CHROMOSOME 13q14 DELETIONS IN MULTIPLE MYELOMA AT
CHRIS-HANI BARAGWANATH HOSPITAL

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Medicine (MMed), in Haematology.

JOHANNESBURG, 2009
DECLARATION

I declare that this is my unaided work, except for some technical and specialised cytogenetic investigations. It is being submitted for the degree of Master of Medicine, in Haematology, to the University of the Witwatersrand. It has not been submitted before for any other degree or examination at this or any other University.

____________________                             ________________
Teboho Pheeha                                                  Date
ETHICS COMMITTEE APPROVAL

Approval for this study was granted by the Ethics Committee for Research on Human Subjects, University of the Witwatersrand (Clearance Certificate Protocol Number – M01-01-12 / Reference Number 14/49 Pheeha).
DEDICATION

This work is dedicated to my parents, my husband Eddie, son Thamaha, and daughters Tshegofatso and Kabelo.
ABSTRACT

Multiple Myeloma (MM) is a malignancy of plasma cells. The incidence worldwide has been reported to be 3-4/100 000 of the population. The exact aetiology is not known, but several factors have been implicated in the aetiology-pathogenesis of the disease.

Chromosomal abnormalities are well documented in MM. Their detection is important, as some of the cytogenetic abnormalities such as the 13q deletion are associated with a poor prognosis. Knowledge of the prognostic factors guides the clinician with respect to the appropriate management of the patient.

Prior to the use of fluorescence in situ hybridisation (FISH) as a technique for detecting cytogenetic abnormalities in MM, progress was slow in this field because of the difficulty of obtaining analysable metaphases in view of the low proliferative activity of plasma cells. FISH has significantly improved the detection rate over conventional cytogenetics.

Objective: The present study set out to determine the proportion of patients with MM who have a detectable chromosome 13q deletion using conventional cytogenetic and FISH analysis. The FISH technique was specifically studied to see if the detection rate of the 13q deletion is improved compared to conventional cytogenetics. Furthermore, the cytogenetic abnormalities detected were correlated with the course of the disease, as well as other parameters of prognostic significance.
Methods: Bone marrow aspiration specimens were obtained from thirty (30) patients with MM. Both newly and previously diagnosed patients were included. The sample size was however reduced to twenty (20) because of the need to optimise the technique and improve signal detection. Conventional cytogenetic and FISH analysis was performed using the LSI D13S319 DNA probe as the test probe, and the centromeric alpha 11 and 18 as control probes. The analysis was carried out by two observers.

Results: In the current study, the detection of chromosomal aberrations was much better with FISH analysis compared to conventional cytogenetics i.e. 25% versus 5%.

Of all the patients with chromosomal aberrations, 25% (5/20) had the specific deletion 13q14 (D13S319). Most of our patients (70%) presented with stage III disease. 60% of those were positive for deletion 13q14 (D13S319), i.e 3/5 patients had stage III disease. However, there was no correlation between disease stage and chromosome status, as the majority of the patients presented with advanced stage disease, irrespective of their chromosomal status. Other factors of prognostic significance such as the haemoglobin level, beta-2 microglobulin and creatinine levels were not found to correlate with the presence of the chromosomal aberration but with disease stage. Furthermore, median survival did not correlate with the presence of the chromosomal abnormality.

Conclusion: FISH analysis improves the detection rate of chromosomal abnormalities in MM compared to conventional cytogenetics. The prevalence of 13q14 deletion in our patient population is lower than that reported in the
literature (25% vs 30-80%). No correlation was found between the presence of the deletion 13q14 (D13S319) and mortality, stage of disease and laboratory parameters that have been associated with poor prognosis in MM i.e. haemoglobin, Beta-2-microglobulin and creatinine levels. The prognostic significance of deletion 13q14 (D13S319) in MM could not be established from this study. A prospective study with a larger sample and using probes covering the whole extent of chromosome 13q may possibly yield different or more conclusive results. The use of other techniques such as gene expression profile analysis would probably be of value in determining the prognostic significance of 13q14 lesions in MM.
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LIST OF ABBREVIATIONS

BJP – Bence Jones Protein
CRP - C–Reactive Protein
CAS – Chronic Antigenic Stimulation
CCT – Combination Chemotherapy
CHBH – Chris-Hani Baragwanath Hospital
DAPI – Diamino-2-phenylindole
DDK1 – Dickkopf1
ESR – Erythrocyte Sedimentation Rate
FGFR-3 – Fibroblast Growth Factor Receptor 3
FISH – Fluorescence In Situ Hybridisation
HB/Hb – Haemoglobin
HHV-8 – Human Herpes Virus 8
Ig - Immunoglobulin
IGFB – Insulin-like Growth Factor Binding Protein
ImiDs – Immunomodulatory drugs
IL - Interleukin
INF - Interferon
KSHV – Kaposi Sarcoma Herpes Virus
LDH – Lactate Dehydrogenase
MGUS – Monoclonal Gammopathy of Undetermined Significance
MM – Multiple Myeloma
OS – Overall survival
RB – Retinoblastoma
RPMI – Roswell Park Memorial Institute
SCT – Stem Cell Transplantation
sFRP – secreted Frizzled receptor like proteins
TNF – Tumour Necrosis Factor
WNT – Wingless type
CHAPTER 1

1.0 LITERATURE REVIEW

1.1 Introduction

Multiple myeloma (MM) is a malignant proliferation of plasma cells, which are terminally differentiated B lymphocytes. MM accounts for approximately 10 to 20% of all haematopoietic malignancies (Cigudosa et al, 1998). On average, the incidence worldwide is reported as 3-4/100 000 of the population (Morgan, 1999; Muir et al, 1987; Alexanian, 1985; Pottern and Blattner, 1985). MM is characteristically a disease of middle and old age. The incidence increases with increasing age and reaches a peak during the seventh decade of life. The median age at diagnosis is 65 years. Myeloma case series from Africa have suggested a younger median age at diagnosis (approximately 5 - 10 years younger) than in the Western world, probably reflecting the younger age structure of the African population (Patel et al, 1992; Mukiibi and Kyobe, 1988). There is a slight male predominance, and the disease occurs twice as often in blacks as it does in caucasians (Muir et al, 1987; Alexanian, 1985; Pottern and Blattner, 1985; Blattner et al, 1979).

The clinical presentation of MM is exemplified by bone pain (especially backache), anaemia, recurrent infections, renal dysfunction, and hypercalcaemia. Other features include pathological fractures, osteopenia, vertebral compression fractures, spinal cord compression, hyperuricaemia, plasmacytommas, abnormal bleeding tendency, hyperviscosity and amyloidosis (Malpas, 1995; Patel, 1994; Kyle, 1990).
The diagnosis of MM is based on the WHO diagnostic Criteria/ International Myeloma Working Group Criteria (see appendix 3 for details). All three of the following criteria are required to establish a diagnosis.

i) M-protein in serum or urine (see figure 1.1)

ii) Bone marrow clonal plasma cells or plasmacytoma

iii) Related organ/tissue impairment (CRAB: hypercalcemia, renal insufficiency, anaemia, bone lesions)

The National Cancer Institute Criteria are detailed in appendix 2.

Figure 1.1 Electrophoresis pattern in Multiple Myeloma

Figure 1.2 Plasma cell morphology
Once the diagnosis of MM is established, the disease is staged according to the Durie and Salmon staging system (Durie and Salmon, 1975 - see Appendix 1).

In addition to the above staging system, a number of other prognostic factors have been developed. These prognostic factors are related to the tumour burden or intrinsic malignancy. Examples of prognostic factors related to the tumour burden include Beta-2 microglobulin, ‘M’ component, percentage of bone marrow plasma cells, osteolytic lesions, haemoglobin and serum calcium. Those that are related to the intrinsic malignancy include: plasma cell labelling index, CRP, IL-6, albumin, genetic alterations/cytogenetic abnormalities, plasma cell phenotype, thymidine kinase, lactic dehydrogenase, immune dysregulation and neopterin (Boccadoro and Pileri, 1995).

The vast majority of patients (approximately 90%) require therapeutic intervention once the diagnosis is established. In such patients therapy can prolong and improve quality of life. The remaining 10% demonstrate an indolent course, with slow progression of disease over many years.

Treatment may be broadly classified as supportive and/or specific. Supportive care is an essential component of the disease. Supportive care is generally directed at the anticipated complications of the disease. Analgesics, allopurinol, increased fluid intake, antibiotics, erythropoietin, haematinics, transfusion of blood and blood products, dialysis and plasmapheresis are examples of supportive care used in the treatment of MM. Hypercalcaemia responds well to hydration, natriuresis, mobilisation, glucocorticoids and importantly to bisphosphonates. Bisphosphonates have pleotropic effects

Most of the specific modalities of therapy used are not curative, except possibly for allogeneic stem cell transplantation. This, together with innovative therapies for MM (see p.5) represent a new treatment paradigm, targeting tumour cells and their microenvironments to achieve greater tumour cytoreduction and potentially a cure (Munshi, 2004).

The standard induction therapy for MM using a combination of oral Melphalan and Prednisone (MP) achieves complete responses in only 5% of patients and improves median survival to 36 months (Barlogie et al, 2004). In 1998, the Myeloma Trialist’s Collaborative Group performed a meta-analysis of data from 6633 patients from 27 randomised trials. They found higher response rates with combination chemotherapy (CCT) than with MP (60% v 53.2%, respectively; P <0.00001), but no significant differences in response duration or overall survival (OS). With high dose melphalan-based autotransplants, especially in a tandem transplant setting, complete response rates exceeding 50% have been reported. At ten years, in the absence of cytogenetic abnormalities (especially abnormalities of chromosome 13, i.e. 13q deletion and hypodiploidy), 25% of patients remain event-free and 40% are alive (Shaughnessy et al, 2003). Although this represents a significant improvement, the development of chemotherapy-resistant disease remains an important therapeutic challenge.

Current research is focusing on attempts at overcoming resistance. New and re-emerging approaches specifically target the mechanisms critical for MM cell growth and survival in the bone marrow microenvironment. Some of
these novel therapeutic agents include thalidomide (Weber et al, 2003; Yakoub-Agha et al, 2002) and its analogue immunomodulatory drugs (ImiDS) e.g. Revlimid (Lenalidomide) (Richardson et al, 2002), the proteosome inhibitor Bortezomib (Richardson et al, 2003) and arsenic trioxide (Munshi et al, 2002), all of which have demonstrated clinical anti-MM activity even in patients with refractory and relapsed disease. Ongoing studies will define the exact role of these agents (i.e. as single agents/combination with other agents such as dexamethasone and where in the course of disease they should be introduced – at diagnosis; post-autotransplants etc.)

1.2 Pathophysiology

Immunoglobulin heavy chain (IgH) analysis reveals that the malignant plasma cells arise from post-germinal centre B lymphocytes that have undergone antigen selection, isotype switch recombination and somatic hypermutation of their Ig (immunoglobulin) genes. The plasma cells then migrate to the bone marrow where they proliferate (van Riet et al, 1998). Interactions with marrow stromal cells facilitate homing and growth of myeloma cells. Stromal cells produce interleukin 6 (IL-6) – an important growth and differentiating factor for plasma cells.

The mechanism by which these cells undergo malignant transformation is not clear. Hallek et al (1998), proposed a multi-step transformation process starting with a normal plasma cell that progresses to Monoclonal Gammopathy of Unknown Significance (MGUS), where the cells are immortalised, but not transformed, and then to intra-medullary myeloma
where the cells have become malignant, and finally to extra-medullary myeloma.

The malignant process may be initiated by an Ig gene translocation related to the Ig recombination process, that results in ectopic expression (dysregulated expression) of an oncogene such as cyclin D1, c-myc etc., caused by the juxtaposition to strong regulatory sequences of the IgH locus and resulting in immortalization of the malignant clone.

Dysregulated c-myc levels may be the first growth stimulatory signal in the development of myeloma. C-myc, together with p53, counteracts pRB and p107 mediated growth arrest signals and thus stimulates cell proliferation. Additionally, the myeloma cells may be susceptible to cytokine-deprived or growth factor-deprived apoptosis. There is early acquisition of chromosomal instability, with frequent multiple trisomies present even in MGUS and monosomy 13 in myeloma.

The second proliferative signal may be activation of the ras/MAPK (mitogen-activated protein kinase) pathway, by secretion of IL-6 and related cytokines from the bone marrow stromal cells in response to adhesion of myeloma cells. Activation of the ras/MAP kinase pathway prevents c-myc induced apoptosis, and results in up-regulation of bcl-2 and other anti-apoptotic factors that prolong survival of myeloma cells. The ensuing constitutive ras activation results in higher expression of bcl-2 and endogenous IL-6 secretion by myeloma cells. The hypermutational process onto ras or a tumor suppressor gene on chromosome 13, results in selection of a single clone for malignant expansion. Activating ras mutations have been noted in 35-50% of MM patients. Most of these involve both N- and K-ras.
Tumours with t(4;14)(p16.3;q32) can have activating mutations of ras or fibroblast growth factor receptor 3 (FGFR3). Mutations of K but not N-ras have been associated with shorter survival (Liu et al, 1996; Bezieau et al, 2001)

In the advanced stages of the disease, loss of p53 function occurs, leading to genomic instability as evidenced by complex karyotypic abnormalities and translocations.

Deletion 17p13 is detected in 5% of the patients at diagnosis and 20-40% in advanced MM or plasma cell leukaemia (Mazars et al, 1992)

The secondary IgH translocation to a variety of loci occurs by mechanisms unrelated to physiologic Ig recombination processes, that may reflect the presence of a more general genomic instability. Further mutations, like p53, lead to stroma-independent growth, and escape of myeloma cells from the bone marrow micro-environment (Bergsagel et al, 1999; Hallek et al, 1998; Feinman et al, 1997).

1.3 Aetiopathogenesis

The exact aetiology of MM is unknown. A number of factors have been suggested as causative. Exposure to ionizing radiation and agriculture are the most common (Patel, 2000; Demers et al, 1993; Shimizu, 1990; Cuzick and De Stavola, 1988; Steineck and Wiklund, 1986; Pottern and Blattner, 1985; Cuzick, 1981). One of the more convincing risk factors for myeloma has been exposure to ionizing radiation. This has been documented in the studies of survivors of the atomic bomb explosions in Hiroshima and Nagasaki, Japan, in 1945 (Ichimaru et al, 1982). Radiation exposures include exposure to ionizing radiation (atomic bomb; high dose) and occupation related radiation
such as nuclear workers and radiologists, and recipients of therapeutic and
diagnostic radiation. In contrast to individuals who were exposed to high dose
irradiation, the excess risk of myeloma in association with low dose radiation
exposure remains controversial. The target of ionising radiation is believed to
be the proto-oncogenes in DNA.

In the reports in which an association with farming has been suggested (see
references above), because of the wide variety of different exposures, it is not
possible to determine whether oncogenic zoonotic viruses, pesticides
(including herbicides and insecticides), agricultural chemicals or some
combination of exposures is responsible for the increased risk of myeloma.

Other associations or specific exposures that may increase myeloma risk
among farmers include dairy and sheep farming, exposure to cattle, poultry
and other farm animals, orchard farming, exposure to grain dust, aflatoxins,
paints and solvents, wood treatment, chemicals used for fencing, engine
exhaust from farm equipment, welding fumes and pollen (Pearce and Reif,
1990; Blair et al, 1985).

Exposure to benzene has also been implicated as an aetiological factor.
Recent data, however, appear to dispute this association (Bergsagel, et al,
1999).

Bergsagel et al (1999), in their review of benzene and myeloma concluded
that in contrast to the strong evidence linking high levels of benzene exposure
to an increased risk of developing acute myelogenous leukaemia, there is no
scientific evidence to support a causal association between exposure to
benzene or other petroleum products and the risk of developing MM.
Chronic antigenic stimulation (CAS) has been suggested to be a risk factor in the development of myeloma. In addition, there is growing evidence that CAS could facilitate the progression of the disease. This may occur via (IL-6) interleukin-6. Overproduction of IL-6 occurs in a number of other diseases such as rheumatoid arthritis, systemic lupus erythematosus, trauma, acute infectious neural disease, cardiac myxoma and transplantation (Wolverkamp and Marquet, 1990). It is plausible that these conditions could be regarded as potential risk factors for the development of myeloma via IL-6 over production. Another pathway by which CAS could be associated with myeloma is through production of a monoclonal protein (M-protein), which may be transient or chronic. Transient production occurs normally in response to trauma, drugs and infections with specific antigens (Haas et al, 1990). Chronic production can occur in response to rheumatoid arthritis, infections, malignancy, neurological and dermatological diseases, chronic liver disease etc. (Passweg et al, 1996; Blade and Kyle, 1995).

Cytokines play an important role in the pathogenesis of MM. Interactions with marrow stromal cells facilitate homing and growth of myeloma cells. The most commonly implicated cytokines are interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNFα), IL-3, IL-6, IL-5, IL-10, IL-21, insulin-like growth factor I (IGF-I), vascular endothelial growth factor (VEGF) and transforming growth factor beta I (TGF-βI) (Chauhan et al, 1996; Urashima et al, 1996; Urashima et al, 1995).

IL-6 may act both as an autocrine and paracrine differentiating factor for plasma cells. IL-6 is a potent stimulator of B-cell differentiation and is essential for the survival and growth of myeloma cells (Klein et al, 1989; Kawano et al,
Additionally, myeloma cells can stimulate stromal and bone cells to produce large amounts of IL-6 (Carter et al, 1990). IL-6 in turn stimulates the myeloma cells as well as cells in the bone marrow microenvironment to produce osteoclast activating cytokines including tumour necrosis factor α and IL-1β. These cytokines upregulate stromal cell secretion of receptor activator of nuclear factor κ B ligand (RANK-L), which stimulates osteoclast production. Excess osteoclastic activity may manifest as lytic bone disease and hypercalcaemia. Secretion of osteoprotegerin, the major inhibitor of RANK-L is reduced (Croucher and Apperley, 1998). In addition Dickkopf-1 which is thought to be produced by myeloma cells or stromal cells in association with myeloma cells inhibits Wnt-signaling, which is important for osteoblast differentiation. This results in inhibition of osteoblast activity in MM (Erming et al, 2003). Other osteoblast inhibitors are also thought to play a role e.g (IGFBP-4) insulin like growth factor binding protein-4, secreted frizzled receptor-like proteins(sFRP-2/3) and IL-7 (Erming et al, 2003)

Recently, human herpesvirus-8/Kaposi sarcoma herpesvirus (HHV-8/KSHV) has been implicated in the aetiopathogenesis of MM (Rettig et al, 1997). Involvement of the virus in the development of myeloma was suggested after detection of KSHV DNA sequences by polymerase chain reaction (PCR) in the dendritic cells of 15/15 patients with MM and 2/8 patients with MGUS, but not from malignant cells or bone marrow dendritic cells from normal individuals or patients with other malignancies (Rettig et al, 1997). The association was biologically plausible as KSHV was found to encode an IL-6 homologue that was capable of stimulating growth and preventing apoptosis of murine and human myeloma cell lines (Burger et al,
1998; Moore et al, 1996). However, following on the initial positive reports, a number of authors have not been able to confirm this association and the role of KSHV in MM remains controversial (Olsen et al, 1998; Tarte et al, 1998). In a local study by Patel et al (2001), the positivity of KSHV DNA sequences in MM adherent cell cultures was found to be 23.5%. This is similar to the background sero-prevalence rate for KSHV in South Africa. Based on these findings, there does not appear to be a clear association between MM and KSHV in the local population.

1.4 Genetics of Multiple Myeloma

The familial occurrence of MM is well recognized. The first documented report was by Mandema and Wildervanck, in 1954. Since then, a number of authors have reported its occurrence, mainly in siblings and first-degree relatives of multiple myeloma patients (Herrinton et al, 1995).

The role of genetic factors in the pathogenesis of the disease is still uncertain, but MM per se is not regarded as an inherited disorder. Genetic factors may be used to explain the differences in incidence between the different ethnic groups. Evidence for the role of genetic factors in the pathogenesis of the disease is based on striking differences in the incidence of monoclonal gammopathy and plasmacytomas in different inbred strains of mice (Potter et al, 1975; Radl and Hollander, 1974); racial differences in the incidence of MGUS (monoclonal gammopathy of undetermined significance) and MM in humans (Riedl and Pottern, 1992); the association of an increased risk of developing myeloma with certain human leucocyte antigens (HLA) (Pottern et al, 1992); and the occurrence of familial MM.
A number of publications have suggested an association of HLA phenotypes with MM. These involve class I (A, B and C), as well as class II (DR) antigens. There is no consistent or specific MM HLA phenotype.

Miller (1974) found that in an analysis of HLA antigens in adults with various forms of haematological malignancies, there was an increase in HLA-5 and HLA-13 in patients with lymphoproliferative disorders, including multiple myeloma, compared to normal controls. Earlier studies of MM patients reported an association with the B locus, in particular HLA-B5; with a significantly elevated risk of developing myeloma (Festen et al, 1976; Mason and Cullen, 1975; Miller, 1974). A significantly higher frequency of HLA-B18 antigen has also been documented in myeloma (Smith et al, 1974; Bertrams et al, 1972). An association with the C locus involving HLA-Cw5 and HLA-Cw6 antigens has also been reported (Leech et al, 1983, Pottern, et al, 1992).

There is a paucity of data available regarding the involvement of the D locus and MM. Muylle et al (1982), in their study of 28 patients, found no association with HLA DR antigens.

Patel et al (2002), in their study of 62 South African black patients with MM, showed a statistically significant association with HLA-B18 and not with any other published (MM associated) HLA phenotype.
1.5 Chromosomal abnormalities in Multiple Myeloma

Chromosomal (cytogenetic) abnormalities are well described in MM. Both numerical and structural abnormalities have been reported (Maslovsky et al, 1999; Bataille and Harousseau, 1997; Dewald et al, 1985). Detection of these abnormalities has been difficult in the past using conventional cytogenetics, due to the lack of adequate analysable metaphases, because of the low proliferation rate of plasma cells. The technical difficulties with culturing and banding contributes to this problem (Zhao et al, 2000). Other factors which compound the problem include the variable pattern of bone marrow infiltration, the complexity of the numerical and structural abnormalities described, and the absence of a common or specific abnormality (Avet-Loiseau et al, 1999). Conventional cytogenetics reveals abnormal karyotypes in only 40% of the patients at diagnosis and 63% of patients with advanced disease (Zojer et al, 2000; Fonseca et al, 1999; Maslovsky et al, 1999; Cigudosa et al, 1998).

Most of the abnormalities are reported in patients with advanced disease (stage III), which might suggest an association of genetic abnormalities with disease progression.

The chromosomal abnormalities that were detected and found to be recurrent in MM include: abnormalities of chromosomes 1, 3, 5, 7, 9, 11, 15, 19, 21, deletions of 13q and reciprocal translocations involving 14q32 (Avet-Loiseau et al, 1999; Garcia-Sanz et al, 1999). Less commonly, abnormalities of other chromosomes such as chromosome 8, 12, 16, 17 and 18 have been associated with myeloma (Fonseca et al, 1999; Tricot et al, 1995; Dewald et al, 1985; Lewis and Mackenzie, 1984).
Deletions of 13q14 and rearrangements of 14q32 (mainly translocations) have been described as the most frequently encountered chromosomal abnormalities in association with multiple myeloma (Zojer et al, 2000; Morgan, 1999; Cigudosa et al, 1998; Bergsagel et al, 1996).

Some chromosomal abnormalities have been associated with a poor prognosis. These includes any translocation, e.g. t(4;14), t(14;16), abnormalities involving 11q, deletions of 13q14 (Fonseca et al, 1999; Cigudosa et al, 1998) and deletions of chromosome 17 (Fonseca et al, 1999). Chromosomal abnormalities associated with a good prognosis and long-term survival are trisomies of chromosomes 6, 9 and 7 (Perez-Simon et al, 1998).

The rate of detection of cytogenetic abnormalities is higher with flow cytometric and FISH (Fluorescence in situ hybridisation) analysis. Abnormalities have been detected in the vast majority (80-90%) of patients with myeloma using FISH, irrespective of the stage of the disease (Tabernero et al, 1996; Drach et al, 1995; Barlogie et al, 1989).

Flow cytometric analysis of DNA content of myeloma cells in G0/1 phase has revealed aneuploidy in 30-80% of the patients (Fonseca et al, 1999; Garcia-Sanz et al, 1999; Barlogie et al, 1989). Based on the flow cytometry aneuploidy data and the FISH analysis, conventional cytogenetics fails to detect the majority of patients with chromosomal abnormalities.

Patients in relapse and progressive disease show a higher frequency (30-60%) of chromosomal abnormalities compared to newly diagnosed patients (Lai et al, 1995; Sawyer et al, 1994; Gould et al, 1988; Dewald et al, 1985). This is in line with the multi-step theory of MM oncogenesis (Hallek et al, 1998).
1.5.1 Chromosome 13 in Multiple Myeloma

Deletion (partial or complete) of chromosome 13q has been shown to be the most frequently encountered chromosomal loss in MM (see figure 1.3). It is detected in 46% of the newly diagnosed and 73% of relapsed patients with MM (Zojer et al, 2000). Shaughnessy et al (2000), reported 13q deletions in 86% of MM patients compared to normal donors. Chang et al (1999), reported 13q deletions in 30% of patients with normal karyotypes on conventional cytogenetics. In a study by Avet-Loiseau et al (2002), monosomy 13 was found to be the most common chromosome 13 abnormality, detected in 92% of their patients. In a study comparing patients with MGUS that progressed to MM and patients with de novo MM, deletion 13q has been reported in 70% of patients with MGUS/MM compared to 40% of patients with de novo MM.

The prevalence of 13q deletion is reported to be 30-55% with interphase FISH (Fonseca et al, 2002; Shaughnessy et al, 2000).

Chromosome 13q deletion appears to be a good candidate event in the malignant transformation of myeloma plasma cells (Avet-Loiseau et al, 1999). The minimal common region of deletion on 13 is thought to be in band 13q14.3, which spans the region containing the RB-1 gene, loci D13S319 and D13S272 (see figure 1.6) (Viguie, 2001; Konigs et al, 2000).

The majority of these deletions are sub-microscopic and only detected during interphase.

The possibility of a putative tumour suppressor gene cannot be excluded, although the minimal deleted region is difficult to demonstrate. A candidate
tumour suppressor gene has been localised to 13q14.3 because this region is also deleted in Chronic Lymphocytic Leukemia and Non-Hodgkins lymphoma.

Figure 1.3 Schematic representation - Chromosome 13q deletion

This gene has however not been characterised and its role in the pathogenesis of multiple myeloma is not known. Chromosome 13q deletion has apparently been associated with IgA subtype myeloma, elevated beta-2 microglobulin level, higher proliferation rate and advanced age (Tricot et al, 1995).

It is recognised that the 13q14 chromosomal abnormality has also been associated with inferior clinical outcome despite treatment (Zojer et al, 2000; Fonseca et al, 1999; Tricot et al, 1995). The prognostic importance of deletion 13q14 is thought not to be in isolation, but in association with a high serum beta-2 microglobulin level, percentage bone marrow plasma cells (Viguie, 2001) and non-hyperdiploidy (Shaughnessy et al, 2003). Avet-Loiseau et al (2002), have however found elevated serum beta-2 microglobulin levels to be more closely associated with 14q32 abnormalities than with 13q14 deletion. Deletion 13q14 in MM is rarely observed as a sole abnormality both in hyperdiploidy and hypodiploidy karyotypes, but has a higher incidence in
hypodiploid forms, such that the prognostic value of deletion 13q14 is thought to be related to the ploidy (Viguie, 2001). Some authors however believe that the two independently confer a poor prognosis (Anthanasios et al, 2002)

The most common chromosomal abnormalities associated with deletion 13q14 are translocations of chromosome 14q32. These abnormalities do not seem to occur randomly, but are rather interconnected in MM.

Based on the various combinations found, MM could be stratified into four groups, viz. patients without 14q32 abnormalities but with deletion 13q14; patients with translocation 14q32 and deletion 13q14; t(4,14) and t(14;16), patients with other abnormalities of 14q and deletion 13q14; and patients with 14q32 abnormalities, but no deletion 13q14.

Figure 1.4 Chromosome 13 ideogram showing the mapping of the probes/genes.
In a study by Shaughnessy et al, using an 11-probe panel spanning the long arm of chromosome 13, deletions were detected in 86% of patients in different combinations.

![Diagram](image)

Figure 1.5 Vysis probe to demonstrate the location of D13S319 with regards to the RB-1 gene.

Heterogeneity was documented for the location, frequency and extent of the regions deleted in all patients. Of the deletions detected 75% were accounted for by the D13S272 locus, and 66% by the D13S31 locus, suggesting their importance in the disease process. Both loci were concurrently deleted in 59% of the patients. D13S319 is located between RB-1 and D13S25.

The RB-1 and D13S319 were deleted in 52% and 70% respectively of newly diagnosed patients.

These two loci (RB-1 and D13S25) appear to be deletion “hot spots” according to some authors (Shaughennessy et al, 2000).

In an earlier study by Zojer et al (2000), the prognostic importance of deletion 13q14 was reported, based on deletions of the RB-1 gene and D13S319 locus, where each was accounted for by 52% and 70% respectively.
This makes it difficult to determine whether the adverse prognostic implications were as a result of the deletions in isolation, or in combination with other loci being deleted.

Deletion of chromosome 13q14 implies both rapid disease recurrence and initial drug resistance (Shaughnessy et al, 2000). In trying to determine the biological implications of deletion 13q14 some groups have shown that deletion 13q14 is frequently associated with lambda type light chain, higher proliferation rate, lower serum monoclonal peak concentration, and increased angiogenesis in some but not all patients (Fonseca et al, 2002, Moreau et al, 2002)

Figure 1.6 The minimally deleted region of 13q14 in CLL
(Adapted from Chang et al, 1999)
1.5.2 Chromosome 14q32 in Multiple Myeloma

Avet-Loiseau et al (1999), reported rearrangements involving the IgH gene at 14q32 in 60-75% of their patients. Nishida et al (1997), reported similar rearrangements in 73% of their patients. Bergsagel and colleagues (1996), have shown that translocations involving the breakpoint at 14q32 to be nearly universal in MM. The rearrangements of 14q32 were found in all tumour cells, which strongly implicated these rearrangements as early events preceding clonal expansion. This translocation involves numerous partners. Those described include chromosomes 11, 4, 8, 16 and 18 (Dewald and Jenkins, 1991; Gould et al, 1988; Dewald et al, 1985). Chromosome 11q13 and 4p16 have been found to be the main partners (Avet-Loiseau, et al 1999; Bergsagel, et al, 1996). These translocations have not been found to form fusion genes, but to cause juxtaposition of oncogenes with promoter regions (Fonseca et al, 1999).

Abnormalities of 14q32 have also been associated with a poor prognosis. It has been shown that t (11;14 ) in particular is associated with a grave prognosis (Fonseca et al, 2002).
1.6 Aims and objectives of the study

The aims and objectives of the study are:

1. To determine if the detection rate of chromosomal abnormalities improves with FISH analysis compared to conventional cytogenetic studies.

2. To determine the proportion/percentage of patients with chromosome 13q14 deletion in the study population, and

3. To correlate the cytogenetic findings with known clinical and laboratory features of prognostic significance in our study population.
CHAPTER 2

2.0 PATIENTS AND METHODS

2.1 Patients

The study population consisted of adults with a confirmed diagnosis of MM. The patients were diagnosed, being treated and followed up by the Clinical Haematology Division, Department of Medicine, Chris-Hani Baragwanath Hospital (CHBH). The study period was from January 1999 to July 2003.

Ethical clearance was obtained from the Committee for Research with Human Subjects (Medical) of the University of the Witwatersrand - Reference Number 14/49.

During this period, all consecutive patients were considered for the study, but only patients with bone marrow aspirate specimens that had adequate tumour representation were selected for the study.

2.2 Materials and Methods

The study had both a prospective (13 patients) and a retrospective (7 patients) arm. Bone marrow aspirate smears were retrieved from storage for the retrospective arm. Two extra bone marrow aspirate smears were prepared and 1-2mls was sent for conventional cytogenetic studies for all the new patients. The sample cultures were collected in RPMI (Roswell Park Memorial Institute) medium with fetal calf serum and antibiotics. Growth was not stimulated as per standard operating procedure.

The pre-hybridisation technique had to be optimised for the older specimens, for FISH preparation.
Some of the strategies employed were, overnight fixation, rehydration, shortening of the diamino-2-phenylindole (DAPI) counterstaining time, extended washing times and repeat hybridisation.

FISH analysis was performed using the LSI* D13S319 spectrum orange, DNA probe specific for the D13S319 locus, purchased from S.A. Scientific Group (Vysis), as the test probe, and a centromeric chromosome 11-alpha and 18-alpha probes as controls. The method as per standard operating procedure involved fixation, dehydration in ethanol series of varying concentration, denaturation, followed by overnight hybridisation in a humidified chamber at 37° Celcius. This was followed by a washing step and Deamino-2-phenylindole (DAPI) counter-staining.

Analysis was carried out using the BX 61 Olympus Flourescence Microscope with computer software for capturing the pictures.

Hybridisation signals were enumerated in 50 to 100 cells. Results were analysed independently by two individuals. The cut off for the number of positive cells was set at five (i.e if signals were detected in less than five cells then the test was regarded as having been unsuccessful, only cases with signals in five or more cells were reported).
CHAPTER 3

3.0 RESULTS

The initial sample size was thirty patients. The number was reduced due to problems encountered with old bone marrow specimens, where hybridisation failed and signal detection was not possible even after optimisation of the technique. Finally, results of twenty of the total number of patients attempted were evaluated. The characteristics and results of the patients in whom conventional cytogenetics was attempted and FISH was successful are depicted in tables 1.1 and 1.2.

There were 15 males and 5 females with a male to female ratio of 3:1. The age ranged from 35 to 77 years with an average of 58 years. Based on the Durie and Salmon staging, four patients presented with stage I, one with stage II and fifteen with stage III disease.

The dominant isotype was IgG (70%) followed by IgA (25%) and 5% light chain disease.

3.1 Cytogenetic studies

Cytogenetic studies were performed on all of the patients in the prospective arm of the study. Success was however limited due to unavailability of metaphases in most patients. The main problem appears to have been failure to achieve growth, irrespective of whether the specimens were fresh or had adequate tumour representation. A successful result was obtained in 1/20 (5%) of the patients studied. The abnormality detected was hyperdiploidy (50-59 chromosomes).
3.2 Fluorescence In Situ Hybridisation (FISH)

With regard to FISH analysis five of the twenty (5/20) patients (25%) were found to be positive for deletion 13q14, locus D13S319. This result is lower than that reported in other studies (Zojer et al, 2000; Chang et al, 1999; Perez-Simon et al, 1998).

Figure 1.7 Interphase cell showing normal signals for chromosome 13q probe, two red 13q and two green signals for the control probe.

Figure 1.8 Interphase cell showing deletion of chromosome 13q14 (D13S319) with only one red signal. The two green signals represent the control probe.
Figure 1.9 The three green signals, demonstrate Trisomy of chromosome 11, which was used as a control probe.

Figure 1.10 Three green signals demonstrate Trisomy 18, which was used as a control probe.
## Table 1.1 Patients Characteristics

| SID | Age Yrs | Gender | WCC x 10^9/l | HB g/dl | Plts x 10^9/l | CRP | BM % Pl Cells | Paraprotein | IgG g/l | IgA g/l | IgM g/l | Urine (BJP) |
|-----|---------|--------|---------------|---------|---------------|-----|---------------|-------------|---------|---------|---------|-----------|-------------|
| 1   | 69      | M      | 6.7           | 8.9     | 187           | 13  | 85%           | 70          | 93.5    | 0.25    | 0.81    | Neg       |
| 2   | 52      | M      | 8.8           | 6.1     | 106           | 150 | 35%           | 51.24       | 2.52    | 50.4    | <0.25   | Pos       |
| 3   | 60      | M      | 5.3           | 6.1     | 215           | *   | 40%           | 64.3        | 3.34    | 88.3    | <0.25   | 0.61      |
| 4   | 55      | M      | 9.8           | 9.4     | 247           | *   | 22%           | 46.0        | 46.0    | 0.79    | 0.62    | 1.69      |
| 5   | 50      | M      | 4.10          | 7.5     | 231           | 55  | 84%           | 21.5        | 6.53    | 0.58    | <0.25   | 6.85      |
| 6   | 54      | F      | 4.89          | 9.4     | 277           | *   | 50%           | 69.2        | 91.8    | 0.51    | 0.38    | Pos       |
| 7   | 35      | M      | 6.6           | 16.6    | 278           | 32  | 42%           | 5.44        | 12.20   | 0.68    | 0.47    | 0.41      |
| 8   | 67      | M      | 5.65          | 10.9    | 231           | *   | 37%           | 46.6        | 58.2    | 0.40    | <0.25   | 0.90      |
| 9   | 60      | M      | 8.9           | 12.5    | 215           | *   | <1%           | 7.94        | 13.2    | 1.04    | 0.61    | 3.2       |
| 10  | 44      | M      | 2.41          | 8.6     | 324           | *   | *             | 38.5        | 42.6    | <0.25   | <0.26   | *         |
| 11  | 55      | F      | 5.84          | 5.2     | 381           | 150 | 89%           | 71.24       | 2.78    | 72.5    | <0.25   | 1.36      |
| 12  | 59      | M      | 4.8           | 9.4     | 186           | 114 | 44%           | 75.2        | 78.4    | <0.25   | 0.63    | Pos       |
| 13  | 60      | M      | 3.5           | 9.2     | 187           | *   | *             | 63.8        | 2.67    | 84.4    | <0.25   | 0.44      |
| 14  | 50      | M      | 4.96          | 5.6     | 93            | 106 | 33%           | 94.2        | 107     | <0.25   | 0.27    | 5.63      |
| 15  | 69      | M      | 7.08          | 6.3     | 290           | *   | 14%           | 69.6        | 113     | <0.25   | <0.25   | 0.09      |
| 16  | 65      | M      | 2.76          | 4.3     | 69            | *   | 70%           | 85.3        | 108     | 0.33    | <0.25   | *         |
| 17  | 65      | M      | 5.11          | 9.8*    | 174           | *   | 60%           | 38.2        | 48.0    | 1.00    | 0.53    | *         |
| 18  | 77      | F      | 9.1           | 9.4     | 224           | *   | 45%           | 43.6        | 77.9    | 0.32    | 0.26    | *         |
| 19  | 54      | F      | 5.8           | 11.9    | 350           | 34  | 15%           | 15.1        | 21.7    | 1.90    | 0.59    | 0.6       |
| 20  | 49      | M      | 9.2           | 7.8     | 419           | *   | *             | 79.3        | 79.3    | *       | Pos      |            |

SID = Study ID; WCC = white cell count; HB = Haemoglobin; CRP = C-Reactive Protein; PLTS = Platelets; BM % Pl Cells = Percentage bone marrow plasma cells; Paraprotein = Paraprotein level; Urine BJP = Urine Bence Jones Protein; Pos= positive; Neg = negative.*Result not available
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Creat = Creatinine; Tprot = Total protein; Alb = Albumin; B2-M = Beta-2 Microglobulin; Cal = Calcium; Stage = Disease stage,
Cyto = Cytogenetic studies, Uns = Unsuccessful; FISH = Fluoresence in situ hybridisation
Pos = positive for deletion 13q14 (D13S319), Neg = negative for the deletion,Tri = trisomy
Table 1.3 Factors of prognostic significance in patients at CHBH.
(Patel et al, 2000)

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LTFU – lost to follow up, Neg – negative, Pos - positive, Tri11- trisomy 11, Tri18 - trisomy18, Surv -Survival, Out-Outcome.
CHAPTER 4

4.0 STATISTICAL ANALYSIS

The statistical analysis was carried out using non-parametric data analysis methods, the U-test of Mann-Whitney and the Fisher exact test. Analyse-it for excel and SigmaStat software were utilised.

The detection rate of chromosomal aberrations improved significantly with FISH compared to conventional cytogenetics (5% vs 25%).

Although both cytogenetics and FISH analysis showed a low detection rate for Chromosome 13q14, compared to other studies, there was a poor measure of agreement between the two methods.

There was no statistically significant difference in mortality between patients with the chromosomal abnormality (13q14, D13S319) compared to those without the chromosomal abnormality (Chi-Square=3.4182 P=0.181), although more patients without the chromosomal abnormality died (66.67%) and two patients with the chromosomal abnormality died. The median survival was 3 months for patients without the 13q14 deletion and 8 months for patients with the 13q14 deletion.

Figure 1.11

However, in view of the small patient numbers studied, definite conclusions cannot be made.

Two of the patients with the chromosomal aberration were lost to follow up, so their survival could not be calculated.
Of the patients where survival could be calculated, two with the chromosomal abnormality had a survival period similar to that of patients without the chromosomal aberration and one patient with the chromosomal aberration and trisomy 18 had prolonged survival (60 months).

Of note is that he presented at a young age (35 years), with early disease (stage-Ia). He also had other favourable prognostic features (i.e. Hb = 16.6g/dl; Beta-2 microglobulin = 1.4; Calcium = 2.29 and normal renal function).

No correlation was found between the stage of the disease and the presence of the chromosomal abnormality (13q14, D13S319). The majority of the patients without the chromosomal abnormality presented with advanced stage disease. Late presentations with advanced stage disease is not unusual in our patient population. This could explain the high mortality rate in these patients.

A positive correlation was found between disease stage and mortality, in that most patients with stage III died compared to patients in stage I and II.

Comparison of laboratory results to disease stage showed statistically significant differences for Creatinine, Beta-2 microglobulin and Haemoglobin level. Patients with disease stage III showed a higher creatinine and Beta-2 microglobulin level and lower haemoglobin levels compared to patients with early stage disease (I and II).
CHAPTER 5

5.0 DISCUSSION

The detection of chromosomal abnormalities in MM has always been a problem, because of the low proliferation rate of the plasma cells. This has however improved significantly with the introduction of the FISH technique, since this does not require metaphases for analysis. We also found that the detection rate of chromosomal abnormalities improved significantly in our patients with FISH analysis. This was attributed to the difficulty to achieve growth and thus failure to obtain analysable metaphases from the cell cultures.

**Chromosome 13q14:** Deletion 13q14, D13S319 was detected in five of the twenty (5/20) patients compared to only one patient where hyperdiploidy (50-59 chromosomes) was detected with cytogenetics studies. This patient did not have a positive FISH result for 13q14,D13S319. The frequency of 25% of 13q14 deletion in our patients is much lower than that reported by other groups in the literature (i.e 30 to 70%), despite the fact that the majority of the patients presented with advanced stage disease. The reasons for the low detection rate of this abnormality in our patients are not entirely clear, but the possibilities include:

- A low prevalence of this abnormality in our patient population.
- The presence of other chromosomal abnormalities e.g chromosomes 14 and chromosome 17 abnormalities which are also known to occur in MM and confer a poor prognosis but were not assessed in this study.
- The different types of probes used by the different study groups. We used a probe specific for D13S319, whereas most of the other studies used probes extending from the RB-1 gene covering a wider region of the chromosome. Chang et al (1999), used a similar probe but reported a slightly higher frequency (30%).

- The variation in the frequency and location of deletions of 13q14 is well documented (J. Shaughnessy et al, 2000). The absence of deletion D13S319 does not exclude other deletions of 13q14. Ideally the whole extent of 13q should be studied in detail to determine the minimum deleted region in MM.

- Technical reasons have to be considered as well. These include:
  i) Very poor yield on conventional cytogenetics
  ii) Inadequate tumour representation in the bone marrow samples.
  iii) Failure of hybridisation because of the aged/ dehydrated specimens, and
  iv) Non-specific representation of the plasma cells due to lack of purification/sorting of the tumour cells.

**Chromosome 11:** Abnormalities of chromosome 11 are said to be common in MM. Four (20%) of our patients showed trisomy of chromosome 11. Two of those also had deletion 13q14 (D13S319). The three patients with trisomy 11 who presented with disease stage III died, and one was lost to follow-up. The specific role of chromosome 11 abnormalities in the pathogenesis of MM is not known. The t (11; 14) has however been associated with a poor prognosis in MM.
Chromosome 18: Abnormalities of chromosome 18 have also been described in MM, including trisomy 18. Trisomy 18 has been found to occur in up to 10% of patients with MM, but apparently not been found to be of prognostic significance. In this study one patient with deletion 13q14 (D13S319) was found to be positive for trisomy of chromosome 18. The significance of this finding is not certain. Generally trisomies are common in MM corresponding to the hyperdiploidy status of these cells, and chromosomal gains are apparently generally associated with poor prognosis (Fonseca et al, 1999)
6.0 CONCLUSION

The occurrence of chromosomal abnormalities is well documented in MM. These may involve a number of chromosomes, and may manifest as deletions, trisomies, monosomies or translocations. These include more importantly abnormalities of chromosomes 11,14,13,17,16. Some chromosomal abnormalities have been found to have prognostic significance.

Deletions of chromosome 13 have specifically been associated with a poor prognosis, despite conventional chemotherapy. It is however not known whether the poor prognosis is as a result of the deletion in isolation, or in association with abnormalities of chromosome 14q32 with the various partners and the hypodiploidy which frequently co-exists.

In this study we also set out to determine whether there was any correlation between the presence of deletion 13q14, D13S319 and other known prognostic factors in our patients.

The prevalence of 13q14 deletion in our patients was lower than that reported in the literature (25% vs 30-80%). There was no correlation found between the presence of deletion 13q14 and mortality, stage of disease and known laboratory parameters associated with poor prognosis in MM.

The prognostic significance of deletion 13q14(D13S319) in MM could not be established from this study.

The limitations of this study includes the small sample size, the unavailability of a built-in control for the specific probe, which necessitated the use of
additional centromeric probes as external controls, which showed abnormalities. The FISH technique also required optimisation for old specimens. This was ultimately achieved, though in a limited number of patients.

The use of fresh specimens and sorting of the tumour cells is recommended to improve yield and quality of results. A probe with a built-in control would be preferable.

A further prospective study with a larger patient sample using probes covering the whole extent of chromosome 13q may yield more conclusive results.

The use of alternative techniques such as analysis of gene expression profiles/signatures to further investigate genes that might be responsible for the poor prognosis in MM should also be considered.

Numerous groups have shown that classification on the basis of gene expression signatures is plausible, with the possibility of making definitive diagnosis upfront, monitoring disease evolution and predicting prognosis.
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APPENDIX 1

Multiple Myeloma – Disease Staging System

The staging system gives an indication of the tumor load and is based on a number of prognostic factors (Durie and Salmon, 1975)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
<th>Measured myeloma cell mass (cell x10^{12} /m^2)</th>
</tr>
</thead>
</table>
| I     | 1. Haemoglobin >10g/dl  
2. Normal Serum Calcium (<12mg/dl) 
3. Normal bone structure (scale 0) or solitary bone plasmacytoma 
4. Low M-component production rate  
a. IgG value <50g/l 
b. IgA value <30g/l 
c. Urine light chain M-component on electrophoresis <4g/24h | < 0.6 (low) |
| II    | Fitting neither stage I or II | 0.6-1.2 (intermediate) |
| III   | 1. Haemoglobin <8.5g/dl  
2. Serum Calcium >12mg/dl  
3. Advanced lytic bone lesions (scale 3) 
High M-component production rates  
IgG >70g/l  
IgA > 50g/l  
Urine light chain M-component on electrophoresis >12g/24hrs  
A - relatively normal renal function (serum creatinine <2mg/dl) 
B - abnormal renal function (serum creatinine >2mg/dl) | > 1.2 (high) |
APPENDIX 2

Criteria for diagnosis of Myeloma

Any 2 major criteria listed below

Major criterion 1 plus minor criterion B, C or D
Major criterion 3 plus minor criterion A or C
Minor criteria A, B and C or A, B and D

MAJOR CRITERIA

1. Plasmacytoma on biopsy
2. > 30% plasma cells in marrow
3. Monoclonal immunoglobulin electrophoretic spike with IgG >3.5 g/dl or IgA > 2 g/dl or kappa or lambda light chain excretion in the urine > 1g /day

MINOR CRITERIA

A. Bone marrow plasmacytosis, with 10-30% plasma cells
B. Monoclonal immunoglobulin present, but at quantitatively lower levels than for a major criteria
C. Lytic bone lesions
D. Depressed normal immunoglobulins IgM<50 mg/dl, IgA < 100 mg/dl, IgG < 600 mg/dl.

( Durie, 1986, Committee of Chronic Leukemia-Myeloma Task Force, National Cancer Institute, 1993)
APPENDIX 3

Criteria for Diagnosis of Myeloma

All three required

M-protein in serum or urine or both

Bone marrow (clonal) plasma cells or plasmacytoma

Related organ or tissue impairment (one or more of the following)

C = hypercalcemia (serum calcium $0.25 \text{ mmol/l}$ above the upper normal limit or $\geq 2.75 \text{ mmol/l}$)

R = renal insufficiency ($\text{creatinine} \geq 173 \text{ umol/l}$ or $2 \text{ mg/dl}$)

A = anaemia (haemoglobin $2 \text{ g/dl}$ below the lower normal limit or haemoglobin $\leq 10\text{ g/dl}$)

B = bone lesions (lytic lesions or osteoporosis with compression fracture)

Other = symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections ($> 2$ episodes in $12$ months)

- No minimal level of serum M-protein or urine M-protein was included in the criteria.

- No minimal levels of clonal bone marrow plasma cells was designated.

- The most critical criterion is the evidence of end organ or tissue impairment.

### Risk Classification in Multiple Myeloma

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<th>High Risk (2-3 years survival)</th>
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