Chapter 1

General Introduction
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Chapter 1

1.1 B-cell differentiation

B-cell malignancies can be derived from different stages of B-cell differentiation. Normal B-cell differentiation begins with precursor B lymphoblasts (blast cells that are the precursors of the entire B-cell line), which undergo immunoglobulin VDJ gene rearrangement and differentiate into mature surface immunoglobulin (sIg) positive (IgM⁺, IgD⁺) naive B-cells. Naive B-cells are small resting lymphocytes that circulate in the blood and also occupy primary lymphoid follicles and follicle mantle zones (so-called recirculating B-cells). Tumors of these cells are usually histologically low grade, clinically indolent, often widespread and leukemic, consistent with the recirculating behavior of normal naive B-cells. On encountering antigen, naive B-cells undergo blast transformation, proliferate, and ultimately mature into IgG or IgA antibody-secreting plasma cells and memory B-cells. Blast cells formed from naive B-cells that have encountered antigen migrate into the center of primary follicles and fill the follicular dendritic cell meshwork, forming the germinal center. Germinal center blast cells are called centroblasts (blasts cells of the germinal center), they are large proliferating cells with vesicular nuclei, one to three prominent, peripheral nucleoli, and a narrow rim of basophilic cytoplasm. Many lack sIg, and also switch off the expression of Bcl2 protein, which makes them susceptible to apoptosis. Centroblasts express CD10 and Bcl6 protein, a nuclear zinc-finger transcription factor that is expressed by both centroblasts and centrocytes, but not by naive or memory B-cells, mantle cells or plasma cells.

In the germinal center, somatic mutations occur in the immunoglobulin variable region gene, which alter the affinity for antigen of the antibody that will be produced by the cell. This results in marked intraclonal diversity in a population of cells derived from only a few precursors. In addition, some cells switch from IgM to IgG or IgA production. Centroblasts mature to centrocytes, which are medium-sized cells with irregular nuclei, inconspicuous nucleoli, and scant cytoplasm. Centrocytes express sIg that has an altered antibody combining site compared with its progenitor cell, because of somatic mutations, and which may have undergone heavy chain class switch. Centrocytes with mutations that result in decreased affinity for antigen rapidly die by apoptosis, while centrocytes with mutations that result in increased affinity are able to bind to antigen trapped on the processes of follicular...
dendritic cells. This process rescues them from apoptosis and they re-express Bcl2 protein. Through these mechanisms, the germinal center reaction gives rise to the better-fitting IgG or IgA antibody-producing cells of the late primary or secondary immune response. Burkitt lymphomas and most large B-cell lymphomas are composed of cells that at least in part resemble centroblasts and that have mutated Ig variable regions, consistent with a derivation from cells that have been exposed to the germinal center. Through interaction with surface molecules on follicular dendritic cells and T-cells, such as CD40 ligand and CD23, centrocytes switch off Bcl6 protein expression, and differentiate into either memory B-cells or plasma cells. Follicular lymphomas are believed to be tumors of germinal center B-cells, in which centrocytes fail to undergo apoptosis because they have a chromosomal rearrangement, t(14;18), that prevents the normal switching off of Bcl2 protein expression. Since they are composed predominantly of centrocytes, which are resting cells, they tend to be indolent.

Memory cells typically reside in the follicle marginal zones (marginal zone B-cells), they have round to slightly irregular nucleoli, moderately condensed chromatin, and a moderate amount of pale cytoplasm. Plasma cells home to the bone marrow, they have condensed chromatin and abundant, basophilic, cytoplasm that contains predominantly IgG or IgA. They lack surface immunoglobulin and pan-B antigens, but express CD79a and CD138. Both memory B and plasma cells have mutated Ig variable genes, but do not continue to undergo mutations. Thus they do not show intraclonal diversity. Marginal zone lymphomas correspond to post germinal center, possibly memory B-cells. Plasma cell myeloma corresponds to a bone-marrow homing IgG or IgA plasma cell.

1.1.1 Plasma cell disorders
Plasma cell disorders are characterized by an accumulation of a clone of plasma cells, in most cases producing homogeneous immunoglobulin product (less than 1% of myelomas is nonsecretory). Clinically several forms of plasma cell disorders can be distinguished.

1.1.1.1 MGUS
Monoclonal gammopathy of unknown significance (MGUS) is typically asymptomatic and stable.
In MGUS the M-protein is <0.3 g/l and the tumor cells comprise no more than 10% of the bone marrow mononuclear cells. Annually 0.6%-3% of patients with MGUS progress to Multiple Myeloma (MM) expressing the same M-protein. At present, there are no unequivocal genetic or phenotypic markers that distinguish MGUS from MM cells. It is not possible to predict if and when a particular MGUS will progress to MM. To what extend intrinsic, either genetic or epigenetic, and/or extrinsic changes in tumor environment (e.g. immune cells) are responsible for progression from MGUS to MM remains unclear.

1.1.1.2 Multiple Myeloma
MM is a malignant proliferation of monoclonal plasma cells in the bone marrow, characterized by skeletal destruction, renal failure, anemia, and hypercalcemia. A clonal plasma cell neoplasm must expand to about $10^9$ cells before it produces enough immunoglobulin to be recognized as a monoclonal Ig “spike” (M-protein) by serum electrophoresis. MM accounts for 10% of all hematological malignancies and can be preceded by premalignant MGUS. The annual incidence is 850 cases in the Netherlands and increases progressively with age.
Smoldering MM, which has a stable intramedullary tumor cell content of >10% but no osteolytic lesions or other complications of malignant MM, has a high probability of progressing to MM. Progression of Smoldering MM to MM can be recognized by the development of osteolytic bone lesions and/or an increasing tumor mass. Further, progression of MM is associated with increasingly severe secondary features (anemia, immunodeficiency, renal impairment), and, in a fraction of patients, the occurrence of tumor in extramedullary locations.

1.1.1.3 Plasmacytoma
Plasmacytomas are clonal proliferations of plasma cells that are identical to those of plasma-cell myeloma but have a localized osseous or extraosseous growth pattern. Solitary plasmacytoma of bone (osseous plasmacytoma) and extramedullary (extraosseous) plasmacytomas each make up about 5% of plasma-cell neoplasms.
The most common sites of osseous plasmacytomas are in marrow areas with the most active hematopoiesis.
Introduction

These include, in descending order of frequency, the vertebrae, ribs, skull, pelvis, femur, clavicle, and scapula. Approximately 80% of extraosseous plasmacytomas occur in the upper respiratory tract, including the oropharynx, nasopharynx, sinuses, and larynx. At 10 years after diagnosis, 55% of patients with osseous plasmacytomas have a diagnosis of MM, and 10% have either local recurrence of the plasmacytoma or another solitary plasmacytoma. In contrast, the development of typical MM occurs in only 10 to 30% of patients with extraosseous plasmacytoma. The treatment of choice for both entities is local radiotherapy given with curative intent (>4000 cGy). This results in long term disease-free survival in approximately 30% of patients with solitary plasmacytoma of bone and 65% of patients with extramedullary plasmacytomas.

1.1.1.4 Plasma cell leukemia
Plasma cell leukemia (PCL), the leukemic variant of malignant plasma cell disorders, is a rare disease and is the least common variant of MM accounting for 2-3% of all plasma cell dyscrasias. The primary form occurs in individuals without preceding MM and presents normally with a rapid clinical course and a short survival. The second form of PCL typically arises as a late manifestation in individuals with MM (in 1-2% of cases of MM). Additional clinical characteristics of this group include a high frequency of extramedullary disease, severe anemia and thrombocytopenia.

1.2 Staging systems
The Durie-Salmon (DS) staging system is the most commonly used staging system for patients with MM. Staging a patient according to the DS system requires results from a bone marrow biopsy, bone survey, serum electrophoresis, and values for hemoglobin, hematocrit and serum calcium. Recently, an International Staging System for MM has been proposed. This staging system divides patients into three stages, based on serum β2-microglobulin (β2-M) and albumin levels (Stage I: serum β2-M < 3.5 and albumin > 3.5; Stage II: serum β2-M < 3.5 and albumin < 3.5, or serum β2-M > 3.5 to 5.5 mg/l). This staging system can stratify patients and predict survival, simply based on the results of these two widely available laboratory tests. How different staging systems correlate with prognosis, will be discussed in the next section.
Table 1.1 International staging system for multiple myeloma

<table>
<thead>
<tr>
<th>Stage</th>
<th>Serum β2-M (mg/l)</th>
<th>Serum albumin (g/dl)</th>
<th>Overall Survival (months)</th>
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</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>&lt; 3.5</td>
<td>&gt; 3.5</td>
<td>62</td>
</tr>
<tr>
<td>Stage 2</td>
<td>3.5-5.5</td>
<td>&lt; 3.5</td>
<td>44</td>
</tr>
<tr>
<td>Stage 3</td>
<td>&gt; 5.5</td>
<td>&lt; 3.5</td>
<td>29</td>
</tr>
</tbody>
</table>

1.3 Prognostic factors
A number of prognostic factors, pertinent to standard dose therapy, have also been shown to be relevant for high-dose therapies. These include β2-M, C-reactive protein (CRP), plasma cell labeling index, lactate dehydrogenase (LDH), cytogenetics and surface markers.

1.3.1 β2-Microglobulin
The serum levels of β2-M, which is part of HLA class I (-A, -B, -C) antigens, are increased in many hematological malignancies. In lymphoproliferative disorders there is generally an association between serum β2-M and tumor load. This relationship is certainly present in MM, where serum β2-M is a powerful prognostic indicator and can be used in stratification and monitoring. A combination of the prognostic factors serum β2-M and serum albumin provide a simple, powerful and reproducible three-stage classification system in MM patients, called the new International Staging System (ISS). The ISS was validated and demonstrated an median survival of 62 months, 44 months and 29 months for respectively stage I, II, and III. The ISS system was further validated by demonstrating effectiveness in patients in North America, Europe, and Asia; both in patients less than and older than 65 years of age; in patients with standard therapy or autotransplantation; and in comparison with the DS staging system.

1.3.2 Interleukin-6 and CRP
Interleukin-6 (IL-6), and serum C-reactive protein (CRP), have been reported to be of prognostic significance in multiple myeloma (MM).
The concentrations of CRP and IL-6 showed a linear association. Logarithmically transformed IL-6, CRP and β2-M were significant variables by univariate survival analysis; by multivariate analysis CRP was a slightly stronger prognostic factor than IL-6 and the only factor of independent prognostic significance.

1.3.3 Cytogenetics

Cytogenetic analysis in MM is hampered by the low proliferate fraction in most cases. Recent studies using cytokine-stimulated bone marrow cultures and fluorescence in situ hybridization (FISH) have increased the proportion of informative cases. Structural and numerical chromosomal abnormalities are described in 20-60% of newly diagnosed patients, with a mean of 30 to 40%. Further structural and numerical chromosomal abnormalities are found at 60 to 70% of patients with progressive disease. Complex karyotypes with multiple chromosomal gains and losses are the most frequent changes, but translocations, deletions, and mutations are all reported. Gains in chromosomes 3, 5, 7, 9, 11, 15, and 19, and losses in chromosomes 8, 13, 14, and X are most common. Among losses, monosomy or partial deletion of chromosome 13 (13q14) is the most common finding, occurring in 15 to 40% of newly diagnosed cases. In one study, patients with a 13q14 deletion had significant reductions in the rate of response to conventional dose chemotherapy (41 vs 79%) and overall survival (24 vs >60 months) compared to patients without this deletion. In this study, this abnormality, which was present in 46% of cases, was the most important independent variable associated with worse outcome. The prognostic implications of cytogenetic abnormalities of chromosome 13 (CA13) and those detected by FISH 13 and other standard laboratory parameters were examined in the first 231 MM patients enrolled in Total Therapy II, an intensive cytotoxic chemotherapy program with tandem autotransplants. Three-year projections of event-free survival (EFS) and overall survival (OS) were 71% and 77% respectively. In comparison EFS and OS were significantly shorter in patients with CA13, FISH 13, LDH ≥ 190 U/l, β2-M ≥4 mg/l and CRP ≥4.0 mg/l. Of note, CA13 was detected in 14% and significantly correlated with FISH 13 (present in 51%), tumor burden, proliferate activity and LDH. Chromosome 1 aberrations are frequently described, the short arm being
preferentially involved in deletions and the long arm in gains. Band 1p21 was found to be frequently deleted, leading to the assumption that a 1p deletion could lead to hemizygosity of at least 1 tumor suppressor gene. Two regions of 1q showed preferential gains: q12 to q22 and q31 to q42; these amplifications could induce the overexpression of one or more oncogenes. Shaughnessy et al demonstrated that altered transcriptional regulation of genes mapping to chromosome 1 may contribute to disease progression and that expression profiling can be used to identify high-risk disease and guide therapeutic interventions (Shaughnessy JD et al. Blood, 2006 Nov 14; [Epub ahead of print]).

Based on these data, it has been proposed that cytogenetics should be part of the initial work-up of patients with MM.17

1.3.4 A Translocation/Cyclin D expression (TC) Classification based on early pathogenic events

A supervised analysis of gene expression profiles provides the basis for a molecular classification of MM. In addition to determining the expression level of cyclin D1, 2, and 3, gene expression profiling can effectively identify MM tumors that overexpress the oncogenes dysregulated by the five recurrent IgH translocations: 11q13 (CYCLIN D1); 6p21 (CYCLIN D3); 4p16 (MMSET & usually FGFR3); 16q23 (c-MAF); and 20q11 (MAFB). These groups (Table 1.2) can be distinguished based on the Ig translocation present, and cyclin D expression: **11q13** (16%) and **6p21** tumors (3%) express high levels of either cyclin D1 or cyclin D3 as a result of an Ig translocation; **D1** tumors (34%) ectopically express low to moderate levels of cyclin D1 despite the absence of a t(11;14) translocation; **D1+D2** (6%) in addition express cyclin D2. D2 tumors (17%), which are a mixture of hyperdiploid and non-hyperdiploid tumors that do not fall into one of the other groups, express increased cyclin **D2** compared to normal PC. Tumors in group **None** (2%) do not have increased expression of a D-type cyclin compared to normal bone marrow PC. **4p16** tumors (15%) express high levels of cyclin D2, and also MMSET (and FGFR3 in approximately 70%) as a result of a t(4;14) translocation; **maf** tumors (7%) express the highest levels of cyclin D2, and also high levels of either c-maf or mafB, consistent with the possibility that both maf transcription factors up-regulate the expression of cyclin D2.

Supervised hierarchical cluster analysis of gene expression profiles demonstrates
that the TC classification identifies homogeneous groups of tumors with distinctive patterns of gene expression, and by corollary, phenotype (Bergsagel PL et al. JCO 2005(23)26(Sep 10) 6333 8).

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary Translocation</th>
<th>Gene(s) at Break point</th>
<th>D Cyclin</th>
<th>Ploidy*</th>
<th>Proli feration Index</th>
<th>Bone Disease (% MRI pos)</th>
<th>Frequency (%)</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6p21</td>
<td>6p21</td>
<td>CCND3</td>
<td>D3</td>
<td>NH</td>
<td>Average</td>
<td>100</td>
<td>3</td>
<td>? Good</td>
</tr>
<tr>
<td>11q13</td>
<td>11q13</td>
<td>CCND1</td>
<td>D1</td>
<td>D=NH</td>
<td>Average</td>
<td>94</td>
<td>16</td>
<td>Good</td>
</tr>
<tr>
<td>D1</td>
<td>None</td>
<td>None</td>
<td>D1</td>
<td>H</td>
<td>Low</td>
<td>86</td>
<td>34</td>
<td>Good</td>
</tr>
<tr>
<td>D1+</td>
<td>None</td>
<td>None</td>
<td>D1+ D2</td>
<td>H</td>
<td>High</td>
<td>100</td>
<td>6</td>
<td>? Poor</td>
</tr>
<tr>
<td>D2</td>
<td>None</td>
<td>None</td>
<td>D2</td>
<td>H=NH</td>
<td>Average</td>
<td>67</td>
<td>17</td>
<td>?</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>NH</td>
<td>Average</td>
<td>100</td>
<td>2</td>
<td>? Good</td>
</tr>
<tr>
<td>4p16</td>
<td>4p16</td>
<td>FGFR3/MMSET</td>
<td>D2</td>
<td>NH&gt;H</td>
<td>Average</td>
<td>57</td>
<td>15</td>
<td>Poor</td>
</tr>
<tr>
<td>Maf</td>
<td>16q23/20q11</td>
<td>c maf/c mafB</td>
<td>D2</td>
<td>NH</td>
<td>High</td>
<td>55</td>
<td>52</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Abbreviations: D, diploid; H, hyperdiploid; NH, non-hyperdiploid

1.3.5 Surface markers

MM plasma cells can be distinguished from normal plasma cells based on the expression of several cell surface markers. Important phenotypic characteristics of plasma cells are the expression of CD138 (Syndecan-1)\(^{18}\), the strong expression of CD38, and the absence or low expression of CD45. The serum level of shed CD138 is an independent prognostic factor,\(^{19}\) probably because it is strongly associated with tumor load. MM plasma cells can also be distinguished from normal plasma cells based on the expression of CD56 and the lack of CD19 expression.\(^{20,21}\)
CD56 is a neural adhesion molecule and expressed in 70-80% cases of MM. Lack of CD56 expression has been shown to be associated with poor prognosis in MM patients treated with conventional chemotherapy. In contrast to reports of CD56 in MM treated with conventional chemotherapy, CD56 negativity was not found to confer a poor prognosis in patients treated with high-dose chemotherapy and autologous stem cell transplantation (ASCT), suggesting that intensive treatment might overcome the adverse influence of CD56 negative MM.

CD19 is a hallmark differentiation antigen of the B-cell lineage and positively regulates antigen receptor signal transduction in mature B-cells. We have previously shown that malignant plasma cells (MM cells) isolated from MM patients lack expression of CD19, while non-malignant plasma cells isolated from healthy donors do express the CD19 antigens. An intriguing observation is the existence of both CD19− and CD19+ plasma cells in some cases of MGUS.

Compared to normal plasma cells, MM plasma cells show overexpression of the CD44v9 variant isoform. The CD44v9 isoform mediates binding to the bone marrow stroma cells, is involved in the induction of IL-6 production by bone marrow stroma cells, and is associated with poor prognosis. Approximately 20% of the MM patients and 50% of plasma cell leukemia patients express the mature B-cell marker CD20. It has been suggested that expression of CD20 on MM plasma cells, reflects a more aggressive subtype. This is based on the fact that CD20 expressing MM cases have a shorter survival compared to those whose MM cells do not express CD20.

Another important MM marker is CD28. CD28 is expressed in the majority of the MM cases, but is lacking on normal plasma cells from tonsil and bone marrow. It has been demonstrated that CD28 is expressed in 19% of the MGUS patients, 41% of the MM patients, and 100% of 13 human MM cell lines. Furthermore, CD28 is expressed in 93% of the extramedullary disease, confirming the results with the MM cell lines which were all derived from the peripheral blood of MM patients with extramedullary relapse.

Recently, we and others have demonstrated that MM plasma cells lack CD27 expression, compared to normal bone marrow derived plasma cells. Analysis of a cross-sectional MM patient group revealed heterogeneous expression of CD27. Interestingly CD27 expression was significantly higher in patients who achieved a complete clinical remission compared to newly diagnosed and relapsed MM patients.
Plasma cells from MGUS patients displayed a homogeneous high CD27 expression. In contrast, CD27 was absent on nine MM human cell lines. These data suggest that loss of CD27 is associated with progression of disease. The differential expression of CD27 on MM plasma cells was confirmed by cDNA microarray analysis. Comparing the mRNA expression of more than 5,000 genes, CD27 was the second most downregulated gene in MM plasma cells compared to normal donor bone marrow plasma cells. Importantly, CD27 was differentially expressed in newly diagnosed MM patients. The expression of CD27 was lowest in the hierarchical clustered subgroup closely resembling human MM cell lines. This group was characterized by the highest prevalence of adverse prognostic factors. In contrast, CD27 expression was highest in the subgroup closely resembling normal plasma cells. In many MM patients at diagnosis we found CD27 positive as well as CD27 negative plasma cells. Allele-specific oligonucleotide real-time PCR analysis on sorted populations showed that both populations can belong to the malignant clone. It is unclear whether CD27 positive plasma cells are derived from clonal CD27 negative plasma cells or whether both populations are derived from separate (clonal) precursor populations. CD27 expression on MM plasma cells significantly correlated with CD19 expression as determined by mean fluorescence intensity analysis, suggesting that expression of CD27 is associated with plasma cell immaturity. The exact role of CD27 and loss of CD27 expression in MM remains to be determined. Strikingly, CD27 was also highly expressed in de novo plasma cell leukemia (PCL), despite its typical aggressive clinical behavior, which suggests that de novo PCL is a distinct disease entity and different from MM.

1.4 Apoptosis

1.4.1 Apoptosis in normal cells

Apoptosis is a well-organized process of pre-programmed cell death. Apoptosis is induced through two distinct pathways: (1) the extrinsic or death receptor pathway composed of tumor necrosis factor (TNF)-family receptors and ligands, and (2) the intrinsic pathway with the release of mitochondrial constituents. Once the apoptotic process is started, it eventually leads to the activation of the caspase cascade. This in turn activates the proteolytic
degradation of a variety of important proteins and leads to the destruction of DNA, resulting in the typical biochemical and morphologic changes characteristic of apoptotic cell death.\(^{35}\) (Figure 1.1)

**Figure 1.1** Three major signaling pathways that mediate apoptosis induction: 1) transcription factor nuclear factor κB (NF κB), 2) mitogen-activated protein kinase (MAPK), and 3) Janus kinase (JAK) signal transducer and activator of transcription 3 (JAK-STAT3) signal transduction. Proapoptotic therapies targeting different points of the signaling pathways are also demonstrated. ER, extracellular regulated; Erk, extra-cellular regulated kinase; Smac/Diablo, second mitochondrial activator of caspases/direct inhibitor of apoptosis protein binding protein with low PI (Yang HH, Ma MH, Vescio RA et al. J Clin.Oncol. 2003;21:4239-47).
1.4.2 Apoptosis in Multiple Myeloma
From the earliest stages of proliferation and somatic hypermutation in the lymph node germinal center, MM cells accumulate genetic lesions including translocations involving the Ig-H switch region, and gross chromosomal abnormalities leading to aneuploidy. Late in the disease, MM cells lose the requirement for the bone marrow microenvironment as they acquire additional mutations involving oncogenes such as myc, ras, and p53. (Figure 1.2)

It has been hypothesized that even during the earliest stages of disease (MGUS), genetic lesions result in an increased apoptotic burden, and the surviving cells must have developed anti-apoptotic mechanisms to counterbalance the death signals. This concept is consistent with the notion that while MM is a disease of deregulated proliferation, early in the disease the labeling index is low (<1%), and an increased survival of malignant plasma cells may be a more important factor for the initial expansion of malignant plasma cells in the bone marrow.36

Figure 1.2 Mechanisms of disease progression in the monoclonal gammopathies.1
1.4.3 Death receptor mediated apoptosis in multiple myeloma

Death receptors 4 and 5 (DR4, DR5) are expressed on MM cell lines and primary MM isolates, and can efficiently activate the extrinsic pathway after binding of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This leads to the rapid activation of caspase 8 that can either directly activate effector caspases 3, 6, or 7 or cleave Bid, leading to amplification of the apoptotic signal by recruiting the mitochondrial pathway. Regulation of TRAIL-induced cell death can also occur at the level of the bone marrow microenvironment. Osteoprotegerin released by osteoblasts and other stromal cells can act as a decoy receptor for TRAIL, thereby blocking its apoptosis-inducing activity. There are five receptors that bind TRAIL. The first two, TRAIL-R1 and TRAIL-R2, contain cytoplasmic death domains that provide pro-apoptotic signals on ligand interaction. The three additional TRAIL receptors (TRAIL-R3 or DcR1, TRAIL-R4 or DcR2, and osteoprotegerin [OPG]), termed decoy receptors, bind TRAIL but fail to provide the pro-apoptotic signal necessary to induce apoptosis. Activation of the death-domain containing TRAIL receptors presents an opportunity to exploit the extrinsic apoptotic pathway to destroy cancer cells. Purified recombinant TRAIL ligand or agonist antibodies that target TRAIL-R1 or TRAIL-R2 offer the potential to utilize the activation of the TRAIL receptors as a means of achieving high therapeutic indices for the treatment of MM patients. These agents have the potential to be used both as single agents and in combination with chemotherapy.

1.4.4 Regulation of the Mitochondrial Pathway of Apoptosis

Myeloma cells are exposed to multiple noxious stimuli with the potential to induce apoptosis, such as chromosomal instability or hypoxia, and those induced by different forms of therapy. These act through various mechanisms depending on the used agent (e.g. Dexamethasone, Melphalan, Thalidomide, Bortezomib). Resistance to apoptosis in these cases ultimately depends on the ability of the MM cells to prevent activation of the mitochondrial pathway of apoptosis. Whether noxious signals are able to activate this pathway is determined by members of the Bcl-2 family. Understanding the molecular functions of these proteins is required for the design of novel therapeutics to overcome the resistance to apoptosis. Bcl-2 family members are divided into
three functional groups; these encode one or more Bcl-2 homology domains (BH1-BH4) and act as inhibitors or inducers of the mitochondrial apoptosis pathway. Anti-apoptotic family members (Bcl-2, Bcl-xl, Mcl-1) are localized to the outer mitochondrial membrane via a hydrophobic carboxy-terminal tail, and regulate the release of apoptotic molecules from the intermembrane space. The apoptosis inducers (e.g., Bax and Bak) encode BH1, BH2, and BH3 domains, and can be induced by apoptotic signals to homo-oligomerize and form pores in the outer membrane, thus permitting efflux of apoptosis-inducing molecules including cytochrome-c, dATP, SMAC/Diablo, and AIF. The mechanism by which mitochondrial outer membrane permeabilization allows efflux of apoptogenic proteins is controversial. However, it has been shown that Bax oligomers can form pores in liposomes that permit the passage of cytochrome-c. Under normal growth conditions, Bak is tethered to the mitochondrial outer membrane, while Bax translocates to the mitochondria in response to apoptosis-induced conformational changes that unmask its carboxy-terminal hydrophobic domain. BH3-only proteins promote apoptosis by monitoring the status of cell “health” from different locations within the cell. Whether a cell undergoes programmed cell death in response to a potential apoptosis-inducing signal, depends on the interactions of the BH3-only proteins with mitochondrial-localized multidomain Bcl-2 family members. If the BH3-only proteins are sequestered by Bcl-2, Bcl-xl, and Mcl-1, apoptosis is prevented. If specific BH3-only family members are able to interact with Bax or Bak, then oligomerization is induced and cytochrome-c is released. Much has been learned about how Bcl-2 and Bcl-xl interact with Bax, Bak, and BH3-only proteins. Recent elegant experiments have led to a revised model for the regulation of the mitochondrial pathway of apoptosis by BH3-only proteins. A critical aspect of this model is based on the relative affinities of distinct BH3-only proteins for anti-apoptotic versus pro-apoptotic Bcl-2 family members, and the relative abundance of each class of protein within the cell. The regulation of the mitochondrial pathway is even more complex, as the Ca\textsuperscript{2+} content of the endoplasmic reticulum has recently been shown to determine a cell's sensitivity to apoptosis. The “mitochondrial pathway of apoptosis” may therefore be regulated by two hits: the Ca\textsuperscript{2+} flux from endoplasmic reticulum stores to the mitochondria, and the induction of mitochondrial outer membrane permeability by BH3-only proteins, both are regulated by multidomain Bcl-2 family members.
1.4.5 The Mitochondrial Pathway in MM

In MM, defects in programmed cell death pathways are frequently caused by imbalances in expression levels of the Bcl-2 family of proteins. It has become clear that every nucleated cell requires protection by at least one pro-survival Bcl-2 homologue, and that the abundance of these guardians regulates tissue homeostasis. MM cells conform to fit in this model; they express Bcl-2, Bcl-xl, and Mcl-1. Both clinical and in vitro data suggest important roles for these proteins in maintaining MM cell survival and in clinical resistance to therapy. Although aberrant switch recombination leading to the activation of multiple translocation partners plays an important role in the pathogenesis of MM, these translocations do not include Bcl-2 family members. Nevertheless, Bcl-2 is expressed in many (but not all) MM cell lines and primary clinical isolates. Bcl-xl is expressed in most cell lines and clinical isolates, and is detected more often at the time of relapse and has been shown to correlate with resistance to chemotherapy. It remains to be determined if Bcl-2, Bcl-xl, and Mcl-1 are purely overlapping in function, or also have distinct activities to promote tumor cell survival in the face of a myriad of apoptotic stimuli. Experimental approaches to ablate expression of Bcl-2, Bcl-xl, and Mcl-1 have begun to address this issue. A number of studies have correlated the induction of MM cell apoptosis with decreased expression of Mcl-1.

The critical role for Mcl-1 as a survival factor in MM has been demonstrated in vitro using antisense oligonucleotides to specifically inhibit Mcl-1 expression. Further, antisense oligonucleotides mediated inhibition of Bcl-xl or Bcl-2 did not induce apoptosis as single agents, even though expression of the molecular targets was shown to be significantly reduced. However, addition of dexamethasone to Bcl-2 antisense oligonucleotides treated cells did promote apoptosis in some cell lines. Signal transducers and activators of transcription (STAT3) was recently shown to be constitutively activated in primary MM cells, and was shown to induce the upregulation of Bcl-xl in the U266 cell line. A number of groups have determined that Mcl-1 is upregulated by IL-6, perhaps through the activity of the STAT3 pathway. However, the role of STAT3 was based on experiments using the tyrosine kinase inhibitor AG490, whose specificity and mechanism of action are not at all completely clear. An analysis of archival BM specimens indicated that while Mcl-1 expression was detected by in situ staining in all
samples, phosphorylated STAT3 was only observed in 48% of the samples.\textsuperscript{60} This supports the notion that in some primary MM cells, Mcl-1 is expressed despite the absence of STAT3 activation. Overall, the data are consistent with our current understanding of MM as a genetically heterogeneous disease, and therefore it is highly likely that the molecular mechanisms regulating Mcl-1 expression will also be heterogeneous.

1.4.6 Future Drug Development Efforts Targeting the Mitochondrial Pathway of Apoptosis

It is likely that Bcl-2, Bcl-xl, and Mcl-1 might be valid therapeutic targets in MM, and through the use of structural biology and high-throughput technologies, a number of low-molecular-weight, cell-permeable compounds will be identified that bind to the BH1-3 hydrophobic pocket of Bcl-2 or Bcl-xl and promote apoptosis by blocking the association with BH3-only proteins. Another approach might be based on the observation that drugs that inhibit mitochondrial functions, such as electron transport, often induce apoptosis and bypass Bcl-2 protective effects. Antimycin A, an inhibitor of complex III of the electron transport chain, was shown to induce apoptosis of Bcl-xl-overexpressing hepatocytes by binding to the hydrophobic groove of Bcl-xl.\textsuperscript{61} Specific inhibitors will be of great value in determining which anti-apoptotic Bcl-2 family members are most important for the survival of MM cells, and whether the functions of anti-apoptotic family members are overlapping or serve unique functions in specific tumor cells. The known genetic heterogeneity of MM suggests that responses to these agents will not be uniform, and that alone or in combination with other therapeutic agents, targeted inhibition of Bcl-2 family members should provide a profound reduction of the apoptotic threshold of tumor cells leading to improved therapeutic outcomes. The possibility of inducing apoptosis of normal cells will require that great care has to be taken in the initial evaluation of these agents. Drugs that simultaneously target all three anti-apoptotic proteins, while lethal to tumor cells, are likely to be very toxic to the host. Specific agents are therefore required.

1.5 Bone disease in Multiple Myeloma

One prominent feature in MM is the occurrence of skeletal events including bone pain, pathological fractures secondary to lytic bone lesions,
and hypercalcemia. Up to 80% of patients with MM present with bone pain, and over 70% of the patients will develop pathologic fractures during the course of their disease.
Myeloma cells grow in the bone marrow, where the micro-environment supports their growth and protects them from apoptosis. The accumulation of myeloma cells within the bone marrow is associated with increased rates of bone turnover. Histomorphometric analyses of bone biopsies from patients with MM have shown that an unbalanced bone remodeling formation was the characteristic feature of patients with osteolytic bone lesions, which on one hand increased osteoclastic resorption and on the other hand lowered bone formation.\textsuperscript{62-64}

A significant increase in both osteoclast activity and the recruitment of new osteoclasts occurs in the close vicinity of myeloma cells. This suggest that bone disease results from local production of an osteoclast activating factor (OAF) secreted by either myeloma cells or bone marrow stromal cells. Recently, two kinds of factors have been identified: NF-κB ligand / osteoprotegerin (RANKL/OPG) system and the chemokine macrophage inflammatory protein-1 (MIP-1).

\subsection{1.5.1 Receptor activation of NF-κB ligand / osteoprotegerin system}
RANKL is expressed by osteoblastic cells and binds to its receptor present on osteoclastic cells, triggering differentiation and activation signals in osteoclast precursors, thereby promoting bone resorption.\textsuperscript{65} OPG is a naturally occurring factor that antagonizes the effects of RANKL, thereby to preserve bone integrity.\textsuperscript{66} Therefore, the ratio between RANKL and OPG (RANKL/OPG) is determining to regulate osteoclast activity and bone resorption. Recent studies have shown that myeloma cells are able to induce increased RANKL expression and decrease OPG production in the bone marrow environment.\textsuperscript{67-69} Several OAFs, including IL-1β, IL-6, and TNF-α, have been reported to be overproduced by stroma in response to MM. However, the RANKL overexpression seems unrelated to these cytokines, since the addition of blocking antibodies against IL-1β, IL-6, and TNF-α in co-cultures did not prevent RANKL upregulation.\textsuperscript{67} The soluble RANKL-inducing factors involved in MM are still unidentified but may implicate IL-7, which is produced by myeloma cells.\textsuperscript{70} In addition to increased expression of RANKL, MM-infiltrated bone marrow exhibit decreased production of the natural
RANKL inhibitor OPG. Inhibition of OPG production at both transcriptional and posttranscriptional levels by myeloma cells is associated with increased expression of RANKL. Finally, the main role of RANKL/OPG axis deregulation in MM-induced osteolysis is highlighted by the high potency of RANKL inhibitors such as OPG or RANK-Fc to prevent both excessive osteoclast development and lytic bone lesion occurrence in different murine myeloma models.68,71,72

1.5.2 Chemokine macrophage inflammatory protein-1
Two different groups have recently shown that the chemokines MiP-1α and MiP-1β significantly participate in myeloma-induced bone disease. The first group found that MiP-1α is overproduced in myeloma bone marrow and the second that both MiP-1α and MiP-1β are secreted by myeloma cells.73,74 The chemokines MiP-1 belong to the RANTES (regulated upon activation normal T-cell expressed and secreted) family and act as chemoattractants and activators of monocytes. Both osteoclast precursors and stromal cells express the receptor for MiP-1α and MiP-1β. It has been demonstrated that MiP-1α as well as MiP-1β induce expression of RANKL in stromal cells and consequently enhance osteoclast formation and resorbing activity.

1.5.3 Direct interaction with osteoclasts
Several studies have shown that myeloma cells enhance osteoclast formation and osteoclast activity via the production of chemokines (i.e. RANKL and MiP-1α) produced by osteoblastic cells. Moreover, studies in mice have demonstrated the dependence of myeloma cells on osteoclast activity and have highlighted the importance of the myeloma-osteoclast loop for sustaining the disease process. Nevertheless, direct interactions between myeloma cells and osteoclasts remain unclear. Like this, the chemokine MiP-1α, in addition to acting through osteoblastic cells to enhance osteoclast activity, may also be a potent osteoclastogenic factor that acts directly on osteoclast precursors that express C-chemokine receptor 5 (CCR5) to induce late stage differentiation.73 Furthermore, the gene coding for Gas6, the ligand for the receptor tyrosine kinase Tyro-3, is overexpressed in plasma cells.75 Tyro-3 is expressed on mature osteoclasts and involved in stimulation of osteoclast mediated bone resorption.
Overproduction of its ligand by plasma cells may be the origin of strong direct interactions between myeloma cells and osteoclasts. These observations suggest that an interdependence could truly exist between myeloma cells and osteoclasts, but further data are needed to sustain this hypothesis.

1.5.4 Decreased bone formation

Histomorphometric studies and biochemical indicators of bone turnover in MM have shown that osteoclast number and function are increased in MM. The key difference in vivo between the presence and absence of lytic lesions is that osteoblasts are fewer and less active in patients with lytic lesions. In the early stages of MM bone formation is increased, reflecting the coupling of bone resorption to bone formation. However, as the disease progresses, bone formation is decreased and this leads to an uncoupling of resorption and formation and consequently rapid bone loss. This suggests that myeloma cells could stimulate osteoblastic function during the early stages of the disease, then inhibit it or even be toxic for these cells during overt expansion of the tumor. Few inhibiting interactions between osteoblasts and MM have been described so far. Recently it has been reported that the production of the potential osteoblast inhibitor Dickkopf-1 (DKK1) by myeloma cells. Actually, DKK1 can block Wnt signaling, an important signaling pathway involved in osteoblast differentiation and function. Overexpression of DKK in MM is associated with lytic bone disease. Other potential means for the interplay between osteoblasts and myeloma cells could be through homophilic binding by the neural cell adhesion molecule (NCAM)/CD56. NCAM is known to be overexpressed by MM cells mainly of kappa subtype, and correlates with the presence of lytic bone lesions. The lack of or weak expression of NCAM by MM cells delineates a subset of MM at diagnosis mainly characterized by a lambda light chain subtype, a lower osteolytic potential and a trend for malignant cells to circulate in the peripheral blood. Of note, NCAM is also strongly expressed by human osteoblasts. Thereby, NCAM-NCAM homophilic binding between MM cells and osteoblasts may induce a decrease in osteoblast function, as described for osteocalcin production. In contrast, these negative interactions between osteoblasts and MM cells lack in CD56+/NCAM negative MM.
1.5.5 Conclusion

RANKL and OPG play an essential role in osteoclast formation and activation, and various bone tumors act through this system to trigger bone resorption. The interaction of MM with stroma results in deregulation of the RANKL/OPG axis, both by increasing RANKL and decreasing OPG.

Disruption of the RANKL/OPG ratio in the bone environment increases osteoclast activity, triggers bone destruction, and promotes tumor growth. Moreover, the chemokines MIP-1 and MIP-1\(\beta\) produced by myeloma cells also enhance osteoclast activity, leading to increased bone resorption. Finally, in vivo use of osteoclast inhibitors (bisphosphonates or specific inhibitors of RANKL) halt MM-induced bone resorption and result in inhibition of myeloma growth and survival. These observations demonstrate a strong interdependence between myeloma cells and osteoclasts: myeloma cells enhance the formation of osteoclasts, whose activity or products, in turn, are essential for the survival and growth of myeloma cells. In line with this concept, a recent study has shown that IL-6 and osteopontin, highly produced by osteoclasts, play a central role in survival and growth of myeloma cells. Indeed, the use of effective osteoclast inhibitors in vivo could break down this vicious circle, by both suppressing bone resorption and decreasing tumor growth. It is tempting to speculate that interfering with bone marrow stroma may inhibit the development of myeloma especially in early or premalignant stages (MGUS) and may inhibit the development of both bone resorption and tumor burden in MM.

1.6 Therapy

After the introduction of melphalan-prednisone as palliative treatment for MM in the early 1960s, little progress was made for decades. With the melphalan-prednisone regimen, approximately 5% of patients attain a complete response (CR), defined as negative immunofixation. The median survival is about 30-36 months, and no more than 5% of patients lived longer than 10 years. In 1992 a meta-analysis was performed to compare survival after treatment with melphalan and prednisolone with that after other combination chemotherapy in patients with multiple myeloma. Overall result of this meta-analysis including 18 trials suggests that there is no difference in efficacy between melphalan-prednisolone and combination chemotherapy. However, the past decade significant progress in the treatment of multiple MM has been seen.
1.6.1 Initial therapy

1.6.1.1 Initial therapy in patients candidate for autologous stem cell transplantation (ASCT)

1.6.1.1.1 VAD regimen
Most newly diagnosed MM patients <65 years of age (or older if fit) are candidates for ASCT. Therefore initial therapy must avoid agents with cumulative myelosuppression in order to permit collection of an adequate number of stem cells. Common pre-ASCT induction regimens have included dexamethasone alone or with vincristine and doxorubicin in the so-called VAD regimen. The VAD regimen produces partial remission (PR) in about 50% of patients and complete remissions (CR) (no evidence of monoclonal protein by electrophoresis and immunofixation and <5% marrow plasma cells) in 5 to 10% of patients. Since its introduction in the 1980s, the VAD regimen has quickly become one of the most commonly used treatment for MM in preparation for stem cell transplantation.

1.6.1.1.2 Thalidomide - dexamethasone
VAD stood the test of time, and became the standard induction regimen for MM in major randomized trials. All this changed with the arrival of Thalidomide. Despite its efficacy, VAD is plagued by the need for a central venous line and because of continuous intravenous infusion of doxorubicin for 4 days or daily peripheral infusions in case of bolus infusion of doxorubicin for 4 consecutive days. There are also substantial questions about the value of vincristine and doxorubicin in the VAD regimen, 2 drugs with negligible single-agent activity in myeloma.
When Thalidomide showed promising activity in relapsed myeloma, it was quickly combined with Dexamethasone in an attempt to develop an oral alternative to the cumbersome VAD regimen. Three phase-2 trials established that the combination of Thalidomide and Dexamethasone can achieve similar or better response rates compared with VAD as initial therapy for myeloma. As a result, the use of VAD as initial therapy has declined substantially. Further, recently, in a matched case-control study of 200 patients, a significantly higher response rate with oral Thalidomide Dexamethasone therapy compared with intravenous VAD was shown (76% vs 52%).
This difference in response rate is almost identical to the difference in response rate between Thalidomide-Dexamethasone and Dexamethasone alone observed in a recent randomized trial, confirming earlier suggestions that most of VAD’s efficacy was due to dexamethasone. However, Thalidomide-Dexamethasone is not without issues either; deep vein thrombosis occurs in over 15% of patients, necessitating prophylactic anticoagulation. Further, peripheral neuropathy is a side effect of Thalidomide. Given the neurotoxicity of vincristine and its questionable activity in myeloma, it would be inappropriate to subject patients to such toxicity up front, thus potentially limiting the future use of Thalidomide and Bortezomib, both of which also have neurotoxic potential. Similarly, there is little reason to subject patients to the cumulative cardiotoxicity of doxorubicin when other alternatives are available. The higher response rate with Thalidomide-Dexamethasone adds more substance to this reasoning by demonstrating that VAD is simply less effective than Thalidomide-Dexamethasone. As a testament to this, none of the four large randomized trials in newly diagnosed MM currently ongoing in the United States use VAD as the initial regimen.

1.6.1.1.3 New agents as part of initial therapy

*Bortezomib*

The proteasome-ubiquitin pathway is a ubiquitous and essential intracellular system that degrades many labile proteins regulating cell cycle, apoptosis, transcription, cell adhesion, angiogenesis, and antigen presentation.\textsuperscript{90-92} Bortezomib (formerly PS-341) is a small molecule that is a potent and selective inhibitor of the 26S proteasome which is the primary component of the protein degradation pathway of the cell. Bortezomib inhibits proliferation and induces apoptosis of human myeloma cell lines. It also inhibits NF-κB activation, overcomes drug resistance and adds to the anti-myeloma activity of Dexamethasone in vitro.\textsuperscript{92} In a phase II study, 54 patients with refractory MM were randomized to receive 1.0 or 1.3 mg/m\textsuperscript{2} of Bortezomib twice weekly for two weeks, every 3 weeks, for a maximum of eight cycles (CREST-trial). Dexamethasone was permitted in patients with progressive or stable disease after two or four cycles, respectively. Patients who received Bortezomib at a dose of 1.3 mg/m\textsuperscript{2} had increased
CR+PR rates (50% vs 33%), prolonged median duration of response (13.7 vs 9.5 months) and median time to progression (11 vs 7 months). Thus, the dose of 1.3 mg/m², twice per week in 3-week cycles, for 8 cycles, seems to be the optimal dose for Bortezomib as single agent or in combination with Dexamethasone.

Bortezomib was evaluated in 256 patients in an open-label, non-randomized, phase II multicenter study conducted in the United States. In the primary efficacy study, 202 patients were entered who had received at least two prior therapies and were progressing on the most recent therapy (SUMMIT-trial). For the 193 patients with measurable disease, the overall response rate was 35%, with 10% having a complete or near-complete response. For responding patients, median duration’s of response and survival were 12 and 16 months, respectively. Toxicity included thrombocytopenia, fatigue and peripheral neuropathy. In this trial, Dexamethasone was added to Bortezomib in patients who had not responded to, or had progressive disease while on Bortezomib.

In a recent phase III trial with 669 patients relapsed MM, Bortezomib showed superior efficacy to high dose Dexamethasone in terms of both overall and time to relapse (APEX-trial). The combined complete and partial response rates were 38% for Bortezomib and 18% for Dexamethasone (P<0.001). The one-year survival rate was 80% among patients taking Bortezomib and 66% among patients taking Dexamethasone (P=0.003). Grade 3 and 4 adverse events were reported in 75% of patients treated with Bortezomib and 60% of those treated with Dexamethasone. The most common side effects of Bortezomib include gastrointestinal symptoms (especially diarrhea), fatigue, fever, cytopenia and peripheral neuropathy. Short-lived and moderate thrombocytopenia occurs in 30% of patients. Peripheral neuropathy, frequently painful, develops in 36% of patients especially in those with prior exposure to neurotoxic agents.

Bortezomib in combination with Dexamethasone was employed in a study of 32 consecutive patients with previously untreated MM, with a complete and near complete response of 25% and an overall response rate of 88% after 6 cycles of therapy. These promising data with upfront Bortezomib have prompted HOVON to initiate HOVON 65. This is a randomized phase III study on the effect of Bortezomib combined with Adriamycin, Dexamethasone (PAD) for induction treatment, followed by High Dose Melphalan with autologous stem cell transplantation. After transplantation patients will receive Bortezomib maintenance therapy for 2 years.
**Lenalidomide**

Lenalidomide (CC-5013) is an immuno-modulatory derivate of Thalidomide with significantly greater in vitro activity than Thalidomide. Phase I results in heavily pretreated patients with relapsed or refractory MM and results of a randomized multicenter phase II study in relapsed refractory MM showed partial response rates in about 25% of patients.\(^\text{87,98}\) The most common adverse effects were grade 3 or higher thrombocytopenia (18%), and neutropenia (28%). Importantly, adverse events commonly observed with Thalidomide such as sedation, constipation, and neuropathy, were not observed.

Many of these patients had been previously exposed to Thalidomide although true Thalidomide resistance was infrequently established. Unlike Thalidomide, Lenalidomide causes myelosuppression which, in the setting of compromised bone marrow reserve due to extensive prior cytotoxic drug exposure, may not be fully reversible.

Two large phase II studies comparing Lenalidomide plus Dexamethasone versus Dexamethasone plus placebo have been completed. Both studies were stopped by their data safety monitoring boards due to superior response rates and time to progression with Lenalidomide plus Dexamethasone. Lenalidomide is an investigational agent, approval by the FDA is pending.

In a Phase III trial for advanced myeloma comparing two different schedules of administration (50 mg x 10 doses and 25 mg x 20 doses q 28 days), high response rates were observed with the more prolonged 25 mg dose schedule. Grade >2 thrombocytopenia was linked to pretreatment platelet count <100,000/µl as a reflection of impaired of hematopoietic reserve.\(^\text{99}\)

The efficacy and safety of oral lenalidomide plus high-dose dexamethasone in patients with relapsed–refractory multiple myeloma was further investigated in two large, randomized, multicenter, double-blind, placebo-controlled studies: the North American trial, multiple myeloma (MM)-009 (n = 354) and the European/Australian trial, MM-010 trial (n = 351). In these trials, patients received either placebo or lenalidomide 25 mg/day for 3 weeks of a 4-week cycle, plus dexamethasone 40 mg/day on days 1–4, 9–12 and 17–20 every 4 weeks for four cycles and then on days 1–4 every cycle thereafter. Primary end points were time to disease progression, clinical response and safety. More than 50% of patients in both studies had received prior treatment with high-dose
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Chemotherapy and SCT, and had failed at least two conventional chemotherapy regimens. Interim analysis of these trials showed that treatment with the combination of lenalidomide and dexamethasone significantly increased response rate and time to progression in patients with multiple myeloma. The median time to progression in patients receiving combination therapy was 15 and 13.3 months in the MM-009 and MM-010 trials, respectively, and in patients receiving dexamethasone alone it was 5.1 months in both trials (p < 0.000001). The overall response rates by Blade criteria were significantly higher in patients who received lenalidomide plus dexamethasone (MM-009: 61.2%; MM-010: 58.0%) than in patients who received placebo with dexamethasone (MM-009: 22.8%; MM-010: 21.7%). Complete responses were achieved in 26.5 and 13.6% of patients, respectively, receiving combination therapy, compared with 4.1 and 4% of patients receiving dexamethasone alone. Based on these data, both studies were stopped by the independent data monitoring committees due to the superior response rates and prolonged time to progression in the lenalidomide–dexamethasone treatment groups (Dimopoulos MA et al. Haematologica 90, 160 (2005); Weber D, presented at 2005 ASCO).

1.6.1.2 Initial therapy in patients not eligible for ASCT
Chemotherapy with alkylating agents is the preferred initial treatment for overt, symptomatic MM in patients in whom hematopoietic stem cell transplantation is not feasible. Of note, peripheral stem cells are best collected before the patients is exposed to alkylating agents if hematopoietic stem cell transplantation is a possible consideration in the future. Melphalan and Prednisone (MP) has been the mainstay of treatment in the older or medically compromised patient population. The oral administration of MP produces an objective response in 50 to 60% of patients and a median survival of two to three years. Various regimens are available: for example: Melphalan 0.15 mg/kg po per day and Prednisone 20 mg three times daily for 7 days, this cycle is repeated every six weeks. Or: Melphalan 0.25 mg/kg po per day with Prednisone 20 mg three times daily for four days every four to six weeks, depending on the hematological tolerance. Of note: Melphalan must be given when the patient is fasting because food reduces its absorption. Unless the disease progresses, at least three courses of MP should be given before it is discontinued for lack of effect.
The natural course of MM is one of progression and, if the patient’s pain is alleviated and there is no evidence of progressive disease the therapeutic regimen is considered beneficial despite the failure to reach an objective response.

A small phase II study from the Italian Multiple Myeloma Study Group compared showed an overall response rate of 93% when the combination of Melphalan, Prednisone and Thalidomide was used in patients older than 65 years previously untreated. These data have been confirmed in a prospective randomized trial involving 200 newly diagnosed patients with MM. This study compared the outcome of MP with MP plus Thalidomide 100 mg daily (MPT). The interim analysis revealed that the overall response rate was 73% with MPT, compared with 48% with MP. The event-free survival at 26 months was 68% for MPT and 32% for the control arm (P<0.001), while the median overall survival had not been reached for either group. Toxicity’s were more common in the MPT group, particularly DVT (19% versus 2%), grade 3-4 infections (13% vs 2%) and grade 1-2 neurotoxicity (35% vs 5%); LMWH prophylaxis for the first 4 months has been recommended.

Interestingly, in both of the above-noted studies, response rates to MPT were similar to those seen after high dose chemotherapy followed by ASCT. However, these results, especially long-term event-free and overall survivals, need to be confirmed before this combination can be recommended outside clinical trial setting. Of note, the HOVON 49 study, which also addresses this question is still open for accrual.

Mateos et al demonstrated that the combination of bortezomib plus MP appears significantly superior to MP in a phase 1/2 trial in 60 untreated MM patients aged at least 65 years (half older than 75 years). VMP response rate was 89%, including 32% immunofixation-negative CRs, of whom half of the IF- CR patients analyzed achieved immunophenotypic remission, even in patients with poor prognostic features.

In the Intergroupe Francophone du Myelome (IFM) 95-01 trial, 488 patients aged 65 to 75 years were randomized between 4 regimens of treatment: melphalan-prednisone, dexamethasone alone, melphalan-dexamethasone, and dexamethasone-interferon alpha. Response rates at 6 months (except for complete response) were significantly higher among patients receiving melphalan-dexamethasone, and progression-free survival was significantly
better among patients receiving melphalan (P < .001, for both comparisons), but there was no difference in overall survival between the 4 treatment groups. Moreover, the morbidity associated with dexamethasone-based regimens was significantly higher than with melphalan-prednisone, especially for severe pyogenic infections in the melphalan-dexamethasone arm and hemorrhage, severe diabetes, and gastrointestinal and psychiatric complications in the dexamethasone arms. Overall, these results indicated that dexamethasone should not be routinely recommended as first-line treatment in elderly patients with MM. In the context of the IFM 95-01 trial, the standard melphalan-prednisone remained the best treatment choice when efficacy and patient comfort were both considered. These results might be useful in the context of future combinations with innovative drugs.\textsuperscript{104}

### 1.6.2 Stem Cell Transplantation’s in Multiple Myeloma

#### 1.6.2.1 High-dose melphalan autotransplant versus standard therapy

The superiority of high-dose therapy with autologous stem cell support over standard dose chemotherapy has been demonstrated in two randomized trials.\textsuperscript{105,106} The Intergrroupe Francophone du Myelome (IFM) was the first, 8 years ago, to demonstrate the superiority of high-dose therapy supported by ASCT compared to conventional chemotherapy for MM. In this randomized IFM90 trial high dose therapy significantly increased the CR rate, the EFS and the OS in patients with newly diagnosed MM up to the age of 65.\textsuperscript{105} Following this publication, the number of ASCT performed worldwide as part of front-line therapy in younger patients increased dramatically. However some concerns remained until last year, when the IFM90 results were fully confirmed by a larger trial published by the British Medical Research Council (MRC). The MRC VII trial demonstrating that MEL 200 mg/m\textsuperscript{2} after Cyclophosphamide, Vincristine, Doxorubicin and Methylprednisolone (C-VAMP) induction was superior to standard combination therapy.\textsuperscript{106} Stringently defined CR was 44% versus 8% (P<0.001); median EFS 32 months vs 20 months (P<0.001), and OS 54 months versus 42 months (P=0.04). The failure of other randomized studies to demonstrate superiority of high-dose over standard-dose therapy may relate to insufficient follow-up, randomization only of responding patients, as well as salvage transplants in the setting of post standard chemotherapy relapse.\textsuperscript{107-110} A randomized controlled trial comparing Melphalan 200 mg/m\textsuperscript{2} with
Melphalan 140 mg/m\(^2\) combined with total body irradiation (TBI) demonstrated the superiority of Melphalan 200 mg/m\(^2\).\(^{111}\) Therefore Melphalan 200 mg/m\(^2\) is currently considered to be the optimal myeloablative conditioning regimen for ASCT in MM. Another important consideration in ASCT is the timing of the ASCT: should the transplant be given upfront or at relapse? A French study intentionally offered delayed ASCT to patients treated conventionally who relapsed or demonstrated resistance in order to address this issue.\(^{112}\) That trial reported higher response rates and longer disease-free intervals and treatment-free intervals in the upfront ASCT, but there were no differences in OS. These results indicate that delayed ASCT is an acceptable strategy for some patients. However, when considering time without symptoms and treatment toxicity, this study showed an advantage for upfront ASCT.

A randomized phase III study on the effect of Thalidomide combined with Adriamycin, Dexamethasone (TAD) and high dose Melphalan in patients with MM was studied in the Netherlands in cooperation with the German Multiple Myeloma Group (GMMG). During induction therapy VAD (arm A) was compared to TAD (arm B). During maintenance therapy standard \(\alpha\)-interferon was compared to Thalidomide (50 mg) until relapse/progression. In the period from November 2001 until April 2005, 1050 patients were included. An interim analysis in March 2005 on 414 patients (enrolled before April 2004) demonstrated an overall response of 80% (with 7% CR) after TAD induction chemotherapy compared to 63% in the standard VAD arm \((P=0.001)\). After high dose Melphalan (200 mg/m\(^2\)) no significant difference was found in overall response with 74% in the TAD arm versus 75% in the standard arm after intention to treat analysis. After a follow-up of 2 year (n=406) no significant difference was found in the OS.

1.6.2.2 Tandem versus single autotransplant

The Arkansas total therapy I phase II trial, which applied tandem ASCT with MEL 200 mg/m\(^2\), effected a median EFS and OS of 43 and 68 months, respectively.\(^{113}\) At 10 years, 15% of the patients remained event-free and 33% were still alive. In an analysis of 515 consecutive, newly diagnosed as well as previously-treated patients, one-fifth remained event-free beyond 7 years.\(^{114}\) The absence of relapses suggests that ‘cure’ is possible with autotransplantation. This supports the original hypothesis that a profound reduction in tumor
mass results in prolonged disease control and, therefore, superior survival. The IFM randomized trial (IFM-94) reported superior EFS and OS with a tandem autotransplant, using Melphalan 140 mg/m² followed by Melphalan 140 mg/m² plus TBI 8 Gy, versus a single cycle with Melphalan 140 mg/m² plus TBI 8 Gy. IFM-94 reported a 7-year overall survival of 42% (58 months) with tandem autotransplants versus 21% (48 months) for a single transplant ($P=0.01$), with a median follow-up of 5 years. The EFS was 30 months with tandem autotransplants versus 25 months for a single transplant ($P=0.03$) On an intention-to-treat basis, CR or very good partial remission was achieved by 42% of patients in the single ASCT group versus 50% in the double ASCT group ($P=0.10$). The probability of 7-year EFS was 10% versus 20% ($P=0.03$) and the probability of 7-year OS was 21% versus 42%, respectively. Interestingly, patients who had more than 90% response after the first ASCT, the OS of this group did not improve by a second ASCT. However, patients with PR after the first ASCT did definitely benefit a second ASCT. Of note, the first ASCT in this study was done after conditioning with melphalan 140 mg/m². Interestingly, an Italian randomized trial (BOLOGNA 96) evaluated single versus tandem autotransplants with Melphalan 200 mg/m² conditioning before the first ASCT. The conditioning of the second ASCT consisted of Melphalan 120 mg/m² plus Busulfan 12 mg/kg. Examination of the first 220 patients enrolled between 1996 and 1999 demonstrated superior EFS (median 34 months vs 25 months, $P=0.05$). With a median follow-up of just 38 months, median OS is similar with 1 and 2 cycles of high-dose therapy (56 months vs 60 months). This study confirmed the data of the IFM-94 study that only patients near in CR benefit from a second ASCT. In the Netherlands a randomized multicenter trial compared the efficacy of intensified treatment followed by myelo-ablative therapy with intensified treatment alone in newly diagnosed MM patients. The results of the final analysis in 441 eligible patients with stage II (22%) and stage III (78%) disease was published in abstract at the ASH 2005. The median age was 55 years (range 31-65). Remission induction consisted of 3-4 cycles of VAD. 63 patients who had an HLA identical sibling were candidates for an allogeneic transplantation. After VAD, patients were randomized to receive Melphalan 140 mg/m² divided in 2 doses of 70 mg/m² (IDM) without stem cell rescue (arm A) or the same regimen followed by myelo-ablative treatment with Cyclophosphamide (120 mg/kg) and TBI with
stem cell transplantation (ASCT, arm B). Peripheral stem cells were mobilized by Cyclophosphamide (4 g/m$^2$) and G-CSF after VAD. Interferon-α-2a was given as maintenance therapy in both arms. Of 441 registered patients, 303 were eligible for randomization. The median follow-up from randomization was 56 months. 81% of patients received both cycles of IDM (79% in arm A and 83% in arm B) and 79% of patients actually received myeloablative therapy followed by ASCT in arm B. Median duration of interferon-α-2a maintenance treatment was 12 months (arm A) vs 7 months (arm B). CR rate was significantly better in arm B (28% vs 13%, $P=0.002$), while the overall response rate (PR plus CR) was not different (90% vs 86%, $P=0.23$). Median EFS from randomization was 22 months (arm B) vs 20 months (arm A) (logrank $P=0.014$). Median PFS was significantly better in patients treated with double intensification (24 vs 23 months, logrank $P=0.032$). TTP was significantly worse in arm A (median 25 vs 33 months, logrank $P=0.001$). The difference for EFS, PFS and TTP between the 2 treatment arms became only evident after 4 years of follow-up. OS was not different between both treatments (median 55 months vs 50 months, logrank $P=0.39$). Multivariate analysis showed that treatment arm A, higher age, hemoglobin >6.2 mmol/l, stage 3 and high serum LDH were significant adverse prognostic factors for EFS. Cytogenetic analysis in 151 patients was abnormal in 37% (45% del 13/13q-, 51% abnormal 1p/q, 33% del 6q, 89% complex abnormalities). Cox regression analysis showed that 1p/q was an independent unfavorable prognostic factor for OS, EFS, PFS and TTP ($P<0.001$), calculated from start VAD. Del 13/13q- was highly correlated with 1p/q abnormalities. By combining β2-microglobulin >3 mg/l with del 13/13q- and 1p/q, prognostic groups were defined with a significant impact on OS ($P<0.000002$), EFS ($P<0.0002$), PFS ($P<0.00006$) and TTP ($P<0.0000002$).

In conclusion, second intensification when added to intensified chemotherapy alone resulted in a superior EFS, PFS and TTP, but not OS. The latter difference is probably due to the high proportion of patients from the control arm who were treated with ASCT at first relapse. It is concluded that double intensive therapy leads to a higher CR rate and a longer PFS.\textsuperscript{110}

1.6.2.3 Allogeneic transplants
The largest series of patients with MM treated with allogeneic myeloablative transplant comes from the EBMT Registry, where data on 690 patients with
MM have been reported. The EBMT Registry analysis examined transplants performed on 334 patients from 1983 to 1993 and 356 patients from 1994 to 1998. Of the patients transplanted during the latter period, 133 (37%) received peripheral blood stem cells (PBSCs) rather than marrow. The most important observation was a marked reduction in transplant-related mortality (TRM) from 46% to 30% between the two time periods. The reduction in mortality was a result of fewer deaths from opportunistic infections and interstitial pneumonias. This was due, in part, to better patient selection with less prior treatment and improvements in supportive care. The improvement in results did not appear to be a result of the introduction of PBSCs. The OS after 3 years improved from 35% during the 1983-1993 periods to 56% during the 1994-1998 period.

An intergroup trial led by Southwest Oncology Group (SWOG) evaluated, in patients up to 55 with an HLA-compatible sibling donor, a primary allo-SCT using Melphalan 140 mg/m² plus TBI 12 Gy after VAD induction. The study was closed due to excessive TRM after enrolling only 38 patients. Remarkably, however, EFS and OS have stabilized beyond 5 years, whereas there have been continued relapses and disease-related deaths among patients registered on autotransplant and standard treatment arms. It is generally accepted that allo-transplantation is the only curative approach for myeloma, mainly based on minimal residual disease studies using polymerase chain reaction analysis. Although molecular remissions are rare after ASCT (7% vs 50% after allo-SCT), there are no convincing clinical data that show a higher ‘cure’ rate with allo-SCT.

Due to small patient numbers and heterogeneity of risk factors in registry data, only a few conventional transplant studies to date have been able to identify a graft versus myeloma effect. Individual case reports have documented a graft versus myeloma effect in association with graft versus host disease when immunosuppression is withdrawn. A recent survey of 25 patients at 15 centers demonstrated CR after one or more donor lymphocyte infusion (DLI) in 7 patients (28%).

Although treatments utilizing high-dose chemoradiotherapy followed by allogeneic SCT are capable of producing remissions and long-term survival for patients with MM, the TRM of 25%-50%, even in “good-risk” patients, limits the application of this approach. Furthermore, since the majority of patients who develop MM are older than 55 years and need closely HLA-matched
members to serve as donors, less than 10% of patients with MM are even eligible for allogeneic-SCT. The demonstrated efficacy of DLI in relapsed allografts suggests that the allogeneic graft versus myeloma effect is a major reason cure can be achieved. This has led to the exploration of low-intensity conditioning regimens, designed more for immunosuppression than cytoreduction, with the aim of establishing consistent donor engraftment with hematopoietic stem cells while minimizing toxicity and damage to normal host tissues. It appears, however, that substantial cytoreduction before allografting is necessary due to a limited graft versus myeloma effect. Preliminary results suggest the tandem auto/nonablative-allogeneic strategy can result in CRs in at least 50% of patients with MM. For example the Seattle experience with this tandem transplantation in 52 patients demonstrated a survival of 78% at 24 months, a overall TRM of 22% and a CR rate of 57%. Of this cohort 46% developed chronic graft versus host disease. It will be important, however, to have longer follow-up of patients transplanted with non-ablative regimens in order to document the durability of these remissions and to document the rates and severity of graft versus host disease.

1.6.3 Second line treatment
Almost all patients with MM who survive their original presentation and treatment eventually relapse. The usual approach to progressive disease has been the use of sequential regimens designed to control the disease with the best quality of life for as long as possible. The mainstay of therapy for such patients includes conventional chemotherapy and corticosteroids, with selected patients undergoing autologous hematopoietic cell transplantation. Agents such as Thalidomide, Bortezomib, and Lenalidomide have shown promising single-agent activity in relapsed MM. Fortunately, the number of options available has increased. Patients who experience a remission lasting several years after a single, or even double, ASCT, may derive benefit from another ASCT. If patients relapse more than six months after the plateau state has been reached, initial chemotherapy should be reinstituted. Most patients will respond the duration and the quality of the response are usually inferior to those of the initial response, and become progressively shorter with each successive regimen.
Patients who are initially or who become refractory to alkylating agent therapy have a low response rate to subsequent chemotherapy and a short survival. Various can be used for relapsing disease, including VAD, Steroids, Thalidomide, Thalidomide combinations, Bortezomib and Arsenicum trioxide. Thalidomide, introduced for the treatment of multiple myeloma in 1998, was the first new agent with reproducible activity in end-stage myeloma since the introduction of alkylating agents and corticosteroids. Thalidomide exerts its anti-myeloma effect through multiple mechanisms including anti-angiogenesis, immunomodulation and induction of apoptosis in tumor cells, as well as its effect on the tumor microenvironment. Although Thalidomide was initially used to treat MM because of its known anti-angiogenetic effects, the mechanism of its anti-MM activity is unclear. Thalidomide effects partial responses in one third of patients with advanced and refractory disease, the majority of whom had received prior autotransplants. Although not influencing initial response frequency, EFS and OS are poor in the presence of cytogenetic abnormalities. Some attained their best response ever on thalidomide. Remarkably, although the median EFS was only 9 months, 28% of patients with normal metaphase cytogenetics remained event-free at 4 years.

In relapsed refractory disease, the combination of Thalidomide plus Dexamethasone yields responses in approximately 50% of patients. The addition of alkylating agents, such as melphalan or cyclophosphamide, to Thalidomide plus Dexamethasone increases the response rate to 75 to 80%. We have used the combination of thalidomide with oral cyclophosphamide (see chapter 4).

Adding Thalidomide to Bortezomib and Dexamethasone for relapsed and refractory disease induced PR in over 55% of patients, of whom 16% achieved a CR or nCR. Median OS and EFS was 22 and 9 months respectively. Unfortunately, abnormal cytogenetics and prior Thalidomide, while not affecting response frequency with this combination therapy, were associated with inferior OS and EFS.

1.7 Supportive care

1.7.1 Recombinant Human Erythropoietin in Multiple Myeloma

In 1990 Ludwig et al published a report on recombinant human erythropoietin
Introduction

(rHuEpo) treatment of 13 anemic patients, some with advanced myeloma. The treatment dose of rHuEpo was 150 U/kg body weight by subcutaneous injection three times per week. The median baseline hemoglobin was 10.2 g/dl, and 11 (85%) of the patients responded to treatment with a hemoglobin rise of >2.0g/dl. The time to response ranged from 3-20 weeks with a median of 5 weeks.

As a result of this initial study, several randomized trials were conducted to establish the effectiveness of rHuEpo in anemic patients with MM. Response was defined as an increase in hemoglobin of >2.0 g/dl above baseline independently of blood transfusion. This was achieved in 58-85% of patients in the treatment arms and in 7-24% in the placebo arms. These differences were statistically significant. The proportion of patients requiring transfusion decreased by approximately 50% in the treatment compared to the placebo arms. Two of these studies randomized patients to different doses of rHuEpo and were able to demonstrate that a starting dose of, or equivalent to, 150 U/kg subcutaneously 3 times per week produced a superior response to a lower starting dose. Doubling the dose to 300 U/kg 3 times per week in non-responders after 4 weeks produces a response in a further quarter of patients. All of the studies confirmed that rHuEpo is safe with a side effect profile similar to that found in the placebo treated arms.

1.7.2 Bisphosphonates

Bisphosphonates, which are potent inhibitors of bone resorption, are widely used in MM-associated hypercalcemia. Placebo-controlled studies, generally including patients with stage III MM, have shown that bisphosphonates, mainly clodronate, pamidronate, and zoledronate, contribute to the long-term control of bone disease. Bisphosphonates reduced the incidence of skeletal events and prevented hypercalcemia. They also alleviated bone pain and improved the patient’s quality of life. But they neither induced bone lesion healing nor improved the survival of patients, presumably because they are initiation at advanced stage of disease.

Interestingly, it has been shown recently that both pamidronate and zoledronate stimulate OPG production by primary human osteoblasts. These observations strongly argue in favor of for the early use of bisphosphonates in MM, to prevent bone disease and decrease tumor growth slow down tumor development.
Chapter 1

The introduction of zoledronic acid, a new-generation bisphosphonate, has greatly extended the use of bisphosphonates in the treatment of patients with bone metastases. On the basis of results from three large, randomized, phase III clinical trials enrolling more than 3,000 patients, zoledronic acid (4 mg via 15 minute infusion) was approved in the United States for the treatment of patients with documented bone metastases from solid tumors in conjunction with standard antineoplastic therapy and patients with multiple myeloma. Zoledronic acid is also approved in Europe for the prevention of skeletal-related events in patients with advanced malignancies involving bone. Bisphosphonate therapy is generally well tolerated but can be associated with increases in serum creatinine.\textsuperscript{136} Also osteonecrosis of the jaw (ONJ) has been described. A retrospective review of 90 MM patients who had dental assessments, included 22 patients with ONJ. ONJ appears to be time-dependent with higher risk after long-term use of bisphosphonates in older MM patients often after dental extractions. No satisfactory therapy is currently available. Trials addressing the benefits/risks of continuing bisphosphonate therapy are needed.\textsuperscript{137} The median number of treatment cycles and time of exposure to bisphosphonates were 35 infusions and 39.3 months for patients with ONJ compared with 15 infusions ($P<0.001$) and 19 months ($P=0.001$), respectively, for patients with no ONJ.\textsuperscript{138}

1.8 Conclusion

The treatment of MM has undergone many changes over the past decade. Intensive treatment with autologous stem cell support has improved the clinical outcome significantly in younger patients. Reduced intensity conditioning regimens have lowered the high treatment-related mortality of myeloablative allogeneic transplantation. New effective anti-myeloma drugs such as Bortezomib and Thalidomide analogues have become available. Therapy guidelines has been developed based on recent phase II and III studies. These include upfront induction therapy followed by autologous transplantation for patients aged up to 65 years and oral Melphalan-Prednisone treatment for patients with several comorbidities and patients over the age of 65 years. Some discussion can be made about the age of 65 as eligibility criteria. Probably the frailty of a patient might be a better selection criteria. Patients under the age of 66 with an HLA-identical donor are candidates for non-myeloablative stem cell transplantation following autologous stem cell transplantation.
For second line treatment, Thalidomide combined with Dexamethasone or combinations of corticosteroids and oral, rather than intravenous, Cyclophosphamide are often used as salvage therapy. A choice which might be depended on the agents used in first line. Younger patients who have relapse after first transplant responding to second-line treatment are candidates for a second transplantation. So after induction therapy in first line, stem cell mobilization must be done for at least two stem cell transplantations. Bortezomib is indicated for those patients refractory to the previous two lines of treatment. All patients should receive long-term bisphosphonates.

1.9 Aim of the thesis
MM is a heterogeneous malignancy. At present, metaphase cytogenetic analysis provides the best tool to discriminate good risk (stroma dependent) and poor risk (stroma independent) myeloma. The therapeutic challenge in good risk MM is to improve outcome without jeopardizing the accomplishments of high-dose Melphalan therapy. As more than 40% of such patients will be alive after more than 10 years, such patients should not be subjected to yet unproven, high risk procedures, such as non-myeloablative allo-SCT. Well-designed translational research should help to determine whether these MM and microenvironment targeting agents can sustain durable responses by interrupting the survival and growth signals provided to MM cells by the microenvironment. In MM, characterized biologically by stroma-independence and reflected by the presence of metaphase cytogenetic abnormalities, rapid post-transplant relapse probably results from less profound subclinical tumor cytoreduction because of genetically acquired drug resistance and more rapid re-growth, possibly facilitated by a hyperactive stroma. DNA microarray studies of highly purified CD138+ myeloma cells and of whole BM biopsy samples are in progress to investigate this question. Based on different prognostic factors like β2-M and cytogenetic abnormalities low and high risk MM patients can be distinguished with different disease and overall survival. In the future different treatment modalities should be studies for these low and high-risk MM patients. However novel strategies like Bortezomib did not influence survival for high risk MM patients so far. The specific aims of this thesis are to explore new treatment options for MM patients.
In **chapter 1** we introduce the different treatment modalities in relation to the biology of MM. The treatment of MM during the last three decades has consisted predominantly of alkylating agents. Long-term survivors are unusual with this treatment modality and the median OS duration is 24-36 months. Further increase in dose intensity can be obtained by performing ASCT which can be facilitated by the use of peripheral blood stem cells isolated following Cyclophosphamide treatment and granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage-CSF (GM-CSF).

In **chapter 2** we have analyzed the effects of VAD-EDAP courses followed by high-dose Melphalan and ASCT in MM patients. The VAD and EDAP regimen was chosen, since this chemotherapy regimen lacks cross-resistance. Further two doses of G-CSF, after high-dose Cyclophosphamide, were studied for peripheral stem cell mobilization. This was done to study whether a lower dose (and consequently lower cost) of G-CSF might have the same mobilization potential compared to higher dose. It is not excluded that a lower dose of G-CSF might give similar results which can further reduce the costs related to the leukapheresis procedure.

In **chapter 3** we have analyzed the results of intensive chemotherapy followed by high-dose Melphalan and ASCT support in three PCL patients and compared the results with the literature. These data suggest that intensive chemotherapy is most relevant for obtaining long-term survival.

In **chapter 4** we have analyzed the efficacy and toxicity of the combined use of Thalidomide and Cyclophosphamide as salvage therapy for patients with refractory or relapsed MM. We questioned whether lower doses of Thalidomide in combination with daily dose of Cyclophosphamide might be an effective regimen with fewer side effects. Low-doses of continuous Cyclophosphamide, also called metronomic scheduling, minimizes toxic side effects and eliminates the obligatory rest periods between cycles, resulting in a continuous suppression of the malignant clone.

In **chapter 5** Bcl-xl expression has been studied in bone marrow biopsies of MM patients before start of treatment and at time of relapse. The heterogeneity in MM patients might be reflected by different expression of pro-apoptotic and anti-apoptotic proteins. Bcl-xl is one of the anti-apoptotic proteins.

We aimed to identify different subgroups of MM by studying the expression of Bcl-xl. Characterization of subgroups based on the expression of Bcl-xl might
be of importance to define MM patients with poor survival, and, consequently, should result in optimal therapy choice. Multivariate analysis was performed between Bcl-xl expression, microvessel density, CRP, β2-M and clinical outcome.

In chapters 6 and 7 the expression of CD27 on malignant plasma cells (CD138⁺ CD38⁺) has been analyzed in a cross-sectional study of normal, MGUS and MM bone marrow samples. CD27 is involved in B-cell differentiation and expressed by a subset of B-cells and by the majority of peripheral T-cells. CD27 is involved in B-cell differentiation. We questioned whether CD27 is aberrantly expressed on malignant plasma cells and might have impact on clinical outcome. We have studied the pattern and prognostic value of CD27 expression in MM.

References
See page 127-148