Asymptomatic Multiple Myeloma – Molecular Background of Progression, Evolution, and Prognosis

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Humanbiologie
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von
Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose
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(Direktor: Univ.-Prof. Prof. h.c. Dr. med. Dr. med. vet. Dr. h.c. Reinhard Schnettler)

Gutachter: Univ.-Prof. Prof. h.c. Dr. med. Dr. med. vet. Dr. h.c. Reinhard Schnettler

Gutachter: Univ.-Prof. Dr. rer. nat. Andre Menke

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Alle offenkundige und heimliche Feindschaft, von Ost und West, von jenseits der See, haben wir bisher ertragen im Bewusstsein unserer Verantwortung und Kraft.

Friedrich Wilhelm Viktor Albert von Preußen, Deutscher Kaiser und König von Preußen,
Berlin, 6. August 1914

For Alfred and Ursula Kossert, all refugees from Prussia and Bohemia, and their children

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1. Introduction

This section comprises five parts; a general introduction into multiple myeloma, and overviews regarding pathogenetic background, treatment principles, as well as determination of disease progression and risk. It closes with aims, work program and methods used in this dissertation within the Sonderforschungsbereich / Transregio TRR79.

1.1 Multiple myeloma - general introduction

Multiple myeloma is characterized by accumulation of malignant plasma cells in the bone marrow, causing clinical signs and symptoms related to bone disease (including hypercalcemia), production of monoclonal protein (renal impairment), and displacement of normal hematopoiesis (anemia, proneness to infection). Before progression to these end organ damages (mnemoniced CRAB-criteria)[92], the disease is termed "asymptomatic" myeloma (AMM). Traditionally synonymously used with "smoldering" myeloma[112,116], the latter designation is now restricted to asymptomatic patients without imminent risk of progression[169]. Asymptomatic myeloma evolves in all patients from a condition termed *monoclonal gammopathy of unknown significance* (MGUS)[117]. The two disease stages are delineated solely by surrogates of tumor mass, i.e. serum monoclonal protein ≥30g/l or urinary monoclonal protein ≥0.5g/die and/or bone marrow plasma cell infiltration of ≥10%[169]. Progression and evolvement of asymptomatic myeloma are thought to be due to an ongoing genetic instability and *de novo* appearance of genetic alterations[147,158] with aberrant plasma cells in monoclonal gammopathy being as of unknown significance regarding their malignant properties[111,113]. Asymptomatic myeloma evolves from MGUS with about 5% and progresses to symptomatic myeloma with 50% probability in 5 years[113,116] with a wide heterogeneity in terms of time to progression for an individual patient – from within months to never in lifetime[112,115]. During the last decade, a widely accepted indication for systemic treatment was only seen once a myeloma patient became symptomatic[92,169]. This was based on lack of prolonging overall survival by earlier treatment attempts, and of biomarkers discerning "high probability" of developing end organ damage[82]. In both regards, the situation has changed: Treatment of asymptomatic myeloma patients has recently shown to prolong progression-free and overall survival[135]. From several biomarkers identifying "imminent" progression[48,79,96,116,153,162,167], the International Myeloma Working Group (IMWG) currently considers clonal bone marrow plasma cell infiltration ≥60%, free light chain (FLC) ratio ≥100, and >1 focal lesion in magnetic resonance imaging (MRI) as sufficiently validated to recommend systemic treatment to prevent end organ damage[169].

1.2 Multiple myeloma - pathogenetic background

1.2.1 Genetic alterations*

BASIC GENETIC ALTERATIONS LEADING TO MYELOMA - HPYERDIPLOIDY AND IGH-TRANSLOCATIONS. Two principal pathways target plasma cell precursors during their maturation and are seen as primary events in multiple myeloma pathogenesis: hyperdiploidy and translocations involving the immunoglobulin heavy chain (IgH) locus (Figure 1.1)[101].

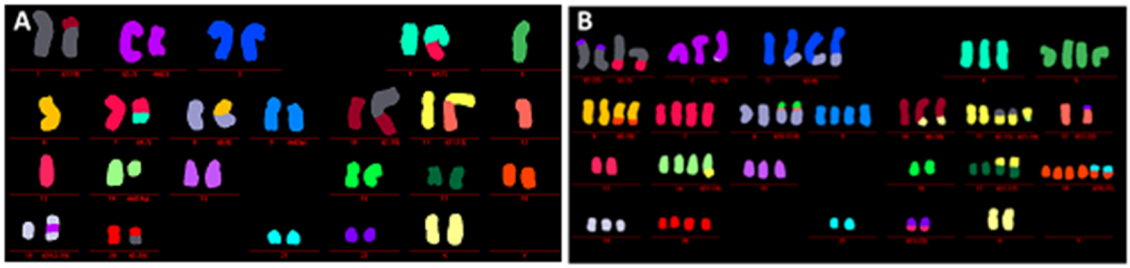


Figure 1.1. Multiple myeloma cells harbor a high median number of chromosomal aberrations and show a high inter-patient variation. Metaphase multicolor-fluorescence in situ hybridization. **A.** Non-hyperdiploid karyotype with several structural (translocations t(1;10), t(2;2), t(4;7), t(6;8), t(11;12), t(19;2;19), t(1;20)), and numerical (deletion of chromosomes or chromosomal regions 5, 13, and 14q, respectively) aberrations. **B.** Hyperdiploid karyotype with multiple gains, characteristically of odd numbered chromosomes, and additional structural aberrations, including recurrent (t(11;14)), and non-recurrent translocations, e.g. t(11;17) and t(1;11). (Figure reference: Prof. Dr. sc. hum. Anna Jauch and Priv-Doz. Dr. med. Dipl.-Phys. Dirk Hose).

Hyperdiploidy represents the gain of odd-numbered chromosomes, most frequently 3, 5, 7, 9, 11, 15, 19, and 21[30]. In hyperdiploid patients, frequently low aberrant expression of *cyclin D1* (*CCND1*), especially in case of a gain of 11q13[83], and overexpression of *CCND2* can be found[101].

The five main *translocations* in multiple myeloma comprise the translocations t(11;14) in 15%, t(6;14) in 2%, t(4;14) in 10-15%, t(14;16) in 2%, and t(14;20) in 2% of myeloma patients, leading to a direct aberrant or overexpression of *CCND1*, *CCND3*, *FGFR3* and *MMSET*, as well as *MAF*, and *MAFB*, respectively[39,152,181,193]. The latter patients likewise show a *CCND2*-overexpression.

UNIFYING PROPERTY - EXPRESSION OF D-TYPE CYCLINS. From the above said and previous work especially by Bergsagel *et al*., a dysregulation of D-type cyclins is seen as unifying property and event in multiple myeloma[20]. The three related proteins cyclin D1, D2, and D3 connect mitogenic and oncogenic pathways with the core cell cycle machinery as ultimate recipient of the oncogenic signals[195]. Normal bone marrow plasma cells express *CCND2* and (arguably) *CCND3* at

* The main part of this, the following and subsection 4.2.2, have been accepted as invited publication for the education session of the 20th European Hematology Association Meeting, Vienna, Austria, 2015, entitled “Clonal architecture of multiple myeloma”, authored by Dr. med. Anja Seckinger and Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose[192].

a low level. Malignant plasma cells aberrantly (i.e. not present in normal plasma cells) express *CCND1*, or overexpress either *CCND2* or *CCND3*. Rarely, both *CCND1* and CCND2, or *CCND3* are expressed[20,101]. These patterns are explained by the underlying genetic alteration. The t(11;14) and t(6;14) places *CCND1* at 11q13 and *CCND3* at 6p21 under the control of the IgH-enhancer, directly leading to a high expression of either *CCND1* or *CCND3*, respectively. In contrast, *CCND2* is almost never overexpressed due to translocations involving the *CCND2* locus at 12p13, or copy number alterations; *CCND2* expression is indirect.

WHEN DO HYPERDIPLOIDY OR TRANSLOCATIONS HAPPEN? Malignant plasma cells resemble terminally differentiated bone marrow plasma cells and, in an individual patient, have undergone the same V(D)J rearrangement and somatic hypermutation[12,209], producing the same monoclonal protein or parts thereof; they are clonal to this regards. Thus, either myeloma cells originate from a bone marrow plasma cell that (re-)acquired the ability to proliferate, or the transformation occurred earlier, when precursors still proliferate, and did not interfere with the maturation to terminally differentiated plasma cells.

For **hyperdiploid** myeloma, it is not known when (and how) the immortalization takes place. **Translocations** originate from the aberrant rejoining of DNA double strand breaks occurring at distinct sites in the genome[173]. Translocations can arise during different time points of the generation of terminally differentiated plasma cells by five mechanisms: 1) aberrant class switch recombination (CSR), 2) aberrant V(D)J rearrangement, 3) homologous recombination, 4) somatic hypermutation, or 5) receptor-revision rearrangement[209]. Investigating 61 samples with IgH-translocation, Walker *et al*. found two thirds showing a breakpoint within the switch regions upstream of the IgH constant genes being generated via CSR in mature B-cells[209]. While in 14 samples with a t(4;14) and five samples with a t(6;14), all were generated through a CSR-mediated mechanism, the frequency of CSR-generated translocations in samples with a t(14;16), or t(11;14), respectively, was 50% only. In six of 29 samples with a t(11;14), and one of four with a t(14;20), the generation of the translocations was mediated via a D_H-J_H rearrangement[209]. The latter occur as early as at the pro-B-cell stage in the bone marrow indicating that at least a subset of translocations in myeloma seems to appear in pre-germinal center cells[209].

FURTHER CHROMOSOMAL ABERRATIONS. As discussed above, IgH-translocations and hyperdiploidy are seen as primary events. A high number of other recurrent aberrations is present[9,85-87,151,152,187,189,207], here exemplified by the most well described deletions of 13q14 (46%), and 17p13 (10%), as well as gains of 1q21 (36%)[151]. All three aberrations have been associated with adverse survival in symptomatic myeloma patients[11,78,103,151,152]. It has however proven difficult to *conclusively* identify genes targeted by these aberrations. Despite candidates,

e.g. on 1q (*CKS1B*, *ANP32E*, *BCL9*, *PDZK1*)[224], 13q14 (*RB*, *DIS3*)[10,32,53,123], and 17p13 (*TP53*)[26,37], regularly the whole chromosomal region is affected. At the same time, indicated chromosomal aberrations very rarely appear as single aberrations[10,27,39,58,152], and the number of adverse aberrations simultaneously present, rather than the individual aberration, has been described to transmit into adverse progression-free and overall survival[27]. Therefore, as for hyperdiploidy, the question is imminent whether it is not a more subtle impact of copy number change with consecutive deregulation of a number of genes in- and outside the region of focus.

SAME AND DIFFERENT PROGNOSTIC IMPACT OF CHROMOSOMAL ABERRATIONS IN ASYMPTOMATIC AND SYMPTOMATIC MYELOMA. We and others have shown that the chromosomal aberrations gain of 1q21, deletion of 17p13, translocation t(4;14), and hyperdiploidy are significantly associated with shorter time to progression from asymptomatic towards therapy-requiring myeloma[153,168]. The first three aberrations are likewise adverse prognostic factors in symptomatic patients regarding progression-free and overall survival[11,151,152]. Hyperdiploidy is different - an *adverse* prognostic factor in asymptomatic myeloma[153], a *positive* predictive factor in symptomatic (treated) patients[107,152]. This implies first that part of the predictive impact of chromosomal aberrations is due to innate and treatment independent properties of myeloma cells (a prognostic factor in strict sense), and part only plays out in interaction with a specific treatment (as e.g. the case for hyperdiploid patients in which the prognostic impact is turned by treatment, i.e. a predictive factor)[151], and secondly that different aberration patterns can lead to the same phenotype, in this case, faster progression. In line with this argumentation, we previously found gains of 1q21 (and deletion of 13q14) to be associated with a higher accumulation (proliferation) rate[87].

DIFFERENT MOLECULAR ENTITIES DEFINED BY GENE EXPRESSION PROFILING. Several attempts have been made to group the plethora of alterations in defined gene expression based subentities[20,28,61,84,194]. John Shaughnessy and colleagues first classified myeloma samples according to their similarity to either plasma cells from individuals with MGUS or myeloma cell lines[222]. The “molecular classification” of the same group is based on unsupervised clustering and prediction of clustered groups[223], whereas the TC-classification by Bergsagel *et al*. is centered on IgH-translocations and CCND-expression[20]. Gene expression profiling also allows grouping of patients in terms of overall survival, i.e. the delineation of 10-25% of “high-risk” patients with very prognosis [43,87,106,136,170,194,219] (see section 1.4.1).

NO UNIFYING MUTATION IN MYELOMA. Whole-exome and whole-genome sequencing revealed 20 to 40 non-synonymous variants per myeloma cell[32,53,123], more than in other hematological malignancies as hairy cell leukemia (n=5)[201] or acute myeloid leukemia (n=8)[121],

but considerably fewer than in solid tumors (e.g. n=540 in non-small-cell lung cancer)[120]. There is **no unifying** mutation in myeloma contrary to other hematologic malignancies, where a common mutation is thought to be the primary driver, e.g. in hairy cell leukemia, the BRAF V600E in all patient samples[201], and MYD88 L265P in 91% of patients with Waldenströms macroglobulinemia[203]. In myeloma, the most frequent mutations are *NRAS* (23%), *KRAS* (26%), *BRAF* mutations (4%), all three in ERK-pathway, *FAM46C* (13%), and *TP53* (8%)[32,53,122]. Other mutations include those on chromosome 13, *DIS3* (10% mutated)[32,53,123,208].

1.2.2 Heterogeneity

INTER-PATIENT HETEROGENEITY has been described above without explicit use of the term: different genetic alterations lead to the same phenotype of plasma cell dyscrasia(s), and those that can do so appear (in different patients). Multiple myeloma is *multiple myelomas* with the unifying genetic feature of leading to an accumulation of terminally differentiated plasma cells.

Besides different individual genetic background related to the two general patterns hyperdiploidy and IgH-translocations and additional aberrations on DNA (chromosomal aberrations; single nucleotide variants, SNV), and RNA-level (changes in gene expression, different gene expression-based molecular entities), i.e. *inter-patient heterogeneity*, heterogeneity also exists within an individual patient: **INTRA-PATIENT HETEROGENEITY**. This can be present in terms of i) a heterogeneity *in loco aspiratio* at presentation, i.e. at the site of clinical bone marrow aspiration, ii) in terms of a *spatial* heterogeneity, e.g. different patterns of genetic alterations in focal lesions vs. random aspirates, and iii) *temporal* heterogeneity, i.e. change of the clonal composition / emergence of new subclones over time, especially evidenced under treatment (discussed in detail in section 4.2.2).

HETEROGENEITY OF THE BONE MARROW MICROENVIRONMENT. Besides changes in the malignant plasma cell population, the corresponding “myelomatous” bone marrow is altered due to factors aberrantly expressed by myeloma cells (e.g. Dickkopf-1[202]), those that are already expressed by normal plasma cells but present in higher abundance due to the accumulation of myeloma cells (e.g. bone morphogenic protein 6 or vascular endothelial growth factor[85,187]), and such expressed by a variety of cells of the (changing) bone marrow microenvironment (Figure 1.2)[16,19-21]. Driven by myeloma cell accumulation, the microenvironment changes over time between early and symptomatic myeloma[131,132,187,189]. This transformation process corresponds with the emanation of bone defects and increased angiogenesis[101]. Presence of different infiltration patterns and bone manifestations, e.g. osteolytic lesions vs. a diffuse infiltration, evidences heterogeneity of the bone marrow microenvironment.

1.2.3 Bone disease

As detailed in section 1.2.2, myeloma cells carry a high number of chromosomal aberrations and alterations of gene expression leading to myeloma cell accumulation, and impact on the bone marrow microenvironment. At the same time, normal plasma cells and myeloma cells of almost all patients depend for survival on interactions with the bone marrow microenvironment; they are thus in bidirectional interaction[101,191].

SPATIAL PATTERNS OF MYELOMA CELL GROWTH AND BONE DEFECTS. Myeloma cells are regularly present all over the hematopoietically active bone marrow, but their growth pattern is different[18]: i) A diffuse distribution in different densities from low to almost complete, ii) a spherical tight accumulation, a focal lesion, and iii) combinations thereof. Osteolytic bone lesions ("bone holes") correspond to focal lesions, but not every focal lesion needs (yet) to present on bone level as osteolytic lesion[211]. It is currently not known what mediates these different growth patterns, but it is tempting to suggest a spatial heterogeneity of the myeloma cell population on RNA- or DNA-level, or both (see section 4.2.2). Alternatively, these patterns could be mediated by different growth kinetics of myeloma cells; e.g. a faster accumulation leading to different growth patterns. Indeed, some patterns, especially the appearance of focal lesions, are associated with faster progression in patients with monoclonal gammopathy or asymptomatic myeloma[79,81,97]. The same holds true if focal lesions persist after initial successful systemic treatment[80]. But also diffuse infiltration visible in whole body MRI (vs. none) is adversely associated with prognosis[139].

CLINICAL RELEVANCE OF MYELOMA BONE DISEASE. Multiple myeloma is the malignant disease most frequently leading to bone lesions[114]. Approximately 80% of myeloma patients develop osteoporosis, lytic bone lesions (osteolyses) or fractures during the course of the disease[114]. Of these, 43% encounter pathological fractures, most often of vertebrae followed by those of the long bones[138,182]. In asymptomatic myeloma patients, occurrence of bone lesions is the most common cause for the initiation of treatment to avoid myeloma induced fractures[153].

Myeloma bone disease represents a threefold **therapeutic problem**: i) *Per se* because of the morbidity, mortality and the accompanying decrease of quality of life associated with resulting pathological fractures[182], ii) as indication to start treatment in otherwise asymptomatic patients (to avoid pathological fractures)[153], or in patients progressing after successful treatment only in terms of enlarging osteolytic lesions, and iii) as *survival space*

("reduit") for myeloma cells during otherwise successful chemotherapeutic treatment and subsequent source of relapse and thus potential obstacle for myeloma cure.

PATHOGENESIS OF BONE LESIONS IN MULTIPLE MYELOMA. Myeloma cells impact on bone turnover by factors increasing number and activity of osteoclasts and simultaneously decreasing number and activity of osteoblasts[63,64,66,71,177,178,202], and destruction of the three-dimensional structure in which bone turnover takes place, i.e. the bone remodeling compartment[5]. They benefit from increasing bone turnover: osteoclasts produce growth- and survival factors as *a proliferation inducing ligand* or *insulin-like growth factor* 1 or liberate these when bone matrix is degraded[101,144,199]. They also stimulate proliferation and survival of myeloma cells via direct contact, e.g. via $\alpha_4\beta_1$-Integrin[1]. The ability to interact with bone remodeling represents already a normal plasma function. Normal plasma cells express factors both stimulating bone resorption, e.g. Annexin A2, and bone formation, e.g. bone morphogenic protein 6[187,189]. Likely this represents a system of "checks and balances" (self-limitation) for the impact of plasma cells on bone turnover, comparable to the interplay of osteoprotegerin and receptor activator of NF-κB ligand being simultaneously produced in osteoblastic cells[179].

HEALING OF MYELOMA INDUCED BONE DEFECTS only appears in successfully treated myeloma patients, i.e. after removing myeloma cells from the lesion. Even then, healing is orders of magnitudes slower than the one of a fracture in normal individuals[54]. Potential reasons are i) *residual myeloma* cells in the bone defect continuing stimulation of bone resorption over bone formation, ii) *"scorched earth"* left over from pathological remodeling and destroyed bone remodeling compartments within the defect region with concomitant *lack of sufficient stimulation* of bone repair[5], iii) ongoing therapy (e.g. lenalidomide maintenance) suppressing bone anabolism[190], and iv) compounds currently used systemically in the treatment of bone defects (i.e. bisphosphonates[141]) act on inhibition of bone resorption without directly fostering bone formation.

1.2.4 Pathogenetic model

Figure 1.2 depicts the pathogenetic model at the beginning of this thesis. Figure 4.1 depicts its modification in relation to the obtained results.

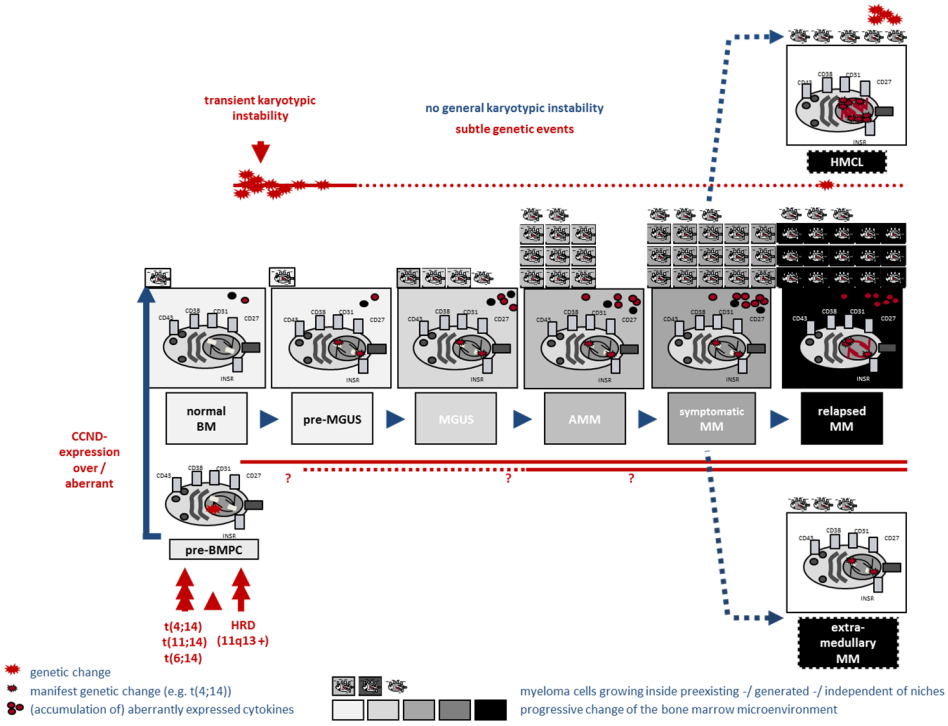


Figure 1.2. Heidelberg model of multiple myeloma pathogenesis, modified from Klein, …, Hose 2011[101]. Two principal pathways target plasma cell precursors (pre-BMPCs): translocations most often involving the IgH-locus, and a hyperdiploid pathway. Both lead to increased cyclin-D (CCND) expression: overexpression (*CCND2*) or aberrant expression (*CCND1*, *CCND3*). Karyotypic instability, assumed by others as principle driver of evolution of AMM from MGUS and subsequent progression to MM (red question marks), is in place only at this time (indicated by red stars). Targeted pre-BMPCs home to the normal plasma cell niche (indicated by a gray box). These cells already have a slightly dysregulated cell cycle ("hijacked normal" plasma cells) and the tendency to accumulate. The bone marrow microenvironment (light-gray box) is unaltered. In pre-MGUS stage, the transformation process of the bone marrow microenvironment begins slowly. Initially, pre-MGUS cells share the niche with normal plasma cells. A further accumulation leads to MGUS/AMM stage without the necessity of further genetic events. The microenvironment is slowly transformed by normal bone marrow plasma cell factors (indicated by the increasingly dark grey) and aberrantly expressed factors (red dots). Aberrant expression is driven mainly by the changing bone marrow microenvironment, not accumulating genetic alterations. Malignant plasma cells populate existing plasma cell niches (light gray boxes), recruit new niches (dark gray boxes) and partially gain independence from the microenvironment (plasma cell without a box). Further accumulation of myeloma cells leads to symptomatic myeloma. The transformation of the microenvironment continues (darkening gray, increased number of (aberrantly) expressed factors) in a positive feedback loop. A further selection pressure to recruit new niches and grow independently of niches is in place. Within this model, open questions are how myeloma cells can spread over the bone marrow, what the role of genetic heterogeneity is, and how it can be explained that some MGUS or AMM-patients remain without disease progression. Human myeloma cell lines (HMCL) can be derived from therapy-requiring or relapsed myeloma, i.e. cells which already gained partial independence of the microenvironment. They are thought to represent a further step of myeloma development. The same holds true for extramedullary myeloma, appearing rarely, even in end-stage patients.

1.3 Treatment of multiple myeloma

During the last decade, an indication for systemic treatment was only seen once a patient became symptomatic[92,169]. This was based on lack of prolonging overall survival by earlier treatment attempts (see Table 1.1), and of biomarkers discerning "high probability" of developing end organ damage (see Table 1.2). In both regards, the situation has changed[135,169].

1.3.1 Symptomatic myeloma

1.3.1.1 Treatment paradigms

The basic distinction is made between patients who can or cannot be treated intensively due to their constitution and co-morbidity. Based on our and published experience, for both survival increased during the last decades[109,204] due to introduction of immunomodulatory drugs (thalidomide, lenalidomide (Revlimid®), pomalidomide (Pomalyst®)), and proteasome inhibitors (bortezomib (Velcade®), carfilzomib (Kyprolis®))[56,74,76,108,126,146,154,159,166,176,198,200,205]; for the first, additionally due to high-dose melphalan treatment and autologous stem cell transplantation.

i) Patients who cannot be treated intensively. For decades, a treatment according to the Alexanian-regimen (melphalan and prednisone, MP) had been the standard of care[115]. This changed to either an inclusion of at least one of the "*novel*" agents, i.e. in the MPT- (melphalan, prednisone, *thalidomide*), MPV- (melphalan, prednisone, *bortezomib* (Velcade))[56,184], or RMP-schedule (e.g. *lenalidomide* (Revlimid), melphalan, prednisone)[156], or, especially in the US, treatment according to the lenalidomide plus dexamethasone (Rd) regimen.

ii) Patients who can be treated intensively. Until a decade ago, standard up-front treatment for patients up to 65-70 years of age had been high-dose therapy with 200mg/m^2 melphalan and autologous stem cell transplantation[6,36] after three to four cycles of induction treatment with e.g. vincristine, adriamycin, and dexamethasone, stem cell mobilization chemotherapy with cyclophosphamide, adriamycin and dexamethasone with growth factor support, and subsequent collection of peripheral blood stem cells. From there, two treatment paradigms have evolved: 1) De-escalation of treatment using novel agents *instead of* high-dose therapy with expected response rates comparable to high-dose therapy, but lower toxicity[21,47]. 2) An intensification of treatment incorporating novel agents in high-dose therapy regimen aiming at higher rates of complete or very good partial remissions[67,75,157] to transmit into long-term remissions with the final aim of a cure in at least some patients[13]. The most intensive concept is exemplified by the "Total Therapy (TT) 3" protocol of Barlogie *et al*.[13] (Figure 1.3).

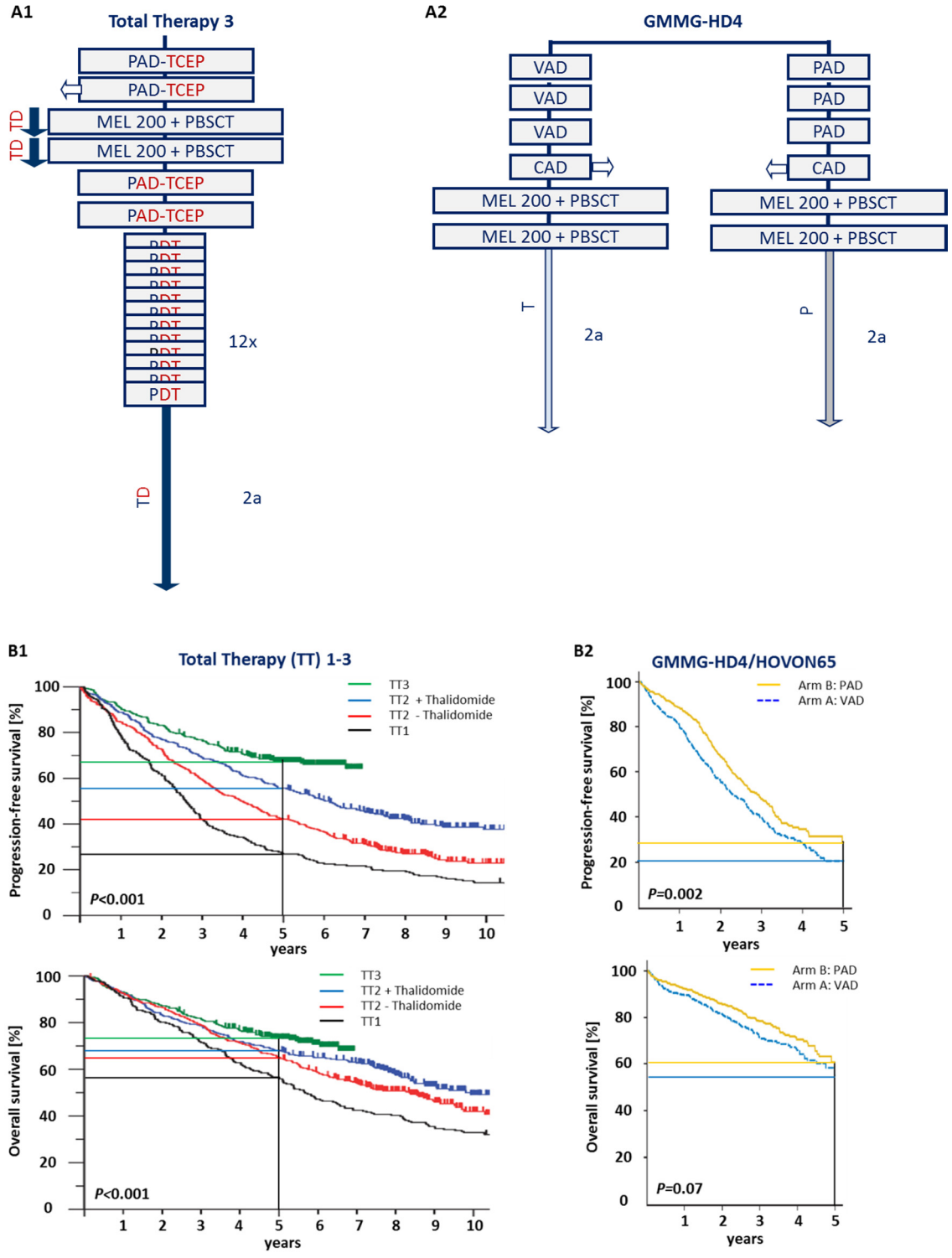


Figure 1.3. Current intensive treatment protocols and achievable survival rates. A1. Total therapy 3 (TT) protocol, University of Arkansas Medical Sciences (UAMS). **A2.** Protocol of the joint **GMMG-HD4 / HOVON-65** trial of the German speaking myeloma multicenter group (GMMG) and the Dutch HOVON-group. **B1.** Results from subsequent generations of TT-trials. Intensification of treatment and inclusion of novel agents improve survival. **B2.** Results of the GMMG-HD4 / HOVON-65 trial. Modified from Usmani *et al.*[205], and Sonneveld *et al.*[197]. VAD, vincristine, adriamycin, dexamethasone; PAD, bortezomib (PS341), adriamycin, dexamethasone; T(D), thalidomide (dexamethasone); TCEP, thalidomide, cyclophosphamide, etoposide, cisplatin; CAD, cyclophosphamide, adriamycin, dexamethasone; Mel200, melphalan 200mg/m^2.

1.3.1.2 Therapeutic questions and challenges

THERAPEUTIC QUESTIONS in myeloma treatment can be summarized under five categories: 1) To **aim** at deep and long-term remission (cure) or disease control, 2) **which** compounds to give, 3) which to **combine**, or give in subsequent lines of treatment, 4) at what stage to **begin** treatment (e.g. high-risk AMM), and 5) when to **re-start** treatment once relapse or disease progression occurred.

The **THERAPEUTIC CHALLENGES** are the following: i) Almost all patients undergo disease progression after initial "successful" treatment; thus, first novel strategies and compounds need to be integrated in myeloma treatment, and secondly treatment needs to be planned with a strategy taking into account multiple subsequent relapses[88], and being aware of the accumulated toxicity of several lines of treatment, e.g. exhaustion of bone marrow reserve. ii) No single compound is active (inducing a partial remission or better[52]) in more than a third of patients[174,175]. Whereas a plethora of novel agents is currently in clinical or preclinical testing (reviewed e.g. in Bianchi *et al*.[23]), no "magic bullet" is in sight[88]. Compound combinations are more effective, but also more toxic[104,142]. iii) The intensity of treatment is, including experience from our group, not further escalatable[13]; a maximal escalation of treatment (e.g. TT3) leads to a remission rate of above 90% and long-term progression-free survival, but nevertheless most patients ultimately relapse. It is thus not possible to add "just another" compound, but only to exchange one, ideally one being inactive in the respective patient. Given that it is currently not possible to predict response to any compound routinely used in myeloma precisely enough allowing clinical use, it is not likely that a completely individual combination ("**individualized treatment**") maximizing efficacy with minimal necessary toxicity will be achievable.

1.3.1.3 Novel strategies

PERSONALIZED TREATMENT. One novel strategy (which was the subject of my habilitation[88]) is to use an active treatment backbone leaving room for additional toxicity, adding compounds which are to a certain extent "targeted". To do so, two strategies can be thought of: a) Identify a general targetable pathway (process), maximizing the percentage of patients in which the compound is active. b) Identifying targets whose expression can be measured and which can be used to treat only patients expressing it (to a certain degree **"personalized" treatment**). Given the potential high toxicity (and eventually mortality) of an extendedly aggressive treatment, one line of thought is to limit its use to those patients with very adverse survival ("high-risk patients"; see section 1.4, **risk-adapted treatment**)[67]. The situation is further complicated as risk scores do not allow the selection of a specific treatment *per se*, and risk-

prone treatments are not necessarily effective in high-risk patients as exemplified by those treated within the intensified TT2 vs. TT3-protocol.

A further strategy introduced by our Sonderforschungsbreich / Transregio TRR79 “Werkstoffe für die Geweberegeneration im systemisch erkrankten Knochen” is **LOCAL TREATMENT** by bone substitute materials. Counter-intuitive at first in a systemic malignancy, the idea is to prevent “local” progression (e.g. one or few growing osteolytic lesions) or potential of progression (e.g. presence of >1 focal lesion) seen as indication for treatment to prevent morbidity and mortality by a fracture-to-be. This concept prerequisites the understanding of determinants and paths of progression (see section 1.5).

EARLY TREATMENT. The **clinical rationale** of treating asymptomatic myeloma patients is to prevent development of end organ damage, which needs to be balanced vs. the prevention of consequences of treatment (side effects and treatment-related mortality, see section 1.3.2, Table 1.1). **Biological rationale** and assumptions are that at earlier stages fewer myeloma cells are present harboring a lower genetic heterogeneity (intra-patient heterogeneity, see sections 1.2.2, 4.2.2), and having induced less potentially irreversible alterations in the bone marrow microenvironment (e.g. bone lesions; see sections 1.2.2, 1.2.3). In this setting, either already a less aggressive treatment (e.g. Rd[135]) might suffice to prolong overall survival, or an intensive treatment might allow long-term remission or even cure (a strategy followed by the group of Landgren *et al*.[118]). The main biological counter argument is that treatment might induce change from a benign (slowly accumulating, non-aggressive) to an aggressive subclone (see section 4.2.2). Despite the final answer needs to be given in clinical trials, knowing more about mechanisms of disease progression seems helpful for their design.

1.3.2 Asymptomatic myeloma

As already mentioned, during the last decade, a widely accepted indication for systemic treatment was only seen once a myeloma patient became symptomatic[92,169], and the standard of care outside clinical trials is still not to treat. This is due to multiple trials failing to show major benefits especially regarding overall survival despite non-neglectable side effects[2,49], summarized in Table 1.1, and lack of biomarkers discerning “high probability” of developing end organ damage. In both regards, the situation has changed, as Mateos *et al*.[135] could show within a phase III trial a benefit in progression-free and overall survival using Rd-treatment in high-risk asymptomatic myeloma patients. A different attempt is made by Landgren *et al*. in a pilot study

using an intensified triple combination regimen (carfilzomib, lenalidomide, dexamethasone) showing initial promise regarding the remission rate induced (see Table 1.1)[118].

The therapeutic questions regarding treatment of asymptomatic myeloma are thus principally the same as in symptomatic myeloma (see 1.3.1.2). An additional challenge to this end is the rate of progression of asymptomatic to symptomatic myeloma of about 50% in 5 years with some patients progressing within months, but other not at all[116]. The benefit of successful treatment, and even cure, needs thus to be balanced against the absence of development of signs or symptoms in a subfraction of patients. It is of high interest to delineate determinants and paths of progression to symptomatic myeloma (see section 1.5).

Table 1.1. Published treatment trials in patients with asymptomatic and smoldering myeloma[14,42,44,68,82,118,128,134,135,149,150,161,165,171,172,213,218]. AMM, asymptomatic multiple myeloma. SMM, smoldering multiple myeloma. PFS, progression-free survival. TTP, time to progression. OS, overall survival. NA, not available. Modified and supplemented from Dispenzieri *et al.*[49]. Successful treatment approaches are highlighted in dark blue.

Therapy	Study type	n	Time to progression	Overall survival	Reference
Initial vs. delayed melphalan + prednisone	Randomized-controlled trial	50 SMM and AMM	12 months	No difference	Hjorth *et al*., 1993
Initial vs. delayed melphalan + prednisone	Randomized-controlled trial	145 AMM	~ 12 months	No difference 64 vs. 71 months	Riccardi *et al*., 1994 and 2000
Delayed melphalan + prednisone	Observational	54 AMM	2-years PFS: 75%	Tumor-specific OS: 80% at 60 months	Peest *et al*., 1995
Pamidronate	Pilot	5 SMM, 7 AMM	2-years TTP: 25%	NA	Martin *et al*., 2002
Pamidronate vs. observation	Randomized-controlled trial	177 SMM	5-years PFS: 53% both arms; SRE: 74% vs. 39% (P=.009)	Median OS 46 vs. 48 months	Musto *et al*., 2003 D'Arena *et al*., 2011
Zoledornate vs. obervation for 1 year	Randomized-controlled trial	163 SMM	TTP: 67 vs. 59 months (P=NS) SRE: 55 vs. 78% (P=.04)	No difference	Musto *et al*., 2008
Thalidomide + pamidronate	Phase II	76 SMM	4-years EFS: 60%	4-years OS: 91%	Barlogie *et al*., 2008
Thalidomide	Phase II	19 SMM, 10 AMM	Median TTP: 35 months	86 months from treatment: 49 months	Musto *et al*., 2001 Dettweiler *et al*., 2010
Thalidomide	Phase II	28 high-risk SMM	NA	NA	Weber *et al*., 2003
Thalidomide + zoledronic acid vs. zoledronic acid	Randomized-controlled trial	68 SMM	29 vs. 14 months	6-years OS: >70%	Witzig *et al*., 2013
Interleukin-1 receptor antagonist ± dexamethasone	Phase II	47 SMM and AMM	37 months	NA	Lust *et al*., 2009
Curcumin vs. placebo	Crossover	17 SMM	NA	NA	Golombick *et al*., 2009
Lenalidomide + dexamethasone for 9 months followed by lenalidomide maintenance for 15 months vs. observation	**Randomized-controlled trial**	**119 SMM**	**2-years PFS: 92% vs. 50% (P<.001)**	**3-years OS: 93 vs. 78% (P=.04)**	**Mateos *et al*., 2013**
Carfilzomib + lenalidomide + dexamethasone for 8 cycles; patients achieving stable disease or better received maintenance therapy with lenalidomide for 2 years	**Phase II**	**12 high-risk SMM**	**No patient progressed while on study**	**All 11 patients who completed 8 cycles of combination therapy obtained nCR or better**	**Landgren *et al*., 2014**

1.4 Determination of disease progression and risk

1.4.1 Symptomatic myeloma

Depending on the treatment approach, the probability of disease progression after up-front treatment (see section 1.3) is in the range of 70% (GMMG-HD4/HOVON65-trial)[197] to 35% (TT3)[15] at 5 years, with 61% to 74% of surviving patients. The survival of an individual patient can however vary between months and over a decade (see Figure 1.3).

Two approaches are applied for risk stratification in symptomatic myeloma, using i) **clinical prognostic factors**[16,17,70], and ii) **molecular profiling**[20,28,43,84,87,106,170,194,219,223]. Regarding the latter, global gene expression profiling (**GEP**) for simultaneous investigation of (almost) all genes expressed in a sample, and interphase fluorescence in situ hybridization (**iFISH**) for the detection of recurrent genetic alterations are clinically used. Experimental techniques include next generation sequencing-based approaches as whole exome or RNA-sequencing[89]. These methods prerequisite a purification of myeloma cells from bone marrow aspirates which can be routinely performed by magnetic activated cell sorting using ferromagnetic beads coupled to antibodies against CD138, the characteristic surface antigen expressed by normal and malignant plasma cells ("CD138-purification", see section 2.2.1, Figure 2.1). Of several clinical adverse prognostic factors[4,29,110,160], most frequently used are the International Staging System (ISS) based on serum beta-2-microglobulin and serum albumin, or the former alone[35,40,41,70]. Both are prognostically independent of chromosomal aberrations or gene expression[10,151]. Though inexpensively obtainable in all patients, neither covers the variance in survival, or identifies patients at very high risk[70]. Assessment of chromosomal aberrations in CD138-purified myeloma cells by **iFISH** is possible in about 93% of therapy-requiring patients[152,153]. iFISH allows a risk stratification with presence of a translocation t(4;14) and/or deletion of 17p13 being the best documented adverse prognostic factors[10,11,31,33,60,99,143], but only a limited number of preselected chromosomal aberrations can be assessed (see section 1.2.1). **GEP** can be routinely used in approximately 80% of therapy-requiring myeloma patients[90,91,188]. GEP-based risk stratification is achieved investigating survival differences between groups representing either "molecular entities"[20,28,84,223], potential targets (e.g. Aurora kinase A[86] or insulin like growth factor 1 receptor[199]), surrogates of biological variables (e.g. proliferation[86,87,194,223]), or directly survival-associated genes[43,194]. Three GEP-based high-risk scores were described using the latter strategy from the UAMS (comprising 70 genes)[194], the Intergroupe Francophone du Myélome (IFM, 15 genes)[43], and our group (Rs-score, 19 genes)[170]. All allow delineating small groups of patients (13%, 25%, and 9%, respectively) with very adverse overall survival in three dependent datasets, including ours.

1.4.2 Asymptomatic myeloma

Within 5 years of observation, half of asymptomatic myeloma patients progress to therapy-requiring myeloma[116]. As for symptomatic myeloma, the probability for disease progression of an individual patient can vary between months to over a decade, or even never (Figure 1.4).

With this dissertation (see section 3.1), grouping prognostic factors is introduced in those being associated with i) plasma cell accumulation (not quantitatively addressed previously), ii) local (e.g. bone marrow plasma cell infiltration ≥60%[167]) or global surrogates of tumor mass (e.g. involved:uninvolved FLC ratio ≥100[119], serum M-protein ≥30g/l[116]), iii) molecular prognostic factors, e.g. chromosomal aberrations, i.e. presence of t(4;14), deletion of 17p13, gain of 1q21, or hyperdiploidy[153,168]; high-risk according to the UAMS70 gene-score[45], or combinations of i - iii), e.g. the Mayo Clinic or the PETHEMA-model[48,162]. Prognostic factors previously assessed by others and us are summarized in Table 1.2.

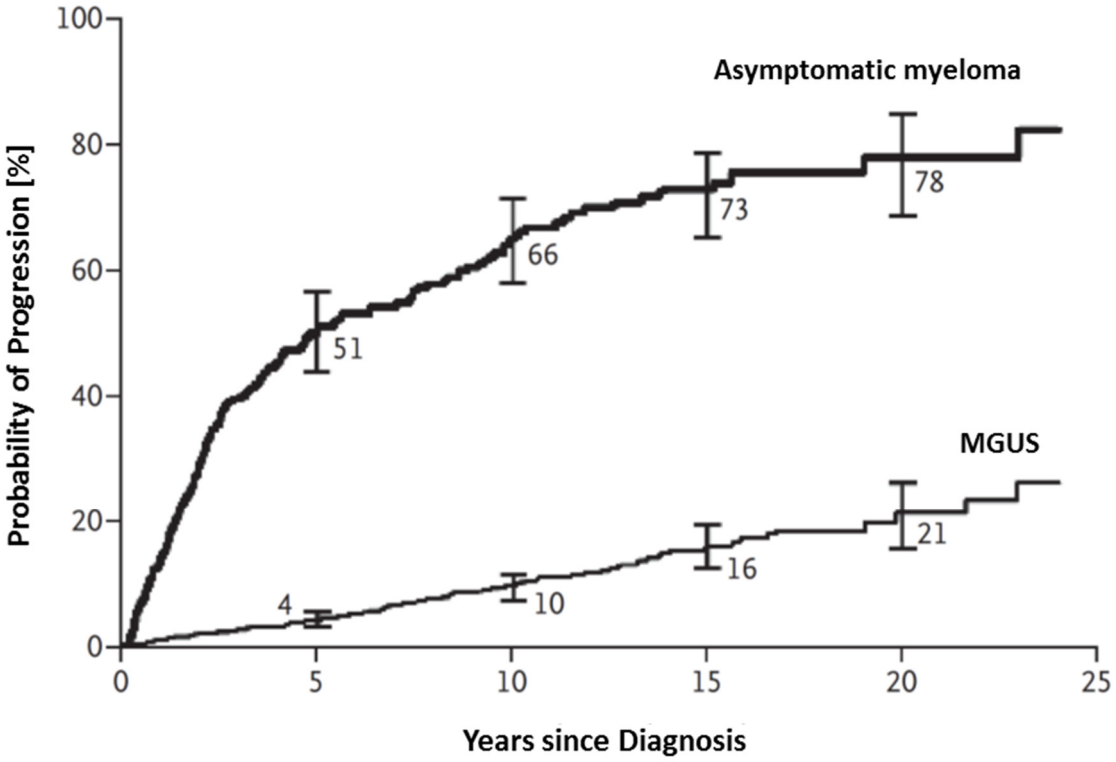


Figure 1.4. Progression of asymptomatic to therapy-requiring multiple myeloma. The overall risk of progression from asymptomatic (smoldering) to therapy-requiring myeloma or primary amyloidosis is 10% per year for the first 5 years, approximately 3% per year for the next 5 years, and 1% per year for the last 10 years, i.e. the latter being comparable to the risk of progression for patients with MGUS (bottom curve)[116]. Modified from Kyle *et al*.[116].

Table 1.2. Prognostic factors for progression of asymptomatic to therapy-requiring myeloma[22,45,46,48,55,79,96,116,119,129,139,148,153,162,164,167,168,180,214,216,217]. AR, accumulation rate. BMPC, bone marrow plasma cells. Mo, months. OS, overall survival. TTP, time to progression. (X), AR not quantitatively assessed. Modified and supplemented from Dispenzieri *et al*.[49].

Prognostic factors	Surrogate					n	Median TTP and OS	Reference
	AR	Tumor mass		Molecular				
		local	global	iFISH	GEP			
Lytic bone lesions, BMPCs >20%		X				71	TTP all: 26 mo, OS: 46 mo; TTP no risk: 39 mo, either risk: 10 mo	Wisloff *et al*., 1991
Lytic bone lesions; protein risk: M-protein > 30g/l or proteinuria > 50 mg/die. Low risk: no factor; intermediate: either protein risk characteristic; high: lytic bone lesions and/or both protein risk characteristics			X			95	TTP all: 26 mo; low risk: 61 mo, intermediate risk: 25 mo, high risk: 10 mo. OS from SMM (from treatment): low risk: 89 mo (31 mo); intermediate risk: 92 mo (31 mo); high risk: 57 mo (41 mo)	Dimopoulos *et al*., 1993
Circulating cells by labeling index (n=14)			X			57	TTP circulating: 9 mo, no circulating: 30 mo	Witzig *et al*. 1994
Hb <12b/l; BMPC >20%, M-protein >30 g/l (IgG) or >25 g/l (IgA): 0 vs. 1 vs. >1 risk factor		X	X			91	TTP all: 48 mo; 0: >50 mo, 1: 26 mo, >1: 6 mo. OS from SMM (from treatment): 0: >70 mo (33 mo), 1: 50 mo (31 mo), >1: 38 mo (32 mo)	Facon *et al*. 1995
Abnormal MRI			X			38	TTP normal MRI: 43 mo, abnormal MRI: 16 mo; variegated: 22 mo, diffuse: 16 mo; focal: 6 mo	Moulopolous *et al*. 1995
M-protein >30 g/l, IgA type, proteinuria >50 mg/die; low: 0, intermediate: 1, high: ≥2			X			101	TTP low: 95 mo, intermediate: 39 mo, high: 17 mo. OS from dexamethasone (from treatment): low: 89 mo (26 mo), intermediate: 87 mo (34 mo), high: 51 mo (32 mo)	Weber *et al*., 1997
Evolving (constant increase in serum M-protein) vs. non-evolving type (long-lasting stable M-protein)	(X)		X			53	Median TTP all: 38 mo, evolving: 16 mo, non-evolving: 47 mo. 2-years (5-years) TTP: evolving: 66% (88%); non-evolving: 12% (58%)	Rosinol *et al*., 2003
M-protein ≥30g/l, BMPC ≥10%; group A: M-protein only, group B: BMPC only, group C: both		X	X			276	3-years TTP (5-years TTP): A: 6% (15%), B: 22% (43%), C: 45% (69%)	Kyle *et al*., 2007
95% aberrant plasma cells according to FACS, immunoparesis of the uninvolved immunoglobulins; neither, either, both criteria		X	X			NA	Median TTP (5-years TTP): neither: not reached (4%), either: 73 mo (46%), both: 23 mo (72%)	Perez-Persona *et al*., 2007
M-protein ≥30g/l, BMPC ≥10%; involved FLC/uninvolved FLC ≥8; 1 high, 2 high, 3 high		X	X			273	2-years TTP (5-years TTP): 1: 12% (25%), 2: 27% (51%), 3: 52% (76%)	Dispenzieri *et al*., 2008
Whole-body MRI: low: no or 1 focal lesion, high: > 1 focal lesion			X			149	Median (2-years TTP): low: not reached (20%), high: 13 mo (70%)	Hillengass *et al*., 2010
PCLI < 1% vs. PCLI ≥ 1%			X			175	2-years TTP (5-years TTP): low: 40% (60%); high: 60% (68%)	Madan *et al*., 2010
BMPC ≥60% (n=21)		X				655	Median TTP (2-years TTP): BMPC ≥60%: 7 mo (95%)	Rajkumar *et al*., 2011
Hb ≤ 12,5 g/dl, M-protein ≥25 g/l, BMPC ≥60% (2,5% of patients)		X	X			397	10-years TTP: 45%; patients with BMPC ≥60% had a 5.6 times higher risk of progression	Rago *et al*., 2012
Involved FLC/uninvolved FLC <100, involved FLC/uninvolved FLC ≥100			X			586	Median TTP (2-years TTP; 5-years TTP): low: not reached (28%; 53%); high: 15 mo (79%; 94%)	Larsen *et al*., 2013
High: slide based > $5x10^6$/l or >5% PC/100 clg MNC; low vs. high		X				91	Median (2-years TTP): low: 57% (24%); high: 12 mo (71%); OS from SMM (from treatment): low: 148 mo (66 mo); high: 49 mo (31 mo)	Bianchi *et al*., 2013

Involved FLC/uninvolved FLC ≥100, BMPC ≥60%		X	X			96	Median TTP: no risk factor: 73 mo; 1 risk factor: 18 mo; both risk factors: 8 mo	Kastritis *et al*., 2013
FISH: low, normal or insufficient; standard: t(11;14), maf translocations, other/unknown translocations, or del13q14; intermediate: trisomies alone; high: t(4;14) or del17p13				X		351	TTP: low: not reached; standard: 54 mo; intermediate: 34 mo; high: 24 mo; OS from SMM (from treatment): low: 135 mo (60 mo); standard: 147 mo (77 mo); intermediate: 135 mo (86 mo); high risk: 105 mo (60 mo)	Rajkumar *et al*., 2013
High-risk FISH: t(4;14), del17p13, or gain 1q21; high tumor mass: M-protein ≥20g/l; FISH and tumor mass: both low risk; FISH high-risk only; tumor mass high-risk only; both high-risk			X	X		246	3-years TTP: both low risk: 8%; FISH high risk only: 30%; tumor mass high risk only: 40%; both high risk: 59%	Neben *et al*., 2013
Serum M-protein >30g/dl, involved FLC >25mg/dl, UAMS70 gene score >-0,26; 0 vs. 1 vs. 2+ risk factors			X		X	331	24-mo estimates TTP (UAMS70 available for n=126 patients): 0: 3%, 1: 29%; 2+: 71%	Dhodapkar *et al*., 2014
Progressive lesion on longitudinal whole-body MRI	(X)		X			63	Radiological progressive disease was associated with a 16.5-fold higher risk of progression compared to patients with stable MRI, regardless of findings in the 1st MRI	Merz *et al*., 2014

1.5 Aim, work program and applied methods

The primary **AIM** of this dissertation within the Sonderforschungsbereich / Transregio TRR79 "Werkstoffe für die Geweberegeneration im systemisch erkrankten Knochen" is to lay a basis for the understanding of the molecular background of evolution, progression and prognosis of asymptomatic myeloma (TP B1, aim 1). The secondary aim is thereby to contribute to the assessment for which patients with asymptomatic or early stage therapy-requiring myeloma (e.g. 1-3 osteolytic lesions as the only symptomatic manifestation) local treatment of osteolytic lesions can be appropriated (TP B1, aim 2).

The **WORK PROGRAM** comprises:

i) The delineation **of determinants of progression** associated with tumor mass, accumulation rate, and molecular characteristics. i.1) A quantitative measure of plasma cell accumulation shall be defined and validated. i.2) Parameters regarding tumor mass will be addressed using published stratifications. Based on these parameters, a combination delineating patients with very low/very high probability of progression to therapy-requiring myeloma shall be defined. i.3) Subsequently, the determinative potential of the novel IMWG-definition delineating AMM in patients with "smoldering" myeloma vs. those with different reasons for imminent progression will be investigated. i.4) **Molecular characteristics** determining progression will be addressed, first in terms of creating a **gene expression-based predictor** for progression of AMM and its validation on therapy-requiring patients, then assessing risk-scores for overall survival of symptomatic patients regarding progression of AMM-patients. Secondly, the impact of **chromosomal aberrations** on progression of AMM will be investigated. i.5) Assessment whether addressed determinants drive progression via different **paths**, i.e. bone disease vs. tumor mass-related causes (e.g. anemia).

ii) The assessment of the **background of molecular characteristics impacting on progression** i.e. in as much their impact can be explained by association with plasma cell accumulation rate and/or tumor mass.

iii) Based on the first two parts **mechanisms of progression and evolution of asymptomatic myeloma are** addressed in terms whether it is necessary to assume an ongoing genetic instability with *de novo* appearing aberrations to explain progression and in turn evolution of AMM from MGUS. Subsequently, it shall be delineated whether a plasma cell at MGUS-stage should be considered already malignant, or not. This is addressed by iii.1) analyzing the percentages of **chromosomal aberrations** in MGUS, asymptomatic and symptomatic myeloma patients and association of differences with disease progression, iii.2) by comparing **gene**

expression of normal bone marrow plasma cells with either MGUS, asymptomatic, or symptomatic myeloma asking at what stage(s) the bulk of changes appears, and iii.3) subsequently between MGUS vs. AMM and AMM vs. symptomatic myeloma. As validation, iii.4) in a longitudinal cohort of patients presenting at AMM and symptomatic myeloma, temporal intra-patient heterogeneity and clonal dynamics is investigated in terms of chromosomal aberrations with special focus on *de novo* gain of progression-associated aberrations and gene expression. iii.5) Subsequently, **growth kinetics** of MGUS and AMM are investigated addressing first how many doublings take place during evolvement and progression of AMM compared to the total number for symptomatic myeloma to evolve, and secondly whether it is possible that a *de novo* appearing aberration in MGUS or AMM drives the respective transitions based on observed doubling time, time to progression, and necessary number of doublings. The section closes with a discussion whether this mechanism *can* therefore be responsible for the majority of progression events.

METHODS used in this dissertation project comprise subjection of CD138-purified plasma cell samples of 2369 consecutive patients with MGUS (n=304), asymptomatic (n=432) and symptomatic myeloma (n=1633) to interphase fluorescence in situ hybridization (n=31898 measurements), and 951 (n=62/259/630) likewise to gene expression profiling. Of these, 65 samples are investigated longitudinally. Serum/urine samples (n=8398) are used to model plasma cell doubling time in AMM and MGUS (n=322 and n=196, respectively). Molecular data are integrated with clinical data including whole-body MRI for assessment of asymptomatic myeloma. Bioinformatics analysis is performed using the software environment R and the Bioconductor project's packages. Collaborations and contributions are depicted in Table 10.1.

2. Patients, samples, and methods

2.1 Patients, healthy donors, and samples

2.1.1 Patients and healthy donors

Within this dissertation, 2369 consecutive patients with monoclonal plasma cell dyscrasias presenting at Heidelberg University Hospital, or being treated within the GMMG-HD4 (ISRCTN64455289)[197] or GMMG-MM5 phase III clinical trials (EudraCT 2010-019173-16)[133,140], and 10 healthy bone marrow donors have been included between January 2002 and May 2014 and followed until February 2015. All patients consented in the analysis of their samples prior to inclusion in the study. The ethic committee of the Medical Faculty of the Ruprecht-Karls-Universität Heidelberg consented in the conduction of the trial and investigations (ethic vote no. 229/2003 and S152/2010). All patients were diagnosed according to standard criteria and response to treatment was assessed[51,52,92].

Table 2.1. Patient characteristics. MGUS, monoclonal gammopathy of unknown significance. AMM, asymptomatic multiple myeloma. MM, therapy-requiring multiple myeloma. ISS, International Staging System. NA, not available. *, not confirmed.

Variable	Level	MGUS		AMM		MM	
		n	%	n	%	n	%
Sex	Male	158	52.0	239	55.3	964	59.0
	Female	146	48.0	193	44.7	669	41.0
Age [years]	≤60	145	47.7	200	46.3	758	46.4
	>60	159	52.3	232	53.7	833	51.0
	NA	0	0.0	0	0.0	42	2.6
Type	IgA	51	16.8	95	22.0	345	21.1
	IgG	232	76.3	305	70.6	951	58.2
	IgD	0	0.0	0	0.0	14	0.9
	Bence Jones	16	5.3	26	6.0	306	18.7
	Double gammopathy	5	1.6	4	0.9	3	0.2
	Asecretory	0	0.0	1	0.2	12	0.7
	Other	0	0.0	1	0.2	2	0.1
Light chain type	Kappa	186	62.0	258	60.3	1091	66.8
	Lambda	115	38.0	169	39.5	529	32.4
	Asecretory	0	0.0	1	0.2	12	0.7
	NA	0	0.0	0	0.0	1	0.06
Plasma cell infiltration [%]	<10	289	95.1	65	15.0	122	7.5
	≥10	10*	3.3	283	65.5	290	17.8
	≥30	0	0.0	62	14.4	398	24.4
	≥60	0	0.0	11	2.5	361	22.1
	NA	5	1.6	11	2.5	462	28.3
Monoclonal protein [g/l]	<20	266	87.5	227	52.5	334	20.5
	≥20	21	6.9	98	22.7	201	12.3
	≥30	1	0.3	81	18.8	834	51.1
	NA	16	5.3	26	6.0	264	16.2
Urinary monoclonal protein [mg/24h]	<500	291	99.0	368	91.3	574	35.2
	≥500	3	1.0	35	8.7	346	21.2
	NA	10	3.3	29	6.7	713	43.7
ISS stage	1	247	81.3	331	76.6	626	38.3
	2+3	35	11.5	70	16.2	926	56.7
	NA	22	7.2	31	7.2	81	5.0

The primary end point was time to progression defined as time from bone marrow assessment to progression to active myeloma due to CRAB-criteria or AL-amyloidosis[92], with either one requiring therapy, or start of treatment because of rapidly increasing M-protein. Accordingly, no patient received myeloma treatment before clinically significant disease progression.

Definition of asymptomatic myeloma was performed according to the IMWG criteria presented in 2003[92] as these were used to define which of the patients to be treated systemically. Potential impact of the current update was assessed and can be seen as first "field testing" and validation of the novel IMWG-criteria[169].

2.1.2 Samples

Normal bone marrow plasma cells from healthy donors **and myeloma cells** were CD138-purified in the Labor für Myelomforschung (LfM) at Heidelberg University Hospital as described below (section 2.2.1.3). CD138-purified plasma cell samples from patients with MGUS (n=304), asymptomatic- (n=432), and symptomatic myeloma (n=1633), were subjected to fluorescence in situ hybridization (section 2.2.2); n=951 (n=62, n=259, and n=630, respectively, as well as n=10 normal plasma cell samples) to global gene expression profiling (section 2.2.3). Expression data are deposited in ArrayExpress (accession numbers: E-MTAB-317, E-TABM-1138). Sixty-five patients were investigated longitudinally at asymptomatic and symptomatic myeloma stage.

The human myeloma cells lines (HMCL) HG1, HG3, HG4, HG5, HG6, HG7, HG8, HG9, HG11, HG12, HG13 were generated in the LfM Heidelberg; the XG-lines XG5, XG6, XG7, XG20, XG21, XG22, XG23, and XG24 were generated by our collaboration partner Prof. Bernard Klein (CHU Montpellier, France)[145,225]. L363, SK-MM-2, LP-1, RPMI-8226, AMO-1, KMS-18, JIM-3, JJN3, KARPAS-620, KMS-12-BM, ANBL-6, KMS-11, MM1S, NCI-H929, KMS-12-PE, MOLP-8, MOLP-2, KMM-1, and EJM were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), or the American Type Culture Collection (Wesel, Germany).

As comparators, FACS-sorted (FACSAria, Becton Dickinson, Heidelberg) peripheral $CD27^+$ memory B-cells (MBC; n=5), and *in vitro* generated polyclonal plasmablastic cells (PPC; n=5) were used as published[94].

Consecutive serum, and urine samples (n=4674, and n=3724, respectively) were available for 322 AMM- and 196 MGUS-patients (Table 2.2 and section 2.2.5).

Table 2.2. Investigations performed. For all included patients, samples were CD138-purified and interphase fluorescence in situ hybridization (iFISH) and gene expression profiling (GEP), respectively, was performed. Different numbers for iFISH analyses are related to availability of sample material. Numbers of serum and urine samples for calculation of doubling time are likewise depicted. MGUS, monoclonal gammopathy of unknown significance. AMM, asymptomatic multiple myeloma. MM, therapy-requiring multiple myeloma. HRD, hyperdiploidy. Note: 11q22/11q23 is depicted as without concomitant t(11;14). NA, not available.

Analysis		n			n
		MGUS	AMM	MM	total
iFISH					
Probes	IgH-Breakapart	278	405	1352	2035
	t(11;14)	294	426	1608	2328
	t(14;16)	208	304	1090	1602
	t(4;14)	257	410	1600	2267
	1q21	290	417	1579	2286
	8p21	255	402	1516	2173
	13q14	303	431	1626	2360
	17p13	294	425	1602	2321
	5p15	219	361	1138	1718
	5q31/5q35	218	362	1137	1717
	9q34	251	396	1462	2109
	11q13	291	424	1589	2304
	11q22/11q23	302	432	1629	2363
	15q22	250	396	1469	2115
	19q13	273	406	1521	2200
	HRD	247	389	1434	2070
				∑	**31898**
GEP					
Populations / Samples	Memory B-cells				5
	Polyclonal plasmablastic cells				5
	Bone marrow plasma cells				10
	Monoclonal gammopathy of unknown significance				62
	Asymptomatic multiple myeloma				259
	Therapy-requiring multiple myeloma				630
	Human myeloma cell lines				38
				∑	**1009**
Doubling time					
Samples	Serum	1617	3057	NA	4674
	Urine	1199	2525	NA	3724
				∑	**8398**

2.2 Methods

In the following section, the sampling strategy as well as the methods used in this dissertation are explained. An overview is given in Figure 2.1.

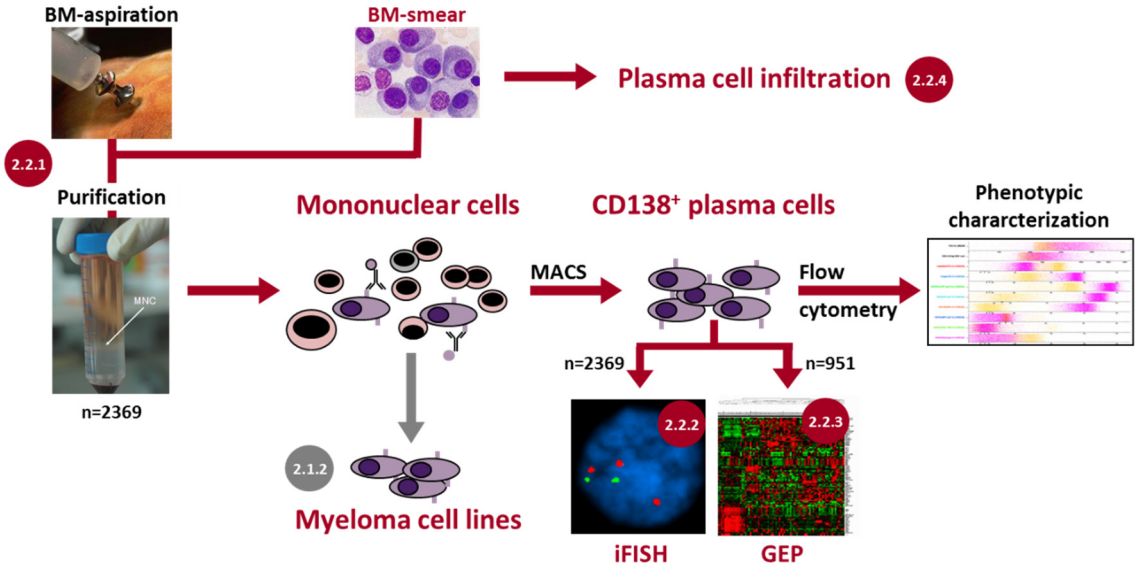


Figure 2.1. Overview sampling and used methods. Three-digit numbers refer to the respective sections in this chapter. BM, bone marrow. MNC, mononuclear cells. iFISH, interphase fluorescence in situ hybridization. GEP, gene expression profiling. MACS, magnetic-activated cell sorting.

2.2.1 Sampling and plasma cell purification

2.2.1.1 Bone marrow aspiration

After written informed consent, 60-80ml of bone marrow was aspirated from the spina iliaca posterior superior after local anesthesia using mepivacain 2% (AstraZeneca, Wedel, Germany). i) Bone marrow was aspirated in a 20ml syringe (Becton Dickinson) prefilled with 2ml sodium citrate (Eifelfango, Bad Neuenahr, Germany) for diagnostic bone marrow smears. ii) Four times 20ml bone marrow was aspirated in 20ml syringes prefilled with 1ml heparin sodium (B. Braun, Melsungen, Germany). After aspiration of each syringe, the aspiration needle (LMV Medizintechnik, Wiesloch, Germany) was moved to another spot within the same anesthetized region (i.e. the needle was withdrawn outside of the bone's compacta, and a further penetration performed). Each aspirate was injected in 20ml IMDM medium (Life Technologies, Darmstadt, Germany) in 50ml plastic-reaction tubes (PRT) (Becton Dickinson).

2.2.1.2 Density gradient centrifugation

The bone marrow medium was mixed by shaking overhead and divided equally in eight 50ml PRTs and filled to 35ml with PBS/EDTA (1xPBS/2mM EDTA). The mixture was then carefully

layered over 15ml Biocoll (Biochrom, Berlin, Germany) per 50ml PRT. Afterwards, centrifugation was performed for 30min at 700g without break. The appearing interphase-ring containing the mononuclear cell fraction (MNC) was carefully removed. The rings were pooled in one 50ml PRT, filled up to 50ml with PBS/EDTA, centrifuged 10min at 450g, the supernatant decanted carefully, and the resulting pellet resuspended. Next, cells were filtered using a 100µm pore size cell strainer (Becton Dickinson) pre-rinsed with 1ml PBS/EDTA in a 50ml PRT. Subsequently, the PRT was filled with PBS/EDTA up to 50ml and centrifuged 10min at 200g. The supernatant was completely removed and the pellet resuspended with 1ml PBS/EDTA. Cells were counted after dilution with "Türks-solution" (Merck, Darmstadt, Germany) using a Neubauer-chamber (Meka Glas, Kaufbeuren, Germany).

2.2.1.3 CD138 purification

After cell counting, MNCs were filled up to 50ml with PBS/EDTA, centrifuged 10min at 200g, and the supernatant was removed completely. Per 10^7 cells 180µl MACS buffer (PBS/EDTA+0,5% bovine serum albumin) and 20µl anti-CD138-microbeads (Miltenyi Biotec, Bergisch Gladbach) were added and incubated for 15min at 4°C. Cell suspension was filled up to 50ml with MACS-buffer and centrifuged 10min at 200g. Afterwards, pelleted cells were resuspended in 2ml MACS-buffer, carefully avoiding any foaming, which would negatively influence the purification result. Separation of CD138-positive cells is performed using an "automated magnetic-activated cell sorter" (autoMACS, Miltenyi Biotec). After purification, negative and positive fraction were counted, pelleted, cells suspended in 700µl RLT-buffer (Qiagen, Hilden), and stored at -80°C.

2.2.1.4 Purity control by flow cytometry

The following tubes were prepared for flow cytometric measurement:

- Bone marrow (BM) before plasma cell purification
  BM IgG/IgG: 10µl BM + 40µl PBS/EDTA + 4µl (IgG/IgG; "Simultest", Becton Dickinson)
  BM CD38/CD138 (CD, cluster of differentiation): 10µl WBM + 40µl PBS/EDTA + 4µl CD38 Fluorescein-Isothiocyanat (FITC, Becton Dickinson) + 4µl CD138 R-Phycoerythrin (PE, Miltenyi Biotec)
- Positive fraction after plasma cell purification
  Positive fraction IgG/IgG: $5x10^4$ cells plus 4µl Simultest
  Positive fraction CD38/CD138: $5x10^4$ cells plus 4µl anti-CD38FITC + 4µl anti-CD138PE
- Negative fraction after plasma cell purification
  Negative fraction IgG/IgG: $5x10^4$ cells plus 4µl Simultest
  Negative fraction CD38/CD138: $5x10^4$ cells plus 4µl anti-CD38FITC + 4µl anti-CD138PE

All tubes were incubated for 15min at 4°C, vortexed, filled with 2ml CellWash (Becton Dickinson), vortexed, pelleted for 5min at 200g, decanted, and remaining drops carefully removed with a cloth. After adding 200µl CellWash, flow cytometry was performed using a FACSCalibur (Becton Dickinson).

2.2.1.6 Cytospins for interphase fluorescence in situ hybridization

Per patient, 10 slides (individually manufactured for the LfM, "Mattrand 10 mm 76x26x1 mm" by Menzel, Brenzinger, Walldorf, Germany) were prepared. The slides were prewashed with 75% ethanol (Roth, Karlsruhe), dried well, and silanisized twice. For the latter, slides were covered with 100µl silane solution (375µl 3-Methacryloxypropyltrimethoxysilane, "bind-silane", Serva, Heidelberg, ad 100ml 100% ethanol *p.a.*), incubated for one minute, and silane removed with a lint-free cloth. Slides were prepared using filter cards and cytospin chambers (all from Hettich, Tuttlingen, Germany). On each slide, 2 spots à $5x10^3$ $CD138^+$ cells in 200µl PBS/EDTA each were spinned at 1500rpm for 3min (Hettich Universal 16). The chamber was demounted and the slides spinned dry 3min at 1500rpm. Slides were then dried 15min at room temperature, fixed in methanol/glacial acetic acid (3:1) at -20°C for 10min and subsequently air-dried. Slides were controlled by light-microscopy and stored at -20°C until use.

2.2.2 Interphase fluorescence in situ hybridization

Assessment of chromosomal aberrations by iFISH was performed on CD138-purified plasma cells in cooperation of the LfM with the "Molekular-cytogenetisches Labor" (Prof. Anna Jauch, Institut für Humangenetik, Heidelberg) using probes for chromosomes 1q21, 5p15, 5q31 or 5q35, 8p21, 9q34, 11q13, 11q22.3 or 11q23, 13q14.3, 15q22, 17p13, 19q13, and translocations t(4;14)(p16.3;q32.3), t(11;14)(q13;q32.3), and t(14;16)(q32.3;q32). Hybridization efficiency was validated on interphase nuclei obtained from peripheral blood and bone marrow of healthy donors. Thresholds for gains, deletions, and translocations were set at 10%. The score of Wuilleme *et al*. was used to assess ploidy using gains of at least two of the three chromosomes 5, 9, and 15[221]. The proportion of malignant plasma cells in an individual patient was determined by the highest percentage of a chromosomal aberration (see below).

iFISH assessment as surrogate of tumor mass. The proportion of clonal (aberrant) plasma cells in an individual patient was determined by the highest percentage of a chromosomal aberration[153]. The measured value is the number of cells carrying a specific aberration (myeloma cells) divided by the number of "all purified cells", i.e. the sum of the number of myeloma cells, normal plasma cells, and "contaminating" non-plasma cells, in a total minimum of 700 counted cells. Thus, the method counts the fraction of myeloma cells within a highly purified sample. The

sensitivity of this method is given by the above defined threshold of 10% for each iFISH aberration. For prognostic evaluation, a threshold of >95% malignant plasma cells was used[153,162]. CD138-purified plasma cells from 65 patients were available longitudinally, i.e. at asymptomatic and symptomatic myeloma stage. Differences regarding absence/presence of chromosomal aberrations between the two stages were assessed as detailed in Table 2.3. For example, in case of a subclonal to clonal switch or the other way round with the percentage of aberrant plasma cells in the given sample being ≥60%, we took as threshold the difference of the percentage of aberrant plasma cells between the two time points + 10% (i.e. the latter being the detection limit in our iFISH analysis). For example: At the time of AMM, our aberration of interest is present in <60% of cells, thus a subclonal aberration, while the percentage of aberrant plasma cells in the sample is ≥60%. At symptomatic myeloma stage, the aberration of interest has now become clonal (≥60% of cells). In case the difference in frequency of our aberration of interest between asymptomatic and symptomatic myeloma is greater or equal our threshold (second but last column), we classified it as subclonal gain of aberration (case 1.1.a); otherwise we would have classified it as no change (case 1.1.b).

Table 2.3. Assessment of changes in chromosomal aberrations in paired samples at asymptomatic and symptomatic myeloma stage. AMM, asymptomatic myeloma. MM, therapy-requiring myeloma. 0, absence of aberration (i.e. normal diploid, no translocation). =, no change. APC, percentage of aberrant plasma cells (i.e. maximal frequency of any aberration in a given sample) according to Neben *et al*.[153]. Clonal, aberration present in ≥60% of cells. Subclonal, aberration present in <60% of cells.

AMM				MM		Δ [%] for respective aberration	Δ for respective aberration
Aberration	**Case**		% clonal plasma cells (APC)	**Aberration**	% aberrant plasma cells		
Present	1.1.a	subclonal	≥60	clonal		≥ ΔAPC + 10	subclonal gain
	1.1.b	subclonal	≥60	clonal		< ΔAPC + 10	=
	1.1.c	subclonal	<60	clonal			=
	1.2	subclonal		subclonal			=
	1.3	subclonal		0			subclonal loss
	2.1.a	clonal		subclonal	≥60	≥ ΔAPC + 10	subclonal loss
	2.1.b	clonal		subclonal	≥60	< ΔAPC + 10	=
	2.1.c	clonal		subclonal	<60		=
	2.3	clonal		clonal			=
	2.4	clonal		0			clonal loss
Absent	3.1	0		subclonal			subclonal gain
	3.2	0		clonal			clonal gain
	3.3	0		0			=

2.2.3 Global gene expression profiling

2.2.3.1 RNA-extraction and quality control

RNA and DNA were extracted from samples stored at -80°C in RLT-buffer (Qiagen) using the AllPrep DNA/RNA Mini kit (Qiagen) according to the manufacturer's instructions. The underlying

principle is a selective binding of RNA (and DNA) to subsequently used centrifugation columns, from which the bound RNA (and DNA) is eluted after several washing steps.

RNA-quantification and quality control were performed using an Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany) according to the manufacturers' instructions. Quantification is achieved by correlation of the measured fluorescence signal with a known standard. RNA quality is controlled by comparing the 28S to 18S RNA peak ratio, (2:1 in non-degraded RNA-samples), or the so-called RNA-integration number (RIN).

2.2.3.2 Gene expression profiling using oligonucleotide arrays

Labeled cRNA was generated from 30-100ng of total RNA following the small sample labeling protocol vII (Affymetrix, Santa Clara, CA, USA). This protocol comprises two amplification cycles (Figure 2.2). It is important to denote that the amplification kit influences the measured gene expression, despite a generally good correlation, and thus needs to be kept stringently constant. Labeled cRNA was fragmented and hybridized on oligonucleotide-DNA microarrays (U133 2.0 Plus). Subsequently, arrays were scanned and the relative fluorescence intensity of single genes (probesets) measured. On the chip, each gene (transcript) is represented by a varying number (11 to over 20) of 25-mer oligonucleotid "probes" ("probes"). Probes are localized in a "probe cell", a quadratic area, containing millions of copies of the same specific oligonucleotide-sequence. Probes are synthesized as pairs of "perfect match", complementary to the target sequence, and "mismatch", in which the 13^{th} base is altered. On the chip, they are arranged as "perfect match" probe-cell with the corresponding homomeric "mismatch" probe-cell. Both together are called a "probe-pair". The individual probe pairs representing a transcript are distributed over the chip. The 11 to 20 probes (probe-cells) representing a transcript are termed "probeset"; for each transcript exists a "perfect-match" probeset and a "mismatch-probeset". For many genes (e.g. *CCND1*) more than one probeset is available on the chip (i.e. more than one set of probes representing one transcript). Not all probes (probesets) on the chip have been synthesized in the right (genomic) orientation. Thus, some of these do not give a signal despite the respective gene (or sequence) being expressed in the respective sample. Regarding the representation of probes over transcripts, probes within the transcripts 3' region are dominating. The **raw expression data,** after their extension ".cel" called "cel-files", represent the primary result of a GEP experiment. These can be normalized using different strategies[122,220] and statistically analyzed (see section 2.2.6.2).

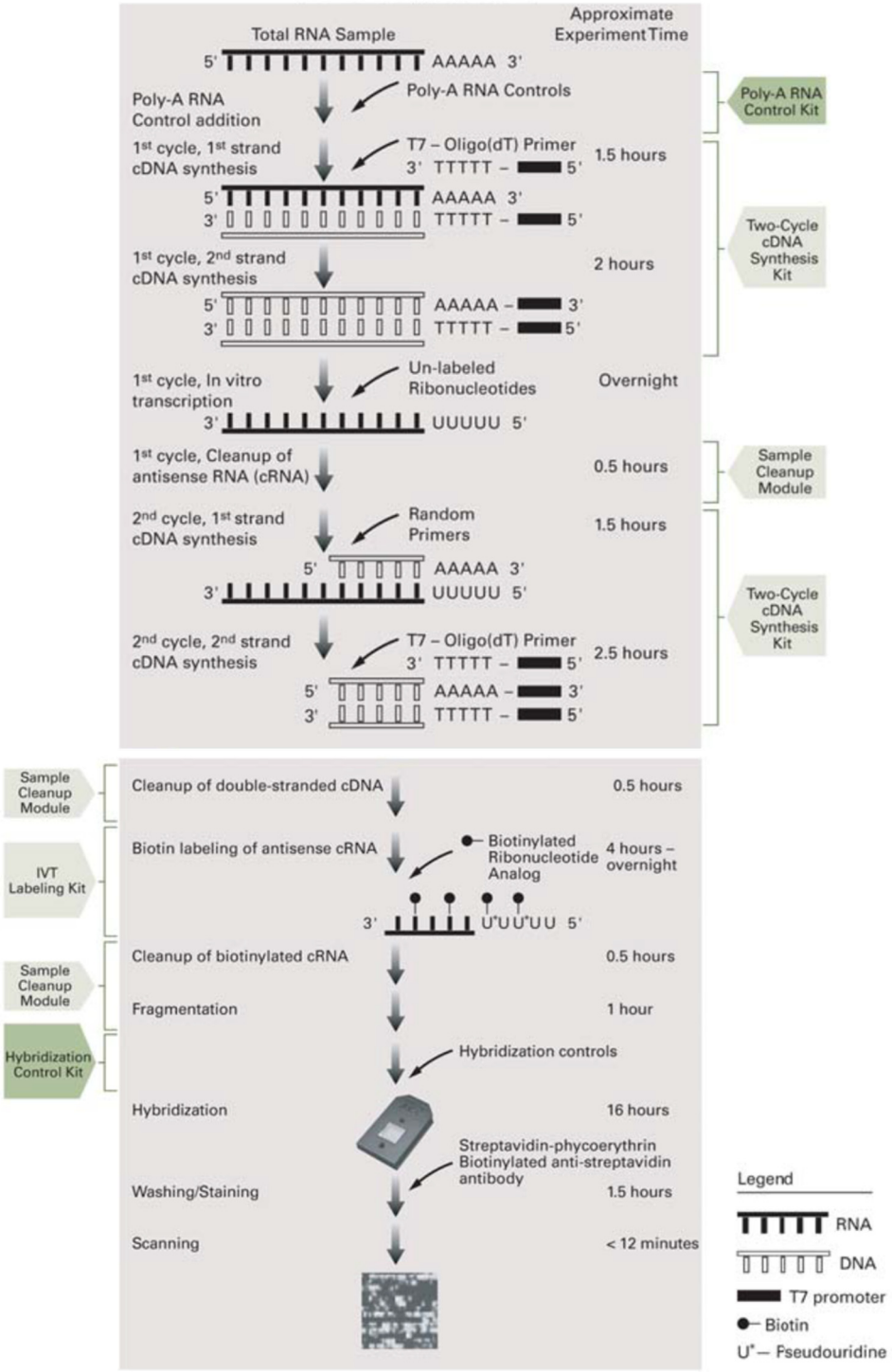


Figure 2.2. Gene expression profiling using U133 2.0 DNA-microarrays and the Affymetrix two cycle target labeling protocol (modified from Affymetrix U133 2.0 manual). **First amplification cycle.** Total RNA (30-100ng) is first reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent *in vitro* transcription (IVT). **Second amplification cycle.** After cDNA synthesis in the first cycle, an unlabeled ribonucleotide mix is used in the first cycle of IVT amplification. The unlabeled cRNA is then reverse transcribed in the first strand cDNA synthesis step of the second cycle using random primers. Subsequently, the T7-Oligo(dT) Promoter Primer is used in the second-strand cDNA synthesis to generate double-stranded cDNA template containing T7 promoter sequences. The resulting double-stranded cDNA is amplified and labeled using a biotinylated nucleotide analog/ribonucleotide mix in the second IVT reaction. The labeled cRNA is then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

2.2.4 Surrogates of tumor mass

Local (i.e. bone marrow plasma cell infiltration, percentage of aberrant (malignant) plasma cells as determined by iFISH[153]), global (i.e. M-protein with thresholds of 30g/l and 20g/l[116], FLC ratio involved:uninvolved of ≥100[119], or immunoparesis as defined by suppression of one or two of the non-involved immunoglobulins below the lower limit of normal[162]), and combined (i.e. according to Kyle *et al*.[116], and the Mayo Clinic model[48]) surrogates of tumor mass were assessed accordingly. The "high tumor mass" group according to Kyle *et al*. includes patients with ≥10% plasma cell infiltration and M-protein levels of ≥30g/l (n=66), all other patients (n=337) were grouped in the "low tumor mass" group due to the number of cases (Table 2.1).

2.2.5 Calculation and modeling of doubling time

Doubling time (DT) as measured in years was calculated as depicted below for patients fulfilling the inclusion and not fulfilling the exclusion criteria.

Inclusion criteria for DT assessment. Consenting patients for which a minimum of two measurements of either serum (for IgA- or IgG-myeloma) and/or urine (for Bence-Jones and predominant light chain myeloma) was available with a minimum time interval between first and last measurement of 1 month. Patients had to fulfill any one or more additional criteria:

1) ≥3 measurements
2) (if only 2 measurements): minimum time interval of first and last measurement ≥3 months.
3) (if only 2 measurements AND minimum time interval of first and last measurement <3months):
   increase of involved heavy chain/M-component >5g/l or 24-hour light chain excretion >200mg (the progress criteria for symptomatic myeloma[52]).

Ad additional criterion 1: Three measurements are requested to reduce the influence of fluctuations of serum/urine measurements by chance on DT, especially if the calculation is based on a short time interval. Ad additional criterion 2: If fewer measurements are available (i.e. 2) the time interval is chosen to be longer. Ad additional criterion 3: This criterion is included to allow assessment of DT for patients with short time to progression (i.e. fast DT). To reduce the number of false positive short doubling time results, in case only 2 measurements were available and the time span was below 3 months, a significant increase was asked for, i.e. using the criteria for disease progression in symptomatic myeloma.

Exclusion criteria for assessment of doubling time. Patients not fulfilling the above mentioned inclusion criteria were excluded.

Five hundred eighteen patients (196 MGUS- and 322 AMM-patients) fulfilled the inclusion criteria and have been included in the analysis. In total, 4674 serum and 3724 urine samples were analyzed, 1614/1199 for MGUS-, and 3057/2525 for AMM-patients (Table 2.2). Mean follow up was 4.8 years for MGUS-, and 3.8 years for AMM-patients, respectively.

2.2.5.1 Modeling of doubling time

Modeling of doubling time was performed by Marcel Mohr, MS, at the Institute of Applied Mathematics, Interdisciplinary Center for Scientific Computing (IWR) at Heidelberg University under the supervision of Prof. Dr. Anna Marciniak-Czochra and Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose.

Alinear dependence of the heavy and light chain concentration on the amount of malignant cells was assumed. This allows getting information about the growth patterns of the malignancy for the patient-specific data sets. An exponential model

$$y = y(t) = be^{at}, \quad a \in R, b > 0 \qquad (1)$$

was used for the concentration (expression) of Ig (LC) y at time t. Exponentials are often used when the rate of change of a quantity is proportional to its initial amount. If the parameter a is positive, the model (1) captures exponential growth of y. If the parameter a is negative, the model (1) represents exponential decay of y. b is the value of y at time t = 0. The doubling time τ in case of a > 0 is given by

$$\tau = \ln(2) / |a| , \quad a \neq 0.$$

Its unit [τ] is given by [τ] = 1/[t] . The doubling time (the half-value time) is defined as the period of time needed for a quantity to double (to halve) in value. In calculations, the absolute value of negative values as output from the program are to be identified as half-value times.

To fit the model (1) to the data, a logarithmic transformation of the data was performed and the transformed data fitted to a linear model, where least squares methods were used. The quality of the exponential fit regarding the (exponential) data is evaluated using the coefficient of determination R^2, which is a ratio of the residual sum of squares and the uncorrected total sum of squares[186]. Calculations and simulations are performed by the computation software program Wolfram Mathematica, Version 9 (Wolfram Research, Oxfordshire, UK).

2.2.5.2 Excel-tool to assess the doubling time

A tool for the calculation of the DT only needing the activity parameter in serum/urine and the date of the measurement was implemented by Marcel Mohr. With help of the DT tool (executable in Microsoft Excel® and Apache Open Office), one can calculate a patient's DT given IgG-, IgA-, or LC-measurements, respectively. Since the value of the calculated DT strongly

depends on the entered data, the program also calculates the standard error of the DT (by using the error propagation formula for a transformed fitting parameter) based on these data. This allows for further evaluation of uncertainties regarding the categorization into one of the defined DT groups.

2.2.6 Statistical analysis

All (other) statistical computations have been performed using R 3.1.1 (http://www.r-project.org/), and Bioconductor 2.14[62]. Differences in clinical parameters, chromosomal aberrations and between defined groups were investigated by Wilcoxon rank-sum test. Correlation was assessed using Pearson's correlation-coefficient or Spearman´s rank correlation. The relationship between categorical variables was assessed using Fisher's exact test. A Jonckheere-Terpstra test was applied to test for ordered differences among groups using the R package clinfun. Depicted logarithms (i.e. log doubling time in Figures 3.12 and 3.13) all have the basis e. An effect was considered as statistically significant if the P-value of its corresponding statistical test was <5%.

2.2.6.1 Survival analysis

Progression-free survival for n=432 asymptomatic myeloma patients was investigated using Cox's proportional hazard model as published[86]. Proportionality of the hazard assumption was tested[69]. Progression rates at 1, 2, 5, and 10 years were calculated. Survival curves and median time to progression (TTP) were computed with nonparametric survival estimates for censored data using the Kaplan-Meier method[57]. Difference between the curves were tested using the G-rho Log-rank test[77]. Landmark analysis for doubling time assessment was performed using measurements until 18 months of follow up from bone marrow aspiration for analysis of TTP by defining an alternative start point (landmark) at 18 months.

2.2.6.2 Analysis of gene expression data

Preprocessing, e.g. background-correction, normalization, grouping of probes in a probeset (see above) of U133 2.0 Plus GeneChip DNA microarrays raw-data (*.cel-files) was performed using the GC-RMA-algorithm (GC-RMA: GC-corrected Robust Microarray Average)[220]. GC-RMA only uses "perfect-match" probesets and normalizes over all chips of a dataset (e.g. cohort of patients). It adjusts background intensities, present because of optical noise (e.g. due to different labeling) and unspecific binding present in GeneChip DNA-microarray data. Background adjusted probe intensities are subsequently converted into a $\log_2$-scaled expression-measure using the quantile-normalization as well as the summation-method (so called "medianpolish"). As two different IVT labeling kits were used, batch correction was

performed using ComBat[93]. Expression profiles of 1014 samples (630 symptomatic multiple myeloma, 259 AMM, 62 MGUS, 38 HMCLs, 10 normal bone marrow plasma cells, 5 MBCs, and 5 PPCs) were analyzed.

Differential gene expression was assessed using empirical Bayes statistics in linear models for microarray data[196]. P-values were adjusted for multiple testing controlling the false discovery rate as defined by Benjamini and Hochberg[19]. Beforehand the number of probesets was reduced using annotation based filtering[25]. Global similarity between disease entities was estimated via RV coefficient using the R package MADE4. For a size corrected analysis of the differences in the disease entities, differential gene expression was measured 10 times with 62 randomly chosen patients each (the size of the MGUS-cohort). Lists of genes present in each of the 10 comparisons were used to create Venn-diagrams, the mean number of differentially expressed genes across the 10 comparisons was assessed. Gene expression differences between paired samples, i.e. at asymptomatic and symptomatic myeloma stage, were measured with a moderated paired t-test.

Gene expression-based assessment of risk (UAMS70[194], EMC92[106], Rs[170], and IFM15[43]) and proliferation[87] as well as classifications of myeloma, i.e. molecular classification[223], TC-[20], and EC-classification[84] were performed as published. Significant differences between entities and groups were assessed using pairwise Fisher-test for count data. For calculation of the UAMS70, EMC92, and the TC-classification, the cohort was normalized with the mas5 algorithm. Thirty seven patients had GEP data available at two different stages (34 at asymptomatic and symptomatic myeloma, used for longitudinal analyses, three at MGUS and symptomatic myeloma, respectively). But for longitudinal analyses, only expression profiles at AMM stage were included.

Principal component analysis was performed. A kind of center of gravity encompassing 10% of expression data of each entity is shown.

The **HDAMM-predictor** for prediction of progression in asymptomatic patients was generated according to a method published by Rème *et al*.[170].

3. Results

3.1 Determinants of progression

Determinants of progression, biologically grouped in those associated with plasma cell accumulation rate, tumor mass, and molecular characteristics, were assessed.

In all, 432 patients with asymptomatic multiple myeloma were included in this analysis. Of these, 150 patients (34.7%) progressed to therapy-requiring myeloma or AL-amyloidosis, respectively, within an estimated median TTP of 4.7 years. Cumulative progression rate after 2 and 5 years totaled 31% and 52%. Of progressing patients, 61.3% progressed due to bone disease, 38.4% due to other, i.e. tumor mass-related causes (e.g. anemia).

3.1.1 Plasma cell accumulation rate

The plasma cell accumulation rate was first assessed by our **gene expression-based proliferation index** (GPI), delineating two significantly different groups in terms of progression (n=259; “medium” GPI, 24% of AMM-patients, vs. ”low” GPI) with 2- and 5-year progression rates of 49% and 67% (medium) vs. 30% and 51% (low), respectively (Figure 3.1A). Unlike symptomatic myeloma, no patient showed a “high” proliferation rate (Table 3.1).

Secondly, to quantitatively assess the accumulation rate of myeloma cells using easily available clinical parameters, the calculation of the **myeloma cell doubling time** using consecutive serum/urine samples (IgA, IgG, and 24-hour light chain excretion, respectively; n=322 patients, n=5582 samples) as in a clinical scenario and fitting a regression model is introduced. To avoid data-adapted thresholds (overfitting), and delineate clinical relevant groups with very fast, fast, intermediate, and almost no accumulation, a geometric row, i.e. 2 (very fast), 4 (fast), <16 years (intermediate), and ≥16 years (slow) was used as cutoff. Patients with a doubling time <2, <4, <16, and ≥16 years each have significantly different progression rates of 80%, 44%, 20%, and 9% at 2 years, and 100%, 83%, 56%, and 15% at 5 years, respectively (Figure 3.1B).

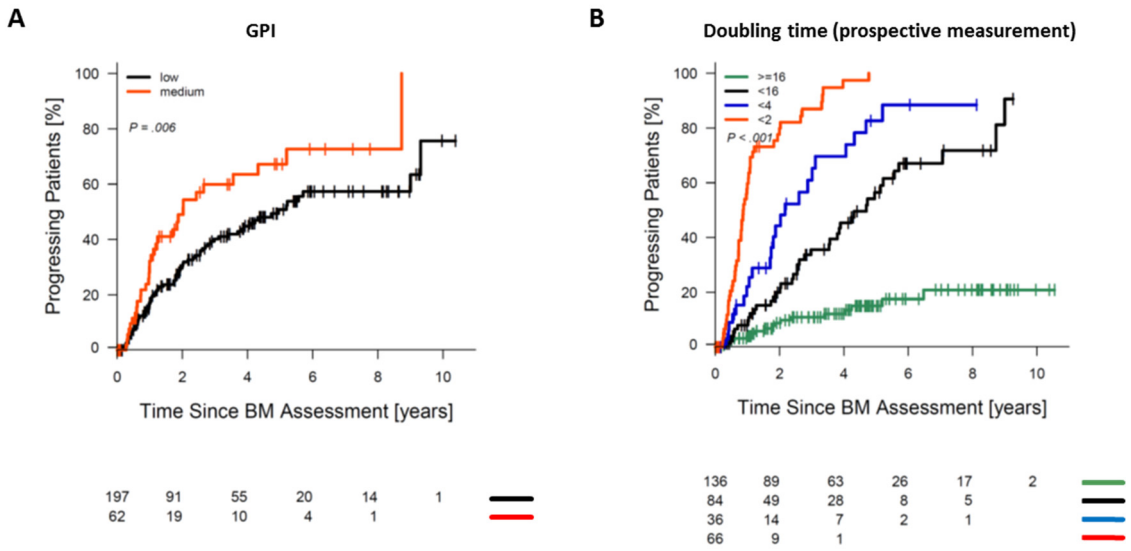


Figure 3.1. Association of progression with the accumulation rate of malignant plasma cells. A. Gene-expression-based proliferation index (GPI). "Medium" proliferation rate (red curve), "low" proliferation rate (black cure). **B.** Doubling time in years, <2 (red curve), <4 (blue), <16 (black), and ≥16 (green), respectively. For all depicted doubling times, significantly different numbers of progressing patients can be delineated with P<.001, P=.003, and P<.001 for the subsequent comparisons between the four groups <2 vs. <4 years, <4 vs. <16 years, <16 vs. ≥16 years, respectively.

As DT-calculation uses parameters during the follow up period, despite it is strongly associated with disease progression, it should not be seen as prognostic factor but as clinical parameter correlative with progression. As confirmation, a landmark analysis balancing the number of measurements and thus eligible patients (see section 2.2.5) vs. loosing especially fast progressing patients (with short doubling time) was performed. The analysis at 18 months shows patients with DT<2 and DT<4 years to have a significantly shorter TTP (Figure 3.2A). Next, the clinical value of progressive assessment of doubling time was addressed in a "what if" scenario. Here only measurements before the date of progression were used, i.e. excluding those at the time of progression. The curves thus represent the TTP from bone marrow aspiration with the DT measured up to this point in time, i.e. the DT on which a clinical decision would have been drawn. TTP regarding doubling time calculated from these values is similar to the one if all values are included (i.e. including progression), as are the obtained hazard ratios of 4.78 vs. 4.86, 7.48, vs. 8.21, and 18.75 vs. 25.17, respectively (Figure 3.2B).

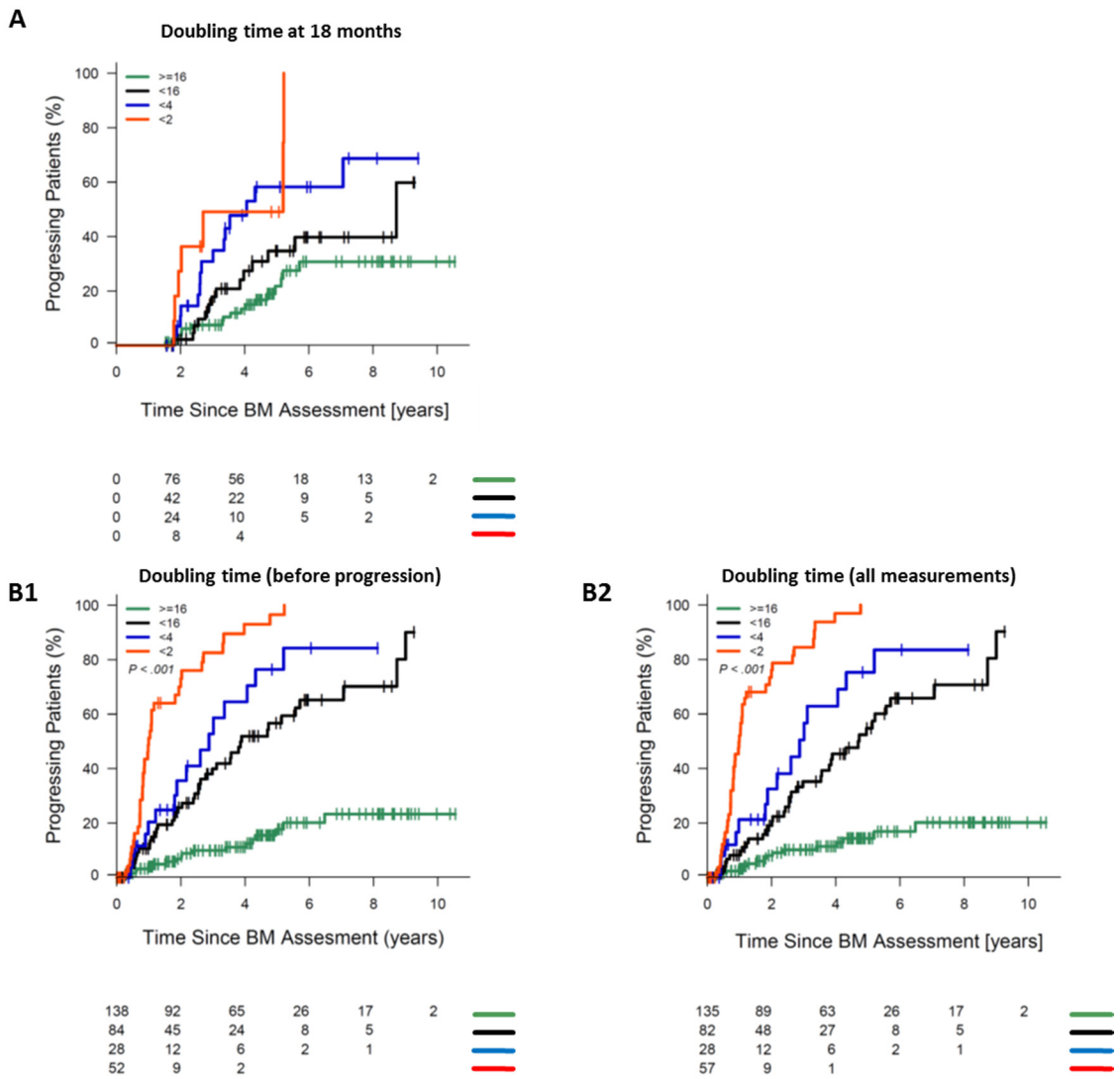


Figure 3.2. Landmark analysis of time to progression to symptomatic myeloma and a "what if" analysis. A. Landmark analysis at 18 months. Shown is the association of progression with doubling time (in years), with <2 (red curve), <4 (blue), <16 (black), and ≥16 (green), respectively. **B.** Results for 302 patients (B1) excluding measurements at the time of progression still fulfilling the inclusion criteria in terms of the number of measurements, and (B2) the same patients using DT-assessment including all available measurement as used for Figure 3.1.

To allow for the calculation of the DT in clinical routine only needing the activity parameter in serum/urine and the date of the findings an easily usable tool was implemented by Marcel Mohr (PhD-student Prof. rer. nat. Anna Marciniak-Czochra and Priv.-Doz. Dr. med. Dipl-Phys. Dirk Hose). With help of the DT tool, one can calculate a patient's DT given IgG-, IgA-, or LC-measurements, respectively (Figure 3.3). Since the value of the calculated DT strongly depends on the entered data, the program also calculates the standard error of the DT based on these data. This allows for further evaluation of uncertainties regarding the categorization into one of the defined DT groups.

Doubling Time Calculation

Insert at least 2 measurements in the colums „Date“ and „Measurement“. Guarantee that the units of the values in „Measurement“ are the same. The tool calculates the Doubling Time (DT) and its Standard Error based on the given data and the fitting results.

1. DT: Click on the green cell (Fit Parameter) and follow the commented instruction. The DT (years) is listed in the green cell „DT (y)“. A negative value has to be interpreted as half-value time.

2. SE: Click on the orange cell (Fit SE) and follow the commented instruction. The SE (years) of the DT is listed in the orange cell „SE (y)“.

# Patient	Date (MM/DD/YY)	Measurement	Time Points	Fit Parameter	DT (y)
1			0	#WERT!	#WERT!
2			0	Fit SE	SE (y)
3			0	#WERT!	#WERT!
4			0		
5			0		
6			0		
7			0		
8			0		
9			0		
10			0		
11			0		
12			0		
13			0		
14			0		
15			0		
16			0		
17			0		
18			0		
19			0		
20			0		

Click on the input field. Drag down the boxes over the available data in the yellow columns „Measurement“ and „Time Points“. Make sure that both boxes are of the same length as the previous boxes. Press ENTER.

Click on the input field. Drag down the boxes over the available data in the yellow columns „Measurement“ and „Extension“. Make sure that both boxes are of the same length. Press ENTER.

Example:

# Patient	Date (MM/DD/YY)	Measurement	Time Points	Fit Parameter	DT (y)
1	08.20.08	35,32	0	1,000397017	4,784202144
2	05.20.09	42,53	273	Fit SE	SE (y)
3	07.22.09	44,75	336	3,69646E-05	0,445525749
4	04.14.10	47,62	602		
5	09.29.10	52,21	770		
6	06.08.11	57,07	1022		
7	03.07.12	60,13	1295		

Figure 3.3. Tool for doubling time calculation. The DT tool (executable in Microsoft Excel® and Apache Open Office) calculates a patient's DT given IgG-, IgA-, or LC-measurements (third column), respectively, and the date of the findings (second column). Since the value of the calculated DT strongly depends on the entered data, the program also calculates the standard error of the DT based on these data. Exemplary data are shown in the lower panel.

3.1.2 Surrogates of tumor mass

Surrogates of tumor mass significantly predict progression, either of global tumor mass, i.e. serum/urine parameters including serum FLC ratio, M-protein, and staging systems combining these factors, or of local tumor mass, i.e. plasma cell infiltration in bone marrow aspirates or percentage of malignant plasma cells assessed by iFISH (Figure 3.4, Table 3.3).

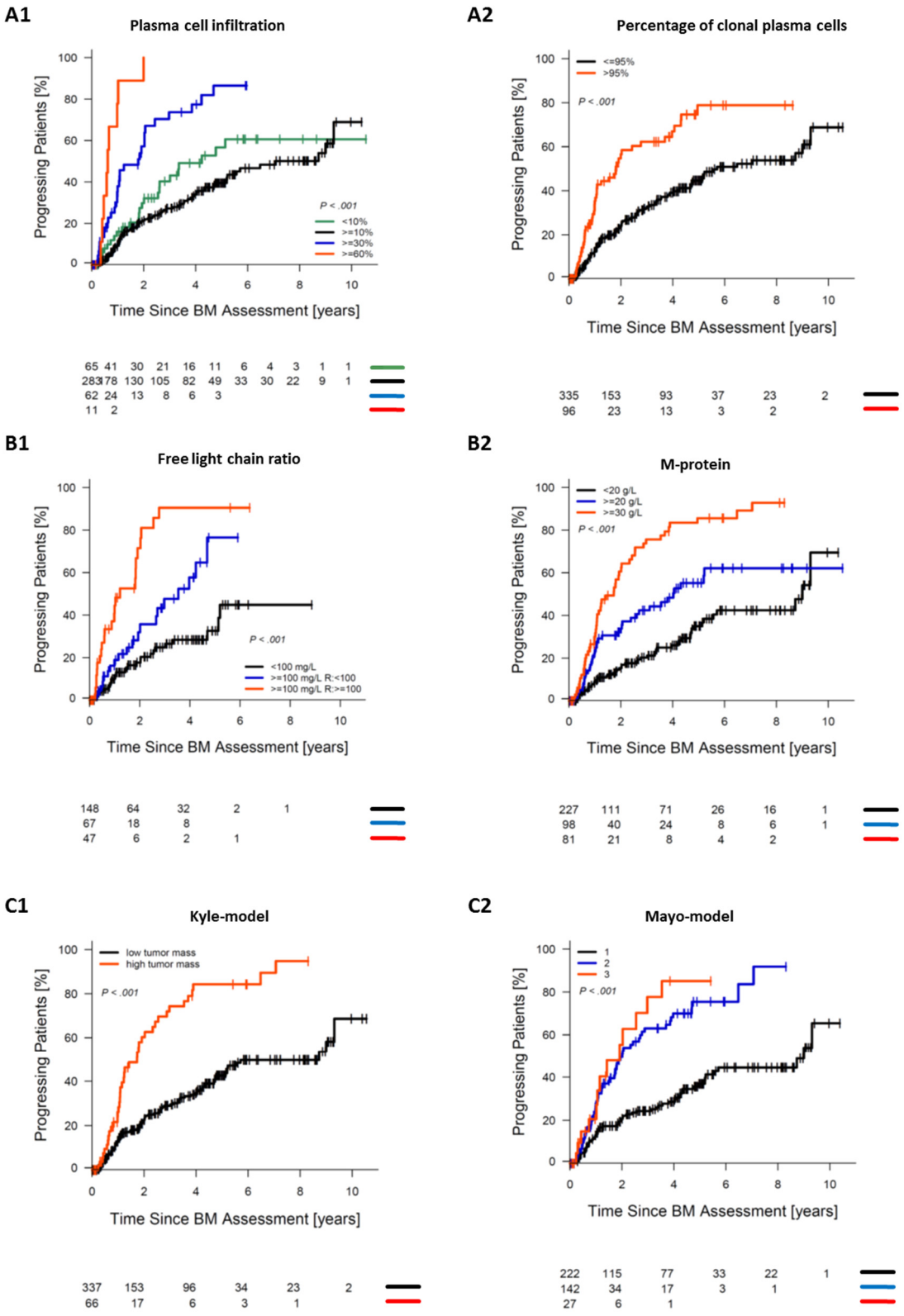


Figure 3.4. Association of progression with surrogates of tumor mass. A. Local surrogates of tumor mass in terms of (A1) bone marrow plasma cell infiltration, and (A2) percentage of clonal (malignant) plasma cells using iFISH. **B.** Global surrogates of tumor mass, i.e. (B1) serum free light-chain ratio, and (B2) serum M-protein levels. **C. Combinations.** Tumor mass surrogates as defined by (C1) Kyle *et al.*[116] with low (black) vs. high tumor mass (red), and (C2) the Mayo-model[48]. Red curve - three risk factors present, blue - two, black - one. For details regarding the different surrogates and their calculation, respectively, please refer to section 2.2.4.

Plasma cell accumulation rate (GPI, DT) and tumor mass surrogates are independent in multivariate analysis (Table 3.4) and a combination delineates patients with very low/very high probability of progression to therapy-requiring myeloma, e.g. patients with doubling rate <4 years and high plasma cell infiltration ≥30% show a 90% progression rate at 2 years (Figure 3.5B; red curve), i.e. above the proposed ultra-high risk definition of >80%[169,212]. Patients with either fast doubling time <4 and low plasma cell infiltration (blue curve) or low doubling rate ≥4 years and high infiltration (≥30%, black curve) likewise progress with a 91% and 77% probability after 5 years, respectively. Patients with a low plasma cell infiltration and low doubling time progress at a fairly constant rate with 30% after 5 years (green curve), those with >100 years and plasma cell infiltration below 30% show a progression rate comparable with MGUS-patients (8% in 5 years) with almost no progression after the first two years.

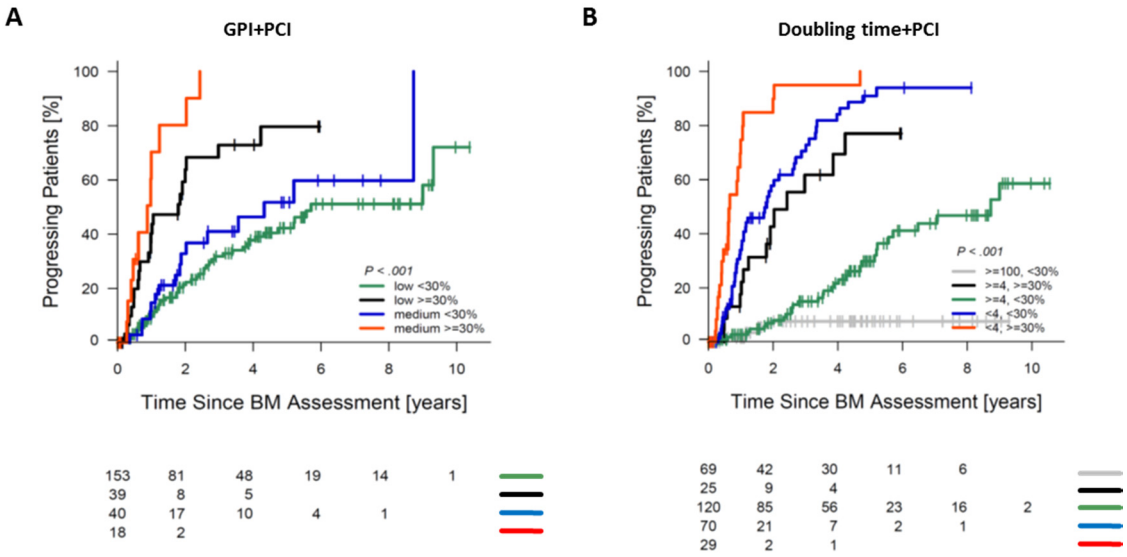


Figure 3.5. Main factors acting independently on progression are tumor mass and accumulation (proliferation) rate. A. Gene expression-based proliferation index (low vs. medium) and bone marrow plasma cell infiltration above/below 30%. All patients with a medium GPI and plasma cell infiltration ≥30% progressed within two years from bone marrow assessment. Subsequent curves show different progression rates, i.e. P=.04 (red vs. black), P=.01 (black vs. blue), and P=.2 (blue vs. green). **B.** Doubling time in years (first number), and bone marrow plasma cell infiltration above/below 30% delineates patients with significantly different probability of progression.

The AMM-cohort according to the novel IMWG-definition was next split in patients with “smoldering” myeloma vs. those with different reasons for imminent progression, i.e. plasma cell infiltration ≥60%, FLC ratio ≥100, ≥1 bone lesion (osteolyses), and/or >1 focal lesion in MRI, vs. “solitary plasmocytoma with minimal bone marrow involvement” (i.e. <10% clonal bone marrow plasma cells). The first two show a 100% and 69% progression rate, respectively, within 2 years to symptomatic myeloma. Unexpectedly, a comparable result could be found for patients with a “solitary plasmocytoma with minimal bone marrow involvement” with a 2-year

progression rate of 69%. Smoldering myeloma patients according to the IMWG 2014 definition have a lower 5-year progression rate of 39% vs. 52% of our total cohort, as well as 10-year progression rate of 64% vs. 73% (Figure 3.6, see also Table 3.3).

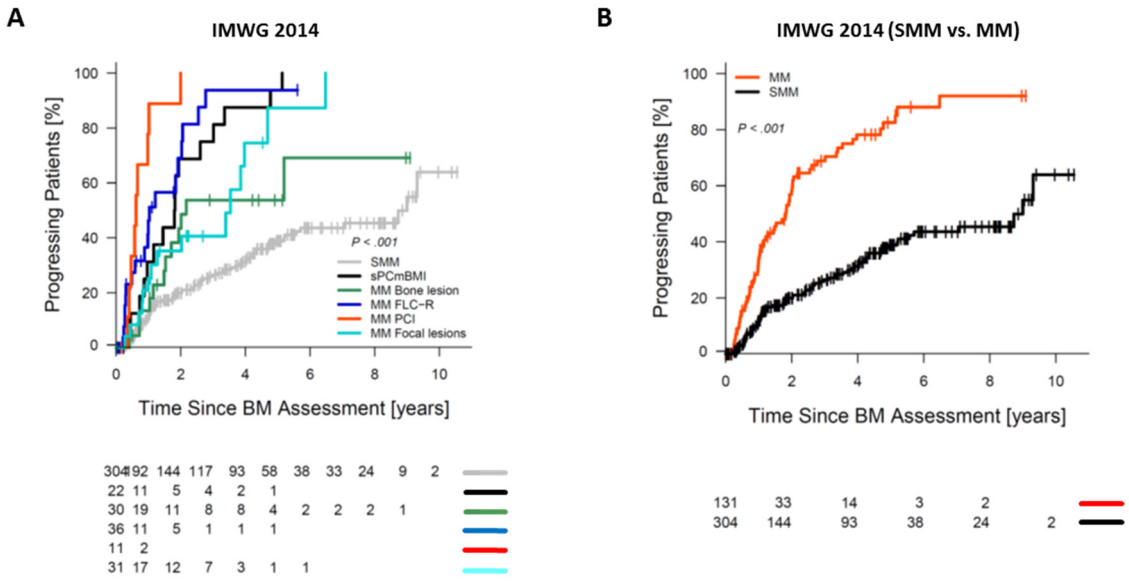


Figure 3.6. "Field testing" and validation of the novel IMWG-definition of smoldering myeloma in our cohort of asymptomatic myeloma patients. A. Patients staged up to myeloma because of plasma cell infiltration (red curve), FLC-ratio (blue), bone lesion (green), or focal lesions in MRI (turquoise), respectively. **B.** TTP for patients termed smoldering multiple myeloma (SMM) according to the IMWG classification vs. all other patients.

3.1.3 Molecular characteristics

The **gene expression-based predictor** for asymptomatic myeloma (HDAMM) developed within this dissertation delineates three groups of patients with significantly different TTP (median TTP: not reached vs. 2.87 vs. 0.97 years) and progression rates of 6%, 37%, and 93% after 2 years, as well as 12%, 65%, and 100% after 5 years, respectively (P<.001 each; Figure 3.7A). It likewise confers prognosis in an independent group of therapy-requiring patients (Figure 3.7B). The other way round, risk scores for overall survival of symptomatic patients (UAMS70-gene score, Rs-score) predict progression of AMM-patients (Figure 3.7C). The IFM15- and EMC92-gene score do not convey prognostic significance. A median/high vs. low HDAMM-score predicts evolution of AMM from MGUS (7/15 vs. 3/44 patients progressing, P=.004), indicating a comparable underlying mechanism.

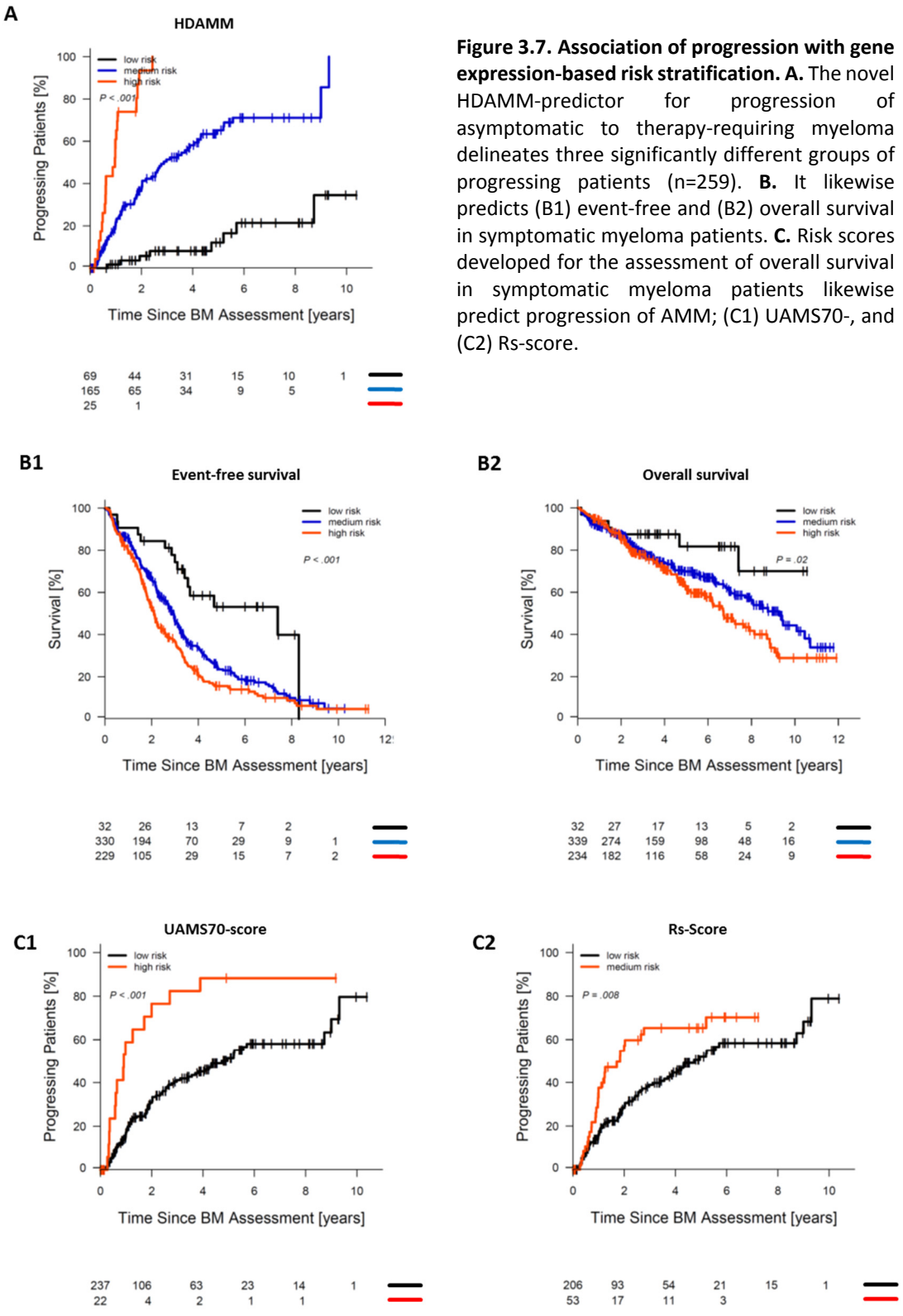


Figure 3.7. Association of progression with gene expression-based risk stratification. A. The novel HDAMM-predictor for progression of asymptomatic to therapy-requiring myeloma delineates three significantly different groups of progressing patients (n=259). **B.** It likewise predicts (B1) event-free and (B2) overall survival in symptomatic myeloma patients. **C.** Risk scores developed for the assessment of overall survival in symptomatic myeloma patients likewise predict progression of AMM; (C1) UAMS70-, and (C2) Rs-score.

Table 3.1. Gene expression-based risk-scores and classifications. Depicted are differences between monoclonal gammopathy of unknown significance (MGUS) and asymptomatic myeloma (AMM) as well as AMM and therapy-requiring myeloma (MM) for gene expression-based assessment of proliferation (GPI), risk assessment (HDAMM-, UAMS70-, and Rs-score), as well as gene expression-based classifications of multiple myeloma, respectively. P-values refer to the respective comparisons. Significant P-values are depicted in red.

	MGUS		Fisher test MGUS vs. AMM	AMM		Fisher test AMM vs. MM	MM	
Number of patients	**59**	**%**	***P*-value**	**259**	**%**	***P*-value**	**588**	**%**
Gene expression-based proliferation index								
high risk	0	0.0%		0	0.0%	**<0.001**	56	9.5%
medium risk	7	11.9%	0.075	62	24.0%	**<0.001**	284	48.3%
low risk	52	88.1%		197	76.4%	**<0.001**	248	42.2%
Risk scores								
HDAMM								
high risk	1	1.7%	0.061	25	9.7%	**<0.001**	232	39.5%
medium risk	14	23.7%	**<0.001**	165	64.0%	**0.029**	327	55.6%
low risk	44	74.6%	**<0.001**	69	26.7%	**<0.001**	29	4.9%
UAMS70 score								
high risk	2	3.4%	0.274	22	8.5%	**<0.001**	154	26.2%
low risk	57	96.6%		237	91.9%		434	73.8%
Rs score								
high risk	0	0.0%	1.0	1	0.4%	**<0.001**	55	9.3%
medium risk	7	11.9%	0.193	52	20.1%	**<0.001**	282	48.0%
low risk	52	88.1%	0.143	206	79.5%	**<0.001**	251	42.7%
Classifications								
Molecular classification								
CD-1	0	0.0%	1.0	3	1.2%	**0.009**	28	4.8%
CD-2	16	27.1%	0.869	66	25.6%	**0.035**	111	18.9%
HY	8	13.6%	0.06	67	26.0%	0.558	165	28.1%
LB	22	37.3%	0.157	72	27.9%	**<0.001**	97	16.5%
MF	9	15.3%	0.072	19	7.4%	**0.022**	21	3.6%
MS	4	6.8%	0.263	32	12.4%	0.661	80	13.6%
PR	0	0.0%	1.0	0	0.0%	**0.547**	86	14.6%
TC-classification								
11q13	5	8.5%	0.078	47	18.2%	0.924	110	18.7%
6p21	1	1.7%	0.562	3	1.2%	0.764	10	1.7%
D1	10	16.9%	0.052	77	29.8%	**0.029**	221	37.6%
D1 + D2	1	1.7%	0.213	17	6.6%	0.573	47	8.0%
D2	14	23.7%	0.469	49	19.0%	**0.003**	65	11.1%
FGFR3	4	6.8%	0.356	31	12.0%	0.822	75	12.8%
MAF	17	28.8%	**0.002**	30	11.6%	0.122	48	8.2%
none	7	11.9%	**0.002**	5	1.9%	1.0	12	2.0%
EC-classification								
EC11	17	28.8%	0.756	83	32.2%	**0.045**	232	39.5%
EC12	9	15.3%	0.289	58	22.5%	0.586	122	20.7%
EC21	29	49.2%	0.053	91	35.3%	0.051	166	28.2%
EC22	4	6.8%	0.475	27	10.5%	0.723	68	11.6%

Regarding impact on progression, three types of **chromosomal aberrations** can be distinguished:

I. Progression-associated aberrations
   - The IgH-translocation t(4;14)
   - Deletions of 13q14, 17p13, and 8p21
   - Gains of 1q21
   - Hyperdiploidy (HRD) and related aberrations (i.e. gains of 5q31/5q35, 9q34, 11q13, 11q22/11q23, 5q22, and 19q13)

II. Aberrations inversely associated with progression
   - The IgH-translocation t(11;14)

III. "Neutral" aberrations in terms of progression
   - The IgH-translocation t(14;16)
   - Presence of (any) IgH-rearrangement

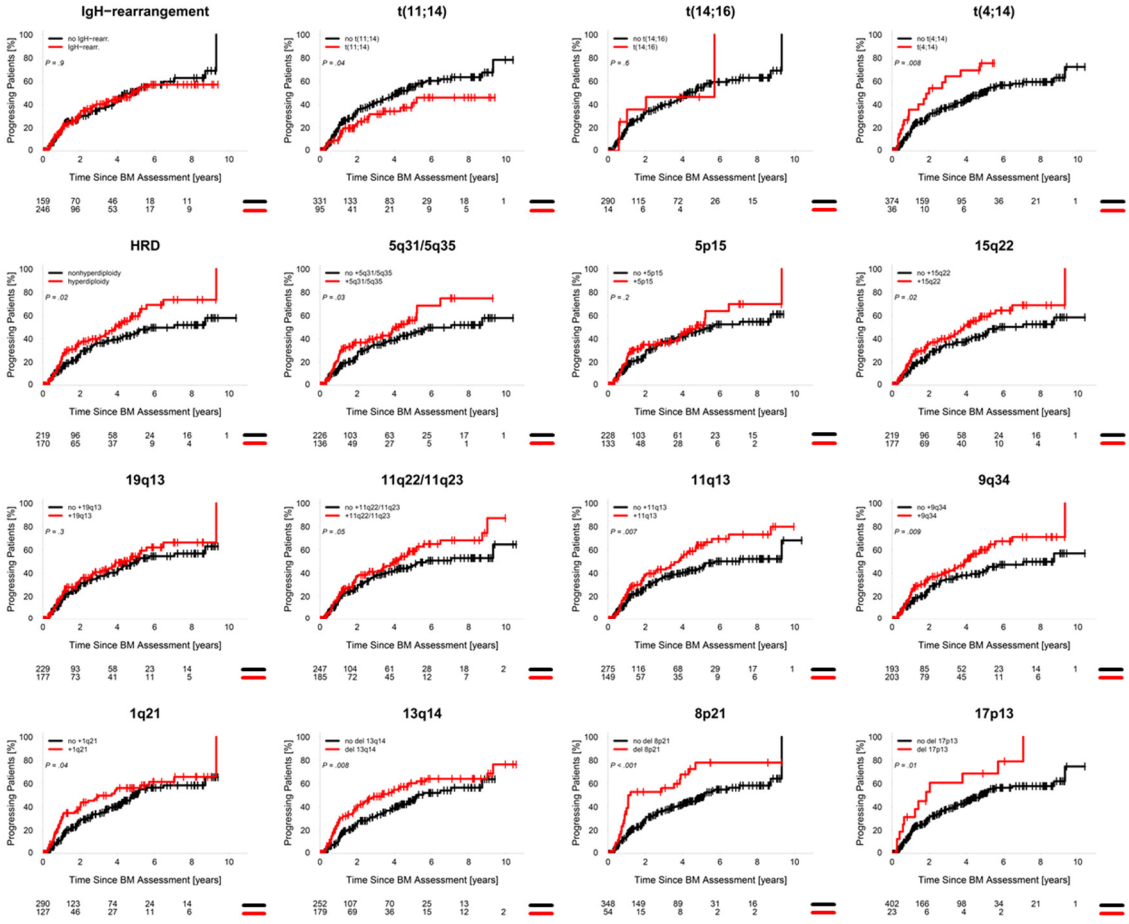


Figure 3.8. Association of progression with chromosomal aberrations. The aberrations gain of 1q21, deletion of 13q14, 17p13, and 8p21, HRD and related aberrations (5p15, 5q31/5q35, 15q22, 19q13, 11q13, 11q22/11q23, 9q34), as well as the translocation t(4;14) are associated with faster progression from asymptomatic to symptomatic myeloma; t(11;14) is associated with slower progression, and presence of any IgH-translocation (IgH-rearrangement) is not associated.

Next it was assessed whether the number of progression-associated aberrations impacts on TTP, which is the case, i.e. a 2- and 5-year progression rate for presence of 0 vs. 1-3 vs. >3 progression-associated aberrations of 16%, 33%, and 79%, as well as 39%, 52%, and 100%, respectively, with hazard ratios of 1.7 and 8.5, respectively (Figure 3.9A, Table 3.3). As in symptomatic myeloma, progression-associated aberrations are associated with each other (Figure 3.9B).

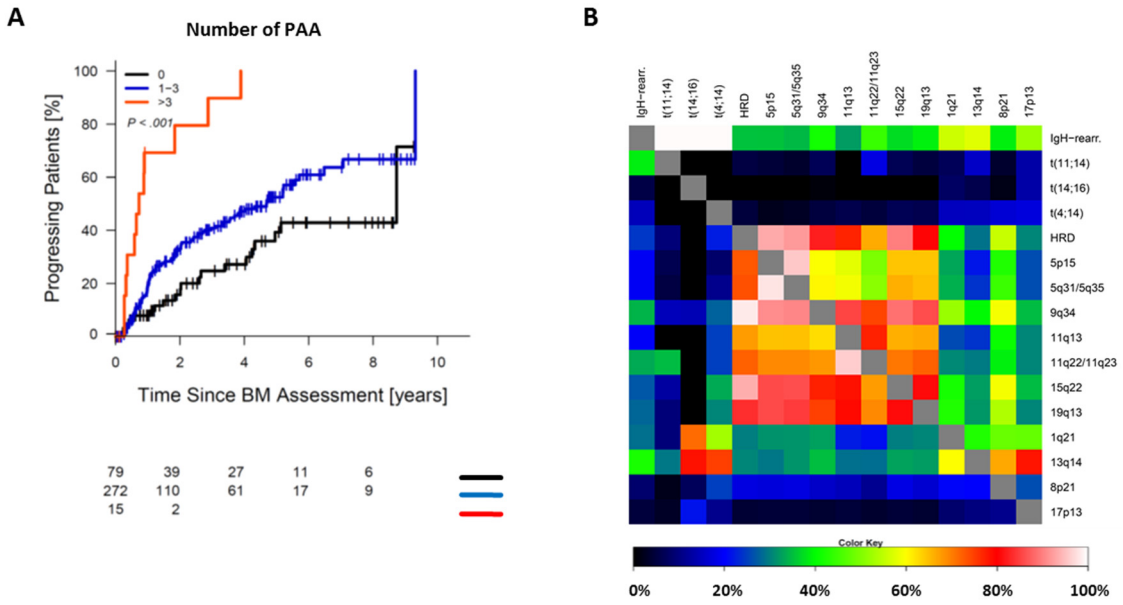


Figure 3.9. Association of progression-associated chromosomal aberrations. A. Aberrations act additively on progression. Number of progression-associated aberrations (PAA), red curve >3, blue curve 1-3, black curve - none. Subsequent groups shows a significantly different progression rate, i.e. P<.001 (>3 vs. 1-3), P=0.02 (1-3 vs. 0), respectively. **B.** Heatmap depicting color coded percentage of simultaneous presence of recurrent chromosomal aberrations. Within each column, the overlap of the named aberration with any of the other aberrations is depicted from 0% (black) to 100% (white). For example, if a deletion 17p13 is present (last column), in 80% of samples simultaneously a deletion of 13q14 can be detected (3rd but last row), red spot.

Concluding this paragraph, it was analyzed whether determinants drive progression via different paths, i.e. bone disease vs. other, tumor-mass related causes (e.g. anemia). The latter is more frequent if higher accumulation rate and/or initially higher tumor mass are present, most strikingly for doubling time of <2 vs. ≥16, a plasma cell infiltration <10% vs. ≥60%, or FLC ratio ≥100. Patients presenting with one osteolytic lesion or focal lesions both progress with 87% probability due to bone lesions (Figure 3.10, Table 3.2).

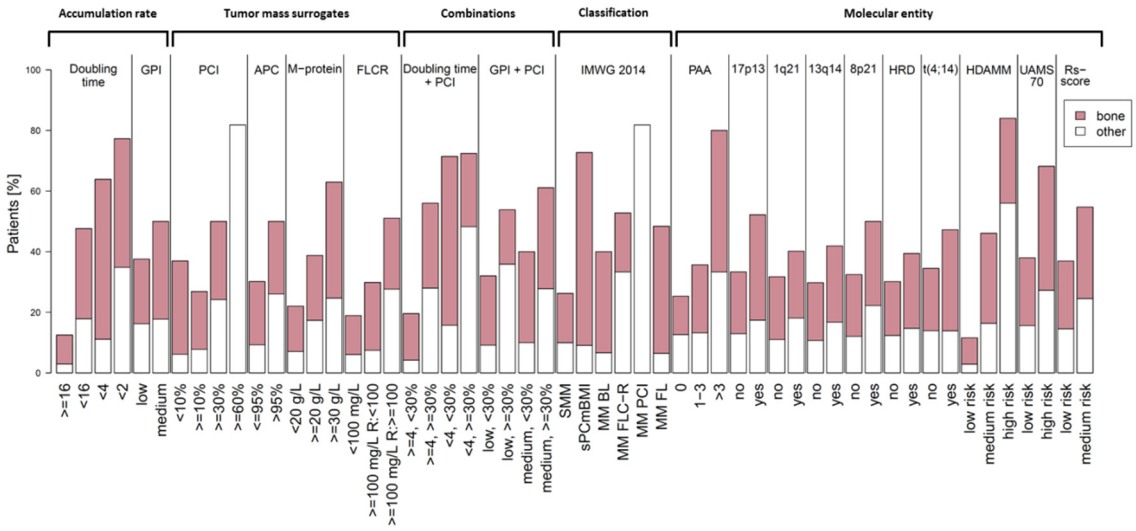


Figure 3.10. Paths of progression. Progression "happens" via two main paths, i) development of bone disease (red area in columns) in 62% of progressing patients, and ii) increasing tumor mass (e.g. displacing hematopoiesis; white area in columns) in 38%. This overall ratio varies with accumulation rate, tumor mass, and molecular entity. The heights of columns correspond to the fraction of progressing patients. For a numerical depiction, see Table 3.2.

Table 3.2. Paths of progression. For a graphical depiction, please refer to Figure 3.10. Significant P-values are depicted in red. PCI, plasma cell infiltration.

Variable	Level	n all	n event	Bone disease		Other causes		P-value
				n	[%]	n	[%]	
Plasma cell accumulation								
GPI	low	197	74	42	56.8%	32	43.2%	0.519
	medium	62	31	20	64.5%	11	35.5%	0.519
Doubling time	>=16	136	17	13	76.5%	4	23.5%	0.415
	<16	84	40	25	62.5%	15	37.5%	0.697
	<4	36	23	19	82.6%	4	17.4%	0.057
	<2	66	51	28	54.9%	23	45.1%	0.063
Tumor mass surrogates								
Plasma cell infiltration	<10%	65	24	20	83.3%	4	16.7%	**0.036**
	>=10%	283	76	54	71.1%	22	28.9%	0.079
	>=30%	62	31	16	51.6%	15	48.4%	0.136
	>=60%	11	9	0	0.0%	9	100.0%	**<0.001**
Free light chain ratio	<100 mg/L	148	28	19	67,9%	9	32,1%	0.618
	>=100 mg/L, R:<100	67	20	15	75,0%	5	25,0%	0.277
	>=100 mg/L, R:>=100	46	23	11	47,8%	12	52,2%	0.069
IMWG 2014 classification	SMM	301	79	49	62.0%	30	38.0%	1
	sPCmBMI	22	16	14	87.5%	2	12.5%	**0.030**
	MM bone lesion	30	12	10	83.3%	2	16.7%	0.133
	MM FLC-R	36	19	7	38.6%	12	63.2%	**0.022**
	MM PCI	11	9	0	0.0%	9	100.0%	**<0.001**
	MM focal lesions	31	15	13	86.7%	2	13.3%	**0.049**
Percentage clonal plasma cells	<=95%	335	101	70	69.3%	31	30.7%	**0.018**
	>95%	96	48	23	47.9%	25	52.1%	**0.018**
Monoclonal protein	<20 g/L	227	50	34	68.0%	16	32.0%	0.281
	>=20 g/L	98	38	21	55.3%	17	44.7%	0.335
	>=30 g/L	81	51	31	60.8%	20	39.2%	0.858
Combinations								
GPI + PCI	low, <30%	153	49	35	71.4%	14	28.6%	0.061
	medium, <30%	40	16	12	75.0%	4	25.0%	**0.004**
	low, >=30%	39	21	7	33.3%	14	66.7%	0.274
	medium, >=30%	18	11	6	54.5%	5	45.5%	0.744
Doubling time + PCI	>=4, <30%	189	37	29	78.4%	8	21.6%	0.096
	>=4, >=30%	25	14	7	50.0%	7	50.0%	0.224
	<4, <30%	70	50	39	78.0%	11	22.0%	**0.049**
	<4, >=30%	29	21	7	33.3%	14	66.7%	**<0.001**
Molecular entities								
Progression-associated aberrations	0	79	20	10	50.0%	10	50.0%	0.327
	1-3	272	97	61	62.9%	36	37.1%	0.405
	>3	15	12	7	58.3%	5	41.7%	1
Deletion 17p13	no	402	134	82	61.2%	52	38.8%	0.768
	yes	23	12	8	66.7%	4	33.3%	0.768
Gain 1q21	no	290	92	60	65.2%	32	34.8%	0.282
	yes	127	51	28	54.9%	23	45.1%	0.282
Deletion 13q14	no	252	75	48	64.0%	27	36.0%	0.737
	yes	179	75	45	60.0%	30	40.0%	0.737
Deletion 8p21	no	348	113	71	62.8%	42	37.2%	0.515
	yes	54	27	15	55.6%	12	44.4%	0.515
HRD	no	219	66	39	59.1%	27	40.9%	0.724
	yes	170	67	42	62.7%	26	37.3%	0.724
t(4;14)	no	374	129	77	59.7%	52	40.3%	0.440
	yes	36	17	12	70.6%	5	29.4%	0.440
HDAMM	low risk	69	8	6	75.0%	2	25.0%	0.467
	medium risk	165	76	49	64.5%	27	35.5%	0.079
	high risk	25	21	7	33.3%	14	66.7%	**0.012**
UAMS70 score	low risk	237	90	53	58.9%	37	41.1%	1
	high risk	22	15	9	60.0%	6	40.0%	1
Rs score	low risk	206	76	46	60.5%	30	39.5%	0.661
	medium risk	53	29	16	55.2%	13	44.8%	0.661

Figure 3.11, as well as Tables 3.3 and 3.4 summarize determinants of progression grouped biologically in plasma cell accumulation rate (DT and GPI), tumor mass (here: bone marrow plasma cell infiltration, percentage of aberrant plasma cells in iFISH, and involved:uninvolved FLC ratio), as well as combinations of both, and as molecular characteristics in terms of presence of progression-associated chromosomal aberrations and gene expression-based risk scores.

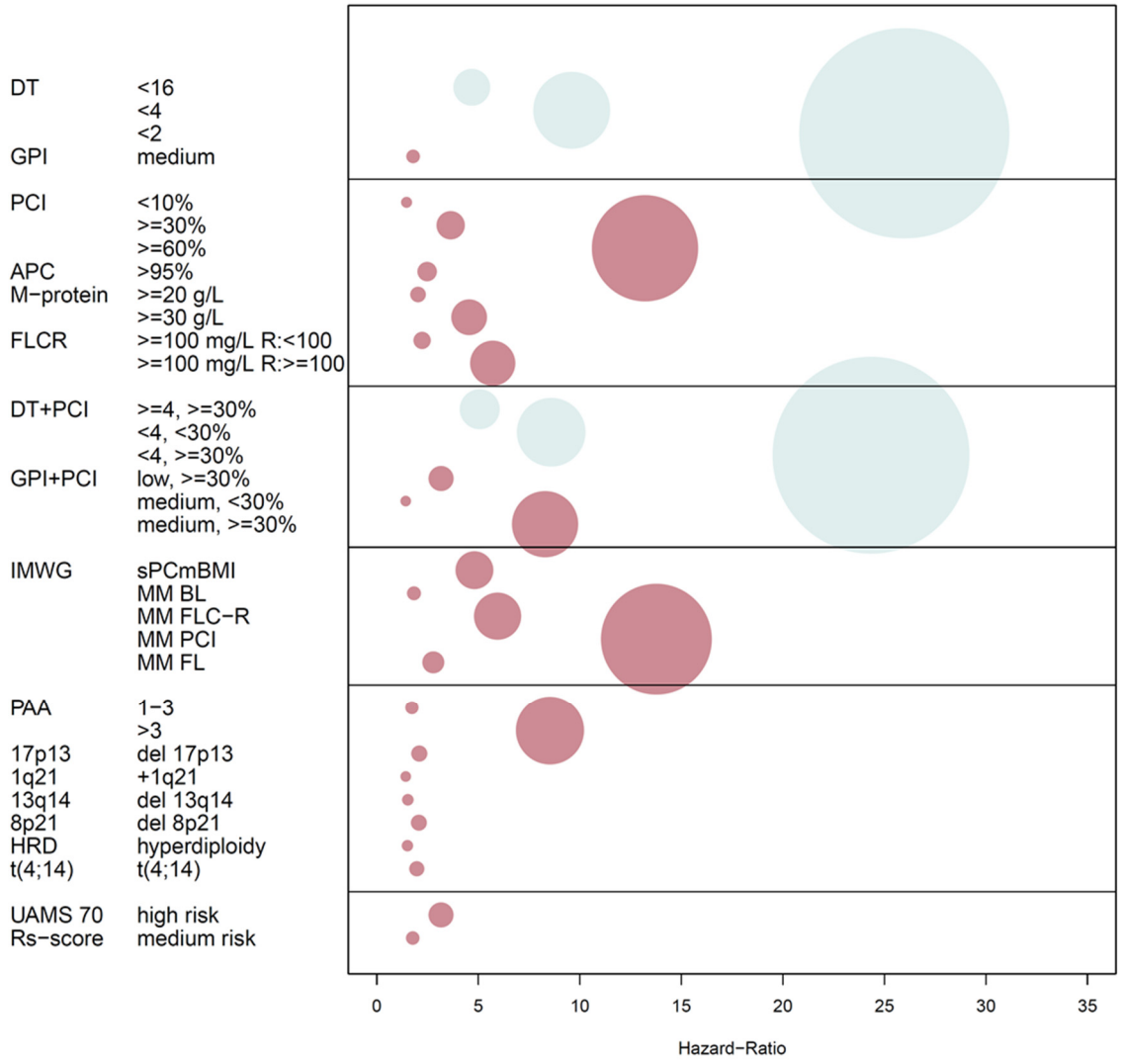


Figure 3.11. Impact of factors associated with progression to symptomatic myeloma. Bubble plot depicting the relation of factors associated with disease progression. The size of the bubble depicts the hazard ratio regarding presence/absence of the respective factor. Of note, as the doubling time is based on prospectively collected serum/urine samples and is not known at the beginning of the follow up period, it should not be interpreted as prognostic factor in statistical sense but a biological variable determining progression (see sections 3.1.1 and 4.1.1 for details). Thus, bubbles including DT are depicted in different color (gray). DT, doubling time. GPI, gene expression-based proliferation index. PCI, plasma cell infiltration. APC, percentage of aberrant (clonal) plasma cells in iFISH. FLCR, free light-chain ratio. IMWG classification: sPCmBMI, solitary plasmocytoma with minimal bone marrow involvement; MM, multiple myeloma because of BL (bone lesions); FLC-R, free light-chain ratio; PCI, plasma cell infiltration; FL, focal lesions. PAA, progression-associated chromosomal aberrations.

Table 3.3. Univariate analysis. Significant P-values are depicted in red. HR, hazard ratio. CI, confidence interval. PCI, plasma cell infiltration.

Variable	Level	HR	CI	*P*-value	Events	Number of patients	TTP rate at 2 years [%]	TTP rate at 5 years [%]	Median TTP
Accumulation Rate									
GPI	low				74	259	30	51	4.96
	medium	1.79	1.2 - 2.7	**0.007**	31	259	49	67	2.03
Doubling time (DT)	>=16				17	322	9	15	NA
	<16	4.68	2.6 - 8.3	**<0.001**	40	322	20	56	4.7
	<4	9.55	5.1 - 18.1	**<0.001**	23	322	44	83	2.17
	<2	25.84	14.4 - 46.4	**<0.001**	51	322	80	NA	0.87
Tumor mass surrogates									
Plasma cell infiltration	>=10%				76	421	21	39	7.07
	<10%	1.47	0.9 - 2.3	0.098	24	421	30	57	4.24
	>=30%	3.64	2.4 - 5.6	**<0.001**	31	421	57	86	1.79
	>=60%	13.21	6.4 - 27.2	**<0.001**	9	421	NA	NA	0.62
Free light chain ratio	<100 mg/L				28	261	18	33	NA
	>=100 mg/L R:<100	2.23	1.3 - 4	**0.006**	20	261	32	76	3.54
	>=100 mg/L R:>=100	5.6	3.2 - 9.8	**<0.001**	23	261	72	91	1.21
IMWG 2014 classification	SMM				79	431	20	39	8.73
	sPCmBMI	4.81	2.8 - 8.3	**<0.001**	16	431	69	94	1.81
	MM bone lesion(s)	1.83	1 - 3.4	0.051	12	431	43	54	2.17
	MM free light-chain ratio	5.95	3.6 - 10	**<0.001**	19	431	69	94	1.03
	MM plasma cell infiltration	13.77	6.7 - 28.3	**<0.001**	9	431	NA	NA	0.62
	MM focal lesions	2.79	1.6 - 4.9	**<0.001**	15	431	35	87	3.54
Monoclonal protein	<20 g/L				50	406	15	35	9.0
	>=20 g/L	2.03	1.3 - 3.1	**0.001**	38	406	34	55	4.02
	>=30 g/L	4.55	3.1 - 6.8	**<0.001**	51	406	61	86	1.73
Immunoparesis	0				32	424	20	36	8.73
	1 or 2	1.99	1.3 - 2.9	**<0.001**	116	424	36	59	3.79
Percentage clonal plasma cells	<=95%				101	431	24	45	5.71
	>95%	2.49	1.8 - 3.5	**<0.001**	48	431	57	79	1.81
Kyle model	low tumor mass				93	403	22	43	8.73
	high tumor mass	3.31	2.3 - 4.8	**<0.001**	40	403	30	84	1.73
Mayo model	1				61	391	21	37	9.0
	2	2.93	2 - 4.2	**<0.001**	61	391	49	75	2.01
	3	3.88	2.1 - 7.1	**<0.001**	13	391	55	85	1.91
ISS	1				118	401	31	52	4.77
	2+3	1.32	0.8 - 2.1	0.232	22	401	41	64	3.54
Combinations									
GPI +PCI	low, <30%				49	250	21	42	5.71
	low, >=30%	3.17	1.9 - 5.3	<0.001	21	250	64	80	1.79
	medium, <30%	1.43	0.8 - 2.5	0.214	16	250	33	52	4.33
	medium, >=30%	8.29	4.2 - 16.5	<0.001	11	250	80	NA	0.88
GPI + M-Protein	low, <30 g/L				38	246	18	35	9.0
	low, >=30 g/L	4.21	2.6 - 6.8	**<0.001**	34	246	62	87	1.74
	medium, <30 g/L	2.12	1.2 - 3.7	**0.008**	19	246	37	55	3.56
	medium, >=30 g/L	7.46	3.4 - 16.4	**<0.001**	8	246	75	NA	1.22
GPI + percentage clonal plasma cells	low, <=95%				47	258	23	44	5.57
	low, >95%	1.79	1.1 - 2.9	**0.019**	26	258	48	69	2.77
	medium, <=95%	1.52	0.9 - 2.7	0.14	17	258	40	54	3.56
	medium, >95%	4.65	2.5 - 8.6	**<0.001**	14	258	69	NA	0.97
GPI + Kyle model	low, low tumor mass				42	239	19	38	9.0
	low, high tumor mass	3.87	2.3 - 6.4	**<0.001**	26	239	65	87	1.73
	medium, low tumor mass	1.93	1.1 - 3.3	0.018	19	239	38	56	3.56
	medium, high tumor mass	5.48	2.1 - 14.1	**<0.001**	5	239	60	NA	1.24
DT + PCI	>=4, <30%				37	313	7	23	NA
	>=4, >=30%	5.07	2.7 - 9.5	**<0.001**	14	313	43	77	2.43
	<4, <30%	8.59	5.5 - 13.4	**<0.001**	50	313	58	91	1.81
	<4, >=30%	24.34	13.7 - 43.3	**<0.001**	21	313	90	NA	0.66
DT + M-Protein	>=4, <30 g/L				29	307	5	20	NA
	>=4, >=30 g/L	6.51	3.8 - 11.2	**<0.001**	26	307	43	78	2.33
	<4, <30 g/L	10.91	6.7 - 17.6	**<0.001**	50	307	59	93	1.71
	<4, >=30 g/L	17.61	9.6 - 32.2	**<0.001**	19	307	84	95	0.82
DT + percentage clonal plasma cells	>=4, <=95%				41	321	8	26	NA
	>=4, >95%	3.39	1.9 - 6.1	**<0.001**	16	321	42	68	2.77
	<4, <=95%	8.96	5.8 - 13.9	**<0.001**	50	321	65	95	1.15
	<4, >95%	8.97	5.3 - 15.2	**<0.001**	23	321	71	90	0.97
DT + Kyle model	>=4, low tumor mass				31	303	6	21	NA
	>=4, high tumor mass	5.33	3 - 9.5	**<0.001**	19	303	41	72	2.55
	<4, low tumor mass	10.11	6.3 - 16.1	**<0.001**	51	303	59	91	1.81
	<4, high tumor mass	25.11	13.2 - 47.9	**<0.001**	17	303	87	NA	0.98

Table 3.3 ff. Univariate analysis.

Molecular entities									
Number of progression-associated aberrations	0				20	366	16	39	8.73
	1-3	1.74	1.1 - 2.8	0.025	97	366	33	52	4.69
	>3	8.53	4.1 - 17.7	<0.001	12	366	79	NA	0.73
Deletion 17p13	no				134	425	30	52	4.73
	yes	2.09	1.2 - 3.8	0.015	12	425	53	69	1.84
Gain 1q21	no				92	417	27	51	4.96
	yes	1.43	1 - 2	0.039	51	417	40	56	3.7
Deletion 13q14	no				75	431	25	46	5.22
	yes	1.54	1.1 - 2.1	0.008	75	431	40	60	3.33
Deletion 8p21	no				113	402	28	48	5.19
	yes	2.07	1.4 - 3.2	<0.001	27	402	53	78	1.09
HRD	no				66	389	26	45	7.07
	yes	1.52	1.1 - 2.1	0.017	67	389	36	59	3.89
t(4;14)	no				129	410	30	51	4.77
	yes	1.97	1.2 - 3.3	0.009	17	410	54	75	1.99
HDAMM	low risk				8	259	6	12	NA
	medium risk	6.69	3.2 - 13.9	<0.001	76	259	37	65	2.87
	high risk	30.32	12.9 - 71.4	<0.001	21	259	93	NA	0.97
UAMS70	low risk				90	259	31	51	4.73
	high risk	3.16	1.8 - 5.5	<0.001	15	259	76	88	0.91
Rs score	low risk				76	259	28	52	4.73
	medium risk	1.78	1.2 - 2.7	0.009	29	259	57	65	1.84

Table 3.4. Multivariate analysis. Significant p-values are depicted in red. HR, hazard ratio. CI, confidence interval. PAA, progression-associated chromosomal aberration.

Variable	n	HR	CI	*P*-value
Gene expression-based proliferation index				
PCI <30% vs. >=30%	250	3.75	2.4 - 5.8	<0.001
GPI: low vs. medium	250	1.76	1.1 - 2.8	0.014
PCI <30% vs. >=30%	225	3.65	2.2 - 6.0	<0.001
GPI: low vs. medium	225	2.09	1.2 - 3.7	0.011
Number of PAA 1-3	225	2.01	1.1 - 3.7	0.028
Number of PAA >3	225	7.44	3.0 -18.6	<0.001
UAMS70 low vs. high risk	225	1.93	1 - 3.8	0.056
Rs score low vs. high risk	225	0.88	0.5 - 1.6	0.665
Doubling time				
PCI <30% vs. >=30%	294	4.56	2.8 - 7.5	<0.001
DT: >=4 vs. <4	294	6.8	4.5 - 10.2	<0.001
PCI <30% vs. >=30%	171	3.61	2.1 - 6.2	<0.001
DT: >=4 vs. <4	171	5.91	3.5 - 9.9	<0.001
Number of PAA 1-3	171	1.97	1.0 - 3.8	0.046
Number of PAA >3	171	5.14	2.0 - 13.2	<0.001
UAMS 70 low vs. high risk	171	1.27	0.6 - 2.5	0.49
Rs score low vs. high risk	171	0.84	0.5 - 1.5	0.545

3.2 Background of molecular characteristics impacting on progression

Next it was addressed in as much the impact of molecular characteristics on TTP can be explained by association with plasma cell accumulation rate and/or tumor mass. “High-risk groups” in gene expression-based risk stratifications and presence of any progression-associated aberration are associated with shorter doubling time, the latter being multiplicative regarding the number of aberrations, 0 vs. 1-3 vs. >3 (Figure 3.12).

Tumor mass surrogates, most strikingly plasma cell infiltration ≥60% or FLC ratio ≥100, are in turn significantly associated with higher DT (Figure 3.13). Figure 3.13B depicts the association of doubling time with the novel IMWG-classification.

“High-risk” groups in gene expression-based scores and progression-associated chromosomal aberrations are to a varying degree associated with higher tumor mass (Figure 3.14).

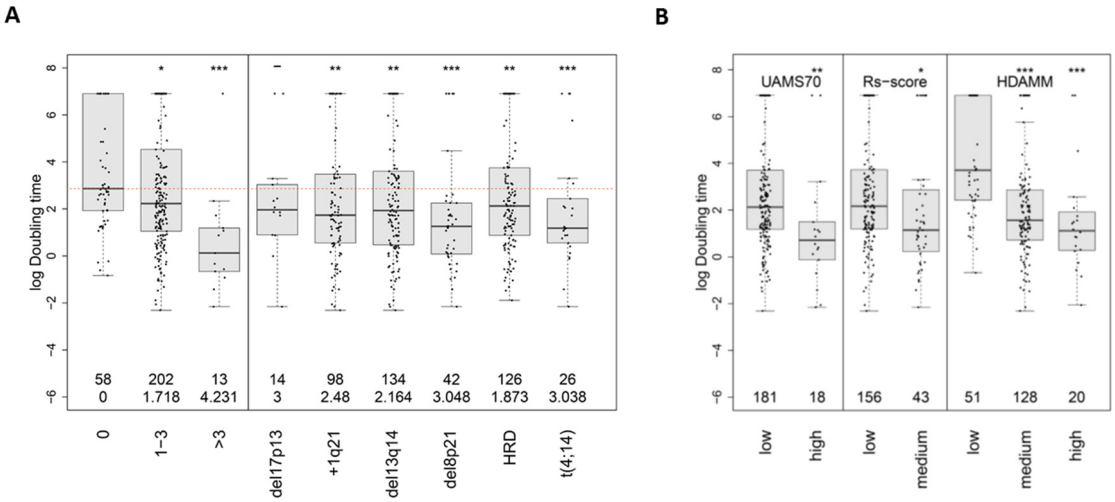
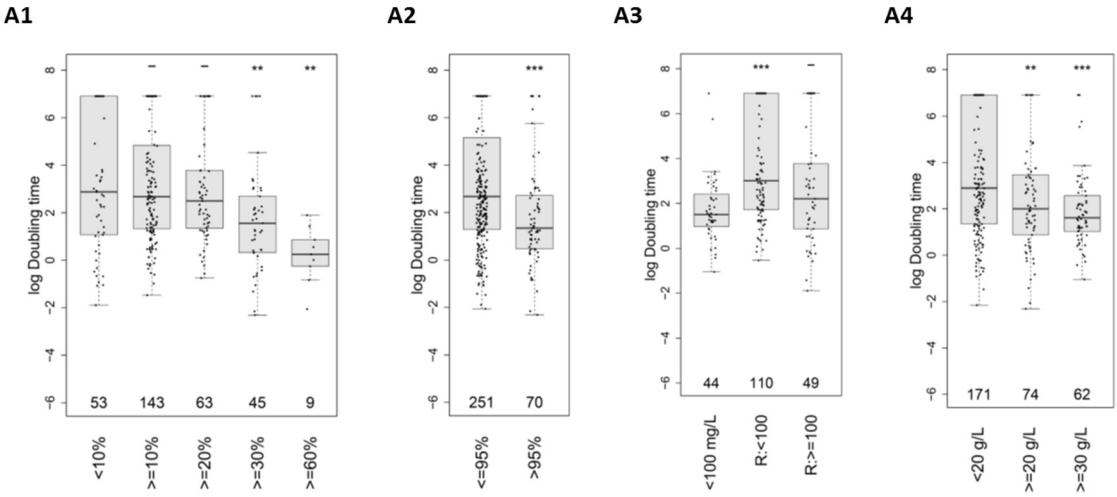


Figure 3.12. Association of chromosomal aberrations and gene expression-based risk stratifications with doubling time of malignant plasma cells. A. Progression-associated aberrations, i.e. deletions 17p13, 13q14, and 8p21, as well as gains of 1q21, hyperdiploidy (HRD) and translocation t(4;14) act multiplicatively on doubling time (P<.001, Jonckheere-Terpstra trend-test). **B.** UAMS70-gene-score, Rs-score, and HDAMM-predictor (n=199 each). At the bottom of the figure, the upper row depicts the number of patients with the respective aberration or combination of aberrations, and risk group respectively. The lower row depicts the mean number of progression-associated aberrations in the respective group. Associations of presence of different aberrations are depicted in Figure 3.9. Significant difference between the groups is depicted by one asterisk (*) for a level of P<0.05, two asterisks (**) for a level of P<0.01, and three (***) for P <0.001.



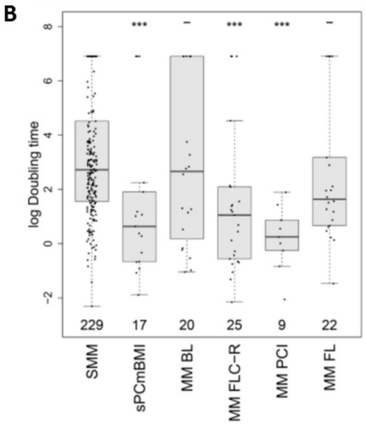
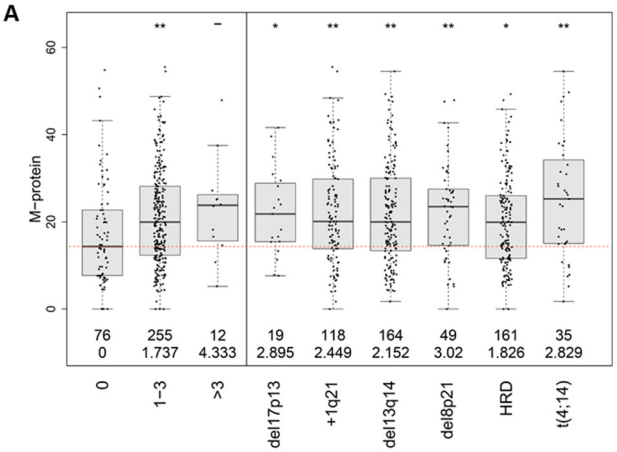


Figure 3.13. Association of doubling time with tumor mass surrogates. A. Association with (A1) bone marrow plasma cell infiltration, (A2) percentage of malignant plasma cells as assessed by iFISH, (A3) FLC ratio (involved:uninvolved), and (A4) different M-protein levels. **B.** Association of the novel IMWG-groups with doubling time. Significant difference between the groups is depicted by one asterisk (*) for a level of P<0.05, two asterisks (**) for a level of P<0.01, and three (***) for P <0.001. (S)MM, (smoldering) multiple myeloma. sPCmBMI, solitary plasmocytoma with minimal bone marrow involvement. MM due to bone lesion(s) (BL), FLC ratio ≥100 (FLC-R), plasma cell infiltration ≥60% (PCI), and focal lesions in MRI (FL).



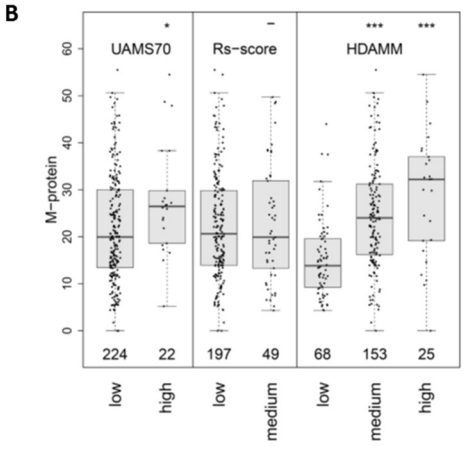


Figure 3.14. Association of chromosomal aberrations and gene expression-based risk stratifications with tumor mass, i.e. M-protein. A. Progression-associated aberrations, i.e. deletions 17p13, 13q14, and 8p21, as well as gains of 1q21, hyperdiploidy (HRD) and translocation t(4;14), as well as. **B.** "high-risk" groups in gene expression-based scores, i.e. UAMS70-gene, Rs-score, and HDAMM-predictor, are to a varying degree associated with higher tumor mass. At the bottom of the figure, the upper row depicts the number of patients with the respective aberration or combination of aberrations, and risk group, respectively. The lower row depicts the mean number of progression-associated aberrations in the respective group. Significant difference between the groups is depicted by one asterisk (*) for a level of P<0.05, two asterisks (**) for a level of P<0.01, and three (***) for P <0.001.

3.3 Mechanisms of progression and evolution of asymptomatic myeloma

Progression to symptomatic myeloma is determined by accumulation rate, tumor mass, and molecular characteristics at asymptomatic stage (section 3.1). Is it necessary to assume an ongoing genetic instability with *de novo* appearing aberrations to explain progression and evolution of AMM from MGUS? And should the plasma cell at MGUS-stage be considered already malignant, or not?

To address this, the percentages of **chromosomal aberrations** was analyzed in MGUS (n=304), asymptomatic (n=432), and symptomatic (n=1633) myeloma patients using iFISH (Table 3.5).

The pattern significantly changes from MGUS to asymptomatic to therapy-requiring myeloma in the same direction. Two basic patterns can be distinguished: First, aberrations increasing in frequency (hyperdiploidy (HRD) and related aberrations, gain of 1q21, deletion of 13q14, 17p13, and 8p21, as well as translocation t(4;14)), secondly, aberrations with constant frequency, i.e. all IgH-rearrangements and t(11;14). All aberrations associated with increasing frequency are also associated with disease progression from AMM to symptomatic myeloma (see Figure 3.8).

All (6/6) aberrations associated with disease progression are significantly less frequent in AMM vs. symptomatic myeloma and MGUS vs. AMM, whereas the fraction of “neutral” aberrations does not change (Table 3.5, Figure 3.15).

Table 3.5. Number and frequency of chromosomal aberrations in MGUS, asymptomatic, and therapy-requiring multiple myeloma. For a graphical depiction, see Figure 3.15. MGUS, monoclonal gammopathy of unknown significance. (A)MM, (asymptomatic) multiple myeloma. TL, translocation.

	MGUS	AMM	MM	MGUS	AMM	MM	MGUS	AMM	MM	MGUS	AMM	MM
	IgH-rearrangement			**t(11;14)**			**t(14;16)**			**t(4;14)**		
Number of patients	278	405	1352	294	426	1608	208	304	1090	257	410	1600
clonal gain/TL	16.2%	44.2%	53.7%	5.8%	17.8%	19.4%	3.4%	4.6%	2.8%	1.9%	8.0%	10.5%
subclonal gain/TL	46.0%	16.5%	9.2%	14.3%	4.5%	1.2%	0.5%	0.0%	0.0%	3.1%	0.7%	0.8%
normal diploid	37.8%	39.3%	37.1%	79.9%	77.7%	79.4%	96.2%	95.4%	97.2%	94.9%	91.2%	88.7%
subclonal loss	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
clonal loss	0.0%	0.0%	0.00%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	HRD			**5q31/5q35**			**5p15**			**15q22**		
Number of patients	247	389	1434	218	362	1137	219	361	1138	250	396	1469
clonal gain/TL	8.5%	29.8%	42.6%	10.6%	28.2%	39.3%	8.7%	27.7%	40.0%	11.2%	33.3%	45.3%
subclonal gain/TL	17.8%	13.9%	10.0%	16.5%	9.4%	6.2%	16.4%	9.1%	6.6%	15.2%	11.4%	7.8%
normal diploid	73.7%	56.3%	47.4%	72.5%	62.4%	52.8%	74.9%	63.2%	52.6%	73.2%	55.3%	46.8%
subclonal loss	0.0%	0.0%	0.0%	0.5%	0.0%	0.4%	0.0%	0.0%	0.4%	0.4%	0.0%	0.1%
clonal loss	0.0%	0.0%	0.0%	0.0%	0.0%	1.3%	0.0%	0.0%	0.4%	0.0%	0.0%	0.0%
	19q13			**11q22/11q23**			**11q13**			**9q34**		
Number of patients	273	406	1521	302	432	1629	291	424	1589	251	396	1462
clonal gain/TL	7.7%	27.8%	39.2%	7.3%	24.3%	40.3%	5.8%	22.9%	35.8%	10.0%	35.4%	49.2%
subclonal gain/TL	20.1%	15.8%	12.3%	16.6%	18.5%	9.0%	13.1%	12.3%	6.7%	20.3%	15.9%	10.7%
normal diploid	71.4%	56.4%	47.8%	76.2%	56.5%	49.6%	81.1%	64.9%	57.2%	69.7%	48.7%	39.9%
subclonal loss	0.7%	0.0%	0.3%	0.0%	0.5%	0.4%	0.0%	0.0%	0.1%	0.0%	0.0%	0.1%
clonal loss	0.0%	0.0%	0.5%	0.0%	0.2%	0.7%	0.0%	0.0%	0.2%	0.0%	0.0%	0.1%
	1q21			**13q14**			**8p21**			**17p13**		
Number of patients	290	417	1579	303	431	1626	255	402	1516	294	425	1602
clonal gain/TL	6.9%	20.4%	28.2%	0.0%	0.5%	0.6%	1.2%	2.5%	2.6%	1.7%	5.9%	5.2%
subclonal gain/TL	7.9%	10.1%	8.5%	0.0%	0.0%	0.4%	1.2%	0.7%	1.6%	4.8%	3.3%	2.5%
normal diploid	84.8%	68.3%	61.7%	72.6%	48.0%	51.0%	94.1%	83.3%	70.0%	92.5%	85.4%	81.2%
subclonal loss	0.3%	0.5%	0.4%	20.5%	15.8%	9.6%	2.0%	5.7%	10.3%	0.7%	3.1%	4.4%
clonal loss	0.0%	0.7%	1.1%	6.9%	25.8%	38.4%	1.6%	7.7%	15.6%	0.3%	2.4%	6.7%

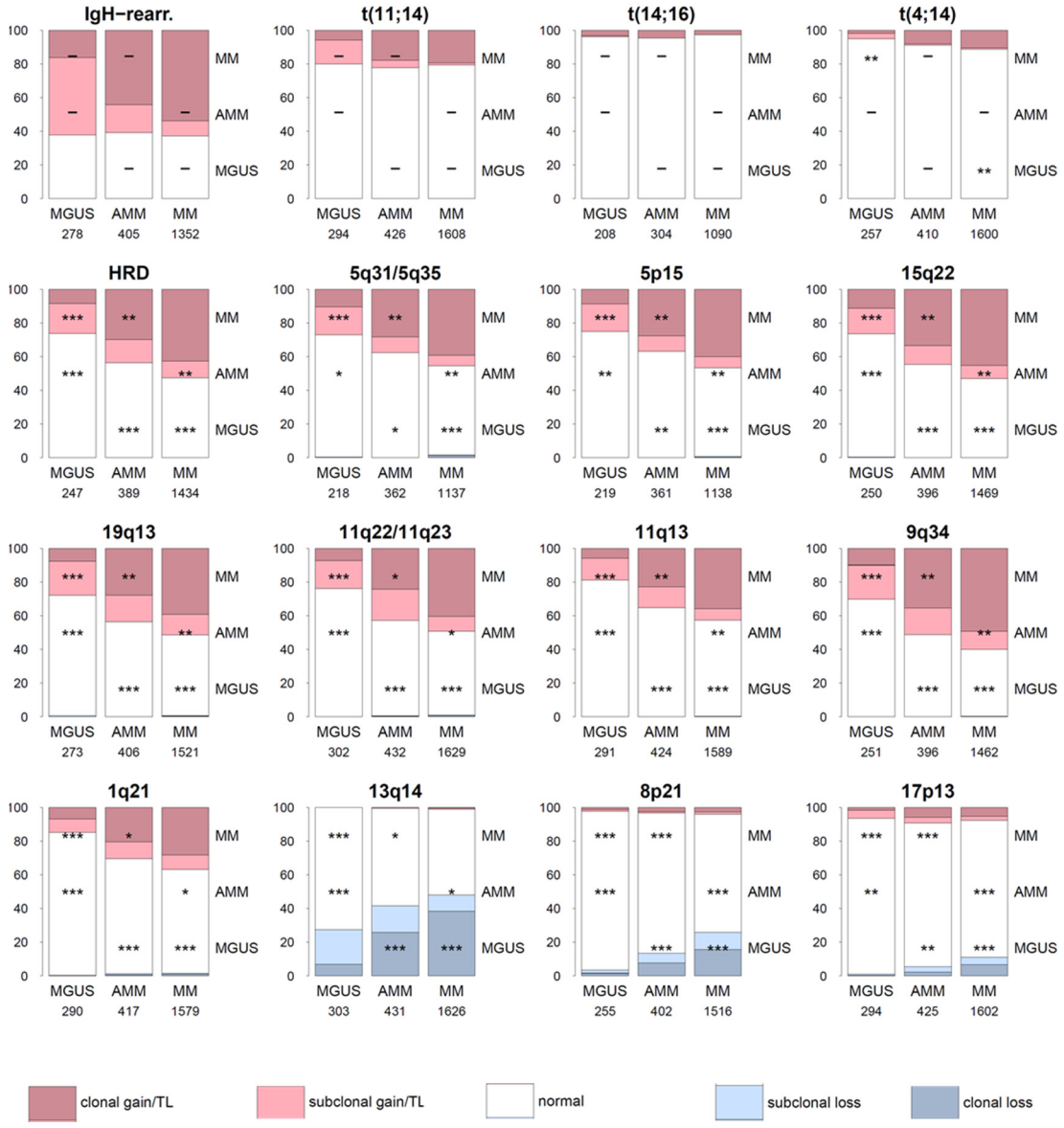


Figure 3.15. Differences in frequency of recurrent chromosomal aberrations in monoclonal gammopathy, asymptomatic and therapy-requiring multiple myeloma. Percentage of patients presenting with the respective aberration. All aberrations associated with increasing frequency are also associated with disease progression from AMM to symptomatic myeloma (see Figure 3.8). The number of patients investigated regarding the specific aberration is depicted within each column. Significant difference between the three groups is depicted by one asterisk (*) for a level of P<0.05, two asterisks (**) for a level of P<0.01, and three (***) for P <0.001. For the translocations (panel 1), white depicts the normal state (no translocation), light red - subclonal presence (>20 and <60% of myeloma cells carry the translocation), and red - clonal (≥60% of myeloma cells carry the translocation). The copy-number (panel 2 to 4) of the respective chromosomal band is coded by different colors. White - normal diploid state (two copies), light red - subclonal gain (>10 and <60% of myeloma cells carry three or more copies), red – clonal gain (≥60% of myeloma cells carry three or more copies), light blue - subclonal loss (>10 and <60% of myeloma cells carry one or less copies), dark blue - clonal loss (≥60% of myeloma cells carry one or less copies). (A)MM, (asymptomatic) multiple myeloma. For a numerical depiction, see Table 3.5.

The percentage of patients carrying at least one progression-associated aberration increases from MGUS (55%) to AMM (78%) to AMM that progressed (85%) to symptomatic myeloma (88%) (Figure 3.16). The increase in frequencies in subsequent stages is explained by association with faster progression.

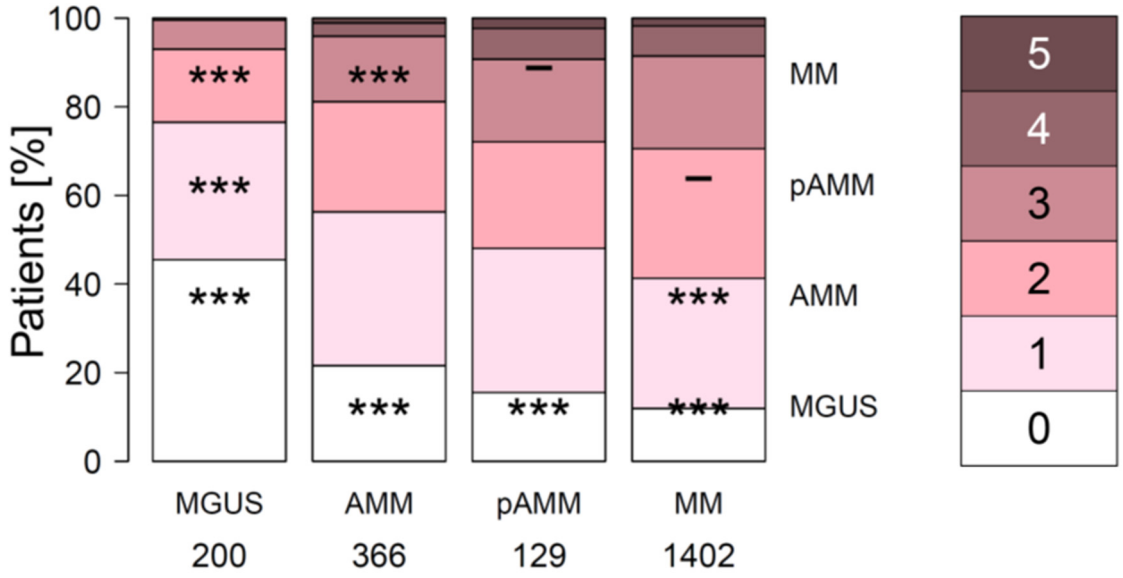


Figure 3.16. Frequency of patients carrying different numbers of progression-associated chromosomal aberrations, i.e. 1q21+, deletion of 13q14, 17p13, and 8p21, as well as HRD, and t(4;14). The color codes the number of progression-associated aberrations in five shades of red from one (lightest) to five (darkest). Total patient numbers differ compared to Table 2.1, as only patients for which all aberrations could be measured were included. pAMM, subfraction of patients with asymptomatic myeloma that progressed (n=129) showing the same pattern as symptomatic myeloma (MM) patients. Significant difference is depicted by one asterisk (*) for a level of P<0.05, two asterisks (**) for P<0.01, and three (***) for P <0.001.

Comparing **gene expression** of normal bone marrow plasma cells (n=10) with either MGUS (n=62), asymptomatic (n=259), or symptomatic myeloma (n=605), 3251, 3279, and 3537 genes were ≥2-fold differentially expressed (Figures 3.17 and 3.18, Table 3.6). The largest differences are seen between normal plasma cells and each of the three entities, not between either MGUS and asymptomatic myeloma or asymptomatic and symptomatic myeloma.

Comparing MGUS vs. AMM and AMM vs. symptomatic myeloma, observed differences for size-adjusted groups are 281 and 242 genes, 11- and 13-fold less than respective comparisons to normal plasma cells (Table 3.6). When analyzing differences within distinct molecular entities, i.e. t(11;14), t(4;14) or HRD, even fewer differences are found (Table 3.6). The bulk of changes in gene expression is thus already apparent at MGUS-stage.

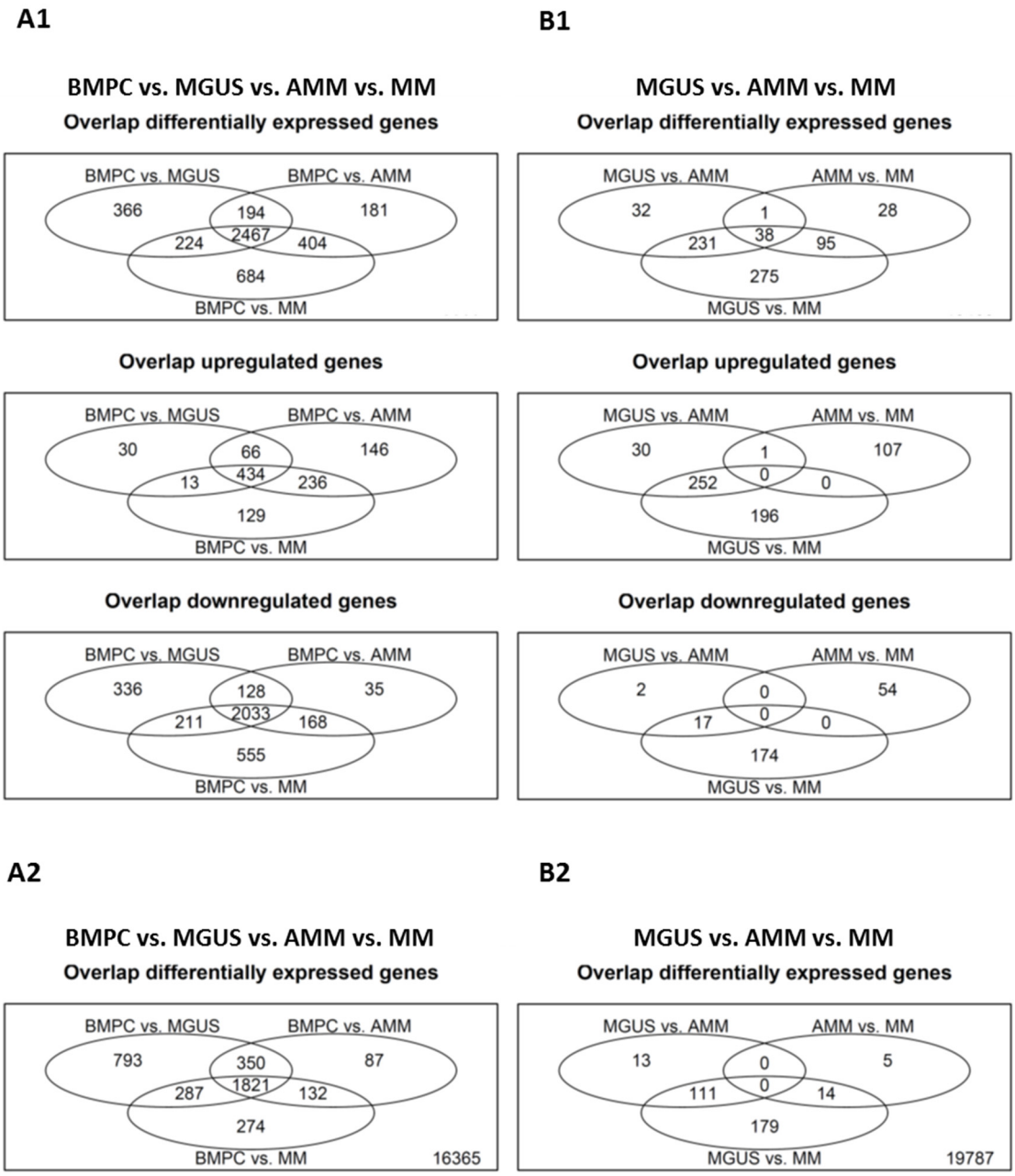


Figure 3.17. Differences in terms of gene expression (Venn-diagram). Genes significantly differentially expressed between **A.** BMPC vs. MGUS vs. AMM vs. MM, and **B.** MGUS vs. AMM vs. MM, respectively. (A1, B1) Analysis for all available patients. (A2, B2) Due to the different size of the cohorts, correction for size with 62 (i.e. the MGUS-cohort size) was performed. For the latter, the median number of 10 randomly selected sets of patients is given. BMPC, normal bone marrow plasma cells. MGUS, monoclonal gammopathy of unknown significance. (A)MM, (asymptomatic) multiple myeloma.

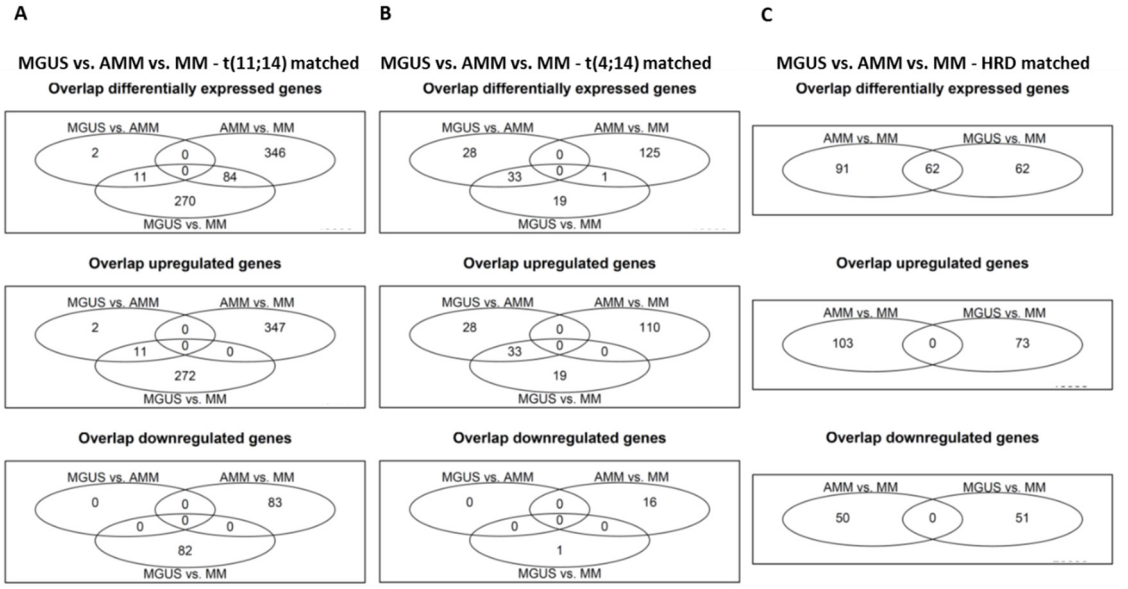


Figure 3.18. Venn-diagrams for matched samples. Comparison between matched samples regarding **A.** t(11;14), **B.** t(4;14), and **C.** hyperdiploidy (HRD) to investigate changes within a disease subentity.

In principal component analysis, the center of gravity of multiple myeloma is shifted to the direction of samples showing either “benign” (PPCs) or “malignant” proliferation (HMCLs), the one for AMM is shifted in the direction of normal plasma cells. MGUS overlaps to a large extend with AMM. Using a generalized correlation matrix for similarity assessment (RV-coefficient), a comparable result can be found (Figure 3.19).

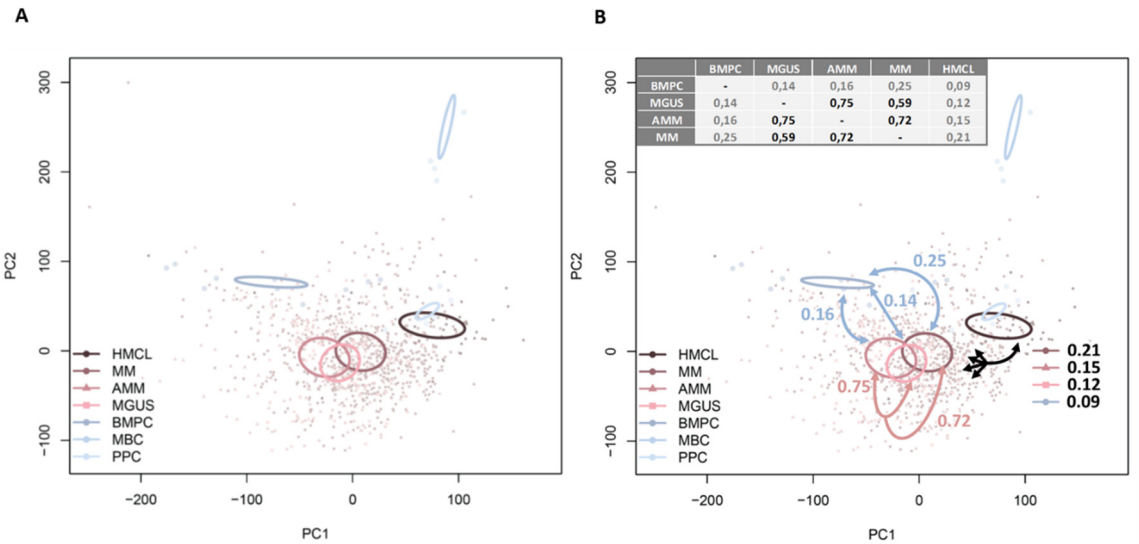


Figure 3.19. Differences in terms of gene expression. Principal component analysis of normal bone marrow plasma cells (BMPC), MGUS, AMM, and symptomatic myeloma in relation to B-memory cells (MBC), proliferating plasmablasts (PPC), and human myeloma cell lines (HMCL) overlaid by the center of gravity. **A.** The center of gravity of MM is shifted to the right, in direction of samples showing either “benign” (PPC) or “malignant” proliferation (HMCL). The center of gravity for AMM is shifted to the left, in direction of normal plasma cells. MGUS (pink) overlaps to a large extend with AMM (light red). **B.** Pairwise assessment of similarity of depicted entities using a multivariate generalization of the squared Pearson correlation coefficient (RV-coefficient). Values range from 0 (no correlation) to 1 (complete correlation). The largest differences are seen between normal plasma cells and each of the three entities.

Table 3.6. Differential gene expression. Genes differentially expressed with a fold change of ≥2 between normal bone marrow plasma cells (BMPC) and MGUS, BMPC and AMM, and BMPC and MM; as well as between plasma cell dyscrasias, i.e. MGUS and AMM, AMM and MM, and MGUS and MM. For the latter, data are also shown after correction for size of cohorts to 62, i.e. the MGUS-cohort size, and also within molecular groups, i.e. patients presenting with t(11;14), t(4;14), or hyperdiploidy (HRD), respectively. The last comparison is between 34 paired (longitudinal) AMM/MM samples.

Differential gene expression	20109 genes Number of differential expressed genes		
	total	upregulated	downregulated
BMPC vs. MGUS vs. AMM vs. MM			
BMPC vs. MGUS	3251	543	2708
BMPC vs. AMM	3246	882	2364
BMPC vs. MM	3779	812	2967
with MGUS, AMM, and MM being size corrected			
BMPC vs. MGUS	3251	543	2708
BMPC vs. AMM	3279	856	2424
BMPC vs. MM	3537	739	2798
MGUS vs. AMM vs. MM			
MGUS vs. AMM	302	283	19
AMM vs. MM	162	108	54
MGUS vs. MM	639	448	191
with MGUS vs. AMM vs. MM being size corrected			
MGUS vs. AMM	281	267	14
AMM vs. MM	242	197	45
MGUS vs. MM	619	439	180
MGUS vs. AMM vs. MM within molecular groups			
t(11;14)			
MGUS vs. AMM	13	13	0
AMM vs. MM	430	347	83
MGUS vs. MM	365	283	82
t(4;14)			
MGUS vs. AMM t(4;14)	61	61	0
AMM vs. MM t(4;14)	126	110	16
MGUS vs. MM t(4;14)	53	52	1
HRD			
MGUS vs. AMM HRD	0	0	0
AMM vs. MM HRD	153	103	50
MGUS vs. MM HRD	124	73	51
AMM vs. MM longitudinal samples			
AMM vs. MM	253	248	5

As do progression-associated chromosomal aberrations, the distribution of GEP-based progression scores significantly shifts to adverse groups from MGUS to AMM to MM (Table 3.1).

Like for iFISH and GEP-based entities, a shift can be observed for frequency of light-chain subtype increasing from MGUS/AMM (5.3%/6.0%) to 18.7% in symptomatic myeloma (P<0.001). Bence Jones myeloma is associated with shorter TTP and significantly shorter doubling time, thus faster progression, not *de novo* appearance (Figure 3.20).

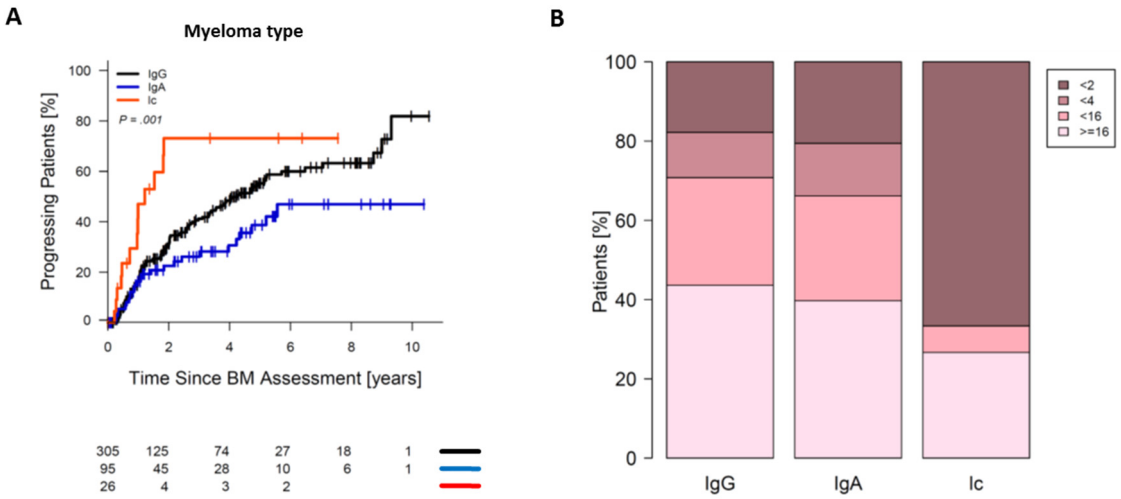


Figure 3.20. Association of progression from asymptomatic to therapy-requiring myeloma with myeloma type. A. Light-chain asymptomatic myeloma (red curve) shows a significantly faster progression to symptomatic myeloma compared to IgG- (black) or IgA-myelomas (blue) with a median TTP of 1.21 vs. 3.54 vs. NA (P<.001). **B.** Consistent with this, 67% of patients presenting with light chain myeloma appear in the group with doubling times <2 years.

Comparing 65 patients with paired iFISH samples at asymptomatic and symptomatic myeloma stage (**longitudinal cohort**), clonal dynamic, i.e. change in any of 15 investigated aberrations, was observed in 26 patients (40%), six of them clonal, and 53 subclonal. Gains and losses of progression-associated aberrations (e.g. del13q14) appear. Eleven patients (17%) showed gains or losses of progression-associated aberrations already present. Only six (9%) patients showed *de novo* appearance of a progression-associated aberration (Figure 3.21); i.e. progression mostly appears without *de novo* gain of progression-associated aberrations. It is thus not necessary to assume an ongoing genetic instability driving disease progression, as increasing frequency of progression-associated aberrations is explained by faster progression of patients carrying them (Figure 3.8).

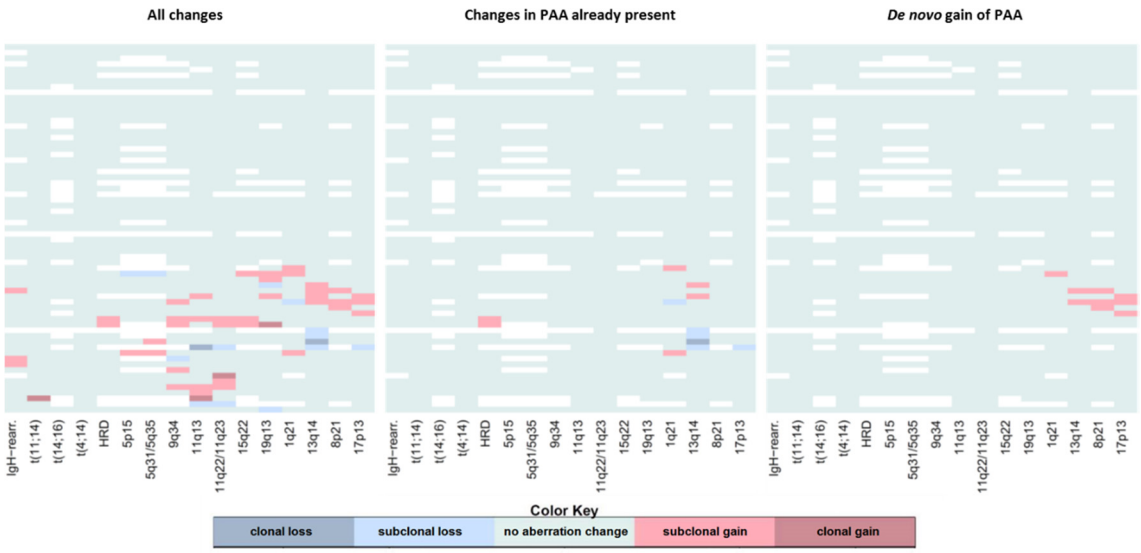


Figure 3.21. Longitudinal (paired) samples – chromosomal aberrations. Differences in terms of chromosomal aberration pattern in paired samples at asymptomatic and symptomatic myeloma stage (n=65). Left panel: Clonal dynamics. Fifty-nine changes in 26 of 65 patients were detected, 6 of them clonal, and 53 subclonal. Central panel: Changes of progression-associated aberrations (PAA) already present. Right panel: *De novo* gain of progression related aberrations can be seen in 6/65 (9%) of patients only.

In 34 of these samples, paired gene expression data were available with 253 genes showing a ≥2-fold difference in expression by paired t-test (Table 3.6). GEP-based risk-scores increased in 41%, 15%, 29%, and 24% of patients regarding GPI, UAMS70-, Rs-, and HDAMM-score, while decreasing in 15%, 6%, 3%, and 3% of patients, respectively (Figure 3.22).

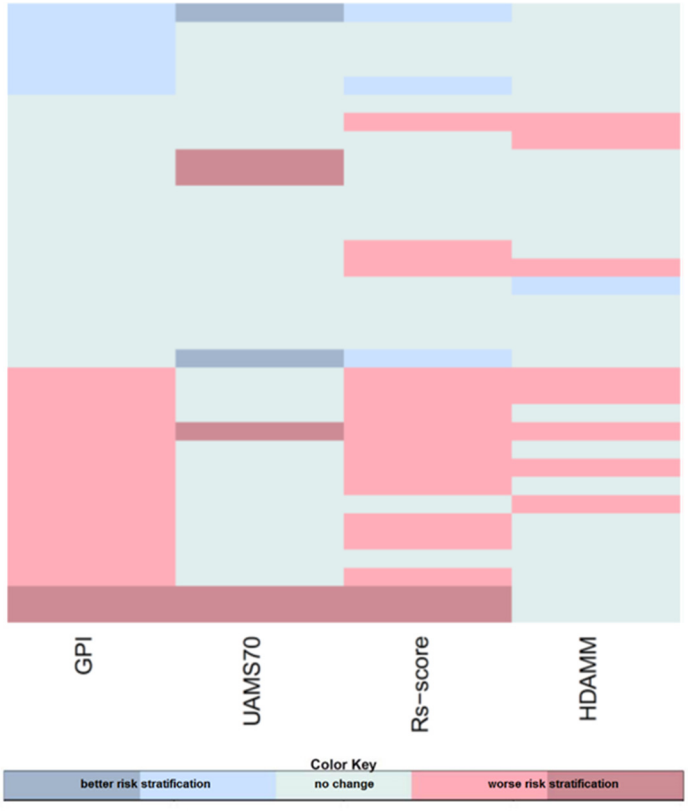


Figure 3.22. Differences in terms of gene expression-based scores in paired samples. For scores with three levels (i.e. GPI, Rs-, HDAMM-score) light red/light blue depicts a change of one level (e.g. from GPI medium to high), dark red/dark blue of two levels (e.g. from GPI low to high). For the UAMS70-score only changes by two levels can appear (i.e. from UAMS70 low to high, or vice versa).

Next, growth kinetics of MGUS and AMM (see section 3.4) regarding myeloma cell accumulation were assessed. Three main patterns can be identified: A fast increase with doubling times of <2 years and <4years, a continuously slower increase with DT between 4 and 16 years, and an infinitesimal (not or almost not visible) increase with DT of ≥16 years (Figures 3.23, 3.24).

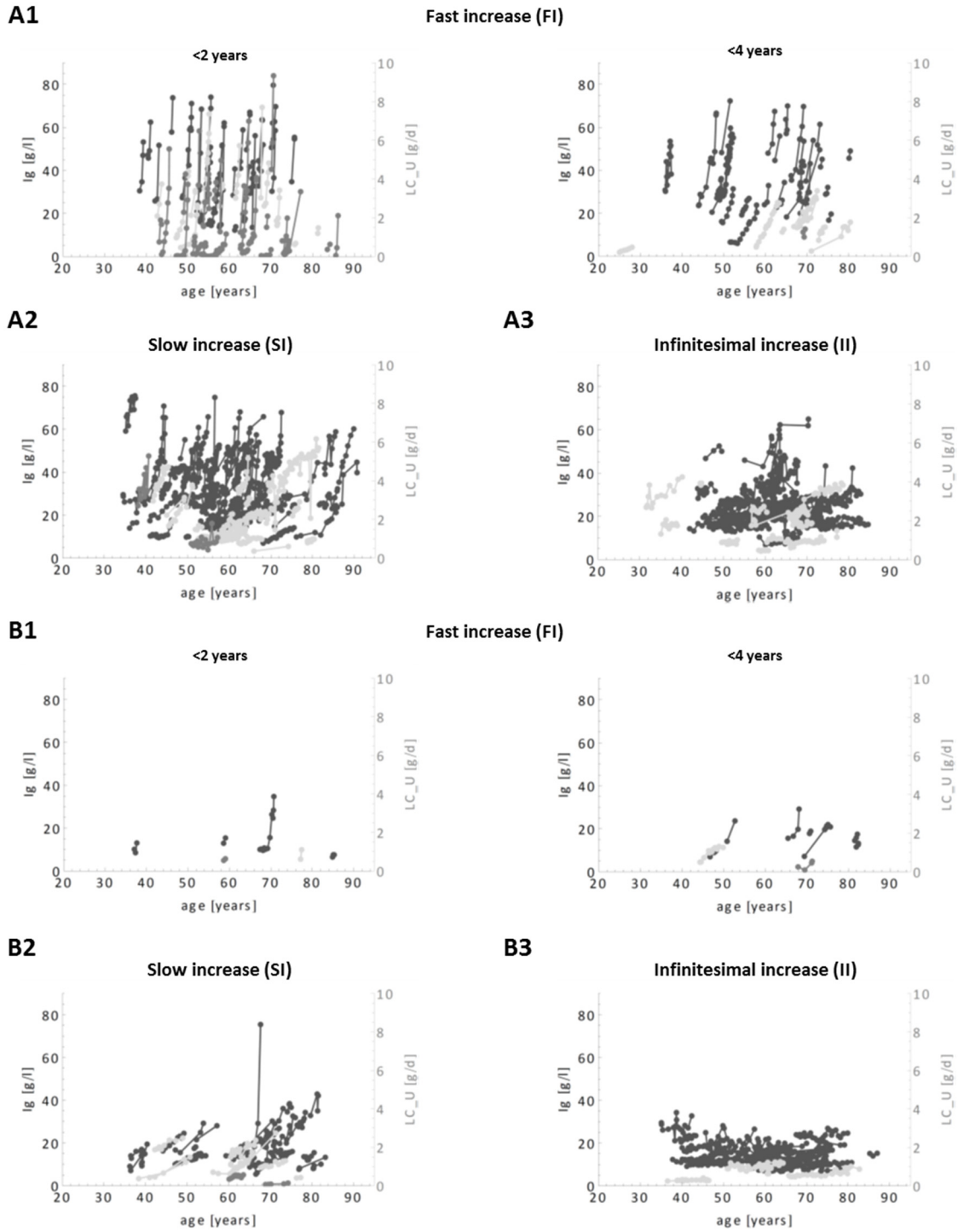


Figure 3.23. Patterns of myeloma cell accumulation in A. asymptomatic myeloma and B. monoclonal gammopathy. (A1) Fast increase (FI, 102/322, 32% of patients), (A2) continuously slower increase (SI, 84/322, 26%), and (A3) infinitesimal increase (II, 136/322, 42%). In patients with monoclonal gammopathy, (B1) 16 of 196 patients (8%) are in the FI-group, (B2) 39 show a SI-pattern (20%), and (B3) 141 of 196 patients (72%) have an infinitesimal increase. IgA, light gray; IgG, dark gray; light chains, medium gray.

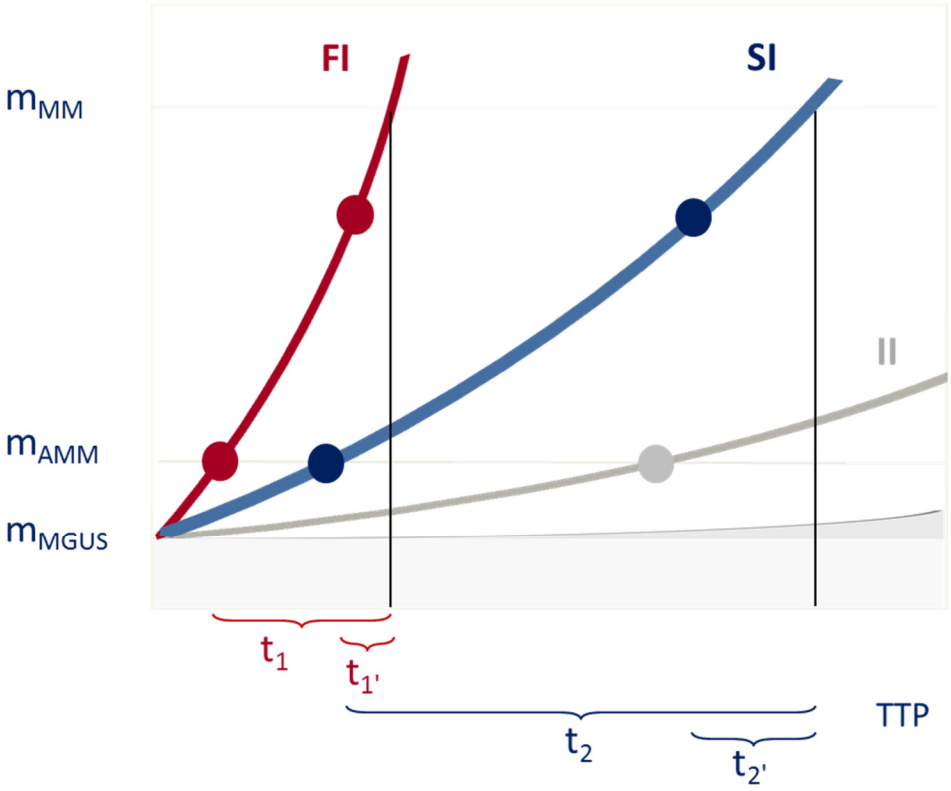


Figure 3.24. Myeloma cell accumulation. Schematic view of accumulation pattern and tumor mass (m) impacting on time to progression (TTP) to symptomatic myeloma. FI (fast increase), SI (slow increase), II- (infinitesimal increase) pattern. If higher tumor mass is present, the patient has already progressed further on his way to symptomatic myeloma, thus the time span is shorter ($t_{1'}$ vs. t_1, $t_{2'}$ vs. t_2).

3.4 Myeloma cell number (tumor mass), and number of doublings between stages

Aim of this section is to answer the following questions: i) How many myeloma cells are present at the different stages MGUS, AMM and MM? ii) How many doublings take place during MGUS-AMM and AMM-MM-transition? iii) Is it possible and likely that a *de novo* appearing aberration in MGUS or AMM drives the respective transitions -based on the observed doubling times, median times to progression and necessary number of doublings?

I) MYELOMA CELL NUMBER (TUMOR MASS) AT DIFFERENT STAGES

i) Determination of myeloma cell number. To approximate the number of myeloma cells (tumor cell mass) in our patients at different stages, two independent assessments were applied: First, using literature data on bone marrow volume and cellularity, the number of cells that fit in the bone marrow space can be calculated and the approximate number of myeloma cells be determined using the plasma cell infiltration measured. Secondly, the formula of Durie and Salmon building the quantitative basis for the respective staging system can be used[50]. The formula is based on a regression model using serum parameters and presence and extent of bone lesions and relating to the relation of individual Ig/light-chain production by myeloma cells *in vitro* and the total production thereof.

Assessment by mean bone marrow volume, cellularity, and measured plasma cell infiltration. The total red bone marrow volume has been assessed already until the 1920ies by several authors using porosity assessment to be 1.6-4.0l [reviewed e.g. by Wetzel[215]]. The cellularity has been measured e.g. by Sandkühler and Gross[185] with a mean cell count in “normal” adults of $4x\ 10^5/mm^3 = 4x\ 10^{11}/l$. Measured values for normal plasma cell numbers were $7.5x10^3/mm^3 = 7.5x10^9/l$. The (weighted) mean cell count in multiple myeloma (n=30 samples with different “sizes of myeloma cells” and corresponding different numbers) was $4.19x\ 10^{11}/l$. Taken together, the total cellular content within the bone marrow can be calculated to be around 10^{12} cells ($6.4x\ 10^{11}$ - $1.6x\ 10^{12}$ cells). The myeloma cell number can then be determined using the percentage of plasma cell (myeloma cell) infiltration.

Assessment by regression formula (Durie formula) using clinical parameters. Salmon and Durie determined the number of myeloma cells in an individual patient measuring the Ig-/light chain production by myeloma cells *in vitro* and the total *in vivo* production to $0.3 - 3.0x\ 10^{12}$ myeloma cells at diagnosis of myeloma corresponding to a tumor mass of 3kg and myeloma becoming

earliest visible in serum electrophoresis at a tumor mass of 40g (i.e. 2.5x 10^{10})[183]. They developed a regression model using clinical parameters to determine the number of myeloma cells[50†]:

Tumor mass [cells * $10^{12}/m^2$] =

0.601+0.283*OL+0.031*LCU -0.058*Hb+0.051*Ca+0.028*M-protein

With osteolyses graded in OL = 0,1,2,3, light chain excretion in urine LCU [g/24h], hemoglobin Hb [g/dl], Ca^{2+} [mg/dl], and M-protein [g/dl].

The model is however skewed at lower tumor masses (for which it was not developed) with an overestimation of myeloma cell number. It e.g. leads to a "tumor mass" of 2.4x 10^{11} in an individual considering normal laboratory values (Hb 14 g/dl, Ca^{2+} 2.24 mmol/l, no M-protein, no osteolyses).

ii) Myeloma cell number at different stages. Symptomatic myeloma. The theoretical range of total myeloma cell number can be estimated to 2x 10^{11} - 6x 10^{12} cells. This range is given by the maximum number of myeloma cells in MGUS (see below, lower limit) and a bone marrow volume completely filled with myeloma cells assessed to 6x 10^{12} using the assessment by Durie/Salmon and a body surface of $2m^2$. Using the bone marrow cellular content and the median plasma cell infiltration of 40% in our MM-cohort (n=1171; Figure 3.25A), the median number of myeloma cells totals 0.8 - 1.6x 10^{12} (depending on the estimated bone marrow volume and cellularity). The median cell number as assessed by the Durie/Salmon-formula yields a comparable result with 1.3x 10^{12} myeloma cells per m^2 (Figure 3.25B). These results are in agreement with 0.3 - 3.0x 10^{12} myeloma cells at diagnosis corresponding to a tumor mass of 3kg calculated by Salmon using the myeloma cells and total Ig/light chain production[183].

Asymptomatic myeloma. The theoretical range of total myeloma cell number can be estimated to 2x 10^{11} - 2x 10^{12} cells. The lower limit is -as for symptomatic myeloma- defined by the maximum number of myeloma cells in MGUS (see below). The upper limit can be approximated by the number of myeloma cells at which myeloma usually leads to clinical signs and symptoms, i.e. using the Durie/Salmon-formula. Whereas the differentiation between the two entities AMM and MM is not made by tumor mass but end organ damage, it is nevertheless extremely unlikely that at the maximum number of myeloma cells in the marrow (i.e. completely filling the bone marrow cavity) no signs as e.g. anemia would be present. The border regarding tumor mass is thus rather ragged between AMM and MM. Using the median plasma cell infiltration in our AMM-cohort of 15% (n=421; Figure 3.25A), a median total number of 1.5 - 3x 10^{11} myeloma

† For clarity of argumentation, this formula is depicted here instead of the methods section.

cells results. The median cell number as assessed by the Durie/Salmon-formula yields 4.2x $10^{11}/m^2$ myeloma cells, i.e. 2-3 times higher (depending on body surface), but thus in the same order of magnitude (Figure 3.25B).

MGUS. The theoretical range of total myeloma cell number can be estimated to 10^8 - 2x 10^{11}. The lower limit is given by the sensitivity of detection of monoclonal protein. Immunofixation or FLC become negative if approximately 0.1% of bone marrow cells are myeloma cells. Considering a bone marrow capacity as detailed above of 2-4l of bone marrow à 5x 10^8 cells/ml, i.e. 1 - 2x 10^{12} cells, 0.1% of these total 10^8 myeloma cells. In other words, MGUS *can* only just become apparent once there are 10^8 myeloma cells (lower limit). The upper limit is given by the definition of MGUS, i.e. a plasma cell infiltration below 10%. With the assumptions detailed above, this totals to a maximum of 2x 10^{11} cells. Using the median plasma cell infiltration in our MGUS-cohort of 5% (n=299), this totals a median number of 5x 10^{10} - 10^{11} myeloma cells.

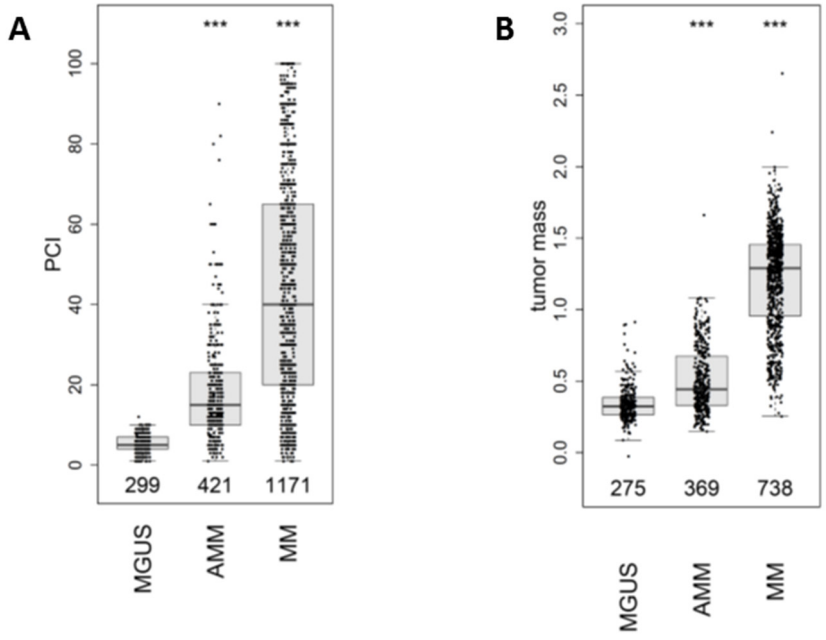


Figure 3.25. Plasma cell infiltration and tumor mass according to Durie and Salmon. A. Plasma cell infiltration in bone marrow aspirates, and **B.** tumor mass according to Durie and Salmon (see above)[50]. MGUS, monoclonal gammopathy of unknown significance. AMM, asymptomatic multiple myeloma. MM, symptomatic multiple myeloma.

Pre-MGUS. This entity can be defined as with a cell number below the detection limit of routine techniques (immunofixation and FLC-assessment), see MGUS, i.e. <10^8 myeloma cells. At the "dawn of MGUS", the entity is theoretically accessible by flow cytometry; given a sensitivity of 10^{-5} (0.01%)[155] a theoretical minimal detectable (total) cell number of 10^7 can be assumed. Even at this stage, before myeloma can become apparent, the number of "myeloma" cells is thus surprisingly high.

II) HOW MANY DOUBLINGS TAKE PLACE DURING MGUS – AMM AND AMM – MM TRANSITION?

When investigating the mechanisms of evolution and progression of AMM, it is helpful to assess the number of doublings and consecutive differences in tumor cell number typically happening between the transitions from MGUS to AMM and AMM to MM. This question can be addressed by at least three ways: First, by measuring the doubling time in relation to the time to progression (median number of doublings), second, by comparing the myeloma cell infiltration and tumor mass between the different stages, and third, using the rate of progression. (The calculations can be performed on a population level and likewise be referred to individual progressing patients.)

i) Patients progressing from MGUS to MM and AMM to MM have median doubling times of 8.0 years (n=15) and 3.3 years (n=131), respectively, while the DT of patients not progressing is 71.6 years for MGUS- (n=181) and 28.4 years for AMM-patients (n=191). The mean number of doublings in progressing patients from MGUS and AMM to MM is 0.62 and 0.92 doublings, respectively (Figure 3.26A).

ii) Considering a myeloma cell number in MGUS vs. AMM vs. MM of 10^{11} vs. $3x\ 10^{11}$ vs. $1.6x\ 10^{12}$ cells as detailed above, a median of 1-2 doublings for MGUS-AMM transition, and 2-3 for AMM-MM transition results. Under the condition that the amount of monoclonal protein produced by an individual myeloma cell in between progression from MGUS to AMM and/or AMM to MM remains stable as shown by Salmon in 1971[183], the relative change in the amount of myeloma cells can also be estimated by the produced monoclonal protein. In doing so, the fractional changes for individuals progressing from MGUS to MM were 1.52 ± 1.14 (n=14), vs. 1.01 ± 0.26, if no progression was observed (n=165). For the AMM to MM transition, the median increase was 1.5 ± 5.92 (n=133), vs. 1.07 ± 3.41 if no progression was observed (n=158; Figure 3.26B). In the case of progressing patients, comparable numbers of doublings are thus obtained.

iii) From the rate of progression from MGUS to AMM of 1% per year and 10% from AMM to MM together with the number of doublings necessary regarding the tumor mass, see above, the corresponding median DT can be approximated to 50-25 years (50% progression after 50 years and 1-2 doubling necessary within this time, upper limit) for MGUS and 2.5-1.8 years for AMM (50% progression in 5 years and 2-3 doublings necessary within this time, upper limit).

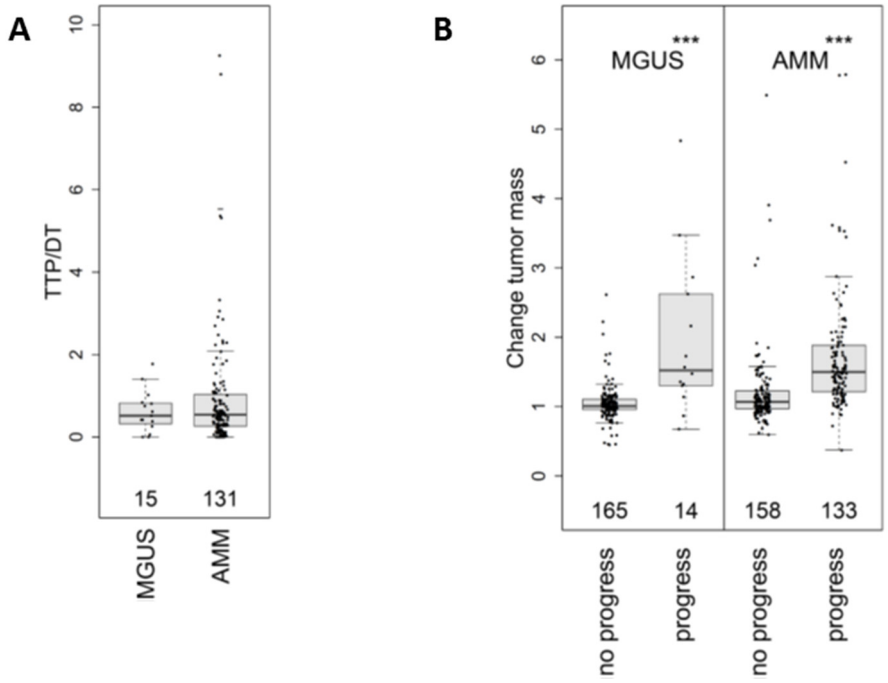


Figure 3.26. Number of doublings and fractional changes in tumor mass in progressing patients. A. Number of doublings, as well as **B.** fractional changes in tumor mass in progressing patients from MGUS and AMM to symptomatic myeloma. MGUS, monoclonal gammopathy of unknown significance. AMM, asymptomatic multiple myeloma.

The median number of doublings in evolution of AMM from MGUS (0.62), and finally progression to MM (0.92) is strikingly low compared to ≈40 for symptomatic myeloma to evolve (2^{40}≈1x 10^{12} cells; Figure 3.26). It is thus not surprising if the bulk of changes is already present at MGUS.

III) IS IT POSSIBLE AND LIKELY THAT A *DE NOVO* APPEARING ABERRATION IN MGUS OR AMM DRIVES THE RESPECTIVE TRANSITIONS?

The next question was whether it is possible that a *de novo* appearing aberration in MGUS or AMM drives the respective transitions based on observed doubling time, time to progression, and necessary number of doublings. Progression at the respective stage would thus start again from one (mutated) cell. To be compatible with the progression rate of AMM and 1x 10^{12} myeloma cells (≈2^{40}), the median DT need to be <0.125 years (5 years/40 doublings), in contradiction with the observed median DT (10.4 years; Figure 3.27). For evolvement of AMM from MGUS to be compatible with progression rate and 1.3x 10^{11} myeloma cells (≈2^{37}), a median DT of <1.4 years (50 years/37 doublings) would be necessary, in contradiction with the observed median DT of 64.9 years (8.0 years for progressing MGUS-patients; Figure 3.27). This mechanism can therefore not be responsible for the majority of progression events, especially those in which DT is slow.

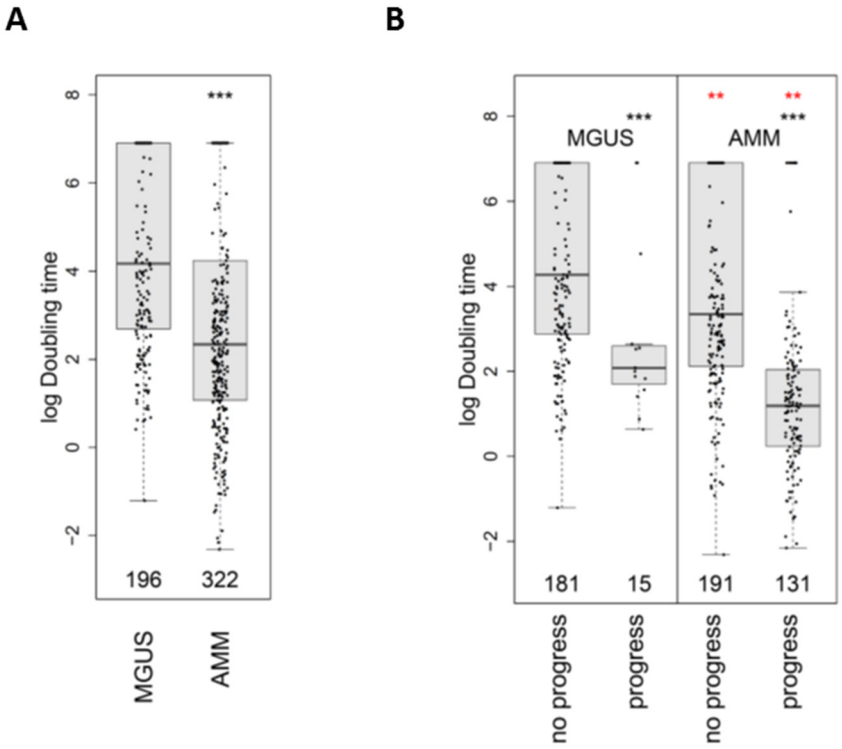


Figure 3.27. Doubling time of patients with monoclonal gammopathy vs. asymptomatic myeloma. A. Median DT in patients with monoclonal gammopathy is 64.9 years vs. 10.4 years in patients with asymptomatic myeloma (P<0.001). **B.** Progressing patients show an even faster DT (P<0.001 each).

In **summary**, there is neither evidence nor necessity to assume ongoing genetic instability driving progression from MGUS to AMM to symptomatic myeloma in the majority of patients. Plasma cells in MGUS already carry “what it takes” to be malignant.

4. Discussion

4.1 Determinants of progression

4.1.1 Factors

WHICH FACTORS DETERMINE EVOLUTION AND PROGRESSION OF ASYMPTOMATIC MYELOMA, AND WHAT IS THEIR MOLECULAR BACKGROUND? Main determinants can be grouped biologically in plasma cell accumulation rate, tumor mass, and molecular characteristics.

ACCUMULATION RATE. Quantitative assessment of plasma cell accumulation rate, i.e. doubling time, as introduced in this dissertation and gene expression-based measurement (GPI) allow delineation of significantly different groups in terms of progression to symptomatic myeloma. These findings are in agreement with the semi-quantitative description of an "evolving" pattern of progressively increasing M-protein level[180]. When used to group patients and assess TTP from bone marrow aspiration, a statistical caveat is that the variable which is used to delineate patients (i.e. doubling time) is progressively measured (as in follow up in clinical practice) and measurement continues in the follow up period from bone marrow aspiration. The myeloma cell doubling time should therefore not be interpreted as prognostic factor in statistical sense but as clinical parameter correlative with progression. The prognostic impact of DT has been validated using a landmark-analysis as well as a clinical "what if" scenario excluding values obtained at the time of progression. The assessment of doubling time by subsequent measurements of serum/urine disease activity parameters as proposed here, using our Microsoft Excel® tool (Figure 3.3), represents the strategy applied in patient care, i.e. drawing clinical consequences from sequential measurement. DT assessed in this way could be used as prospective assessment of the accumulation rate of myeloma within the grading very fast/fast/intermediate/very slow, i.e. quantification of the clinically assessed increase. In full agreement with the IMWG for prognostic factors[169], it is suggested only to use DT as clinical parameter for decision making once it has been reported by two further independent groups.

SURROGATES OF TUMOR MASS. In contrast, tumor mass surrogates determined "locally" in bone marrow aspirates or "globally" (e.g. serum M-protein) are well described prognostic factors as detailed in Table 1.2[48,116,153,162], which was validated in our analysis.

CLINICAL DETERMINANTS OF IMMEDIATE PROGRESSION. Combinations of tumor mass and accumulation rate. As shown here, both parameters, i.e. doubling time and surrogates of tumor mass, are independent. In the recently published IMWG-criteria, an attempt has been made based on literature review to redefine asymptomatic myeloma with imminent progression risk,

which is then termed “multiple myeloma” and treatment recommended[169]. Likewise, patients in former Durie-Salmon stage IA with one osteolytic lesion[50] and <10% clonal plasma cells have been excluded and been made a separate entity, i.e. solitary plasmocytoma with minimal bone marrow involvement. As shown here, indeed the remaining “smoldering myeloma” patients have a lower risk of progression. Patients with “solitary plasmocytoma with minimal bone marrow involvement” however have a progression rate comparable to the previous definition and one might consider not taking them as separate group. The analysis presented here can thus be seen as first “field testing” and validation of the novel **IMWG-criteria**[169].

MOLECULAR CHARACTERISTICS. With the one exception of hyperdiploid myeloma, turning from bad to neutral or good[10,153], the same progression-associated chromosomal aberrations are prognostically adverse in asymptomatic and symptomatic myeloma[153,168]. In agreement with recent data for therapy-requiring patients[27], progression-associated aberrations act additive on TTP with a certain interchangeability of aberrations and prominence of the number rather than the individual aberration. The pathogenetic background is their association with plasma cell accumulation, multiplicative regarding the number of aberrations present. Gene expression-based risk-scores for therapy-requiring myeloma confer prognosis in AMM, i.e. the UAMS70-gene score, in agreement with a previous publication[45], and the Rs-score. The other way round, the HDAMM-predictor for AMM conveys prognosis in symptomatic patients, being the first gene expression-based score for non-symptomatic patients developed. This means that the same mechanisms responsible for disease progression in AMM, i.e. intrinsic properties of myeloma cells, are also responsible for at least part of the mechanism driving progression under treatment in symptomatic patients. In multivariate analysis, plasma cell accumulation rate, tumor mass, progression-associated chromosomal aberrations and GEP-based predictors are independent.

4.1.2 Background of molecular determinants of progression

Much of the impact of molecular characteristics on TTP can be explained by association with plasma cell accumulation rate and/or tumor mass. “High-risk groups” in gene expression-based risk stratifications and presence of any progression-associated aberration are associated with shorter doubling time, the latter being multiplicative regarding the number of aberrations, and to a varying degree also with higher tumor mass.

Tumor mass surrogates, most strikingly a plasma cell infiltration ≥60% or a FLC ratio ≥100, are in turn significantly associated with higher DT.

4.1.3 Paths of progression

Determinants drive progression via different paths, i.e. bone disease vs. other, tumor-mass related cause (e.g. anemia). The latter is more frequent if higher accumulation rate and/or initially higher tumor mass are present, most strikingly for doubling time of <2 vs. ≥16, a plasma cell infiltration <10% vs. ≥60%, or FLC ratio ≥100. Patients presenting with one osteolytic lesion or focal lesions both progress with 87% probability due to bone lesions, of importance for application of local treatment strategies (section 4.4.2).

4.2 Evolvement and progression of AMM

In this section, first, a synthesis regarding the findings in terms of mechanisms of evolution and progression of asymptomatic myeloma will be presented (4.2.1), followed by a discussion whether the reported findings are in agreement with published data (4.2.2), and answering whether progression and evolution is driven by an ongoing genetic evolution (4.2.3). Next it is discussed whether myeloma cells at MGUS-stage should already be considered malignant (4.2.4), and closes with a discussion how the reported findings can be used to assess initial events, founder cells, myeloma cell spread and thereby alternatively explain how clonal heterogeneity can be present and maintained (4.2.5). Based on this, an updated pathogenetic model of myeloma is presented in section 4.3.

4.2.1 Mechanism of evolution and progression of AMM

Progression according to these findings is driven by an initial “given set” of aberrations present directly after or during generation of myeloma founding cells. Malignant plasma cells with progression-associated aberrations and thus shorter doubling time progress faster through the natural course of myeloma (“time-lapsed myeloma”). Exactly the fraction of MGUS patients having been “given” a shorter DT by their set of aberrations progresses whereas the “median typical MGUS” with DT of 64.9 years almost never progresses. The individual patient remains at a longer or shorter time in each of the compartments depending on the number of progression-associated aberrations in his myeloma cells, in agreement with the observed frequencies of different progression-associated aberrations within the respective compartments (Figures 3.15, and 3.16). In symptomatic patients, there is thus an “enrichment” (higher frequency) of (faster progressing) patients harboring any and especially combinations of these aberrations, as shown not by prospective gain in an individual patient, but an accumulation of a population of patients carrying these aberrations (Figures 3.12, 3.15, and 3.16, Table 3.5).

This likewise explains effortlessly the bulk of differences in gene expression being present already at MGUS-stage, as the relative sparseness of gain of progression-associated aberrations in longitudinally investigated patients (Figures 3.17, 3.18, and 3.19, Table 3.6).

In this scenario, in patients evolving, the initial set of aberrations would just need to confer accumulation, e.g. at least in part by aberrant or increased CCND-expression (see section 1.2.1). Myeloma cells are to this end special in that they are "already at home" at their final location from the beginning, i.e. contrary to solid tumors (e.g. adenoma – carcinoma sequence in colon cancer)[38], they do not need to acquire additional features vs. their normal counterpart to move there.

4.2.2 Genetic intra-patient heterogeneity and progression

To discuss whether the reported findings are in agreement with published data about genetic intra-patient heterogeneity, a new terminology of describing genetic heterogeneity is introduced[‡]. Based on this, the main seemingly "obvious" counter argument as part of the current pathogenetic model is addressed, the presence of subclonal aberrations and clonal heterogeneity[147]. Does this not by itself mean that an ongoing genetic instability and evolution need to be present to drives progression?

4.2.2.1 Genetic heterogeneity

Besides different individual genetic background related to the two general patterns hyperdiploidy and IgH-translocations and additional aberrations on DNA (chromosomal aberrations; SNVs), and RNA-level (changes in gene expression, different gene expression-based molecular entities), i.e. *inter-patient heterogeneity*, heterogeneity also exists within an individual patient: *intra-patient heterogeneity*. This can be present in terms of i) a heterogeneity *in loco aspiratio* at presentation, i.e. in the site of clinical bone marrow aspiration, ii) in terms of a *spatial* heterogeneity, e.g. different patterns of genetic alterations in focal lesions vs. random aspirates, and iii) *temporal* heterogeneity, i.e. change of the clonal composition / emergence of new subclones over time, especially evidenced under treatment.

4.2.2.2 Heterogeneity *in loco aspiratio* at presentation

Several lines of evidence exist for the presence of heterogeneity *in loco aspiratio* (at the site of bone marrow aspiration). By iFISH, chromosomal aberrations can be present in different

[‡] The main part of this and subsection 1.2 have been accepted as invited publication for the education session of the 20th European Hematology Association Meeting, Vienna, Austria, 2015, entitled "Clonal architecture of multiple myeloma", authored by Dr. med. Anja Seckinger and Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose[192].

percentages within the malignant plasma cell population of a given patient. Whereas IgH-translocations as t(4;14) or ploidy state (hyperdiploidy) usually appear in the majority of myeloma cells, the frequency of malignant plasma cells in which a deletion 13q14 can be detected varies between 20% and 100%[3,130]; the same holds true for deletion of 17p13 or gains of 1q21[39]. If one chromosomal aberration appears in ≥60% of myeloma cells whereas another only in a smaller percentage of this population, a "subclonal aberration" is present[85,187]. Their appearance is a sign for an evolution of the malignant plasma cell clone, in which the subclonal aberration appeared after the clonal aberration (temporal heterogeneity, see below)[39]. Using next generation sequencing and analyzing seven serial myeloma cell samples from an individual patient at diagnosis, remission, four relapses, and ultimately plasma cell leukemia, Keats *et al*. were able to show different (sub)clones being present at myeloma diagnosis[100]. In this patient with a t(4;14), longitudinal array comparative genomic hybridization and iFISH analyses revealed the presence of two major clones with a dominant one being present in 72% of cells as well as two major subclones that emerged from the first. While one major subclone was already present at diagnosis in 11% of cells, the second one seemed to have emerged at a later time point, i.e. after treatment was initiated, or was below the threshold at diagnosis. One subclone differed by only six copy number alterations (2 lost and 4 gained) from its parental clone, while the second subclone showed 13 lost and 39 gained copy-number abnormalities[100]. In agreement with this, genetic analysis of sorted single cells in patients with t(11;14) revealed the presence of two to six major clones at presentation[137]. Combining SNP-based array data and whole-exome sequencing, Walker *et al*. also found evidence for a clonal heterogeneity in samples of patients with t(4;14) (n=10) vs. those presenting with t(11;14) myeloma (n=12)[208]. Focusing on mutations in the RAS pathway, they found *NRAS* or *KRAS* mutations to be present in 32-96% and 20-72% of malignant plasma cells, respectively. Although seen as driver aberration, RAS mutations were thus not always present in the dominant clone but only in minor fractions of the myeloma cell population, and a subfraction of patients[32,123,208]. In case a RAS mutation was present in a given sample, there was evidence for the simultaneous presence of ≥1 subclone[208]. These findings were confirmed in a larger series of myeloma patients, showing mutations often to be present in subclonal fractions only with numerous clones being predicted to be present at diagnosis[123]. Taken together, there is convincing evidence for the presence of aberrations at a subclonal level.

4.2.2.3 Spatial heterogeneity

Different bone marrow infiltration patterns of myeloma cells can be simultaneously present in the same patient[18,79,211], i.e. focal lesions and/or diffuse spread over the bone marrow, tempting to assume a connection between the existence of several subclones and different manifestation

patterns, i.e. the presence of a *spatial heterogeneity*. Evidence is given by Zhou *et al*. comparing myeloma cells from random (pelvic) aspirates (RA) with those from computer-assisted tomography-guided fine needle aspiration from MRI-defined focal lesions (FL)[227]. Using conventional karyotyping, four patterns of chromosomal abnormalities can be distinguished: 1) chromosomal abnormalities detected in both, RA and FL, i.e. RA+/FL+ (n=75, 18% of patients), 2) RA+/FL- in 16% of patients (n=67), 3) RA-/FL+ in 18% of patients (n=77), and 4) absence of chromosomal abnormalities in both, RA-/FL- in 48% of patients (n=200). Furthermore, also discordances in chromosomal abnormalities, i.e. structural or numerical aberrations being unique to only one of the two sites, was observed in 48 of 75 patients (64%) of the first category. Regarding event-free and overall survival, the 75 patients showing chromosomal abnormalities in both sites (RA+/FL+) exhibited the worst outcome with seven-year estimates of 23% and 28%, respectively. The other groups had comparable event-free survival, while overall survival was inferior in patients with RS+/FL- (n=67). In agreement with a worse outcome and differences in laboratory parameters (e.g. higher lactate dehydrogenase level in the RA+/FL+ group), 52% of patients in the RA+/FL+ group were assigned to be high-risk according to the UAMS70 risk-score of the same group[194] vs. 27% in the 49 patients with RA+/FL- vs. 6% among the 50 patients with RA-/FL+, and 4% of the 130 patients with RA-/FL- for whom gene expression data were available[227]. This study has shown that there *can* be a difference. It will be interesting to gain further insight in the genetic architecture of samples from random aspirates vs. those from focal lesions and their association with the infiltration pattern. Corresponding analyses using high-resolution techniques are currently under way.

4.2.2.4 Temporal heterogeneity

DOES THE COMPOSITION OF THE MYELOMA CELL CLONE AT ONE SITE CHANGE OVER TIME?

Treatment associated changes. Recent data provided evidence for the presence of a *temporal clonal heterogeneity*. Analyzing seven serial samples as described above, Keats *et al*. were able to show different patterns of subclonal composition, i.e. subclones gained or lost dominance during the course of the disease, *"clonal tides",* with different treatment regimens exerting varying selection pressures on the cells[100]. Egan *et al*. conducted whole-genome sequencing at four time points during tumor evolution of the same patient at diagnosis, first relapse, second relapse, and plasma cell leukemia[53]. Fifteen SNVs were shown to be present at all four time points (*AFF1*, *ATXN1*, *COL2A1*, *CORO1A*, *CNGA3*, *CSMD3*, *LTB*, *MAGI1*, *MSL1*, *KCNIP4*, *KRT9*, *LRRC4C*, *MYPN*, *RNF145*, *TYRP1*), thus sharing a common progenitor. Six SNVs were only detectable at alternating time points, i.e. diagnosis and second relapse (*ACER1*, *C12orf42*, *DOKS*, *PARD3B*, *PPFBP1*, *ZNF557*), seven SNVs could be only observed in first relapse (*ATXN1*, *CACNA1S*,

DSC1, *PCDH7*, *PTPRD*, *TLR9*, *TUBB6*), and seven uniquely at the last time point, i.e. plasma cell leukemia (*BIRC5*, *RB1*, *ZKSCAN3*, *SUB1*, *TNN*, *TUBB8*, *ZKSCAN3*, *ZNF521*). The first relapse and the plasma cell leukemia sample did not share any unique SNV beyond the ones observed at all four time points (see above). *PDE4DIP* was found uniquely at diagnosis.

In 28 paired samples from symptomatic patients treated with different chemotherapeutic regimens analyzed by array comparative genomic hybridization, a median number of 23 copy-number abnormalities per sample was identified[100]. The number of copy-number abnormalities increased significantly during the course of the disease with a mean of 19.7 at baseline to 26.3 detected at the second time point. A mean of 16.8 copy-number abnormalities were shared between both time points indicating a certain clonal relation. Three different phenotypic patterns of *temporal tumor types* can be distinguished according to Keats *et al*.: 1) Genetically stable tumors, especially in patients with a more favorable hyperdiploid myeloma, with no detectable changes between paired samples from a given patient (35.7% of patients). 2) A linear evolutionary path in 21.4% of patients characterized by newly acquired copy-number abnormalities at the second time point, and 3) a changing clonal dominance (42.9% of patients) with gains and losses of copy-number abnormalities including the reappearance of bi-allelic deleted regions suggesting the existence of different subclones at diagnosis. Interestingly, patients with high-risk myeloma, i.e. presence of a translocation t(4;14), t(14;16), t(16;20), or deletion 17p13, showed significantly more copy-number abnormalities over time. Presence of deletion 17p13 was also accompanied by a higher number of copy-number abnormalities at the time of diagnosis[100].

In conclusion, there is evidence that with treatment the subclonal composition in a given patient *can* shift[100,210]. Is the observed change in turn driven by treatment? The pro arguments are that first treatment represents a very substantial additional selection pressure, i.e. regularly killing a high proportion of myeloma cells, and thus changing the cellular composition of the bone marrow microenvironment. A different responsiveness of subclones could easily explain the change in subclonal composition. The alternate hypothesis that was suggested to us in review of our EHA-education manuscript[192] was that there could be a continuously ongoing process of change in the subclonal composition of the bone marrow myeloma cell population, which in turn leads to different responsiveness. Within this concept, the change of subclonal composition would be driven by an association with the accumulation rate of plasma cells: faster growing subclones would outgrow the respective slower growing subclones, simultaneously without impact on responsiveness to treatment, as otherwise treatment would drive the clonal composition. For this process to happen, in each subsequent relapse the accumulation rate of

plasma cells would need to be higher compared to the previous, i.e. a continuous selection to higher accumulation rates. If such a mechanism exists, it would need to be present in untreated patients. As it was shown in this dissertation that this cannot be the regular mechanism for disease progression, and based on published evidence (see below), this can now reasonably be excluded. As already written previously, given the huge additional selection pressure of treatment, independently it does not seem likely that what was observed is just the image of clonal dynamics independent of treatment.

Changes associated with disease progression. Without treatment, only "natural" selection pressures, e.g. competition for myeloma survival niches or faster accumulation, are present; the situation in MGUS-AMM-MM transition. *What evidence for changes exists here?* First, in cohort studies (transversal samples), an increased frequency of aberrations later associated with disease progression, e.g. 1q21[153], has been found in none of 14 individuals with MGUS, 43% (206/479) of newly diagnosed, 72% (32/45) of relapsing myeloma patients, and 93% (21/23) of myeloma cell lines[73]. A further example is t(4;14)[58]. These finding are traditionally interpreted as chromosomal aberrations appearing in higher frequencies at later stages to be associated with an evolution of the malignant plasma cell clone during different stages of plasma cell dyscrasias, "multi-step-model"[210]. However, all chromosomal aberrations detected in symptomatic myeloma *can* be detected already in MGUS or asymptomatic myeloma[7,8,24,34,59,98,153,168]. In *longitudinal samples*, gains and losses of aberrations have been found[124,125,210]. Examples comprise whole-exome sequencing data of paired samples as asymptomatic- and symptomatic myeloma (n=4 patients) by Zhao *et al*. showing no newly acquired SNVs in three patients, while in the fourth patient only one new SNV of unclear significance was detected in a single gene (*BBOX1*)[226]. Although the degree of loss of heterozygosity (LOH) was greater at baseline in samples from progressing patients (n=4) vs. those who did not progress (n=6), there was no shared pattern. In addition, the LOH pattern was largely maintained with only few changes in the samples at the time of progression identified, but for two patients with newly acquired regions with LOH[226]. A comparable result has been found by Walker *et al*. who, but for inactivating mutations in *RUNX2* and acquired translocations into *BRCA2* and *UNCSD*, "could not identify truly acquired genetic abnormalities" between paired AMM and MM samples "despite thoroughly checking for coding SNVs, indels and copy number abnormalities"[210]. Whereas there is convincing evidence that, also without treatment, changes in the clonal composition at least at the level of SNV can appear, these have as of yet not convincingly been associated with disease progression.

This is in agreement with an initial analysis of our cohort of longitudinal patients (data not shown). There is thus published evidence by two independent groups using whole-exome sequencing that progression from asymptomatic to symptomatic myeloma *can* appear without driving genetic alterations. Of course this does not exclude that clonal progression *can* be present in *a subset* of patients.

4.2.3 Is progression driven by ongoing clonal evolution?

There is neither evidence nor necessity to assume ongoing genetic instability *driving* progression from AMM to symptomatic myeloma (section 4.2.2, Figures 3.21, 3.22, and 3.24). Given that most in terms of disease progression "happens" not during MGUS to AMM to MM transition but before MGUS *can* become apparent (see sections 3.4, and 4.2.1) there is no reason why further accumulation of plasma cells cannot be driven by the very same mechanism from MGUS onwards to asymptomatic and further to therapy-requiring myeloma.

From the said above, there is previous evidence for *possible* clonal change, and clonal evolution if selection pressure is applied by treatment, but not for clonal *evolution driving* progression, in perfect agreement with our findings.

4.2.4 When to call a plasma cell malignant - or drop "us"

There are three lines of evidence why plasma cells in monoclonal gammopathy should be considered malignant and consecutively "of unknown significance" be dropped from MGUS. First, as shown here, the bulk of differences between normal and malignant plasma cells is already present at MGUS-stage. Second, the same molecular determinants of progression (changes in gene expression, progression-associated chromosomal aberrations) for AMM are already present in MGUS. Third, whereas per definition the tumor mass (number of myeloma cells) is lower in MGUS vs. AMM, and consecutively more doublings are necessary to reach a critical tumor mass, 28% (55%) of MGUS-patients already show DT<16 years (<100 years) and thus a considerable accumulative potency (Figures 3.23, 3.24, and 3.25).

4.2.5 Initial events - founder cells, spread and heterogeneity

NUMBER OF FOUNDER CELLS. The implications of the measured doubling time and calculated number of doublings for an extrapolation to initial events has not yet been addressed. **Is it possible that myeloma evolved from a single myeloma cell harvesting in the bone marrow?** In the following, we conservatively assume each myeloma cell to divide and none to die, thus the actual number of doublings and concomitant accumulation rate needs to be even higher. If one considers one founding cell, 40 doublings ($2^{40} \approx 1x\ 10^{12}$ cells) are regularly necessary for symptomatic myeloma to evolve. Of these, 36 doublings ($2^{36} \approx 6.8x\ 10^{10}$) have happened *before* MGUS regularly does (in our cohort, section 3.4) and 27 ($2^{27} \approx 1.3\ 10^{8}$) before it *can* become apparent. Thus, 90% of growth in terms of doubling would have happened before MGUS regularly becomes apparent (36/40 doublings). Given the observed median doubling time in MGUS (i.e. 64.9 years) and the number of doublings to reach the tumor mass of symptomatic myeloma (40), it would take 64.9x40=2596 years for symptomatic myeloma to develop from one founder cell, which is naturally not possible. In this scenario, only patients with DT below 2 years (80 years for 40 doublings) could reasonably be thought to progress to symptomatic myeloma, which is not the case (Figures 3.1, 3.23, and 3.24).

To explain this, two scenarios are possible: First, the initial doubling time has been higher and slowed down, e.g. because of reaching the maximum number of doublings due to maintenance of shrinking telomeres. Only those patients with still "high enough" DT would progress further to AMM. Or secondly, the initial number of founder cells is higher. **What would be a reasonable number of founding cells to assume?** If one conservatively considers 50 years as time-frame (early initiating event between 0 and 20 years of age and consequently MGUS between 50 and 70 years), with a DT of 16 years, this would leave time for 3 (48 years) to 4 (64 years) doublings, to reach 2^{36} - $2^{37} \approx 6.8x\ 10^{10}$ - $1.4x\ 10^{11}$ cells, i.e. a starting number of $6.8x\ 10^{6}$ - $1.4x\ 10^{8}$ cells. Is this a sensible number? Indeed, this would be in agreement with the number of plasma cells generated physiologically in an initial antigen response, 10^{7}, of which 10^{6} (10%) remain in the memory phase. Radbruch *et al*.[163] report up to 30 of these adoptions per year to happen, i.e. 30 waves of plasma cells hitting the bone marrow.

IMPLICATIONS FOR EXPLAINING STATIC NUMBER OF MYELOMA CELLS, SPREAD, AND CLONAL HETEROGENEITY. It is thus tempting to hypothesize that the myeloma initiating event takes place during one of the 30 normal novel plasma cell generation cycles before the amplification leading to the burst of 10^{7} early plasma cells. If a slightly higher proliferation rate is present from the beginning (e.g. due to the initiating aberrations already impacting at this time) the burst

could lead to a higher number of founding cells, e.g. 10^8 - 10^9 cells in the initial burst; even the number at which MGUS becomes apparent (see also below). As the growth conditions are different during the burst (as in the generation of normal plasmablasts[94,95]), this could easily explain why some AMM and many MGUS patients remain with a rather **static number of myeloma cells,** neither progressing nor showing a continuous increase in cell number (e.g. DT >100 years) – because a high initial burst lead to this number.

These pre-myeloma cells home to different sites all over the bone marrow as do normal plasma cells, explaining **myeloma cell spread** without the need of re-entering circulation.

The observed **clonal heterogeneity** despite no overt growth advantage of the different clones could thereby be easily explained: Despite myeloma cells originate from a single ancestral cell hit before the "burst" (as they all produce the same immunoglobulin or parts thereof), heterogeneity in terms of subtle (e.g. point mutations) or gross (e.g. deletion of chromosomes or parts thereof, e.g. del13q14) appear *during this burst* due to errors during replication, i.e. at a time when proliferation is naturally present during plasma cell development (see section 1.2.1). The number of cells of a specific subclone is thus less (or for others not at all) driven by the impact on proliferation of the respective aberration in this phase, which still needs not be the same as in a later "bone marrow plasma cell like" myeloma cells, but rather the time during the expansion when it happens. As said, this easily explains that myeloma cells need not carry aberrations associated with a certain growth-advantage to explain clonal diversity. Of course, some (but very few) of these can indeed confer a growth advantage, but in these cases, clonal diversity would hardly be visible at the time of diagnosis. Thus, somewhat counter-intuitive, clonal diversity speaks *against* an *ongoing* genetic instability as otherwise a clone harboring a growth advantage would outgrow the minor clones.

4.3 Revised model of myeloma pathogenesis

In the following, findings within this study are and integrated in the current model of progression and evolution of multiple myeloma[101] (Figure 1.2).

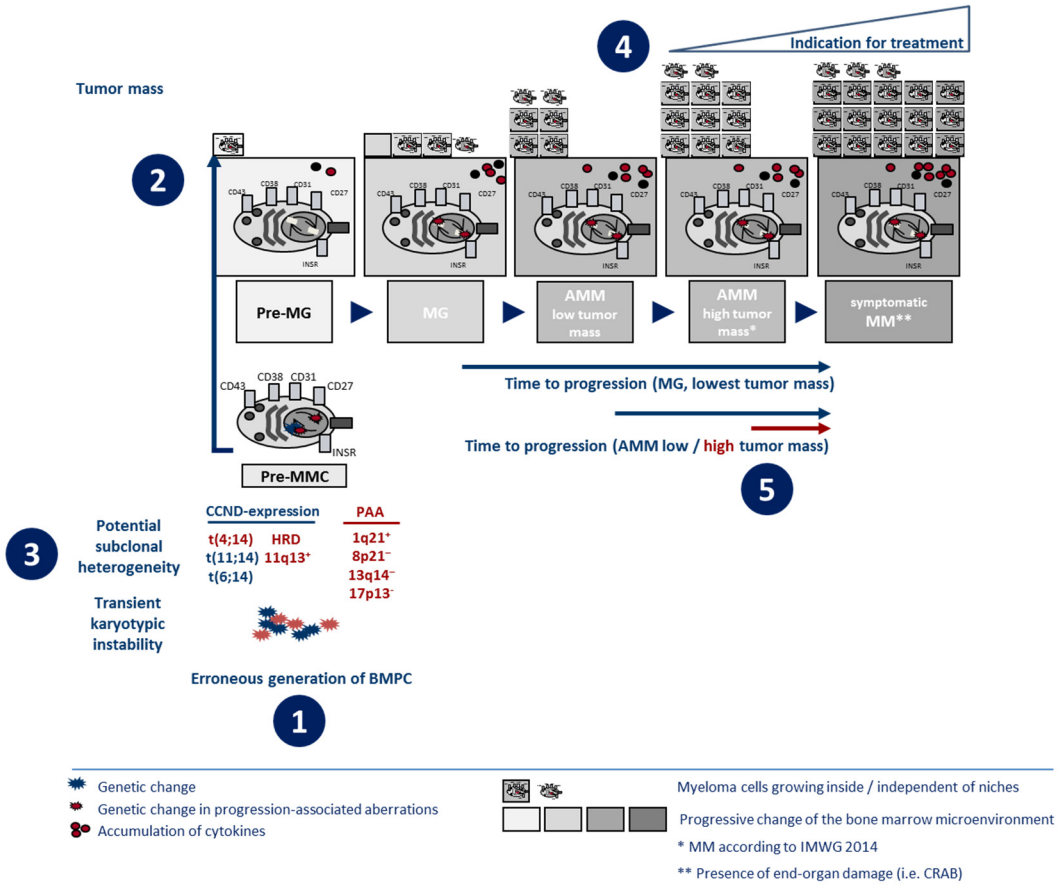


Figure 4.1. Revised model of myeloma pathogenesis. See text below. MG, monoclonal gammopathy. (A)MM, (asymptomatic) multiple myeloma. PAA, progression-associated aberrations.

INITIAL EVENT. (1) Generation of normal bone marrow plasma cells is an erroneous process. Errors occur before or during the expansion leading to 10^7 or more initial pre-myeloma cells harvesting in the bone marrow. The process follows the one in generation of terminally differentiated plasma cells producing antibodies against novel antigens, occurring about 30 times per year[163], i.e. as one of the 30 waves of plasma cells hitting the bone marrow (section 4.2.5). Pre-myeloma cells home throughout the bone marrow, as do normal bone marrow plasma cell precursors, explaining the **SPREAD** of systemic myeloma **(2)**.

CLONAL HETEROGENEITY. **(3)** Despite myeloma cells originate from a single ancestral cell hit before the "burst" (as they all produce the same immunoglobulin or parts thereof), heterogeneity in terms of subtle (e.g. point mutations) or gross (e.g. deletion of chromosomes

or parts thereof, e.g. del13q14) appears *during this burst* due to errors during replication, i.e. at a time when proliferation is naturally present during plasma cell development. The number of cells of a specific subclone is thus less (or for others not at all) driven by the impact on proliferation of the respective aberration in this phase, which still need not be the same as in a later "bone marrow plasma cell like" myeloma cells, but rather the time during the expansion when it happens. This explains that myeloma cells can carry subclonal aberrations not conferring a growth advantage (clonal diversity). Of course, some (but very few) of these can indeed confer a growth advantage, but in these cases, clonal diversity would hardly be visible at the time of diagnosis. Given the number of doublings to reach MGUS-stage, clonal competition is in most cases decided already at MGUS-stage. Thus, somewhat counter-intuitive, clonal diversity speaks *against* an *ongoing* genetic instability as otherwise a clone harboring a growth advantage would outgrow the minor clones.

ACCUMULATION OF MYELOMA CELLS is driven by the initial set of aberrations that appeared during or before the clonal expansion of pre-myeloma cells analogously to normal pre-BMPC. The initial pattern of aberrations is driven on a random background by e.g. the transcriptional activity (e.g. IgH-loci, and frequent break points[72,102]). Only if the aberration pattern **(4)** is associated with plasma cell accumulation, the respective aberration can "grow out". Only patterns fulfilling this criterion appear as "non-random" aberrations in MGUS, AMM and MM, explaining why the majority of chromosomal aberration patterns present are associated with plasma cell accumulation, and in turn almost all (progressing) AMM patients carrying one of the aberrations associated with plasma cell accumulation (Figures 3.12, and 3.16). Aberrations fulfilling this criterion yield aberrant or over-expression of D-type cyclins[20] (e.g. t(4;14), hyperdiploidy), are associated with (higher) doubling time e.g. deletions 13q14 or 8p21, gain of 1q21, or both (e.g. t(4;14)).

The **SPEED OF PROGRESSION** is mainly driven by the doubling time, which is in turn dependent on number of progression-associated aberrations (on either DNA or RNA-level, see also Figures 3.9 and 3.12), and tumor mass at the time of diagnosis (5): if the patient presents at higher tumor mass, he has already progressed further on his way to symptomatic myeloma, thus the time span is shorter, see also Figure 3.24. Some AMM and many MGUS patients remain with a rather **STATIC NUMBER OF MYELOMA CELLS** because a high initial burst has led to this number, without aberrations conferring significant growth.

There is **NEITHER EVIDENCE NOR NEED FOR ONGOING CLONAL EVOLUTION** to explain plasma cell accumulation and disease progression: the prominent clone drives evolvement of asymptomatic and subsequent progression to therapy-requiring myeloma in the vast majority

of patients. A new (sub)clone can emerge, but this is a very rare event, and in the vast majority of patients, plasma cell accumulation is continuous and driven by the initial set of aberrations. In evolution and progression of AMM, doubling-times (MGUS-AMM: 18.0 years, AMM-MM: 5.1 years) and number of doublings (MGUS-AMM: 0.62, AMM-MM: 0.92) are incompatible with common *de novo* appearance of progression-driving aberrations.

PLASMA CELLS AT MGUS STAGE SHOULD ALREADY BE CONSIDERED MALIGNANT AND ”US” BE DROPPED FROM MGUS. They already carry the bulk of changes in gene expression compared to normal bone marrow plasma cells, the same set of progression-associated aberrations, and our gene expression-based predictor for progression of AMM likewise confers progression of MGUS. Furthermore, as depicted above, only a median number of 0.62 doublings happens during MGUS-AMM.

4.4 Clinical implications

In the introduction (section 1.3.1), the current therapeutic questions have been summarized focusing on which compounds to give, which to combine or give subsequently. Two further questions impacted by the results of this dissertation are when to start treatment and a potential application of local treatment in a systemic disease.

4.4.1 Clinical implications on early treatment

The **clinical rationale** of **treating asymptomatic myeloma** patients is to prevent development of end organ damage, which needs to be balanced vs. the prevention of consequences of treatment (side effects and treatment-related mortality, see section 1.3, Table 1.2). **Biological rationale** and assumptions are that at earlier stages fewer myeloma cells are present harboring a lower genetic heterogeneity (intra-patient heterogeneity, see section 1.2.2, 4.2.2), and having induced less potentially irreversible alterations in the bone marrow microenvironment (e.g. bone lesions, see section 1.2.3). In this setting, either already a less aggressive treatment (e.g. Rd[135]) might suffice to prolong overall survival, or an intensive treatment might allow long-term remission or even cure; a strategy followed by the group of Landgren *et al*.[118]. The main biological counter argument is that treatment might induce change from a benign (slowly accumulating, non-aggressive) to an aggressive subclone both in terms of accumulation rate and decreased response to treatment. The **findings** presented in this dissertation impact on the biological rationale in the following way: Given the median number of doublings between e.g. AMM and symptomatic myeloma or MGUS and AMM (section 3.4), compared to the total number of doublings necessary for myeloma to evolve (section 3.4), and the lack of necessity of *de novo* aberrations to appear to drive progression (section 3.3), it is not plausible that a significantly higher genetic intra-patient heterogeneity is present in asymptomatic compared to symptomatic myeloma, thus this can no longer be taken as argument for earlier treatment. As always, the final answer needs to be given in clinical trials comparing overall survival using the differently intensive flavors of early vs. delayed treatment.

The clinical rationale of **treating disease progression early** is similar, balancing prevention of "consequences" of myeloma, i.e. (further) end organ damage, vs. preventing consequences of treatment (side effects, here especially those preventing further treatment in subsequent disease progression, e.g. exhaustion of bone marrow microenvironment, and treatment-related mortality). Biologically, in both scenarios fewer cells are present compared to the level present in symptomatic myeloma when treatment is traditionally initiated, so the same concepts apply.

4.4.2 Clinical implications for local treatment

Local treatment of myeloma bone lesions by bone substitute materials is a concept introduced by my group within our Sonderforschungsbreich / Transregio TRR79.

As introduced in section 1.2.3, myeloma bone disease represents a threefold **therapeutic problem**: i) *Per se* because of the morbidity, mortality and the accompanying decrease of quality of life associated with resulting pathological fractures[182], ii) as indication to start treatment in otherwise asymptomatic patients (to avoid pathological fractures), or in patients progressing after successful treatment only in terms of enlarging osteolytic lesions, and iii) as *survival space* ("reduit") for myeloma cells during otherwise successful chemotherapeutic treatment and subsequent source of relapse and thus potential obstacle for myeloma cure.

Counterintuitive at first in a systemic malignancy, the idea is to locally prevent "local" progression (e.g. 1-3 growing osteolytic lesions) or potential of progression (e.g. presence of >1 focal lesions) seen as indication of treatment to prevent morbidity and mortality by a fracture-to-be. As shown here, indeed patients with presence of one bone lesion (single bone lesion with minimal bone marrow involvement) down-staged by the IMWG have a comparably high rate of progression to symptomatic myeloma (69% in 2 years, 94% in 5 years vs. 31% and 52% for all AMM-patients, see Figure 3.6), the same holds true for patients presenting with more than one focal lesion. Indeed, in both cohorts of patients, 87% progress due to bone related cause (Table 3.2, Figure 3.10). The **potential indication** to this regards are thus in treatment of early stage patients (see above) and early treatment of relapse. A further concept is to eliminate the myeloma cell "reduit", i.e. the osteolytic lesion as potential source of relapse (Figure 4.2).

Local treatment can be applied conventionally by radiation or by application of bone substitute materials. For an overview on radiotherapy in multiple myeloma, please refer to Krause *et al*.[105]. The advantages of the first method are that it is non-invasive, generally leading to local tumor control, and there is no (known) cross-resistance between sensitivity against radiation and chemotherapeutic agents used in myeloma. The disadvantage are off-target effects, especially if more osteolytic lesions are targeted, e.g. suppression of hematopoiesis. A further disadvantage is that radiation leaves "scorched earth" in the lesion, which further hinders already fundamentally reduced bone formation and thus the healing of bone lesions. Local treatment by bone substitute materials on the other hand would allow local tumor control, stimulation of bone formation, and, after remodeling, a *reconstitutio ad integrum*, re-instating normal bone structure (stability) and hematopoiesis (increasing the hematological reserve), and, potentially returning the bone marrow microenvironment to a normal state, being thus

potentially less fertile soil for myeloma cell regrowth. It might likewise eliminate the myeloma cell "reduit".

Bone substitute materials developed within the TRR79 in collaboration with my laboratory comprise bortezomib-releasing calcium phosphate bone cements (TP M2; Prof. Dr. rer. nat. Michael Gelinsky), and bortezomib-releasing composites based on collagen, silica, and calcium phosphate phases (TP M3; Dr. rer. nat. Thomas Hanke). Proteasome inhibitors (e.g. bortezomib) are an ideal compound as they are not only killing myeloma cells but also *in vitro* simultaneously reducing activity of osteoclasts and simulating osteoblast differentiation[65,127,206]. A third strategy are functionalized hydrogels presenting simultaneously β1-integrin selective ligands and bone morphogenic protein 6 to stimulate osteoblast function and induce myeloma cell apoptosis based on previous results of my laboratory[187] (TP M9; Dr. med. dent. Dr. rer. nat. Elisabetta Ada Cavalcanti-Adam, Dr. med. Anja Seckinger).

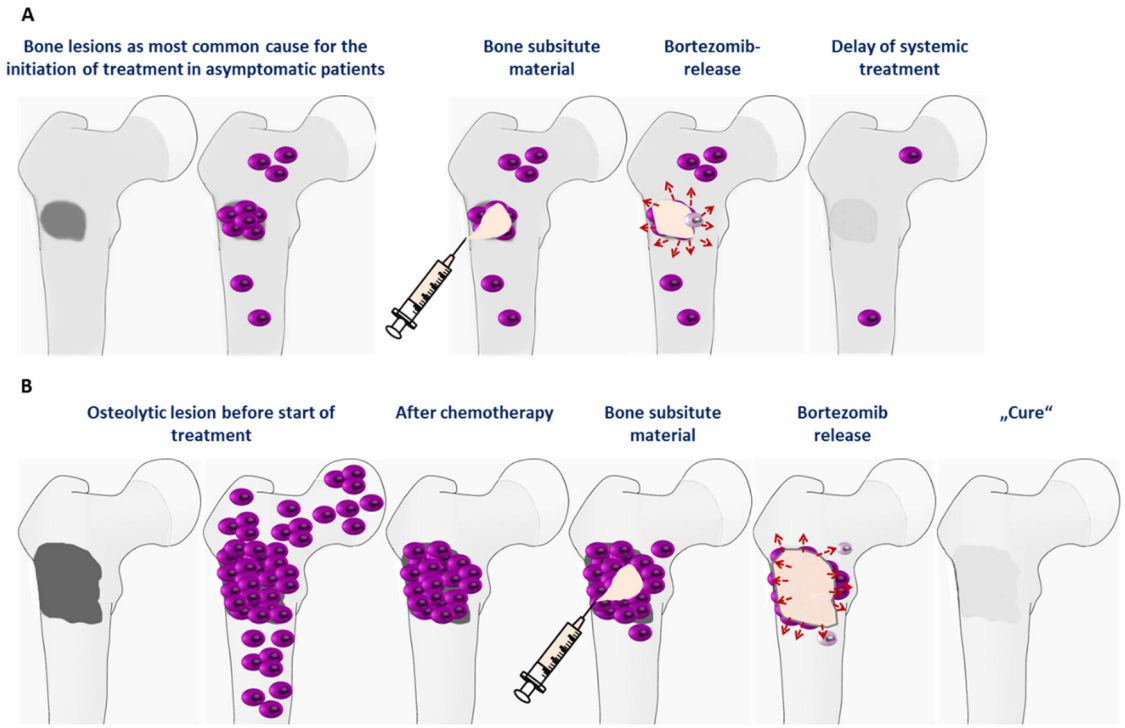


Figure 4.2. Local treatment approach using bortezomib-releasing bone substitute materials. A. Therapeutic approach in asymptomatic myeloma patients (i.e. delay of necessity of systemic treatment or cure in some patients; prevention of fractures), and consecutively in **B.** symptomatic patients after successful chemotherapeutic treatment (i.e. prevention of progressive disease or cure in some patients; enabling of bone healing). Red arrows, bortezomib-release.

4.5 Discussing aim and work program

The primary **AIM** of this dissertation within the Sonderforschungsbereich / Transregio TRR79 "Werkstoffe für die Geweberegeneration im systemisch erkrankten Knochen" was to lay a basis for the understanding of the molecular background of evolution, progression and prognosis of asymptomatic myeloma (TP B1, aim 1). The secondary aim, thereby to contribute to the assessment for which patients with asymptomatic or early stage therapy-requiring myeloma (e.g. 1-3 osteolytic lesions as the only symptomatic manifestation) local treatment of osteolytic lesions can be appropriated (TP B1, aim 2).

The **WORK PROGRAM** comprised:

i) The successful delineation **of determinants of progression** associated with plasma cell tumor mass, accumulation rate, and molecular characteristics, a classification as result of this dissertation. i.1) A quantitative measure of plasma cell accumulation (doubling time) using easily obtainable clinical variables has been defined and validated. Plasma cell doubling time is a clinically strongly predictive factor for progression to symptomatic myeloma. It likewise allows the investigation of the association of molecular parameters (e.g. chromosomal aberrations) with plasma cell accumulation. It is especially beneficial in delineating slow progression. We also provide a tool for its clinical application. An independent measure of plasma cell accumulation, the GPI, likewise delineates a group of AMM patients with significantly faster progression. i.2) Parameters regarding tumor mass are significantly associated with disease progression as would be expected. Accumulation rate and tumor mass are independent in a multivariate analysis. Based on these parameters (plasma cell infiltration and DT/GPI) a group with almost no progression after 2 years of follow up could be identified, as patients with high tumor mass (e.g. plasma cell infiltration ≥60%) or DT<2 years almost all progressing within 2 years. i.3) Subsequently, the determinative potential of the novel IMWG-definition delineating AMM in patients with "smoldering" myeloma vs. those with different reasons for imminent progression was addressed. Indeed, groups no more belonging to smoldering myeloma show a significantly faster progression to symptomatic myeloma, surprisingly including the down-staged group of solitary plasmocytoma with minimal bone marrow involvement. The only of these subgroups showing 80% progression within 2 years however is the one with ≥60% plasma cell infiltration. The analysis can thus be seen as first "field testing" of the novel classification. 4) **Molecular characteristics** determining progression have been addressed. First, a **gene expression-based predictor** for progression of AMM has been created and successfully validated on therapy-requiring patients. Risk-scores for overall survival of symptomatic patients (UAMS70-gene score, Rs-score) likewise proved to be prognostic regarding progression of AMM-patients.

This can be interpreted that part of the factors determining progression in symptomatic patients under treatment are intrinsic features of myeloma cells, i.e. independent of treatment. Secondly, the impact of **chromosomal aberrations** on progression of AMM was investigated, extending previous work by a new classification of chromosomal aberrations regarding association with progression, identifying an aberration inversely associated with progression (t(11;14)), and one previously not associated with progression (deletion 8p21). i.5) Of special interest also regarding the secondary aim of this dissertation was the finding that different determinants drive progression via different **paths**, i.e. bone disease vs. tumor mass-related causes (e.g. anemia).

ii) The assessment of the **background of molecular characteristics impacting on progression** showed that a large part of their impact can be explained by association with plasma cell accumulation rate (DT) and/or tumor mass.

iii) Based on the first two parts, **mechanisms of progression and evolution of asymptomatic myeloma** have been addressed especially whether it is necessary to assume an ongoing genetic instability with *de novo* appearing aberrations to explain progression and in turn evolution of AMM from MGUS, which is not the case. At the same time, plasma cells at MGUS-stage should be considered already malignant. This has been addressed by iii.1) analyzing the percentages of **chromosomal aberrations** in MGUS, asymptomatic and symptomatic myeloma patients and association of differences with disease progression, showing those aberrations increasing in frequency in each subsequent compartment that are associated with plasma cell accumulation, and iii.2) by comparing **gene expression** of normal plasma cells with either MGUS, asymptomatic, or symptomatic myeloma showing the bulk of changes already appearing at MGUS stage, with iii.3) less differences between MGUS vs. AMM and AMM vs. symptomatic myeloma. As validation, iii.4) in a longitudinal cohort of patients presenting at AMM and symptomatic myeloma, temporal intra-patient heterogeneity and clonal dynamics with special focus on *de novo* gain of progression-associated aberrations was investigated, showing a *de novo* gain in only 9% of patients. iii.5) Subsequently, **growth kinetics** of MGUS and AMM have been investigated showing that a surprisingly low number of doublings takes place during evolvement of AMM from MGUS (0.62) and progression to MM (0.92) compared to the total number for symptomatic myeloma to evolve. Based on this analysis and the observed median DT in evolution (18.0 years) and progression of AMM (5.1 years) it is not possible that a *de novo* appearing aberration in MGUS or AMM drives the respective transitions based on observed doubling time, time to progression, and necessary number of doublings. This mechanism *cannot* therefore be responsible for the majority of progression events.

5. Conclusions

Accumulation rate, tumor mass, and molecular characteristics determine progression to symptomatic myeloma. Progression-associated chromosomal aberrations are multiplicatively associated with myeloma cell doubling time, explaining their increasing frequency from MGUS to asymptomatic- to symptomatic myeloma. Their number, rather than single aberrations, determines progression. The developed GEP-based HDAMM-score predicts progression of asymptomatic, symptomatic and MGUS-patients, as scores for symptomatic patients predict AMM-progression. The bulk of altered gene expression is already present in MGUS-patients with minor subsequent differences to asymptomatic and symptomatic myeloma. Longitudinal patient samples rarely (9%) show *de novo* appearance of progression-associated aberrations. In evolution and progression of asymptomatic myeloma, doubling times (MGUS-AMM: 18.0 years; AMM-MM: 5.1 years) and number of doublings (MGUS-AMM: 0.62; AMM-MM: 0.92) are incompatible with common *de novo* appearance of progression-driving aberrations.

Evolution and progression of asymptomatic myeloma can be explained by accumulation rate, tumor mass and molecular characteristics without necessity of de novo appearance of genetic alterations. Plasma cells at MGUS-stage should be considered malignant and the letters “US” be dropped from “MGUS”.

6. Summary

6.1 Summary (English)

Background

Asymptomatic multiple myeloma (AMM) evolves from monoclonal gammopathy of unknown significance (MGUS) and progresses to symptomatic myeloma characterized by end organ damage. Here, three main questions are addressed: i) Which factors determine evolution and progression of asymptomatic myeloma, and what is their molecular background? ii) Is progression driven by ongoing molecular (clonal) evolution? iii) When to call a plasma cell "malignant"?

Methods

CD138-purified plasma-cell samples of 2369 consecutive patients with MGUS (n=304), asymptomatic (n=432) and symptomatic myeloma (n=1633) were subjected to interphase fluorescence *in situ* hybridization (n=31898 measurements), and of these n=951 (n=62 MGUS, n=259 AMM, and n=630 symptomatic myeloma) likewise to gene expression profiling (GEP). Sixty-five patients were followed longitudinally. Serum/urine samples (n=8398) allowed modelling plasma cell doubling time in AMM and MGUS (n=322, and n=196, respectively).

Results

Accumulation rate, tumor mass, and molecular characteristics determine progression to symptomatic myeloma. Progression-associated chromosomal aberrations are multiplicatively associated with myeloma cell doubling time, explaining their increasing frequency from MGUS to asymptomatic- to symptomatic myeloma. Their number, rather than single aberrations, determines progression. The developed GEP-based HDAMM-score predicts progression of AMM-, symptomatic and MGUS-patients, as scores for symptomatic patients predict AMM-progression. The bulk of altered gene expression is already present in MGUS-patients with minor subsequent differences to AMM and symptomatic myeloma. Longitudinal patient samples rarely (9%) show *de-novo-appearance* of progression-associated aberrations. In evolution and progression of AMM, doubling times (18.0/5.1 years) and number of doublings (0.62/0.92) are incompatible with common *de novo* appearance of progression-driving aberrations.

Conclusions

Evolution and progression of AMM can be explained by accumulation rate, tumor mass and molecular characteristics without necessity of *de novo* appearance of genetic alterations. Plasma cells at MG*US*-stage should be considered malignant and the letters "US" be dropped from "MGUS".

6.2 Summary (German)

Hintergrund

Das asymptomatische Multiple Myelom entwickelt sich aus einer Monoklonalen Gammopathie *unklarer Signifikanz* (MGUS) und progrediert zum symptomatischen Myelom, welches durch Endorganschäden charakterisiert ist. Hier wurde untersucht: i) Welche Faktoren determinieren Evolution und Progression des asymptomatischen Myeloms und was ist deren molekularer Hintergrund? ii) Wird die Progression durch eine anhaltende molekulare (klonale) Evolution bedingt? iii) Wann sollte eine Plasmazelle als „maligne" bezeichnet werden?

Methoden

CD138-aufgereinigte Plasmazellproben von 2369 konsekutiven Patienten mit MGUS (n=304), asymptomatischem Myelom (AMM; n=432) und symptomatischem Myelom (MM; n=1633) wurden mittels Interphase Fluoreszenz *in situ* Hybridisierung (n=31898 Untersuchungen), n=951 ebenfalls mittels globaler Genexpressionsanalysen (n=62 MGUS, n=259 AMM und n=630 MM Proben), sowie 65 longitudinal untersucht. Serum- und Urinproben (n=8398) ermöglichten die Modellierung der Plasmazell-Verdopplungszeit bei AMM (n=322) und MGUS (n=196).

Ergebnisse

Akkumulationsrate, Tumormasse und molekulare Charakteristika determinieren die Progression zum symptomatischen Myelom. Progressionsassoziierte chromosomale Aberrationen wirken multiplikativ auf die Verdopplungszeit von Myelomzellen, was deren zunehmende Häufigkeit von MGUS, AMM zum symptomatischen Myelom erklärt. Entscheidender als die Einzelaberration ist deren Gesamtzahl. Der entwickelte GEP-basierte HDAMM-Score prädiktiert die Progression von AMM, symptomatischem Myelom und MGUS-Patienten, Scores für symptomatischen Patienten die AMM-Progression. MGUS-Patienten zeigen bereits den Hauptteil alterierter Genexpression, mit nur kleinen Unterschieden zu AMM und symptomatischen Patienten. Longitudinale Patientenproben weisen selten (9%) *de novo* progressionsassoziierte chromosomale Aberration auf. Verdopplungszeit (18,0/5,1 Jahre) und Zahl der Verdopplungen (0,62/0,92) während Evolution bzw. Progression des AMM sind in Widerspruch zum regelhaften *de-novo* Auftreten progressionsbedingender Aberrationen.

Schlussfolgerung

Evolution und Progression des AMM können durch Akkumulationsrate, Tumormasse und molekulare Charakteristika erklärt werden ohne Notwendigkeit des *de novo* Zugewinns genetischer Alterationen. Plasmazellen im MGUS-Stadium sollten als maligne angesehen und die beiden Buchstaben „US" in MGUS weggelassen werden.

7. List of frequently used abbreviations

AMM ..... asymptomatic multiple myeloma
BMPC ..... (normal) bone marrow plasma cell
CA ..... chromosomal aberrations
CSR ..... class switch recombination
DT ..... doubling time
FL ..... focal lesion
FI ..... fast increase
FLC ..... free light chain (assay)
GEP ..... (global) gene expression profiling
GMMG ..... German-speaking myeloma multicenter group
GPI ..... gene expression-based proliferation index
HMCL ..... human myeloma cell line
HRD ..... hyperdiploidy
iFISH ..... interphase fluorescence in situ hybridization
IgH ..... immunoglobulin heavy chain
IgL ..... immunoglobulin light chain
II ..... infinitesimal increase
IMWG ..... International Myeloma Working Group
ISS ..... International Staging System
LfM ..... Labor für Myelomforschung
LOH ..... loss of heterozygosity
MBC ..... memory B-cells
MGUS ..... monoclonal gammopathy of unknown significance
MM ..... (therapy-requiring) multiple myeloma
MNC ..... mononuclear cell
MRI ..... magnetic resonance imaging
OS ..... overall survival
PAA ..... progression-associated chromosomal aberrations
PFS ..... progression-free survival
PPC ..... polyclonal plasmablastic cells
PRT ..... plastic reaction tube
RA ..... random aspirate
SI ..... slow increase
SNV ..... single nucleotide variant
TTP ..... time to progression

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10. Appendix – Contributions

Table 10.1. Contributions to the scientific work depicted in this dissertation.

SUBJECT	ITEM	CONTRIBUTION	ASSOCIATION / COOPERATION
CONCEPTION	Dissertation	**PD Dr. med. Dipl.-Phys. Dirk Hose**	1, 2, 3
	Dissertation within the TRR79	**PD Dr. med. Dipl.-Phys. Dirk Hose**	1, 2, 3
		Univ.-Prof. Prof. h.c. Dr. med. Dr. med. vet. Dr. h.c. Reinhard Schnettler	4
PATIENTS, SAMPLES AND METHODS			
Patients			
Presenting within clinical routine	Clinical, administrative and organizational responsibility	**Univ.-Prof. Dr. med. Anthony D. Ho** Prof. Dr. med. Hartmut Goldschmidt	2 2,3
presenting in clinical trials of the GMMG	Clinical, administrative and organizational responsibility	Prof. Dr. med. Hartmut Goldschmidt	2,3
Methods			
Sampling and plasma cell purification	Scientific, administrative and organizational responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose**	1, 2, 3
	Performing bone marrow aspiration	Responsible physician	2
	Performing purification	Technicians Labor für Myelomforschung	1
	Data analysis and interpretation	**PD Dr. med. Dipl.-Phys. Dirk Hose** Dr. med. Anja Seckinger	1, 2, 3
Interphase fluorescence in situ hybridization	Scientific responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose** Prof. Dr. sc. hum. Anna Jauch	1, 2, 3 5
	Administrative and organizational responsibility	**Prof. Dr. sc. hum. Anna Jauch**	5
	Performing iFISH	Technicians Molekularzytogenetisches Labor	5
	Data analysis and interpretation (regarding scientific project)	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger** Prof. Dr. sc. hum. Anna Jauch	1, 2, 3 1, 2, 3 5
Gene expression profiling	Scientific responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose**	1, 2, 3
	Administrative and organizational responsibility	**Prof. Dr. Bernard Klein**	6
	Performing GEP	Véronique Pantesco **PD Dr. med. Dipl.-Phys. Dirk Hose et al.**	6 1, 2, 3
	Data analysis and interpretation (regarding scientific project)	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3
Clinical data	Scientific responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3
	Data collection	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger** Sybille Seyfried	1, 2, 3 1, 2, 3 1
	Data analysis and interpretation	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3
Calculation and modeling of doubling time	Scientific responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3
	Modeling	**Marcel Mohr** Prof. Dr. rer. nat Anna Marciniak-Czochra	7
	Data analysis and interpretation	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3
Statistical analysis	Scientific responsibility	**PD Dr. med. Dipl.-Phys. Dirk Hose** Thomas Hielscher	1, 2, 3 8
	Performing analyses	Susanne Lipp Martina Emde PD Dr. med. Dipl.-Phys. Dirk Hose	1 1 1, 2, 3
	Data analysis and interpretation	**PD Dr. med. Dipl.-Phys. Dirk Hose** **Dr. med. Anja Seckinger**	1, 2, 3

1. Labor für Myelomforschung (Head: Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose), Medizinische Klinik V, Universitätsklinikum Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
2. Medizinische Klinik V (Medical director: Univ.-Prof. Dr. med. A.D. Ho), Universitätsklinikum Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
3. Sektion Multiples Myelom (Section head: Prof. Dr. med. Hartmut Goldschmidt), Medizinische Klinik V, Universitätsklinikum Heidelberg and Nationales Centrum für Tumorerkrankungen (NCT), Im Neuenheimer Feld 460, 69120 Heidelberg, Germany.
4. Klinik und Poliklinik für Unfall-, Hand- und Wiederherstellungschirurgie - Operative Notaufnahme Universitätsklinikum Gießen und Marburg GmbH, Standort Gießen and Institut für Infektiologie und Pathogenomik (Medical director: Univ.-Prof. Prof. h.c. Dr. med. Dr. med. vet. Dr. h.c. Reinhard Schnettler), Justus-Liebig-Universität Gießen, Rudolf-Buchheim-Straße 8, 35392 Gießen Germany.
5. Molekular-zytogenetisches Labor (Head: Prof. Dr. sc. hum. Anna Jauch), Institut für Humangenetik (Medical head: Univ.-Prof. Dr. med. Claus R. Bartram), Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany.
6. CHU de Montpellier, Hôpital Saint-Eloi, Institute for Research in Biotherapy (Head: Prof. Dr. Bernard Klein), 80, av. Augustin Fliche, 34295 Montpellier Cedex 5, France.
7. Applied Analysis and Modelling in Biosciences Group (Head: Prof. Dr. rer. nat. Anna Marciniak-Czochra), Institut für Angewandte Mathematik, BIOQUANT and IWR, Universität Heidelberg, Im Neuenheimer Feld 294, 69120 Heidelberg, Germany.
8. Abteilung für Biostatistik (Head: Prof. Dr. rer. nat. Annette Kopp-Schneider), Deutsches Krebsforschungszentrum Heidelberg, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany.

11. List of publications

11.1 Original publications in first or last authorship

1. **D. Hose**, T. Rème, T. Meissner, J. Moreaux, A. Seckinger, J. Lewis, V. Benes, A. Benner, M. Hundemer, T. Hielscher, J.D. Shaughnessy, Jr., B. Barlogie, K. Neben, A. Kramer, J. Hillengass, U. Bertsch, A. Jauch, J. de Vos, J.F. Rossi, T. Mohler, J. Blake, J. Zimmermann, B. Klein, H. Goldschmidt.
   Inhibition of aurora kinases for tailored risk-adapted treatment of multiple myeloma.
   Blood. 113 (2009) 4331-4340.
   Peer-Review, Impact-Factor: **9,775**

2. **D. Hose**, J. Moreaux, T. Meissner, A. Seckinger, H. Goldschmidt, A. Benner, K. Mahtouk, J. Hillengass, T. Rème, J. de Vos, M. Hundemer, M. Condomines, U. Bertsch, J.F. Rossi, A. Jauch, B. Klein, T. Mohler.
   Induction of angiogenesis by normal and malignant plasma cells.
   Blood. 114 (2009) 128-143.
   Peer-Review, Impact-Factor: **9,775**

3. A. Seckinger, T. Meissner, J. Moreaux, H. Goldschmidt, G.M. Fuhler, A. Benner, M. Hundemer, T. Rème, J.D. Shaughnessy, Jr., B. Barlogie, U. Bertsch, J. Hillengass, A.D. Ho, V. Pantesco, A. Jauch, J. de Vos, J.F. Rossi, T. Mohler, B. Klein, **D. Hose**.
   Bone morphogenic protein 6: a member of a novel class of prognostic factors expressed by normal and malignant plasma cells inhibiting proliferation and angiogenesis.
   Oncogene. 28 (2009) 3866-3879.
   Peer-Review, Impact-Factor: **8,559**

4. J. Caers*, **D. Hose***, [*shared], I. Kuipers, T.J. Bos, E. van Valckenborgh, E. Menu, E. De Bruyne, H. Goldschmidt, B. van Camp, B. Klein, K. Vanderkerken.
   Thymosin β4 has tumor suppressive effects and its decreased expression results in poor prognosis and decreased survival in multiple myeloma.
   Haematologica. 95 (2010) 163-167.
   Peer-Review, Impact-Factor: **5,868**

5. **D. Hose**, T. Rème, T. Hielscher, J. Moreaux, T. Meissner, A. Seckinger, A. Benner, J.D. Shaughnessy, Jr., B. Barlogie, Y. Zhou, J. Hillengass, U. Bertsch, K. Neben, T. Mohler, J.F. Rossi, A. Jauch, B. Klein, H. Goldschmidt.
   Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma.
   Haematologica. 96 (2011) 87-95.
   Peer-Review, Impact-Factor: **5,868**

6. T. Meissner, A. Seckinger, T. Rème, T. Hielscher, T. Mohler, K. Neben, H. Goldschmidt, B. Klein, **D. Hose**.
   Gene expression profiling in multiple myeloma–reporting of entities, risk, and targets in clinical routine.
   Clin. Cancer Res. 17 (2011) 7240-7247.
   Peer-Review, Impact-Factor: **8,193**

7. A. Seckinger, T. Meißner, J. Moreaux, D. Depeweg, J. Hillengass, K. Hose, T. Rème, A. Rösen-Wolff, A. Jauch, R. Schnettler, V. Ewerbeck, H. Goldschmidt, B. Klein, **D. Hose**.
Clinical and Prognostic Role of Annexin A2 in Multiple Myeloma.
Blood. 120 (2012) 1087-94.
Peer-Review, Impact-Factor: **9,775**

8. A. Yordanova*, **D. Hose*** [*shared], K. Neben, M. Witzens-Harig, I. Gütgemann, M.S. Raab, T. Moehler, H. Goldschmidt, I.G. Schmidt-Wolf.
Sorafenib in patients with refractory or recurrent multiple myeloma.
Hematol. Oncol. 31 (2013) 197-200.
Peer-Review, Impact-Factor: **2,355**

9. K. Neben, A. Jauch, T. Hielscher, J. Hillengass, N. Lehners, A. Seckinger, M. Granzow, M.S. Raab, A.D. Ho, H. Goldschmidt, **D. Hose.**
Progression in Smoldering Myeloma Is Independently Determined by the Chromosomal Abnormalities del(17p), t(4;14), gain 1q, Hyperdiploidy, and Tumor Load.
J Clin Oncol. 31 (2013) 4325-32.
Peer-Review, Impact-Factor: **17,879**

10. V. Wagner*, **D. Hose*** [*shared], A. Seckinger, L. Weiz, T. Meißner, R. Rème, I. Breitkreutz, K. Podar, A.D. Ho, H. Goldschmidt, A. Krämer, B. Klein, M.S. Raab.
Preclinical efficacy of sepantronium bromide (YM155) in multiple myeloma is conferred by down regulation of Mcl-1.
Oncotarget. 5 (2014) 10237-50.
Peer-Review, Impact-Factor: **6,627**

11. N. Weinhold, T. Meissner, D.C. Johnson, A. Seckinger, J. Moreaux, A. Försti, B. Chen, J. Nickel, D. Chubb, A.C. Rawstron, C. Doughty, N.B. Dahir, D.B. Begum, K. Young, B.A. Walker, P. Hoffmann, M.M. Nöthen, F.E. Davies, B. Klein, H. Goldschmidt*, G.J. Morgan*, R.S. Houlston*, **D. Hose***¥, K. Hemminki* [*shared PI status; ¥ corresponding author].
The 7p15.3 (rs4487645) association for multiple myeloma shows strong allele-specific regulation of the MYC-interacting gene CDCA7L in malignant plasma cells.
Haematologica. 100 (2015) e110-3.
Peer-Review, Impact-Factor: **5,868**

12. S. Fichtner*, **D. Hose***, [*shared], M. Engelhardt, T. Meißner, B. Neuber B, F. Krasniqi, M. Raab, S. Schönland, A.D. Ho, H. Goldschmidt, M. Hundemer.
Association of antigen-specific T-cell responses with antigen expression and immunoparalysis in multiple myeloma.
Clin Cancer Res. 21 (2015) 1712-21.
Peer-Review, Impact-Factor: **8,193**

11.2 Original publications in coauthorship

13. F.W. Cremer, J. Bila, I. Buck, M. Kartal, **D. Hose**, C. Ittrich, A. Benner, M.S. Raab, A.C. Theil, M. Moos, H. Goldschmidt, C.R. Bartram, A. Jauch.
Delineation of distinct subgroups of multiple myeloma and a model for clonal evolution based on interphase cytogenetics.
Genes Chromosomes Cancer. 44 (2005) 194-203.
Peer-Review, Impact-Factor: **3,836**

14. F.W. Cremer, M. Kartal, **D. Hose**, J. Bila, I. Buck, F. Bellos, M.S. Raab, M. Brough, A. Moebus, H.D. Hager, H. Goldschmidt, M. Moos, C.R. Bartram, A. Jauch.
High incidence and intraclonal heterogeneity of chromosome 11 aberrations in patients with newly diagnosed multiple myeloma detected by multiprobe interphase FISH.
Cancer Cancer Genet. Cytogenet (Cancer Genetics since 2011). 161 (2005) 116-124.
Peer-Review, Impact-Factor: **2,417**

15. J. Moreaux, F.W. Cremer, T. Rème, M. Raab, K. Mahtouk, P. Kaukel, V. Pantesco, J. de Vos, E. Jourdan, A. Jauch, E. Legouffe, M. Moos, G. Fiol, H. Goldschmidt, J.F. Rossi, **D. Hose**, B. Klein.
The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature.
Blood. 106 (2005) 1021-1030.
Peer-Review, Impact-Factor: **9,775**

16. K. Mahtouk, **D. Hose**, T. Rème, J. de Vos, M. Jourdan, J. Moreaux, G. Fiol, M. Raab, E. Jourdan, V. Grau, M. Moos, H. Goldschmidt, M. Baudard, J.F. Rossi, F.W. Cremer, B. Klein.
Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells.
Oncogene. 24 (2005) 3512-3524.
Peer-Review, Impact-Factor: **8,559**

17. S. Wenisch, K. Trinkaus, A. Hild, **D. Hose**, K. Herde, C. Heiss, O. Kilian, V. Alt, R. Schnettler.
Human reaming debris: a source of multipotent stem cells.
Bone. 36 (2005) 74-83.
Peer-Review, Impact-Factor: **4,461**

18. K. Trinkaus, S. Wenisch, C. Siemers, **D. Hose**, R. Schnettler.
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Der Unfallchirurg. 108 (2005) 650-656.
Peer-Review, Impact-Factor: **0,608**

19. M. Hundemer, S. Schmidt, M. Condomines, A. Lupu, **D. Hose**, M. Moos, F.W. Cremer, C. Kleist, P. Terness, S. Belle, A.D. Ho, H. Goldschmidt, B. Klein, O. Christensen.
Identification of a new HLA-A2-restricted T-cell epitope within HM1.24 as immunotherapy target for multiple myeloma.
Exp. Hematol. 34 (2006) 486-496.
Peer-Review, Impact-Factor: **2,806**

20. J. Moreaux, **D. Hose**, T. Rème, E. Jourdan, M. Hundemer, E. Legouffe, P. Moine, P. Bourin, M. Moos, J. Corre, T. Mohler, J. de Vos, J.F. Rossi, H. Goldschmidt, B. Klein.
CD200 is a new prognostic factor in multiple myeloma.
Blood. 108 (2006) 4194-4197.
Peer-Review, Impact-Factor: **9,775**

21. K. Mahtouk, F.W. Cremer, T. Rème, M. Jourdan, M. Baudard, J. Moreaux, G. Requirand, G. Fiol, J. de Vos, M. Moos, P. Quittet, H. Goldschmidt, J.F. Rossi, **D. Hose**, B. Klein.
Heparan sulphate proteoglycans are essential for the myeloma cell growth activity of EGF-family ligands in multiple myeloma.
Oncogene. 25 (2006) 7180-7191.
Peer-Review, Impact-Factor: **8,559**

22. S. Wenisch, K. Trinkaus, A. Hild, **D. Hose**, C. Heiss, V. Alt, C. Klisch, H. Meissl, R. Schnettler.
Immunochemical, ultrastructural and electrophysiological investigations of bone-derived stem cells in the course of neuronal differentiation.
Bone. 38 (2006) 911-921.
Peer-Review, Impact-Factor: **4,461**

23. M. Condomines, **D. Hose**, P. Raynaud, M. Hundemer, J. de Vos, M. Baudard, T. Moehler, V. Pantesco, M. Moos, J.F. Schved, J.F. Rossi, T. Rème, H. Goldschmidt, B. Klein.
Cancer/Testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis.
J. Immunol. 178 (2007) 3307-3315.
Peer-Review, Impact-Factor: **5,362**

24. J. Moreaux, **D. Hose**, M. Jourdan, T. Rème, M. Hundemer, M. Moos, N. Robert, P. Moine, J. de Vos, H. Goldschmidt, B. Klein.
TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines.
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Remarks:
Impact factors are based on the Journal Citation Report Science Edition 2013 (Thomson Reuters).

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Gießen, den 22. Mai 2015 Priv.-Doz. Dr. med. Dipl.-Phys. Dirk Hose