAD and 51% in PAD (OR = 1.58, 95% CI = 1.06–2.35, P = 0.03). PFS was median 31 vs. 36 months (HR = 0.80, 95% CI = 0.62–1.04, P = 0.09). OS was 54 vs. 70% at 5 years (HR = 0.69, 95% CI = 0.46–1.04, P = 0.07).

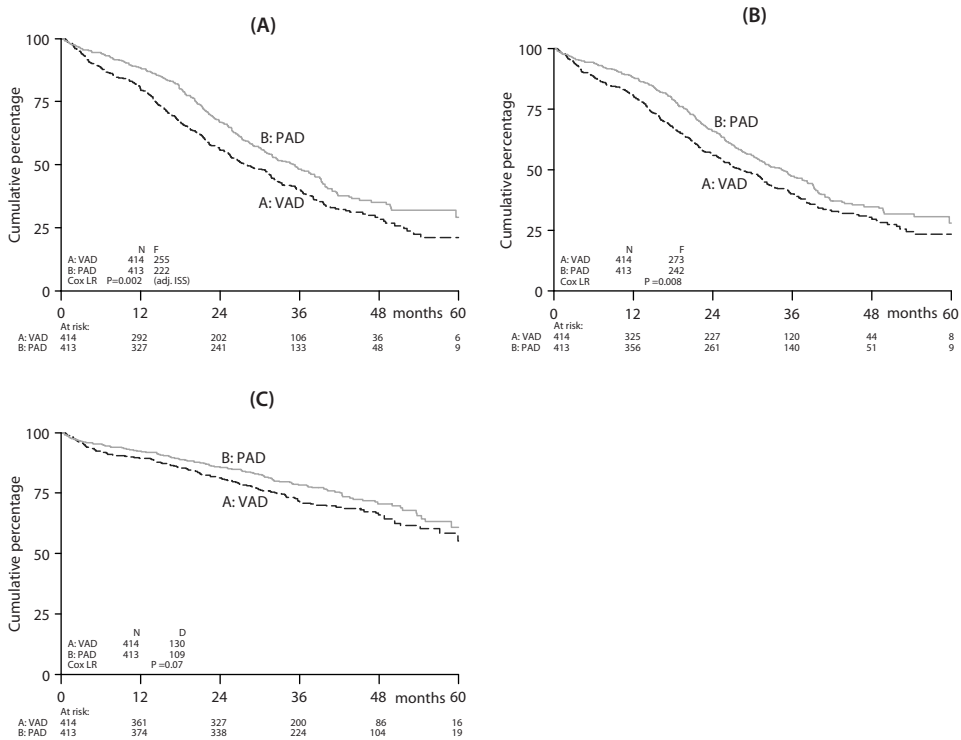

Figure 2. Kaplan-Meier survival curves among patients with multiple myeloma, according to randomized treatment. **(A)**: Progression free survival (censored at AlloSCT); **(B)**: Progression free survival (not censored at AlloSCT); **(C)**: Overall survival. N denotes number of patients; F, number of failures (i.e. progression, relapse or death); and D, number of deaths.

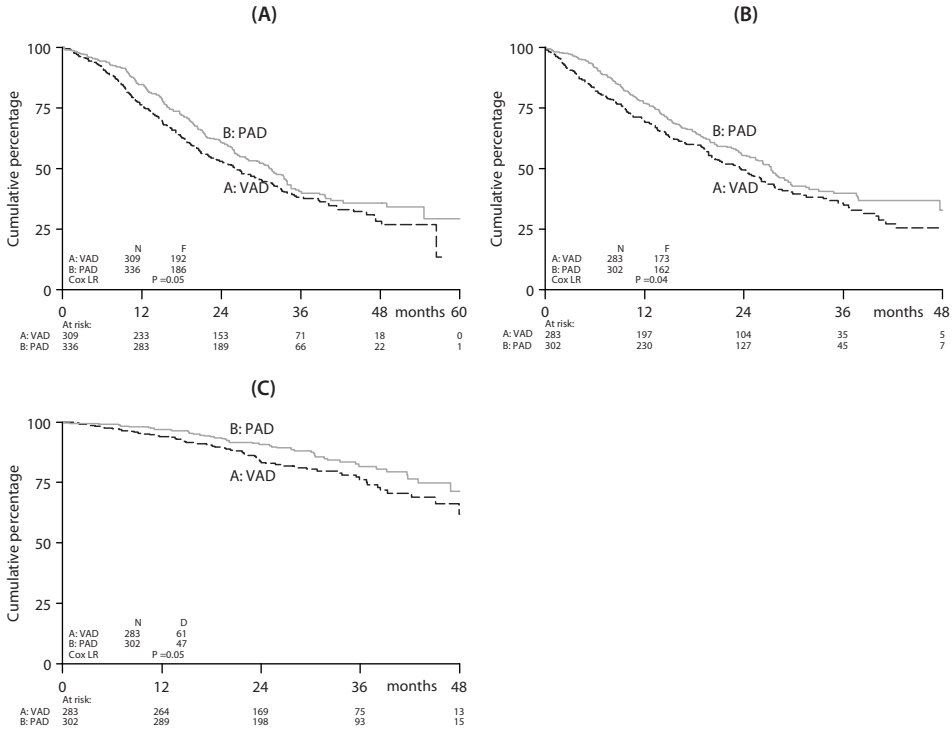

Figure 3. Landmark analysis of progression-free survival (PFS; censored at allogeneic stem-cell transplantation) and overall survival among 585 patients (283 in the vincristine, doxorubicin, and dexamethasone [VAD] arm and 302 in the bortezomib, doxorubicin, and dexamethasone [PAD] arm) with multiple myeloma who had received at least one treatment with high-dose melphalan and who were still progression free at 12 months, according to treatment arm. (A) PFS from last high-dose melphalan; (B) PFS (censored at allogeneic stem-cell transplantation); (C) overall survival. Cox LR, Cox logistic regression; D, number of deaths; F, number of treatment failures (ie, progression, relapse, or death). survival. Cox LR, Cox logistic regression; D, number of deaths; F, number of treatment failures (ie, progression, relapse, or death).

Bortezomib resulted in a superior outcome in patients with elevated serum creatinine. In these patients both median PFS (13 vs. 30 months; HR = 0.45, 95% CI = 0.26–0.78, p = 0.004) and OS (21 vs. 54 months; HR = 0.33, 95% CI = 0.16–0.65, p < 0.001) dramatically improved with bortezomib as compared to VAD/thalidomide (Fig 4). In patients with normal serum creatinine, PFS (median 31 vs. 35 months; HR = 0.80, 95% CI = 0.66–0.97, p = 0.02) remained superior in the PAD arm, but OS was very similar between both arms (59 vs. 62% at 5 years; HR = 0.94, 95% CI = 0.71–1.25, p = 0.94).

Subgroup analyses were also performed for FISH abnormalities, i.e., del(13q), t(4;14) and del(17p). The numbers of patients tested per FISH probe are listed in Table 1. In these

analyses patients with abnormal FISH were compared with all patients without the specific abnormality. In patients with del(13q), a negative impact on PFS was observed in both treatment arms (Figure 5A). OS in patients with this deletion was similar to no del(13q) in the PAD arm and significantly better than VAD (median OS:49 vs. 59 months; HR = 0.60, 95% CI = 0.42–0.87, p = 0.007; Figure 5B). t(4;14) was associated with worse PFS (HR = 1.76, 95% CI = 1.32–2.36, p < 0.001) and OS (HR = 2.13, 95% CI = 1.45–3.15, p < 0.001). Although PAD achieved better results in patients with t(4;14), this did not reach statistical significance (Figure 5C+D).

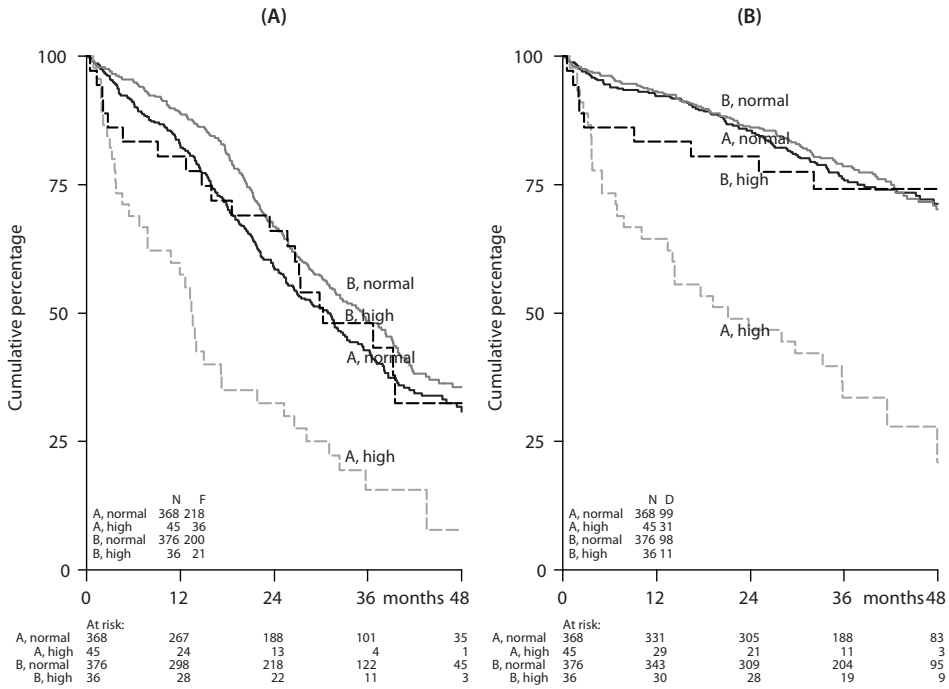

Figure 4. Kaplan-Meier survival curves of progression free survival (PFS) and overall survival (OS) according to treatment arm within subgroups according to creatinine level at presentation. (A) PFS in patients with creatinine ≤ 2 mg/dL (VAD:blue, PAD:green) or > 2 mg/dL (VAD:red, PAD:black) (B) OS in patients with creatinine ≤ 2 mg/dL (VAD:blue, PAD:green) or > 2 mg/dL (VAD:red, PAD:black) (see page 248 for colour figure).

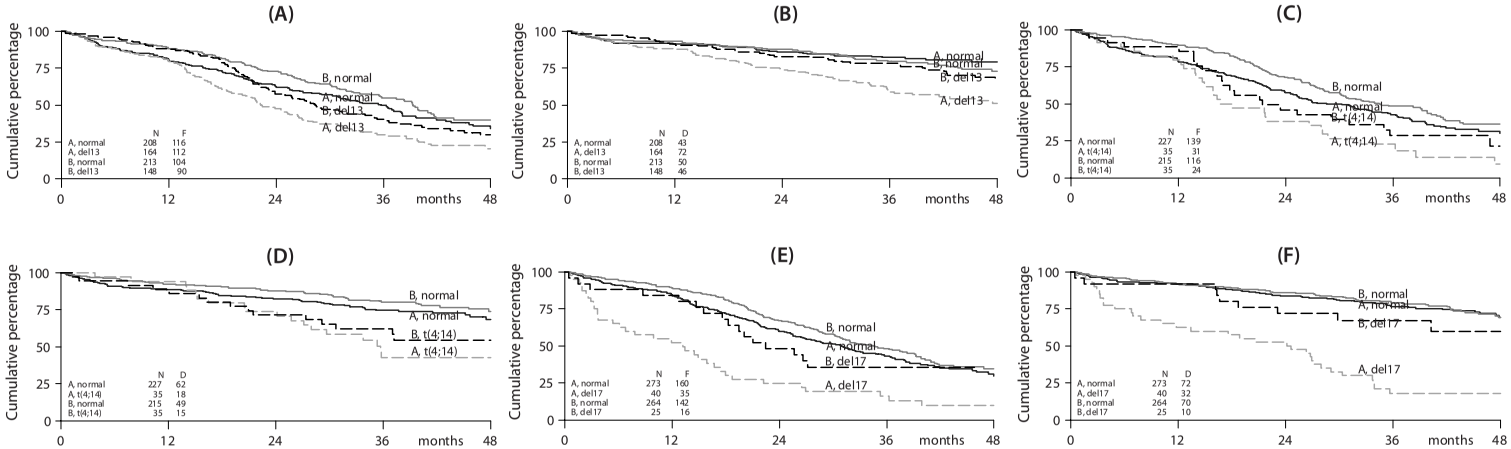

Figure 5. Kaplan-Meier survival curves of progression free survival (PFS) and overall survival (OS) according to treatment arm within subgroups according to del(13/13q) or t(4;14) or according to del(17p). In all figures patients without data regarding the specific abnormality have been excluded. **(A)** PFS in patients without del(13q) (VAD:blue, PAD:green) or with del(13q) (VAD:red, PAD:black) **(B)** OS in patients without del(13q) (VAD:blue, PAD:green) or with del(13q) (VAD:red, PAD:black) **(C)** PFS in patients without t(4;14) (VAD:blue, PAD:green) or with t(4;14) (VAD:red, PAD:black) **(D)** OS in patients without t(4;14) (VAD:blue, PAD:green) or with t(4;14) (VAD:red, PAD:black) **(E)** PFS in patients without del(17p) (VAD:blue, PAD:green) or with del(17p) (VAD:red, PAD:black) **(F)** OS in patients without del(17p) (VAD:blue, PAD:green) or with del(17p) (VAD:red, PAD:black) (see page 249 for colour figure).

Table 5. Safety profile and toxicities.

	VAD (N = 414)				**PAD (N = 413)**			
	VAD Induction (N = 411)		Thalidomide Maintenance (N = 270)		PAD Induction (N = 410)		Bortezomib Maintenance (N = 229)	
Any AE – no. of patients (%)	401	(98)	260	(96)	400	(98)	222	(97)
AE grade 3–4 – no. (%)	220	(54)	123	(46)	258	(**63**)†	110	(48)
AE classified as SAE – no. (%)	148	(36)	61	(23)	187	(**46**)†	77	(**34**)†
AE leading to discontinuation, dose reduction or delay of Bortezomib – no. (%)	n.a.	n.a.	n.a.	n.a.	112	(27)	81	(35)
Death from AE – no. (%)	9	(2)	0	(0)	7	(2)	0	(0)
	All grades	Grade 3–4	All grades	Grade 3–4	All grades	Grade 3–4	All grades	Grade 3–4
Hematological toxicities - (%)								
Anemia	24	7	15	1	28	8	**27**†	1
Neutropenia	2	1	4	1	4	3	2	0
Thrombocytopenia	18	5	19	2	**39**$	**10***	**37**$	4
Infections	49	21	61	18	56	26	**75**†	24
Herpes Zoster	0	0	1	0	**2***	0	2	0
Non-hematological toxicities – (%)								
Wasting, Fatigue	28	4	20	1	27	4	20	1
GI symptoms	59	7	40	4	**67***	**11***	48	5
Cardiac disorders	24	5	13	2	27	8	19	3
Thrombosis	5	3	1	1	6	4	1	1
Peripheral Neuropathy – (%)	26	10	**53**$	8	**37**$	**24**$	33	5

Abbreviations: AE, adverse event (including infection); SAE, serious adverse event
Bold numbers indicate a statistically significant higher proportion compared to the other arm
* $P < 0.05$
† $P < 0.01$
$ $P < 0.001$

DISCUSSION

This randomized multicenter trial in patients with MM who were eligible for high-dose therapy demonstrates that bortezomib during induction and maintenance treatment results in a significantly better response, quality of response, PFS, and in a multivariate analysis also OS. In this trial no randomization for maintenance was performed, and consequently this cannot be independently assessed. Instead, the hypothesis was tested whether bortezomib before and after high-dose therapy may result in prolonged PFS. Maintenance treatment with bortezomib for 2 years was much better tolerated than thalidomide maintenance, with less patients stopping prematurely. Bortezomib maintenance significantly improved

nCR+CR rate, from 31% to 49%. A landmark analysis indicated that patients with at least nCR had a better PFS and OS. The subgroup analyses show that the superior outcome with bortezomib is predominantly accomplished in patients with high-risk disease, i.e. myeloma-related renal failure and del(17p). Patients without the high-risk characteristics seem to have less difference of PFS between the treatment arms. In addition, OS was better in the GMMG group, which performed standard double HDM with ASCT in 69% of patients in contrast to HOVON (single HDM). Previous trials have not clearly demonstrated a survival benefit of double versus single HDM[37,38]. Since the trial was not powered to compare single versus double HDM, the question remains open.

Other studies have explored induction treatment with bortezomib in 2 or 3 drug combinations such as bortezomib, dexamethasone (BD), with cyclophosphamide (VCD), thalidomide (VTD), or lenalidomide (VRD) in newly diagnosed patients. All observed a higher VGPR or CR rate and/or an improvement of PFS but not OS as compared with standard treatment.[25,39-42] The IFM trial was not designed to evaluate OS.[39] In the GIMEMA and UAMS trials no difference of OS was achieved, respectively.[25,40] In Total Therapy 3 bortezomib was given from induction through consolidation and maintenance, resulting in a 5-year OS of 72%.[43] A phase 2 trial by the French IFM group demonstrated the safety and the impact of bortezomib early after HDM conditioning. [39] Recently, bortezomib consolidation after high-dose melphalan was investigated.[26] Monotherapy of 20 injections over 21 weeks was well tolerated (mean total dose 82%), increased ≥ VGPR rate from 39% to 70%, and resulted in a longer median PFS from 20 to 27 months.

The Spanish Pethema group used VTD induction without consolidation, showing a post-ASCT CR rate of 46%.[44] In GIMEMA, VTD induction and consolidation with high-dose melphalan resulted in an improved CR rate from 19% after induction to 42% after second ASCT and 49% after consolidation.[25] These results indicate that post-ASCT consolidation with bortezomib may increase CR or VGPR. Maintenance treatment with bortezomib, as performed in the current trial may offer the same CR/nCR and an improved PFS and OS.

Bortezomib-emergent peripheral neuropathy (BiPN) was the prevalent toxicity during induction, preventing a substantial number of patients from starting maintenance. In those who started maintenance, BiPN grade 3–4 occurred in 5%. More patients were unable to complete thalidomide maintenance treatment. Hence, the lower percentage of failures in the bortezomib arm may have contributed to the better PFS/OS in that group. Prolonged administration of bortezomib in the two-weekly schedule seems feasible, and therefore it is important to prevent BiPN during the induction phase, enabling patients to continue into maintenance. The tolerability of the two-weekly schedule is in line with the reported weekly schedule.[4] In addition, subcutaneous administration of bortezomib may further improve tolerability.[45] Recently, a significant prolongation of PFS and/or OS was demonstrated with lenalidomide maintenance after high-dose therapy.[23,24] Future trials will address the optimal choice for maintenance treatment.

Our subgroup analyses revealed that bortezomib was superior for CR/nCR and for PFS and OS in patients presenting with renal insufficiency, almost to the level of patients without this organ failure. While the safety and efficacy of bortezomib in renal failure has been observed in phase 2 studies,[46-48] this large trial prospectively defines the benefit of bortezomib in this high-risk group of patients.

The previously reported better outcome with bortezomib of patients with t(4;14) was not confirmed. Although bortezomib treatment resulted in better PFS and OS, the outcome remained inferior compared to patients without the abnormality in both treatment arms. Bortezomib significantly improved PFS and OS in patients with del(13q) and more importantly in del(17p), identifying a potential effective treatment option in this high-risk group. In the recent trial of the French IFM group, using bortezomib plus dexamethasone for induction, no improvement of PFS was observed in patients with del(17p), indicating that induction treatment with bortezomib may not be sufficient and that longer treatment may be required for this better outcome.[17] As a plateau in PFS and OS is not yet observed, long-term follow-up is warranted. In conclusion, bortezomib used during induction and maintenance improves response, PFS and OS in patients with MM.

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CHAPTER 3

GENE EXPRESSION PROFILING FOR MOLECULAR CLASSIFICATION OF MULTIPLE MYELOMA IN NEWLY DIAGNOSED PATIENTS

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ABSTRACT

To identify molecularly defined subgroups in multiple myeloma, gene expression profiling was performed on purified CD138$^+$ plasma cells of 320 newly diagnosed myeloma patients included in the Dutch-Belgian/German HOVON-65/GMMG-HD4 trial. Hierarchical clustering identified 10 subgroups; 6 corresponded to clusters described in the University of Arkansas for Medical Science (UAMS) classification, CD-1 (n = 13, 4.1%), CD-2 (n = 34, 10.6%), MF (n = 32, 10.0%), MS (n = 33, 10.3%), proliferation-associated genes (n = 15, 4.7%), and hyperdiploid (n = 77, 24.1%). Moreover, the UAMS low percentage of bone disease cluster was identified as a subcluster of the MF cluster (n = 15, 4.7%). One subgroup (n = 39, 12.2%) showed a myeloid signature. Three novel subgroups were defined, including a subgroup of 37 patients (11.6%) characterized by high expression of genes involved in the nuclear factor kappa light-chain-enhancer of activated B cells pathway, which include *TNFAIP3* and *CD40*. Another subgroup of 22 patients (6.9%) was characterized by distinct overexpression of cancer testis antigens without overexpression of proliferation genes. The third novel cluster of 9 patients (2.8%) showed up-regulation of protein tyrosine phosphatases *PRL-3* and *PTPRZ1* as well as *SOCS3*. To conclude, in addition to 7 clusters described in the UAMS classification, we identified 3 novel subsets of multiple myeloma that may represent unique diagnostic entities.

INTRODUCTION

Multiple myeloma (MM), a disease characterized by the accumulation of terminally differentiated antibody-secreting plasma cells (PCs), is an incurable malignancy with a median overall survival of 3 to 4 years. Disease sequelae include immunodeficiency, anemia, hypercalcemia, renal failure, and lytic bone lesions.[1]

On the basis of (cyto) genetics, myeloma can roughly be divided in nonhyperdiploid and hyperdiploid myeloma. Nonhyperdiploid myeloma is present in 40% of cases and is characterized by recurrent translocations involving the immunoglobulin heavy chain gene at 14q32, resulting in transcriptional activation of *CCND1*, *CCND3*, *MAF*, *MAFB*, or *FGFR3/MMSET*. Hyperdiploid myeloma is characterized by trisomies of multiple odd chromosomes (3, 5, 7, 9, 11, 15, 19, and 21).[2-4] Together with t(11;14), hyperdiploidy confers a relatively favorable prognosis, whereas *MAF*, *MAFB*, or *FGFR3/MMSET* activation and deletion of chromosome 13 and/or 17 are associated with a poor prognosis.[5-10]

Several groups have reported gene expression profiles determined by RNA microarray technology in patients with newly diagnosed MM.[11-16] Two major genetic classification systems have been developed, the translocation and cyclin D (TC) classification and the University of Arkansas for Medical Sciences (UAMS) molecular classification of myeloma. The TC classification distinguishes 8 subgroups on the basis of overexpression of genes deregulated by primary immunoglobulin H translocations and transcriptional activation of cyclin D genes.[2] Use of the UAMS molecular classification of myeloma led to the identification of 7 tumor groups characterized by distinct gene expression profiles, including translocation clusters MS [t(4;14)], MF [t(14;16)/t(14;20)], and CD-1/2 [(t(11;14) and t(6;14)], as well as a hyperdiploid cluster (HY), a cluster with proliferation-associated genes (PR), and a cluster mainly characterized by a low percentage of bone disease (LB).[15] Here, we report the hierarchical clustering determined by gene expression profiles in 320 primarily white, Northern European patients with newly diagnosed MM included in a multicenter phase 3 trial.

METHODS

Patients

Bone marrow PC samples were obtained from newly diagnosed patients with MM who were included in a large multicenter, prospective, randomized phase 3 trial (Dutch-Belgian Cooperative Trial Group for Hemato-Oncology [HOVON-65]/GMMG-HD4), trial EudraCT Nr 2004-000944-26. This trial included patients with Salmon & Durie stage II or III who were 18 to 65 years of age. Patients with amyloidosis or monoclonal gammopathy of undetermined significance were excluded. Informed consent to treatment protocols and sample

procurement was obtained for all cases included in this study, in accordance with the Declaration of Helsinki. Use of diagnostic tumor material was approved by the institutional review board of Erasmus MC.

Myeloma cell purification and RNA isolation

PC purification of bone marrow samples from included patients was performed in 11 centers in The Netherlands, Germany, and Belgium that were equipped to perform PC purification. PCs were separated by the use of positive magnetic cell sorting selection with CD138 magnetic microbeads (Miltenyi Biotec B.V.). Next, purified samples were analyzed for purity and viability by flow cytometric analysis (FACSCalibur and CellQuest Software; BD Biosciences) with CD138-PE (Beckman Coulter), annexin-fluorescein isothiocyanate (NeXins Research), and 7-amino-actinomycin D (Beckman Coulter). Protocols for PC purification and fluorescence-activated cell sorting analysis were equal in all centers. Purified PCs were stored in RLT buffer at -80°C until collection. RNA isolation was performed at the Erasmus Medical Center and at the University of Heidelberg. Only samples with a monoclonal PC purity greater than 80% were used for analysis. RNA was isolated from purified PCs by the use of a DNA/RNA prep kit (QIAGEN). RNA concentration was measured by use of the NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA quality and purity was assessed by use of the RNA 6000 pico or nano assay (Agilent 2100 Bioanalyzer; Agilent Technologies).

Gene expression profiling and array analysis

RNA processing, target labeling, and hybridization to gene expression arrays were performed exclusively in the Erasmus Medical Center. Biotin-labeled cRNA was obtained by the use of the 2-Cycle Eukaryotic Target Labeling Assay (Affymetrix). A total of 15 µg of fragmented, biotin-labeled cRNA was hybridized to Affymetrix GeneChip Human Genome U133 plus 2.0 arrays according to standard Affymetrix protocol (Affymetrix Inc).

Quality controls of arrays that used GeneChip Operating Software included scaling factor and percentage of genes present. Arrays with a scaling factor difference of less than 3 and more than 20% genes present were analyzed further. Raw data from selected gene expression arrays (CEL-files) were preprocessed by the use of GCRMA in Partek Genomics Suite, version 6.4 (Partek). Final quality control of arrays included relative log expression and normalized unscaled standard errors (NUSEs) from the AffyPLM package (http://www.bioconductor.org). Arrays showing a NUSE value greater than 1.05 and aberrant relative log expression plots were excluded from analysis. Microarray data presented in this work have been deposited in the Gene Expression Omnibus (National Center for Biotechnology Information) and are accessible through GEO Series accession number GSE19784 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19784).

Cluster analysis

GCRMA-normalized expression data were imported in Omniviz software version 6.1 (BioWisdom). In Omniviz, the exponential values were taken of the GCRMA-derived log2 intensity values, and because GeneChips do not reliably discriminate between values less than 30, all intensity values less than 30 were set to 30. The level of expression for every probe set was determined relative to the geometric mean and log transformed (base 2). The 5% (2730) most variable probe sets from the total were selected by the use of a cut-off of log2 geometric mean less than -5.12 or more than 5.12 (reflecting up- or down-regulation) in at least one patient (supplemental Table 1). Hierarchical clustering of average linkage with the centered correlation metric was performed by the use of BRB-array tools version 3.6.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html). The dendrogram obtained was compared with translocation status, and robustness (R) indices (BRB-array tools) were calculated to give an indication concerning the reproducibility of the clusters. To determine expression signature of clusters, each cluster was compared with the remaining clusters by use of the Class Comparison option with the following settings: *P* less than .001 and false discovery rate less than 5% (BRB-array tools).

Prediction analysis of micro-arrays

To validate clusters, a method of nearest-shrunken centroid classification that uses prediction analysis of microarrays in R version 2.6.0 (PAMr package in R Version 2.6.0) was used.[17] Validation of clusters was performed in an independent dataset, GSE2658 [NCBI GEO], generated by the UAMS, which included 559 newly diagnosed MM patients. The dataset containing the 5% most variable genes, 2730 genes, was used (supplemental Table 1). Sensitivity (Sn), specificity (Sp), positive predictive values, and negative predictive values were calculated.

In addition, validation analysis to confirm all identified clusters was performed by use of the CEL files of 2 independent datasets, the APEX/SUMMIT/CREST dataset,[18] and the UAMS dataset.[15] CEL files were normalized by the use of our normalization methods, sample, and gene selection criteria as described.

An extensive description of the method of prediction analysis of translocations t(4;14), t(11;14), and t(14;16)/t(14;20) is outlined in the supplemental data (Document 1). In brief, samples with available fluorescence in situ hybridization (FISH) data were randomly divided in a training set (2/3) and a test set (1/3). Training set and test set were separately normalized. For the training set, the 5% most variable genes, 2730 genes, were generated by the use of the method described previously (supplemental Table 2). These 2730 probe sets were subsequently used in the test set. Percentage correctly classified samples, Sn, Sp, positive predictive value, and negative predictive value were calculated.

Cytogenetic analysis and FISH

FISH analysis was performed in 304 patients. In addition, karyotyping data were available for 119 patients. In nonpurified PC samples (n = 125) at least 200 interphase nuclei per sample were analyzed by the use of epi-fluorescence microscopy and image analysis software, with in several cases a preceding analysis of selected myeloma cells determined by light chain counterstaining or morphology. In CD138-purified PC samples (n = 179), 100 nuclei were evaluated by the use of an epifluorescence microscope (Leica Microsystems). Hybridization efficiency was validated on PCs obtained from bone marrow of a healthy donor; thresholds for gains, deletions, and translocations were set at 10%.

Interphase FISH analysis was performed as previously described.[6,19] Detection of numerical changes was performed by the use of commercial 2-color probes chromosome loci 1q21/8p21, 11q23/13q14, 9q34/22q11, 6q21/15q22, and17p13/19q13 (Poseidon Probes; Kreatech) or by the use of alpha satellite probes for centromere regions of chromosome 9 and 11 (CEP 9 and CEP 11; Vysis; Abbott Molecular). The combination of trisomies 9, 11, and 15 was found to be predictive of hyperdiploidy.[8] Hyperdiploid MM was defined by presence of trisomy of 2 of these chromosomes (trisomy 9 and 11, 11 and 15, or 9 and 15) or all of them (trisomy of chromosomes 9, 11, and 15), as determined by FISH and/or karyotyping data.

Translocations t(11;14)(q13;q32), t(4;14)(p16;q32), and (14;16)(q32;q23) were determined by the use of LSI IGH/CCND1, LSI IGH/FGFR3, and LSI IGH/MAF probes, respectively (Vysis; Abbott Molecular) or commercial 2-color probe sets for detection of translocations t(11;14)(q13;q32), t(4;14)(p16;q32) (both Poseidon Probes; Kreatech) and t(14;16) (q32;q23) (Vysis). A t(14;20)(q32;q12) with 14q32 IGH gene rearrangement was confirmed by FISH by the use of 14q32 immunoglobulin H rearrangement probe, LSI IGH DC, and whole chromosome paint 14 and 20 probes, wcp14 and wcp 20 (Vysis; Abbott Molecular). Conventional karyotyping was performed as described previously.[20]

RESULTS

Identification of expression signatures

A total of 320 bone marrow aspirates from newly diagnosed patients were obtained upon inclusion in the HOVON-65/GMMG-HD4 trial for gene expression profiling. Comparison of baseline clinical characteristics of this subset of patients showed no significant difference between characterized subset and the whole patient group in the trial (supplemental Table 3). The sample clustering presented by a dendrogram with 5 major branches and 11 clusters is shown in Figure 1. Translocation status and robustness (R) indices per cluster are shown in supplemental Table 4. The top 10 genes ($P < .001$, false discovery rate < 5%) showing the greatest fold change per cluster in comparison with remaining clusters are shown in

Tables 1 and 2, and the top 50 genes are shown in supplemental Table 5. Of the 11 clusters found, 10 were characterized in detail. The remaining cluster, consisting of 9 samples with 41 differentially expressed genes, was excluded from analysis because no clear signature could be determined. Six of the identified clusters corresponded well to the published UAMS classification and were therefore named accordingly.[15]

Samples harboring t(11;14) and/or overexpression of *CCND1* were divided into 2 clusters, CD-1 and CD-2. A relatively low frequency of t(11;14) (33%) was found in the CD-1 cluster in our study, which is low compared with previous reports.[15] Still, this cluster was characterized by high *CCND1* expression and by overexpression of argininosuccinate synthetase 1 *ASS1*, inhibin beta E *INHBE*, and nidogen 2 *NID2* as has been described previously. B-cell markers *MS4A1 (CD20)*, *VPREB3*, *CD79A*, and *BANK1* defined cluster CD-2 (Table 1; supplemental Table 5). *CD20* expression has been associated with presence of t(11;14),[21] which is consistent with the high percentage of t(11;14) observed in this cluster in comparison to cluster CD-1.

The MS cluster was characterized by translocation t(4;14), deregulating *FGFR3* and *MMSET*, present in 96% of patients in this cluster. Other notable overexpressed genes include desmoglein *DSG2*, *CCND2*, selectin L (lymphocyte adhesion molecule 1) *SELL*, and serpin peptidase inhibitors *SERPINE2* and *SERPINI1* (Table 1; supplemental Table 5). This cluster showed a significantly greater percentage of patients with 1q21 amplification (61%, compared with 8% to 50% in the remaining clusters; $P < .001$; Figure 2).

The MF cluster contained 32 samples, of which 7 harbored a confirmed t(14;16) or t(14;20). *c-MAF*, which is deregulated by t(14;16), and *MAFB*, deregulated by t(14;20), were observed only in a subset of patients, which clustered separately within this MF cluster. The remaining samples in the MF cluster clustered with these samples on the basis of overexpression of downstream targets of *MAFB* and/or *c-MAF*: *RND3*, *CCND2*, and *ITGB7* (supplemental Figure 1). [22] *FRZB* and *DKK1*, both WNT inhibitors of which the presence is associated with osteolytic lesions in myeloma patients, were among the top down-regulated genes (Table 1; supplemental Table 5).[23,24] Our analysis of both subsets separately revealed an even stronger signature for the MF subcluster (supplemental Table 6). Clinical features such as elevated lactate dehydrogenase and thrombocytopenia were predominantly present in the MF subcluster and significantly greater in comparison with the remaining clusters, 47% versus 0% to 46% ($P = .01$) and 35% versus 0% to 21% ($P < .001$; supplemental Table 7). The remaining subset of 15 samples lacking translocations and clustering together only on the basis of downstream targets showed a gene signature with the top overexpressing genes corresponding to those overexpressed in the UAMS LB cluster, *CST6*, specific for the UAMS LB cluster, as well as *RASGRP1* and *PHACTR3* (supplemental Table 6). The MF cluster showed the lowest percentage of patients with bone lesions, 52% versus 62% to 100% in the remaining clusters ($P = .004$). This percentage was even lower in the LB subcluster, 50% versus 53% to 100% ($P = .04$; supplemental Table 7).

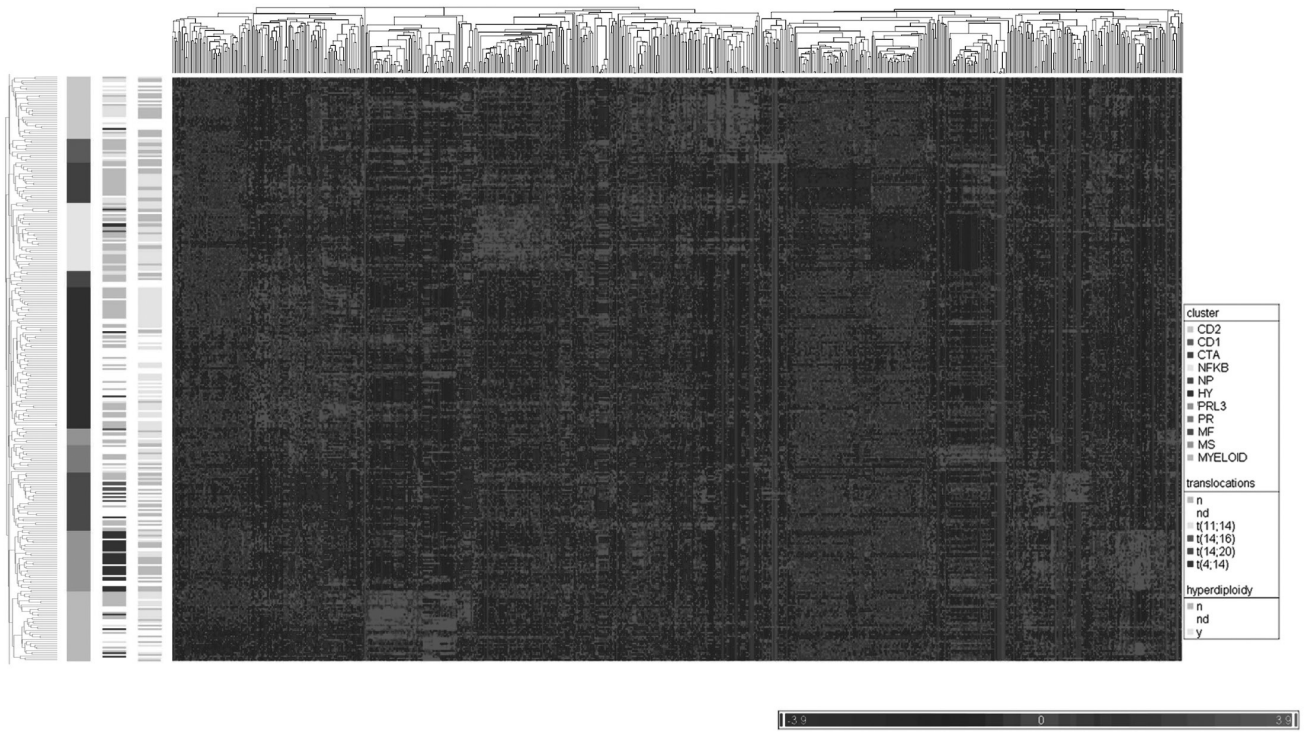

Figure 1. Dendrogram and heatmap. Vertical dendrogram shows sample clustering with 5 major branches and 11 distinct clusters; the dendrogram is cut at 11 clusters. First column,11 clusters: CD2, CD-2 cluster; CD1, CD-1 cluster; CTA, CTA cluster; NF-κB, NF-κB cluster; NP, no clear profile; HY, HY cluster; PRL3, PRL3 cluster; PR, PR cluster; MF, MF cluster; MS, MS cluster; Myeloid, Myeloid cluster. Translocations are shown in the second column: t(11;14), yellow; t(4;14), blue; t(14;16) or t(14;20), red; no translocation, green; and not determined, white. The third column indicates hyperdiploidy: y, hyperdiploidy (yellow); n, no hyperdiploidy (green) and nd (not determined; white). Horizontal dendrogram shows clustering of genes. The heatmap shows the spectrum of expression values, the log2 expression value from the geometric mean for each gene is indicated by a color, with red representing positive expression (up-regulation) and blue representing negative expression (down-regulation) of a gene (see page 250 for colour figure).

Table 1. Top 10 fold up-regulated genes.

Cluster/ probe set	Gene symbol	Fold change
CD-2		
208711_s_at	*CCND1*	78.43
228592_at	*MS4A1*	52.20
208712_at	*CCND1*	34.84
223823_at	*KCNMB2*	21.04
235518_at	*SLC8A1*	17.57
220068_at	*VPREB3*	13.64
210356_x_at	*MS4A1*	13.49
228599_at	*MS4A1*	13.16
225842_at	*PHLDA1*	12.81
217418_x_at	*MS4A1*	11.31
CD-1		
230493_at	*SHISA2*	29.08
235278_at	*MACROD2*	17.77
210587_at	*INHBE*	13.97
218589_at	*P2RY5*	12.52
206760_s_at	*FCER2*	12.17
207076_s_at	*ASS1*	11.14
223823_at	*KCNMB2*	11.07
206759_at	*FCER2*	9.78
221911_at	*ETV1*	9.33
225285_at	*BCAT1*	8.83
CTA		
210387_at	*HIST1H2BG*	7.59
206102_at	*GINS1*	5.01
208955_at	*DUT*	4.94
238021_s_at	*hCG_1815491*	4.79
238762_at	*MTHFD2L*	4.58
206834_at	*HBB,HBD*	4.57
227212_s_at	*PHF19*	4.55
202016_at	*MEST*	4.45
203213_at	*CDC2*	4.22
238022_at	*hCG_1815491*	4.16

Cluster/ probe set	Gene symbol	Fold change
NF-κB		
214230_at	*CDC42*	19.17
202643_s_at	*TNFAIP3*	11.73
211032_at	*COBLL1*	10.87
1557257_at	*BCL10*	10.20
1559249_at	*ATXN1*	9.52
208622_s_at	*EZR*	8.62
230082_at	*LOC100133660*	8.57
1554229_at	*C5orf41*	8.05
238633_at	*EPC1*	7.96
1552542_s_at	*TAGAP7*	7.84
HY		
222943_at	*GBA3*	8.28
219954_s_at	*GBA3*	5.78
219463_at	*C20orf103*	5.15
203153_at	*IFIT1*	5.08
214329_x_at	*TNFSF10*	5.01
202687_s_at	*TNFSF10*	4.80
212843_at	*NCAM1*	4.57
205051_s_at	*KIT*	4.55
202688_at	*TNFSF10*	4.49
206609_at	*MAGEC1*	4.43
PRL3		
200953_s_at	*CCND2*	19.28
206574_s_at	*PTP4A3*	15.66
209695_at	*PTP4A3*	15.11
204469_at	*PTPRZ1*	9.73
227697_at	*SOCS3*	5.93
217865_at	*RNF130*	5.89
209183_s_at	*C10orf10*	4.40
218788_s_at	*SMYD3*	4.11
228051_at	*KIAA1244*	3.82
219195_at	*PPARGC1A*	3.69

Table 1. *Continued*

Cluster/ probe set	Gene symbol	Fold change	Cluster/ probe set	Gene symbol	Fold change
PR			**MS**		
232231_at	*RUNX2*	14.43	204379_s_at	*FGFR3*	153.02
210432_s_at	*SCN3A*	7.64	222777_s_at	*WHSC1*	45.72
206640_x_at	*[multiple gene symbols]*	7.21	200953_s_at	*CCND2*	35.57
203213_at	*CDC2*	7.04	217901_at	*DSG2*	23.53
206102_at	*GINS1*	6.80	201387_s_at	*UCHL1*	22.40
208235_x_at	*GAGE12F, GAGE12*	6.76	212190_at	*SERPINE2*	21.45
201292_at	*TOP2A*	6.74	222778_s_at	*WHSC1*	20.47
207739_s_at	*[multiple gene symbols]*	6.50	217963_s_at	*NGFRAP1*	17.83
214070_s_at	*ATP10B*	6.39	209053_s_at	*WHSC1*	16.52
201291_s_at	*TOP2A*	6.38	212771_at	*C10orf38*	16.29
MF			**Myeloid**		
200953_s_at	*CCND2*	17.72	206111_at	*[multiple gene symbols]*	12.54
212724_at	*RND3*	15.84	205033_s_at	*DEFA1, DEFA3, LOC7*	10.01
211986_at	*AHNAK*	12.55	215051_x_at	*AIF1*	9.24
200951_s_at	*CCND2*	11.03	201137_s_at	*HLA-DPB1*	8.33
226875_at	*DOCK11*	6.95	207269_at	*DEFA4*	7.95
202207_at	*ARL4C*	6.77	202917_s_at	*S100A8*	7.93
205590_at	*RASGRP1*	6.70	213975_s_at	*LYZ*	7.84
204589_at	*NUAK1*	5.95	205950_s_at	*CA1*	7.77
218935_at	*EHD3*	5.54	215193_x_at	*[multiple gene symbols]*	7.66
226961_at	*PRR15*	5.23	203645_s_at	*CD163*	7.64

Per cluster, the top 10 up-regulated genes are shown. The first column shows the cluster and probe set IDs, the second column shows the gene symbols, and the third column indicates the fold change differences of per probe set in the specific cluster versus the remaining clusters. CTA indicates cancer testis antigens; HY, hyperdiploid cluster; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; and PR, proliferation-associated genes

Six clusters were characterized by high frequencies of hyperdiploidy, ranging from 57% to 94% (supplemental Table 8). One of these clusters showed up-regulation of erythroid and myeloid markers as well as genes involved in cell-mediated immune response, humoral immune response, and antigen presentation. This cluster was indicated as the myeloid cluster. No distinct clinical features characterized this cluster, as was observed in the UAMS classification regarding the low percentage of patients having an immunoglobulin A subtype, B2M, and renal injury. However, bone marrow PC percentage before and after PC

purification was significantly lower in this cluster in comparison with the remaining clusters, 30% versus 50% ($P = .008$) and 87% versus 91% ($P < .001$), respectively. The lower level of bone marrow plasmacytosis at diagnosis also was observed in the UAMS myeloid cluster.

Table 2. Top 10 fold down-regulated genes.

Cluster/ probeset	Gene symbol	Fold change
CD-2		
219463_at	*C20orf103*	0.10
200953_s_at	*CCND2*	0.13
205159_at	*CSF2RB*	0.14
205968_at	*KCNS3*	0.15
226333_at	*IL6R*	0.15
204489_s_at	*CD44*	0.16
201387_s_at	*UCHL1*	0.18
212063_at	*CD44*	0.18
238021_s_at	*hCG_1815491*	0.19
219954_s_at	*GBA3*	0.20
CD-1		
201005_at	*CD9*	0.08
205352_at	*SERPINI1*	0.17
205239_at	*AREG, LOC727738*	0.18
222392_x_at	*PERP*	0.19
213737_x_at	*GOLGA9P*	0.26
211071_s_at	*MLLT11*	0.26
227067_x_at	*NOTCH2NL*	0.26
204647_at	*HOMER3*	0.27
208078_s_at	*SNF1LK*	0.28
CTA		
200643_at	*HDLBP*	0.07
201024_x_at	*EIF5B*	0.10
212586_at	*CAST*	0.11
203675_at	*NUCB2*	0.11
200977_s_at	*TAX1BP1*	0.12
224567_x_at	*MALAT1*	0.12
211968_s_at	*HSP90AA1*	0.12
200595_s_at	*EIF3A*	0.13
210645_s_at	*TTC3*	0.13
219221_at	*ZBTB38*	0.14

Cluster/ probeset	Gene symbol	Fold change
NF-κB		
206978_at	*CCR2 ,FLJ78302*	0.07
212731_at	*ANKRD46*	0.07
200768_s_at	*MAT2A*	0.10
231576_at		0.11
202797_at	*SACM1L*	0.12
209296_at	*PPM1B*	0.12
213005_s_at	*KANK1*	0.13
201503_at	*G3BP1*	0.14
201664_at	*SMC4*	0.15
201098_at	*COPB2*	0.19
HY		
200953_s_at	*CCND2*	0.12
219799_s_at	*DHRS9*	0.18
203186_s_at	*S100A4*	0.19
201029_s_at	*CD99*	0.19
225673_at	*MYADM*	0.20
201666_at	*TIMP1*	0.22
224009_x_at	*DHRS9*	0.22
205229_s_at	*COCH*	0.23
200951_s_at	*CCND2*	0.23
223952_x_at	*DHRS9*	0.24
PRL3		
201721_s_at	*LAPTM5*	0.04
212063_at	*CD44*	0.04
204489_s_at	*CD44*	0.08
216438_s_at	*TMSB4X,TMSL3*	0.16
208892_s_at	*DUSP6*	0.19
208690_s_at	*PDLIM1*	0.20
225282_at	*SMAP2*	0.28
201432_at	*CAT*	0.34
201300_s_at	*PRNP*	0.40

Table 2. *Continued*

Cluster/ probeset	Gene symbol	Fold change
PR		
215001_s_at	*GLUL*	0.10
206150_at	*CD27*	0.11
209201_x_at	*CXCR4*	0.13
235400_at	*FCRLA*	0.13
211919_s_at	*CXCR4*	0.14
205671_s_at	*HLA-DOB*	0.15
209619_at	*CD74*	0.15
228284_at	*TLE1*	0.15
210889_s_at	*FCGR2B*	0.16
235372_at	*FCRLA*	0.16
MF		
204602_at	*DKK1*	0.05
226702_at	*CMPK2*	0.06
202391_at	*BASP1*	0.10
203698_s_at	*FRZB*	0.11
216576_x_at	*IGKC, IGKV1-5, LOC647506*	0.12
215176_x_at	*LOC100130100*	0.12
202688_at	*TNFSF10*	0.13
225681_at	*CTHRC1*	0.13
203697_at	*FRZB*	0.13
242625_at	*RSAD2*	0.14
MS		
208712_at	*CCND1*	0.12
203698_s_at	*FRZB*	0.19
203697_at	*FRZB*	0.19
228592_at	*MS4A1*	0.20
201721_s_at	*LAPTM5*	0.20
221969_at		0.21
206609_at	*MAGEC1*	0.22
204794_at	*DUSP2*	0.23
226068_at	*SYK*	0.23
243780_at		0.25
Myeloid		
200730_s_at	*PTP4A1*	0.42
218826_at	*SLC35F2*	0.43
210942_s_at	*ST3GAL6*	0.47
227189_at	*CPNE5*	0.48
206445_s_at	*PRMT1*	0.50
214359_s_at	*HSP90AB1*	0.51
209457_at	*DUSP5*	0.51
204790_at	*SMAD7*	0.51
211967_at	*TMEM123*	0.52
226612_at	*FLJ25076*	0.52

Per cluster, the top 10 down-regulated genes are shown. The first column shows the cluster and probe set IDs, the second column shows the gene symbols, and the third column indicates the fold change differences of per probe set in the specific cluster versus the remaining clusters. CTA indicates cancer testis antigens; HY, hyperdiploid cluster; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; and PR, proliferation-associated genes.

The HY cluster showed hyperdiploidy in 94% of cases. This group was characterized by up-regulation of death receptor *TNFSF10* (*TRAIL*); interferon-induced genes such as *IFIT1*, *IFIT3*, and *IFI27*; WNT antagonists *FRZB* and *DKK1*; glucosidase; beta; acid 3 (cytosolic) *GBA3*; and *MYC* proto-oncogene.

Two predominantly hyperdiploid clusters showed up-regulation of cancer testis antigens (CTA; supplemental Table 8). These include *MAGEA3*, *MAGEA6F*, *MAGEA12*, *PAGE1*, and *GAGE12F*. The presence calls of some CTA genes have been reported to correlate with significantly shorter event-free survival, such as *CTAG1B*, *CTAG2*, *MAGEA1*, *MAGEA2*, *MAGEA3*,

and *MAGEA6*.[25] The latter 2 were among the top 50 up-regulated genes in both clusters. In addition, cases with the 15% highest values of the high-risk index were predominantly observed in these clusters ($P < .001$). The high-risk index is determined on the basis of the published 17 gene model, which has been linked to early disease-related death (supplemental Figure 2).[26] The difference between these 2 clusters was determined on the basis of overexpression of genes involved in cell cycle and proliferation in one of the clusters (Table 1; supplemental Table 5), with a significantly greater proliferation index (PI), on the basis of the calculated median expression of 11 genes associated with proliferation: *TOP2A*, *BIRC5*, *CCNB2*, *NEK2*, *ANAPC7*, *STK6*, *BUB1*, *CDC2*, *C10orf3*, *ASPM*, and *CDCA1* ($P < .001$; supplemental Figure 3).[27] This cluster was named PR cluster, described before by Zhan et al..[15] The other CTA overexpressing cluster was mainly characterized by CTA genes and therefore named CTA cluster. Overlapping characteristics between the CTA and PR cluster were the overexpression of Aurora kinase A (*AURKA*), recently reported to be associated with a greater proliferation rate and poor outcome, which was significantly greater in both clusters in comparison with the remaining clusters ($P < .001$) and even greater in the PR compared with the CTA cluster ($P = .2$; supplemental Figure 4).[28,29] Also *BIRC5*, another recently described gene of which the presence call has been associated with lower event-free survival and overall survival (OS) in newly diagnosed MM patients, was observed among the top 50 up-regulated genes in PR and CTA cluster. [30] The CTA cluster has not been described as a distinct entity before and is therefore proposed as a new cluster.

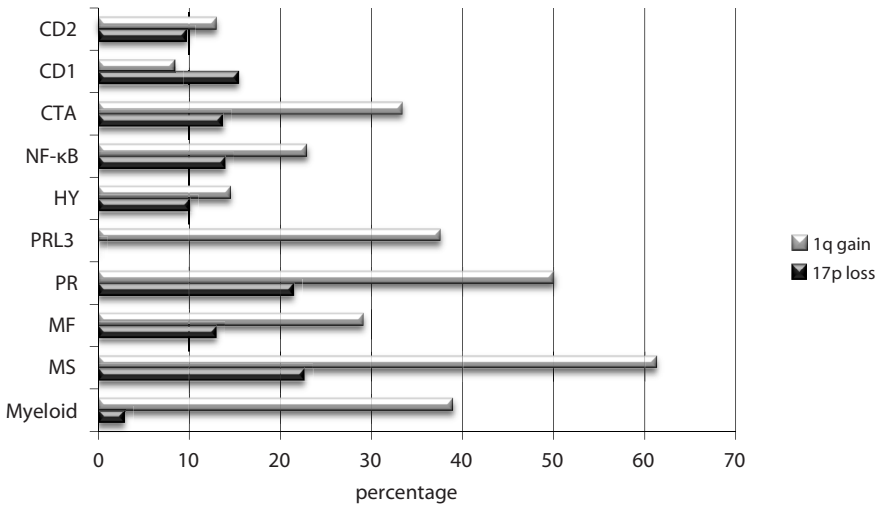

Figure 2. 1q gain and 17p loss. Percentage of patients per cluster showing 1q gain (dark gray bar) and 17p loss (light gray bar).

The second new cluster was characterized by clear differential expression of genes involved in the nuclear factor κB (NF-κB) pathway. Highly expressed NF-κB genes include *BCL10*, *TNFAIP3*, *IL8*, *GADD45B*, *NFKNIE*, *TNFIP1*, *NFKBIZ*, *IL2RG*, *CD40*, and *CD74* (Table 1; supplemental Table 5). In addition, the NF-κB index as reported by Keats et al on the basis of the mean expression level of 4 probe sets corresponding to *CD74*, *IL2RG*, and *TNFAIP3* (2x), as well as the NF-κB index published by Annunziata et al, based on the mean expression of 11 probe sets (*BIRC3*, *TNFAIP3*, *NFKB2*, *IL2RG*, *NFKBIE*, *RELB*, *NFKBIA*, *CD74*, *PLEK*, *MALT1*, and *WNT10A*) were significantly greater in this cluster compared with the other clusters ($P < .001$; Figure 3A-B).[31,32]

On the basis of these characteristics, this cluster was termed NF-κB cluster. Regulators of the NF-κB pathway were further analyzed. *CD40* and *NIK* (NF-κB-inducing kinase) expression are both involved in activation of NF-κB signaling. Only *CD40* expression was significantly greater ($P < .001$), whereas the tumor necrosis factor receptor-associated factor 3 *TRAF3*, a negative NF-κB regulator, showed significantly lower expression in the NF-κB cluster ($P = .004$; Figure 3C,E).

The third new cluster consisted of 9 cases, and only 27 genes were differentially expressed, including overexpression of protein tyrosine phosphatase *PTP4A3* (*PRL3*), protein tyrosine phosphatase, receptor-type, Z polypeptide 1 *PTPRZ1*, and suppressor of cytokine signaling 3 *SOCS3*. In lieu of any other characteristic, this cluster was termed PRL3 cluster. Chromosomal characteristics include hyperdiploidy in 75% of patients in this cluster; 1q gain was observed in 38% of patients. However, no 17p loss was observed. Strikingly, all patients in this cluster exhibited bone lesions. Furthermore, this cluster had the greatest percentage of patients in International Staging System stage I, 67% versus 19% to 57% in remaining clusters ($P = .062$). Expression levels of certain important genes in different clusters, such as *MMSET*, *FGFR3*, *CCND1*, *INHBE*, *ASS1*, *VPREB3*, *MS4A1*, *NUAK1*, and *RND3* were successfully verified by quantitative reverse transcription polymerase chain reaction (supplemental Figure 5).

Validation in independent datasets and comparison to TC and UAMS classification

To validate clusters described here, we used the dataset upon which the UAMS classification is based (GSE2658 [NCBI GEO]). We performed prediction analysis of microarrays analysis of corresponding clusters using the UAMS cluster definitions (Table 3; supplemental Table 9).[15] High Sn and Sp values were found for the classifiers of clusters CD-2, MS, MF, and HY, with Sn varying from 84% to 97% and Sp from 91% to 100%. Lower Sn was observed with classifiers for clusters CD-1 and PR. The classifier for the myeloid cluster consisting of 87 probe sets yielded the lowest Sn and Sp. The CTA, NF-κB, and PRL3 cluster were novel clusters and could therefore not be validated by use of the UAMS cluster definitions.

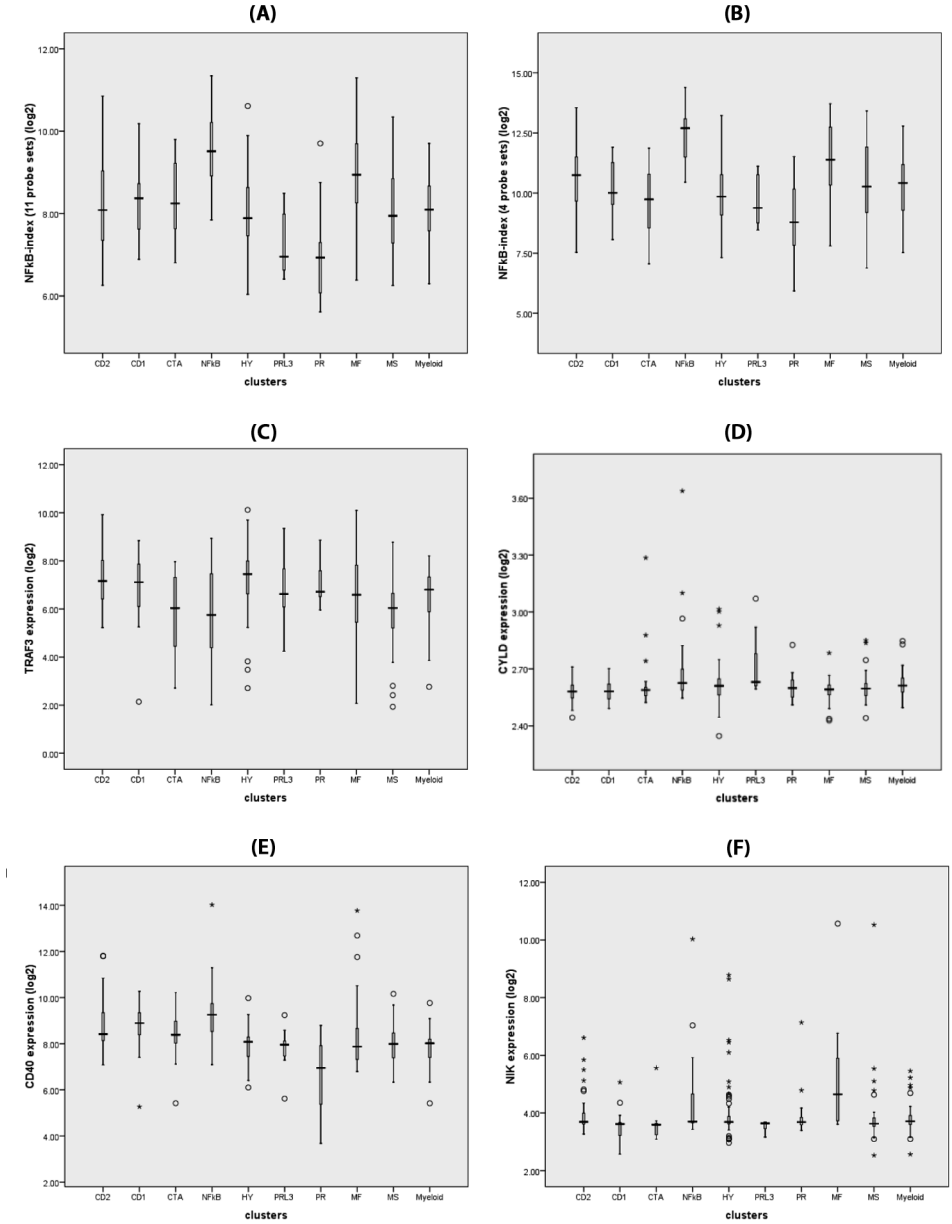

Figure 3. NF-κB index and regulators of NF-κB activity. **(A)** NF-κB index determined by Annunziata et al.[31] on the basis of the mean expression of 11 genes (*BIRC3*, *TNFAIP3*, *NFKB2*, *IL2RG*, *NFKBIE*, *RELB*, *NFKBIA*, *CD74*, *PLEK*, *MALT1*, *WNT10A*). **(B)** NF-κB index determined by Keats et al.[32] on the basis of the mean expression of 4 genes (CD74, *IL2RG*, and *TNFAIP3*, 2x). Expression (log2) per cluster of negative regulators of the NF-κB pathway: **(C)** *TRAF3* and **(D)** *CYLD*. Expression (log2) of positive regulators of NF-κB pathway: **(E)** *CD40* and **(F)** *NIK*.

Table 3. Validation of clusters: PAM analysis generating classifiers for clusters validated in independent dataset GSE2658.

	CD1 cluster	CD2 cluster	MF cluster	MS cluster	PR cluster	HY cluster	Myeloid cluster
Samples in training set*	13	34	32	33	15	77	39
Samples in validation set (GSE2568)#	28	60	37	68	47	116	145
No. of probes in classifier	106	45	35	8	86	56	87
PPV, %	57.60	80.60	97.10	94.30	61.70	76.70	58.50
NPV, %	97.60	98.30	98.90	99.40	95.10	94.60	81.70
Sn, %	67.90	90.00	89.20	97.10	61.70	84.80	42.80
Sp, %	96.40	96.30	99.70	98.80	95.10	91.30	89.40

Per cluster, the number of samples and total number of samples in the training set and test set are shown, the number of probe sets in the classifier, and the PPV, NPV, Sn, and Sp.
NPV indicates negative predictive value; PAM, prediction analysis of microarrays; PPV, positive predictive value; PR, proliferation-associated genes; Sn, sensitivity; and Sp, specificity.
* The total of all samples in the training set was 320.
#The total of all samples in the validation set was 414 except in the Myeloid cluster, where the total was 559.

In addition, our clustering was compared with the TC classification,[12] and UAMS classification.[15] To this end, TC criteria were used to assign the samples to TC classes and the published top 50 up-regulated and top 50 down-regulated probe sets that defined the 7 UAMS clusters to cluster our dataset (Tables 4 and 5). The MF subcluster, as defined previously, corresponded well to the Maf TC class; the MS cluster corresponded well to the 4p16 TC class. Samples from our CD-1/2 clusters corresponded to 11q13 and D1 classes.

Because of the limited nature of the TC classification, the classes did not compare with any of our other clusters. Regarding the UAMS classification, we confirmed the 7 described clusters. In addition, we identified a cluster showing a high NF-κB index and overexpression of *BCL10*, which was observed among the top up-regulated genes in our NF-κB cluster. Furthermore, we observed that HOVON-65/GMMG-HD4 samples originally present in the NF-κB cluster were now shifted to this extra cluster, which therefore probably represents the NF-κB cluster.

For additional validation of our classification, including the novel described clusters, we used 2 independent datasets, i.e., the UAMS data and a separate set of data from relapsed MM cases included in the APEX/SUMMIT/CREST trials to which we applied our normalization methods and gene selection criteria.[18]

From the UAMS dataset, 548 CEL files were made available. After performing quality control with NUSE, 10 arrays were excluded. The 2730 most variable genes of the remaining 538 samples were selected as described. A total of 1255 genes overlapped with the HOVON-65/GMMG-HD4 gene set. Clustering resulted in the identification of the translocation

clusters, HY, PR, and myeloid cluster (supplemental Figure 6). We identified an NF-κB cluster with up-regulation of genes involved in the NF-κB pathway such as *TNFAIP3*, *CFLAR*, *NFKB2*, *PLEK*, *IL2RG*, and *CD74* and a high NF-κB index, and additionally genes up-regulated in the UAMS LB cluster such as *CST6*, *PHACTR3*, *RASGRP1*, *IL6R*, *BIK*, and *EDN1*. This cluster clustered next to the MF cluster with subsequent up-regulation of *RND3*, *AHNAK*, *CCND2*, and *ARL4C*. Down-regulated genes included *CCR2*, *TNFSF10*, *DKK1*, *FRZB*, and interferon-induced genes. This cluster consists of UAMS LB and contaminated samples. Furthermore we identified a PRL3 cluster on the basis of overexpression of *PRL3* and *SOCS3*. No separate CTA cluster was identified. On the basis of the 100 up/down-regulated genes characterizing the CTA cluster, we observed that 7% samples (n = 37) with highest/lowest expression of these genes were found mainly within the UAMS PR cluster (n = 15), MS (n = 5), HY (n = 5) and contaminated cluster (n = 5; data not shown).

Table 4. Confusion matrices comparing with TC classification: H65 samples assigned to TC classes on the basis of TC criteria.

	11q13	D1	D2	D1 + D2	6p21	maf	4p16	None	Class error rate
CD–1	4	6	1	2	0	0	0	0	0.69
CD–2	25	9	0	0	0	0	0	0	0.26
MS	0	0	0	0	0	0	33	0	0.00
MF	0	0	5	0	0	12	0	0	0.29
HY	1	66	0	6	0	2	0	2	NA
PR	0	8	5	0	1	0	0	1	NA
LB	0	3	9	1	0	1	0	1	NA
NF-κB	1	23	6	1	0	1	1	4	NA
CTA	1	8	8	2	1	0	0	2	NA
PRL3	0	4	5	0	0	0	0	0	NA
Myeloid	1	13	13	5	0	0	6	1	NA
NA	0	5	3	0	1	0	0	0	NA

Comparing HOVON-65/GMMG-HD4 and TC classification: HOVON-65/GMMG-HD4 samples are assigned to TC classes on the basis of expression of *CCND1*, *MMSET*, *FGFR3*, *ITGB7*, *c-MAF*, and *CCND3* expression according to TC criteria. In lieu of normal bone marrow samples, for assignment of samples to D1, D1 + D2, and D2 classes, the threshold expression value of 30, as explained in "Methods," was used as a reference value for normal bone marrow. Class error rate is depicted in the last column. CTA indicates cancer testis antigens; HOVON, Dutch-Belgian Cooperative Trial Group for Hemato-Oncology; HY, hyperdiploid cluster; LB, low percentage of bone disease; NA, class error rate not determined because there were no corresponding subgroups; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; PR, proliferation-associated genes; and TC, translocation and cyclin D.

Table 5. Confusion matrices comparing with UAMS classification: HOVON 65 samples clustered with 50 up- and 50 down-regulated UAMS probe sets.

	CD–1	CD–2	MS	MF	HY	PR	LB	NF-κB	Class error rate
CD–1	8	0	0	0	1	0	4	0	0.38
CD–2	1	28	0	0	1	0	4	0	0.18
MS	0	0	31	0	0	1	0	1	0.06
MF	0	0	0	17	0	0	0	0	0.00
HY	1	0	0	2	63	4	6	1	0.18
PR	2	0	0	2	2	9	0	0	0.40
LB	0	0	0	2	0	0	13	0	0.13
NFB	0	8	0	1	1	1	0	26	0.30
CTA	0	5	0	0	5	8	0	4	NA
PRL3	0	0	0	0	0	4	3	2	NA
Myeloid	4	0	7	12	4	5	1	6	NA
NA	3	0	0	0	1	0	5	0	NA

Comparing HOVON-65/GMMG-HD4 and UAMS classification: The top 50 up- and top 50 down-regulated genes of all 7 UAMS clusters were used to cluster the HOVON-65/GMMG-HD4 samples. NF-κB is marked because the gene signature was very weak, on the basis of the expression of BCL10 and high NF-κB index.
CTA indicates cancer testis antigens; HOVON, Dutch-Belgian Cooperative Trial Group for Hemato-Oncology; HY, hyperdiploid cluster; LB, low percentage of bone disease; NA, class error rate not determined because there were no corresponding subgroups; NF-κB, nuclear factor kappa light-chain-enhancer of activated B cells; PR, proliferation-associated genes; and UAMS, University of Arkansas for Medical Sciences.

The APEX/SUMMIT/CREST dataset consisted of 264 gene expression profiles of relapsed MM patients; all of the U133A and B arrays used showed good NUSE values. Gene selection by the criteria used in the present study yielded 2248 probe sets. The overlap with HOVON-65/GMMG-HD4 gene set was 1002 genes. Again, the translocation clusters, HY, PR, and myeloid cluster were identified (supplemental Figure 7). In addition we detected an NF-κB cluster with up-regulation of NF-κB related genes such as *TNFAIP3*, *IL2RG*, *CFLAR*, *NFKBIA*, *LMNA*, and *KLF6* but also genes up-regulated in the UAMS LB cluster, such as *PHACTR3*, *RASGRP1*, *IL6R*, and *CST6* and genes frequently up-regulated in the MF cluster, such as *AHNAK*, *CCND2*, and *ARL4C*. Furthermore, we identified a PRL3 cluster on the basis of overexpression of *CCND2*, *PRL3*, and *PTPRZ1* and a CTA-like cluster. The CTA like cluster was defined by a different CTA profile than observed in the CTA cluster in our dataset, with up-regulation of *SSX3*, *SSX4B*, and *MAGE2B*.

A classifier for translocations

Samples with available FISH data were used to develop class predictors for translocations. Results are shown in Table 6 and more in detail in supplemental Table 10. For translocation

t(11;14) the lowest classification error generated a classifier of only 5 probe sets among which multiple probe sets of *CCND1* and *KCNMB2*, yielding a Sn of 83% and Sp of 97%. For translocation t(4;14) a 25-probe set classifier generated a Sn of 100% and Sp of 97%. Because samples with t(14;16) and t(14;20) clustered together, a combined t(14;16)/t(14;20) classifier of 18 probe sets was generated, which yielded a Sn of 100% and Sp of 99%.

Table 6. Validation of translocations: PAM analysis generating classifiers to predict translocations t(11;14), t(4;14), and t(14;16)/t(14;20).

	t(4;14)	t(11;14)	t(14;16)/t(14;20)
Samples in training set			
Translocations	26	24	5
Total	153	143	143
Samples in test set			
Translocations	11	13	4
Total	80	75	73
No. of probes in classifier	25	5	18
PPV, %	84.6	83.3	80.0
NPV, %	100.0	96.8	100.0
Sn, %	100.0	83.3	100.0
Sp, %	97.1	96.8	98.5
Correctly classified, %	97.5	94.7	98.6

Per translocation, the number of samples harboring the specific translocation and total number of samples in the training set and test set, the number of probe sets in the classifier, and the PPV, NPV, Sn, and Sp are shown.
NPV indicates negative predictive value; PAM, prediction analysis of microarrays; PPV, positive predictive value; Sn, sensitivity; and Sp, specificity.

DISCUSSION

Gene expression profiling was performed on 320 bone marrow PCs obtained at diagnosis from primarily white North European patients included in the HOVON-65/GMMG-HD4 trial. The objective of this randomized, open-label, phase 3 trial was to evaluate the efficacy of bortezomib in newly diagnosed MM cases.[33]

Unsupervised hierarchical clustering resulted in a subdivision in 10 clusters, of which 3 novel clusters have not been described previously. These are the NF-κB, CTA, and PRL3 clusters. The NF-κB cluster was characterized by hyperdiploidy in 66% of cases, demonstrated clear differential expression of genes involved in the NF-κB pathway. A subgroup characterized by genes involved in NF-κB signaling and anti-apoptosis was previously

reported in an analysis restricted to hyperdiploid myeloma samples.[16] files determined by RNA microarray technology in patients with newly diagnosed MM. NF-κB signaling is crucial in the pathogenesis of myeloma, involving both inactivating and activating mutations that primarily result in constitutive activation of the noncanonical NF-κB pathway.[31,32] Cases with high expression values of probe sets corresponding to NF-κB genes *CD74*, *IL2RG*, and *TNFAIP3* show a better response to bortezomib but no change in progression-free survival (PFS), whereas patients with low *TRAF3* expression show a better response to bortezomib and a prolonged PFS.[32] The NF-κB index determined by *CD74*, *IL2RG*, and *TNFAIP3* was significantly greater in our NF-κB cluster. In keeping with this finding, negative regulators of NF-κB signaling showed reduced expression, for instance, *TRAF3*, whereas genes involved in stimulating NF-κB activity, for instance, *CD40*, were found to be increased. The unexpectedly high expression of *CYLD* in the NF-κB cluster, a negative regulator of the NF-κB pathway, may be explained by the effect of inflammation stimuli, such as tumor necrosis factor-α, that activate the NF-κB pathway, which in turn could induce *CYLD*.[34] High NF-κB activity also characterized the MF cluster, in which predominantly overexpression of *NIK* was observed. In conclusion, various mechanisms appear to be responsible for the high NF-κB activity observed in the NF-κB and MF cluster.

The second distinct novel subgroup observed here is the CTA cluster. This resembles the PR cluster concerning the presence of poor prognostic markers such as CTA genes, the highest percentage of patients with an extreme high-risk index, and overexpression of *AURKA* and *BIRC5*. Although proliferation associated genes such as *AURKA* and *BIRC5* as well as cell cycle genes such as *CDC2* and *CDC42* were among the top up-regulated genes in the CTA cluster, the CTA cluster showed a significantly lower PI in comparison with the PR cluster. An explanation could be the absence of several genes representing the PI. Alternatively, the fold change difference of present genes between the CTA cluster and the remaining clusters was lower than the fold change difference in PR cluster versus remaining clusters. Besides features such as a greater percentage of 1q gain and a significantly greater PI, no clinical features distinguished the CTA from the PR cluster. This CTA cluster might represent a group of samples going through a transition phase from hyperdiploid myeloma to a PR signature. Evidence for this comes from the comparison with the UAMS classification, in which CTA characterizing genes did not cluster samples to one cluster but were found among samples in the UAMS PR, MS, HY, and contaminated cluster.

Overexpression of protein tyrosine phosphatases *PRL3*, *PTPRZ1*, and *SOCS3* characterized the third novel cluster, the PRL3 cluster. Greater *PRL3* expression was found in bone marrow PCs from patients with newly diagnosed monoclonal gammopathies than in PCs from healthy donors and significantly greater in the UAMS PR, LB and MS groups. Silencing of *PRL3* by siRNA impaired SDF-1–induced migration of MM cells, but no influence on cell-cycle distribution or cell proliferation was observed.[34] *PTPRZ1* is involved in the regulation of protein phosphorylation and plays a critical function in signal transduction, cell growth,

differentiation, and oncogenesis.[35,36] *SOCS3* is a cytokine-inducible negative regulator of cytokine signaling. The expression of this gene is induced by various cytokines, including interleukin-6 (IL-6), IL-10, and interferon gamma (IFN-γ). Transfection of myeloma cell lines with *SOCS3* showed protection from growth suppression by IFN-α. IL-6 induced by IFN-α may play an important role in the growth and survival of myeloma cells, and up-regulated *SOCS3* by IL-6 may be at least partially responsible for the IL-6-mediated inhibition of IFN-α signaling in myeloma cells.[37-39] *PRL-3* overexpression in mammalian cells was reported to inhibit angiotensin-II–induced cell calcium mobilization and promote cell growth. Absence of poor prognostic factors such as 17p loss, combined with low values for high-risk index, proliferation index, and *AURKA* expression, suggests patients within this cluster may have less severe disease ($P \leq .001$). Indeed, the frequency of International Staging System I was markedly greater in this cluster than in the other clusters.

Comparison with existing classifications confirmed the 7 clusters described in the UAMS classification, CD–1, CD–2, MS, MF, HY, PR, and LB.[15] Furthermore, Zhan et al.[15] reported a group of cases with a myeloid signature that was excluded from further analyses. The patients in this so-called contaminated cluster showed less disease activity and performed better on treatment, with significantly prolonged event-free survival and OS. We retained the group of patients with a myeloid signature in the gene expression analysis. These samples clustered together, clinically characterized by a significantly lower level of bone marrow plasmacytosis.

We validated our classification by applying our sample and gene selection criteria to 538 UAMS raw data files representing newly diagnosed MM cases and 264 APEX/SUMMIT/CREST raw data files representing relapsed MM cases. Sample clustering resulted in confirmation of clusters CD1, CD2, MS, MF, HY, PR, and in addition a myeloid cluster in both datasets. In both sets we observed a combined NF-κB/LB cluster, showing overexpression of genes involved in the NF-κB signaling pathway, but also of *PHACTR3*, *RASGRP1*, *IL6R*, and downstream targets of *MAF/MAFB*. In our clustering, LB samples were found as a subcluster of the MF cluster, on the basis of expression of *MAFB* and *c-MAF* downstream targets. This LB subcluster might represent a subgroup corresponding to an earlier stage of disease, as suggested by the lack of poor prognostic markers, such as thrombocytopenia and elevated LDH. The HOVON-65/GMMG-HD4 NF-κB cluster and LB subcluster were observed as 2 distinct clusters; except for a high NF-κB index, no overlap in differentially expressed genes or percentage of bone lesions was observed. Merging of these 2 clusters in independent datasets might be possible on the basis of a weaker expression of NF-κB-related genes showing lower fold changes relative to LB cluster genes and *MAF/MAFB* downstream targets. However, the presence of a cluster mainly characterized by an NF-κB index cannot be disputed.

We also confirmed the PRL3 cluster on the basis of the overexpression of at least *PRL3* among the top 10 genes showing the highest fold change difference, and *SOCS3*, *CCND2*, and/or *PTPRZ1*. A CTA-like cluster was found in the APEX dataset characterized by a different

CTA expression profile compared with our CTA cluster. No CTA cluster was detected in the UAMS dataset; samples with a CTA signature clustered within the PR, HY, MS, and myeloid cluster. Although the CTA cluster showed a high robustness index of 0.77 (range 0–1) in our dataset, we were not able to consistently confirm this cluster. Population as well as technical differences might play a role in this. Despite these differences as well as differences in myeloma status, PC selection procedures, and technical procedures, we were able to divide myeloma patients into 9 robust and consistent clusters. Naturally, changing the gene list does influence the size and composition of clusters. The 5% most variable gene list was selected on the basis of the division of translocations over the clusters.

In the UAMS classification, the PR, MS, and MF clusters were defined as high-risk groups with a significantly lower PFS and OS.[15] In agreement to this report, we demonstrated associations between clusters PR, MS, MF, the novel cluster CTA, and poor prognostic factors, such as increased high-risk index and elevated LDH. Future analyses will evaluate the prognostic impact of the current defined clusters in the HOVON-65/GMMG-HD4 trial.

Finally, the ability to predict the primary translocations is important for diagnostic purposes. Because these classifiers were found to be robust, the development of methods that complement or even replace FISH techniques will be relevant and subject to future studies. In conclusion, the classification described here showed good correlation to the previously described classifications in MM. Yet, 3 new clusters were identified, one of which signifies the involvement of NF-κB signaling in MM.

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CHAPTER 4

IMPACT OF BORTEZOMIB TREATMENT ON SURVIVAL IN MOLECULAR SUBGROUPS OF NEWLY DIAGNOSED MULTIPLE MYELOMA

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ABSTRACT

Novel agents such as thalidomide, lenalidomide, and bortezomib have substantially improved outcome of patients with multiple myeloma (MM). However MM remains an incurable malignancy with a variable overall survival (OS) ranging between a few months to more than 10 years, with 30% reaching 5 year survival after diagnosis. We have previously published a myeloma classification based on correlated gene expression profiles of newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial (GSE19784). This trial compared bortezomib based treatment versus conventional treatment. Here, we evaluated the impact of bortezomib on survival in relation to cluster designation. In patients treated conventionally, i.e. without bortezomib, a significant difference was found between all clusters for both overall survival (OS) and progression free survival (PFS) ($p < 0.001$, for both). The clusters MS, MF and PR demonstrated the shortest survival time both for OS and PFS. In contrast, no significant difference was observed in survival time between clusters of patients treated with a bortezomib based treatment. Of bortezomib treated patients, those with PR cluster gene expression still demonstrated the poorest OS and PFS, but the survival of both MF and MS clusters was clearly improved.

INTRODUCTION

Multiple myeloma (MM) is an incurable disease characterized by accumulation of malignant monoclonal plasma cells in the bone marrow. Median overall survival (OS) varies widely between patients. Prognostic markers, such as serum β2-microglobulin (B2M) and albumin, together constituting the international staging system (ISS), delineate patients into three distinct risk categories.[1]

In addition, MM can be cytogenetically divided into hyperdiploid and nonhyperdiploid MM, with the latter category demonstrating a high proportion of translocations involving the immunoglobulin heavy chain at chromosome 14q32. Together with translocation t(11;14), involving *CCND1*, hyperdiploid MM has a relatively favorable prognosis as compared to nonhyperdiploid MM. Translocation t(4;14), t(14;16) and t(14;20) are considered to be high risk genetic aberrations. These high-risk groups are also recognized in our classification (based on the HOVON-65/GMMG-HD4 set) as well as in a predating classification of the UAMS group. The UAMS group has described poor prognosis for MS, MF and PR in a study of newly diagnosed MM patients included in TT2, existing of multiple phases of chemotherapy, with or without the addition of thalidomide. The HOVON-65/GMMG-HD4 trial, which we used for generating our classification as well as generating a high risk signature, compared bortezomib based induction with conventional induction treatment, followed by high-dose melphalan and maintenance treatment (GSE19784).[2] Among the 10 defined clusters in the classification, we identified 3 novel clusters that were not identified before by the UAMS classification.[3] Here, we report the prognostic impact of bortezomib treatment in the different clusters compared with standard treatment.

MATERIALS AND METHODS

Patients and procedures

833 patients were included in a large prospective, randomized, phase III trial (HOVON-65/GMMG-HD4). Patients were randomly assigned to three cycles of induction treatment with vincristine, doxorubicin, and dexamethasone (VAD), or bortezomib, doxorubicin, and dexamethasone (PAD). Both groups received high-dose melphalan with autologous stem-cell rescue followed by maintenance treatment with thalidomide (group assigned to VAD) or bortezomib (group assigned to PAD) for 2 years.[2] This study was approved by the Ethics Committees of the Erasmus University MC, the University of Heidelberg and the participating sites. Informed consent to treatment protocols and sample procurement was obtained for all cases included in this study, in accordance with the Declaration of Helsinki. Use of diagnostic tumor material was approved by the institutional review board of Erasmus MC.

Gene expression profiling, assessment of outcome and statistical analysis

The gene expression dataset GSE19784 was used, derived from patients included in the HOVON-65/GMMG-HD4 trial.[3] 320 patients were included in the molecular classification and follow-up data were available for 319 patients.

PFS was calculated from randomization until progression, relapse or death, whichever came first. Patients who received a non-myeloablative AlloSCT were censored at the date of AlloSCT. OS was measured from randomization until death from any cause. Patients alive at the date of last contact were censored. The median follow-up was 41 months.

Survival analysis was performed using the SPSS software. Kaplan Meier analysis was performed using the log rank test to assess for significance in survival time between clusters.

RESULTS AND DISCUSSION

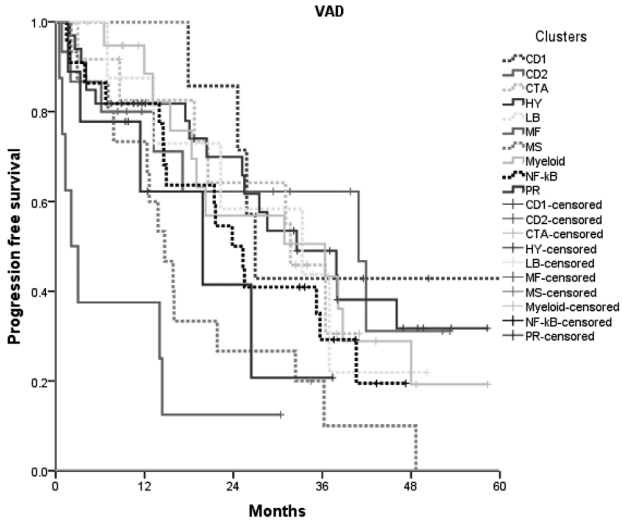
Our published myeloma classification (EMC classification) consisted of 10 main clusters including CD–1, CD–2, MS, PR, HY, MF, Myeloid, NF-κB, CTA, and PRL–3. The MF cluster could be further subdivided in a LB subcluster, and a MF subcluster. In addition, one cluster did not have a clear gene expression signature, i.e. no profile (NP) cluster. In the following, both subclusters and clusters are denoted as clusters (i.e. the MF subcluster is considered as the MF cluster).

Survival analysis was performed on the bortezomib treated patients and the conventionally treated patients, with exclusion of the clusters containing less than 10 patients (i.e. PRL–3 and NP cluster).

Patients treated with conventional treatment demonstrated a significant difference in survival time depending on the cluster designation as shown in Figure 1A and C. Strikingly, patients treated with a bortezomib based treatment did not demonstrate a significant difference in survival time between different clusters (Figure 1B and D). The lack of significant difference in bortezomib treated patients is mostly due to the improved survival of patient groups with poor prognosis with conventional treatment, as outlined below.

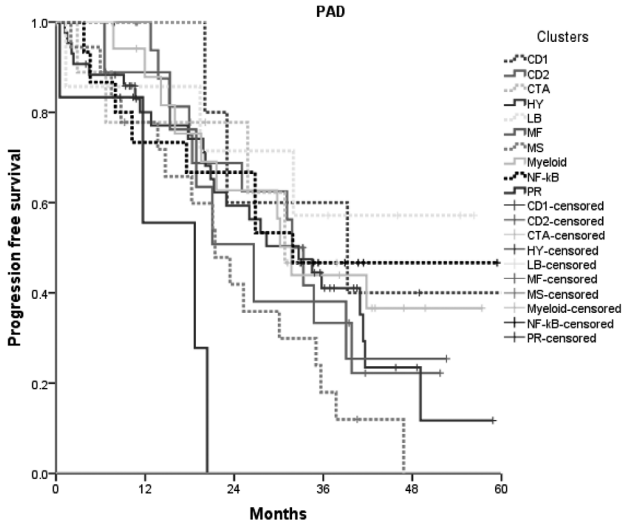
In the conventional treatment arm, the MF cluster, consisting of 5% of the patients in this study, demonstrated the shortest median PFS and OS of all the clusters (2 and 4 months, respectively). In marked contrast, in the bortezomib based treatment arm the MF cluster demonstrated a median PFS of 27 months and a median OS of 54 months, which showed the most striking improvement in survival from conventional to bortezomib based treatment. Following the MF cluster, the median PFS of the MS cluster (10% of studied population) was 15 months in the conventional treatment arm, compared to 31 months median survival on average for all other clusters (excluding MS and MF). PFS of the MS cluster was 6 months longer in the bortezomib treatment arm. For OS, the difference was more obvious with a median OS limited to 30 months for conventionally treated patients and median OS not reached for bortezomib treated patients.

(1A)



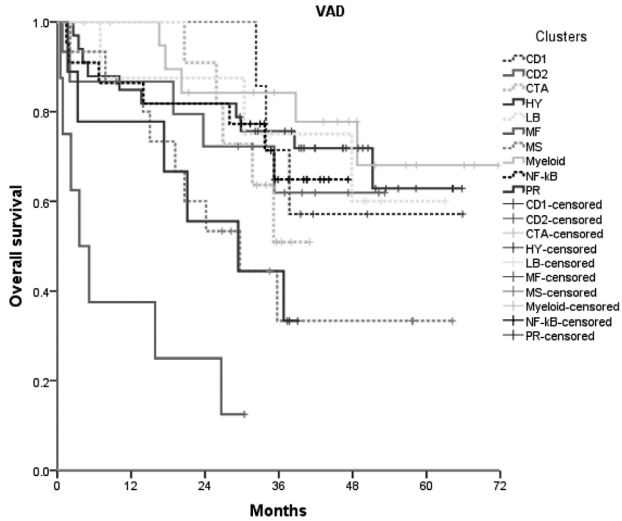
clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	7	15	12	33	8	8	15	21	22	9
Median PFS (months)	27	41	31	33	33	2	15	36	24	20

(1B)



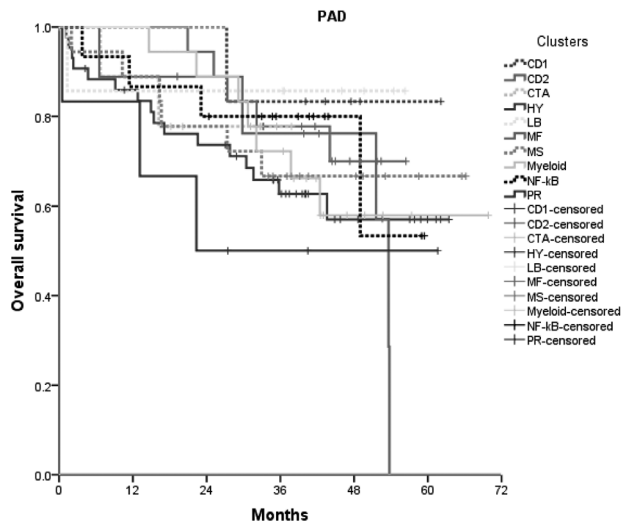
clusters	CD-1	CD-2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	6	18	10	44	7	9	18	18	15	6
Median PFS(months)	39	32	31	33	NR	27	21	32	32	19

(1C)



clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	7	15	12	33	8	8	15	21	22	9
Median PFS(months)	NR	NR	NR	NR	NR	4	30	NR	NR	29

(1D)



clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	6	18	10	44	7	9	18	18	15	6
Median PFS(months)	NR	NR	NR	NR	NR	54	NR	NR	NR	22

Figure 1. Kaplan Meier curves. **(A)** 12 clusters and PFS following treatment with VAD, **(B)** 12 clusters and PFS following treatment with PAD, **(C)** 12 clusters and OS following treatment with VAD, **(D)** 12 clusters and OS following treatment with PAD. NR=Not reached (see pages 251 and 252 for colour figure).

In the conventionally treated patients, the third cluster with the shortest median PFS, following MF and MS, was the PR cluster with median PFS of 20 months. Whereas both MF and MS demonstrated a clear benefit of bortezomib treatment, the PR cluster demonstrated a PFS which is virtually unchanged (19 months). In terms of OS, this cluster showed a median survival of 29 months in conventionally treated patients whereas the median was 22 months in bortezomib treated patients.

Since we identified a cluster characterized by genes involved in the NF-κB pathway, the NF-κB cluster (12% of studied population), we were particularly interested in the survival data of this cluster following treatment with bortezomib. Bortezomib, a proteasome inhibitor, is thought to exert its actions in part through this pathway.[4,5] Furthermore, it was observed that NF-κB activity was among the significant gene sets relatively highly expressed in samples from responsive patients to bortezomib.[6] The NF-κB cluster demonstrated a median PFS of 24 months in conventionally treated patients compared to 32 months in bortezomib treated patients. Other clusters which demonstrate longer median PFS in bortezomib treated patients compared to conventionally treated patients were CD–1 and LB, comprising 4% and 5% of patients respectively (see Figure 1A and B).

The clusters which demonstrate benefit from bortezomib treatment include poor prognostic clusters MS and MF, and clusters CD–1, LB and NF-κB. In total, these clusters comprise 36% of this patient population. On the other hand, PR patients (5%) did not demonstrate an improvement on bortezomib treatment. Other clusters with shorter median PFS after bortezomib compared to treatment with conventional drugs, included CD-2 (11% of patients, 32 months vs. 41 months, respectively) and Myeloid (12%, 32 months vs. 36 months, respectively). Finally, and importantly, two clusters demonstrated no difference in median PFS if treated conventionally or using Bortezomib. These were the novel CTA cluster and the hyperdiploid cluster (comprising 7% and 24%, respectively). It must be stressed that these analyses concern small groups of patients, and that this analysis must be confirmed in larger datasets.

In conclusion, we observed a clear effect of bortezomib on the poor prognostic clusters MF and MS, while the PR cluster remained a poor prognostic cluster regardless of treatment used.

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CHAPTER 5

GENE EXPRESSION PROFILING AND CORRELATION WITH OUTCOME IN CLINICAL TRIALS OF THE PROTEASOME INHIBITOR BORTEZOMIB

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Blood. 2007 Apr 15;109(8):3177-88.

ABSTRACT

The aims of this study were to assess the feasibility of prospective pharmacogenomics research in multicenter international clinical trials of bortezomib in multiple myeloma and to develop predictive classifiers of response and survival with bortezomib. Patients with relapsed myeloma enrolled in phase 2 and phase 3 clinical trials of bortezomib and consented to genomic analyses of pretreatment tumor samples. Bone marrow aspirates were subject to a negative-selection procedure to enrich for tumor cells, and these samples were used for gene expression profiling using DNA microarrays. Data quality and correlations with trial outcomes were assessed by multiple groups. Gene expression in this dataset was consistent with data published from a single-center study of newly diagnosed multiple myeloma. Response and survival classifiers were developed and shown to be significantly associated with outcome via testing on independent data. The survival classifier improved on the risk stratification provided by the International Staging System. Predictive models and biologic correlates of response show some specificity for bortezomib rather than dexamethasone. Informative gene expression data and genomic classifiers that predict clinical outcome can be derived from prospective clinical trials of new anticancer agents.

INTRODUCTION

Multiple myeloma is an incurable malignancy that originates in the antibody-secreting bone marrow plasma cells. Median survival is approximately 3 to 4 years, but the clinical course is highly variable and difficult to predict.[1,2] Therefore, there is a need to better define patient-specific treatment strategies for the use of both standard and novel therapies.

A number of clinical and laboratory features provide prognostic information, including age, performance status, tumor burden, tumor proliferative index, and hemoglobin and platelet levels, as well as serum β-2 microglobulin, albumin, creatinine, lactic dehydrogenase, and calcium.[3-8] Some of these factors relate to the patient's status, whereas others reflect aspects of the tumor. A recent multivariate analysis of data from 10 000 patients identified serum albumin and ß-2 microglobulin as a reliable prognostic tool, referred to as the International Staging System (ISS).[1] The ISS is valid for patients of different age groups and geographies, and with respect to the 2 most common myeloma treatments of the past decade, standard-dose chemotherapy and high-dose therapy (HDT) followed by stem cell rescue.[1] However, therapeutic choices for myeloma have become increasingly complex as new active agents have emerged,[9,10] and their optimal use either alone or in combination with standard chemotherapy or HDT remains to be defined. As indicated in the ISS study, standard clinical prognostic factors were unable to reliably identify the highest risk patients most in need of novel therapies (defined as those with < 12 months overall survival [OS]).[1]

It is anticipated that genomics will help provide more precise prognostic and predictive tools.[11-13] However, the practicality, utility, and challenges of prospective genomic research have only recently been explored.[14] In addition, it remains unclear whether the strategies used to define prognostic genomic classifiers[15] can be used to develop classifiers that predict outcome for a specific therapy.

Various molecular analyses suggest that myeloma, like other cancers, is composed of distinct subtypes that have somewhat different molecular pathologies and prognoses.[13,16,17] For instance, cytogenetic studies reveal that approximately 60% to 80% of myeloma cases exhibit rearrangements of the *IGH* heavy chain locus, with 40% involving 5 recurrent translocations.[13] Patients with the t(11;14)(q13;q32) translocation experience superior survival on treatment with HDT, whereas those with t(4;14)(p16;q32) exhibit a relatively poor survival.[18-25] More specifically, although t(4;14)(p16;q32) tumors initially respond to therapy, they relapse quickly and are insensitive to alkylator salvage treatment.[13,19] Deletion of chromosome 13 occurs in tumors with and without IgH translocations[26] and is a significant poor prognostic factor, regardless of therapy or age.[26-28]

Furthermore, distinct gene expression patterns are associated with most of the molecular subtypes of myeloma,[14,16,29-32] and these patterns are now being associated with disease prognosis.[14] A recent genomic analysis of 231 myeloma cases identified 8 distinct tumor subtypes, defined via assessment of cyclin D status and other genes frequently involved

in IgH translocations (referred to as TC subtypes, for **T**ranslocation and **C**yclin D).[16] Despite these and other molecular advances, it remains unclear how to match disease subtypes appropriately with standard myeloma therapies or the use of new agents.

To assess the technical feasibility of conducting prospective pharmacogenomics research in myeloma and, if possible, to develop and independently validate a genomic classifier of efficacy to a specific single agent, we generated gene expression data during the course of national and international phase 2[33,34] and phase 3[35] clinical trials of a novel agent, the proteasome inhibitor bortezomib (VELCADE; Millennium Pharmaceuticals, and Johnson & Johnson Pharmaceutical Research & Development). We report here the microarray results from those trials. This is the first report demonstrating the prospective development and independent validation of a genomic classifier that predicts clinical response between myeloma patients treated with a new agent (bortezomib) or an active control drug (high-dose dexamethasone; Dex).

METHODS

Sample collection, enrichment, data generation, and array quality control

On collection of patients' bone marrow aspirate, myeloma cells were enriched via negative selection. The RosetteSep procedure (Stem Cell Technologies, Vancouver, BC, Canada) uses a cocktail of cell-type–specific antibodies (as described in Tai et al.[36]) to deplete nonplasma cells (see Document S1 for details, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Myeloma cells were collected and frozen. In the international studies, the first 2 samples from each site were collected and subjected to RNA isolation so that feedback on quantity and quality could be provided; ultimately, phase 2 and 3 trials provided a similar percentage of informative samples. Control samples included bone marrow–derived normal plasma cells (PCs), neutrophils, T cells, and CD71+ erythroid cells (AllCells, Berkeley, CA).

Total RNA was isolated using a Qiagen RNAeasy isolation kit (Qiagen, Valencia, CA) and quantified by spectrophotometry; samples with at least 0.5 μg were labeled for gene expression profiling in 2 batches (Document S1), using the Affymetrix GeneChip microarray system (Affymetrix, Santa Clara, CA). A standard T7-based amplification protocol (Affymetrix) was used to convert 2.0 μg RNA (if available) to biotinylated cRNA. cRNA for each sample was hybridized to the U133A/B arrays in triplicate; operators, chip lots, clinical sites, and scanners (GeneArray 3000; Affymetrix) were controlled throughout. Data processing used Affymetrix MAS5.0. Quality control metrics determined by Affymetrix and Millennium Pharmaceuticals included the percentage present (> 25%), scale factor (< 14), β-actin 3′/5′ ratio (< 15), and background (< 120) (Table S1). Samples falling outside these metrics were excluded from subsequent analysis.

The myeloma purity score examines expression of genes described as highly expressed in myeloma cells and their normal plasma precursor cells (205692_s_at CD38 antigen *[P45]*; 201286_at syndecan-1 *[SDC1]*; 201891_s_at β-2 microglobulin *[B2M]*; 211528_x_at *B2M*) compared with genes expressed highly in erythroid cells (37986_at erythropoietin receptor *[EPOR]*; 209962_at *EPOR*; 205838_at glycophorin A *[GYPA]*), neutrophils (203948_s_at myeloperoxidase *[MPO]*; 203591_s_at colony-stimulating factor 3 receptor *[CSFR3]* [granulocyte]; 204039_at CCAAT/enhancer binding protein α*[CEBPA]*; 214 523_at CCAAT/enhancer binding protein ε*[CEBPE]*), or T cells (209603_at GATA binding protein 3 *[GATA3]*; 209604_s_at GATA binding protein 4 *[GATA4]*; 205456_at CD3E antigen, ε polypeptide). Myeloma score = expression of myeloma markers / expression of (erythroid + neutrophil + T cell) markers. The data set is available at Gene Expression Omnibus (http://www.ncbi.gov/geo/).

Clinical studies and efficacy

The APEX phase 3 trial (039) was conducted at 93 centers in the United States, Canada, Europe, and Israel from June 2002 to October 2003.[35] A total of 669 patients with myeloma who had relapsed following 1 to 3 prior therapies were randomly assigned to treatment with bortezomib 1.3 mg/m^2 or high-dose Dex; Dex patients who experienced progressive disease (PD) were permitted to crossover to receive bortezomib in a companion study (040). The 040 study also directly enrolled 263 patients who had more than 3 prior therapies; these "non-crossover" patients were also eligible for pharmacogenomics research. The SUMMIT phase 2 trial (025) enrolled 202 patients with relapsed and refractory myeloma at 14 centers in the United States.[34]

Patients received bortezomib 1.3 mg/m^2 for no more than 8 cycles. The CREST phase 2 trial (024) had a similar design, except the 54 enrolled patients had either relapsed or refractory disease, and they received bortezomib 1.0 or 1.3 mg/m^2.[33] Phase 2 investigators had the option to add Dex 20 mg if patients had suboptimal response; however, clinical and genomic studies report activity of single-agent bortezomib by censoring outcome data at the time of adding Dex.

Review boards at all participating institutions approved the studies; all patients provided written informed consent. Additional consent was provided for pharmacogenomics analysis. The studies were conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation Good Clinical Practice guidelines.

Outcome definitions

Clinical response was treated as a categorical variable, whereas OS was treated as a censored continuous-time variable. OS (days) was assessed from the date patients received their first dose of study drug, without regard to other subsequent therapies. Patients were classified as achieving complete response (CR), partial response (PR), minimal response (MR), no

change (NC), or PD, using European Group for Bone Marrow Transplantation criteria.[37] In brief, PD requires 25% increase in paraprotein, whereas MR, PR, and CR require at least 25%, 50%, and 100% decreases, respectively. NC is the absence of response or progression but, in this study, required at least 2 measures of stable disease. The efficacy data of the genomics subset were manually reviewed to reconfirm classifications (Document S1).

Data analysis

Only the 9200 probe sets with strongest between-sample variance relative to their in-sample replicate variance were retained for further analysis (B.B., E.K., G.M., manuscript in preparation). Repeated expression measurements on a given sample were summarized by the log of their median value.

Gene set enrichment analysis (GSEA)

Analysis used GSEA software (version 1.0; Broad Institute, http://www.broad.mit.edu/gsea/) and C2 curated functional gene sets from the Molecular Signature Database (MSigDB)[38] (Document S1). Analysis was performed on the full set of bortezomib samples from all trials, as well as on samples from individual trials, including 039 bortezomib and 039 Dex separately. Gene sets satisfying the default multiple hypothesis testing threshold (FDR q value < .25) and having nominal P values no more than .025 were identified (56 associated with response [CR/PR/MR; R] and 16 associated with PD). These were classified as to whether they also had a less stringent (nominal $P \leq .05$), but consistent, association with the phenotype in analysis of samples from at least 2 individual trials. Gene sets were then ranked, first by consistent association with phenotype, then by FDR q value.

Analysis of clinical response

Differential expression of genes with respect to clinical response was assessed by 2-sided t test with unequal variances. Predictive models were built using a linear predictor score[39] on the top 100 differentially expressed genes. To assess the accuracy of the predictive modeling method on the entire dataset, a standard bootstrap procedure was used,[40] in which the data were repeatedly divided into separate training and test sets. Each time, genes were selected, and a predictive model was built on the training set; the model was then applied to the test set to assess accuracy, sensitivity (Sn), and specificity (Sp). To determine whether predictive accuracy differed significantly from what might be expected at random, outcome values were repeatedly randomly permuted among samples, and the bootstrap procedure was reapplied. Empirical distributions of accuracies for true and permuted outcomes were then compared.

Analysis of OS

We used the Cox proportional hazards model to assess strength of association of individual probe sets with OS. Predictive models were built using the method of Bair and Tibshirani,[41] as follows. The 100 probe sets most strongly associated with outcome were selected for the model. We computed the top 2 principal components of these genes' expression on the training samples. Test data were mapped onto the space defined by the principal component vectors, and Cox modeling was used to assess strength of association of the transformed test data with outcome. For visualization of the models using Kaplan-Meier curves, the linear predictor score from the Cox model was used to divide test samples in equally sized high- and low-risk groups.

RESULTS

Sample collection and genomic data generation in multicenter clinical trials

The phase 2 and phase 3 clinical trials of bortezomib for the treatment of myeloma included a research component to investigate the feasibility of pharmacogenomics in a prospective setting; tumor samples were provided from 89 centers in 12 different countries. A pretreatment bone marrow aspirate was collected during routine screening procedures. The percentage of tumor cells in aspirates varied from approximately 5% to greater than 75%. All samples were therefore subject to an enrichment procedure in an effort to increase tumor content to at least 60% to 80%, a level consistent with prior genomic studies of cancer biology and outcome.[14,42,43] Fluorescence cell sorting analysis (FACS) of samples before and after enrichment demonstrated that enrichment could yield samples of 80% to 90% tumor cells (Figure 1A). FACS analyses were not practical at all participating centers. Therefore, we assessed sample purity via analysis of a myeloma purity score derived from microarray data. Samples with low tumor cell purity were excluded from further analyses (Figure 1B).

Sample attrition was observed at each step in the process of generating gene expression data (Table 1). Approximately 60% of samples exhibited RNA quantity and quality adequate for hybridization. Of these samples, about 85% generated high-quality microarray data, and 85% passed the assessment of tumor-cell enrichment with the myeloma purity score. Results were generally consistent between trials (Table 1). The bortezomib dataset consists of 169 patients evaluable for response and 188 evaluable for OS, whereas the Dex dataset has 70 and 76 evaluable for response and OS, respectively. The details for each trial are provided in Table S2.

For each trial we examined a series of clinical and prognostic variables to ensure that the subsets of patients with genomic data were representative of the general trial populations (Table 2). No bias was observed with regard to age, sex, number of prior therapies, or myeloma isotype. For some trials the response rate, time to progression (TTP), or survival

values of the genomics subset were indicative of a worse outcome. Although serum albumin and serum β-2 microglobulin were elevated in the genomics subset of trial 025, this was not observed in other trial data. However, the genomics subset of each trial did exhibit a higher baseline tumor burden in the bone marrow aspirate (Table 2), indicating that successful sampling is partly related to extent of marrow disease. The data suggest that genomic subsets are reasonable representations of study populations as a whole, except for an overrepresentation of patients with greater tumor burden. Because of differences in entry criteria, there are differences between the trial populations in terms of median number of prior therapies, time from diagnosis, and response rate (Table 1; Table S3). For example, trial 025 enrolled relapsed patients who were refractory to their last prior therapy (median number of prior lines of therapy, 6), whereas trial 039 specified 1 to 3 prior lines of therapy. Accordingly, the median number of prior lines of therapy in genomics subsets of trials 025 and 039 are 6 and 2, respectively.

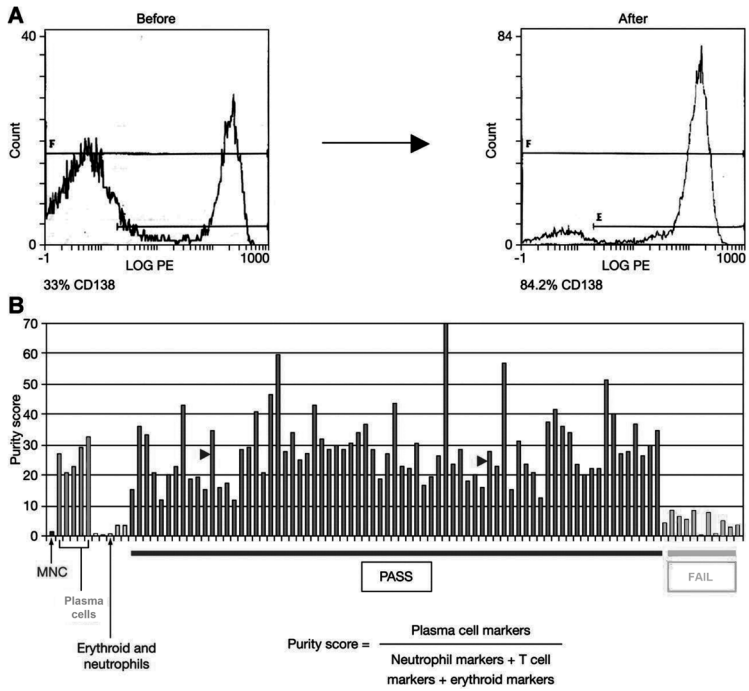


Figure 1. Bone marrow aspirate enrichment procedure effectively depletes nontumor cells. **(A)** Bone marrow aspirate samples before and after enrichment were subject to CD138 staining and FACS analysis. **(B)** Myeloma purity score is elevated in control plasma cell samples (> 90% pure) relative to bone marrow mononuclear cells (MNCs), neutrophils, and erythroid cells. Two enriched patient samples of 84% and 91% tumor purity by FACS analysis had scores of 35 and 28, respectively (blue arrows). A score of at least 10 (at least 3-fold elevated relative to the score for nonplasma cell types) was set as a threshold for further analysis (see page 253 for colour figure).

Table 1. Sample attrition in the process of generating gene expression data in the 024, 025, 039, and 040 trials, and the number of response- and survival-evaluable samples obtained from each trial.

	Trial 024	Trial 025	Trial 039	Trial 040
No. of patients	54	202	669	263†
Phase	Phase 2	Phase 2	Phase 3	Phase 3 companion
Disease/prior therapy entry criteria	Relapsed or refractory	Relapsed and refractory	1-3 prior lines	> 3 prior lines
Patients providing consent for pharmacogenomics analysis,* no. (%)	32/54 (59)	126/202 (62)	505/669 (75)‡	213/263 (81)‡
Samples collected, no. (%)	29/32 (91)	121/126 (96)	459/669 (69)	205/263 (78)
Samples that passed RNA QC, no. (%)	17/29 (59)	76/121 (63)	190/459 (41)	72/205 (35)
Samples that passed Affymetrix hyb QC, no. (%)	12/17 (71)	65/76 (86)	173/190 (91)	63/72 (88)
Samples that passed purity analysis, no. (%)	10/12 (83)	54/65 (83)	156/173 (90)	58/63 (92)
Response evaluable, no.	7	39	141 (71 bortezomib, 70 Dex)	52
Survival evaluable, no.	7	44	156 (80 bortezomib, 76 Dex)	57

CR indicates complete response; PR, partial response; QC, quality control.
* In trials 039 and 040, based on the informed consent of all patients in trial; not all patients who consented had a sample collected; see following row (Samples collected).
†Non-crossover population.
‡Consent for whole genome analysis.

Tumor samples were collected between 2001 and 2004; microarray hybridizations were performed in 2 batches separated by 9 months. Replicate hybridizations allowed us to assess within-patient reproducibility and between-patient variations prior to selecting the approximately 9200 most differentially expressed probe sets for further analysis.

Comparison of dataset with published myeloma biology

Our genomics approach differs from that of prior myeloma studies [14,30,32,44] in that samples were collected at multiple sites and subjected to a negative-selection procedure to enrich for tumor cells. Therefore, we closely examined how the data might have been influenced by demographic, clinical, and technical parameters, using unsupervised hierarchical clustering. Figure 2A shows a dendrogram of 264 myeloma patient samples and 6 normal plasma cell control samples. Patients with different age, sex, and myeloma isotype were randomly distributed (Figure 2A) across these groups. Further, there was no significant clustering of samples that originated at the same clinical center.

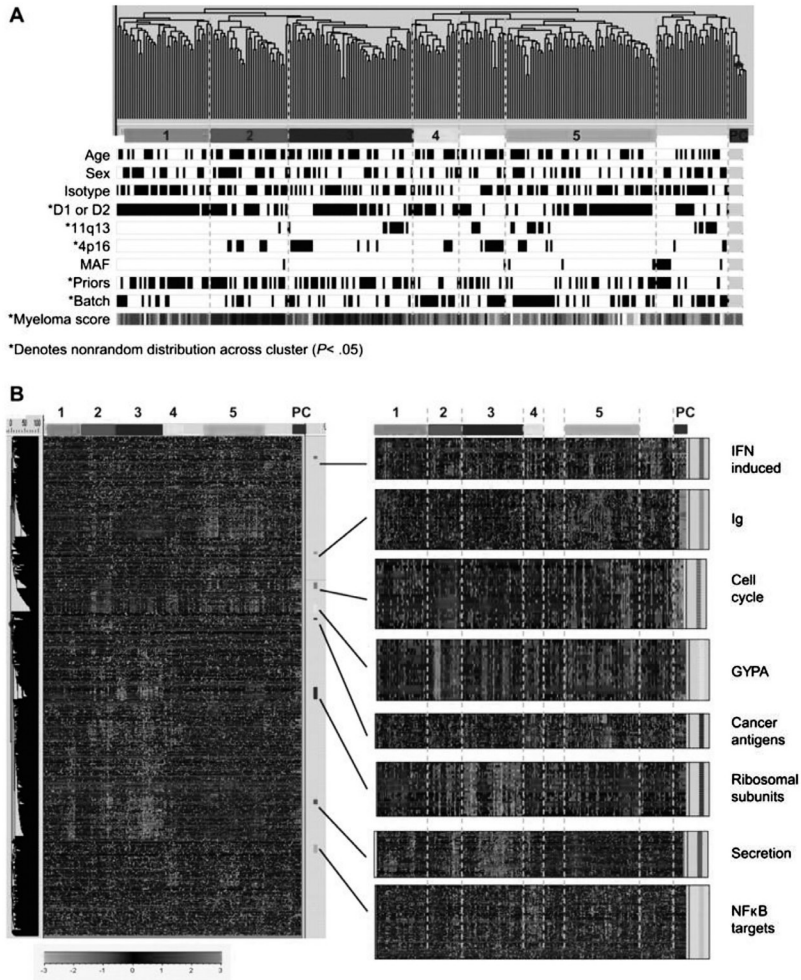


Figure 2. Sample relationships are influenced by clinical and gene-expression characteristics. Two hundred sixty-four myeloma patient samples and 6 normal plasma cell control samples were subject to unsupervised hierarchical clustering based on 9174 differentially expressed probe sets. Highly related branches (labeled groups 1-5) were identified by setting a fixed similarity metric (GeneMaths software; Applied Maths, Austin, TX) and requiring at least 12 samples for membership; unlabeled samples comprise various smaller groups. **(A)** Patient attributes are encoded below the sample dendrogram. Attributes with nonrandom distribution ($P < .05$) are indicated by asterisks. Black is associated with age older than 60 years, female sex, IgG isotype, 1 or 2 prior therapies, hybridization batch 1 (trials 024, 025, and 040), and low purity score. White is associated with age 60 years and younger, male sex, other isotypes, 3 or more prior therapies, hybridization batch 2 (trial 039), and high purity score. **(B)** An overview of the 9174 differentially expressed probe sets, with an expansion of specific functional groups (see page 254 for colour figure).

Table 2. Baseline and disease characteristics and efficacy data in the total populations in the 025, 039, and 040 trials and in the pharmacogenomics/nonpharmacogenomics cohorts from each trial

Variable	Overall	Nongenomics	Genomics	*P*, genomics vs. non-genomics
Trial 025				
Overall survival, d*	518.0 (434.0–643.0)	533.0 (441.0–787.0)	434.0 (237.0–628.0)	.06
Time to progression, d*	158.0 (119.0–213.0)	210.0 (154.0–377.0)	83.0 (60.0–139.0)	.03
Response rate, CR + PR, %†	30.1	30.0	30.2	> .999
Response rate, CR + PR + MR, %†	40.4	40.7	39.5	> .999
Albumin level, g/L‡	35.2 (34.43–36.01)	35.8 (34.87–36.64)	33.4 (31.62–35.07)	.01
Platelet count, x 109/L‡	170.5 (157.11–183.84)	170.7 (155.22–186.27)	169.5 (142.72–196.28)	.91
C-reactive protein level, mg/L‡	13.0 (10.56–15.43)	12.0 (9.45–14.45)	16.5 (9.85–23.11)	.32
β-2 Microglobulin level, µg/mL‡	5.6 (3.97–7.16)	4.9 (3.91–5.91)	7.8 (1.57–14.01)	.63
Prior lines, n‡	4.5 (4.27–4.81)	4.5 (4.24–4.82)	4.6 (3.90–5.24)	.71
Age at randomization, y‡	60.2 (58.87–61.45)	60.5 (59.05–61.97)	58.9 (56.03–61.74)	.47
Male, %†	60.5	59.1	65.9	.49
IgG, %†	47.9	46.8	52.3	.61
Plasma cells in bone marrow aspirate, %‡	35.7 (30.9–40.5)	30.0 (24.8–35.1)	53.8 (43.9–63.7)	.001
Trial 039§				
Overall survival, d*	773.0 (692.0–912.0)	836.0 (694.0–NA)	685.0 (565.0–NA)	.25
Time to progression, d*	147.0 (126.0–168.0)	148.0 (127.0–170.0)	127.0 (97.0–171.0)	.46
Response rate, CR + PR, %†	28.2	27.2	31.7	.29
Response rate, CR + PR + MR, %†	40.5	39.6	43.4	.44
Albumin level, g/L‡	38.6 (38.16–39.04)	38.8 (38.30–39.22)	38.1 (36.97–39.17)	.43
Platelet count, x 109/L‡	193.8 (187.61–200.00)	197.9 (190.85–204.99)	180.4 (167.63–193.14)	.02
C-reactive protein level, mg/L‡	11.1 (9.41–12.78)	9.9 (8.35–11.40)	15.0 (9.87–20.19)	.12
β-2 Microglobulin level, µg/mL‡	5.2 (4.82–5.63)	5.2 (4.70–5.65)	5.4 (4.64–6.15)	.13
Prior lines, n‡	2.0 (1.88–2.04)	1.9 (1.84–2.01)	2.1 (1.89–2.24)	.25

Table 2. *Continued*

Variable	Overall	Nongenomics	Genomics	*P*, genomics vs. non-genomics
Age at randomization, y‡	61.0 (60.26–61.77)	60.8 (59.96–61.65)	61.7 (60.05–63.38)	.32
Male, %†	58.1	57.8	59.0	.85
IgG, %†	57.9	56.7	61.5	.31
Plasma cells in bone marrow aspirate, %‡	31.2 (28.9–33.4)	28.4 (25.9–30.9)	39.6 (35.0–44.2)	.001
Trial 040				
Overall survival, d*	355.0 (277.0–434.0)	349.0 (216.0–464.0)	377.0 (336.0–486.0)	.76
Time to progression, d*	161.0 (137.0–203.0)	168.0 (140.0–234.0)	158.0 (119.0–204.0)	.19
Response rate, CR + PR, %†	29.1	25.3	42.9	.02
Response rate, CR + PR + MR, %†	37.7	32.2	57.1	.002
Albumin level, g/L‡	35.9 (35.13–36.67)	35.9 (35.02–36.73)	36.0 (34.23–37.69)	.69
Platelet count, x 109/L‡	150.6 (137.55–163.57)	154.7 (139.42–169.99)	137.8 (112.63–162.89)	.30
C-reactive protein level, mg/L¶	NA	NA	NA	
β-2 Microglobulin level, μg/mL¶	NA	NA	NA	
Prior lines, n¶	> 3 (4.00–4.00)	> 3 (4.00–4.00)	> 3 (4.00–4.00)	> .999
Age at randomization, y‡	58.2 (56.89–59.41)	58.5 (57.04–60.00)	57.0 (54.62–59.45)	.33
Male, %†	61.5	62.6	57.9	.53
IgG, %†	57.1	59.8	49.1	.17
Plasma cells in bone marrow aspirate, %‡	44.1 (39.0–49.2)	37.9 (31.9–43.8)	62.5 (54.1–70.9)	.001

To convert β-2 microglobulin level from micrograms per milliliter to nanomoles per liter, multiply micrograms per milligrams by 85.

CR indicates complete response; PR, partial response; MR, minimal response; NA, not available; IgG, immunoglobulin G myeloma subtype.

* Median time to event; 95% CI in parentheses; *P* value from log-rank test.

† *P* value from Fisher exact test.

‡ Mean; 95% CI in parentheses; *P* value from Wilcoxon rank sum test.

§ Data for patients receiving bortezomib or Dex.

¶ No more detailed information collected.

However, a nonrandom distribution was observed for clinical study, number of prior therapies, array hybridization batch, myeloma purity score, and, consistent with a recent

report,[14] myeloma TC subtype. Because several of these factors are interrelated (e.g., patients from trial 039 had fewer prior therapies and their samples were hybridized in one batch), it was difficult to discern which factors influence the clustering. We investigated the influence of prior therapies by examining the distribution of samples from trial 025, which have a varied number of prior therapies and were hybridized in a single batch. In fact, patients from trial 025 in groups 1 to 3 had fewer lines of prior therapy (mean = 3.7) than those in groups 4 to 5 (mean = 5.1) ($P = .053$), suggesting that distribution of these samples is at least in part influenced by the extent of prior therapy.

Analysis of gene expression patterns within this dataset revealed several features in common with previously reported studies of myeloma (Figure 2B). These include a reduced expression of genes associated with immune function *(IGH, IGL)*[32] and heterogeneous overexpression of cancer antigens, interferon-induced genes, and genes involved in protein synthesis and proliferative pathways.[30,32,45,46] We also noted differential expression of various genes related to protein secretion and endoplasmic reticulum stress, as well as NF-κB transcription targets (Figure 2B).

Recently, a study highlighted the overexpression of D-type cyclins and other common IgH translocation targets and suggested that newly diagnosed myeloma comprises 8 distinct TC subtypes.[47] These TC subtypes were also observed in this dataset of relapsed/refractory patients collected from multiple clinical centers (Figure 3A). Notably, the frequencies of each TC subtype were very similar in the 2 different datasets (Figure 3B). Figure 3A highlights additional hallmark features of myeloma gene expression, including loss of *FGFR3* expression in a subset of t(4;14)(p16;q32)–positive samples[48] and correlation between overexpression of c-MAF transcription factor and the c-MAF target gene cyclin D2[44] Together these observations indicate that the current genomic dataset, derived from national and international clinical trials, is consistent with previously described data.

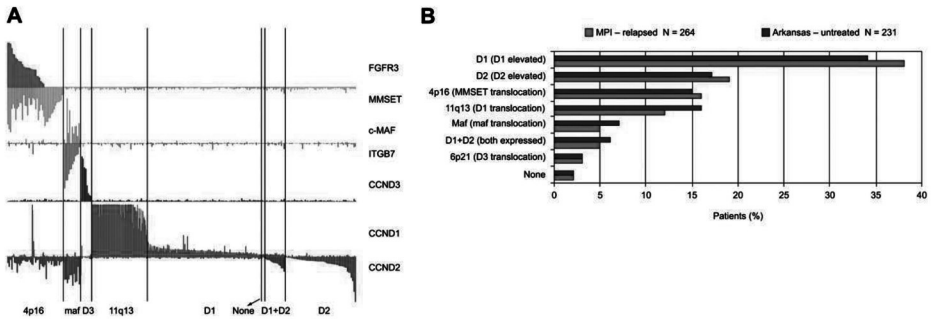


Figure 3. All samples assigned to TC subtypes based on expression of D cyclins and translocation target genes (n = 264). **(A)** The TC subtypes of 264 relapsed myeloma samples are shown. The y-axis shows normalized expression level of each gene; subtypes were determined as in Bergsagel et al.[47] **(B)** A comparison of the TC subtype frequency for relapsed patients in Millennium Pharmaceuticals (MPI)

studies (green) and for newly diagnosed patients (blue) as defined at the University of Arkansas[47] (see page 255 for colour figure).

We noted that a subset of samples express genes generally detected in erythroid or myeloid lineages, including *GYPA* and *CD14* (Figure 2B). The origin of this expression pattern remains unclear. However, such expression has been noted in both normal plasma cells and myeloma cells after positive selection,[14] and in such studies this expression was associated with better prognosis on treatment with HDT.[14]

Can pretreatment gene expression predict response?

Response rates in the various bortezomib trials are shown in Table 2. To investigate whether information in pretreatment tumor samples could predict whether patients would respond to bortezomib, we first used a bootstrap approach, in which samples were repeatedly split into random train and test sets. The mixing of patients from different trials minimized the influence of known and unknown confounding variables. To best distinguish any predictive signal and interpret subsequent biology we initially focused on patients who had either PD or R. A linear predictor classifier[39] to distinguish PD and R was developed in each training set and evaluated on the held-out test data. As shown in Figure 4A, the median test-set accuracy was 70.2% (mean = 69.8%); this accuracy exceeded the accuracy obtained when sample labels in the training set were permuted (mean = 53.6%, 95th percentile = 69.4%).

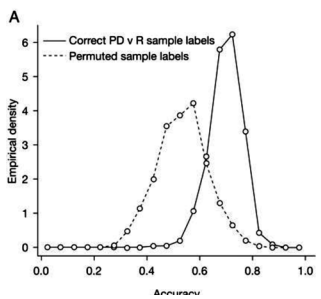
Because data came from several multisite studies with different patient populations, we next assessed whether a predictor developed with data from one study could be validated on another. Using samples from the earliest trial (025), a classifier was developed to distinguish PD and R, and bootstrap validation within trial 025 suggested the classifier should have significant accuracy on other similar data (73% average accuracy, 95% of test sets showing > 55% accuracy). However, this classifier exhibited an overall accuracy of 55% (Sn = 58%, Sp = 47%; $P = .77$) on testing in the bortezomib arm of trial 039, and 57% (Sn = 64%, Sp = 48%; $P = .41$) in the Dex arm. Lack of significance with the samples from trial 039 as an independent test set may relate to differences in patient populations enrolled in these distinct trials (notably, the higher response rate to bortezomib in trial 039), the relatively small sample size of the training set, disease heterogeneity, or a combination of these factors.

We next built a response classifier using data from both the 025 and 040 trials (67 samples from patients with R or PD) and tested it on data from trial 039. As shown in Figure 4B, response in the bortezomib arm was predicted with an overall accuracy of 75%, (Sn = 92%, Sp = 33%; $P = .033$). However, response prediction for the Dex arm was 57% (Sn = 79%, Sp = 32%; $P = .53$), suggesting that the classifier has some specificity for bortezomib. The 100 probe sets comprising this classifier are listed in Table S4. Finally, we obtained similar results when these predictive analyses included patients with NC, who were grouped with PD patients to form a nonresponse (NR) category. Although an 025 trial NR versus R classifier

was unable to significantly predict outcome of the test set from trial 039, an 025 + 040 trials NR versus R classifier exhibited 63% ($P = .03$) and 54% ($P = .3$) overall accuracy when tested in the bortezomib and Dex arms of trial 039, respectively (Table S5). Median bootstrap accuracy of the NR versus R predictor was also 63% (mean = 63.1%); this accuracy exceeded that obtained when the sample labels were permuted (median = 49.2%, mean = 49.4%, 95th percentile = 62.7%; Figure S1). Similar accuracy was noted when the number of probe sets used in the classifier was varied from 50 to 500 (data not shown).

Although we have used the permutation approach and the Fisher exact test to establish that our predictions are significant, it is also common to compare prediction accuracies with that of the best constant model (which is 72% for trial 039 bortezomib arm, and 68% for the bootstrap of all bortezomib-treated patients). Our PD versus R models do not significantly outperform the best constant model. However, this appears to be due to the response classes being unbalanced: a subsampling analysis (Figure S2) shows that our prediction method significantly outperforms the best constant model in the case of equal numbers of PD and R patients. The more-balanced NR versus R prediction also shows significant accuracy (63%) compared with the best constant model (46%; see Document S1 for details).

In summary, we observed a statistically significant prediction of response when data were combined across all the studies or from 2 studies, but not with the 025 study alone.



B

039 Bortezomib arm

		Actual R	Actual PD	Total		
Predicted	R	35	10	45	78%	PPV
	PD	3	5	8	63%	NPV
Total		38	15	53		
		92% Sens	33% Spec	Overall accuracy = 75%		

$P = .033$

039 Dex arm

		Actual R	Actual PD	Total		
Predicted	R	22	17	39	56%	PPV
	PD	6	8	14	57%	NPV
Total		28	25	53		
		79% Sens	32% Spec	Overall accuracy = 57%		

$P = .53$

Figure 4. Prediction scores. **(A)** Data from all bortezomib-treated patients analyzed in bootstrap; the empirical distributions of prediction accuracies for all test sets are shown. Note that the median value of the accuracies for the correctly labeled samples (70.2%) is higher than 95% of the accuracies for the permuted sample labels (95th percentile = 69.4%). Thus, the 2 distributions are significantly different. **(B)** A classifier for trials 025 and 040 was used to predict the response of patients receiving bortezomib and patients receiving Dex in trial 039. Accuracy of response prediction for bortezomib-treated patients is significant ($P < .033$; 75% overall accuracy) but not significant ($P = .53$; 57% overall accuracy) for patients treated with Dex. No significant accuracy is observed when all test samples are simply predicted as the most popular response category ($P > .999$).

Genes and pathways associated with response

A number of the probe sets in the 025 + 040 trials classifier (Table S4) represent genes of known function. Among those overexpressed in PD are ribosomal *(RPS7, RPS13)*, mitochondrial *(COX7C, UQCRH)*, ER stress *(SERP1)*, DNA repair *(APEX1, REC14)*, and cancer-associated *(NRAS, NPM1)* genes. Those overexpressed in patients achieving R include components of the PI3 kinase pathway *(PIK3R1, DAPP1)* and other signaling molecules *(TYROBP, RRAGC, LYK5)*.

We further examined the biology of bortezomib sensitivity by applying GSEA,[49] an algorithm that correlates all approximately 18 000 genes represented on the arrays with a phenotype (R or PD) and highlights known or experimentally annotated sets of genes that are enriched in these phenotypes. This analysis included bortezomib data from trials 024 and 039 as well as that of the samples from 025 + 040 trials. The most significant gene sets relatively highly expressed in samples from responsive patients are shown in Table 3. These include adhesion, cytokines, NF-κB activity, and hypoxia gene sets. Gene sets elevated in samples from patients classified as PD (Table 3) include protein synthesis, mitochondrial function and RNA transcription/splicing. Among the NF-κB targets correlated with R were *IL8*, *IL15*, *CXCL5*, *CFLAR*, *ICAM*, and *NFKB2*, suggesting that expression of a subset of NF-κB targets characterizes myeloma cells more sensitive to bortezomib. This is consistent with various preclinical studies of bortezomib's mechanism of efficacy, showing inhibition of NF-κB signaling and subsequent apoptosis of myeloma[50,51] and other cells[52,53] on treatment with bortezomib.

Several gene sets elevated in samples from patients achieving R encode adhesion molecules, indicating that more adhesive myelomas may be sensitive to bortezomib. This interpretation is also supported by preclinical experiments showing that fibronectin adhesion increases sensitivity of myeloma cells to Bortezomib[54] while reducing sensitivity to melphalan and doxorubicin.[55] Interestingly, on analysis of the smaller datasets from the 039 trial, several gene sets highlighted in Table 3 are strongly correlated with R or PD ($P < .05$) in the bortezomib arm but not the Dex arm; these include brentani cell adhesion and cytokine pathway (Table 3, associated with R), and translation factors and ribosomal proteins (Table 3, associated with PD). Such results imply that these pathway observations are specific to bortezomib.

Can pretreatment gene expression predict survival?

The 039 randomized trial demonstrated superior OS with bortezomib versus Dex (30 versus 24 months; $P = .027$; 22-month median follow-up, 44% events occurred).[56] A significant TTP and survival advantage was also observed at a preplanned interim analysis, at which time all patients were permitted to receive bortezomib and 62% of the patients in the Dex arm subsequently received single-agent bortezomib.[35]

Table 3. Gene sets associated with response (R) and progressive disease (PD).

Gene set*	Description*	All studies, raw fdr	Trial 039	
			Bortezomib, raw npv	Dex, raw npv
Associated with R†				
BRENTANI_CELL_ ADHESION‡	Cancer-related genes involved in cell adhesion and metalloproteinases	0.046	0.0373	NC
MYELIN_DOWN_LE‡	Genes down-regulated in Egr2Lo/Lo mice (mutations in the transcription factor Egr2) with expression altered after sciatic nerve injury	0.057	0.0047	0.1446
PASSERINI_ADHESION‡	Genes associated with cellular adhesion that are differentially expressed in endothelial cells of pig aortas from regions of disturbed flow	0.088	0.0657	0.5820
cytokinePathway‡	Intercellular signaling in the immune system occurs via secretion of cytokines, which promote antigen-dependent B- and T-cell response	0.093	0.0149	NC
KRAS_TOP100_ KNOCKDOWN_ CORDERO‡	Genes up-regulated in K-ras knockdown vs control in a human cell line	0.103	0.0722	0.3936
MOUSE_DENA_UP‡	Genes up-regulated in hepatoma induced by diethylnitrosamine	0.105	0.0684	NC
ST_MYOCYTE_AD_ PATHWAY‡	Cardiac myocytes have a variety of adrenergic receptors that induce subtype-specific signaling effects	0.108	0.0209	0.0618
MYC_293_DOWN‡	Genes down-regulated by MYC in 293T (transformed fetal renal cell)	0.163	0.0401	0.4203
ST_ADRENERGIC‡	Adrenergic receptors respond to epinephrine and norepinephrine signaling	0.175	0.3021	0.2914
HYPOXIA_UP_ MANALO‡	Genes up-regulated in human pulmonary endothelial cells under hypoxic conditions or after exposure to AdCA5 (constitutively active HIF-1α)	0.186	0.1941	0.6324
lairPathway‡	The local acute inflammatory response is mediated by activated macrophages and mast cells or by complement activation	0.188	0.0860	NC
NFKB_UP_HINATA‡	Genes up-regulated by NF-κB	0.201	0.0794	0.2811
Smooth_muscle_ contraction‡	NA	0.227	0.0124	NC
Striated_muscle_ contraction‡	NA	0.233	0.0108	0.3853
TNFA_HEPATO_UP	Genes up-regulated by TNFA in Hc cells (normal hepatocyte)	0.084	0.6158	0.7953

Table 3. *Continued*

Gene set*	Description*	All studies, raw fdr	Trial 039	
			Bortezomib, raw npv	Dex, raw npv
RAS_STROMA_DOWN_CROONQUIST	Genes down-regulated in multiple myeloma cells with N-ras-activating mutations versus those cocultured with bone marrow stromal cells	0.096	0.0804	NC
IL6_STROMA_UP_CROONQUIST	Genes up-regulated in multiple myeloma cells exposed to the pro-proliferative cytokine IL-6 versus those cocultured with bone marrow stromal cells	0.099	0.1527	0.7804
MOUSE_CIP_UP	Genes up-regulated in hepatoma induced by ciprofibrate	0.100	0.0608	NC
Statin_Pathway_PharmGKB	NA	0.159	0.0451	0.9095
BRENTANI_CYTOSKELETON	Cancer-related genes also related to the cytoskeleton	0.185	0.0409	0.6597
Associated with PD§				
Translation_Factors‡	NA	0.048	0.0294	NC
Ribosomal_Proteins‡	NA	0.111	0.0115	NC
ELECTRON_TRANSPORT_CHAIN	Genes involved in electron transport	0.109	0.3554	0.6618
VOXPHOS	Oxidative phosphorylation	0.118	0.3116	0.6303
Electron_Transport_Chain	NA	0.118	0.3535	0.6561
MRNA_PROCESSING	Genes involved in mRNA processing	0.126	0.0916	0.0713
Oxidative phosphorylation	NA	0.157	0.4757	0.9730
MRNA SPLICING	Genes involved in mRNA splicing	0.166	0.1780	0.0247
RNA_transcription_Reactome	NA	0.202	0.2380	0.0351
mRNA processing	NA	0.207	0.0978	0.0137
IFNG_5ENDOTHELIAL_DOWN	Genes down-regulated by interferon-γ in colon, dermal, iliac, aortic, and lung endothelial cells	0.212	0.2767	0.4042
MITOCHONDRIA	Mitochondrial genes	0.216	0.5342	0.3284
HUMAN_MITODB_6_2002	Mitochondrial genes	0.217	0.5601	0.3699
eif2Pathway	Eukaryotic initiation factor 2 (EIF2) initiates translation by transferring Met-tRNA to the 40S ribosome in a GTP-dependent process	0.219	0.8587	NC

Table 3. *Continued*

Gene set*	Description*	All studies, raw fdr	Trial 039	
			Bortezomib, raw npv	Dex, raw npv
GOLDRATH_HP	Genes up-regulated in CD8+ T cells undergoing homeostatic proliferation (HP) versus naive CD8+ T-cell populations; these genes are not up-regulated versus effector or memory cell population	0.224	0.1534	0.5239
CELL_CYCLE_ REGULATOR	Obsolete by GO; was not defined before being made obsolete	0.226	0.0596	0.1615

Top-scoring gene sets from GSEA analysis of the full set of bortezomib samples are shown, along with corresponding FDR statistical scores. The nominal *P* values for the 2 arms of the 039 trial, used to assess the extent to which the gene set associations were treatment specific, are also shown.
NA indicates no description available; NC, no correlation with phenotype.
* Gene set name and description from Molecular Signature Database.39
† Top 20 gene sets are listed.
‡ Gene sets showing generally consistent phenotype association based on analysis of individual trials are shown in ranked order.
§ All 16 gene sets are listed.

We used gene expression data from patients in 025 + 040 trials to develop a survival classifier[41] that was then tested with data from the 039 trial. As shown in Figure 5A, this gene expression classifier stratified the patients in trial 039 receiving bortezomib into high- and low-risk groups that were significantly associated with risk of death ($P < .001$). The classifier also effectively stratified the patients enrolled in the Dex arm of trial 039 ($P < .001$; Figure 5B). It is possible this survival classifier and the underlying probe sets may be prognostic of survival independent of the specific therapy administered. However, there may be some specificity for bortezomib (as observed with the response classifier) that is masked by the subsequent use of bortezomib in the majority of patients enrolled in the Dex arm. Additional analyses and comparisons with other myeloma pharmacogenomics datasets will be required to address these possibilities.

To determine whether the pretreatment gene expression provides data not already captured by prognostic clinical variables, we assessed the survival of patients predicted to be high- or low-risk by ISS.[1] These risk groups are relevant for various myeloma therapies[1] and also discern high/low risk in the 039 trial patients (data not shown). As shown in Figure 5C-D, the gene expression classifier enables further, significant stratification in patients identified as low risk (ISS = 1; Figure 5C) and high risk (ISS = 2-3; Figure 5D) respectively, indicating that clinical staging and genomic information are not redundant but are likely to be complementary.

The probe sets comprising this survival classifier (Table S6) do not overlap with the response classifier. This is not surprising, because the survival and response end points are only partially related. Overexpression of adhesion-related genes *(CDH1, CD36)* are correlated with longer survival, suggesting there may be biologic consistencies, but a more detailed examination of response and survival pathways will be required.

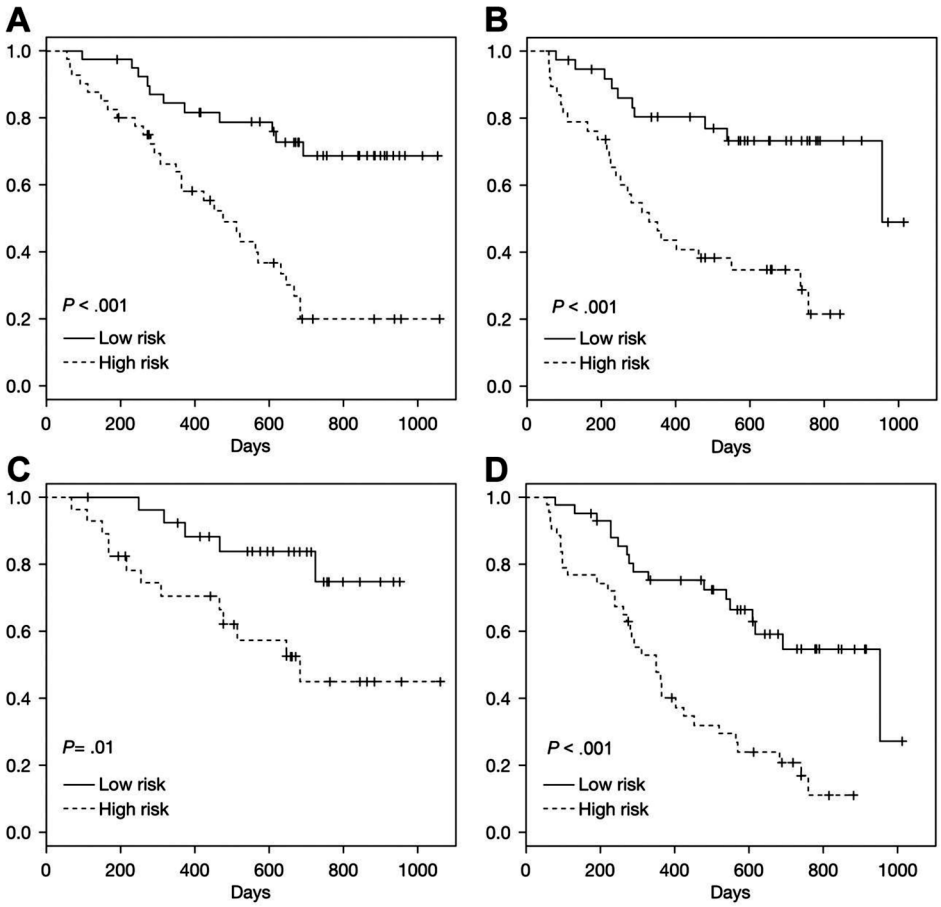


Figure 5. Prediction of survival using Super PC. A survival classifier based on 025 + 040 trials was used to identify high- and low-risk groups within an independent test dataset derived from 039 patients. Kaplan-Meier analyses of the actual survival of these predicted high-/low-risk patient groups is shown for test set **(A)** trial 039 bortezomib, **(B)** trial 039 Dex, **(C)** ISS = 1 for patients from 039 trial (bortezomib or Dex), **(D)** ISS = 2 to 3 for patients from 039 trial (bortezomib or Dex).

DISCUSSION

Clinical genomics offers great promise to improve cancer diagnosis, prognosis, and treatment selection. However, this type of research requires large datasets derived from well-characterized, uniformly treated patients with appropriate end point data.[12,41,57] This study describes a myeloma gene expression dataset derived from large prospective clinical trials, and the lessons from this research highlight both the challenges and advantages of the implementation of similar research in the future.

The first challenge was sample attrition (Table 1). Inadequate RNA, because of insufficient tumor sampling or RNA degradation, precluded use of approximately 50% of collected samples. Across these myeloma trials, patient consent, sample acquisition, and data generation/quality control produced only limited losses; however, even small losses at each stage compounded the attrition issue.

Second, the necessary analysis of data from multiple clinical trials and comparisons between trials were made more difficult because of differences between trials. Patients in the phase 2 trials had experienced more prior therapy and were less responsive than patients enrolled in the phase 3 trial (Table 1; Table S3).[33-35] It will be interesting to compare further these data from relapsed patients with data from newly diagnosed myeloma. The near identical frequency of TC subtypes in both relapsed and newly diagnosed myeloma (Figure 3B) indicates the TC categories do not define any subgroup of patients that is rapidly lost after first-line therapy; other ways of comparing these datasets may reveal such high-risk patient types. A final caveat to future studies is the time required for prospective research. In this example, despite bortezomib's rapid advance to phase 3 trials in myeloma, more than 4 years elapsed between the initial sample collection in phase 2 trials and the genomic analysis of the updated survival data from the phase 3 trial.

Despite such issues and differences in purity methodologies, these clinical trials yield a myeloma dataset consistent with a previous single-center study (Figure 3).[47] The data are primarily derived from patients who were subsequently treated with bortezomib but includes a subset of control patients whose treatment was Dex.

We identified a pretreatment gene expression pattern and predictive classifier that is significantly associated with subsequent response to bortezomib but not Dex. Although the association with response appears to be subtle, the significance is supported by bootstrap analyses as well as testing of independent data.[57,58] This comparison of predictive accuracy for bortezomib and Dex is not complicated by the previously mentioned confounding variables (extent of prior therapies and prognostic features) because the independent test data derives from patients enrolled in a randomized study that controlled for the number of prior therapies and β-2M levels in each study arm.[35] The apparent specificity suggests that there are distinct subsets of patients sensitive to bortezomib or Dex and that these subsets can be distinguished by pretreatment tumor gene expression. A distinct genomic

classifier based on OS also showed statistical significance when tested with independent data. However, at this time, we cannot determine whether this association is specific for bortezomib treatment, because the majority of patients in the Dex arm were subsequently treated with bortezomib.

An overview of the gene sets significantly associated with response to bortezomib (Table 3) highlighted pathways, such as NF-κB activity and cell adhesion, whose functions were already clearly implicated as relevant to bortezomib activity *in vitro*.[50-54] This overlap between genomic analyses of clinical specimens and preclinical model systems is encouraging and suggests that some preclinical systems may provide relevant information regarding the drug sensitivity of patients. Many of the pathways associated with PD regulate protein biosynthesis and mitochondrial function, which could relate to protein load in secretory myeloma cells [59,60] or the status of mitochondrial apoptotic pathways.[61] Consistent with the response prediction, some of these pathways (e.g., adhesion- and cytokine-related gene sets) appeared to be bortezomib specific when data for bortezomib versus Dex were compared (Table 3). It will be important to induce and/or inhibit these pathways in model systems to test whether their activity confers sensitivity or resistance to bortezomib, Dex, and/or other anticancer agents.

We note that the survival classifier described here captures outcome-related information that is distinct from clinical prognostic variables (e.g., serum albumin and β-2M) as demonstrated by the significant capacity to discern risk groups within the high- as well as the low-risk ISS groups (Figure 5). Studies in lymphoma have drawn similar conclusions.[62] Multivariate analyses to integrate the genomic and clinical variables are being investigated; it is hoped that merging these complementary data will enable a better understanding of both clinical trial populations and individual patients.

The predictive accuracy required of a clinical diagnostic for myeloma treatment has not yet been defined. Requirements may vary according to disease stage, therapeutic options (single-agent versus combination regimens), and whether therapy is likely to achieve disease control or cure. Although the classifier described here is promising, further refinement is necessary before it can be considered for clinical use in predicting patient response to single-agent bortezomib in the relapsed setting. The 75% overall accuracy (92% Sn, 33% Sp) might be improved with more patient samples, or it may be that there is not adequate information in the RNA levels of pretreatment, purified myeloma samples to make a significantly more accurate prediction. Additional research is needed to assess the relevance of these genomic predictors in newly diagnosed myeloma and in the context of multiagent therapy that is fundamental to more-effective treatment of myeloma. Key data for such analyses will emerge from genomic research in other large clinical trials, including Total Therapy 2 and 3,[14,32] as well as the ongoing HOVON cooperative trial comparing vincristine, doxorubicin, and Dex with bortezomib, doxorubicin, and Dex as induction therapy in newly diagnosed patients. These analyses will help to rapidly highlight the patient groups that benefit from drug combinations as well as those still in need of novel therapies.

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CHAPTER 6

A GENE EXPRESSION SIGNATURE FOR HIGH-RISK MULTIPLE MYELOMA

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ABSTRACT

There is a strong need to better predict survival of patients with newly diagnosed multiple myeloma (MM). Since gene expression profiles (GEPs) reflect the biology of MM in individual patients, we built a prognostic signature based on GEPs.

GEPs obtained from newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial (n = 290) were used as training data. Using this set, a prognostic signature of 92 genes (EMC-92-gene signature) was generated by supervised principal components analysis combined with simulated annealing.

Performance of the EMC-92-gene signature was confirmed in independent validation sets of newly diagnosed (TT2, n = 351; TT3, n = 142; MRC-IX, n = 247) and relapsed patients (APEX, n = 264). In all sets, patients defined as high-risk by the EMC-92-gene signature show a clearly reduced overall survival with hazard-ratios (HR) of 3.4 (95% CI:2.19–5.29) for the TT2 study, HR:5.23 (2.46–11.13) for the TT3 study, HR:2.38 (1.65-3.43) for the MRC-IX study and HR:3.01 (2.06–4.39) for the APEX study ($p<0.0001$ in all studies). In multivariate analyses this signature was proven independent of currently used prognostic factors.

The EMC-92-gene signature is better or comparable to previously published signatures. This signature contributes to risk assessment in clinical trials and could provide a tool for treatment choices in high-risk multiple myeloma patients.

INTRODUCTION

Multiple myeloma (MM) is characterized by accumulation of malignant monoclonal plasma cells in the bone marrow. Median overall survival (OS) for newly diagnosed patients treated with high dose therapy varies from 4 to 10 years.[1,2]

The International Staging System (ISS), based on serum β_2-microglobulin and albumin, is widely used as a prognostic system for patients with newly diagnosed MM. ISS has been confirmed as a solid prognostic factor in clinical trials.[1] Additional clinical factors to define high-risk disease have not been consistently reproduced, with the exception of extensive disease represented by renal failure and plasma cell leukemia.[2,3] In addition to ISS, cytogenetic aberrations such as deletion of 17p (del(17p)), translocations t(4;14) and t(14;16) were shown to be associated with an adverse prognosis. The combination of prognostic markers t(4;14), del(17p) and ISS enabled further delineation of patients into prognostic subgroups.[4]

A strategy to include genetic characteristics of MM is the translocation and cyclin D (TC) classification, which distinguishes 8 subgroups based on genes which are deregulated by primary immunoglobulin H translocations and transcriptional activation of cyclin D genes.[5]

Subsequently, the University of Arkansas for Medical Sciences (UAMS) generated a molecular classification of myeloma based on gene expression profiles of patients included in their local trials. The UAMS molecular classification of myeloma identifies seven distinct gene expression clusters, including the translocation clusters MS, MF, and CD-1/2, a hyperdiploid cluster (HY), a cluster with proliferation-associated genes (PR), and a cluster characterized by low percentage of bone disease (LB).[6] More recently, we extended this classification based on the HOVON-65/GMMG-HD4 prospective clinical trial and identified additional molecular clusters, i.e. NF-κB, CTA and PRL3.[7] Because these clusters were discriminated based on disease specific gene expression profiles (GEP), we and others hypothesized that they may be relevant for therapy outcome. Indeed, the UAMS defined clusters MF, MS and PR were found to identify high-risk disease in the Total Therapy 2 trial.[6]

Several survival signatures were developed based on samples from clinical trials, such as the UAMS-70, the related UAMS-17 and the recently published UAMS-80 signature which have value in prognostication of MM.[8-10] Other signatures include the Medical Research Council (MRC) gene signature based on the MRC-IX trial, the French Intergroupe Francophone du Myélome (IFM) signature and the Millennium signature based on relapse patients.[11-13] Recently, a GEP based proliferation index was reported.[14] So far, none of these signatures have been introduced in general clinical practice.

The additional and independent prognostic significance of a prognosticator based on gene expression has been acknowledged in mSMART (Mayo Stratification for Myeloma And Risk-adapted Therapy). Hereby, a high-risk MM population can be defined for which alternative treatment is proposed although this has not been validated in prospective clinical trials.[15]

The aim of the present study was to develop a prognostic signature for overall survival of MM patients. This investigation was prospectively included as a secondary analysis of a randomized clinical trial for newly diagnosed, transplant-eligible patients with multiple myeloma (HOVON-65/GMMG-HD4).

MATERIALS AND METHODS

Patients

As training set the HOVON-65/GMMG-HD4 study (ISRCTN64455289) was used. Details of the training set are given in the supplemental document A.[16] Informed consent to treatment protocols and sample procurement was obtained for all cases included in this study, in accordance with the Declaration of Helsinki. Use of diagnostic tumor material was approved by the institutional review board of the Erasmus Medical Center. Arrays used for analysis passed extensive quality controls, as described previously.[7] Of the 328 gene arrays deposited at the NCBI-GEO repository, clinical outcome data was available for 290 patients (accession number: GSE19784).

Four independent datasets were used as validation of which both survival data were available as well as GEPs of purified plasma cells obtained from bone marrow aspirates of myeloma patients. The datasets Total Therapy 2 (UAMS-TT2; n = 351; GSE2658; NCT00573391), Total Therapy 3 (UAMS-TT3; n = 142; E-TABM-1138; NCT00081939) and MRC-IX (n = 247; GSE15695; ISRCTN68454111) were obtained from newly diagnosed patients. The APEX dataset (n = 264; GSE9782; registered under M34100-024, M34100-025 and NCT00049478/ NCT00048230) consisted of relapsed myeloma cases (see supplemental document A).[11,17-23]

Gene expression pre-processing

To allow gene expression analysis in the HOVON-65/GMMG-HD4, plasma cells were purified from bone marrow aspirates obtained at diagnosis, using immunomagnetic beads. Only samples with a plasma cell purity of ≥ 80% were used. Gene expression was determined on a Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array.

To allow for validation across different studies, only probe sets present on both the U133 Plus 2.0 and the U133 A/B platforms were included (n = 44754). Probe sets having an expression value below the lowest 1% bioB hybridization control in more than 95% of the samples are excluded. This resulted in 27680 probe sets to be analyzed. All data were MAS5 normalized, log2 transformed and mean-variance scaled, using default settings in the Affy package in Bioconductor.[24]

The normalized validation gene expression data sets were downloaded from the repositories NCBI-GEO (APEX, MRC-IX, UAMS-TT2) and ArrayExpress (UAMS-TT3). Datasets UAMS-TT2, UAMS-TT3 and MRC-IX were generated using the U133 Plus 2.0 platform whereas

the Affymetrix HG U133 A/B platform was used in the APEX study. The IFM dataset was not included in our analysis due to an incompatible, custom platform.

The strong batch effect that exists between these GEP studies was successfully removed by ComBat using the non-parametric correction option.[25] APEX was run on a different array platform with an incomplete overlap in probe sets with the other datasets, and as a result ComBat correction was applied in two separate runs with one run for all analyses involving the APEX data set and an additional run for all other analyses.

Survival signature

The HOVON-65/GMMG-HD4 data were used as a training set. GEP and PFS data were combined for building a GEP based survival classifier. PFS was used for generating a classifier for OS since PFS was the primary endpoint of the HOVON-65/GMMG-HD4 study and PFS demonstrated a higher number of events compared to OS (179 PFS vs. 99 OS events in total in the HOVON-65/GMMG-HD4). All evaluations of the signature are based on OS data in training and validation sets. Analyses were performed using R with the survival package for survival analyses.[26] Out of 27680 probe sets tested, 1093 probe sets were associated to PFS in univariate Cox regression analyses (FDR < 10%; for probe sets and survival data see supplemental document B). Subsequently, this set was used as input into a supervised principal component analysis (SPCA) framework in combination with simulated annealing (see supplemental documents A and B).[27] This analysis yielded a model of 92 probe sets, termed the EMC-92 signature. The survival signature is a continuous score, i.e. the sum of standardized expression values multiplied by the probe set specific weighting coefficient (Table S1 and R-script, supplemental document C). High-risk disease was defined as the proportion of patients with an overall survival of less than two years in the training set.

Validation of the EMC-92 signature

A multivariate Cox regression analysis was performed for patients with available covariates. Covariates with < 10% of the data missing were used as input in a backward stepwise selection procedure ($P < 0.05$).

The EMC-92 signature together with seven previously described, external signatures for OS in multiple myeloma have been analyzed in a pair-wise comparison using a multivariate Cox regression analysis. This analysis was performed for all pair-wise comparisons on the pooled datasets excluding the training sets for the signatures being tested. The models were stratified for study.

Pathway analysis

Pathway analysis was performed using the 92 genes corresponding to the EMC-92 signature as well as the 1093 genes generated by univariate PFS analysis (FDR< 10%) with the probe sets used as input for the analysis as a reference set (n = 27680; Ingenuity Systems®, www.

Ingenuity.com). P-values were derived from right-tailed Fisher exact tests and corrected for multiple testing by a Benjamini-Hochberg correction.[28]

RESULTS

The EMC-92 signature

GEPs obtained from newly diagnosed MM patients were analyzed in relation to survival data, in order to generate a classifier to distinguish high-risk from standard-risk disease. We used the HOVON-65/GMMG-HD4 data as a training set.[7] After filtering for probe set intensity, using internal Affymetrix control probe sets, 27680 probe sets were analyzed in a univariate Cox regression analysis with progression free survival (PFS) as survival endpoint. This resulted in 1093 probe sets associated with PFS with a false discovery rate of < 10% (supplemental document B). Based on these 1093 probe sets, a supervised principal components analysis based model was built in which simulated annealing was applied to generate the optimal model settings in a 20-fold cross-validation. The final predictive model consisted of 92 probe sets with specific weighting coefficients. The sum of normalized intensity values multiplied by this weighting is the output of the signature. This model was termed the EMC-92 signature. A positive weighting coefficient indicates that increased expression contributes to a higher value for the EMC-92 signature value and thus a higher risk for poor survival. The majority of the probe sets are annotated genes (n = 85, with one of the genes represented by two probe sets). The remaining probe sets are open reading frames (n = 3), expressed sequence tags (n = 2) and one additional probe set without annotation. Several known cancer genes are among these genes, of which *FGFR3* (weighting coefficient: 0.06), *STAT1* (weighting coefficient: 0.05) and *BIRC5* (weighting coefficient: 0.02) were described in detail in relation to myeloma (Table S1; all supplemental tables are given in the Supplemental document A).[29-31]

To define a high-risk population, the cut-off threshold for the continuous signature score was set to a value of 0.827 based on the proportion of patients in the training set that had an overall survival of less than two years (63 out of 290 patients (21.7%); Figure S2).

Four independent validation datasets were available: UAMS-TT2, UAMS-TT3, MRC-IX and APEX. Gene expression datasets UAMS-TT2 and TT3 consisted of 351 and 142 transplant-eligible patients whereas the MRC-IX dataset contained both transplant-eligible and non-transplant-eligible MM patients (n = 247). In the APEX dataset, GEPs of 264 relapse patients were collected. The results of the EMC-92 signature in the validation sets are shown in Figure 1 and Table S2. In the UAMS-TT2 dataset, the EMC-92 signature identified a high-risk population of 19.4% with a hazard-ratio of 3.40, 95% confidence interval (CI) = 2.19–5.29 ($P = 5.7 \times 10^{-8}$). In the UAMS-TT3, 16.2% of patients were identified as high-risk with a hazard-

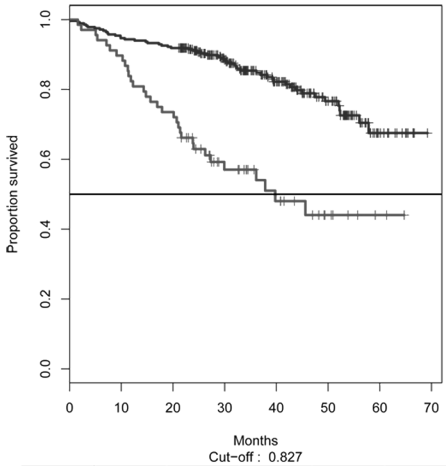
ratio of 5.23, 95% CI = 2.46–11.13, ($P = 1.8 \times 10^{-5}$). In the MRC-IX dataset, 20.2% of patients were identified as high-risk with a hazard-ratio of 2.38, 95% CI = 1.65-3.43, ($P = 3.6 \times 10^{-6}$). The high-risk signature was able to identify patients with significantly shorter survival in both the transplant-eligible and non-transplant-eligible patients included in the MRC-IX study. In non-transplant-eligible patients, 23.9% high risk patients were identified with a hazard-ratio of 2.38, 95% CI = 1.47–3.86, ($P = 4.3 \times 10^{-4}$), whereas 16.8% of transplant-eligible patients were high-risk with a hazard-ratio of 2.54, 95% CI = 1.43–4.52 ($P = 1.5 \times 10^{-3}$; Figure 1c and d). The signature was not restricted to newly diagnosed patients, as 16.3% of patients included in the APEX relapse dataset were designated high-risk with a hazard-ratio of 3.01, 95% CI = 2.06–4.39, ($P = 1.26 \times 10^{-8}$; Figure 1e and 2d).

To assess the relation between EMC-92 signature outcome and treatment, we evaluated whether there is evidence for differences in survival between treatment arms in the high-risk group or standard-risk group. Within the high-risk patients of the HOVON-65/GMMG-HD4 trial, the survival of bortezomib treated patients was longer than patients treated with conventional chemotherapy (VAD) (30 months compared to 19 months), albeit not significant (p = 0.06; number of bortezomib treated patients: 26 vs. 37 in the VAD arm). Within the high-risk patients of MRC-IX, no difference was observed between the treatment arms (p = 0.5: MRC-IX non-transplant eligible: CTDa n = 14 vs. MP n = 12) and p = 1.0 (MRC-IX transplant eligible; CTD n = 16 vs. CVAD n = 7). For the standard risk patients no differences in survival between treatment arms were found in either trial.

Multivariate analysis was performed in the training set and in the APEX and MRC-IX validation sets, for which information on a large number of variables were available. This showed that in addition to the EMC-92 signature, del(17p) was an independent predictor in HOVON-65/GMMG-HD4. Furthermore, in both HOVON-65/GMMG-HD4 and in the APEX multivariate analysis, a component of the ISS was an additional independent prognostic predictor (beta-2-microglobulin for the HOVON-65/GMMG-HD4 set and serum albumin for the APEX data set). Trial specific covariates were seen in each multivariate analysis such as sub-study in the APEX dataset and the MP treatment arm in the MRC-IX set. In conclusion, in all three datasets of newly diagnosed and relapse MM patients the EMC-92 signature performed as the strongest predictor for survival after inclusion of available covariates (Table 1). For univariate associations to survival see Table S3.1-S3.3.

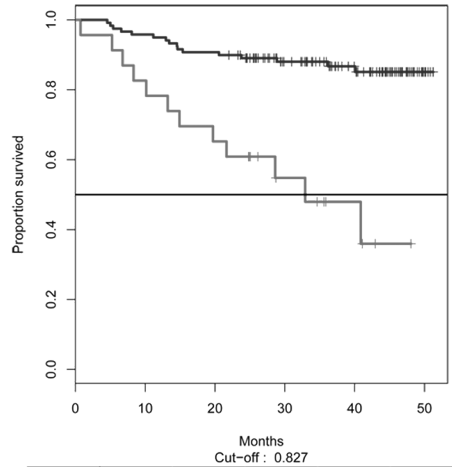
Using the nearest neighbor classification method, all patients in the validation sets were classified into molecular clusters based on the HOVON-65/GMMG-HD4 classification.[7] A clear enrichment of the MF, MS, PR clusters and decreased proportion of the HY cluster was found in the pooled high-risk populations of all validation sets (Table S4).

(1A)



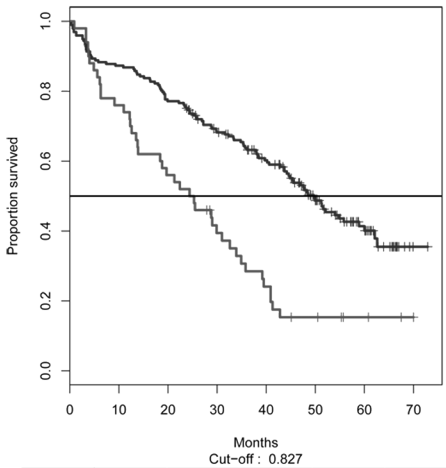
Group	N	Events	HR	Wald P	Median
Standard	283	53	1.00		
High	68	32	3.40	5.7e−08	39.8

(1B)



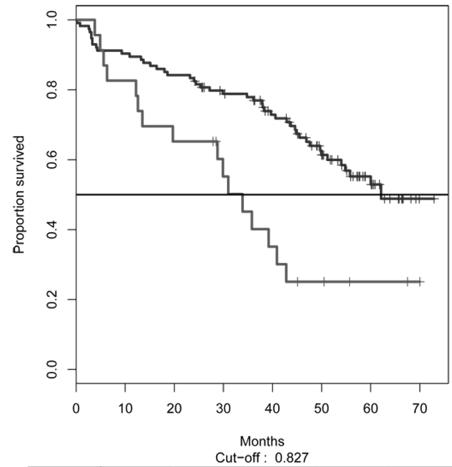
Group	N	Events	HR	Wald P	Median
Standard	119	16	1.00		
High	23	12	5.23	1.8e−05	32.9

(1C)

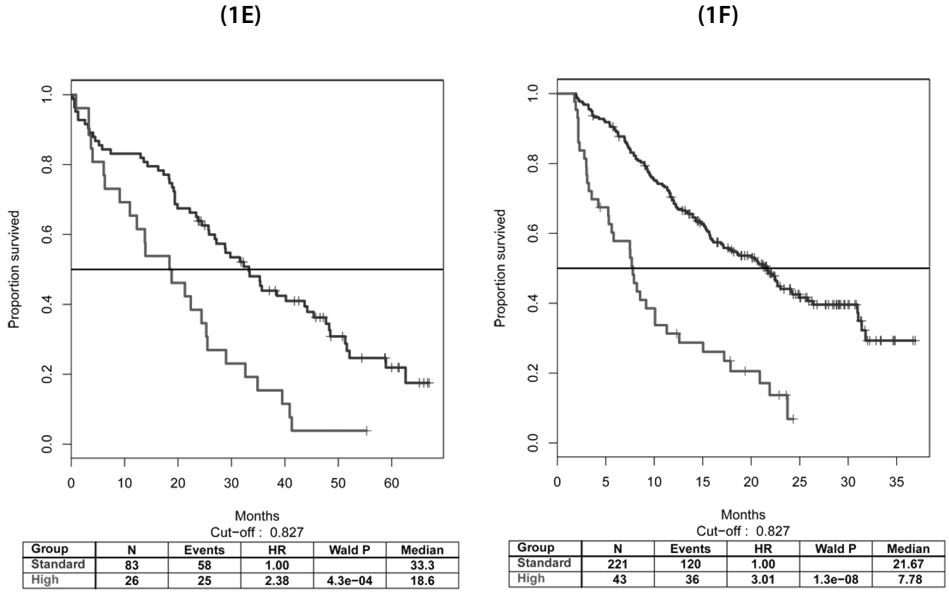


Group	N	Events	HR	Wald P	Median
Standard	197	104	1.00		49.7
High	50	41	2.38	3.6e−06	24.4

(1D)



Group	N	Events	HR	Wald P	Median
Standard	114	46	1.00		62.1
High	23	16	2.54	1.5e−03	33.9



Group	N	Events	HR	Wald P	Median
Standard	83	58	1.00		33.3
High	26	25	2.38	4.3e−04	18.6

Group	N	Events	HR	Wald P	Median
Standard	221	120	1.00		21.67
High	43	36	3.01	1.3e−08	7.78

Figure 1. Kaplan-Meier overall survival curves for EMC-92 signature defined high-risk patients versus standard-risk patients in five validation sets. The cut-off value is fixed at 0.827 based on proportion of patients with OS <2 years in the HOVON-65/GMMG-HD4 set. In the MRC-IX one patient had an unknown treatment status and was disregarded in figures c and d. **(A)** UAMS Total Therapy 2. **(B)** UAMS Total Therapy 3. **(C)** MRC-IX. **(D)** MRC-IX transplant-eligible patients. **(E)** MRC-IX non-transplant-eligible. **(F)** APEX. N, number of patients; Events, number of events; HR, hazard ratio; Wald P, p value for equality to standard-risk group; Median, median survival time (see pages 255 and 256 for colour figures).

Comparison to published gene signatures

We set out to evaluate the performance of the EMC-92 signature in relation to available GEP based prognostic signatures for OS in multiple myeloma. To this end, the following signatures were evaluated: UAMS-70, UAMS-17, UAMS-80, IFM-15, gene proliferation index (GPI-50), MRC-IX-6 and, MILLENNIUM-100.[9-14]

These signatures were evaluated as continuous variables as well as using the cut-off values as published (Figure 2A-E, Figure S2 and supplemental documents A and B). Overall, the performance of the EMC-92 signature is robust, consistent and compares favorably to previously published signatures. Specifically, the EMC-92, UAMS, MRC-IX and GPI-50 signatures demonstrated significance in all validation sets tested both for the dichotomized and the continuous values of the signatures. Significance was reached in 3 out of 5 studies for the IFM-15 signature using a dichotomized model, whereas the MILLENNIUM-100 signature had significant performance in the dichotomized model in 1 out of 4 independent studies. Thus, performance was less robust for the IFM-15 and MILLENNIUM-100 signatures.

Although the proliferation index GPI-50 was found to be significant in all validation sets tested, the proportion of high-risk patients was much lower compared to the proportion found using either the EMC-92 or the UAMS-80 signatures. Ranked, weighted high-risk proportions are GPI: 10.0%, UAMS-17: 12.4%, UAMS-70: 13.0%, MRC-IX-6: 13.3%, EMC-92: 19.1% and UAMS-80: 23.4%. To determine which signature best explained the observed survival, pair-wise comparisons were performed. For every comparison the EMC-92 is the strongest predictor for OS tested in an independent environment (Figure 3 and Table S9).

Table 1. Multivariate analysis for the EMC-92-gene in the HOVON-65/GMMG-HD4 (1.1), APEX (1.2) and MRC-IX (1.3). Covariates that were non-missing in more than 90% of the patients were included. Variants were selected into the model by a backward stepwise approach ($p \leq 0.05$).

1.1

	EMC-92-gene (Cut-off: 0.827)		
	HOVON-65/GMMG-HD4 (n = 290)		
	Hazard-ratio	(95% CI)	Wald P
EMC-92-gene [1/0]	3.44	(2.20–5.37)	5.1E-08
β2m [≥ 3.5mg/L]	2.42	(1.48–3.35)	4.1E-04
Del(17p) [1/0]	2.23	(1.36–3.68)	1.6E-03
WHO [> =1]	2.07	(1.30–3.29)	2.1E-03

Likelihood ratio test = 95.8 on 4 df, p = 0 n = 257, number of events = 93; 33 observations deleted due to missingness

Available covariates: Del(17p)[1/0], Del(13p)[1/0], 1q Gain[1/0], Age[yr], Age[≥ 60 yr], Bortezomib treated[1/0], ISS=2[1/0], ISS=3[1/0], Female[1/0], Creatinine[mg/dL], Creatinine[< 20 mg/dL], β2m[mg/L], β2m[≥ 3.5mg/L], β2m[≥ 5.5mg/L], Serum albumin[g/L], Serum albumin[≤ 3.5 g/L], LDH[>ULN], IgA[1/0], IgG[1/0], Light Chain Disease[1/0], k light chain[1/0], Diffuse osteoporosis[1/0], Hemoglobin[mmol/L], Hemoglobin[< 6.5 mmol/L], Hemoglobin[< 5.3 mmol/L], Calcium[mmol/L], Calcium[> 2.65mmol/L], WHO[>=1], WHO[>=2], WHO[>=3], WHO[=4]

1.2

	EMC-92-gene (Cut-off: 0.827)		
	APEX (n = 264)		
	Hazard-ratio	(95% CI)	Wald P
EMC-92-gene [1/0]	2.42	(1.62–3.61)	1.50E-05
Serum albumin [g/L]	0.95	(0.93–0.98)	1.20E-04
Age [≥ 60 yr]	1.73	(1.23–2.43)	1.60E-03
IgG [1/0]	0.64	(0.46–0.90)	1.00E-02
studyAPEX [1/0]	0.58	(0.41–0.82)	1.80E-03

Likelihood ratio test = 64.5 on 5 df, p = 1.43e-12 n = 250, number of events = 150; 14 observations deleted due to missingness

Available covariates: Age [yr], Age [≥ 60 yr], Age [≥ 65 yr], Bortezomib treated [1/0], Female [1/0], Black [1/0], White [1/0], IgA [1/0], IgG [1/0], Light chain [1/0], studyCREST [1/0], studySUMMIT [1/0], studyAPEX [1/0], studyAPEXprogressive [1/0], Serum albumin [g/L], Serum albumin [≤ 3.5 g/L], Priorlines

Table 1. *Continued*

1.3			
	EMC-92-gene (Cut-off: 0.827)		
	MRC-IX (n = 247)		
	Hazard-ratio	(95% CI)	Wald P
EMC-92-gene [1/0]	2.48	(1.69–3.64)	3.4E-06
Age [yr]	1.04	(1.02–1.07)	3.0E-05
Hemoglobin [mg/L]	0.86	(0.79–0.95)	1.8E-03
MP treatment	1.63	(1.09–2.44)	1.8E-02

Likelihood ratio test = 74.8 on 4 df, p = 2.11e-15 n = 246, number of events = 145; 1 observation deleted due to missingness.

Available covariates: Del(13q)[1/0], IgH split[1/0], Hyperdiploid[1/0], t(4;14)[1/0], t(11;14)[1/0], t(14;16)[1/0], t(14;12)[1/0], t(6;14)[1/0], Del(17p)[1/0], 1qGain[1/0], Female[1/0], Bone disease[1/0], Albumin[g/L], Albumin[≤ 3.5g/L], Hemoglobin[mg/L], Hemoglobin[< 8.5 mg/L], Hemoglobin[< 10.5 mg/L], Calcium[mmol/L], Calcium[> 2.65mmol/L], Creatinine[mg/dL], Creatinine[< 20 mg/dL], WHO[>=1], WHO[>=2], WHO[>=3], WHO[=4], Age[yr],Age[≥ 60 yr], Age[≥ 65yr], Intensive treatment[1/0], CVAD treatment[1/0], CTD treatment[1/0], MP treatment[1/0], CTDA treatment[1/0]

There is a varying degree of overlapping probe sets between all signatures (Figure S3). Seven out of fifty probe sets present in the GPI-50 overlap with the EMC-92 signature (*BIRC5, FANCI, ESPL1, MCM6, NCAPG, SPAG5* and *ZWINT*). One of the six MRC-IX genes (*ITM2B*) is also seen in the EMC-92. Overlap between EMC-92 and the remaining signatures is limited (EMC92 vs. UAMS17/70: *BIRC5* and *LTBP1*; EMC-92 vs. MILLENNIUM-100: *MAGEA6* and *TMEM97* and EMC-92 vs. IFM-15: *FAM49A*).

Combined risk classifiers

The performance of the EMC-92 signature was in line with the UAMS signatures, although they were derived from quite different patient populations. The intersection of high-risk patients between the EMC-92 and UAMS-70 signatures was ~8% of the total population on the pooled datasets that were independent of both our training set and the UAMS-70 training set (i.e. MRC-IX, TT3 and APEX; Table S11). Approximately 13% of patients were classified as high-risk by either one of these signatures. The intersecting high-risk group had the highest hazard-ratio as compared to the intersecting standard-risk group (HR = 3.87, 95% CI = 2.76-5.42, $P = 3.6 \times 10^{-15}$). Patients classified as high-risk by either signature, showed an intermediate risk, i.e. with an HR of 2.42, 95% CI = 1.76-3.32, for the EMC-92 signature ($P = 5.1 \times 10^{-8}$) and an HR of 2.22, 95% CI = 1.20-4.11, for the UAMS-70 signature ($P = 1.1 \times 10^{-2}$; Table S12). To test whether there is evidence for better performance if outcomes of two dichotomous predictors are merged, we took the models made in the pair-wise comparison (Table S9) and tested these in a likelihood-ratio test against a single signature outcome

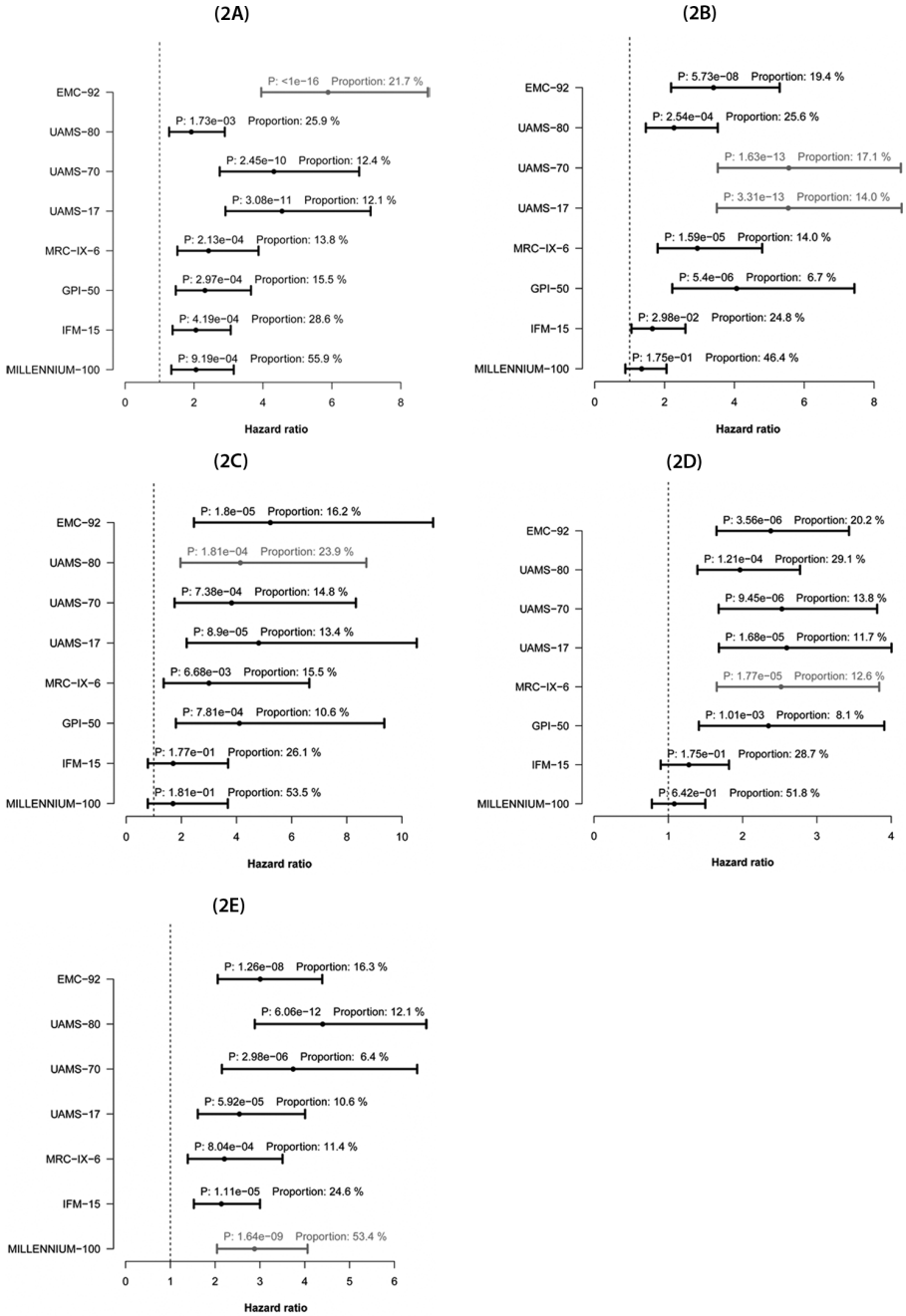


Figure 2. Performance per signature in available datasets. For every signature the hazard ratio (high-risk versus standard-risk) is shown with 95% confidence interval. Grey lines indicate results on training set. **(A)** HOVON-65/GMMG-HD4. **(B)** UAMS-TT2. **(C)** UAMS-TT3. **(D)** MRC-IX. **(E)** APEX. P, p value for equal survival in high and standard-risk groups; Proportion, proportion of high risk defined patients.

model. Merging the EMC-92 with UAMS-80 ($p = 2.19 \times 10^{-3}$), UAMS-17 ($p = 9.36 \times 10^{-3}$), GPI-50 ($p = 2.95 \times 10^{-2}$), MRC-IX-6 ($p = 1.58 \times 10^{-2}$) and UAMS-70 ($p = 3.96 \times 10^{-2}$) demonstrated a better fit to the data than any of the single models (Table S10).

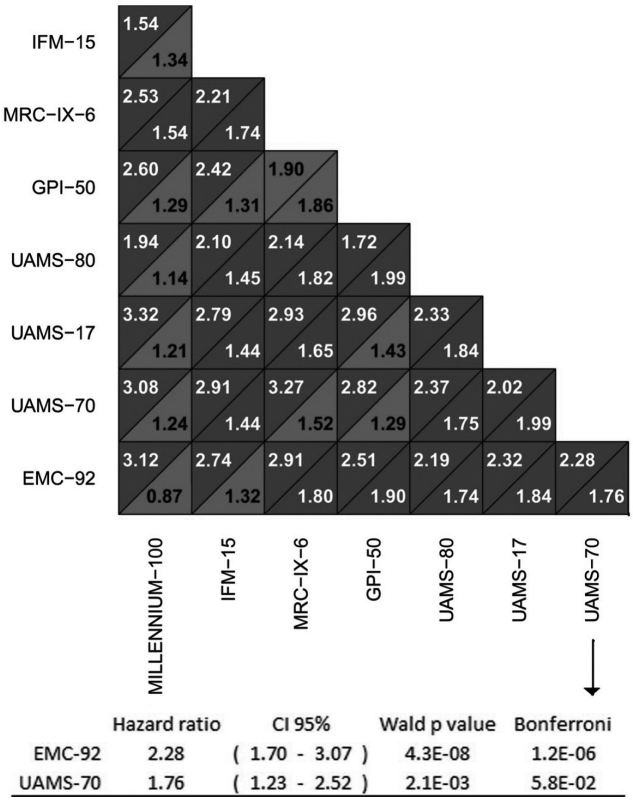


Figure 3. Pair-wise comparison for all signatures. To find the signature best fitting the underlying datasets, Cox regression models (high-risk versus standard-risk) were made for all pair-wise signatures. These models are based on pooled independent datasets (i.e. excluding training sets) and stratified for study. The two paired hazard ratios associated with the signatures derived per model are shown in the two cells within the square panels. Only hazard ratios within one panel can be compared because these are based on the same dataset. Dark gray cells indicate significance hazard ratios (Bonferroni-Holm corrected P-value); light gray cells denote non-significant findings. For the bottom right panel (i.e. UAMS-70 vs. EMC-92 signatures) the underlying model is given. All other models can be found in Table S9.

EMC-92 signature and FISH

To compare the high-risk populations composition as defined by the EMC-92 and the UAMS-70 signatures, cytogenetic aberration frequencies in both populations were determined using an independent set for which cytogenetic variables were known, i.e. MRC-IX (Figure 4 and Table S13). As expected, poor prognostic cytogenetic aberrations 1q gain, del(17p), t(4;14), t(14;16), t(14;20) and del(13q) were enriched in the high-risk populations (Figure 5), whereas the standard risk cytogenetic aberrations such as t(11;14) were diminished in the high-risk populations. In contrast, only 15% (6 out of 39) of MRC-IX cases with high-risk status as determined by the EMC-92 signature showed absence of any poor prognostic cytogenetic aberrations, as opposed to 44% (74 out of 168) in standard risk cases ($P = 1.8 \times 10^{-3}$). Similarly, of the UAMS-70 defined high-risk patients 4% (1 out of 23) did not have any poor prognostic cytogenetics, whereas of the UAMS-70 defined standard risk patients this proportion was 43% (79 out of 183) ($P = 5.3 \times 10^{-3}$).

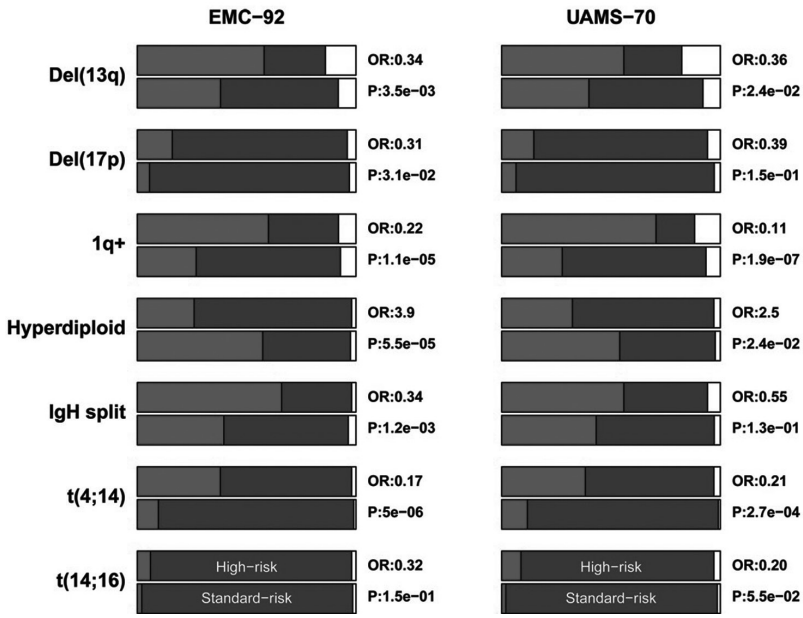


Figure 4. Distributions of high-risk and standard-risk patients per FISH marker in the MRC-IX dataset. Distribution of FISH markers within the high-risk (top panels) and standard-risk (bottom panels) groups for the EMC-92 and UAMS-70 signatures. The EMC-92 and UAMS-70 identified 50 and 42 patients out of 247 as high-risk, respectively. OR, Odds-ratio ; P, Fisher exact *p*-value; light gray, presence of an aberration; dark gray, absence of an aberration; white, missing data. Details are given in Table S13.

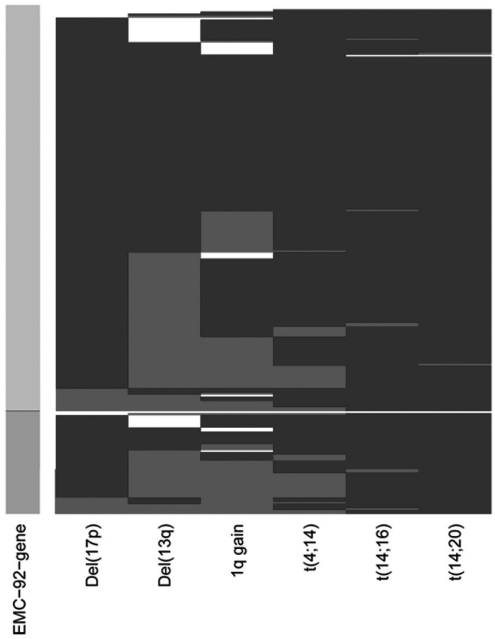


Figure 5. Poor prognostic cytogenetic aberrations in comparison to the EMC-92 signature in MRC-IX patients. Each horizontal line represents one patient. The first column denotes the distinction between high-risk (in light gray, n = 50) and standard risk (in dark gray, n = 197). Columns 2 to 7 represent cytogenetic aberrations as shown. Light gray, presence of an aberration; dark gray, absence and white, missing data. More than half of the EMC-92 standard risk patients are affected by one or more poor FISH markers.

DISCUSSION

Here we report on the generation and validation of the EMC-92 signature, which was based on the HOVON-65/GMMG-HD4 clinical trial. Conventional prognostic markers such as ISS stage and adverse cytogenetics have been augmented by signatures based on gene expression in order to increase accuracy in outcome prediction in MM. More accurate prognosis may lead to the development of treatment schedules which are specifically aimed at improving survival of high-risk MM patients. Prognostic signatures for MM include the UAMS-70, the UAMS-17, the UAMS-80, the IFM-15, the gene proliferation index (GPI-50), the MRC-IX-6 and the MILLENNIUM-100 signatures.

For clinical relevance, a signature must have both the ability to separate risk groups as clearly as possible and to predict stable groups of relevant size. The EMC-92 signature meets both criteria. In all validation sets a high-risk group of patients can be significantly

determined and the proportion of high-risk patients is stable across the validation sets. The validation sets represent different drug regimens, including thalidomide (MRC-IX, TT2) and bortezomib (APEX, TT3). Also the signature is relevant to both transplant eligible (e.g. TT3) and non-transplant eligible patients (subset of MRC-IX) as well as newly diagnosed (e.g. TT2) and relapsed patients (APEX). In contrast, the predictions of the IFM-15 and MILLENNIUM-100 signatures in the validation sets fail to reach significance in independent data sets such as MRC-IX and TT3. The differences in gene expression platform may have contributed to this. Indeed, the IFM signature is based on a custom cDNA based gene expression platform, rather than the Affymetrix GeneChips which have become common for MM GEP studies.[32] The cDNA platforms have been reported to be difficult to compare to the Affymetrix oligonucleotide platform.[12] Although the MILLENNIUM signature was generated using Affymetrix GeneChips, the use of an earlier version of this platform may have contributed to the limited performance of this signature.[11] The performance of the EMC-92 signature is comparable to the UAMS derived signatures, MRC-IX-6 and the GPI-50, as measured by the significance of prediction in validation sets. For the UAMS-70 and GPI-50 the proportion high-risk patients appears more variable, which may hinder clinical interpretation, especially when the high-risk proportion is less than 10%. Importantly, pair-wise comparisons of all the signatures evaluated in this paper demonstrated that the EMC-92 has the best fit to the observed survival times in independent sets.

Strikingly, we found that performance can be improved by simply combining signatures (e.g. EMC-92 with UAMS-80). However, this analysis is only an indication of the possibilities of combining signatures, and future work involving more complex combined signatures is in progress.

It is important to note that the genes within the signature reflect optimal performance of the signature rather than a biological definition of survival in MM. The initially selected 1093 probe sets which were found to be associated with PFS in univariate testing, are more likely to give a good representation of myeloma biology, as indicated for instance by the protein synthesis related pathways. Although an extended biological discussion is outside the scope of this paper, a number of interesting genes are included in the signature. *BIRC5* was found in 4 signatures evaluated in this paper: EMC-92, UAMS-17, UAMS-70 and the GPI-50. This gene is a member of the inhibitor of apoptosis gene family, which encodes negative regulatory proteins that prevent apoptotic cell death, and up-regulation has been described to be associated with lower EFS and OS in newly diagnosed MM patients.[11,12,31] Other important myeloma genes include *FGFR3* and *STAT1*. *FGFR3* is deregulated as a result of translocation t(4;14), which is an adverse prognostic cytogenetic event.[30] *FGFR3*, a transmembrane receptor tyrosine kinase, is involved in the regulation of cell growth and proliferation.[33] *STAT1*, important component of the JAK/STAT signaling, is involved in multiple pathways, including apoptosis induced by interferon signaling.[29]

A clear enrichment of the long arm of chromosome 1 was observed in the 1093 probe sets in this study. Previously the importance of chromosome 1 was reported for the UAMS-70 signature. Genes on 1q in the UAMS-70 signature include *CKS1B* and *PSMD4*, both of which were not in the EMC- 92 signature, although *CKS1B* was found to be associated with PFS in our set and thus in the 1093 set.[9,10] The EMC-92 signature did contain 9 genes on 1q of which *S100A6* has been described in relation to 1q21 amplification in MM and other cancer types.[34] This may also be part of the explanation why, despite the use of the same GEP platform, the overlap between different signatures is limited. Indeed, multiple genes are found within the 1q21 amplicon with downstream factors possibly over-expressed as a result of this. Which gene will be linked most significantly to survival in a specific set is most likely due to factors such as variability in datasets, to which population differences and differences in used techniques may contribute.

Other reasons may be found in the difference in treatment strategies used, in which other genes could be responsible for adverse prognosis.

To characterize the high-risk group in depth, we have demonstrated that in the MRC-IX study, high-risk patients are enriched for poor cytogenetic aberrations 1q gain, del(17p), t(4;14), t(14;16), t(14;20) and del(13q). Still more than half of the patients in the standard risk group showed one or more poor prognostic cytogenetic markers indicating that the occurrence of a single poor-risk marker does not have very strong prognostic value.

Clinical use of a gene signature (UAMS-70) has recently been incorporated in the mSMART risk stratification, which additionally includes FISH, metaphase cytogenetics, and plasma cell labeling index. The mSMART risk stratification is the first risk stratification system adjusting treatment regimens according to risk status, although this has not been validated in prospective clinical trials.[15,35] Ultimately clinical use of any signature must be proven to be of use in prospective clinical trials, which allow treatment choice based on risk assessment. This will result in clinical guidelines to improve treatment of patients with a poor PFS and OS on novel therapies. For practical application of the EMC-92 signature it is essential to stress that this signature has not been designed for classification of a single patient. However, collection of a set of more than ~25 patients will result in reliable prediction, and each additional patient can be predicted as soon as it is tested.

In conclusion, we developed a risk signature highly discriminative for patients with high-risk versus standard-risk MM, irrespective of treatment regime, age and relapse setting. Use of this signature in the clinical setting may lead to a more informed treatment choice and potentially better outcome for the patient.

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CHAPTER 7

CANCER TESTIS ANTIGENS IN NEWLY DIAGNOSED AND RELAPSE MULTIPLE MYELOMA: PROGNOSTIC MARKERS AND POTENTIAL TARGETS FOR IMMUNOTHERAPY

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ABSTRACT

Background: In multiple myeloma, expression of cancer testis antigens may provide prognostic markers and potential targets for immunotherapy. Expression at relapse has not yet been evaluated for a large panel of cancer testis antigens, which can be classified by varying expression in normal tissue: restricted to testis, expressed in testis and brain and not restricted but selectively expressed in testis.

Design and methods: Evaluation of cancer testis antigen expression was performed in newly diagnosed multiple myeloma cases (HOVON-65/GMMG-HD4 trial; n = 320) and in relapse cases (APEX, SUMMIT, CREST trials; n = 264). Presence of expression using Affymetrix GeneChips was determined for 123 cancer testis antigens, of which 87 had a frequency of more than 5% in the newly diagnosed and relapsed patients and were evaluated in detail.

Results: For 58 out of 87 cancer testis antigens tissue restriction was known. A significantly lower frequency of presence calls in the relapsed compared to newly diagnosed cases was found for 3 out of 13 testis restricted genes, 2 out of 7 testis/brain restricted genes and 17 out of 38 testis selective genes. *MAGEC1, MAGEB2* and *SSX1* were the most frequent testis-restricted cancer testis antigens in both data sets. Multivariate analysis demonstrated that presence of *MAGEA6* and *CDCA1* were clearly associated with shorter progression free survival, and presence of *MAGEA9* with shorter overall survival in the set of newly diagnosed cases. In the set of the relapse cases, presence of *CTAG2* was associated with shorter progression free survival and presence of *SSX1* with shorter overall survival.

Conclusions: Relapse multiple myeloma reveals extensive cancer testis antigen expression. Cancer testis antigens are confirmed as useful prognostic markers in newly diagnosed MM patients and in relapse MM patients.

INTRODUCTION

Improvements in the treatment of multiple myeloma (MM) have resulted in significantly improved survival, which is limited to 4-5 years.[1-3] For younger MM patients treatment consisting of high-dose chemotherapy with autologous stem-cell transplant (ASCT) is available, often including novel agents both pre-transplant (induction treatment) and post-transplant (maintenance treatment).[4] Residual disease after treatment is an important issue, for which specific therapeutic approaches such as immunotherapy may be of value.[5-7] Immunotherapy of cancer types such as melanoma and non-small lung cancer has demonstrated the clinical relevance of this treatment approach.[8] Optimized peptide and DNA vaccination protocols demonstrate ongoing improvements in immunotherapeutic intervention.[9,10] A critical requirement for immunotherapy is that tumor associated antigens (TAAs) are expressed in tumor cells when disease re-emerges after therapy. To this end, we have evaluated the gene expression of an important family of TAAs, the cancer testis antigens (CTAs) in relapse samples and we have compared this to expression in newly diagnosed MM cases. CTA expression after treatment has been shown for a limited number of CTAs including *PASD1*, *CTAG1B* and *MAGEC1/CT7*.[11-15] In addition, in MM, expression of CTA genes has been shown to be strongly correlated to clinical outcome, i.e. presence of CTA expression has been linked to shorter survival.[16] Similarly, in other tumor types, CTA expression has been linked to prognosis.[17,18] Prognostic implications of CTAs post-therapy have not been evaluated systematically. Three expression patterns have been defined for CTAs: expression restricted to testis, restricted to testis and brain and expression in other tissues but strong expression in testis (testis-selective).[19] Evaluation of CTAs in relation to tissue restriction is highly relevant to issues of likelihood of side-effects of immunotherapy. Here, the expression of 123 CTAs, spanning these categories, was evaluated further in relapse cases and in newly diagnosed MM.[20-22]

DESIGN AND METHODS

Patients

Bone marrow aspirates of newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial were processed as described previously.[22] This trial is a large multicenter, prospective, randomized phase III trial, evaluating bortezomib as front-line treatment (EudraCT nr 2004-000944-26; registered at www.trialregister.nl as NTR213.[20] Gene expression profiles of the APEX, SUMMIT and CREST trials were collectively described.[21] These multicenter trials (USA, Canada, Europe and Israel) evaluated bortezomib in relapsed MM. Number of prior therapies in these trials ranged from 1 to 14, with a median of 3. Plasma cells were obtained from bone marrow aspirates as described.[21] Informed consent was obtained for all cases

included in this study, in accordance with the Declaration of Helsinki. The approval of local ethics committees was obtained.

RNA isolation and microarray processing

For HOVON-65/GMMG-HD4, RNA from samples with a plasma cell purity > 80% was extracted using the RNeasy kit (Qiagen). After a double in vitro transcription reaction, biotinylated cRNA was hybridized to Affymetrix GeneChip HG U133 plus 2.0 arrays. 320 cases passed quality controls and were included (GSE19784).[22] Similarly, amplification, hybridization (Affymetrix HG U133 A/B) and quality control were applied to the APEX/SUMMIT/CREST samples.[21] Based on the quality control measure Normalized Unscaled Standard Errors (NUSE) obtained using the AffyPLM package (Bioconductor), the replicate chip with the lowest NUSE value was used for the analysis. The APEX/SUMMIT/CREST dataset provided 264 cases with gene expression profiles, in which a gene expression based myeloma purity score was used to exclude samples with an apparently low purity (GSE9782).[21]

Preprocessing and gene selection

The raw data files (CEL-files) were analyzed using the mas5 calls algorithm available in the affy package (Bioconductor).[23] This resulted in a presence call for a specific probe set for a specific patient, or an absence call. Here the frequency of presence calls per probe set for both the newly diagnosed and relapse patient sets was reported. The CTA list (n = 253) was obtained from the current version of the CT database, a CTA classification website initiated by the Ludwig Institute for Cancer Research and the Laboratório Nacional de Computação Científica.[8,24] Using Affymetrix annotation in combination with the online gene compendium GeneCards V3, forty-eight genes did not have corresponding probe sets, with the gene symbol present (n = 37) or absent (n = 11) in GeneCards (Table S1).[25] Based on the CT database, twelve genes were excluded from further analysis, either due to being splicing variants of other genes (which are reported here) or due to overlapping and essentially being other genes (*LAGE-1b, XAGE-3b, CT16.2, CTAGE-2, MMA1b* (splicing variants), *GAGE3, CT47B1,SPANXE, BAGE2, BAGE3, BAGE4, BAGE5*). This resulted in 193 genes out of 253 genes were found to be represented by probe sets on either the U133A and B chip (n=173) or were present on the U133 Plus 2.0 chip (n = 193, 20 present only on U133 Plus 2.0.[25] Based on normal testis expression (GSE1133) and the CT database, probe sets/genes not expressed in normal testis were excluded, which resulted in the exclusion of twenty-four genes (U133 A and B) and 22 overlapping genes for the U133 Plus 2.0 chip (Table S1; GSE1133). Due to the genetic proximity of a number of genes, 26 genes on U133AB and 29 genes on U133 Plus 2.0 were represented by probe sets corresponding to closely related CTA genes. Examples include the GAGE cluster of genes of which 16 genes are represented by 2 probe sets (Table S1). For genes with more than one probe set, the probe set with the highest percentage of present calls was used. For five genes with discrepancies between the two types of chips, i.e.

positive difference between probe sets in one platform and a negative difference between probe sets in another platform, arbitrarily the probe set with the highest presence call on the U133AB chip was used (Table S2). Finally, 142 probe sets (171 genes) on U133 Plus 2.0 and 123 probe sets (149 genes) on U133AB were evaluated. To avoid reporting identical findings (i.e. different genes but same probe sets), the results will be presented per probe set with the most prominent gene given (Table S1). Therefore, the set of 123 probe sets was used to represent 123 genes for comparison in presence frequency between newly diagnosed and relapse cases, and an overlapping set of 142 genes were reported on for the newly diagnosed patients (i.e. 142–123 = 19 genes exclusively reported for newly diagnosed patients). To set a filter for general prevalence of CTAs, a 5% presence call frequency cut-off was used for the populations tested. Genes with a presence call frequency of below 5% were reported in the Supplemental Tables. 87 CTA genes had a presence call frequency of more than 5% and were discussed in the main body of this report. It should be noted that a presence call for any given gene using the mas5 algorithm represents a robust level of quantifiable mRNA.

For 94 genes out of 142 genes newly diagnosed cases determined on the U133 Plus 2.0 chip, expression in normal tissue has been determined.[19] Twenty-one were classified as testis-restricted, 64 as testis-selective and 9 as testis/brain restricted. For the 123 genes on the U133AB chip: 17 were testis-restricted, 58 testis-selective and 9 testis/brain restricted.[19]

Presence of CTA expression in normal plasma cells was evaluated using the GSE6477 data set.[26] Presence calls were determined as described above using the mas5 algorithm. Due to the U133A chip used in this data set, the number of genes evaluated was restricted to 82 (Table S6).

Statistical analysis

Differences in frequency of presence calls were evaluated with the Fisher Exact test with Benjamini-Hochberg correction.[27] Survival analysis was performed in newly diagnosed cases (HOVON-65/GMMG-HD4 trial) as well as in relapse samples, restricted to samples belonging to the APEX trial (n = 156). Presence of CTA genes was analyzed in relation to progression free survival and overall survival. In the HOVON-65/GMMG-HD4 trial, to prevent bias caused by patients receiving allogeneic stem cell transplantation, these patients were censored where appropriate. For 229 HOVON-65/GMMG-HD4 cases clinical follow-up data was available. Kaplan-Meier analysis was applied with log-rank test. To correct for multiple testing Benjamini-Hochberg correction was performed with false discovery rate of 5%.[27] Genes significantly associated with progression free survival and overall survival were further analyzed by Cox regression analysis by backward elimination. For the HOVON-65/GMMG-HD4 study, ISS stage and cytogenetic covariates were included (1q gain, 17p loss and translocations t(4;14), t(11;14) and t(14/20)/t(14;16)). For the APEX study, ISS stage was combined with TC classification, which was used as a substitute for cytogenetic covariates.

TSPY1 was excluded for survival analysis, since it is expressed only in males. To avoid analysis of too many small groups, genes with a presence frequency overall of > 5% and< 95% were evaluated by survival analysis (n = 84, HOVON-65/GMMG-HD4; n = 61, APEX study; Table S1).

Correlation analysis

To evaluate the correlation between CTAs, cluster analysis was performed using 57 genes with known tissue restriction, presence frequency of >5% (Table 1A, B; Table S1) and excluding *TSPY1* (located on Y). Cluster 3.0 and TreeView software by Eisen et al. was used, which is available through the BRB-analysis tool.[28] After median centering of the genes, the clustering was performed using the uncentered Pearson correlation combined with the complete linkage option. Clusters were characterized by determining the differential expression of genes within a specific cluster versus all other clusters using the ClassComparison tool ($p < 1*10^{-7}$).

RESULTS

Expression of CTAs in MM patients

Expression of CTA genes was evaluated in MM at diagnosis (n = 320) and at relapse (n = 264). Newly diagnosed cases were taken from the HOVON-65/GMMG-HD4 trial, and relapse cases were taken from the combined APEX/SUMMIT/CREST trials. Patient characteristics are provided in the Supplementary Information (Table S3). A significant difference in age distribution was found as a result of the HOVON-65/GMMG-HD4 inclusion criteria which stipulate participation of patients younger than 65 years old only whereas there was no age restriction for the APEX/SUMMIT/CREST trials. In addition, and in agreement to a more advanced disease state, thrombocytopenia was more frequent in relapse cases compared to newly diagnosed cases.

Based on the CT database (see Design and Methods for details), 123 CTA genes were available for evaluation in both newly diagnosed and relapse patients. For 87 of these 123 CTAs, a frequency of more than 5% was found in one of the study populations (Table 1, Table S4). The genes with low presence frequency are presented in supplemental Table S5. For 58 of the 87 genes, the tissue expression restriction in normal tissue has been evaluated previously.[19] The expression categories, i.e. restricted to testis (TR), restricted to testis and brain (TBR) and testis selective (TS) are given (Table 1, Table S1, S4, S5). *MAGEC1, MAGEB2* and *SSX1* were the most frequent TR CTAs in both data sets, present in 71%, 47% and 30% in newly diagnosed patients, respectively and present in 61%, 28% and 30% in relapse patients, respectively. At least one of these 3 genes was found in 266 out of 320 (83%) newly diagnosed cases, compared to 188 out of 264 (71%) relapse cases (Figure S1). One or more

of the top 3 TBR genes are found in almost all cases both in newly diagnosed cases (98%) and in relapse cases (99%) with *FAM133A* present in 86% and 79% of newly diagnosed and relapse cases, respectively (Table 1A). A vaccine strategy simultaneously targeting these 3 top TBR genes would be expected to of relevance to almost all relapse MM cases. Strikingly, TS genes *SPAG9, CASC5* and *PBK* were expressed in more than 85% of relapse cases (99.6%, 89.4% and 86.4%, respectively).

Three CTA expression categories were apparent: increased, decreased or similar frequency of expression in the relapse cohort as compared to the newly diagnosed cohort. Significantly higher frequency of presence calls in relapse cases compared to newly diagnosed cases was found for none of the TR genes, 3 out of 7 TBR genes and 10 out of 38 TS genes (Table 1, Table S4). Most pronounced increase in the TBR genes was noted for *GAGE4* and *GAGE8*, with a 4-fold increase from 16.6% and 15%, respectively, in newly diagnosed MM cases to 71.2% and 61.4%, respectively, in relapse MM cases. Assessing the frequency of high expression, within the cases with a present call, *GAGE4* and *GAGE8* both demonstrated a much lower proportion of cases with high expression in relapse cases compared to newly diagnosed cases. For TR gene *TEX14*, a decreased frequency of expression is coupled to a higher frequency of high expression cases in relapse compared to newly diagnosed cases (Table S6).

Decrease of CTA expression in relapse cases is of particular interest for immunotherapy, as decreased expression may prohibit use of specific CTAs as vaccine targets. A significantly lower frequency of presence calls in relapse cases compared to newly diagnosed cases was found for 3 out of 13 TR genes, 2 out of 7 TBR genes and 17 out of 38 TS genes (Table 1). For instance, TR gene *MAGEB2* was found in 47% of newly diagnosed cases and in 27% of relapse cases. It is important to stress here that 75% (15 out of 20) of the most important putative immunotherapeutic targets, i.e. TR/TBR genes, demonstrate unchanged or higher expression frequencies in relapse cases compared to newly diagnosed cases. A lower proportion, but still 55% (21/38) of TS genes are found in equal or increased frequency in relapse cases compared to newly diagnosed cases.

For the genes without known normal tissue restriction, the majority, 20/29, demonstrate a significantly reduced frequency in relapse cases whereas only 2 genes are significantly higher in relapse cases compared to newly diagnosed MM cases (Table S4). *SPAG4, TCC52* and *RQCD1* expression is found in > 95% of both newly diagnosed and relapse cases.

Overall, 58 genes with known tissue restriction and 29 genes without known tissue restriction were evaluated in both newly diagnosed and relapse MM cases, and 45 out of 87 genes were found to remain at comparable level or demonstrate increased presence frequency (52%). Finally, a subset of genes was not represented by the probe sets available on the U133AB platform used for relapse cases, and therefore only assessed in newly diagnosed cases. These genes are listed in Table S4. *CPXCR1* (TR; 52%) and *CCDC36* (TBR; 56%) are the most frequently found genes in this category in the newly diagnosed cases.

Table 1. Presence of CTA gene expression in newly diagnosed and relapsed MM patients. All testis-restricted, testis/brain-restricted and top 5 of testis-selective CTAs with expression presence of more than 5% in either newly diagnosed or relapse MM population are given first (first 25 genes). The last 19 genes in this table represent genes only present on platform U133Plus2.0.

Gene symbol	Probe set	Tissue restriction	Newly diagnosed		Relapse		
			n	%	n	%	*p*-value
MAGEC1	206609_at	testis-restricted	228	71.3	160	60.6	**0.008**
MAGEB2	206218_at	testis-restricted	151	47.2	73	27.7	**<0.0001**
SSX1	206626_x_at	testis-restricted	97	30.3	78	29.5	0.9
MAGEA1	207325_x_at	testis-restricted	70	21.9	42	15.9	0.07
TSPY1	207918_s_at	testis-restricted	34	10.6	36	13.6	0.3
MAGEA2	214603_at	testis-restricted	30	9.4	22	8.3	0.8
TEX14	221035_s_at	testis-restricted	23	7.2	8	3	0.03
SSX2	210497_x_at	testis-restricted	21	6.6	17	6.4	1
PAGE2	231307_at	testis-restricted	19	5.9	6	2.3	0.04
MAGEB1	207534_at	testis-restricted	17	5.3	10	3.8	0.4
MAGEB4	207580_at	testis-restricted	17	5.3	3	1.1	**0.01**
SPANXC	220217_x_at	testis-restricted	16	5	8	3	0.3
SSX3	211670_x_at	testis-restricted	9	2.8	15	5.7	0.1
FAM133A	239481_at	testis/brain-restricted	276	86.3	209	79.2	0.03
CTNNA2	205373_at	testis/brain-restricted	194	60.6	70	26.5	**<0.0001**
CTAGE1	220957_at	testis/brain-restricted	180	56.3	242	91.7	**<0.0001**
MAGEC2	220062_s_at	testis/brain-restricted	93	29.1	25	9.5	**<0.0001**
GAGE4	208155_x_at	testis/brain-restricted	53	16.6	188	71.2	**<0.0001**
GAGE8	207086_x_at	testis/brain-restricted	48	15	162	61.4	**<0.0001**
MAGEA9	210437_at	testis/brain-restricted	35	10.9	15	5.7	0.03
SPAG9	212470_at	testis-selective	320	100	263	99.6	0.5
CTAGE5	215930_s_at	testis-selective	306	95.6	128	48.5	**<0.0001**
PBK	219148_at	testis-selective	301	94.1	228	86.4	**0.002**
ZNF165	206683_at	testis-selective	266	83.1	36	13.6	**<0.0001**
JARID1B	211202_s_at	testis-selective	264	82.5	89	33.7	**<0.0001**
CASC5	228323_at	testis-selective	238	74.4	236	89.4	**<0.0001**
CEP290	205250_s_at	testis-selective	218	68.1	135	51.1	<0.0001
OIP5	213599_at	testis-selective	218	68.1	98	37.1	**<0.0001**
CCDC110	230900_at	testis-selective	207	64.7	48	18.2	**<0.0001**
MPHOSPH1	205235_s_at	testis-selective	196	61.3	20	7.6	**<0.0001**
MORC1	220850_at	testis-selective	184	57.5	86	32.6	**<0.0001**
ACRBP	223717_s_at	testis-selective	144	45	20	7.6	**<0.0001**
MAGEA6	214612_x_at	testis-selective	144	45	130	49.2	0.3
FATE1	231573_at	testis-selective	126	39.4	93	35.2	0.3
SPA17	205406_s_at	testis-selective	122	38.1	24	9.1	**<0.0001**

Table 1. *Continued*

Gene symbol	Probe set	Tissue restriction	Newly diagnosed		Relapse		
			n	%	n	%	*p*-value
MAGEA3	209942_x_at	testis-selective	121	37.8	125	47.3	**0.02**
SSX4	210394_x_at	testis-selective	110	34.4	78	29.5	0.2
TSGA10	220623_s_at	testis-selective	109	34.1	13	4.9	**<0.0001**
SPO11	222259_s_at	testis-selective	89	27.8	3	1.1	**<0.0001**
MAGEA5	214642_x_at	testis-selective	82	25.6	37	14	**0.001**
DDX43	220004_at	testis-selective	77	24.1	42	15.9	**0.02**
TPTE	220205_at	testis-selective	77	24.1	90	34.1	**0.01**
XAGE1	220057_at	testis-selective	65	20.3	66	25	0.2
SPACA3	243621_at	testis-selective	61	19.1	67	25.4	0.07
CTAG1B	211674_x_at	testis-selective	60	18.8	43	16.3	0.4
CTAG2	215733_x_at	testis-selective	54	16.9	38	14.4	0.4
MAGEA12	210467_x_at	testis-selective	49	15.3	89	33.7	<0.0001
C21orf99	237794_at	testis-selective	47	14.7	103	39	**<0.0001**
CABYR	219928_s_at	testis-selective	41	12.8	2	0.8	**<0.0001**
LDHC	207022_s_at	testis-selective	36	11.3	0	0	**<0.0001**
DSCR8	241224_x_at	testis-selective	33	10.3	24	9.1	0.7
LEMD1	229927_at	testis-selective	28	8.8	27	10.2	0.6
ROPN1	233203_at	testis-selective	28	8.8	44	16.7	**0.01**
CRISP2	210262_at	testis-selective	20	6.3	7	2.7	0.05
SYCP1	206740_x_at	testis-selective	18	5.6	11	4.2	0.5
MAGEA4	214254_at	testis-selective	10	3.1	15	5.7	0.2
SPINLW1	206318_at	testis-selective	5	1.6	53	20.1	**<0.0001**
PIWIL2	220686_s_at	testis-selective	2	0.6	61	23.1	**<0.0001**
SPAG4	219888_at	not available	320	100	257	97.3	**0.004**
TCC52	224789_at	not available	319	99.7	264	100	1
RQCD1	213179_at	not available	318	99.4	264	100	0.5
TMEFF1	205122_at	not available	296	92.5	94	35.6	**<0.0001**
TMEFF2	224321_at	not available	288	90	251	95.1	0.03
GPATCH2	242224_at	not available	272	85	180	68.2	**<0.0001**
KIAA0100	201728_s_at	not available	258	80.6	246	93.2	**<0.0001**
CEP55	218542_at	not available	247	77.2	48	18.2	**<0.0001**
TTK	204822_at	not available	220	68.8	134	50.8	<0.0001
LOC130576	228360_at	not available	216	67.5	71	26.9	**<0.0001**
IGSF11	228375_at	not available	195	60.9	33	12.5	**<0.0001**
CDCA1	223381_at	not available	187	58.4	148	56.1	0.6
SPEF2	232745_x_at	not available	184	57.5	64	24.2	**<0.0001**
NOL4	206045_s_at	not available	174	54.4	112	42.4	**0.005**

Table 1. *Continued*

Gene symbol	Probe set	Tissue restriction	Newly diagnosed		Relapse		
			n	%	n	%	*p*-value
TMEM108	223524_s_at	not available	154	48.1	11	4.2	**<0.0001**
ELOVL4	219532_at	not available	147	45.9	10	3.8	**<0.0001**
PEPP2	220952_s_at	not available	146	45.6	115	43.6	0.7
PTPN20A	215172_at	not available	106	33.1	17	6.4	**<0.0001**
PRAME	204086_at	not available	102	31.9	100	37.9	0.1
ODF1	214485_at	not available	91	28.4	12	4.5	**<0.0001**
GPAT2	235557_at	not available	85	26.6	43	16.3	**0.003**
ARX	238878_at	not available	80	25	14	5.3	**<0.0001**
ANKRD45	236421_at	not available	69	21.6	7	2.7	**<0.0001**
CCDC62	231567_s_at	not available	65	20.3	41	15.5	0.2
IMP-3	203820_s_at	not available	64	20	19	7.2	**<0.0001**
LOC440934	230844_at	not available	61	19.1	0	0	**<0.0001**
ODF2	225617_at	not available	47	14.7	69	26.1	**0.001**
MAEL	229475_at	not available	36	11.3	14	5.3	**0.01**
SPANXB2	220921_at	not available	27	8.4	5	1.9	0.0004
MAGEB3	207579_at	testis-restricted	9	2.8	8	3	1
CXorf48	221121_at	testis-restricted	8	2.5	6	2.3	1
DKKL1	220284_at	testis-restricted	0	0	0	0	NA
SPANXA1	220922_s_at	testis-restricted	0	0	0	0	NA
PASD1	240687_at	testis/brain-restricted	11	3.4	6	2.3	0.5
HORMAD1	223861_at	testis/brain-restricted	7	2.2	0	0	0.02
NLRP4	242334_at	testis-selective	12	3.8	3	1.1	0.06
TDRD1	221018_s_at	testis-selective	11	3.4	8	3	0.8
TULP2	206733_at	testis-selective	9	2.8	11	4.2	0.5
SYCE1	233084_s_at	testis-selective	7	2.2	0	0	**0.02**
ADAM2	207664_at	testis-selective	5	1.6	0	0	0.07
TDRD6	232692_at	testis-selective	5	1.6	0	0	0.07
RBM46	244351_at	testis-selective	4	1.3	1	0.4	0.4
BRDT	206787_at	testis-selective	3	0.9	3	1.1	1
HSPB9	230510_at	testis-selective	3	0.9	0	0	0.3
MAGEA8	210274_at	testis-selective	3	0.9	5	1.9	0.5
ADAM29	221337_s_at	testis-selective	2	0.6	0	0	0.5
MAGEA11	210503_at	testis-selective	2	0.6	0	0	0.5
NXF2	220981_x_at	testis-selective	2	0.6	0	0	0.5
TSP50	220126_at	testis-selective	2	0.6	0	0	0.5
AKAP3	207344_at	testis-selective	0	0	0	0	NA

Table 1. *Continued*

Gene symbol	Probe set	Tissue restriction	Newly diagnosed		Relapse		
			n	%	n	%	*p*-value
ARMC3	240275_at	testis-selective	0	0	2	0.8	0.2
KLKBL4	231287_s_at	testis-selective	0	0	0	0	NA
TAF7L	220325_at	testis-selective	0	0	0	0	NA
TEX15	221448_s_at	testis-selective	0	0	0	0	NA
TSSK6	224409_s_at	testis-selective	0	0	0	0	NA
LY6K	223687_s_at	not available	11	3.4	6	2.3	0.5
TEX101	223906_s_at	not available	9	2.8	6	2.3	0.8
IL13RA2	206172_at	not available	8	2.5	1	0.4	0.05
CT45A5	235700_at	not available	4	1.3	4	1.5	1
DPPA2	240301_at	not available	3	0.9	3	1.1	1
PRM1	206358_at	not available	3	0.9	0	0	0.3
AKAP4	207019_s_at	not available	2	0.6	1	0.4	1
LOC348120	231132_at	not available	2	0.6	0	0	0.5
LOC196993	214418_at	not available	0	0	0	0	NA
PRM2	210122_at	not available	0	0	0	0	NA
CPXCR1	1560493_a_at	testis-restricted	165	51.6			
MAGEB6	1552858_at	testis-restricted	24	7.5			
Cxorf61	1559258_a_at	testis-restricted	23	7.2			
DDX53	1555357_at	testis-restricted	13	4.1			
CCDC36	1569690_at	testis-selective	180	56.3			
CCDC33	1563090_at	testis-selective	61	19.1			
CTCFL	1552368_at	testis-selective	27	8.4			
FMR1NB	1552906_at	testis-selective	24	7.5			
SLCO6A1	1552745_at	testis-selective	5	1.6			
COX6B2	1553367_a_at	testis-selective	1	0.3			
FAM46D	1552461_at	not available	26	8.1			
ODF4	1552408_at	not available	14	4.4			
CT45A1	1567912_s_at	not available	10	3.1			
CALR3	1552421_a_at	not available	3	0.9			
POTE15	1553474_at	not available	3	0.9			
OTOA	1553432_s_at	not available	1	0.3			
SPATA19	1559138_a_at	not available	1	0.3			
CAGE1	1563787_a_at	not available	0	0			
ODF3	1553051_s_at	not available	0	0			

Presence of expression in normal plasma cells was analyzed (GSE6477).[26] Due to restrictions of this data set, 82 genes were evaluated here (Table S7). A number of genes, such as *SPAG4* and *SPAG9*, were present in all 15 normal plasma cell samples, whereas the majority (70%) was present in none of the cases. Out of 12 testis-restricted genes shown in Table 1A, *MAGEB2* showed presence in 4 out of 15 normal cases and *MAGEC1* and *SPANXC* were present in 1 out of 15 cases. The remaining 9 evaluated genes were not present in any of the 15 normal plasma cell cases analyzed (Table S7).

Prognostic impact of CTA genes

CTA expression was analyzed in relation to progression free survival (PFS) and overall survival (OS; see Design and Methods). Univariate Kaplan Meier analysis, evaluated by log-rank testing, generated a set of CTA genes with prognostic value for PFS and OS for newly diagnosed cases and for relapse cases (Table S8). Based on this analysis, both in newly diagnosed and in relapse patients, *SSX1* was found to be prognostic for both PFS and OS, suggesting universal value for this marker. Multivariate analysis indicated *MAGEA6* and *CDCA1* to be prognostic factors for PFS in the newly diagnosed cases, independent of cytogenetic factors and ISS. Similarly, *MAGEA9* constituted an independent prognostic factor in terms of overall survival (Figure 1). In the relapse cases, *CTAG2* (PFS) and *SSX1* (OS) were found to be independent of the TC classification, used as a substitute marker for cytogenetic markers, and ISS (Figure 1). Correlation between different CTA genes was evaluated using cluster analysis of 57 CTA genes (i.e. >5%, known tissue restriction and without *TSPY1*). This analysis resulted in 15 clusters (Figure S2A). Cases belonging to clusters 5, 12 and 15 had a shorter overall survival and progression free survival in newly diagnosed cases. Top overrepresented genes in these clusters combined are *XAGE1*, *MAGEA12* and *SSX1*. Multivariate analysis showed that the prognostic value of cluster 5, 12 and 15 for newly diagnosed cases was independent of cytogenetic factors and ISS only in case of progression free survival (Figure S2B). For relapse cases, cluster 15 alone, with top overrepresented genes being *CTAG2, SSX4* and *CTAG1B*, was associated with poor overall survival and progression free survival (Figure S2C, D). Cluster 15 is an independent prognostic factor using multivariate analysis incorporating the TC classes and ISS.

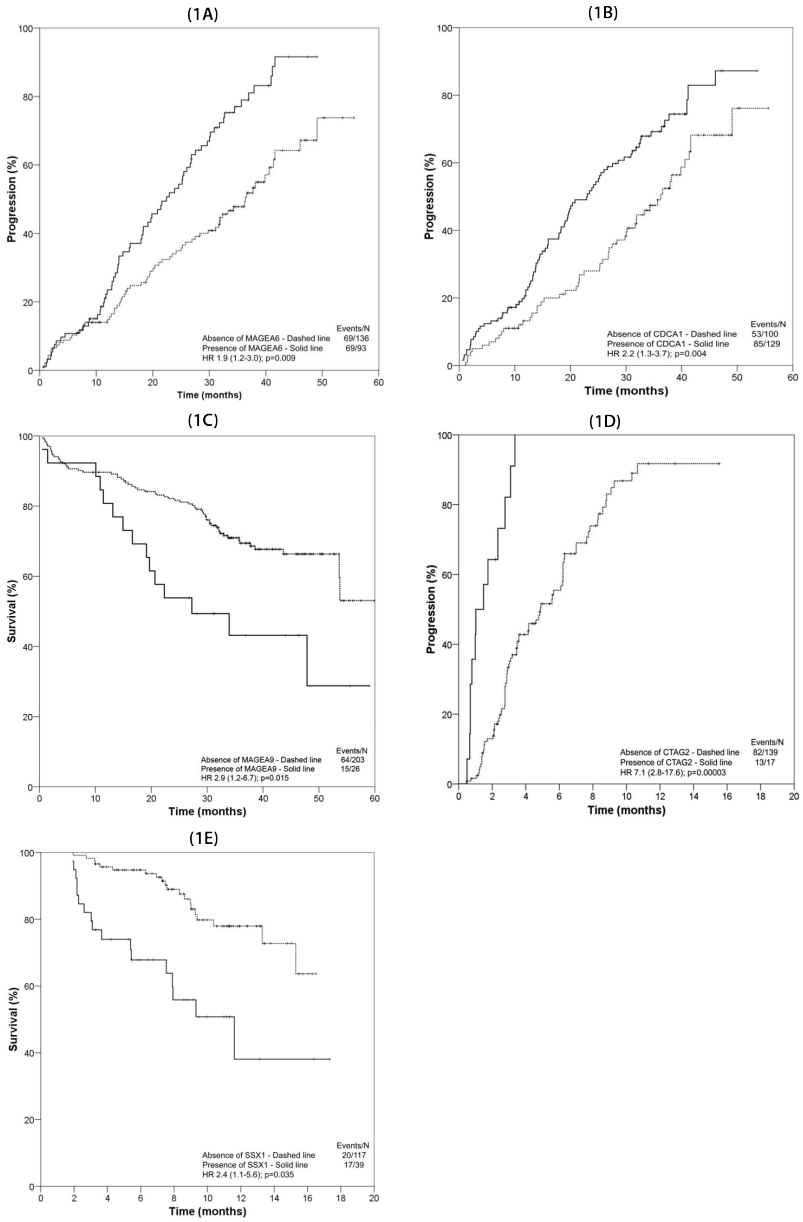

Figure 1. Survival analysis according to CTA gene expression status in the HOVON-65/GMMG-HD4 trial and in the APEX trial. Presence and absence of gene expression are indicated by solid lines and dashed lines, respectively. In HOVON-65/GMMG-HD4 presence of MAGEA6 (Panel A) and CDCA1 **(B)** are prognostic for a significantly shorter PFS. Presence of CTA MAGEA9 **(C)** is prognostic for a significantly worse survival (overall survival, OS) in the newly diagnosed HOVON-65/GMMG-HD4 patients. In the APEX trial of relapse patients, presence of CTAG2 is prognostic for shorter PFS **(D)** and SSX1 for shorter OS **(E)**. The prognostic value of all five CTAs shown is independent of ISS and cytogenetic covariates/TC class (see text). Hazard ratios (HR), 95% confidence intervals and p-values, adjusted for ISS, are shown.

DISCUSSION

The CTA mRNA expression profile in relapse MM cases was determined and compared to the profile in newly diagnosed cases. Forty-five out of 87 CTAs demonstrated either increased or equally expressed in the relapse cohort. Although two of three of the top CTA genes with testis restricted expression demonstrate significantly reduced frequency of expression in relapse cases, the proportion of samples expressing one of the top three genes in relapse cases is still 71%, which compares favorably to the 83% in newly diagnosed cases. In a study by Atanackovic et al.,[13] the frequency of *MAGEC1, MAGEA3, MAGEC2* and *SSX2* expression was determined in a set of myeloma cases: 65%, 52%, 43% and 12%, respectively. The expression presence in our study was lower for most of these genes (i.e. 71%, 38%, 29% and 7%, respectively). However, as indicated below our results corresponded well to a previous report on CTA expression as determined using Affymetrix GeneChips in newly diagnosed cases only.[16] The difference between studies may be attributed to differences in techniques used. CTA expression in myeloma has been evaluated in large patient sets but emphasis has so far been on newly diagnosed patients.[13,16,29,30] Compared to the study of Condomines et al.,[16] presence of gene expression frequency differed with no more than 2-fold in 73% of overlapping genes, when comparing to the frequencies found in newly diagnosed patients in our study. Of these, 12 are highly correlated with a difference in frequency of no more than 1.5 fold, including *MAGEC1, MAGEA5* and *SSX3* (61%, 26% and 3% in our study and 66%, 22% and 3% in the study of Condomines et al.).[16] In some cases, different probe sets belonging to the same gene may explain the difference between expression frequencies found between the two studies.

Comparison of 13 CTAs in treated and untreated MM has previously been reported for a small study group.[14] In that study, CTA expression was evaluated by RT-PCR on non-purified samples,[14] whereas we have reported on GeneChip data on purified plasma cells. Despite these clear differences, some similarities in CTA expression pattern were found. Overall, the expression frequency determined in our study on purified plasma cells was higher (1.2–1.3 fold), but the change between newly diagnosed and previously treated was comparable in direction in 8 out of 10 genes.

The testis-restricted antigen MAGEC1 was confirmed in this study as an important antigen in MM. Presence of *MAGEC1*, also referred to as *CT7*, is high post-therapy, and was observed in 61% of relapse cases. Expression of *MAGEC1* by Q-PCR has also been reported to correlate with disease burden following therapy.[13] A small series of 10 cytospins derived from purified CD138-positive tumor cells was stained for MAGEC1 by immunohistochemistry (IHC) (data shown in Figure S3); a clear correlation was observed for gene presence call and protein expression. In fact, *MAGEC1* presence calls were found only in cases with more than 50% of the tumor cells positive for MAGEC1 by IHC. The heterogeneity of *MAGEC1* expression in MM, i.e. level of MAGEC1 positive cells by IHC, has recently been reported

to be correlated to survival and proliferation.[15] A linked mRNA and protein expression has now been reported for multiple CTAs, where suitable antibodies for specific CTA detection are available.[12,31,32] Protein expression is naturally a requirement for immunotherapy for epitopes to be presented for CTL recognition, and further studies need to assess this aspect in detail for the targeted CTAs.

SSX1, another notable testis restricted gene, was found to be expressed in equal frequencies in relapse cases compared to newly diagnosed cases. When assessed in newly diagnosed MM, co-expression of *SSX1,2,4,5* was found to predict reduced survival, of which *SSX2* alone has been reported to yield the strongest association with reduced survival.[33] Here we find *SSX1* to be the sole CTA, using univariate log rank analysis, which was found to be correlated to shorter overall survival and progression free survival in both independent cohorts (i.e. newly diagnosed: HOVON-65/GMMG-HD4 and relapse MM: APEX trial).

For immunotherapy, genes with the most restricted expression pattern in normal tissue, i.e. the TR category, represent the most suitable targets.[8] Very limited expression in normal plasma cell samples was found for TR genes in this study, with presence of *MAGEB2* expression in 4 out of 15 samples representing the highest value. Presence of expression in normal plasma cells indicates that a gene does not comply fully with the previously reported tissue restriction pattern, in this case TR. For *CTAGE1*, in the TBR category, presence of expression was found in almost all normal plasma cell samples, and its status as TBR gene may therefore be questioned. In addition, CTA genes with almost universal expression in MM, such as *SPAG9*, demonstrated also very high frequency of expression in normal plasma cells. *SPAG9* is present in all but one sample in this study, and has been described to mediate JNK signaling.[34,35] For immunotherapy, expression in normal tissues raises concerns of generating autoimmune responses following vaccination. Still, some TS antigens, like MAGEA3, are targeted in current immunotherapeutic protocols.[36,37] It is of interest that not all TS antigens demonstrated expression in normal plasma cell samples.

In total, 15 out of 87 CTA genes demonstrate a reduction of 30% or more in the relapse set compared to the newly diagnosed cases. The majority of these genes are found either in the set of TS genes (7/38) or in the set without known tissue restriction (7/29). The most pronounced differences were found in *ZNF165* (70% reduction; TS) and *TMEFF1* (57% reduction; no tissue restriction known), which emphasizes the importance of monitoring the presence of antigens post-therapy.

Clearly, our study does not allow for analysis of the patterns of CTA expression in the same patient longitudinally. However, our data indicate that a large number of CTAs, but importantly not all, are prevalent in relapse MM patients and offers potentially suitable targets for immunotherapy. Atanackovic et al. reported on the longitudinal analysis of 4 CTA genes in 17 patients during treatment, and in most cases the expression of these genes persisted from initial presentation to relapse.[13] Only *MAGEC2* demonstrated a clear reduction in terms of fold-change in our study and is also the gene with the highest proportion of decrease in the study of Atanackovic et al..[13]

The relation between CTA expression and its prognostic value is underlined by the expression of *SSX1* mentioned above. Moreover, multivariate analysis identifies *SSX1* as an independent prognostic factor associated with poor overall survival in relapse cases, with *CTAG2* prognostic for progression free survival in this set. For newly diagnosed cases, *MAGEA6* and *CDCA1* were independent prognostic factors for progression free survival, and *MAGEA9* is an independent risk factor in terms of overall survival. CDCA1 is a cell division protein, and forms a part of protein complex associated with the centromere. The prognostic impact of this gene correlates well with the known poor prognosis of MM with high proliferation.[38] Previous studies confirm the presence call of *CTAG2* and *MAGEA6* to be of prognostic importance in terms of event free survival alongside with presence call of *MAGEA3, MAGEA1, MAGEA2* and *CTAG1B*.[16] Others have demonstrated prognostic impact of *MAGEC1*, *SSX2* and *CT45*.[13,16,34,39,40] A recently published signature for high-risk disease contains the genes *GAGE1* and *GAGE12*, which further confirms the relation between CTA genes and prognosis.[41]

Cluster analysis further confirms the correlation between different CTAs and especially those located on chromosome X. In cluster 15, part of the prognostically important set of clusters in the newly diagnosed MM cases and the most important cluster in relapse cases, the top 10 overrepresented genes are all derived from chromosome X.

In our classification of MM we have described a new cluster in MM: the CTA group. This cluster demonstrated expression of CTA genes but without concomitant expression of proliferation genes, setting it apart from the proliferation cluster originally identified by Zhan et al.[22,42] Future studies will expand on the prognostic value and underlying biology of this classification. It is also important to consider CTA expression in relation to treatment using demethylating agents.[43] Despite the clear advantage of potentially derepressing genes such as *p53*, the effects on inducing CTA expression must be taken into account. Also drug regimens currently in use in MM may have effect on methylation status. Indeed, bortezomib treatment has been demonstrated to have effects on gene demethylation.[44,45] In our study, however, gene expression analysis in both newly diagnosed and in relapse patients was performed prior to bortezomib treatment.

Although the function of CTA genes is generally not characterized extensively, repression of *MAGEC1* and *MAGEA3* has been shown to result in increased apoptosis in myeloma cell lines.[46] A functional role for CTAs in MM ties in with the finding of CTA expression post-relapse in this study. Finally, CTA expression has been reported in cancer stem cells, among others in melanoma and glioma.[47,48] In MM, a putative cancer stem cell has been reported, and may be CD19+CD138-ve.[49-51] The putative importance of CTA genes in these cells is subject to future investigation. In conclusion, evaluating a large panel of CTA genes suggests that many of these antigens are relevant to tumor cells at relapse, offer putative immunotherapeutic targets and have value as prognostic markers. Future studies are aimed at validating CTA genes as risk factors in MM progression as well as analyzing optimal targets for immunotherapy.

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CHAPTER 8

HIGH CEREBLON EXPRESSION IS ASSOCIATED WITH BETTER SURVIVAL IN PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA TREATED WITH THALIDOMIDE MAINTENANCE

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ABSTRACT

Recently, cereblon (*CRBN*) expression was described to be essential for the activity of Thalidomide and Lenalidomide. We investigated if clinical efficacy of Thalidomide in multiple myeloma is associated with *CRBN* expression in myeloma cells. Patients with newly diagnosed multiple myeloma were included in the HOVON-65/GMMG-HD4 trial, in which post-intensification treatment in one arm consisted of daily Thalidomide 50 mg for 2 years. Gene expression profiling, determined at the start of the trial, was available for 96 patients who started Thalidomide maintenance. In this patient set, increase of *CRBN* gene expression was significantly associated with longer progression free survival ($p = 0.005$) and longer overall survival ($p = 0.04$). In contrast, no association between *CRBN* expression and survival was observed in the arm with Bortezomib maintenance. We conclude that *CRBN* expression may be associated with clinical efficacy of Thalidomide.

INTRODUCTION

Induction treatment for multiple myeloma (MM) was greatly improved with the introduction of Thalidomide, Bortezomib and Lenalidomide.[1-4] Attention is now shifting towards improving consolidation and maintenance therapy, with the aim to improve both depth and duration of response, and ultimately extended progression free survival (PFS) and overall survival (OS).[5] Thalidomide and Lenalidomide represent immunomodulatory drugs (IMiD) with variable efficacy during maintenance after high-dose therapy and in the non-transplant setting.[6-8] So far, there are no biomarkers for prediction of outcome following Thalidomide and/or Lenalidomide treatment. *CRBN* was recently identified as the target gene responsible for the teratogenic effects of Thalidomide.[9] In addition, it was shown that *CRBN* levels are critical for the antitumor activity of Lenalidomide and Thalidomide in *in vitro* model systems and in Lenalidomide resistant patients.[10] Here we report that *CRBN* expression is associated with outcome of Thalidomide maintenance in newly diagnosed MM patients.

PATIENTS AND METHODS

Patients and procedures

In the HOVON-65/GMMG-HD4 trial patients, aged 18–65 years with newly diagnosed MM, were randomly assigned to VAD induction, intensification with high-dose melphalan (HDM) and autologous stem cell transplantation (ASCT), followed by maintenance therapy with Thalidomide, or PAD (Bortezomib, Doxorubicin and Dexamethasone), HDM and ASCT, followed by maintenance with Bortezomib. The maximum duration of maintenance therapy in both arms was 2 years.[11] Patients randomized to VAD received maintenance with Thalidomide 50 mg daily for 2 years, starting 4 weeks after HDM. This study was approved by the Ethics Committees of the Erasmus University MC, the University of Heidelberg and the participating sites. All patients gave written informed consent and the trial was conducted according to the European Clinical Trial Directive 2005 and the Declaration of Helsinki.

Response assessments and endpoints

Clinical characteristics were registered at diagnosis. Cytogenetic studies were performed as described.[12] For this sub-analysis, PFS and OS were measured from start of the maintenance treatment. For PFS, progression was used as endpoint and for OS, death from any cause. Patients alive at the date of last contact were censored. Evaluation of response is described in detail in Supplemental table 4.

Gene expression profiling and statistical analysis

The gene expression dataset GSE19784 was used, which was derived from patients included in the HOVON-65/GMMG-HD4 trial.[11,13] *CRBN* expression was assessed using the intensity value of the probe sets coding for *CRBN*: 218142_s_at and 222533_at. Both probe sets were combined using the method of Dai et al..[14] Presence calls for *CRBN* expression were determined using the PANP algorithm, using standard settings (PANP reference manual, Bioconductor website).[15] Details of the quantitative RT-PCR are given in supplemental Figure 3. Multivariate Cox regression analysis was performed to assess the value of *CRBN* as a prognostic factor in relation to the International Staging System (ISS) and high-risk cytogenetics, as described.[11]

RESULTS AND DISCUSSION

Patients and Response

833 patients were enrolled in the HOVON65/GMMG-HD4 trial. Of the patients randomized to the VAD arm, 77/347 (22%) went off protocol after HDM because of Allo-SCT (n = 21, 6%), persisting toxicity (n = 11, 3%) or other reasons (n = 45, 13%), while 270 (78%) patients started Thalidomide maintenance treatment. Normal completion of Thalidomide maintenance was achieved in 73/270 (27%) patients. Eleven of 270 Thalidomide maintenance patients underwent Allo-SCT and were not considered in this sub-analysis. Out of the remaining 259 patients, GEP and survival data were available for 96 patients. Baseline characteristics between this subgroup (n = 96) and the remainder (n = 163) were comparable (Table S1). Presence calls were determined for the *CRBN* probe sets, and *CRBN* expression was found to be present in ≥99% of cases. A significant correlation was found between *CRBN* gene expression measured by microarray and qRT-PCR (Spearman's rho: 0.67; $p = 0.002$, n = 18, Supplemental Figure 3). *CRBN* expression was evaluated in relation to the EMC-clustering: the CTA cluster demonstrated a significantly higher *CRBN* expression compared to the other evaluated clusters (Bonferroni-Holm corrected p = 0.01, Supplemental Figure 2).[16] In univariate Cox regression analysis, *CRBN* expression was significantly associated with PFS (hazard ratio (95% CI): 0.68 (0.52–0.89); $p = 0.005$) and with OS (hazard ratio (95% CI): 0.65 (0.43–0.97); $p = 0.04$; Table 1A and B). Kaplan-Meier analysis was used solely for visualization with CRBN expression split in 2 or 4 groups using median or quartile intensities: patients with *CRBN* expression above the median demonstrated longer PFS compared to patients with *CRBN* levels below the median ($p = 0.009$; Figure 1A and B; quartile intensities: Supplemental figure 4). In addition, an optimal CRBN cut-off was calculated (Supplemental table 2). For this calculation, the PFS data were used which prohibits use of this cut-off in this dataset for any analyses related to PFS. In contrast, the median expression value is arbitrarily chosen, and was used for analysis in relation to response upgrade. Multivariate

Table 1. Univariate Cox regression analysis of CRBN expression in relation to PFS (**A**) and OS (**B**); Multivariate Cox regression analysis of CRBN expression in relation to PFS (**C**) and OS (**D**); High-risk cytogenetics is defined as having del(17p) and/or 1q gain and/or t(4;14).HR, hazard ratio; 95%CI, 95% confidence interval of hazard ratio.

A

	HR	95%CI	*P-value*
CRBN expression	0.68	0.52–0.89	0.005

B

	HR	95%CI	*P-value*
CRBN expression	0.65	0.43–0.97	0.04

C

Covariate	HR	95%CI	*P-value*
CRBN expression	0.66	0.45–0.96	0.03
ISS2	2.35	1.15–4.82	0.02
ISS3	2.55	1.21–5.36	0.01
High-risk FISH	2.82	1.59–5.00	0.0004

D

Covariate	HR	95%CI	*P-value*
CRBN expression	0.75	0.43–1.32	0.32
ISS2	4.66	1.38–15.81	0.01
ISS3	5.49	1.67–18.08	0.005
High-risk FISH	3.65	1.53–8.70	0.003

Cox regression analysis was performed using the covariates ISS, continuous *CRBN* levels and high-risk cytogenetics, defined as having del(17p) and/or 1q gain and/or t(4;14). Higher *CRBN* levels remained significantly related to longer PFS, but not OS, with a hazard ratio of 0.66 ($p = 0.03$) and 0.75 ($p = 0.3$), respectively (Table 1C and D). No significant correlation was found between any of these covariates and *CRBN* but it is of note that lower *CRBN* expression was found in ISSIII compared to either ISSI or ISSII (Bonferroni corrected $p = 0.10$; Kruskal Wallis test). The CRBN gene is positioned on chromosome 3. Chromosome 3 trisomies are frequently found in patients with hyperdiploidy and indeed *CRBN* levels were significantly higher in hyperdiploid patients compared to non-hyperdiploid patients ($p = 0.005$). In a multivariate Cox regression analysis, however, *CRBN* levels, but not hyperdiploidy, were found to be related to PFS ($p = 0.006$ and $p = 0.8$, respectively; data not shown). *CRBN* expression was not associated with an upgrade of response, i.e. improvement of response during Thalidomide maintenance ($p = 0.3$, Supplemental Table 4). In order to determine if *CRBN* expression was specifically relevant for the outcome of Thalidomide treatment, we also examined the relation between *CRBN* expression and survival in patients treated with Bortezomib maintenance. No association was observed between *CRBN* expression and PFS/OS after Bortezomib maintenance (Figure 1C and D). For validation of these results, the MRC-IX study was evaluated.[18] Only 30 patients with gene expression were available who received Thalidomide during maintenance but not during induction. This subset was too small to allow solid analysis of the relation between *CRBN* expression and Thalidomide during maintenance. Finally, *CRBN* forms an E3 ubiquitin ligase complex with proteins DDB1 and CUL4A.[9] This complex has been suggested to be involved in regulation of beta-catenin activity, which in turn affects downstream targets such as CCND1 and C-MYC.

CRBN was also described to bind to AMPK alpha 1(PRKAA1) and to the large conductance Ca^{2+} activated potassium channel KCNMA1.[17] Assessment of expression levels of these genes in a multivariate model with *CRBN* levels demonstrated that *CCND1* and *CRBN* were independently related to longer PFS (Supplemental table 3). A relation with PFS was not found for either *CCND1* or *CRBN* in the patients treated with Bortezomib in the maintenance phase.

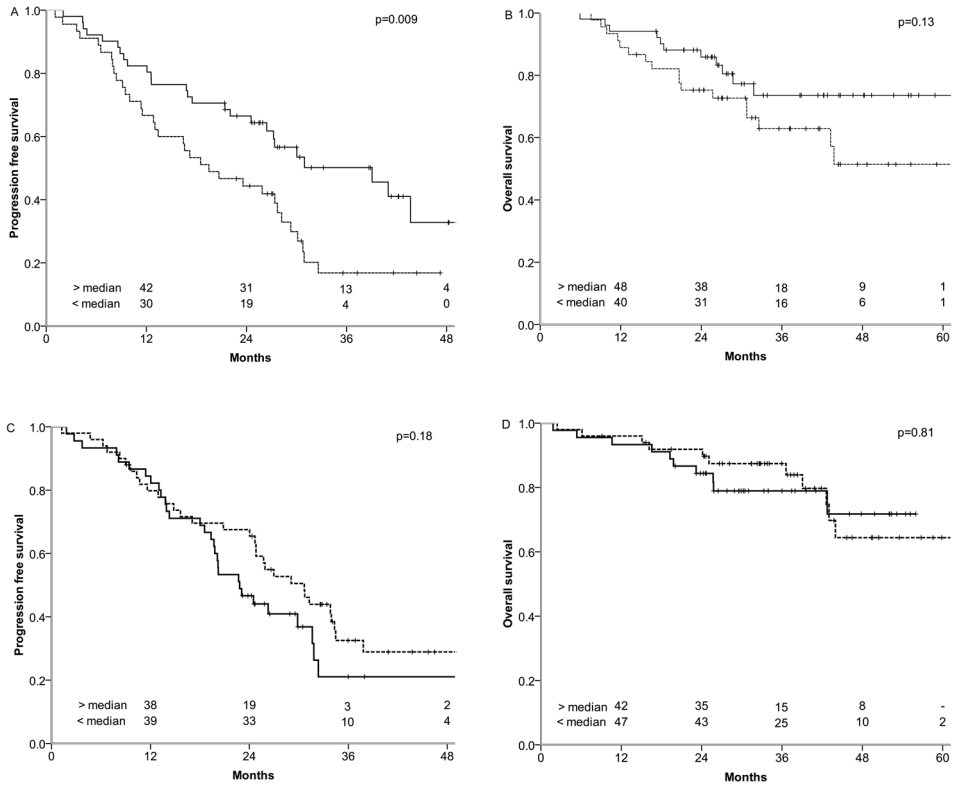

Figure 1. CRBN expression in HOVON-65/GMMG-HD4: relation to PFS and OS Kaplan-Meier curves of *CRBN* expression in relation to survival in Thalidomide treated patients (**A**, **B**) and in relation to Bortezomib treated patients (**C**, **D**). Left panels, PFS; panels on the right, OS. *P*-values in right hand corner of each panel are log rank *p*-values. Broken lines indicate *CRBN* expression levels below the median and solid lines indicate expression levels above the median. Remaining patients at risk are shown above the X-axis (at 1, 2, 3 and 4 years, PFS; at 1, 2, 3, 4 and 5 years, OS). The median CRBN expression was determined on the combined data of both Thalidomide and Bortezomib treated patients: 45 out of 96 patients were below the median in the Thalidomide set whereas 50 out of 95 were below the median in the Bortezomib set.

In conclusion, we observed that higher expression of *CRBN* was associated with increased PFS and OS during maintenance treatment with Thalidomide, but not in patients with Bortezomib maintenance. This corresponds well to the report of reduced *CRBN* expression in > 85% of MM patients who were Lenalidomide-resistant.[10] Our observations warrant analysis of the predictive effect of *CRBN* expression in newly diagnosed and relapse/refractory patients treated with IMiDs as part of induction and consolidation treatment.

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SUPPLEMENTAL DATA

Supplemental Table 1. Baseline Patient and Disease Characteristics. Percentages are given; percentage of known, i.e. cases with known characteristics add up to 100%. Percentages of unknown are calculated over the number of patients in the subset (i.e. 96 vs 163).

Characteristics	Thalidomide maintenance N = 96	Other N = 163	P-value
Median age, years (range)	58 (38–65)	56 (29–65)	0.55##
Male sex – no. of patients (%)	55%	57%	0.80#
WHO performance stage (% of known)			
0	49	45	
1	42	43	
2	8	9	0.96#
3	1	2	
Unknown	0	1	
ISS stage (% of known)			
I	42	43	
II	35	32	
III	23	26	0.85#
Unknown	4	14	
M-protein isotype (% of known)			
IgA	23	25	
IgG	61	54	
IgD	0	2	
IgE	0	0	
LCD	16	18	
IgM	0	1	0.63#
Unknown	0	0	
Kappa	67	67	
Lambda	33	33	1.00#
Unknown	0	0	
Creatinin, mg/dL– no. (% of known)			
≤ 2 mg/dL	91	95	
> 2 mg/dL	9	5	0.20#
Unknown	0	1	
Number of skeletal lesions (% of known)			
0	20	27	
1	7	5	
2	4	4	
≥ 3	70	63	0.55#
Unknown	4	3	
Serum LDH (% of known)			
≤ ULN	82	85	
> ULN	18	15	0.60#
Unknown	2	2	

Supplemental Table 1. *Continued*

Characteristics	Thalidomide maintenance N = 96	Other N = 163	P-value
Genetic abnormalities (% of known)			
Del(13q)	49	42	0.29#
Unknown	1	17	
t(4;14)	14	14	1.00#
Unknown	18	44	
t(11;14)	13	16	0.54#
Unknown	8	34	
Del(17p)	9	9	1.00#
Unknown	1	38	
Gain (1q)	21	18	0.57#
Unknown	2	39	
Median β_2-microglobulin – mg/L	3.2	3.1	0.65##
Median hemoglobin – mmol/L	6.8	6.7	0.97##
Median calcium – mmol/L	2.3	2.3	0.91##

Abbreviations: WHO, World Health Organization; ISS, International Staging System; LDH, serum lactodehydrogenase; BM, Bone Marrow. ULN, upper limit of normal

#Fisher's Exact *p*-value

##Mann-Whitney U *p*-value

Supplemental table 2. Maxstat analysis for determining the optimal cut-off of *CRBN* expression in relation to progression free survival of Thalidomide treated patients during maintenance in the HOVON-65/GMMG-HD4 study. The value of 10.22 is statistically most optimal, the *p*-value is corrected for multiple testing which is inherent to the Maxstat method. For comparison, the median value, arbitrarily used in the paper, is a *CRBN* expression log2 value of 11.00.

M	p-value	estimated cut-off
2.824	0.06	10.22
maxstat package in r. command: m<- maxstat (y=time, x=data[,"expression"], smethod = "LogRank", pmethod = "min", iscores = TRUE, minprop = 0.1, maxprop = 0.9)		

Supplemental table 3. Multivariate Cox regression model including *CRBN, KCNMA1, DDB1, CUL4A, PRKAA1, CCND1, MYC, IRF4* and *CTNNB1* (PFS). HR, hazard ratio; 95%CI, 95% confidence interval of hazard ratio. For *CUL4A*, probe sets 201423_s_at and 201424_s_at were combined using the method of Dai et al.[14] For *AMPK alpha 1* probe sets 209799_at, 225984_at and 225985_at were combined, and for *CCND1* 208711_s_at and 208712_at. Probe sets of *CRBN* are given in the Patients and Methods section. Probe sets for the other genes: *KCNMA1* (221584_s_at), *DDB1* (208619_at), *MYC* (202431sat), *IRF4* (204562_at) and *CTNNB1* (201533_at). Bold indicates significance.

	HR (95% CI)	*P-value*
CRBN	0.66 (0.46–0.95)	0.03
KCNMA1	0.97 (0.83–1.12)	0.7
DDB1	0.90 (0.59–1.38)	0.6
CUL4A	1.12 (0.80–1.57)	0.5
PRKAA1	1.06 (0.69–1.64)	0.8
CCND1	0.89 (0.81–0.97)	0.008
MYC	1.06 (0.92–1.21)	0.4
IRF4	1.06 (0.83–1.36)	0.6
CTNNB1	0.90 (0.67–1.21)	0.5

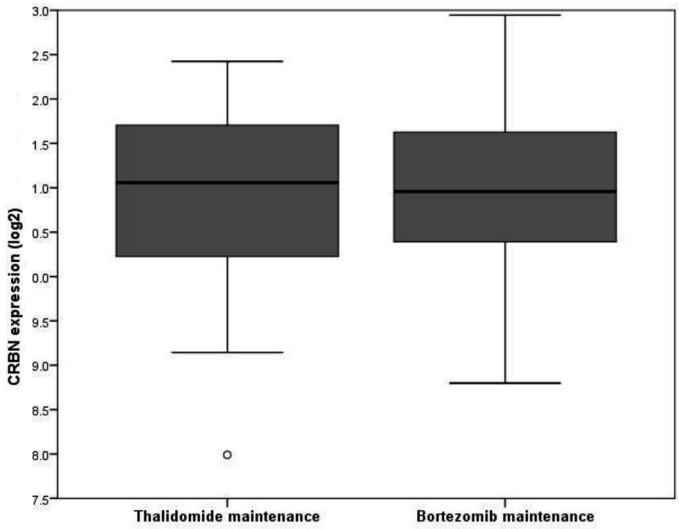
Supplemental Table 4. Response upgrade in patients with Thalidomide maintenance in relation to *CRBN* expression. Evaluation of response was performed according to modified European Group for Blood and Marrow Transplantation (EBMT) criteria, including nearCR (nCR) and Very Good Partial Response (VGPR) as in the International Myeloma Working Group (IMWG) uniform response criteria.[19] Response was assessed after first and second transplantation and at 2 month intervals during maintenance. For patients receiving tandem ASCT, the response state after the second transplantation was taken for analysis. Upgrade of response was defined as any improvement of response compared to the response determined following HDM (from <PR to ≥PR or <VGPR to ≥VGPR or <nCR to ≥nCR or <CR to CR). 11 out of 96 patients demonstrated CR after HDM and after Thalidomide maintenance and were excluded. No relation between response upgrade and median *CRBN* expression was found (**A**). Patients with response upgrade are described in detail in **B**.

A

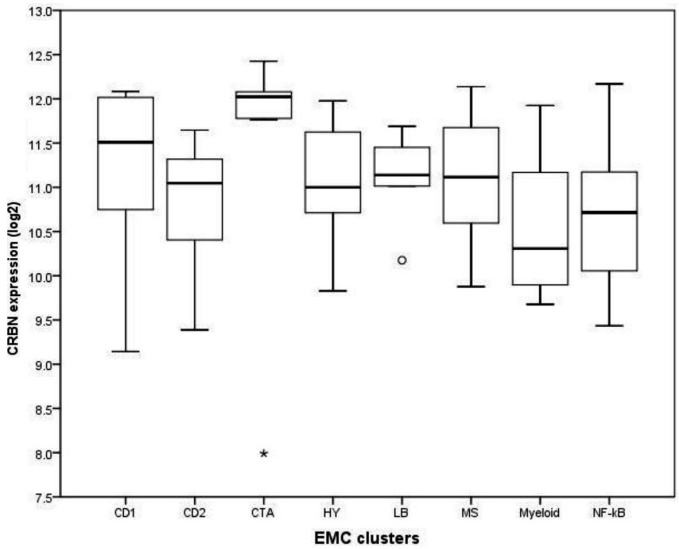
	Cereblon expression		
	< median	**> median**	
Response upgrade	14	25	
No response upgrade	22	24	
			p-value = 0.3

B

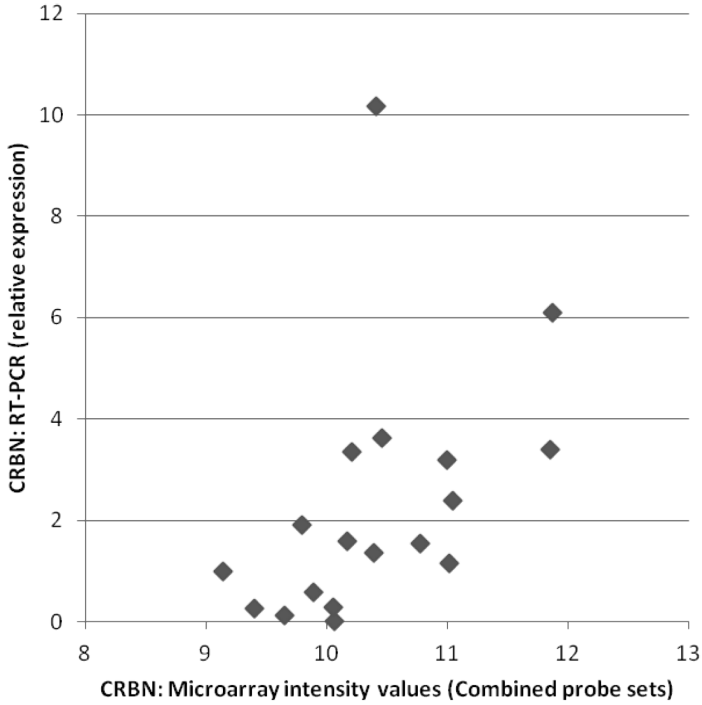
Response		**Cereblon expression**	
HDM	**Thalidomide**	**< median**	**> median**
NCR	CR	0	3
VGPR	CR	1	6
PR	CR	2	5
≤ MR	CR	1	0
VGPR	NCR	0	3
PR	NCR	1	3
≤ MR	NCR	0	0
PR	VGPR	8	3
≤ MR	VGPR	0	0
≤ MR	PR	1	2
		14	25



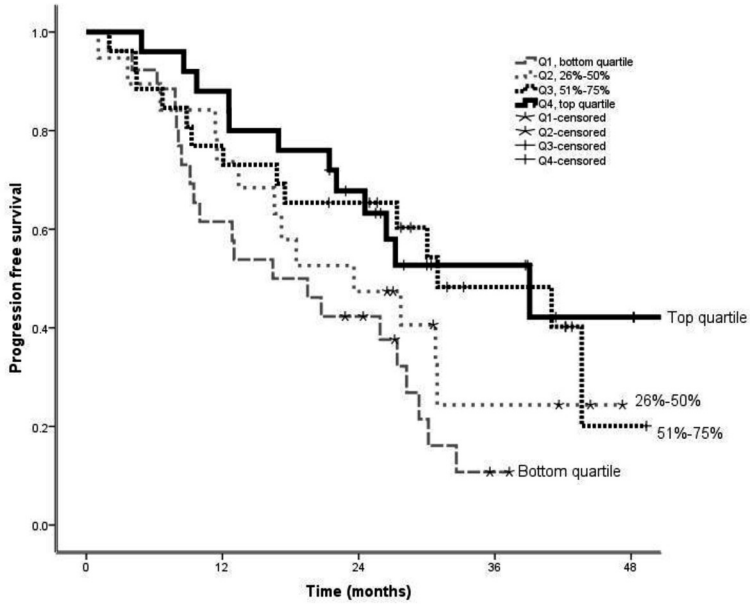
Supplemental Figure 1. Box-plot visualization of *CRBN* expression in patients with Thalidomide maintenance **(left)** and with Bortezomib maintenance **(right)**. Box represents 25% value (bottom hinge) and 75% value (top hinge), the inner fences extend to 1.5x the height of the box, bold line represents medians, circle indicates an outlier.



Supplemental Figure 2. *CRBN* expression in patients with Thalidomide maintenance treatment in the HOVON-65/GMMG-HD4 trial in relation to EMC-clusters.[16] Clusters PR, PRL3, MF and the cluster without clear gene expression profile were excluded due to a low number of cases (<5). Boxes represent 25% value **(bottom hinge)** and 75% value **(top hinge)**, the inner fences extend to 1.5x the height of the box, lines within the boxes represent the medians, circle and asterisk indicate outliers (asterisk is extreme outlier within that cluster, with a value of more than 3x the height of the box lower). Kruskal-Wallis analysis comparing each cluster against the remaining clusters was performed, resulting p-values were corrected for multiple testing by Bonferroni correction. CTA cluster demonstrated significantly higher expression **(*p = 0.01*).**



Supplemental Figure 3. Relation between RT-PCR and microarray intensity values. Correlation was determined using Spearman's correlation test. The correlation coefficient is 0.67 (p = 0.002, n = 18). Forward primer 250*CRBN*1145x10f (5'-GCCGGCCTTCTACAGAACACAGC-3') and reverse primer 251*CRBN*11302x11r (5'-GGGCAACAGAGCAGATCGCGT-3') were used. GAPDH primers, forward: 60FWD (5'-GAAGGTGAAGGTCGGAGT-3') and reverse: 61REV (5'-GAAGATGGTGATGGGATTTC-3'). Using quantitative RT-PCR, GeneChip intensity values were confirmed on cDNA of a subset of patients (n = 18). cDNA was generated as described previously (17). GAPDH was used to correct for difference in input material. Quantitative RT-PCR was performed using Dynamo 2x reaction buffer in an Applied Biosystems 7500 Fast Real-Time PCR System, according to the recommendations of the manufacturers (Finnzymes, Finland; Applied Biosystems, Carlsbad, USA).



Supplemental Figure 4. *CRBN* expression divided in quartiles in relation to progression free survival. It must be stressed that this figure is meant for visualization only; please see the continuous Cox regression analysis presented in Table 1. Analysis was performed on patients who received Thalidomide maintenance in the HOVON-65/GMMG-HD4 study. Log-rank p-value for this analysis is 0.03.

CHAPTER 9

MECHANISMS OF PERIPHERAL NEUROPATHY ASSOCIATED WITH BORTEZOMIB AND VINCRISTINE IN PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA: A PROSPECTIVE ANALYSIS OF DATA FROM THE HOVON-65/GMMG-HD4 TRIAL

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ABSTRACT

Background: Bortezomib-induced peripheral neuropathy is a dose-limiting toxicity in patients with multiple myeloma, often requiring adjustment of treatment and affecting quality of life. We investigated the molecular profiles of early-onset (within one treatment cycle) versus late-onset (after two or three treatment cycles) bortezomib-induced peripheral neuropathy and compared them with those of vincristine-induced peripheral neuropathy during the induction phase of a prospective phase 3 trial.

Methods: In the induction phase of the HOVON-65/GMMG-HD4 trial, patients (aged 18-65 years) with newly diagnosed Salmon and Durie stage 2 or 3 multiple myeloma were randomly assigned to three cycles of bortezomib-based or vincristine-based induction treatment. We analyzed the gene expression profiles and single-nucleotide polymorphisms (SNPs) of pretreatment samples of myeloma plasma cells and peripheral blood, respectively. This study is registered, number ISRCTN64455289.

Findings: We analyzed gene expression profiles of myeloma plasma cells from 329 (39%) of 833 patients at diagnosis, and SNPs in DNA samples from 369 (44%) patients. Early-onset bortezomib-induced peripheral neuropathy was noted in 20 (8%) patients, and 63 (25%) developed the late-onset type. Early-onset and late-onset vincristine-induced peripheral neuropathy was noted in 11 (4%) and 17 (7%) patients, respectively. Significant genes in myeloma plasma cells from patients that were associated with early-onset bortezomib-induced peripheral neuropathy were the enzyme coding genes *RHOBTB2* (upregulated by 1.59 times; p=4.5 $\times$ 10^{-5}), involved in drug-induced apoptosis, *CPT1C* (1.44 times; p = 2.9 $\times$ 10^{-7}), involved in mitochondrial dysfunction, and *SOX8* (1.68 times; p = 4.28 $\times$ 10^{-13}), involved in development of peripheral nervous system. Significant SNPs in the same patients included those located in the apoptosis gene *caspase 9* (odds ratio [OR] 3.59, 95% CI 1.59–8.14; p = 2.9 $\times$ 10^{-3}), *ALOX12* (3.50, 1.47–8.32; p = 3.8 $\times$ 10^{-3}), and *IGF1R* (0.22, 0.07–0.77; p = 8.3 $\times$ 10^{-3}). In late-onset bortezomib-induced peripheral neuropathy, the significant genes were *SOD2* (upregulated by 1.18 times; p = 9.6 $\times$ 10^{-3}) and *MYO5A* (1.93 times; p = 3.2 $\times$ 10^{-2}), involved in development and function of the nervous system. Significant SNPs were noted in inflammatory genes *MBL2* (OR 0.49, 95% CI 0.26–0.94; p = 3.0 $\times$ 10^{-2}) and *PPARD* (0.35, 0.15–0.83; p = 9.1 $\times$ 10^{-3}), and DNA repair genes *ERCC4* (2.74, 1.56–4.84; p = 1.0 $\times$ 10^{-3}) and *ERCC3* (1.26, 0.75–2.12; p = 3.3 $\times$ 10^{-3}). By contrast, early-onset vincristine-induced peripheral neuropathy was characterized by upregulation of genes involved in cell cycle and proliferation, including *AURKA* (3.31 times; p = 1.04 $\times$ 10^{-2}) and *MKI67* (3.66 times; p = 1.82 $\times$ 10^{-3}), and the presence of SNPs in genes involved in these processes – e.g., *GLI1* (rs2228224 [0.13, 0.02–0.97, p = 1.18 $\times$ 10^{-2}] and rs2242578 [0.14, 0.02–1.12, p = 3.00 $\times$ 10^{-2}]). Late-onset vincristine-induced peripheral neuropathy was associated with the presence of SNPs in genes involved in absorption, distribution, metabolism, and excretion – e.g., rs1413239 in *DPYD* (3.29, 1.47–7.37, 5.40 $\times$ 10^{-3}) and rs3887412 in *ABCC1* (3.36, 1.47–7.67, p = 5.70 $\times$ 10^{-3}).

Interpretation Our results strongly suggest an interaction between myeloma-related factors and the patient's genetic background in the development of treatment-induced peripheral neuropathy, with different molecular pathways being implicated in bortezomib-induced and vincristine-induced peripheral neuropathy.

INTRODUCTION

Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) is a boronic acid dipeptide, which specifically inhibits the chymotryptic site of the 26S proteasome. In patients with newly diagnosed myeloma, bortezomib in combination with conventional drugs resulted in high rates of complete response and very good partial response.[1-4] This drug is generally well tolerated; however, one of its most frequent and potentially disabling side-effects is the development of a painful, sensory peripheral neuropathy,[5-7] often requiring dose modification or discontinuation of bortezomib, which negatively affects clinical endpoints and quality of life.[8] Grade 1 and 2 bortezomib-induced peripheral neuropathy can arise in 27–75% of patients with recurrent multiple myeloma and in 25–33% of those with newly diagnosed multiple myeloma, whereas grade 3 and 4 peripheral neuropathy might affect 0-30% of patients with recurrent disease and 0-18% of those with newly diagnosed disease.[9] In most patients, this side-effect is reversible and does not seem to be affected by the number or type of previous treatments.[7] Bortezomib-induced peripheral neuropathy results from axonal degeneration,[10,11] often occurring within the first cycles of treatment, and does not seem to increase after the fifth cycle of bortezomib.[7]

Little is known about the mechanism of bortezomib-induced peripheral neuropathy, but a multifactorial pathogenesis seems likely. Damage to mitochondria and endoplasmic reticulum through activation of apoptosis has been seen in dorsal root ganglia of mice given bortezomib.[11] Additionally, mechanisms such as dysregulation of mitochondrial calcium homoeostasis,[12] autoimmune factors and inflammation,[13] and blockade of nerve-growth-factor-mediated neuronal survival through inhibition of the activation of nuclear factor κB (NF-κB)[6] could contribute to bortezomib-induced peripheral neuropathy. Evidence that multiple myeloma is also implicated in peripheral neuropathy was described by Ropper and Gorson in 1998.[14] Baseline neuropathy is present in 15-20% of patients with newly diagnosed myeloma,[15,16] which might be of both axonal and demyelinating subtypes.[14] The role of myeloma-related factors in peripheral neuropathy related to treatment is not clear. Bortezomib-induced peripheral neuropathy was noted at higher frequencies in patients with multiple myeloma than in those with solid tumours.[17] Richardson and colleagues characterized the possible role of myeloma-related factors in bortezomib-induced peripheral neuropathy using plasma cells from patients with multiple myeloma.[16] Additionally, we have noted that inherited single-nucleotide polymorphisms (SNPs) are associated with a

higher probability of developing thalidomide-induced or bortezomib-induced peripheral neuropathy (Corthals SL, unpublished data). We therefore analyzed myeloma-related gene expression and inherited patient variations as indicators of the potential risk of developing treatment-related peripheral neuropathy. We investigated whether particular molecular profiles were specific for early-onset versus late-onset bortezomib-induced peripheral neuropathy and compared these with genetic profiles associated with early-onset versus late-onset vincristine-induced peripheral neuropathy to elucidate molecular differences associated with the development of peripheral neuropathy after the different treatments.

METHODS

Patients

833 patients (aged 18–65 years) with newly diagnosed Salmon and Durie stage 2-3 multiple myeloma were enrolled in a prospective, randomized phase 3 trial (HOVON-65/GMMG-HD4; EudraCTnr2004-000944-26) in 75 centers in the Netherlands, Germany, and Belgium.[4] Patients were excluded if they had amyloidosis or monoclonal gammopathy of unknown significance, and baseline peripheral neuropathy of grade 2 or more.

The trial was done in accordance with the Declaration of Helsinki, and was approved by a medical ethics review committee. We obtained written informed consent from the patients for treatment and sample procurement.

Procedures

Patients were randomly assigned to three cycles of induction treatment with vincristine 0.4 mg intravenously on days 1–4, doxorubicin 9 mg/m^2 intravenously on days 1–-4, and dexamethasone 40 mg orally on days 1–4, 9–12, and 17–20 or bortezomib 1.3 mg/m^2 intravenously on days 1, 4, 8, and 11, doxorubicin 9 mg/m^2 intravenously on days 1-4, and dexamethasone 40 mg orally on days 1–4, 9–12, and 17–20. Stem-cells were mobilized by use of cyclophosphamide 1000 mg/m^2 intravenously on day 1, doxorubicin 15 mg/m^2 intravenously on days 1–4, dexamethasone 40 mg orally on days 1–4, and granulocyte colony-stimulating factor (filgrastim) 10 µg/kg per day subcutaneously, divided in two doses per day, from day 5 until last stem cell collection. After induction therapy, patients received one or two cycles of high-dose melphalan (200 mg/m^2 intravenously) with autologous stem-cell rescue followed by maintenance treatment with thalidomide (50 mg per day orally; group assigned to vincristine-based induction treatment) or bortezomib (1.3 mg/m^2 intravenously once every 2 weeks; group assigned to bortezomib-based induction treatment) for 2 years. Treatment was not masked for physicians and patients.

Severity of neuropathy was graded at baseline and after each treatment cycle by use of the National Cancer Institute's Common Toxicity Criteria for Adverse Events criteria (version

3.0).[18] All data were analyzed centrally. No neurological assessment was undertaken to objectify peripheral neuropathy. Since grade 1 peripheral neuropathy could easily be missed or misinterpreted, and because it does not include pain or interfere with the activities of daily life, we decided that grade 1 peripheral neuropathy was not clinically significant enough for the molecular analysis and therefore cases of this grade were excluded. Furthermore, the dose-modification guidelines established during the SUMMIT,[5,6] CREST,[5] and APEX[19] trials did not recommend discontinuation of bortezomib or dose modifications when grade 1 bortezomib-induced peripheral neuropathy occurred. We did not routinely assess data for diabetes and vascular disease. Development of peripheral neuropathy after the first cycle of induction treatment is described as early onset, and after two to three cycles of induction treatment as late onset. Vincristine-induced peripheral neuropathy was used as a reference when we assessed the incidence and severity of bortezomib-induced peripheral neuropathy.

RNA isolation and microarray processing was done as previously described.[20] Microarray data presented in this report have been stored in the Gene Expression Omnibus database (National Center for Biotechnology Information, Bethesda MD, USA), accession number GSE19784. Gene expression arrays were done with RNA extracted from myeloma plasma cells that were purified (≥ 80%) from the extra bone marrow aspiration taken at diagnosis and met the criteria for quality.[20]

DNA was extracted from peripheral blood nucleated cells or CD138-negative bone marrow cells and quantified by use of the Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE, USA). Samples were genotyped by use of the Affymetrix Targeted Genotyping (Affymetrix, Santa Clara, CA, USA) custom built panel, with 3404 SNPs, selected with a hypothesis-driven strategy, targeting genes and SNPs for which associations or putative functional effects have been noted (Corthals SL, unpublished data).

Statistical analysis

For differences in incidence of baseline and grade 2-4 peripheral neuropathy after one cycle and after two to three cycles of bortezomib-based and vincristine-based treatment, χ^2 analysis was done with a two-sided p value of 0.05. For gene expression data, class comparison of groups of arrays was done with one-way ANOVA in Partek Genomics Suite (version 6.4), followed by multiple-test correction with a false discovery rate of less than 0.05.

For SNP genotyping data, deviations from Fisher's exact *t* test for Hardy-Weinberg equilibrium at $p < 0.00001$ and bias in missing data were controlled for each SNP. SNPs with a minor allele frequency of less than 5% and a call rate of less than 80% were removed from further analysis. To assess SNP associations with treatment-related peripheral neuropathy and calculation of odds ratios (ORs), a Cochran-Armitage trend test and a Fisher's *t* exact test were done. We assessed the genomic inflation factor λ based on the median χ^2 for each analysis with PLINK (version 1.07).[21] To account for multiple testing, 10 000 permutation

tests were done with the max(T) permutation procedure with PLINK. To assess the effect of non-synonymous SNPs associated with bortezomib-induced peripheral neuropathy and vincristine-induced peripheral neuropathy, SNPs were characterized by use of the prediction program Sorting Intolerant From Tolerant (version 4.0.3).

Analysis of the gene and SNP sets for peripheral neuropathy associated with bortezomib and vincristine was done by use of Ingenuity Pathway Analysis software (version 8.7).

This study is registered as an International Standard Randomized Controlled Trial, number ISRCTN64455289.

RESULTS

We did gene expression arrays for 329 (39%; 170 treated with bortezomib, 159 treated with vincristine) of 833 patients included in the trial, and SNP profiles for samples taken from 369 (44%; 186 treated with bortezomib, 183 treated with vincristine) patients. Simultaneous gene expression and SNP data were obtained for 185 patients; only SNP data were available for 184 patients, and only gene expression data were available for 144 patients. The baseline clinical characteristics of 513 patients included in this study were not different from the whole patient group included in the trial (webappendix p 1).

Table 1 shows the incidence of peripheral neuropathy at baseline and after treatment with bortezomib-based and vincristine-based induction treatments in 500 of 513 patients who were fully assessable and had a minimum follow-up of 40 months. The median time to development of bortezomib-induced peripheral neuropathy was 42 days (range 0–137). Cumulative dose of bortezomib given before development of peripheral neuropathy was 13 mg/m^2. 52 patients (21%) developed grade 1 bortezomib-induced peripheral neuropathy, and 34 (14%) developed grade 1 peripheral neuropathy before progressing to a higher grade. When patients developed peripheral neuropathy, the dose of bortezomib was adjusted according to the established guidelines for dose modification.[5-7,19] Median time to development of vincristine-induced peripheral neuropathy was 37 days (range 0–171). Cumulative dose of vincristine given before development of peripheral neuropathy was 4 mg. 60 (24%) patients developed vincristine-induced peripheral neuropathy, and 18 (7%) developed grade 1 peripheral neuropathy before progressing to a higher grade. When patients developed vincristine-induced peripheral neuropathy, vincristine was discontinued and supportive treatments such as pregabalin were used. Overall, baseline peripheral neuropathy was noted in only a small number of patients (Table 1). The proportion of patients developing late-onset bortezomib-induced peripheral neuropathy was significantly higher than that of patients with late-onset vincristine-induced peripheral neuropathy (Table 1).

Table 1. Incidence of baseline, bortezomib-induced, and vincristine-induced peripheral neuropathy.

	bortezomib + AD (n = 250)	**VAD (n = 250)**	***p-value***
Baseline PNP	8 (3%)	13 (5%)	*(p=.37)*
Early onset BiPN / ViPN (after one cycle)	BiPN	ViPN	
PNP grade 2–4	20 (8%)	11 (4%)	*(p=.27)*
PNP grade 2	10 (50%)	9 (82%)	
PNP grade 3	7 (35%)	1 (9%)	*(p=.18)*
PNP grade 4	3 (15%)	1 (9%)	
Late onset BiPN/ ViPN (after cycle 2–3)			
PNP grade 2–4	63 (25%)	17 (7%)	*(p<.0001)*
PNP grade 2	31 (49%)	11 (65%)	
PNP grade 3	24 (38%)	6 (35%)	*(p=.72)*
PNP grade 4	8 (13%)	0	

Gene expression arrays for 15 patients developing early-onset grade 2–4 bortezomib-induced peripheral neuropathy were compared with arrays of patients who did not develop bortezomib-induced peripheral neuropathy (table 2). Grade 2–4 early-onset bortezomib-induced peripheral neuropathy was characterized by 19 differentially expressed genes (false discovery rate < 0.05). The genes showing the highest changes in the gene expression arrays are shown in table 2, and the complete number of differentially expressed probe sets are shown in the webappendix pp 2–3. The genes showing the highest change in expression included *RAPH1* (involved in signal transduction), *FRMD4B*, *MFHAS1* (possibly an oncogene regulated by NF-κB or tumor necrosis factor), and *DDIT4L* (a DNA-damage inducible transcript; table 2). Genes that might play a direct part in bortezomib-induced peripheral neuropathy are transcription regulator *SOX8* (involved in development of peripheral nervous system), and *CPT1C* and *RHOBTB2* (webappendix pp 2-3). Ingenuity pathway analyses of gene and SNP sets showed enrichment of genes implicated in the canonical pathway of signaling mediated by AMP-activated protein kinase (AMPK), including *CPT1C, CKM*, and *PIK3CG* (three of 156 genes involved in AMPK signaling were upregulated, $p = 7.33 \times 10^{-5}$).

Gene expression arrays for 44 patients with grade 2–4 late-onset bortezomib-induced peripheral neuropathy were characterized by 27 differentially expressed genes, using the same false discovery rate as for early onset (webappendix pp 2–3), and showed a different pattern of gene expression to that in early-onset bortezomib-induced peripheral neuropathy, without overlap (Table 2). *RASGRP1* showed the highest change in patients with late-onset bortezomib-induced peripheral neuropathy compared with patients without this side-effect (Table 2). Furthermore, we noted upregulation of genes involved in transcription regulation, including *TRERF1*, *TRPS1*, and *MDM2*. We noted enrichment of genes involved in the development and function of the nervous system, including *SOD2* and *MYO5A*.

Table 2. Differentially expressed genes in early-onset and late-onset bortezomib-induced and vincristine-induced peripheral neuropathy.

	Gene name	Gene description	Factor difference in expression	*p value*
Grade 2–4 peripheral neuropathy (n = 15) versus no peripheral neuropathy (n = 134) after one cycle of bortezomib				
225189_s_at	*RAPH1*	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	2·24	$3{\cdot}04 \times 10^{-2}$
235014_at	*LOC147727*	Hypothetical protein LOC147727	2·15	$1{\cdot}91 \times 10^{-2}$
1569872_a_at	*LOC650392*	Hypothetical protein LOC650392	1·98	$9{\cdot}65 \times 10^{-4}$
213056_at	*FRMD4B*	FERM domain containing 4B	1·74	$8{\cdot}42 \times 10^{-3}$
227984_at	*LOC650392*	Hypothetical protein LOC650392	1·71	$1{\cdot}19 \times 10^{-3}$
225478_at	*MFHAS1*	Malignant fibrous histiocytoma amplified sequence 1	1·68	$5{\cdot}34 \times 10^{-9}$
226913_s_at	*SOX8*	SRY (sex determining region Y)-box 8	1·68	$4{\cdot}28 \times 10^{-13}$
204810_s_at	*CKM*	Creatine kinase, muscle	1·67	$1{\cdot}11 \times 10^{-30}$
1569871_at	*LOC650392*	Hypothetical protein LOC650392	1·65	$1{\cdot}77 \times 10^{-19}$
228057_at	*DDIT4L*	DNA-damage-inducible transcript 4-like	1·59	$5{\cdot}59 \times 10^{-20}$
Grade 2–4 peripheral neuropathy (n = 44) versus no peripheral neuropathy (n = 78) after two or three cycles of bortezomib				
205590_at	*RASGRP1*	RAS guanyl releasing protein 1 (calcium and DAG regulated)	2·97	$2{\cdot}14 \times 10^{-2}$
204527_at	*MYO5A*	Myosin VA (heavy chain 12, myoxin)	1·93	$3{\cdot}21 \times 10^{-2}$
235065_at	..	..	1·57	$3{\cdot}19 \times 10^{-2}$
205422_s_at	*ITGBL1*	Integrin, β-like 1 (with EGF-like repeat domains)	1·44	$1{\cdot}35 \times 10^{-3}$
228113_at	*RAB37*	RAB37, member of RAS oncogene family	1·41	$3{\cdot}69 \times 10^{-2}$
210321_at	*GZMH*	Granzyme H (cathepsin G-like 2, protein h-CCPX)	1·37	$3{\cdot}19 \times 10^{-2}$
226969_at	*MTR*	5-methyltetrahydrofolate-homocysteine methyltransferase	1·34	$4{\cdot}26 \times 10^{-2}$
204072_s_at	*FRY*	Furry homolog (*Drosophila*)	1·31	$4{\cdot}94 \times 10^{-2}$
236442_at	*DPF3*	D4, zinc and double PHD fingers, family 3	1·30	$3{\cdot}38 \times 10^{-2}$
243329_at	..	..	1·30	$4{\cdot}26 \times 10^{-2}$
Grade 2–4 peripheral neuropathy (n = 9) versus no peripheral neuropathy (n = 129) after one cycle of vincristine				
208235_x_at	*GAGE7*	G antigen 7	11·55	$3{\cdot}21 \times 10^{-3}$
206640_x_at	*GAGE12I*	G antigen 12I	11·46	$4{\cdot}29 \times 10^{-3}$
207739_s_at	*GAGE2C*	G antigen 2C	7·76	$1{\cdot}62 \times 10^{-3}$
208155_x_at	*GAGE6*	G antigen 6	6·88	$1{\cdot}06 \times 10^{-5}$
206897_at	*PAGE1*	P antigen family, member 1 (prostate associated)	6·76	$4{\cdot}29 \times 10^{-2}$

Table 2. *Continued*

	Gene name	Gene description	Factor difference in expression	*p value*
216063_at	*HBBP1*	Haemoglobin, β pseudogene 1	6·24	$4{\cdot}04 \times 10^{-2}$
207086_x_at	*GAGE4*	G antigen 4	6·16	$3{\cdot}29 \times 10^{-5}$
206626_x_at	*SSX1*	Synovial sarcoma, X breakpoint 1	5·93	$2{\cdot}61 \times 10^{-2}$
207912_s_at	*DAZ1*	Deleted in azoospermia 1	5·86	$1{\cdot}06 \times 10^{-4}$
214957_at	*ACTL8*	Actin-like 8	4·93	$1{\cdot}32 \times 10^{-10}$
Grade 2–3 peripheral neuropathy (n = 10) versus no peripheral neuropathy (n = 103) after two or three cycles of vincristine				
210632_s_at	*SGCA*	Sarcoglycan, alpha (50 kDa dystrophin-associated glycoprotein)	4·08	$3{\cdot}35 \times 10^{-2}$
210992_x_at	*FCGR2C*	Fc fragment of IgG, low affinity IIc, receptor for (CD32)	2·49	$3{\cdot}57 \times 10^{-2}$
241991_at	..	..	1·80	$3{\cdot}35 \times 10^{-2}$
206771_at	*UPK3A*	Uroplakin 3A	1·59	$2{\cdot}94 \times 10^{-2}$
241365_at	..	..	1·57	$3{\cdot}35 \times 10^{-2}$
236266_at	*RORA*	RAR-related orphan receptor A	1·53	$2{\cdot}94 \times 10^{-2}$
214059_at	*IFI44*	Interferon-induced protein 44	1·51	$4{\cdot}92 \times 10^{-6}$
230477_at	..	..	1·48	$2{\cdot}94 \times 10^{-2}$
237322_at	*MIAT*	Myocardial infarction associated transcript (non-protein coding)	1·45	$2{\cdot}94 \times 10^{-2}$
239239_at	..	..	1·33	$2{\cdot}94 \times 10^{-2}$

First column is the probe-set identification numbers. Genes were ranked from highest to lowest change; the first ten genes with the highest changes are shown.

All significant SNPs (permuted $p < 0.01$) associated with grade 2–4 early-onset bortezomib-induced peripheral neuropathy are shown in table 3 (values of permuted $p < 0.05$ are shown in webappendix pp 4–10). Several SNPs associated with early-onset bortezomib-induced peripheral neuropathy were located in *caspase 9* (rs4646091, rs2020895, rs2020903, rs4646032, and rs4646034). Other highly associated SNPs were located in genes *RDM1*, *ALOX12*, *IGF1R*, and *LSM1* (Table 3). Pathway analysis of these associated genes showed enrichment of genes involved in cell death (14 genes, $p = 5{\cdot}25 \times 10^{-3}$-$4{\cdot}93 \times 10^{-2}$), DNA repair (14 genes, $p = 5.25 \times 10^{-3}$-4.93×10^{-2}), and development and function of the nervous system (four genes, $p=2.01 \times 10^{-3}$).

The SNPs that were characteristic of late-onset bortezomib-induced peripheral neuropathy were mainly located in DNA repair genes, such as *ERCC3, ERCC4, ATM, BRCA1, EXO1*, and *MRE11A* (Table 3; webappendix pp 4–10). Pathway analysis showed enrichment of associated SNPs located in genes involved in the development and function of the

nervous system (three genes, $p = 3.35 \times 10^{-3}$-1.69×10^{-2}) and in inflammatory disease (26 genes, $p = 2.09 \times 10^{-3}$-4.95×10^{-3}).

Table 3. Single-nucleotide polymorphisms associated with bortezomib-induced and vincristine-induced peripheral neuropathy.

	Chromosome	Gene	Single-nucleotide polymorphism type	Odds ratio (95% CI)	*p value*	Permuted *p value*
Grade 2–4 peripheral neuropathy (n = 13) versus no peripheral neuropathy (n = 147) after one cycle of bortezomib						
rs2251660	17	*RDM1*	Coding non-synonymous	3·65 (1·55–8·57)	$9{\cdot}06 \times 10^{-4}$	$2{\cdot}40 \times 10^{-3}$
rs4646091	1	*CASP9*	Intron	3·59 (1·59–8·14)	$1{\cdot}43 \times 10^{-3}$	$2{\cdot}90 \times 10^{-3}$
rs1126667	17	*ALOX12*	Coding non-synonymous	3·50 (1·47–8·32)	$2{\cdot}95 \times 10^{-3}$	$3{\cdot}80 \times 10^{-3}$
rs434473	17	*ALOX12*	Coding non-synonymous	3·50 (1·47–8·32)	$2{\cdot}95 \times 10^{-3}$	$4{\cdot}10 \times 10^{-3}$
rs7823144	8	*LSM1*	Intron	4·11 (1·48–11·39)	$2{\cdot}30 \times 10^{-3}$	$7{\cdot}60 \times 10^{-3}$
rs1879612	15	*IGF1R*	Intron	0·22 (0·07–0·77)	$9{\cdot}42 \times 10^{-3}$	$8{\cdot}30 \times 10^{-3}$
rs1029871	3	*NEK4*	Coding non-synonymous	0·30 (0·11–0·81)	$8{\cdot}31 \times 10^{-3}$	$9{\cdot}30 \times 10^{-3}$
Grade 2–4 peripheral neuropathy versus (n=49) no peripheral neuropathy (n=80) after two or three cycles of bortezomib						
rs1799800	16	*ERCC4*	Intron	2·74 (1·56–4·84)	$5{\cdot}16 \times 10^{-4}$	$1{\cdot}00 \times 10^{-3}$
rs1799801	16	*ERCC4*	Coding synonymous	2·48 (1·43–4·28)	$8{\cdot}85 \times 10^{-4}$	$1{\cdot}10 \times 10^{-3}$
rs2300697	2	*SRD5A2*	Intron	0·63 (0·37–1·05)	$4{\cdot}80 \times 10^{-2}$	$2{\cdot}90 \times 10^{-3}$
rs1059293	21	*IFNGR2*	Untranslated, intron	2·30 (1·37–3·87)	$8{\cdot}97 \times 10^{-4}$	$3{\cdot}20 \times 10^{-3}$
rs2276583	2	*ERCC3*	Locus	1·26 (0·75–2·12)	$3{\cdot}87 \times 10^{-1}$	$3{\cdot}30 \times 10^{-3}$
rs189037	11	*ATM*	Locus, untranslated	0·53 (0·32–0·89)	$2{\cdot}32 \times 10^{-2}$	$3{\cdot}60 \times 10^{-3}$
rs10501815	11	*MRE11A*	Intron, TagSNP	3·27 (1·39–7·74)	$4{\cdot}41 \times 10^{-3}$	$4{\cdot}20 \times 10^{-3}$
rs664677	11	*ATM*	Intron	0·57 (0·34–0·96)	$4{\cdot}36 \times 10^{-2}$	$5{\cdot}90 \times 10^{-3}$
rs664982	11	*ATM*	Intron	0·51 (0·30–0·85)	$1{\cdot}72 \times 10^{-2}$	$6{\cdot}20 \times 10^{-3}$
rs6131	1	*SELP*	Coding non-synonymous	0·43 (0·23–0·83)	$6{\cdot}69 \times 10^{-3}$	$6{\cdot}30 \times 10^{-3}$
rs1130499	7	*PTPRN2*	Coding non-synonymous	0·43 (0·23–0·79)	$6{\cdot}23 \times 10^{-3}$	$6{\cdot}60 \times 10^{-3}$
rs4722266	7	*STK31*	Coding non-synonymous	0·29 (0·12–0·74)	$5{\cdot}66 \times 10^{-3}$	$8{\cdot}30 \times 10^{-3}$
rs2267668	6	*PPARD*	Intron	0·35 (0·15–0·83)	$9{\cdot}30 \times 10^{-3}$	$9{\cdot}10 \times 10^{-3}$
Grade 2–4 peripheral neuropathy versus (n = 7) no peripheral neuropathy (n = 151) after one cycle of vincristine						
rs7739752	6	*PPARD*	Intron	13·43 (3·90–46·22)	$6{\cdot}34 \times 10^{-7}$	$8{\cdot}00 \times 10^{-4}$
rs2288087	9	*ALDH1A1*	Intron, TagSNP	7·62 (1·68–34·65)	$1{\cdot}40 \times 10^{-3}$	$1{\cdot}50 \times 10^{-3}$
rs1494961	4	*HEL308*	Coding non-synonymous	6·67 (1·47–30·32)	$2{\cdot}30 \times 10^{-3}$	$2{\cdot}60 \times 10^{-3}$
rs6901410	6	*PPARD*	Intron	9·67 (2·65–35·30)	$7{\cdot}75 \times 10^{-3}$	$6{\cdot}00 \times 10^{-3}$
rs6902123	6	*PPARD*	Intron	9·67 (2·65–35·30)	$7{\cdot}75 \times 10^{-3}$	$6{\cdot}00 \times 10^{-3}$
rs2274407	13	*ABCC4*	Coding non-synonymous	7·15 (2·02–25·31)	$2{\cdot}94 \times 10^{-4}$	$6{\cdot}10 \times 10^{-3}$

Table 3. *Conntinued*

	Chromo-some	Gene	Single-nucleotide polymorphism type	Odds ratio (95% CI)	*p value*	Permuted *p value*
rs909253	6	*LTA*	Intron	4·67 (1·52–14·34)	$3{\cdot}09 \times 10^{-3}$	$6{\cdot}60 \times 10^{-3}$
rs6457816	6	*PPARD*	Intron	8·89 (2·46–32·17)	$1{\cdot}40 \times 10^{-4}$	$7{\cdot}30 \times 10^{-3}$
rs1041981	6	*LTA*	Coding non-synonymous	4·52 (1·47–13·88)	$3{\cdot}58 \times 10^{-3}$	$7{\cdot}40 \times 10^{-3}$
rs3803258	13	*SLC10A2*	Untranslated	4·30 (1·45–12·74)	$3{\cdot}51 \times 10^{-3}$	$7{\cdot}40 \times 10^{-3}$
rs3749442	3	*ABCC5*	Coding synonymous	4·64 (1·5–14·05)	$2{\cdot}72 \times 10^{-3}$	$9{\cdot}60 \times 10^{-3}$
Grade 2–3 peripheral neuropathy (n = 14) versus no peripheral neuropathy (n = 104) after two or three cycles of vincristine						
rs10515114	5	*CART*	Locus	4·62 (1·68–12·72)	$7{\cdot}92 \times 10^{-4}$	$2{\cdot}90 \times 10^{-3}$
rs6873545	5	*GHR*	Intron	0·09 (0·01–0·69)	$3{\cdot}44 \times 10^{-3}$	$3{\cdot}60 \times 10^{-3}$
rs3734354	6	*SIM1*	Coding non-synonymous	3·30 (1·39–7·82)	$2{\cdot}31 \times 10^{-3}$	$5{\cdot}10 \times 10^{-3}$
rs11688	1	*JUN*	Coding synonymous	5·00 (1·80–13·91)	$9{\cdot}10 \times 10^{-4}$	$5{\cdot}20 \times 10^{-3}$
rs4129472	5	*GHR*	Intron	0·11 (0·01–0·80)	$6{\cdot}46 \times 10^{-3}$	$5{\cdot}20 \times 10^{-3}$
rs1413239	1	*DPYD*	Intron, TagSNP	3·29 (1·47–7·37)	$3{\cdot}03 \times 10^{-3}$	$5{\cdot}40 \times 10^{-3}$
rs1045020	5	*SLC22A5*	Untranslated	4·80 (1·83–12·61)	$1{\cdot}48 \times 10^{-3}$	$5{\cdot}40 \times 10^{-3}$
rs9885672	6	*KIAA0274*	Coding non-synonymous	3·89 (1·62–9·33)	$2{\cdot}05 \times 10^{-3}$	$5{\cdot}60 \times 10^{-3}$
rs3887412	16	*ABCC1*	Intron, TagSNP	3·36 (1·47–7·67)	$3{\cdot}31 \times 10^{-3}$	$5{\cdot}70 \times 10^{-3}$
rs6886047	5	*GHR*	Intron	0·10 (0·01–0·72)	$3{\cdot}97 \times 10^{-3}$	$6{\cdot}10 \times 10^{-3}$
rs1236913	9	*PTGS1*	Coding non-synonymous	5·40 (1·79–16·28)	$1{\cdot}43 \times 10^{-3}$	$6{\cdot}30 \times 10^{-3}$
rs2644983	16	*ABCC1*	Intron, TagSNP	4·22 (1·69–10·50)	$2{\cdot}27 \times 10^{-3}$	$6{\cdot}60 \times 10^{-3}$
rs1042713	5	*ADRB2*	Coding non-synonymous	0·23 (0·08–0·69)	$5{\cdot}30 \times 10^{-3}$	$7{\cdot}20 \times 10^{-3}$
rs1966265	5	*FGFR4*	Coding non-synonymous	3·47 (1·51–7·94)	$3{\cdot}40 \times 10^{-3}$	$7{\cdot}30 \times 10^{-3}$
rs2308327	10	*MGMT*	Coding non-synonymous	3·38 (1·33–8·58)	$3{\cdot}69 \times 10^{-3}$	$7{\cdot}30 \times 10^{-3}$
rs5759197	22	*BZRP*	Intron	2·93 (1·31–6·53)	$6{\cdot}32 \times 10^{-3}$	$7{\cdot}60 \times 10^{-3}$
rs1005658	22	*BZRP*	Locus	3·14 (1·39–7·08)	$6{\cdot}04 \times 10^{-3}$	$8{\cdot}50 \times 10^{-3}$
rs7441774	4	*UGT2B7*	Intron	3·60 (1·40–9·23)	$6{\cdot}61 \times 10^{-3}$	$9{\cdot}60 \times 10^{-3}$

The genetic profile of myeloma plasma cells from nine patients who developed grade 2–4 early-onset vincristine-induced peripheral neuropathy showed overexpression of the genes for testis cancer antigens, of which the *GAGE* genes were mainly upregulated (Table 2).

The gene profiles of ten patients who developed grade 2 or 3 late-onset vincristine-induced peripheral neuropathy showed only ten differentially expressed genes, including *RORA* and *IFI44* (Table 2).

Table 3 shows SNPs significantly associated with early-onset vincristine-induced peripheral neuropathy. Four of the most highly associated SNPs (rs7739752, rs6901410, rs6902123, and rs6457816) were located in the transcription factor *PPARD*. Additionally, an

intronic (rs909253) and a coding non-synonymous SNP (rs1041981) in *LTA* were significantly associated with early-onset vincristine-induced peripheral neuropathy. Other significant SNPs were located in genes for transporter enzymes *ABCC4*, *ABCC5*, and *SLC10A2*, oxidizing enzyme *ALDH1A1*, and *GLI1I* (Table 3; webappendix pp 4–10). Pathway analysis showed enrichment of associated SNPs located in genes involved in cellular growth and proliferation (four genes, $p=1.14\times10^{-2}$-4.95×10^{-2}).

Some intronic SNPs in the dihydropyrimidine dehydrogenase gene *DPYD* and some in the ABC transporter gene *ABCC1* were associated with late-onset vincristine-induced peripheral neuropathy (Table 3). Pathway analysis showed that most significant SNPs (permuted $p < 0.05$) were located in genes for absorption, distribution, metabolism, and excretion (six genes, $p = 2.06 \times 10^{-2}$-4.18×10^{-2}).

DISCUSSION

The genetic profiles of patients with early-onset bortezomib-induced peripheral neuropathy suggest the involvement of genes involved in transcription, apoptosis, and AMPK-mediated signaling. The possible role of AMPK-mediated signaling is of particular interest because this enzyme functions by stimulating the signaling pathways that replenish cellular ATP supplies in response to low glucose, hypoxia, ischemia, or heat shock, which might be triggered in myeloma cells in response to bortezomib. *CPT1C* codes for an enzyme found in neuron mitochondria that is involved in transport of hydrophobic fatty acid chains into mitochondria, and plays a part in mitochondrial dysfunction. It might also have an important role in bortezomib-induced peripheral neuropathy, since damage to mitochondria and endoplasmic reticulum through activation of a mitochondrial-based apoptotic pathway by bortezomib was noted in dorsal root ganglia of mice given bortezomib.[11] *RHOBTB2*, encodes another enzyme implicated here, has been shown to be upregulated during drug-induced apoptosis, being mainly dependent on E2F1.[22] Knockout of *RHOBTB2* with small interfering RNAs has been shown to delay the onset of drug-induced apoptosis.[22] *RASGRP1* is involved in many processes, including apoptosis and calcium-ion binding, which are potentially interesting for its role in bortezomib-induced peripheral neuropathy. The presence of polymorphisms in the apoptosis gene *caspase 9*, which plays an important part in bortezomib-induced apoptosis, suggests the possible contribution of this enzyme to early-onset peripheral neuropathy.[23,24] One of the most significant SNPs (rs1029871) might have a role in the splicing regulation of *NEK4*, which is involved in the regulation of cell cycle and cell division. Furthermore, SNPs in enriched pathways like DNA repair and nervous system development and function were associated with early-onset bortezomib-induced peripheral neuropathy.

Late-onset bortezomib-induced peripheral neuropathy was associated with genes involved in the development and function of the nervous system. We noted upregulation of the superoxide dismutase gene *SOD2* in myeloma plasma cells; *SOD2* is regulated by tumor necrosis factor α and NF-κB, and is known to have a role in the survival of neurons. Patients with diabetes and a polymorphism in the *SOD2* gene, leading to reduced *SOD2* activity, have been shown to be at increased risk of developing diabetic peripheral neuropathy.[25] The protective effect of *SOD2* might be eliminated with bortezomib-induced apoptosis, which might trigger a susceptibility to oxidative stress in treated patients. Three SNPs associated with late-onset bortezomib-induced peripheral neuropathy were located in *SERPINB2* (plasminogen activator inhibitor-2). *SERPINB2,* with *SERPIN-1* (plasminogen activator inhibitor-1), tissue-type plasminogen activator, and urokinase-type plasminogen activator, has been shown to be induced in dorsal root ganglion neurons after peripheral axotomy in mice.[26] These serpins might also act as autocrine or paracrine regulators of plasminogen-activator-mediated nerve regeneration processes.[26] The associated SNPs might affect *SERPINB2* expression through their effect on splicing regulation. Besides genes involved in development of the nervous system, proinflammatory genes might play an important part in the pathogenesis of late-onset bortezomib-induced peripheral neuropathy, based on the presence of intronic SNPs in *MBL2* and *PPARD* (Corthals SL, unpublished data), and of about 30% of SNPs with reported inflammatory roles. The hypothesis that the DNA repair pathway is involved in bortezomib-induced peripheral neuropathy, and that this side-effect might be caused by the inability to repair neuronal damage (Corthals SL, unpublished data), could be substantiated by the presence of SNPs in *BRCA1* (rs16941 and rs799917). These non-synonymous SNPs might have an effect on the phosphorylation state of a protein, which has been shown to abolish the P871L phosphorylation site in *BRCA1*.[27] Therefore, early-onset and late-onset bortezomib-induced peripheral neuropathies were both associated with a myeloma genetic profile that was characterized by genes involved in the development of the nervous system; however, apoptosis was also a characteristic for the development of early-onset bortezomib-induced peripheral neuropathy. Genetic polymorphisms in genes involved in nervous system development and DNA repair play a part in both the early and late onset of this side-effect.

A comparison of the molecular profiles of bortezomib-induced peripheral neuropathy and vincristine-induced peripheral neuropathy showed no overlap in associated genes or SNPs. Genes involved in cell cycle and proliferation were mainly associated with early-onset vincristine-induced peripheral neuropathy, both in the analyses of genetic pathways and SNPs. Additionally, involvement of proinflammatory genes in early-onset vincristine-induced peripheral neuropathy was substantiated by the finding of SNPs in *PARP1* and *LTA*, and two SNPs in *GLI1* (rs2228224 and rs2228226), which both encode an aminoacid change; rs2228226 has been shown to affect *GLI1* activity, thereby affecting the inflammatory response.[28]

Genes implicated in drug absorption, distribution, metabolism, and excretion have been shown to be involved in chemotherapy-induced peripheral neuropathy.[29] In accordance with this finding, an association was noted for late-onset vincristine-induced peripheral neuropathy with nine intronic SNPs in *ABCC1*; vincristine is known to be a substrate of the protein coded for by this gene.

In conclusion, this study provides the first large dataset in which the contribution of both the inherited genetic constitution of the host (patient) and the tumor (myeloma) to the development of bortezomib-induced peripheral neuropathy has been reported. We identified molecular factors that are associated with bortezomib-induced peripheral neuropathy in patients with newly diagnosed multiple myeloma. Genes for apoptosis contribute to early-onset bortezomib-induced peripheral neuropathy, whereas genes that have a role in inflammatory pathways and DNA repair contribute to the development of late-onset peripheral neuropathy, indicating that distinct genetic factors are involved in the development of early-onset and late-onset forms of this side-effect. Bortezomib-induced and vincristine-induced peripheral neuropathy arise through different molecular mechanisms. Our findings strongly suggest an interaction between myeloma-related factors and the patient's genetic background in the development of bortezomib-induced peripheral neuropathy. Profiles of genetic risk might be used in future to identify patients with an increased risk of bortezomib-induced peripheral neuropathy.

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CHAPTER 10

GENERAL ASPECTS AND MECHANISMS OF PERIPHERAL NEUROPATHY ASSOCIATED WITH BORTEZOMIB IN PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA

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ABSTRACT

Introduction of the proteasome inhibitor bortezomib (Velcade®, Millennium Pharmaceuticals, Inc., The Takeda Oncology Company, Cambridge, Massachusetts) has substantially improved outcomes for patients with multiple myeloma (MM), and has become one of the cornerstones of current anti-myeloma treatment regimens. However, with the introduction of bortezomib it has become clear that peripheral neuropathy (PN) is one of the most frequent, potentially disabling non-hematological complications of bortezomib, often requiring dose modification or discontinuation, with a potential negative impact on clinical endpoints and quality of life. To find a balance between maximal benefit of bortezomib treatment, while maintaining quality of life, it is necessary to minimize toxicity. In this part of the issue concerning bortezomib for treatment of MM, we discuss all aspects of bortezomib induced peripheral neuropathy (BiPN), and elaborate on the mechanisms underlying the development of BiPN.

INTRODUCTION

MM is characterized by clonal expansion of malignant plasma cells in the bone marrow, the presence of a monoclonal protein in serum and/or urine, and the presence of related organ or tissue impairment. More than a decade ago, the treatment of multiple myeloma patients under 65 years of age consisted of chemotherapy in combination with corticosteroids, followed by high dose melphalan and autologous stem cell transplantation (ASCT). Patients ineligible for ASCT received treatment with melphalan and prednisone. Since the introduction of novel agents, such as thalidomide and bortezomib, CR rates, PFS, and OS, have substantially improved. However, with these novel agents different adverse events were reported. From the time thalidomide and bortezomib entered clinical trials, a striking high percentage of patients were reported to develop polyneuropathy (PN).

Clinical presentation

Peripheral neuropathy is defined as any form of damage, inflammation, or degeneration of peripheral nerves, implying that it is not limited to sensory nerve damage. Patients can also present with symptoms or signs of motor or autonomic nervous system damage, or both, although sensory neuropathy and neuropathic pain are the dominant features of BiPN.

Sensory neuropathy includes characteristic symptoms such as hyperesthesia, hypoesthesia, and paresthesias (tingling), usually in a distal stocking and- glove distribution over the hands and feet.

Neuropathic pain, which can be a sharp pain or burning sensation, may occur at rest, is usually localized in the soles of the feet, but occasionally also presents in the fingers and the palmar sides of the hands. It is mainly caused by small fibre damage and is associated with altered heat and cold sensation.[1]

Additionally, sensory BiPN can result in areflexia and loss of proprioception, placing patients at risk for injury through ataxia and gait disturbance.[2]

Motor symptoms are rare and if present, they mostly occur in the context of severe sensory peripheral neuropathy. Symptoms include muscle cramps, muscle atrophy, or loss of strength in distal muscles.

Autonomic symptoms, such as orthostatic hypotension, bradycardia and constipation can occasionally occur and are induced by damage to small fibres.[3,4] To illustrate BiPN, the differences with thalidomide induced neuropathy are depicted in table 1. This issue concerns different aspects in treatment with bortezomib, thalidomide will not be further discussed here.

Table 1. Presenting symptoms of TiPN and BiPN.[48]

peripheral neural tract involved	presenting symptoms	thalidomide	bortezomib
Sensory	hypo-esthesia paresthesia: numbness, tingling, pin-prick sensation hyperesthesia	common	common
	ataxia, gait disturbance	Rare	rare
	neuropathic pain	rare	common
Motor	weakness	rare	rare
	Muscle cramps, fasciculations	common	rare
Autonomic	gastro-intestinal	constipation	constipation
	others	impotence bradycardia	orthostatic hypotension

Incidence of BiPN

BiPN has been well documented from the time of the initial clinical introduction of bortezomib in (heavily) pretreated relapsed/refractory multiple myeloma (MM) patients included in phase 2, SUMMIT an CREST, and phase 3 trials, APEX.[5-7] A total of 587 patients in these trials receiving standard doses of bortezomib were assessed for PN. By contrast with the phase 2 studies, where 22 of 256 (9%) patients had NCI-CTC grade 3 PN at baseline, patients with grade 2 or higher PN at baseline were excluded in the APEX trial. BiPN grade 1–4 occurred in 84 of 228 (37%) and 124 of 331 (37%) of patients receiving bortezomib 1.3 mg/m2, and in six of 28 (21%) of patients receiving bortezomib 1.0 mg/m2, with grade 3 in 9–13%, and grade 4 in less than 1% of patients (Table 2).[8,9]

In the IFM trial for newly diagnosed patients treated with bortezomib in the induction regimen followed by autologous stem cell transplantation, BiPN occurred in 46% of patients, with up to 7% being grade 3 or 4.[10] In the HOVON-65/GMMG-HD4 trial including newly diagnosed transplant candidates, in which bortezomib was given as induction treatment as well as during maintenance, BiPN was observed in 37% of patients during induction treatment, with 24% concerning grade 3-4 BiPN.[11]

In elderly patients, bortezomib in combination with melphalan and prednisone (VMP) was associated with 13% grade 3-4 and 44% overall BiPN. [12]

Interestingly, when bortezomib was combined with other potentially neurotoxic agents, such as thalidomide, this did not further increase the rate of treatment induced PN. Peripheral neuropathy grade 3-4 occurred in 23/236 (10%) of newly diagnosed MM patients treated with bortezomib, thalidomide and dexamethasone (VTD).[13] In 14% of relapsed/refractory MM patients treated with bortezomib, lenalidomide and dexamethasone (VRD), grade 3 BiPN was observed.[14] Grade 3 to 4 sensory neuropathy was reported in 8% of

untreated multiple myeloma patients ineligible for autologous stem-cell transplantation receiving VMPT-VT and in 5% of patients receiving VMP; neuralgia was reported in 4% of patients receiving VMPT-VT and 3% of patients receiving VMP.[15]

Table 2. Incidence of BiPN.

Trial	Schedule		All BiPN %	BiPN gr 3-4 %	Reference
SUMMIT, CREST	Bortezomib	256	35	13	[5,6,8]
APEX	Bor vs Dex	331 vs. 336	37 vs. 9	8 vs < 1	[7,9]
IFM 2005-01	Bor/Dex vs VAD	239 vs. 239	46 vs. 28	7 vs. 2	[10]
HOVON-65/ GMMGH-D4	PAD vs VAD	373 vs. 371	37 vs. 26	26 vs.10	[11]
VISTA	VMP vs MP	340 vs. 337	44 vs. 5	13 vs. 0	[12]
M. Cavo	VTD vs VD	236 vs. 238	34 vs. 14	10 vs. 2	[13]
M. Dimopoulos	VRD vs RD	49 vs. 50	NR	14 vs. NR*	[14]
A. Palumbo	VMPT-VT vs VMP	250 vs. 253	NR	8 vs. 5	[15]

NR, not reported; dex, dexamethasone; bor, bortezomib; VAD, vincristine/doxorubicin/dexamethasone; PAD, bortezomib/ doxorubicin/dexamethasone; VMP, bortezomib/melphalan/prednisone; MP, melphalan/prednisone; VTD, bortezomib/ thalidomide/dexamethason; VD, bortezomib/dexamethason; VRD bortezomib/lenalidomide/dexamethason; RD, lenalidomide/dexamethason; VMPT-VT, bortezomib/melphalan/prednisone/thalidomide- bortezomib/thalidomide.
* patients with previous ≥ grade 2 PN received RD, whereas patients with previous neuropathy ≤ grade 2 received VRD

Risk factors for BiPN

Baseline PN is present in 10–37% of patients with myeloma. This can be attributed to comorbidity (e.g. diabetes mellitus (DM)), previous neurotoxic treatment or multiple myeloma related PN).[16-19] It is of major importance to identify predisposing factors for treatment induced PN. Baseline PN and co-morbidities such as the presence of DM, alcohol abuse, vitamin deficiencies, and viral infections at baseline condition may elicit or aggravate symptomatic PN. Several studies addressed the question which predisposing factors could affect development of BiPN, however conflicting results have been reported.

In the APEX trial age, number and/or type of prior therapies, baseline glycosylated hemoglobin level, or DM history did not increase BiPN incidence and severity.[9] Another study found that the risk of bortezomib-related PN was greater in patients who had PN and DM at baseline.[16] In the VISTA trial, baseline PN was the only consistent risk factor for any BiPN, for ≥ grade 2 BiPN, and for ≥ grade 3 BiPN.[20]

In this context, pharmacogenetic studies building on the newest technologies might become helpful for risk stratification of treatment-related neurotoxicity.[18,21,22]

Mechanisms of BiPN

Although neurons are post-mitotic cells, they are highly susceptible to the toxic effects of anticancer drugs. The main targets for treatment emergent neurotoxicity in MM are the neuronal cell bodies within the dorsal root ganglion (DRG) and their axons extending in the extremities (Figure 1). Bortezomib mainly causes direct DRG toxicity, and axonopathy to a lesser extent. Concerning the mechanism of BiPN, a multifactorial pathogenesis seems likely. Mitochondrial and endoplasmic reticulum damage in both Schwann and satellite cells has been observed in sciatic nerve and DRG of rats given bortezomib;[23] bortezomib is known induce apoptosis in MM cells through activation of the mitochondrial based ("intrinsic") apoptotic pathway.[1] Another mechanism by which bortezomib could contribute to PN is dysregulation of mitochondrial calcium homeostasis.[24] Disruption of the intracellular calcium homoeostasis in nerves can promote depolarization and spontaneous discharge, causing pain and other abnormal sensations.[25] Other factors involved in BiPN include autoimmune factors and inflammation,[26] and blockade of nerve-growth-factor-mediated neuronal survival through inhibition of the activation of nuclear factor κB (NF-κB).[6]

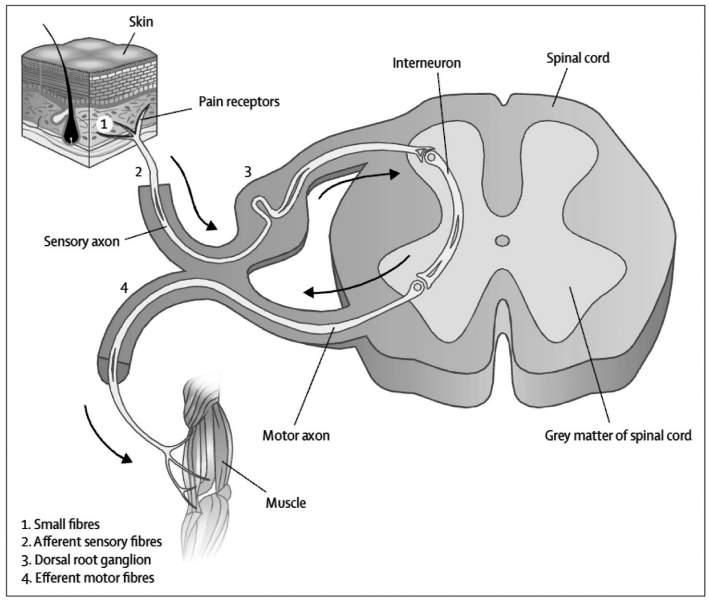

Figure 1. Potential targets for thalidomide-induced and bortezomib-induced peripheral neuropathy. Adapted from: Delforge M. et al., Lancet Oncology, 2011.[38]

In addition, MM itself is associated with peripheral neuropathy. Approximately 54 to 67% of MM patients show abnormalities on neurophysiological examination at baseline,[17,18]

however PN is a dominant feature in 10-37% of patients and mainly concerns a sensory or sensorimotor PN.[17-19] Furthermore, BiPN was noted at higher frequencies in patients with multiple myeloma than in those with solid tumours.[27]

The role of myeloma related factors in peripheral neuropathy related to treatment is not clear. Richardson and colleagues characterized the possible role of myeloma-related factors in BiPN using baseline gene expression profiles (GEPs) of plasma cells from 25 patients with MM, 9 patients with and 16 patients without treatment-emergent PN. The GEPs that distinguish patients with treatment-emergent PNP did not involve genes with a clear etiological link to PNP development. Although not significant due to the small number of patients, they observed gene profiles involved in protein translation, ribosomal proteins, and cell-surface markers.[18]

We have addressed the role of both the myeloma plasma cell as well as constitutional genetic variability in BiPN development. GEPs of purified plasma cells obtained from 170 patients at baseline and single nucleotide polymorphisms (SNP) profiles of peripheral blood (PB) from 186 patients, all treated with bortezomib were determined. Patients who developed BiPN grade 2–4 or 3–4 were compared to patients who did not develop BiPN. Although separately analyzed, both GEPs in plasma cells and SNP profiles in PB revealed genes and pathways involved in the etiology of PN. Genes involved in the development and function of the nervous system, *SOD2,* SNPs in genes involved in dorsal root ganglion neurons, *SERPINB2*, but also SNPs in DNA repair genes and proinflammatory genes were overrepresented in patients with BiPN.[21] This study emphasized the contribution of both the inherited genetic constitution of the host (patient) and the tumor (myeloma) to the development of BiPN.

A pharmacogenomic analysis from the phase III HOVON-65/GMMG-HD4 and IFM 2005–1 trials which compared conventional treatment with bortezomib based induction regimens, found one significant SNP in het *CYP17A1* gen, involved in steroid biosynthesis. Although not significant after multiple testing correction, 56 SNPs were associated with BiPN; pathway analysis of these SNPs showed involvement of neurological disease (21 genes), among which *NEFL, PON1, PTGS2* and *ABCG2*.[22]

Similarly, an independent pharmacogenomic analysis of the VISTA trial identified an association between time to onset of PN and the immune gene *CTLA4*, and the same association was shown in a confirmatory analysis of data from the IFM trial mentioned above.[28] Efforts are under way to investigate whether genome wide screening of single nucleotide polymorphisms can be used to identify patients at risk of developing BiPN.

Diagnosis and measurement of PN

Awareness of BiPN is crucial for early detection and appropriate intervention, especially for a small subset of patients who may develop severe peripheral neuropathy shortly following start of treatment with bortezomib. In daily clinical practice, reliable, uniform and

simple methods for screening and grading of PN are needed. One of the most widely used grading systems for assessing neurotoxicity are the National Cancer Institute's Common Toxicity Criteria for Adverse Events (NCI-CTCAE). Most of the published data concerning BIPN were based on use of either the NCI-CTC version 2.0 or 3.0 criteria,[29] or the 11-item functional Assessment of Cancer Therapy Neurotoxicity (Ntx-FACT/GOG) subscale,[30] which are both current standards of neurotoxicity assessment. Other widely used algorithms are Eastern Cooperative Oncology Group criteria,[31] Ajani,[32] and the World Health Organization criteria.[33] A disadvantage of all these methods is that they do not include assessment of neuropathic pain, a key sign of BiPN. However, recently, an update of the NCI-CTC criteria was released, NCI-CTC version 4.0, which now also includes the grading of neuralgia (Table 3).[34] Another limitation in using the above algorithms is that the degree of PN is subjective and dependent on patients' reporting, thereby resulting in variances in interpreting clinical aspects and poor reliability. Limitations also result from intra- and interobserver variation of scales.[35] In addition to symptom assessment, a regular and focused clinical neurological examination including the evaluation of sensation (touch, pain, temperature, vibration, proprioception), distal muscle strength, ankle reflexes, and supine versus upright blood pressure is recommended.

Table 3. Definition of PN according to NCI-CTC criteria version 4.0.

ADVERSE EVENT	1	2	3	4
Peripheral motor neuropathy	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Moderate symptoms; Limiting instrumental ADL	Severe symptoms; limiting self care ADL; assistive device indicated	Life-threatening consequences; urgent intervention indicated
Peripheral sensory neuropathy	Asymptomatic; loss of deep tendon reflexes or paresthesia	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self care ADL	Life-threatening consequences; urgent intervention indicated
Neuralgia	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self care ADL	-

More complex algorithms such as the Total Neuropathy Score (TNSr), which combines the assessment of symptoms with electrophysiological measurements, such as nerve conduction studies (NCS) and needle electromyography, result in higher sensitivity for the detection and specification of neurological damage.[36] The value of these electrophysiological measurements to detailed clinical examination for the detection and

follow-up of treatment-related peripheral neuropathy in MM is still a matter of debate. First of all, electrophysiological measurements are difficult to apply in routine clinical practice. Second, discrepancies between neurophysiological and clinical results in BiPN are common: sensory neuropathic symptoms may be out of proportion to objectively measurable signs at baseline, as was noted during treatment in three patients, who developed symptomatic treatment emergent small-fiber neuropathy (characterized by burning aching pain, paresthesia, and allodynia) during the study, in whom NCS and quantitative sensory testing showed no change, a phenomenon which is typical of small-fiber involvement.[8] The explanation for the discrepancy between NCS and clinical observation is that in BiPN preferentially small diameter sensory fibers are affected and NCS is not very sensitive for detection of damage to these fibers.[4,8]

Management of BiPN

Currently, there's no curative treatment for BiPN, therefore prevention is the preferred approach to maintain quality of life and preserve future options for anti-myeloma treatment. All MM patients should be clinically assessed for signs and symptoms of peripheral neuropathy before initiation of a neurotoxic drug. Patients with pre-existing peripheral neuropathy should be monitored closely during initial treatment and prompt dose modification according to guidelines is important to prevent development of more serious neurologic complications and aid reversibility. Careful clinical assessment at each visit, with neurologic examination in case of suspected PN and neurophysiological testing in specific situations, is the best approach for detection and follow-up of drug-induced peripheral neuropathy. This is a multidisciplinary task requiring the awareness of patients, nurses, hematoma-oncologists, and neurologists.

Dose-modification guidelines for the management of BiPN, have been based on experience during the first trials with single agent bortezomib, CREST and SUMMIT trials.[5,6] In these trials the effect of dose adjustment/discontinuation was well established. 90 out of 256 patients included in SUMMIT or CREST trial experienced BiPN, of which 35 had BiPN grade 3–4. In all 256 patients, neuropathy led to dose reduction in 12% and discontinuation in 5%. Of 35 patients with neuropathy grade 3–4 and/or neuropathy requiring discontinuation, resolution to baseline or improvement occurred in 71%. The median duration from the last dose to resolution or improvement of peripheral neuropathy was 47 days (range, 1 to 529 days).[8]

Guidelines for bortezomib dose modification and/or discontinuation were subsequently validated in the APEX and VISTA trial.[9,20] Overall, 124/331 (37%) patients included in the APEX trial had treatment-emergent peripheral neuropathy, including 30 (9%) with grade ≥ 3; Of patients with grade ≥ 2 peripheral neuropathy, 58/91 (64%) experienced improvement or resolution to baseline at a median of 110 days, including 49/72 (68%) who had dose modification versus 9/19 (47%) who did not. Survival outcome did not appear adversely affected in patients who had dose modification due to peripheral neuropathy.[9]

In the VISTA trial, 47% of patients receiving VMP developed peripheral neuropathy, including 19% grade 2 and 13% grade ≥ 3 (< 1% grade 4). The BiPN incidence was dose-related and reached a plateau at a cumulative bortezomib dose of approximately 45 mg/m^2. Median time to onset of BiPN was 2.3 months. BiPN was reversible; 79% of events improved by at least one NCI CTC grade within a median of 1.9 months and 60% completely resolved within a median of 5.7 months, with reversibility similar in responding and non-responding patients.[20]

More recently, multi-agent studies have shown that, in addition to dose reduction, weekly dosing may prevent the progression of PN and reduce severe PN and did not appear to affect efficacy.[37] Weekly bortezomib dosing is therefore considered to be an effective strategy to prevent further worsening of PN once patients have developed BiPN grade 1. Bortezomib dose modification guidelines have recently been updated and are based on NCI-CTC version 3.0, table 4 and 5.[38] For elderly patients, frequently having additional comorbidities, adjustment in dose and frequency of bortezomib administration is recommended, shown in table 6.

Table 4. Definition of PN according to NCI-CTC criteria version 3.0.

ADVERSE EVENT	1	2	3	4
Peripheral motor neuropathy	Asymptomatic, weakness on exam/testing only	Symptomatic weakness interfering with function, but not interfering with ADL	Weakness interfering with ADL; bracing or assistance to walk (e.g., cane or walker) indicated	Life-threatening; disabling (e.g., paralysis)
Peripheral sensory neuropathy	Asymptomatic; loss of deep tendon reflexes or paresthesia (including tingling) but not interfering with function	Sensory alteration or paresthesia (including tingling), interfering with function, but not interfering with ADL	Sensory alteration or paresthesia interfering with ADL	Disabling

Once PN has resolved, there is no increased risk for cumulative BiPN upon retreatment with bortezomib-containing regimens.

Furthermore, a recent published trial randomized 222 relapsed MM patients to subcutaneous administration of (SC, n = 148) or intravenous administration (IV, n = 74) of bortezomib. ORR after 4 cycles were identical in both arms, 42%, en after 8 cycles 52%, with CR rates in 10% (SC) en 12% (IV). After a median follow-up of 11.8 months no significant difference was observed in time to progression (TTP) (median 10.4 vs. 9.4 months) en OS

(1-years OS 72.6% vs. 76.7%) in SC vs. IV bortezomib. More importantly, BiPN grade 1–4 percentages (38% vs. 53%), grade ≥ 2 (24% vs. 41%), en grade ≥ 3 (6% vs. 16%) BiPN were significantly lower in the SC vs. IV administration of bortezomib.[39]

Table 5. Dose-modification guidelines for bortezomib-induced neuropathy.

BiPN, NCI CTCAE score	Bortezomib dose modification guidelines
Grade 1	If the patient is on a biweekly schedule: reduce current bortezomib dose by one level‡ or prolong dosing interval to once weekly If the patient is already on a weekly schedule: reduce current bortezomib dose by one level‡
Grade 1 with neuropathic pain or grade 2	If the patient is on a biweekly schedule: reduce current bortezomib dose by one level‡ or prolong dosing interval to once weekly If the patient is already on a weekly schedule: reduce current bortezomib dose by one level‡ or consider temporary discontinuation of bortezomib. If the neuropathy resolves to grade 1 or better, once weekly treatment with reduced bortezomib dose may be restarted if the benefit-to-risk ratio is favorable
Grade 2 with neuropathic pain or grade3	Discontinue bortezomib, if the neuropathy resolves to grade 1 or better, once weekly treatment with reduced bortezomib dose may be restarted if the benefit-to-risk ratio is favorable
Grade 4	Discontinue bortezomib

Summary of Product Characteristics (SPC) guidelines were modified according to expert opinion and clinical practice in reference centres. ‡Bortezomib dose reductions: standard dose = 1.3 mg/m^2; dose reduced by one level = 1.0 mg/m^2; dose reduced by two levels = 0.7 mg/m^2.

Table 6. Dose guidelines for bortezomib in elderly patients.

	< 65 yr	65–75 yr	> 75 yr
Bortezomib	1.3 mg/m^2 twice weekly	First cycle: 1.3 mg/m^2 twice weekly Next cycles:1.3mg/m^2 once weekly	1.3 mg/m^2 once weekly

Pharmacological interventions to prevent BiPN have been proposed based on theoretical models, extrapolation from trials of different diseases, and the assumption that they are non-toxic, including vitamin B, antioxidants such as vitamin E, alpha-lipoic acid, glutathione, and supplements with glutamine or acetyl-L-carnitine.[2,40] However, there are no prospective randomized studies, and the general use of these compounds to prevent BiPN warrants caution. In fact, the administration of pyridoxine (Vitamin B6) can cause additional sensory neuropathy in patients with impaired renal function and in association with a protein-

deficient diet.[41,42] Vitamin C may interfere with bortezomib metabolism and may also abrogate bortezomib-mediated inhibition of proteasome activity.[43,44]

Drugs to alleviate bortezomib induced neuropathic pain in multiple myeloma include: gabapentin or pregabalin, tricyclic antidepressants, serotonin and norepinephrine reuptake inhibitors, carbamazepine, and opioid-type analgesics.[40] The efficacy of these drugs is mainly based on uncontrolled studies, case reports, personal experience, or most often, on studies in other indications such as painful diabetic neuropathy and postherpetic neuralgia, but not on studies in BiPN. Local application of lidocaine or menthol-containing analgesic cream can temporarily alleviate bortezomib-induced neuropathic pain,[2,45] whereas other compounds, such as capsaicin, can be harmful and should be used with care. Simple emollients such as cocoa butter are non-toxic and might be helpful for symptom relief. Furthermore, patients may be advised to wear loose-fitting clothes and shoes and to keep feet uncovered in bed. Patients with autonomic dysfunction should rise cautiously and avoid demanding physical tasks. Patients suffering from severe peripheral neuropathy are disabled in the exertion of daily tasks, and in addition to pharmacological intervention, they may benefit may benefit from physical exercise and physiotherapy.[2] The only effective approach in BIPN is to adhere to dose modification guidelines to prevent treatment-emergent BIPN and allow treatment to continue.

Future perspectives

BiPN is one of the most frequent, but also one of the most feared dose-limiting side effects of bortezomib. Yet, for many patients, the probability of prolonged remission depends on the treatment with bortezomib alone or combined with other (potentially neurotoxic) anti-myeloma agents. Repetitive use of the (potentially neurotoxic) anti-myeloma agents during successive stages of their disease is inevitable. It is therefore of major importance to prevent BiPN in order to allow the treatment to be continued and completed. The prevention of severe BiPN by close monitoring, use of uniform, simple and clear scales for screening and grading of PN, such as the NCI-CTC or the Ntx-FACT/GOG systems and most importantly by using dose-reduction algorithms defines the standard of care.

Also novel measures to prevent drug induced PN are undertaken. Recently, subcutaneously administration of bortezomib was reported to significantly decrease the percentage of BiPN, while showing almost identical response and survival outcomes, however follow-up is short.[39]

Furthermore, second generation proteasome inhibitors which have less PN , such as carfilzomib, with almost no grade 3–4 PN, and NPI-0052 (marizomib), are being evaluated in clinical trials.[46,47]

Besides PN at baseline, no consistent risk factors for development of BiPN are established. Therefore, pharmacogenomic studies are the future for better identification of patients with an increased risk of bortezomib-induced PN.

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GENERAL DISCUSSION

In the previous decade, data on differences in biology of multiple myeloma (MM) started to emerge, beginning with the cytogenetic distinction between nonhyperdiploid and hyperdiploid myeloma. The introduction of microarray technology, enabling the measurement of expression of thousands of genes at once, provided further insight in this complex, heterogeneous disease, which will be discussed here.

Alongside these discoveries, the introduction of novel agents such as thalidomide, bortezomib and lenalidomide have impressively improved outcome in MM patients. Despite the considerable progress that has been made in the treatment of MM, still a percentage of patients experience early relapse and are at risk to die from treatment failure. Furthermore, with the introduction of novel agents, different side effects were reported. Polyneuropathy (PN) is the most important toxicity with respect to thalidomide and bortezomib treatment. The objectives of this thesis were:

I) Molecular profiling of MM to gain insight in the heterogeneity of MM, described in chapters 3 and 4.
II) Development of a gene expression based signature to identify MM patients at risk for treatment failure, described in chapters 5 and 6
III) Determine molecular factors associated with response or treatment failure, described in chapters 7 and 8.
IV) Identification of molecular characteristics of MM patients who develop bortezomib induced peripheral neuropathy (BiPN), described in chapters 9 (and 10).

I. MOLECULAR CLASSIFICATIONS OF MM

In addition to the translocation/ cyclin D (TC) classification,[1] and the UAMS classification by Zhan et al.,[2] we describe a classification in chapter 3, which was based on hierarchical clustering of gene expression profiles from newly diagnosed MM patients included in a multicenter phase 3 trial (HOVON65/GMMG-HD4).[3] We confirmed the clusters described in the UAMS classification, and defined three novel subgroups, named according to the genes that were overexpressed: an NF-κB cluster, characterized by high expression of genes involved in the nuclear factor-kappa B (NF-κB) pathway, a CTA cluster, based on cancer testis antigen (CTA) genes, and a PRL-3 cluster which showed upregulation of genes encoding for protein tyrosine phosphatases *PRL-3* and *PTPRZ1* as well as *SOCS3*.

The key questions are: how robust are the novel clusters, and what is their added value in terms of clinical features, biology, and prognosis?

As a measure for robustness, a robustness index (RI) was calculated for the novel clusters. The CTA cluster showed the highest RI, which was comparable to the RI of the t(4;14)/MS cluster, followed by the NF-κB and PRL-3 cluster. In addition, we assessed robustness by validating these novel clusters in independent datasets, for which we applied our sample and gene selection criteria to 2 independent datasets, and performed hierarchical clustering. Clusters with a PRL-3 and an NF-κB profile were reproduced. However, a CTA cluster, despite its high RI, could not be identified in these independent data sets.

Apart from observing a tendency towards increased bone disease in the PRL3 cluster, none of the other evaluated, clinical characteristics demonstrated a clear relation to the novel identified clusters. In addition, known poor prognostic markers, such as del(17p) and 1q gain, were not specifically overrepresented in any of the novel clusters, although we did observe that none of the samples in the PRL-3 cluster harbored a del(17p). We have to keep in mind that the PRL-3 cluster is a small cluster of 9 samples, and these findings have to be assessed in larger cohorts of patients

Biology of clusters

In terms of biology, the activation of NF-κB has been widely considered to be one of the most important deregulated signaling pathways in MM, resulting in activation of transcription of anti-apoptotic factors, cytokines, growth factors, and cell adhesion molecules important for the growth and survival of myeloma plasma cells. The role of mutations in a wide range of NF-κB genes contributing to NF-κB activation was shown, including in a recent whole genome sequencing study in MM.[4-6] Overexpression of CTA genes characterized the CTA cluster. Expression of CTAs, a family of tumor associated antigens (TAAs), is normally restricted to adult testicular germ cells but aberrant CTA expression has been found in various tumors.[7-9] While the functions of many of these CTAs are still unknown, recent studies showed that these proteins are likely involved in cell cycle progression and regulation, transcriptional control, cell survival and apoptosis. Studies on the expression of CT antigens have also shown that epigenetic events, such as DNA methylation, are the primary mechanism regulating the expression of CT antigens in germ cells and transformed cells.[10] Furthermore, CTA genes have been observed in association with a poor prognosis which will be discussed in more detail below.[11]

Due to the small size of the PRL-3 cluster, validation in larger sets is paramount to allow for further characterization of this cluster. Still, genes highlighted in comparison of this cluster to other clusters, have been found to be important in MM and in other cancers. *PRL-3* has been shown to affect SDF-1 mediated migration of MM plasma cells. In addition, this gene was shown to be a prognostic factor in AML, and was reported in solid tumors in relation to increased aggressiveness.[12-14]

Effect of bortezomib on clusters

In chapter 4 we investigated the effect of the novel agent bortezomib on survival of the clusters. The PRL-3 cluster was excluded since the number of patients was too small to allow for reliable survival analysis. Based on the mechanism of action of bortezomib, which is thought to exert its actions in part through the NF-κB pathway,[15,16] and the findings that overexpression of genes involved in activation of the NF-κB pathway were associated with response to bortezomib,[6,17] we were interested in the effect of bortezomib on the NF-κB cluster. The NF- κB cluster was one of the 5 clusters showing an improvement of outcome following bortezomib. Given that the other 4 clusters showed an even higher increase in PFS, probably other molecular factors and mechanisms besides NF-κB activity play an important role in outcome following treatment with bortezomib, such as the unfolded protein response.[18,19] The CTA cluster, on the other hand, characterized by CTA genes, well known markers associated with a poor prognosis,[11] was expected to confer for a poor prognosis. Bortezomib did not improve PFS in this cluster, however the median PFS of 31 months and a median OS not reached, did not represent a particularly poor PFS or OS following VAD or bortezomib based treatment. The CTA cluster resembles the poor prognostic PR cluster, which will be discussed below, in the expression of CTA genes. The difference in prognostic impact may indicate that the presence of proliferation and cell cycle genes characterizing the PR cluster, confer for the poor prognosis observed with this cluster.

In summary, despite the absence of prognostic value, the clusters PRL-3 and NF-κB represent reproducible clusters which can be of potential clinical interest. For the novel clusters, and for the clusters PR, LB, and myeloid cluster, the underlying genetic and/or epigenetic factors that contribute to these clusters are still unknown.

Therefore, larger datasets need to be evaluated to determine the value of these novel clusters in relation to clinical parameters such as survival and treatment options as well as in relation to genetic and epigenetic characterization.

Another question related to the clusters concerns the effect of treatment on high-risk clusters.

Patients with translocation t(4;14), clustering for the largest part in the MS cluster, are a classical poor prognosis group in MM.[5,20,21] In chapter 4 we have demonstrated that poor prognosis associated with this risk factor as well with the t(14;16)/t(14;20) translocations/ MF cluster demonstrate greatly improved outcomes following bortezomib treatment. This is consistent with data from the HOVON65/GMMG-HD4 trial, showing an improvement of PFS and OS for patients with t(4;14) treated with bortezomib, as opposed to conventional treatment.[22] In contrast, the PR cluster which performed poorly on conventional treatment did not appear to benefit from bortezomib based treatment.

Classifiers for translocations

A further use of gene expression profiling in MM is to improve or complement detection of samples harboring a translocation. For this purpose, we developed classifiers for translocations t(4;14), t(14;16)/t(14;20) and t(11;14). The signatures demonstrated good accuracy and may in future be used to determine translocation status. The gene expression based translocation detection can only be implemented if gene expression profiling yields information which exceeds the prognostic value of FISH. Future research will show whether FISH or gene expression profiling, or possibly the combination of both methods will be the most accurate for both risk assessment and for determining the underlying aberration.

The currently conducted multicenter phase 3 trial (HOVON95/EMN02) with a targeted enrollment of 1500 patients will include standard cytogenetic evaluation of chromosomal aberrations on purified myeloma plasma cells at diagnosis. Side studies of this trial will include gene expression profiling, in which one of the objectives will be to validate defined clusters using hierarchical clustering and nearest neighbor and increase robustness of small clusters using a larger cohort of patients. Furthermore, addition of novel techniques such as the genome-wide human SNP Array 6.0 and whole genome sequencing (WGS) will allow for further characterization of clusters and samples in more detail.

Clinical implications

Finally, the ultimate question is whether these findings have any implications in clinical practice.

Until recently, treatment of MM was stratified only for age and fitness of the patient. Data from different trials have shown that patients harboring poor prognostic cytogenetic markers, such as t(4;14), t(14;16) and del(17p), still perform poorly on thalidomide based treatment,[23,24] however were shown to benefit from proteasome inhibition.[25,26] Recently, consensus has been reached that bortezomib based regimens should be the treatment of choice, at least for patients with a t(4;14) and del(17p). In addition, these patients will proceed to allogeneic stem cell transplantation in first relapse. Future research will demonstrate whether gene expression based classification can contribute to this risk-based treatment stratification.

II. GENE BASED SIGNATURES FOR IDENTIFICATION OF HIGH-RISK MM

Molecular profiling generated substantial knowledge of the biology of MM in individual patients and diversity between MM patients with prognostic implications, as described above for clusters in the different classification systems. Therefore, developing a gene expression signature to predict outcome of treatment was the rational next step.

For clinical relevance, a gene expression signature identifying high-risk disease must have both the ability to separate risk groups as clearly as possible and to predict stable groups of relevant size, with the ultimate goal to guide treatment choice.

MILLENNIUM-100 risk signature

One of the first attempts to develop molecular signatures able to predict response and survival in MM is described in chapter 3. These signatures were based on microarray data from relapsed/ refractory patients included in international phase 2,[27,28] and phase 3 clinical trials,[29] which studied the efficacy of bortezomib. The response predictor able to significantly predict response to bortezomib, contained 100 probe sets of which ribosomal, mitochondrial, ER stress, DNA repair, and cancer-associated genes were among those associated with progressive disease.

The gene expression classifier for prediction of survival (MILLENNIUM-100) identified a high-risk and low-risk group in patients receiving bortezomib, with the high-risk group showing a significant lower OS. In addition, the survival classifier further stratified patients within the different ISS groups. Noteworthy, CTAs were overrepresented in the classifier. Other genes in the classifier which were inversely associated with poor survival included genes involved in cell adhesion, and normal plasma cell, myeloid and erythroid markers.

EMC-92 high-risk signature

In chapter 5, we describe a gene expression based signature, EMC-92 high-risk signature, to predict high-risk MM. This signature was validated in 4 independent datasets and compared with published signatures in their ability to predict high-risk MM. The EMC-92 was able to identify a high-risk group of patients with a significantly lower OS in all 4 validation sets. These validation sets represented different drug regimens (thalidomide and bortezomib), newly diagnosed and relapsed patients, and transplant eligible and ineligible patients. The proportion of high-risk patients identified in the validation sets was found to be stable and of a relevant size, 16.2%–20.2%. Furthermore, no treatment specificity for the EMC-92 signature was observed between EMC-92 defined high-risk patients in the different treatment arms.

The 92 genes in the signature and initially selected 1093 probe sets showed enrichment of genes in cell cycle, protein synthesis, and cancer pathways. The finding of enrichment in cell cycle pathways may reflect proliferation status of the MM plasma cells, while protein synthesis may relate to the role of unfolded protein response (UPR) in production of large quantities of proteins by myeloma plasma cells.[18,30,31].

Until now, 7 high-risk signatures have been developed to predict high-risk MM patients. In addition to the aforementioned MILLENNIUM-100 these are UAMS-70, UAMS-17, UAMS-80, IFM-15, GPI-50, and MRC-IX-6.[17,32-36] When we compared the EMC-92 to the 7 published high-risk signatures, we observed that the performance of the EMC-92 is comparable to

the UAMS derived signatures, MRC-IX-6 and the GPI-50, as measured by the significance of prediction in validation sets. The IFM-15 and MILLENNIUM-100 failed to reach significance in all data sets, which could be due to differences in gene expression platform or use of an earlier version of the Affymetrix platform. While the EMC-92 signature was able to predict stable groups of relevant size, the UAMS-70 and GPI-50 selected proportions of high-risk patients of variable size, and in some data sets of less than 10%. Most importantly, when we compare all high-risk signatures directly to each other by means of pair-wise comparisons, the EMC-92 demonstrated to have the best fit to the observed survival times in independent sets. Only limited overlap in probe sets between the 8 signatures was observed. Interestingly, *BIRC5* was found in 4 signatures: EMC-92, UAMS-17, UAMS-70 and the GPI-50. More likely, overlap between signatures will be observed at the level of pathways rather than of individual probe sets and genes.

Evaluation of known poor cytogenetic markers, which were available in the MRC-IX study, showed enrichment of 1q gain, del(17p), t(4;14), t(14;16), t(14;20) and del(13q) in EMC-92 defined high-risk patients. Still, more than half of the patients in the standard risk group showed one or more poor prognostic cytogenetic markers. Concerning the impact of clusters, a clear enrichment of the MF, MS, PR clusters and a decreased proportion of the HY cluster was found in the pooled high-risk populations of all validation sets, confirming their prognostic impact. Multivariate analysis showed that the EMC-92 performed as the strongest predictor for survival after inclusion of available covariates, including del (17p) and components of international staging system (ISS).

Use in clinical practice

Use in clinical practice has been realized for the UAMS-70, which is now incorporated in the Mayo Stratification for Myeloma And Risk-adapted Therapy (mSMART). The mSMART guides adjustment of treatment regimens according to risk status, based on UAMS-70, FISH, metaphase cytogenetics, and plasma cell labeling index. However, the mSMART has not been validated in prospective clinical trials.[37]

Ultimately, clinical use of any signature must be proven to be of use in prospective clinical trials, which allow treatment choice based on risk assessment.

The EMC-92-gene high-risk signature identified a group of patients that despite the use of novel agents, including bortezomib, showed a significantly lower PFS and OS. For the patients identified by the EMC-92 high-risk signature we need to search for more intensive or alternative treatment options, which could range from use of consolidation and maintenance therapy, use of combinations of 2 or more novel agents, consolidation treatment, early switch to allogeneic stem cell transplantation or experimental therapy and search for novel targets for therapy. We will validate this signature in the HOVON95/EMN02, a randomized phase III study comparing high dose melphalan (HDM) vs. bortezomib, melphalan, prednisone (VMP), followed by consolidation with 2 novel agents

vs. no consolidation, both followed by lenalidomide maintenance. Besides validation, this protocol can answer some of the questions, such as the effect of HDM vs. VMP, and the role of consolidation with 2 novel agents in survival of high-risk patients.

Ultimately, such trials will result in clinical guidelines to improve treatment of patients with a poor PFS and OS on novel therapies.

For practical application of the EMC-92 signature it is essential to stress that this signature has not been designed for classification of a single patient. However, collection of a set of ~25 patients will result in reliable prediction, and each additional patient can be predicted as soon as it is tested. For now, centralized use, in centers equipped to perform gene expression profiling, and facilitated to perform the analyses and interpret the data, is needed to obtain a sufficient number of patients and essential for consistent performance of this classifier. Further research is being performed to improve this signature to enable prediction per patient.

III. PROGNOSTIC MOLECULAR MARKERS

Cancer testis antigens

In the molecular classification presented in chapter 3, we identified 2 clusters that were characterized by expression of CTA genes, i.e. the PR cluster and the CTA cluster. These CTA genes included *MAGEA3, MAGEA6, PBK, MAGEA12*, *PAGE1,* and various *GAGE* genes. *MAGEA6* was also found among genes constituting the EMC-92 signature. The presence calls of some CTA genes have been reported to correlate with significantly shorter EFS, such as *CTAG1B*, *CTAG2*, *MAGEA1*, *MAGEA2*, *MAGEA3*, and *MAGEA6*.[11] Furthermore, CTA genes were found to be overrepresented in the MILLENIUM100 high-risk signature.

CTAs represent an important family of tumor associated antigens (TAAs), and continued expression after treatment may allow for the development of (immuno)therapy. [7-9]

In chapter 7, we have therefore focused on the presence of CTAs in newly diagnosed and relapsed patients. This study underlined the importance of CTA genes in terms of prognosis, and gave an indication of which CTAs may be suitable for immunotherapy. Still, the protein expression of only one CTA was assessed and this deserves further study.

The shortcoming of this study is that we could not perform this analysis of patterns of CTA expression in the same patients longitudinally. This will be assessed in future studies evaluating the same patients at different time points. In relation to CTA genes, it is of interest to note that a CTA mutation was identified in 2/38 patients in the first WGS study published in MM, suggestive of importance in MM.[5]

Cereblon

The rationale to evaluate *cereblon (CRBN)* expression in MM patients in association with response to thalidomide and survival came from a recent published paper by Ito et al. who identified *CRBN* as a primary target of thalidomide teratogenicity,[38] followed by a paper showing that *CRBN* was required for the antimyeloma activity of lenalidomide and pomalidomide.[39] We have performed a subanalysis in order to evaluate the clinical value of *CRBN* expression in myeloma treatment. The HOVON-65/GMMG-HD4 trial design included thalidomide in the maintenance setting. We therefore performed a landmark analysis of PFS and OS from start of thalidomide maintenance, and investigated the association with *CRBN* expression. We observed that increase of *CRBN* expression was significantly associated with a longer PFS and OS. We performed this analysis as a set-up for future research in trials with IMiDs as part of the induction regimen. HOVON-87, an ongoing phase 3 trial which will enroll 668 newly diagnosed patients not eligible for autologous stem cell transplantation, will compare 2 IMiDs, thalidomide and lenalidomide, both in combination with melphalan and dexamethasone. This trial includes a research component to perform genomic analysis in a prospective setting. We will investigate the role of *CRBN* in MM patients treated with thalidomide or lenalidomide treatment in relation to outcome, in which it will be interesting to analyze the dependence and extent of influence of *CRBN* expression in response to and outcome following thalidomide vs. lenalidomide and whether low *CRBN* expression can be used to predict non-responsiveness or a reduced survival following treatment with IMiDs.

IV. MOLECULAR CHARACTERISTICS IN ASSOCIATION WITH BIPN

With the introduction of bortezomib in clinical trials, it soon became apparent that a markedly high percentage of patients suffered from peripheral neuropathy (PN) following treatment with bortezomib.[27-29] PN has become one of the most frequent, potentially disabling complications of bortezomib. Furthermore, it was notable that a small subset of patients developed severe PN after a short time interval following start of treatment with bortezomib.

Besides PN at baseline, no consistent risk factors for development of bortezomib induced polyneuropathy (BiPN) were established.[40-42] Therefore, we performed a pharmacogenomic study to evaluate molecular factors involved in treatment-related neurotoxicity. SNP analysis, i.e. studying inherited variability in relation to PN was combined with gene expression analysis of myeloma. For the former, DNA was obtained from peripheral blood, whereas the latter was performed on purified myeloma cells.

For SNP analysis the custom designed Bank On A Cure (BOAC) SNP chip was used.[43] The rationale for using gene expression profiling of MM in relation to PN, is that MM itself has been found to be associated with PN. In fact, symptoms of sensory or sensorimotor

PN at diagnosis were reported in 10-15%, and in one study even in 37% of patients.[41,44-46] In addition, BiPN was noted at higher frequencies in patients with MM than in those with solid tumours.[47] Hence, processes specific to MM may result in the development of PN and possibly in modifying drug induced PN.

The analysis described in chapter 9, demonstrated comparable genes and pathways in gene expression profiles of myeloma plasma and in SNP profiles in peripheral blood cells to be associated with PN.

Gene expression profiles in plasma cells and SNPs in peripheral blood associated with early-onset BiPN (after one cycle of bortezomib induction treatment) included genes involved in apoptosis, transcription, and AMP-activated protein kinase (AMPK)-mediated signaling. Late-onset BiPN (after 2 or 3 cycles of bortezomib induction treatment) was associated with development and function of the nervous system as well as pro-inflammatory genes. Both early and late-onset BiPN were characterized by genes and SNPs involved in nervous system development and DNA repair.

These findings suggest a possible role of these markers in identification of patients at risk and their involvement in development of BiPN. These data have to be validated in future trials to further investigate their role in prediction or pathogenesis.

The shortcomings of a custom designed SNP panel are that only a selection of SNPs and pathways is represented and inherently, there is no genome wide coverage. This impedes detection of SNPs with an as yet unknown impact on MM and drug metabolism, which represented the two most important criteria for development of the BOAC chip. Furthermore this also restricted a combined analysis of SNPs and gene expression, investigating the direct relationship between SNPs in genes and gene expression.

We are currently using the Affymetrix Genome-Wide Human SNP Array 6.0® (SNP6.0), which will be used to identify SNPs associated with MM features or treatment related complications, and combined with gene expression data to identify expression quantitative trait loci (eQTLs).

Measures for prevention of BiPN

Alongside the efforts to investigate risk factors, clinically and at a molecular level for BiPN, other important measures were undertaken to prevent PN. One of the remarkable discoveries was the subcutaneous (sc) administration of bortezomib. Comparing intravenous (iv) and sc administration of bortezomib, sc bortezomib was reported to significantly decrease the percentage of BiPN, while showing almost identical response and survival outcomes.[48]

Furthermore, second generation proteasome inhibitors which induce less PN, such as carfilzomib, with almost no grade 3–4 PN, and NPI-0052 (marizomib), are being evaluated in clinical trials.[49,50]

Although new proteasome inhibitors and/or sc use of bortezomib will reduce the risk of PN, it will remain of interest to define the causes of this toxicity for two reasons: first,

PN development under these new drug regimens is much reduced but not absent, and a simple test may prove to be of significant benefit to both patient and health practitioner if high grade, sudden onset PN can be avoided. Secondly, new treatments are not optimized as yet, and may give rise to a higher PN occurrence than initially expected.

More importantly, however, examining the genetic constitution of MM patients will lead to increased insight in a wide range MM related features or other treatment related complications. Moreover, if technical obstacles can be overcome, this analysis may allow genetic risk factors to be identified for the development of MM.

FUTURE PERSPECTIVES

Studying MM in a prospective, multicenter phase 3 trial may lead to further insights by utilizing an optimal combination of cytogenetic data, molecular profiling and clinical data, such as adverse events, response and survival. This combination was realized in HOVON65/GMMG-HD4 and it will be important to continue gaining insight performing side studies in future trials, such as the HOVON95/EMN02. These side studies will include validation of the results which were collected in this thesis, and will allow for further characterization of myeloma using gene expression profiling, copy number analysis and genome wide SNP analysis, as well as use of novel technologies, such as whole exome sequencing (WES) and whole genome sequencing (WGS). Following these studies, in vitro and in vivo experiments are required to validate genes and pathways which will be found to be of interest. Altogether, these studies may ultimately lead to further elucidation of the molecular basis of multiple myeloma.

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SUMMARY

Until about a decade ago, multiple myeloma (MM) was considered a single disease entity. This changed with the discovery of recurrent translocations, and MM was divided into two groups, hyperdiploid (HRD) and non-hyperdiploid (NHRD) MM. The arrival of novel technologies, such as micro-arrays for molecular profiling enabled further categorization of MM in subgroups, with different disease characteristics and differences in prognosis. Considerable progress has been made in MM treatment with the introduction of novel agents such as thalidomide and bortezomib. A major draw-back of these agents concerned the occurrence of serious adverse events, particularly peripheral neuropathy (PN).

Chapter 1 presents a general introduction to MM with emphasis on pathogenesis and prognosis, focusing on novel technologies such as molecular profiling. Furthermore, conventional and novel treatment strategies are outlined.

Chapter 2 describes the results of the multi-center, phase III trial, HOVON-65/GMMG-HD4, comparing bortezomib in induction and post-intensification vs. conventional chemotherapy and thalidomide post-intensification in newly diagnosed MM patients. The side studies described in Chapters 3, 4, 6, 7, 8 and 9 are based on the data and samples collected as part of this trial.

In **Chapter 3** we present a clustering of MM samples based on correlated gene expression profiles (GEPs). 10 clusters were characterized in detail; for one cluster no clear signature could be determined. We confirmed 7 clusters described in the University of Arkansas for Medical Science (UAMS) classification: CD-1, CD-2, MF, MS, PR, and HY; with one subgroup showing a previously recognized myeloid signature. Three novel subgroups were defined, including a subgroup characterized by high expression of genes involved in the NF-κB pathway, termed NF-κB cluster. Another subgroup was characterized by distinct overexpression of cancer testis antigens (CTA) without overexpression of proliferation genes, i.e. the CTA cluster. The third novel cluster showed up-regulation of protein tyrosine phosphatases *PRL-3* and *PTPRZ1* as well as *SOCS3,* termed PRL-3 cluster. Furthermore, gene predictors for translocations t(4;14), t(14;16)/t(14;20), and t(11;14) were developed, which were able to classify samples harboring a specific translocation with good accuracy.

In **Chapter 4** we investigated the prognosis of the different clusters as defined in Chapter 3 and evaluated the impact of bortezomib treatment on outcome of these clusters. Following conventional treatment, progression free survival (PFS) and overall survival (OS) was significantly different between clusters, while no significance was observed between clusters in terms of PFS and OS following bortezomib. This was mostly due to clusters showing a poor outcome following conventional treatment, which improved substantially following bortezomib treatment, i.e. MF and MS cluster. The PR cluster did not benefit from

bortezomib treatment and remained a poor prognostic cluster regardless of treatment used.

In **Chapter 5** the development and performance of predictive gene classifiers is described for response and survival following treatment with bortezomib in patients with relapsed myeloma enrolled in phase 2 (SUMMIT and CREST) and phase 3 (APEX) clinical trials of bortezomib. This study, to which we contributed by performing part of the analyses, was done by Mulligan and colleagues.

In **Chapter 6** we built a high-risk signature on GEPs obtained from newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial. This high-risk gene signature, consisting of 92 genes (EMC-92-gene signature), was validated in independent datasets of newly diagnosed and relapsed patients, the latter described in Chapter 5. In all sets, patients defined as high-risk by the EMC-92-gene signature showed a significant reduced OS. In multivariate analyses EMC-92-gene signature was proven independent of currently used prognostic factors. Comparing the EMC-92-gene signature to the 7 high-risk signatures that have been developed until now, we observed a good performance for the EMC-92 signature. Also, the EMC-92 signature selected a high-risk group of relevant size. Moreover, directly comparing all high-risk signatures to each other by means of pair-wise comparisons demonstrated the EMC-92 signature to have the best fit to the observed survival times in independent sets.

Chapter 7 revolves around the expression of an important family of tumor associated antigens (TAAs), the cancer testis antigens (CTAs) in MM at diagnosis and at relapse. The incentive for this research was that continued expression after treatment may allow for the development of (immuno)therapy. The Chapter focused on CTA genes in terms of prognosis, and gave an indication of which CTAs may be suitable for immunotherapy. The protein expression of one of these CTAs, i.e. *MAGEC1,* was assessed.

The rationale behind the research described in **Chapter 8** comes from the recent identification of cereblon *(CRBN)* as a primary target of thalidomide teratogenicity. Furthermore, a subsequent paper showed that *CRBN* played an important role in the mechanism of action of thalidomide and IMiDs as antitumor agents. As a set up for future research in trials with IMiDs as part of the induction regimen, we determined *CRBN* expression in relation to outcome on thalidomide maintenance in patients included in the HOVON-65/GMMG-HD4. We observed that patients showing higher expression of *CRBN* showed a significantly better PFS and OS compared to patients with low expression of *CRBN*. This finding was not observed in patients with bortezomib maintenance.

Chapters 9 and **10** focus on one of the most serious non-hematological side effects limiting the optimal use of bortezomib, i.e. peripheral neuropathy (PN). In **Chapter 9** we analyzed GEPs of myeloma plasma cells from patients at diagnosis, and single nucleotide polymorphisms (SNPs) in DNA of peripheral blood samples from patients using a custom designed Bank On A Cure (BOAC) SNP chip. We investigated GEPs and SNPs in genes

in association with occurrence of bortezomib-induced peripheral neuropathy (BiPN) and vincristine-induced peripheral neuropathy (ViPN). GEPs in plasma cells and SNPs in peripheral blood associated with early-onset BiPN (after one cycle of bortezomib induction treatment) included genes involved in apoptosis, transcription, and AMP-activated protein kinase (AMPK)-mediated signaling. Late-onset BiPN (after 2 or 3 cycles of bortezomib induction treatment) was associated with genes and SNPs in genes involved in development and function of the nervous system as well as pro-inflammatory genes. Both early and late-onset BiPN were characterized by genes and SNPs involved in nervous system development and DNA repair. Finally, we demonstrated that both the inherited genetic constitution of the patient as well as the molecular characteristics in myeloma plasma cells are associated with the development of BiPN.

Chapter 10 represents an overview of BiPN, including clinical presentation, risk factors, and management of BiPN.

SAMENVATTING

Multipele myeloom (MM; ziekte van Kahler) werd lang beschouwd als één ziekte-entiteit. Dit veranderde ongeveer 10 jaar geleden met de ontdekking van frequente translocaties, waarmee MM werd verdeeld in twee groepen, hyperdiploid (HRD) en niet-hyperdiploid (NHRD) MM. De komst van nieuwe technologieën, zoals genexpressie arrays voor moleculaire profilering, maakte het mogelijk om MM patiënten verder onder te verdelen in subgroepen, met verschillen in ziekte karakteristieken en verschillen in prognose. Met de introductie van nieuwe middelen, zoals thalidomide en bortezomib, werd substantiële vooruitgang geboekt in de behandeling van MM. Een groot probleem van deze middelen betrof echter de ontwikkeling van ernstige toxiciteit, in het bijzonder perifere neuropathie (PN).

Hoofdstuk 1 bevat een algemene introductie over MM met nadruk op pathogenese en prognose, waarbij gefocust wordt op nieuwe technologieën zoals moleculaire profilering. Daarnaast worden de conventionele en nieuwe behandelingsstrategieën uiteengezet.

Hoofdstuk 2 beschrijft de resultaten van een multicenter, fase 3 studie, HOVON-65/GMMG-HD4, waarin bortezomib in inductie en post-intensificatie behandeling wordt vergeleken met conventionele chemotherapie en thalidomide post-intensificatie in nieuw gediagnosticeerde MM patiënten. De onderzoeken beschreven in hoofdstukken 3, 4, 6, 7, 8 en 9 maakten onderdeel uit van deze klinische studie en zijn gebaseerd op de data en samples verkregen van patiënten die werden geïncludeerd in deze studie.

In **hoofdstuk 3** presenteren we een clustering van MM samples gebaseerd op gecorreleerde genexpressie profielen (GEPs). 10 clusters werden gekarakteriseerd in detail; één extra cluster toonde geen duidelijk gen profiel. We bevestigden de 7 clusters beschreven in de University of Arkansas for Medical Science (UAMS) classificatie: CD-1, CD-2, MF, MS, PR, en HY, en een additionele subgroep gekarakteriseerd door een myeloid/normaal plasmacel profiel. Drie nieuwe subgroepen werden geïdentificeerd, waaronder een subgroep gekarakteriseerd door hoge expressie van genen betrokken in de NF-κB signaleringsroute, genaamd NF-κB cluster. Een andere subgroep werd gekarakteriseerd door overexpressie van kanker testis antigenen (CTA) zonder overexpressie van proliferatie genen, het CTA cluster. Het derde cluster toonde overexpressie van proteïne tyrosine fosfatasen *PRL-3* en *PTPRZ1* evenals *SOCS3,* genaamd PRL-3 cluster. Daarnaast werden genetische indices ontwikkeld voor translocaties t(4;14), t(14;16)/t(14;20), en t(11;14), waarmee het mogelijk werd samples met een van de genoemde translocaties te classificeren met goede nauwkeurigheid.

In **hoofdstuk 4** beschrijven we het onderzoek naar de prognose van de verschillende clusters, zoals gedefinieerd in hoofdstuk 3, en de impact van bortezomib op overleving in deze clusters. Na conventionele behandeling werd een significant verschil in progressie

vrije overleving (PFS) en overleving (OS) gezien tussen de clusters. Volgend op bortezomib behandeling verdween de significantie in PFS en OS tussen de clusters. Dit werd vooral veroorzaakt doordat de clusters die een slechte overleving toonden na conventionele behandeling een substantiële verbetering van overleving lieten zien na bortezomib behandeling, dit zijn de MF en MS cluster. Het PR cluster toonde geen verbetering van overleving na bortezomib en behield zijn negatief prognostische waarde onafhankelijk van behandeling.

In **hoofdstuk 5** wordt de ontwikkeling en het functioneren beschreven van prognostische genetische indices voor respons en overleving na bortezomib behandeling in patiënten met recidief/refractair MM geïncludeerd in fase 2 (SUMMIT en CREST) en fase 3 (APEX) klinische studies. Dit onderzoek, waarin wij hebben bijgedragen door een gedeelte van de analyses te doen, werd verricht door Mulligan en collega's.

In **hoofdstuk 6** ontwikkelden we een hoog-risico index op basis van GEPs van nieuw gediagnosticeerde MM patiënten geïncludeerd in de HOVON-65/GMMG-HD4 studie. Deze hoog-risico index, bestaande uit 92 genen, werd gevalideerd in onafhankelijke datasets van nieuw gediagnosticeerde en recidief patiënten, de laatste set is beschreven in hoofdstuk 5. In alle sets toonden de patiënten gedefinieerd als hoog-risico met de EMC-92-gen index een significant kortere OS. In multivariate analyses bleek de EMC-92-gen index onafhankelijk van bestaande prognostische factoren. Wanneer we de EMC-92-gen index vergeleken met de 7 hoog-risico indices die tot nu toe zijn ontwikkeld, observeerden we dat de EMC-92 gen index in staat was een hoog-risico groep van relevante grootte uit te selecteren. Bovendien, als we alle hoog-risico indices direct met elkaar vergeleken door middel van paarsgewijze vergelijking, bleek de EMC-92-gen index de best mogelijk voorspelling van OS te geven in de onafhankelijke datasets.

Hoofdstuk 7 draait om de expressie van een belangrijke familie van tumor geassocieerde antigenen (TAAs), de kanker testis antigenen (CTAs) in MM bij diagnose en bij recidief. De gedachte achter dit onderzoek was dat persisterende expressie na behandeling mogelijkheden zou kunnen bieden voor ontwikkeling van (immuno)therapie. Het hoofdstuk richt zich op CTA genen in termen van prognose, en geeft een indicatie welke CTAs geschikt zouden zijn voor immunotherapie. De eiwit expressie van één van deze CTAs, *MAGEC1,* werd bepaald.

De rationale achter het onderzoek beschreven in **hoofdstuk 8** komt van de recente ontdekking van cereblon *(CRBN)* als een directe target van thalidomide teratogeniciteit. Deze ontdekking werd gevolgd door een onderzoek waarin werd aangetoond dat *CRBN* een belangrijke rol speelt in het mechanisme van antitumor activiteit van thalidomide en IMiDs. Als een opzet voor toekomstig onderzoek naar de rol van *CRBN* expressie in studies waarin IMiDs deel uitmaken van de inductie behandeling, bepaalden we *CRBN* expressie in relatie met overleving volgend op thalidomide onderhoudsbehandeling in patiënten geïncludeerd in de HOVON-65/GMMG-HD4. We observeerden dat patiënten met hoger dan

mediane *CRBN* expressie een significant betere PFS en OS hadden vergeleken met patiënten met lager dan mediane *CRBN* expressie. Deze observatie werd niet gezien in patiënten met bortezomib als onderhoudsbehandeling.

Hoofdstuk 9 en **10** richten zich op één van de meest ernstige niet-hematologische bijwerkingen, die optimaal gebruik van bortezomib limiteren, namelijk perifere neuropathie (PN). In **hoofdstuk 9** analyseerden we GEPs van myeloma plasmacellen van patiënten bij diagnose, evenals single nucleotide polymorphismen (SNPs) in DNA van perifeer bloed samples van patiënten, gebruik makend van een speciaal voor dit type onderzoek ontworpen Bank On A Cure (BOAC) SNP chip. We onderzochten GEPs en SNPs in genen in associatie met optreden van bortezomib-geinduceerde perifere neuropathie (BiPN) en vincristine-geinduceerde perifere neuropathie (ViPN). GEPs in plasma cellen en SNPs in perifeer bloed geassocieerd met vroeg optreden van BiPN (na één cyclus van bortezomib inductie behandeling) betroffen genen betrokken in apoptose, transcriptie en AMP-geactiveerde proteïne kinase (AMPK)-gemedieerde signaleringsroute. Laat optreden van BiPN (na 2 of 3 cycli van bortezomib inductie behandeling) was geassocieerd met genen in ontwikkeling en functie van het zenuwstelsel evenals pro-inflammatoire genen. Zowel vroeg als laat optreden van BiPN werd gekarakteriseerd door genen en SNPs in genen betrokken in het zenuwstelsel en DNA repair. Hiermee demonstreerden we uiteindelijk dat zowel de genetische constitutie van de patiënt als de moleculaire karakteristieken in myeloma plasmacellen geassocieerd zijn met de ontwikkeling van BiPN. **Hoofdstuk 10** representeert een overzicht van BiPN, inclusief klinische presentatie, risico factoren en beleid bij optreden van BiPN.

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CURRICULUM VITAE

Annemiek Broijl is geboren op 13 augustus 1976 te Warnsveld. Zij heeft in 1994 haar VWO diploma behaald aan het Stedelijk Lyceum te Zutphen. In september van dat jaar is zij gestart met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Haar afstudeeronderzoek deed zij op de afdeling hematologie van de Erasmus Universiteit Rotterdam onder leiding van dr. R. E. Ploemacher en heeft zij afgerond in 1998. Van 1998 tot 2000 heeft zij onderzoek gedaan op de afdeling experimentele hematologie aan de Technion universiteit in Haifa onder leiding van dr. S. Merchav. Na het behalen van het arts examen in 2002, is zij in 2003 aangevangen met de opleiding inwendige geneeskunde (prof. dr. H.A.P. Pols en prof. dr. J.L.C.M. van Saase). In 2005 begon zij haar promotie onderzoek op de afdeling hematologie van de Erasmus Universiteit Rotterdam onder leiding van prof. dr. P. Sonneveld. In de periode 2003 tot 2012 ronde zij achtereenvolgens de opleiding tot internist, de specialisatie tot hematoloog en uiteindelijk haar promotie onderzoek af, waarvan de resultaten zijn beschreven in dit proefschrift. Momenteel is zij werkzaam als internist-hematoloog in het Erasmus Medisch Centrum.

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ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse event
ASCT	autologous stem cell transplantation
BiPN	Bortezomib induced polyneuropathy
BM	Bone marrow
B2M	β_2-microglobulin
BOAC	Bank On A Cure
CI	Confidence interval
CR	Complete response
CRAB	Hypercalcemia, renal insufficiency, anemia and bone lesions
CTA	Cancer testis antigen
CTD	Cyclofosfamide, thalidomide, dexamethason
CT-L	Chymotrypsin-like activity
CVAD	Cyclofosfamide-VAD
EBMT	European Group for Blood and Marrow Transplantation
ECM	Extracellular matrix
EFS	Event free survival
FACS	Fluorescence cell sorting analysis
FISH	Fluorescence in situ hybridization
GEP	Gene expression profile
GEO	Gene Expression Omnibus
GIMEMA	Gruppo Italiano Malattie EMatologiche dell'Adulto
GMMG	German-Speaking Myeloma Multicenter Group
GSEA	*Gene set enrichment analysis*
HDAC	Histone deacetylase
HDACi	HDAC inhibitors
HDM	High dose melphalan
HDT	High dose therapy
HOVON	Dutch-Belgium Hemato-Oncology Group
HR	Hazard ratio
HMCL	Human myeloma cell line
HRD	Hyperdiploid MM
IFM	Intergroupe Francophone du Myélome
IgH	Immunoglobulin heavy chain
IMWG	International Myeloma Working Group
ISS	International staging system

IMiD	Immunomodulatory drug
IMWG	International myeloma working group
LMWH	Low molecular weight heparin
mAB	Monoclonal antibody
MAF	Minor allele frequency
MGUS	Monoclonal of undetermined significance
MM	Multiple myeloma
MP	Melphalan, prednisone
MPT	Melphalan, prednisone, thalidomide
MPR-R	Melphalan, prednisone, lenalidomide, lenalidomide maintenance
MR	Minor response
MRC	Medical Research Council
mSMART	Mayo Stratification for Myeloma And Risk-adapted Therapy
NCI-CTCAE	National cancer institute's common toxicity criteria for adverse events
nCR	near CR
NC	No change
NCS	nerve conduction studies
NR	No response
NHRD	Nonhyperdiploid MM
NUSE	Normalized, unscaled standard error
OS	Overall survival
OR	Odds ratio
ORR	Overall response rate
PAD	Bortezomib, adriamycin, dexamethasone
PAM	prediction analysis of microarrays
PCL	Plasma cell leukemia
PC	Plasma cell
PD	Progressive disease
PFS	Progression free survival
PI	Proliferation index (gene based)
PN	Polyneuropathy
PR	Partial response
R	Response
Rd	Lenalidomide plus low-dose dexamethasone
RD	Lenalidomide plus high-dose dexamethasone
ROTI	Related organ or tissue impairment
SAE	Severe adverse event
SD	Stable disease
SMM	Smoldering myeloma

SNP	Single nucleotide polymorphism
Sn	Sensitivity
Sp	Specificity
SPCA	supervised principal component analysis
TAA	Tumor associated antigen
TBR	testis and brain restricted
TC	Translocation/ cyclin D classification
TD	Thalidomide, dexamethasone
TAD	Thalidomide, adriamycin, dexamethasone
TiPN	Thalidomide induced polyneuropathy
TR	testis restricted
TS	testis selective
TTP	Time to progression
UAMS	University of Arkansas for Medical Sciences
UPR	Unfolded protein response
VAD	Vincristine, adriamycin, dexamethasone
VD	Bortezomib, dexamethasone
VGPR	Very good partial response
VMP	Bortezomib, melphalan, prednisone
VRD	Bortezomib, lenalidomide, dexamethasone
VTD	Bortezomib, thalidomide, dexamethasone
VTE	Venous thrombo-embolic event
WGS	Whole genome sequencing
WES	Whole exome sequencing

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Annemiek Broyl	PhD period: may 2005 – may 2010
Erasmus MC Department: Hematology	Promotor and supervisor: Prof. Dr. P. Sonneveld
Research School: Molecular Medicine	

1. PhD training

	Year	Workload ECTS
Specific courses (e.g. Research school, Medical Training)		
– SNPs and Human diseases (MolMed)	2005	2
– Analysis of microarray gene expression data (MCG)	2005	1
– Molecular Medicine (MolMEd)	2005	0.5
– Symposium Microarrays from Bench to Bedside	2005	0.5
– The Workshop of applied bioinformatics (MolMed)	2007	0.5
Seminars and workshops		
– Workshop on gene expression profiling	2005	1
Presentations		
– 6 Hematology presentations	2005-2009	3
– 1 journal club	2005	0.2
– 1 CTMM presentation	2010	0.3
(Inter)national conferences		
– European Hematology Association (oral presentation)	2005	4
– American Society of Hematology (poster presentation)	2006	2
– European Hematology Association (poster presentation)	2007	4
– XIth International Myeloma Workshop (oral presentation)	2007	4
– 12th Molecular Medicine Day (poster presentation)	2008	0.5
– Wetenschapsdagen Interne Geneeskunde Erasmus MC (poster presentation)	2008	0.5
– Nederlandse Internisten dagen (oral presentation)	2008	1
– Dutch Hematology Congress	2009	1
– XIIth International Myeloma Workshop (poster presentation)	2009	2
– American Society of Hematology (poster presentation)	2009	2
– Dutch Hematology Congress	2010	2
– European Hematology Association (oral presentation)	2010	4
– IV International Workshop On Myeloma Pharmacogenomics & Novel Therapeutics (oral presentation)	2010	2
– American Society of Hematology (2 oral presentations)	2010	4
– XIIIth International Myeloma Workshop (poster presentation)	2011	2
– V International Workshop On Myeloma Pharmacogenomics & Novel Therapeutics	2011	1
– Dutch Hematology Congress	2012	1
Other		
– Invited speaker, paper selected for top publication, Nederlandse Internisten Vereniging (NIV) dagen	2011	1
– Invited speaker, 13e regionale nascholing hematologie voor internisten, hematologen, hematologen in opleiding en internisten in opleiding	2011	1
– Invited speaker, MM Masterclass Dordrecht	2011	0.5
– Invited speaker, IKNL netwerkdagen Vlissingen	2012	0.5
– Writing application for Zon MW	2004	3
– Writing and receiving European Hematology Association clinical research grant	2005	3
– Writing application for Multiple Myeloma research Foundation	2006	4
– Research visit, Heidelberg, Germany	2006	2
– Research visit, Department of Haemato-oncology, the institute of Cancer Research, Sutton, Surrey, UK	2007	2
2. Teaching		
Lecturing		
Zorgacademie, onderwijs verpleegkundigen	2010-2012	1
TOTAL		**64**

COLOUR FIGURES

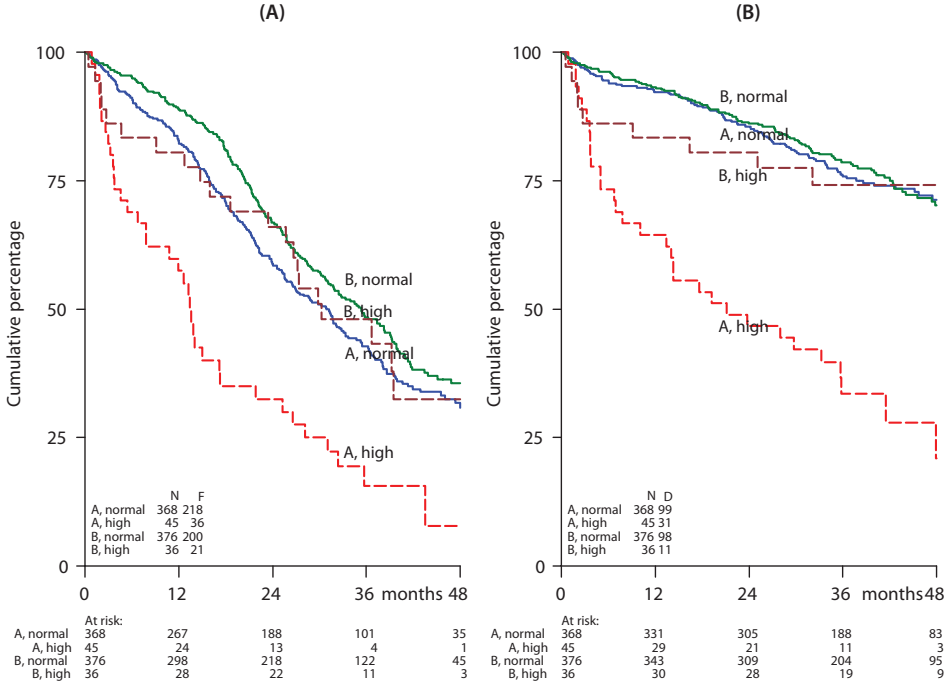


Figure 2.4. Kaplan-Meier survival curves of progression free survival (PFS) and overall survival (OS) according to treatment arm within subgroups according to creatinine level at presentation. (A) PFS in patients with creatinine ≤ 2 mg/dL (VAD:blue, PAD:green) or > 2 mg/dL (VAD:red, PAD:black) (B) OS in patients with creatinine ≤ 2 mg/dL (VAD:blue, PAD:green) or > 2 mg/dL (VAD:red, PAD:black) (see page 59).

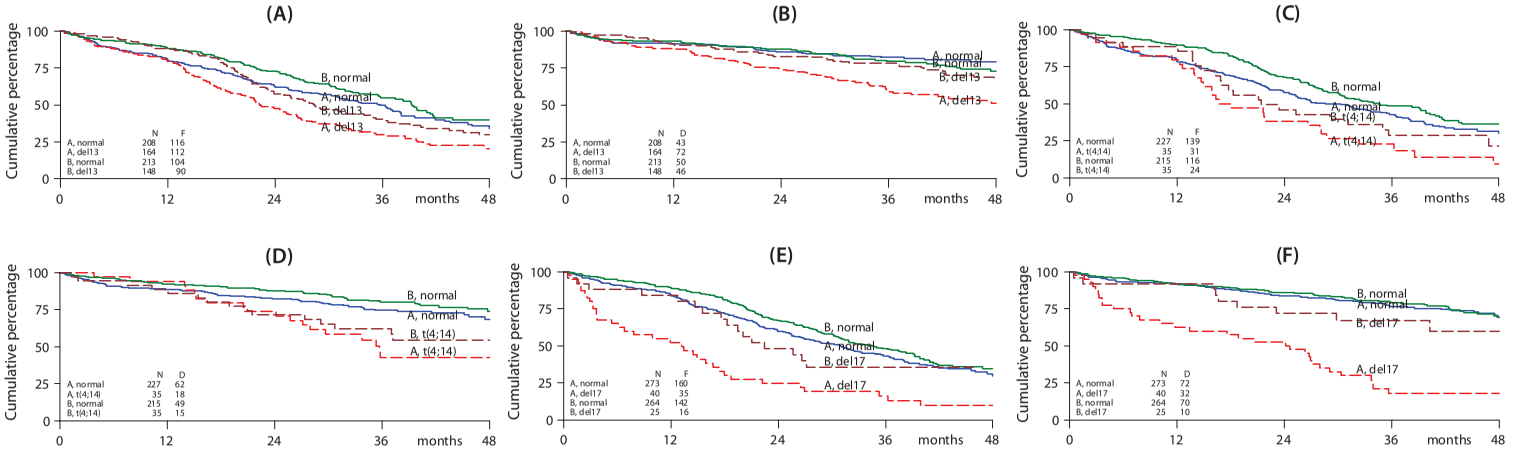


Figure 2.5. Kaplan-Meier survival curves of progression free survival (PFS) and overall survival (OS) according to treatment arm within subgroups according to del(13/13q) or t(4;14) or according to del(17p). In all figures patients without data regarding the specific abnormality have been excluded. **(A)** PFS in patients without del(13q) (VAD:blue, PAD:green) or with del(13q) (VAD:red, PAD:black) **(B)** OS in patients without del(13q) (VAD:blue, PAD:green) or with del(13q) (VAD:red, PAD:black) **(C)** PFS in patients without t(4;14) (VAD:blue, PAD:green) or with t(4;14) (VAD:red, PAD:black) **(D)** OS in patients without t(4;14) (VAD:blue, PAD:green) or with t(4;14) (VAD:red, PAD:black) **(E)** PFS in patients without del(17p) (VAD:blue, PAD:green) or with del(17p) (VAD:red, PAD:black) **(F)** OS in patients without del(17p) (VAD:blue, PAD:green) or with del(17p) (VAD:red, PAD:black) (see page 60).

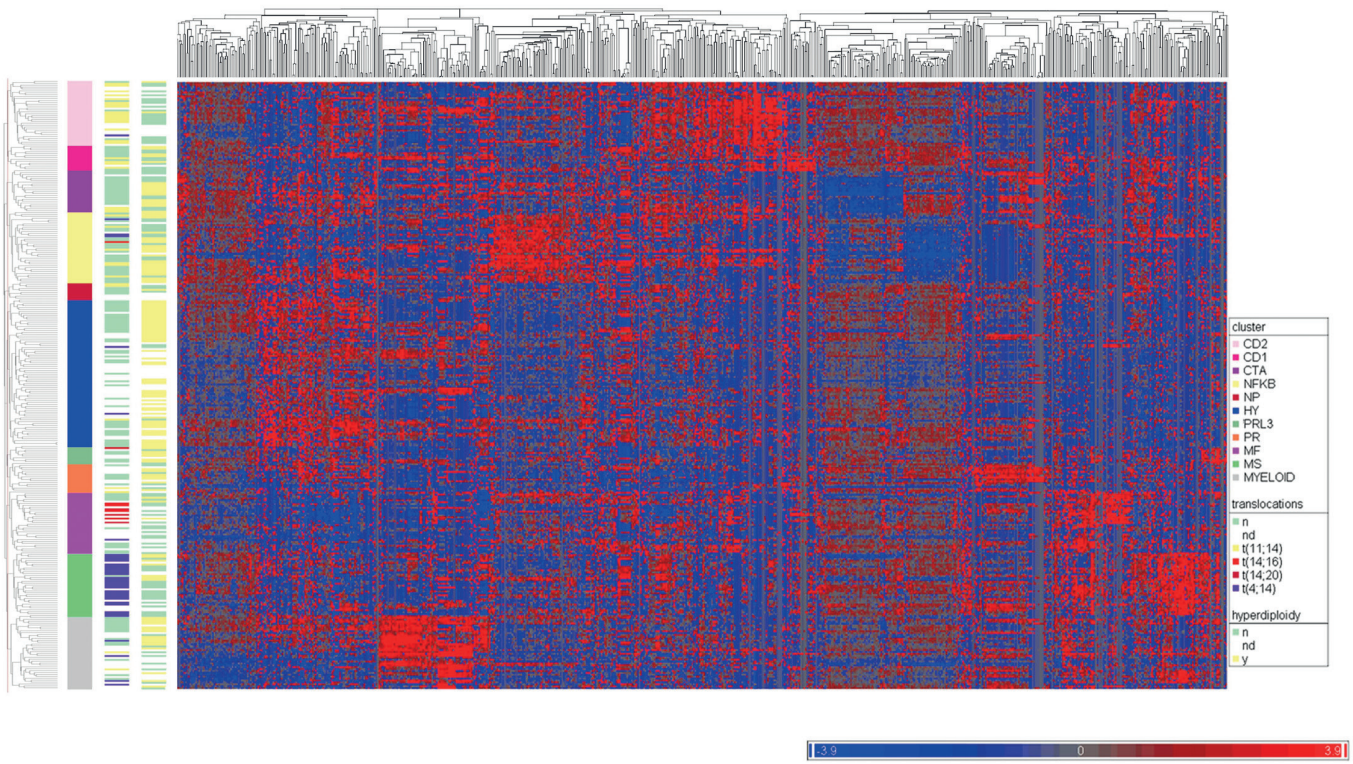
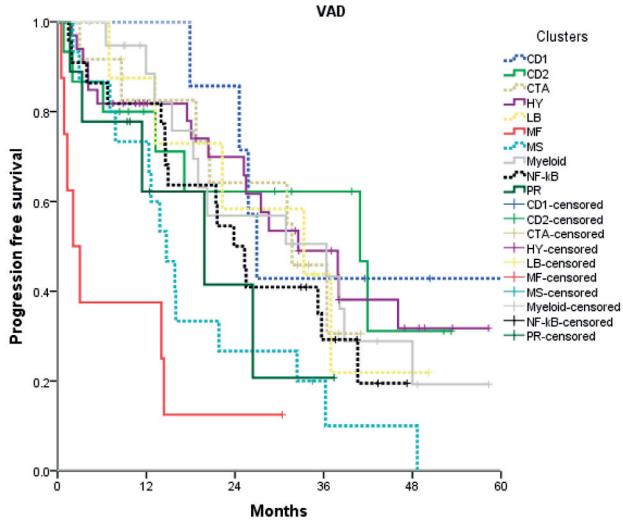


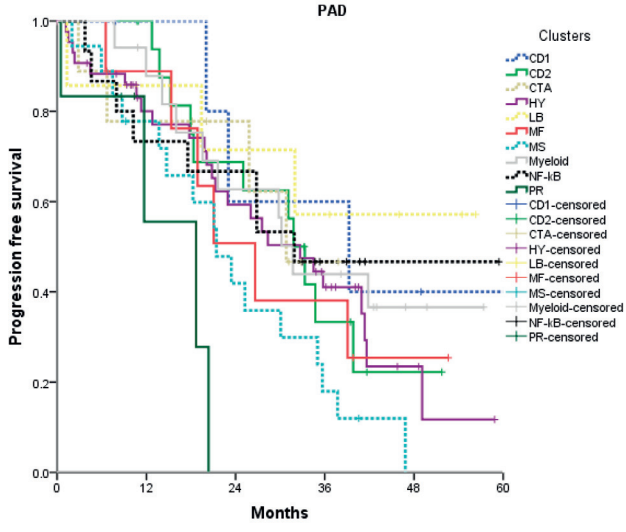
Figure 3.1. Dendrogram and heatmap. Vertical dendrogram shows sample clustering with 5 major branches and 11 distinct clusters; the dendrogram is cut at 11 clusters. First column,11 clusters: CD2, CD-2 cluster; CD1, CD-1 cluster; CTA, CTA cluster; NF-κB, NF-κB cluster; NP, no clear profile; HY, HY cluster; PRL3, PRL3 cluster; PR, PR cluster; MF, MF cluster; MS, MS cluster; Myeloid, Myeloid cluster. Translocations are shown in the second column: t(11;14), yellow; t(4;14), blue; t(14;16) or t(14;20), red; no translocation, green; and not determined, white. The third column indicates hyperdiploidy: y, hyperdiploidy (yellow); n, no hyperdiploidy (green) and nd (not determined; white). Horizontal dendrogram shows clustering of genes. The heatmap shows the spectrum of expression values, the log2 expression value from the geometric mean for each gene is indicated by a color, with red representing positive expression (up-regulation) and blue representing negative expression (down-regulation) of a gene (see page 74).

(4.1A)



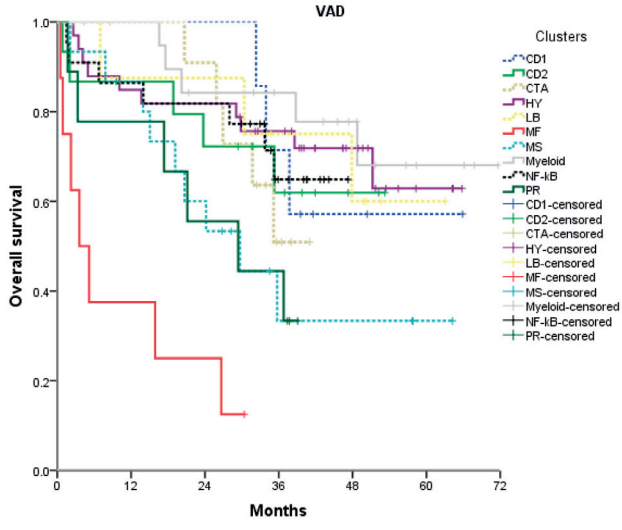
clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	7	15	12	33	8	8	15	21	22	9
Median PFS (months)	27	41	31	33	33	2	15	36	24	20

(4.1B)



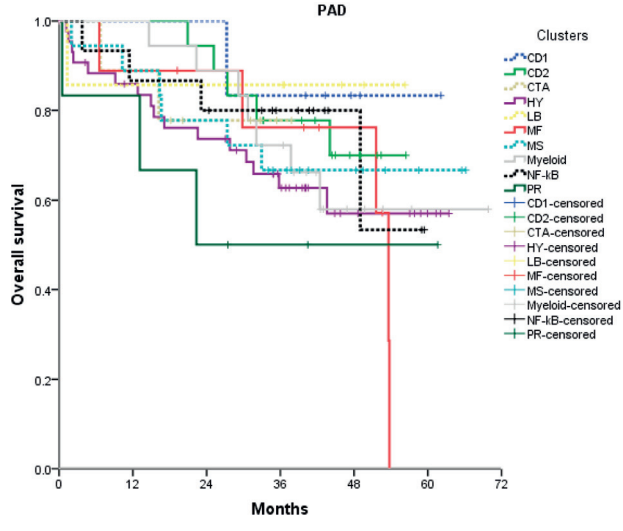
clusters	CD-1	CD-2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	6	18	10	44	7	9	18	18	15	6
Median PFS(months)	39	32	31	33	NR	27	21	32	32	19

(4.1C)



clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	7	15	12	33	8	8	15	21	22	9
Median PFS(months)	NR	NR	NR	NR	NR	4	30	NR	NR	29

(4.1D)



clusters	CD–1	CD–2	CTA	HY	LB	MF	MS	Myeloid	NF-κB	PR
N	6	18	10	44	7	9	18	18	15	6
Median PFS(months)	NR	NR	NR	NR	NR	54	NR	NR	NR	22

Figure 4.1. Kaplan Meier curves. **(A)** 12 clusters and PFS following treatment with VAD, **(B)** 12 clusters and PFS following treatment with PAD, **(C)** 12 clusters and OS following treatment with VAD, **(D)** 12 clusters and OS following treatment with PAD. NR=Not reached (see pages 95 and 96).

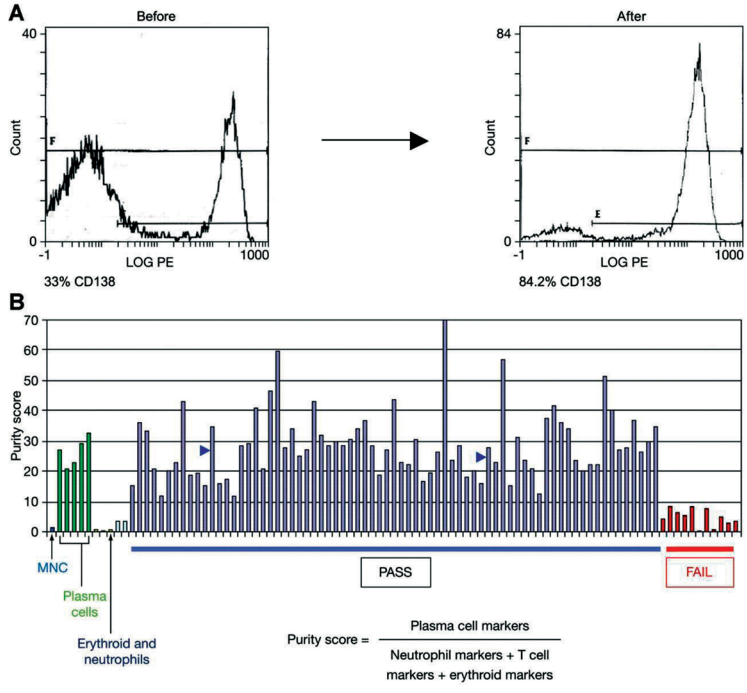


Figure 5.1. Bone marrow aspirate enrichment procedure effectively depletes nontumor cells. **(A)** Bone marrow aspirate samples before and after enrichment were subject to CD138 staining and FACS analysis. **(B)** Myeloma purity score is elevated in control plasma cell samples (> 90% pure) relative to bone marrow mononuclear cells (MNCs), neutrophils, and erythroid cells. Two enriched patient samples of 84% and 91% tumor purity by FACS analysis had scores of 35 and 28, respectively (blue arrows). A score of at least 10 (at least 3-fold elevated relative to the score for nonplasma cell types) was set as a threshold for further analysis (see page 106).

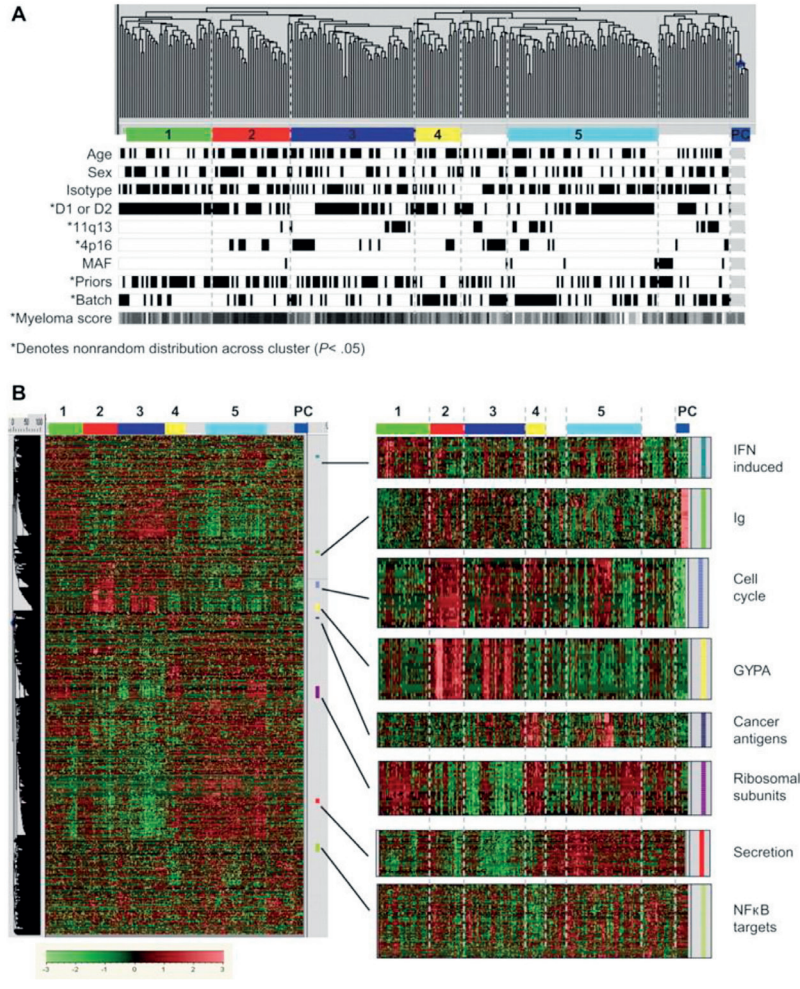


Figure 5.2. Sample relationships are influenced by clinical and gene-expression characteristics. Two hundred sixty-four myeloma patient samples and 6 normal plasma cell control samples were subject to unsupervised hierarchical clustering based on 9174 differentially expressed probe sets. Highly related branches (labeled groups 1-5) were identified by setting a fixed similarity metric (GeneMaths software; Applied Maths, Austin, TX) and requiring at least 12 samples for membership; unlabeled samples comprise various smaller groups. **(A)** Patient attributes are encoded below the sample dendrogram. Attributes with nonrandom distribution ($P < .05$) are indicated by asterisks. Black is associated with age older than 60 years, female sex, IgG isotype, 1 or 2 prior therapies, hybridization batch 1 (trials 024, 025, and 040), and low purity score. White is associated with age 60 years and younger, male sex, other isotypes, 3 or more prior therapies, hybridization batch 2 (trial 039), and high purity score. **(B)** An overview of the 9174 differentially expressed probe sets, with an expansion of specific functional groups (see page 106).

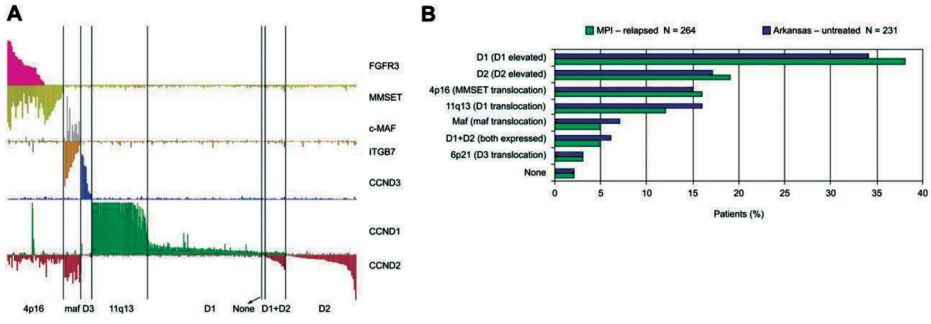
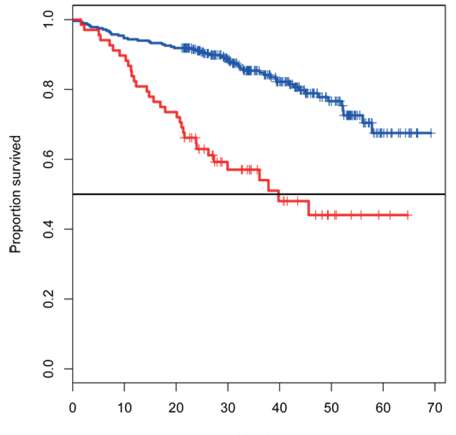


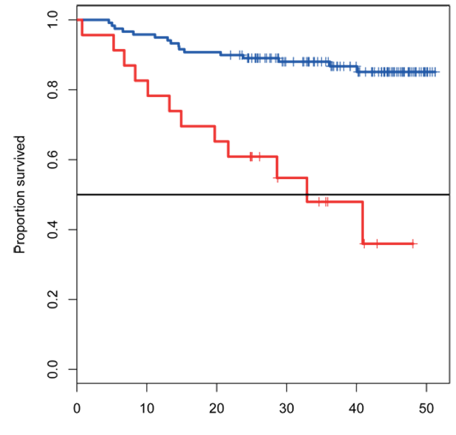
Figure 5.3. All samples assigned to TC subtypes based on expression of D cyclins and translocation target genes (n = 264). **(A)** The TC subtypes of 264 relapsed myeloma samples are shown. The y-axis shows normalized expression level of each gene; subtypes were determined as in Bergsagel et al.[47] **(B)** A comparison of the TC subtype frequency for relapsed patients in Millennium Pharmaceuticals (MPI) studies (green) and for newly diagnosed patients (blue) as defined at the University of Arkansas[47] (see page 255 for colour figure).

(6.1A)



Group	N	Events	HR	Wald P	Median
Standard	283	53	1.00		
High	68	32	3.40	5.7e−08	39.8

(6.1B)



Group	N	Events	HR	Wald P	Median
Standard	119	16	1.00		
High	23	12	5.23	1.8e−05	32.9

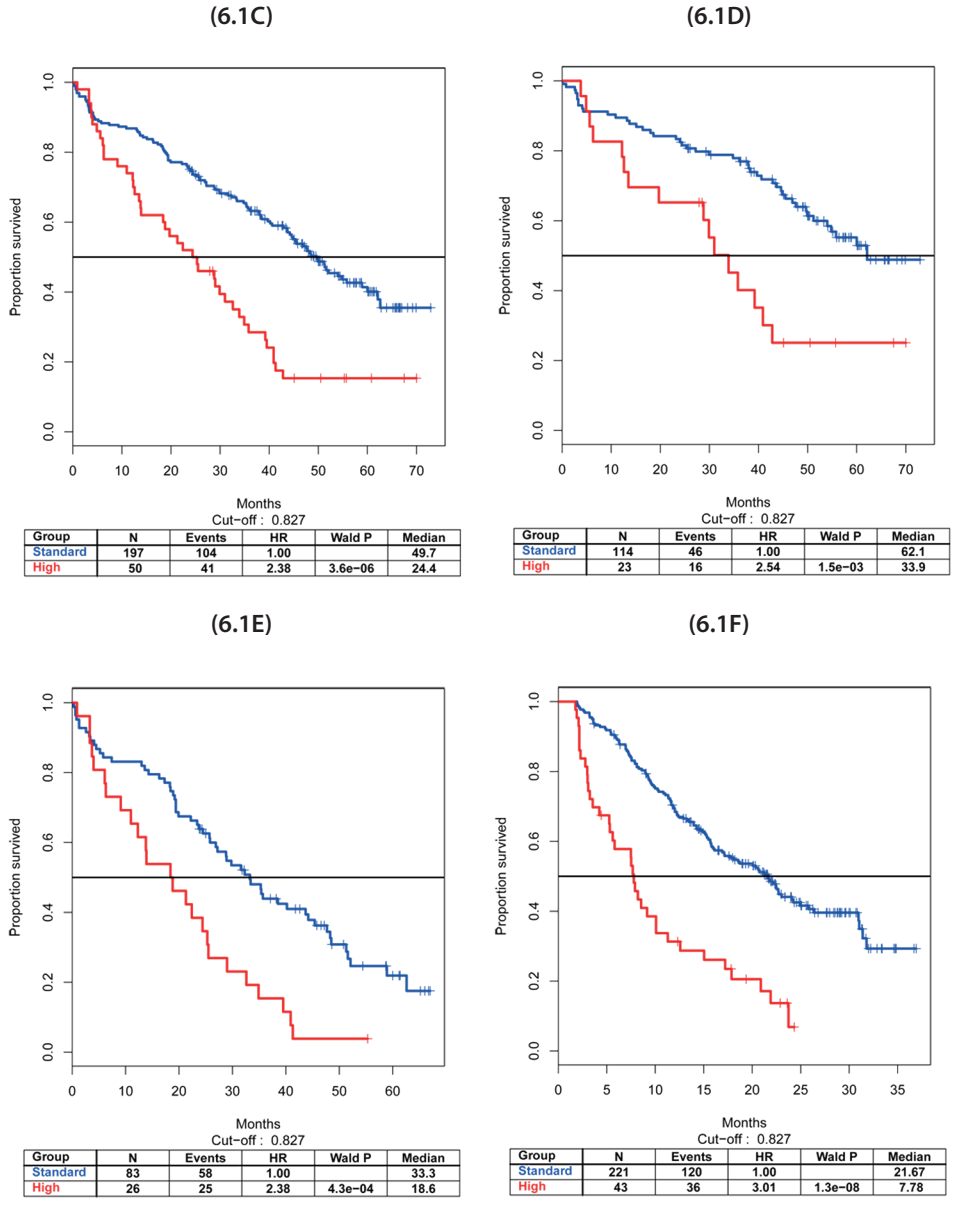


Figure 6.1. Kaplan-Meier overall survival curves for EMC-92 signature defined high-risk patients versus standard-risk patients in five validation sets. The cut-off value is fixed at 0.827 based on proportion of patients with OS <2 years in the HOVON-65/GMMG-HD4 set. In the MRC-IX one patient had an unknown treatment status and was disregarded in figures c and d. **(A)** UAMS Total Therapy 2. **(B)** UAMS Total Therapy 3. **(C)** MRC-IX. **(D)** MRC-IX transplant-eligible patients. **(E)** MRC-IX non-transplant-eligible. **(F)** APEX. N, number of patients; Events, number of events; HR, hazard ratio; Wald P, p value for equality to standard-risk group; Median, median survival time (see pages 132 and 133).