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# **Genetic Analyses of Multiple Myeloma and Related Plasma Cell Dyscrasias**

**Therese Nilsson**



**LUND UNIVERSITY**

**Department of Clinical Genetics**

**2004**

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To my parents

*“Lascia ch’io pianga la dura sorte,  
E che sospiri la libertá  
Il duol infranga queste ritorte  
De’ miei martiri sol per pietà”*

From Rinaldo by G. F. Händel

## LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, referred to in the text by their Roman numerals.

- I Nilsson T, Lenhoff S, Turesson I, Rylander L, Mitelman F, Westin J, Höglund M, Johansson B.** Cytogenetic features of multiple myeloma: impact of gender, age, disease phase, culture time, and cytokine stimulation. *Eur J Haematol* 2002; 68: 345-353.
- II Nilsson T, Höglund M, Lenhoff S, Rylander L, Turesson I, Westin J, Mitelman F, Johansson B.** A pooled analysis of karyotypic patterns, breakpoints and imbalances in 783 cytogenetically abnormal multiple myelomas reveals frequently involved chromosome segments as well as significant age- and sex-related differences. *Br J Haematol* 2003; 120: 960-969.
- III Nilsson T, Nilsson L, Lenhoff S, Rylander L, Åstrand-Grundström I, Strömbeck B, Höglund M, Turesson I, Westin J, Mitelman F, Jacobsen SEW, Johansson B.** MDS/AML-associated cytogenetic abnormalities in multiple myeloma and monoclonal gammopathy of undetermined significance: evidence for a frequent de novo occurrence and multipotent stem cell involvement of del(20q). *Genes Chromosomes Cancer*, in press 2004.
- IV Nilsson T, Lenhoff S, Rylander L, Höglund M, Turesson I, Mitelman F, Westin J, Johansson B.** High frequencies of chromosomal aberrations in multiple myeloma and MGUS in direct chromosome preparation. *Submitted*.
- V Nilsson T, Lenhoff S, Rylander L, Höglund M, Turesson I, Mitelman F, Westin J, Johansson B.** Multiple myeloma and MGUS cultured as solid tumors: a novel cytogenetic approach to investigate plasma cell dyscrasias. *Manuscript*.



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## ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BJP	Bence Jones protein
BM	bone marrow
B2M	beta-2-microglobulin
CGH	comparative genomic hybridization
CML	chronic myeloid leukemia
COBRA	combined binary ratio labelling
DCP	direct chromosome preparation
DS	Durie & Salmon (MM stages)
FISH	fluorescence in situ hybridization
GM-CSF	granulocyte/monocyte-colony stimulating factor
HCD	heavy chain disease
Ig	immunoglobulin
<i>IGH</i>	immunoglobulin heavy chain gene
<i>IGK</i>	immunoglobulin kappa light chain gene
<i>IGL</i>	immunoglobulin lambda light chain gene
IL	interleukin
K	kappa
L	lambda
M component	monoclonal component
MDS	myelodysplastic syndromes
M-FISH	multicolor fluorescence in situ hybridization
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
PB	peripheral blood
PC	plasma cell
PCD	plasma cell dyscrasia
PCL	plasma cell leukemia
PCLI	plasma cell labelling index
S	serum
SKY	spectral karyotyping
SMM	smoldering multiple myeloma
U	urine
WM	Waldenström's macroglobulinemia

## PREFACE

Numerous clinical, morphologic, and immunophenotypic studies have revealed that hematologic malignancies constitute a highly heterogeneous group of disorders, characterized by different acquired chromosomal and molecular genetic abnormalities. Since the discovery of the Philadelphia chromosome in chronic myeloid leukemia in 1960, it has become increasingly appreciated that several chromosomal rearrangements are strongly associated with distinct subtypes. To date, almost 33 000 cytogenetically abnormal hematologic malignancies have been reported, yielding a wealth of information of both biologic and clinical importance. Thus, karyotypic investigations, today often supplemented with molecular genetic analyses, have become an integral part of the diagnostic and prognostic evaluation of malignant hematologic disorders, in particular the acute leukemias.

However, little is known about type of chromosomal changes and their clinical impact in the plasma cell dyscrasias (PCDs) monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), even though the incidences of these conditions are quite high, as compared to the acute leukemias. The reasons for the relative lack of karyotypic data in PCDs are manifold, including difficulties in culturing the neoplastic cells, resulting in normal karyotypes in most cases. Considering that molecular genetic and fluorescence in situ hybridization (FISH) studies identify abnormalities in the vast majority of PCDs, the normal karyotypes identified by G-banding generally do not represent dividing neoplastic cells.

The aims of the present thesis, which is based on five articles, were to investigate PCDs, mainly MM and MGUS, by chromosome banding and FISH analyses in order to identify characteristic abnormalities in these disorders and to compare different culture methods, with the goal to increase the cytogenetic success rate in PCDs.

The thesis is divided into two major sections. The first gives an overview of the different techniques used to investigate genetic changes, the clinical aspects of MM and MGUS, and the pathogenetic and prognostic importance of genetic findings in these disorders. The second section summarizes the objectives, materials and methods, and results of the present thesis.

Lund, April 2004

## INTRODUCTION

### **The bone marrow – the cells and their function**

The red bone marrow (BM) in adults occupies the cavities of the iliac crest, vertebrae, skull, ribs, sternum, scapulae, and the proximal parts of humerus and femur (Figure 1). It is the site of the production of the red blood cells (erythrocytes), platelets (thrombocytes), and the white blood cells (leukocytes). The erythrocytes carry oxygen to the tissues, the thrombocytes are involved in coagulation, and the myeloid and lymphoid leukocytes, i.e. neutrophils, eosinophils, basophils, monocytes, and B and T lymphocytes, take part in our defense against, e.g. infections. The production, number, function, and release of these blood cells are carefully regulated through a complex interaction between the stem cells and the progenitors, and the microenvironment in the BM (Jandl, 1996).



Figure 1. The blood cells are produced in the red BM, distributed within the skeletal cavities (denoted in black).

The production of blood cells – the hematopoiesis – is characterized by a strictly controlled proliferation and differentiation of the different hematopoietic cell populations, a process related to the capability of the stem cells to self-replicate as well as to produce mature progeny. The term proliferation refers to the process of cell replication, also denoted cell cycle, whereas differentiation is the process by which the cells acquire their specialized function. Various factors may affect the BM. For example, bleeding, infection, and hypoxia increase the number of the individual cell types (Jandl, 1996).

### **Bone marrow neoplasia**

Neoplastic transformation of the BM cells results in hematologic malignancies, i.e. leukemias and related disorders. Several studies have revealed that the initiating, leukemogenic event occurs at the stem cell or precursor cell levels (Takahashi et al., 1998; Nilsson et al., 2000, 2002), but that, most likely, several “hits” are needed for the transformed clone to evolve into a clinically manifest disease (Biernaux et al., 1995; Bose et al., 1998; Corral et al., 1996).

The neoplastic process is closely associated with acquired genetic abnormalities, both chromosomal changes as well as molecular genetic abnormalities, which are found in the leukemic cells (Look, 1997; Rabbitts, 2001; Mitelman et al., 2004a). In 1960, the first disease-specific genetic aberration in a hematologic malignancy was reported, namely the Philadelphia (Ph) chromosome in chronic myeloid leukemia (CML) (Nowell & Hungerford, 1960). Since then, close to 33 000 cytogenetically abnormal malignant hematologic disorders have been published (Mitelman et al., 2004a). These karyotypic analyses have revealed several recurrent chromosomal abnormalities, which have been further characterized at the molecular level, disclosing genes that, if structurally rearranged or otherwise deregulated, are intimately associated with the leukemogenic process (Mitelman et al., 2004b). At the same time, the clinical significance of these aberrations has become increasingly appreciated, and cytogenetic as well as molecular genetic investigations are today an integral part in the diagnostic and prognostic evaluation of, particularly, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and myelodysplastic syndromes (MDS) (Le Beau et al., 1986; Grimwade, 2001; Harrison, 2001a). For example, genetic studies are today mandatory for proper risk assessment and treatment considerations in treatment trials of childhood ALL (Harrison, 2001b).

During the last few years, promising results have been obtained by a drug, Gleevec, developed to target specifically the BCR/ABL1 fusion protein encoded by the t(9;22)(q34;q11), i.e. the Ph chromosome (Mauro & Druker, 2001), and trials are ongoing as regards the possibility to inhibit the effect of another leukemia-associated gene rearrangement, i.e. *FLT3* abnormalities (Levis et al., 2002; Smith et al., 2004). These examples clearly indicate that cytogenetic and molecular genetic analyses most likely will play an increasingly important role in the management of hematologic malignancies.

### **Methods to identify neoplasia-associated genetic changes**

Among the many different methods and techniques available to investigate and to identify neoplasia-associated genetic abnormalities, chromosome banding, fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), reverse transcriptase-polymerase chain reaction (RT-PCR), and,

very recently, cDNA microarray analyses have proved particularly fruitful, and these methods will be briefly described below.

Cytogenetic investigations, using conventional G-banding, are still the golden standard to screen for chromosomal aberrations (Figure 2), and are today used routinely for diagnostic and prognostic purposes in several hematologic malignancies, in which more than 350 recurrent balanced chromosomal abnormalities have been identified to date (Mitelman et al., 2004b). However, there are some shortcomings of this technique. First, the requirement of dividing cells obviously poses a problem if the proliferation indices of the neoplastic cells are low. Second, the sometimes poor chromosome morphology may preclude detailed karyotypic descriptions (van den Berghe, 1990) (Figure 3). Third, some chromosomal abnormalities, such as the  $t(12;21)(p13;q22)$  in childhood ALL (Romana et al., 1994), are cryptic, and for this reason cytogenetic studies are today often supplemented with FISH and/or molecular genetic analyses.

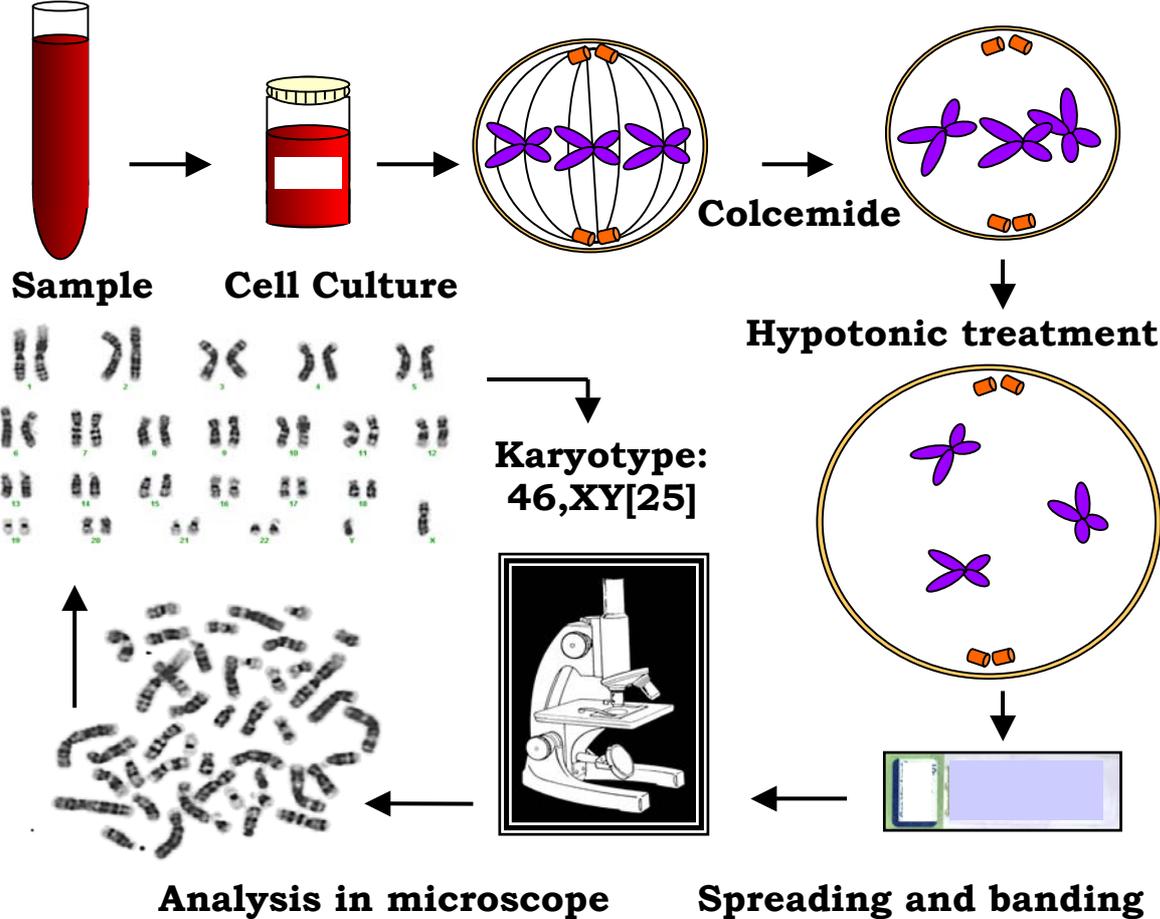


Figure 2. Cytogenetic investigations include cell culture, harvesting using Colcemide, which results in an accumulation of metaphases, hypotonic treatment, increasing the spreading of the chromosomes, fixation and spreading, chromosome banding, and finally analysis in microscope.

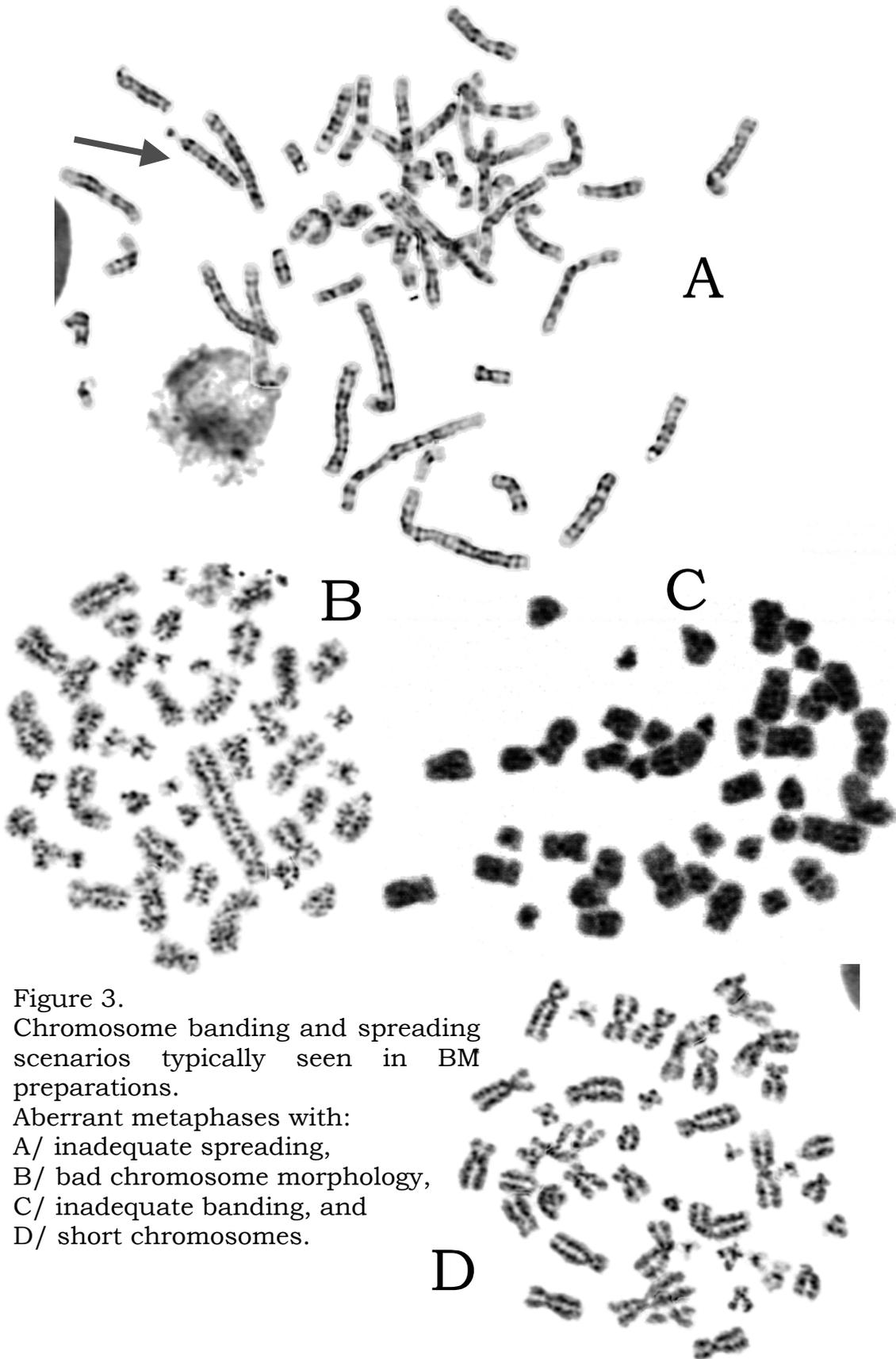


Figure 3.  
 Chromosome banding and spreading  
 scenarios typically seen in BM  
 preparations.  
 Aberrant metaphases with:  
 A/ inadequate spreading,  
 B/ bad chromosome morphology,  
 C/ inadequate banding, and  
 D/ short chromosomes.

FISH, which was developed and introduced in the late 1980s (Pinkel et al., 1988), has revolutionized cytogenetic analyses, enabling identification of submicroscopic abnormalities, detailed characterization of complex chromosome changes, as well as detection of aberrations also in non-dividing cells (Gisselsson et al., 2000; Cuneo et al., 2002; I, Schoch et al., 2002; Dewald et al., 2003; III, IV, V), using various types of probes (Figure 4). In the mid and late 1990s, FISH was further developed, making it possible to label all chromosome pairs in unique colors, i.e. multicolor-FISH (M-FISH), something that can be achieved by different techniques, e.g. spectral karyotyping (SKY) (Schröck et al., 1996), multi-fluor FISH (Speicher et al., 1996), and combined binary ratio labelling (COBRA) FISH (Tanke et al., 1999). These methods have proved particularly useful in the analyses of complex karyotypes (Ashman et al., 2002; Barbouti et al., 2002; Cohen et al., 2002; Tchinda et al., 2003). Although FISH undoubtedly increases the sensitivity, it should be stressed that it cannot replace chromosome banding; it should be considered a supplement (Harrison et al., 2003).

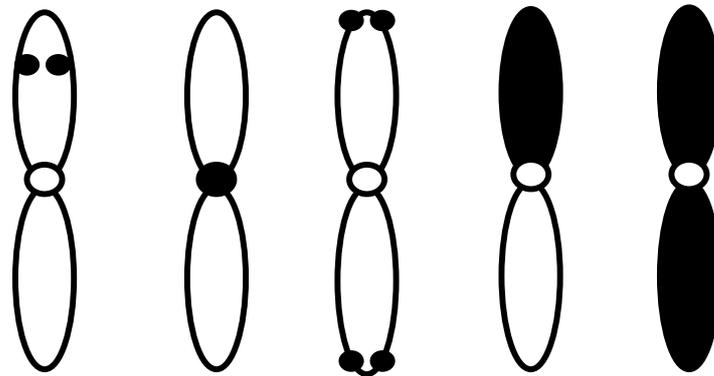


Figure 4. Examples of different FISH probes (from left to right): locus specific, centromeric, subtelomeric, partial chromosome painting, and whole chromosome painting.

The main advantage of CGH, a method that produces a map of DNA sequence copy number as a function of chromosomal location throughout the entire genome (Kallioniemi et al., 1992), i.e., identifies genomic imbalances, is that tumor tissues – fresh, frozen, or paraffin-embedded – can be analyzed, i.e. prior cell culture or dividing cells are not required (Speicher et al., 1995; McNeil & Reid 2000). Solid tumors may be difficult to culture, and CGH has hence proved particularly useful for genetic analyses of such tumors (Knuutila et al., 1999). Because CGH

only detects imbalances, its value in malignant hematologic disorders, many of which are characterized by balanced translocations and inversions (Mitelman et al., 2004b), is quite limited. Recent data suggest that balanced structural chromosomal abnormalities may be equally important in solid tumors (Mitelman et al., 2004b), a finding which would argue against CGH as the sole method to identify genetic changes also in solid tumors. Another disadvantage of CGH is that imbalances <10 Mb are not identifiable. However, recent progress in CGH array has made it possible to detect smaller deletions and amplifications (du Manoir et al., 1995; Joos et al., 1995; Hurst et al., 2004). Furthermore, in order to obtain representative data, the investigated tissues must harbor at least 50% tumor cells, which often makes tumor cell enrichment a prerequisite.

Most neoplasia-associated translocations and insertions have been shown to result in fusion genes (Mitelman et al., 2004a,b), which are easily detectable by RT-PCR that specifically amplifies such chimeras (Panagopoulos et al., 2001). Although RT-PCR often is used for diagnostic purposes, its main value – considering its high sensitivity – may well be for follow-up studies, i.e. to evaluate and quantitate minimal residual disease in hematologic malignancies (Uzunel et al., 2003).

During the last few years, the cDNA microarray technology, enabling the expression pattern of thousand of genes to be identified in a single experiment (DeRisi et al., 1996; Schena et al., 1996), has yielded exciting and promising data of both diagnostic and prognostic significance in hematologic disorders as well as in solid tumors (Gruvberger et al., 2001; Armstrong et al., 2002; Pomeroy et al., 2002). However, it may still take some time before microarray analyses become integrated in clinical practice, considering the present lack of a universal standard method, the high cost, the possible need to purify samples before processing, and the limitation to identify intra-tumor heterogeneity (Shaughnessy & Barlogie, 2003a).

All the above-mentioned techniques have been essential for detecting and characterizing genetic changes in various hematologic malignancies, in particular ALL, AML, CML, and MDS (Mitelman et al., 2004a). As regards the plasma cell dyscrasias (PCDs), however, which are as common as the acute leukemias (The National Board of Health and Welfare, 2003), fewer studies using these methods have been undertaken, and for

this reason our knowledge of genetic changes in PCDs is relatively limited.

## PLASMA CELL DYSCRASIAS – AN OVERVIEW

The PCDs constitute a broad spectrum of diseases characterized by clonal proliferation and accumulation of cells producing monoclonal immunoglobulins (M component) and include monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), smoldering MM (SMM), plasma cell leukemia (PCL), Waldenström's macroglobulinemia (WM), POEMS syndrome, plasmacytoma, heavy chain disease (HCD), and amyloidosis. The following sections focus mainly on the most common subtypes, namely MGUS and MM.

### **History of multiple myeloma**

The disease entity multiple myeloma was described in the late 1940s (reviewed in Bergsagel et al., 1999). However, MM as such has of course afflicted people throughout the ages. In fact, evidence for MM has been found in Egyptian mummies, dating back to approximately 1500-500 BC, displaying characteristic MM-associated skeletal changes (reviewed in Zink et al., 1999).

One of the very first clinical case reports of MM was made already in the 1850s by Dr. William Macintyre who wrote the following about a patient of his (reviewed in Kyle, 1996): *“Mr. M, a highly respectable tradesman, aged 45, placed himself under my care on the 30th of October, 1845. He was then confined to the house by excruciating pains of the chest, back, and loins, from which he had been suffering, more or less, for upwards to twelve months.”* Because edema had been noticed, a urine specimen was sent to a colleague in London with the letter *“Dear Doctor Jones. The tube contains urine of very high specific gravity. When boiled it becomes slightly opaque. On the addition of nitric acid, it effervesces, assumes a reddish hue, and becomes quite clear; but as it cools, assumes the consistence and appearance which you see. Heat reliquifies it. What is it?”*. Dr Bence Jones, the receiver of the letter, was at this time in his career a leading physician. He was, for example, well acquainted with Florence Nightingale, who regarded him the best “chemical doctor” in London (Kyle, 1996). In response to the question raised by Macintyre, Bence Jones emphasized the importance of looking for the same phenomenon in other cases of softening of the bone. In honor of his

microscopic studies of the urine, the term Bence Jones proteinuria (BJP) was later introduced for the protein identified in the urine in patients with PCDs (Kyle, 1996). However, it was not until the mid 1950s that the chemical composition of BJP was determined. Using immunoelectrophoresis, Korngold and Lipari discovered that BJP consisted of parts of monoclonal immunoglobulins (Kyle, 1994a). Today, BJP is known to involve the light chains of the immunoglobulins, the kappa (K) or lambda (L) – named after Korngold and Lipari, respectively (Kyle, 1994a).

In 1873, Von Rustizky was the first to refer to “multiple myeloma” when, performing an autopsy, he found eight separate tumors in the BM. He described the cells of the tumors as round, with the size of white cells and with the nucleus located peripherally, near the cell membrane (reviewed in Kyle, 1996). In 1900, Wright (the scientist giving his name to the Wright’s stain used for chromosome banding) described that the tumor cells consisted of plasma cells (PCs), suggesting that these were the cells from which MM originates (reviewed in Lee et al., 2002).

### **Diagnostic criteria of plasma cell dyscrasias**

Below, the definitions of the following PCD subtypes will be briefly outlined: MM, MGUS, SMM, PCL, WM, POEMS syndrome, plasmacytoma, HCD, and primary amyloidosis.

MM is a progressive hematologic malignancy involving long-lived PCs that originate from the post-germinal center. One or several of the following features are usually present: M component ( $>30$  g IgG/l or  $>10$  g IgA/l), urinary excretion of  $>1.0$  g K or L light chains/24 h, increased frequency (10% or more) of clonal PCs within the BM, anemia, lytic bone lesions, renal insufficiency, and hypercalcemia. MM is in about 50% of the cases preceded by MGUS (Greipp & Lust, 1996; Östenborg & Mellerstedt, 1996b).

MGUS is characterized by an M component ( $<30$  g/l). No related organ or tissue impairments, e.g. bone lesions, and no evidence of MM, or other B-cell proliferative disorders, should be found in MGUS, and the BM infiltration of PCs should be less than 10%. The majority of patients with MGUS live with this condition without any symptoms. However, MGUS may transform, at a rate of about 1% per year, to MM (Greipp & Lust, 1996; Östenborg & Mellerstedt, 1996b; Kyle et al., 2002; International Myeloma Working Group, 2003).

SMM is characterized by an M component (often >30 g/l) and by 10% or more PCs in the BM, similar to MM. However, no other MM-associated features are found. Thus, SMM may be grouped in-between MGUS and MM. Patients with SMM can have a stable disease for several years, without any need for treatment (Greipp & Lust, 1996; Östenborg & Mellerstedt, 1996b).

PCL is subdivided into primary or secondary PCL. Primary PCL presents de novo as a leukemia and is characterized by a replacement of the BM by immature PCs (above 90%), enlarged liver and spleen, severe anemia and bleeding. However, osteolytic lesions are rarely found. Secondary PCL occurs as a terminal phase in a few percent of MM patients, when the PCs exceed 20% of the white blood cell count in the peripheral blood (PB) (Jandl, 1996).

The Swedish physician Jan Waldenström described WM in 1944. WM is a chronic lymphoproliferative disorder, with cells of lymphoid, plasmacytoid, or intermediate morphology, producing an M component consisting of IgM proteins (Jandl, 1996; Owen et al., 2003). Although very rare, IgM-associated MM cases have been reported (De Gramont et al., 1990; Dierlamm et al., 2002; Avet-Loiseau et al., 2003b). Thus, a finding of monoclonal IgM does not necessarily imply WM. There are currently no universally accepted criteria for the diagnosis of WM (Owen et al., 2003).

POEMS syndrome consists of polyneuropathy (P), organomegaly (O), endocrine (E) dysfunction, monoclonal (M) gammopathy, and skin (S) changes (Dispenzieri et al., 2003).

Plasmacytoma may be extramedullary, i.e. isolated plasma cell tumors of soft tissues, or a solitary lytic lesion in the skeleton (with less than 5% PCs) in a patient with no other features suggesting MM (Greipp & Lust, 1996).

HCD is characterized by an accumulation of variably shortened immunoglobulin heavy chains, with the production of HCD proteins including incomplete versions of the gamma, alpha, and micro heavy chain classes (Jandl, 1996).

The characteristic feature of primary amyloidosis is the deposition of monoclonal free light chains, which have been partly degraded into fragments. The accumulation of these aberrant proteins, which most commonly are derived from the L chains, may cause a severe multisystemic disease (Jandl, 1996).

### **Clinical features of multiple myeloma**

As mentioned above, the hallmark of MM and other PCDs is the finding of an M component. There are five major classes of immunoglobulins (Ig) produced by MM cells: IgG (55-60%), IgA (20-25%), IgM (0.5-1%), IgD (1-2%), and IgE (less than 0.01%). In most cases, whole Ig are secreted by the dysfunctional PCs. However, there may be a discrepancy in the production of the heavy and light chains leading to an imbalance, with an excess of free light chains – K or L – excreted in the urine (BJP). Occasionally, the transformed PCs do not secrete any Ig, a condition called non-secretory MM (Östenborg et al., 1996a; Dierlamm et al., 2002; Avet-Loiseau et al., 2003b; International Myeloma Working Group, 2003;).

The clinical signs and symptoms in MM vary depending on disease stage, most commonly defined by the system proposed by Durie and Salmon in 1975 (Durie & Salmon, 1975) – DS I, DS II, and DS III. Skeletal destructions/osteolytic lesions (Figure 5) are a characteristic feature of MM, being found in 70% of all cases. The lower back, ribs, and spine are the most commonly affected areas, and at diagnosis most patients have bone lesions to an extent directly related to the mass of the tumor. The lesions are due to an unbalanced process between the cells reabsorbing bone (osteoclasts) and the cells producing bone (osteoblasts). The skeletal lesions and their accompanying hypercalcemia give rise to asthenia, cachexia, bone pain, fractures, compression of the spinal cord, and renal insufficiency, and are major causes of morbidity (Jandl, 1996). Anemia, resulting in fatigue, is common during the course of MM, and is caused by the malignant cells infiltrating the BM, and/or from inadequate erythropoietin responsiveness. Bacterial infections are recurrent and are due to deficiencies in both the humoral and cellular immunity. Renal failure is one of the most serious adverse complications of MM, and is caused by accumulation of immunoglobulins as well as deposition of calcium in the kidneys, leading to obstruction and inflammation. Neurological symptoms are most commonly related to the effect of the tumor mass, e.g. compression of the spinal cord or the nerves, but can also be due to hypercalcemia, hyperviscosity, or depositions of amyloids (Jandl, 1996).

At present, MM is incurable. However, it should be stressed that MM is clinically quite heterogeneous, with survival ranging from a few months to more than 10 years (Samson, 1996; Barlogie et al., 2004).

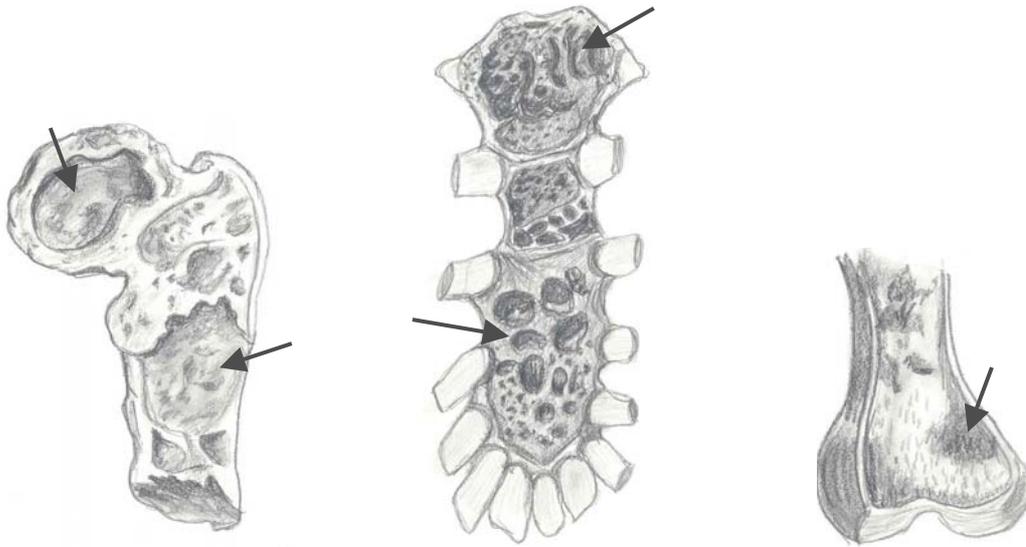


Figure 5. Osteolytic lesions (arrows) in femur (left and right) and sternum (middle).

### **Epidemiology of MGUS and multiple myeloma**

MGUS is the most frequent PCD subtype. The incidence increases with age and is estimated to be 10-20% in the population above 80 years (Ögmundsdottir et al., 2002; Kyle & Rajkumar, 2003). Although the M component usually is quite stable in MGUS patients – the previous name of this condition was “benign monoclonal gammopathy” – almost 10% are diagnosed with MM or another PCD within 5 years (Bladé et al., 1992). Furthermore, nearly 1/3 of all newly diagnosed MM patients have a previous history of MGUS (Kyle et al., 1994b). Thus, MGUS and MM are most likely two different phases of the same disease process. The fact that MM and MGUS harbor similar genetic abnormalities (see below) adds further evidence that they are closely related.

Approximately 6 new MM cases per 100 000 are diagnosed each year (The National Board of Health and Welfare, 2003), with the large majority being above the age of 60 (Turesson et al., 1984; Bergsagel, 1996a). However, rare cases of MM among early teenagers have been described (Powari et al., 2002). MM as well as MGUS are more common among men (Bergsagel, 1996a). There seem to be some geographic/ethnic frequency differences in MM incidences. For example, MM accounts for 31% and 13% of all hematologic malignancies in African Americans and Caucasians, respectively, and the lowest incidences are found in the Chinese and Japanese populations (Bergsagel, 1996a).

## **Etiology of MGUS and multiple myeloma**

As for most neoplastic disorders, the etiology of MGUS and MM is unknown. However, numerous risk factors for MM have been proposed, including chronic immune stimulation, autoimmune disorders, occupational exposure to pesticides, herbicides, or dioxin, and prolonged use of certain hair coloring products (Bergsagel, 1996a). MM has also been associated with exposure to Agent Orange, used during the Vietnam War, and the incidence of MM was increased among survivors of the atomic bombs at Hiroshima and Nagasaki (Bergsagel, 1996a). Furthermore, a number of viruses have been suggested to play a role, e.g. patients with HIV have a 4.5-fold increased likelihood of developing MM (Yee et al., 2001) and the human herpes virus 8 has been associated with MM, MGUS, and other PCDs (Said et al., 1997; Cathomas, 2000).

Familial clusters of MGUS and MM as well as MM among married couples have been described. In addition, healthy relatives to MM patients have been reported to have an elevated incidence of Ig abnormalities, without any other signs of PCD. No major differences regarding gender, age, distribution of M components, clinical/laboratory data, or prognosis between familial and non-familial MM have, in general, been observed (Lynch et al., 2001).

## **PATHOGENESIS OF MULTIPLE MYELOMA AND MGUS**

### **B-cell development**

The normal B cell precursors need to undergo three steps of differentiation in order to produce specific Ig. The first step occurs in the BM, with the constant (C), joining (J), variable (V), and diversity (D) gene segments rearranging somatically to produce an immunoglobulin heavy chain (*IGH*) gene. This is followed by a similar rearrangement of the light chain genes, i.e. K or L. The first Ig produced is IgM, appearing on the surface of B cells. The second step, somatic hypermutation of the antigen binding regions, takes place in the germinal centers of the lymph nodes and enhances the specificity and the binding stringency of the antibodies. The follicular dendritic cells present antigens with a high affinity to certain B cells, thus initiating a mechanism for these cells to escape programmed cell death (apoptosis) and to continue the differentiation into memory B cells. The third step occurs when the memory cells undergo *IGH* switching, i.e.

exchange IGH C regions, changing the function of the Ig. Whereas the pre-switched B cells express IgM, the post-switched B cells may express IgG, IgA, IgD, or IgE. These latter cells can migrate to the BM, where they adhere to, and interact with, the stromal cells and differentiate into long-lived PCs, which survive for approximately 30 days (Hallek et al., 1998).

The malignant PCs in MM are long-lived, mainly localized to the BM, have a very low plasma cell labelling index (PCLI). It is generally believed that the initial neoplastic event in MM occurs in a single post-germinal center B cell, but evidence for transformation of a pre-switched B cell has also been reported (Hallek et al., 1998; Seidl et al., 2003).

### **Non-genetic pathogenetic factors**

Granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-alpha, and interleukin 3 (IL-3) are some of the growth factors that have been implicated in the survival, proliferation, and differentiation of MM cells (Hallek et al., 1998). However, IL-6 is perhaps the most important cytokine in MM pathogenesis. IL-6 induces growth of the MM cells both in vivo and in vitro. The cells excreting IL-6 include stromal cells, osteoblasts, and osteoclasts, which act in a paracrine manner on the MM cells. Furthermore, MM cells can produce IL-6 themselves. Thus, IL-6 seems to stimulate the neoplastic cells in both an autocrine and a paracrine fashion. The IL-6 binds to cell surface receptors, which combine with a 130 kDa transmembrane glycoprotein that activates intracellular signaling pathways, leading to an increased MM cell population firstly by stimulation of MM cell division and secondly by prevention of apoptosis (Hallek et al., 1998).

### **Cytogenetic abnormalities**

The first chromosome studies of PCDs were performed in the 1960s (Bottura, 1963; Lewis et al., 1963; Houston et al., 1967). Although some acquired cytogenetic changes were identified, lack of chromosome banding techniques in this pre-banding era precluded any detailed karyotypic descriptions. All this changed when the various banding methods were developed and introduced in the early 1970s (Caspersson et al., 1970).

To date, cytogenetic aberrations have been reported in approximately 1 000 MM, 100 PCL, 70 WM, and 20 MGUS (Mitelman et al., 2004a). Although the number of abnormal MM

may seem impressive, it is disproportional in relation to other hematologic malignancies. For example, more than 10 000 AMLs and close to 7 000 ALLs with chromosomal changes have been published (Mitelman et al., 2004a). Considering that the incidence of MM is quite similar to acute leukemias (The National Board of Health and Welfare, 2003) there is obviously a relative lack of cytogenetic data in MM. The discrepancy is partly due fewer PCD samples being referred for cytogenetic investigation compared to the acute leukemias. However, the main obstacles have been – and still continue to be – difficulties in performing karyotypic analyses of MM. In fact, only 30-50% MM are karyotypically abnormal, with some – but not all – studies reporting a larger fraction of cytogenetically aberrant cases in DS II and III as compared to the early stage DS I (Weh et al., 1993; Zandecki et al., 1994; Calasanz et al., 1997a; Hernández et al., 1998; Gutierrez et al., 2000; I). The frequencies of cytogenetically abnormal MGUS cases in the small series reported to date range between 0% to 65% (Calasanz et al., 1997a; Lloveras et al., 2002; I; IV; V). Considering that analyses with interphase FISH and molecular genetic techniques reveal chromosomal abnormalities in close to 100% of MM cases and in the majority of MGUS cases, the normal karyotypes are most likely not representative of the neoplastic clone (Drach et al., 1995a,b; Flactif et al., 1995; Tabernero et al., 1996; Avet-Loiseau et al., 1997; Nishida et al., 1997; Zandecki et al., 1997; IV; V). Despite these methodological shortcomings, based on the presently available cytogenetic data some specific or characteristic chromosomal abnormalities or karyotypic patterns are now emerging in MM.

A recent review of the cytogenetic features of MM (II) showed that the largest karyotypic subgroup is hyperdiploid (39%) and that hypo- and pseudodiploidy are equally common (27% and 24%, respectively), whereas tri- and tetraploidy is quite rare (10%). Interestingly, hyperdiploidy was shown to be more common in elderly patients. However, the underlying mechanisms for this age-related difference are presently unknown. The number of published cytogenetically abnormal MGUS cases is very low (less than 20), but available literature data suggest that the majority are pseudodiploid (Mitelman et al., 2004a).

The cytogenetic complexity, i.e. number of chromosomal abnormalities, of MM is quite pronounced (Figure 6). In fact, the median number of aberrations per MM is eight, with close to 75% displaying three or more chromosome changes (II). Such

karyotypic complexity is quite rare in other hematologic malignancies, with the exception of the malignant lymphomas (Mitelman et al., 2004a). In contrast to ploidy levels, the number of chromosomal changes in MM does not seem to vary in relation to age (II). Most of the published MGUS cases harbor only one or two anomalies (Mitelman et al., 2004a).

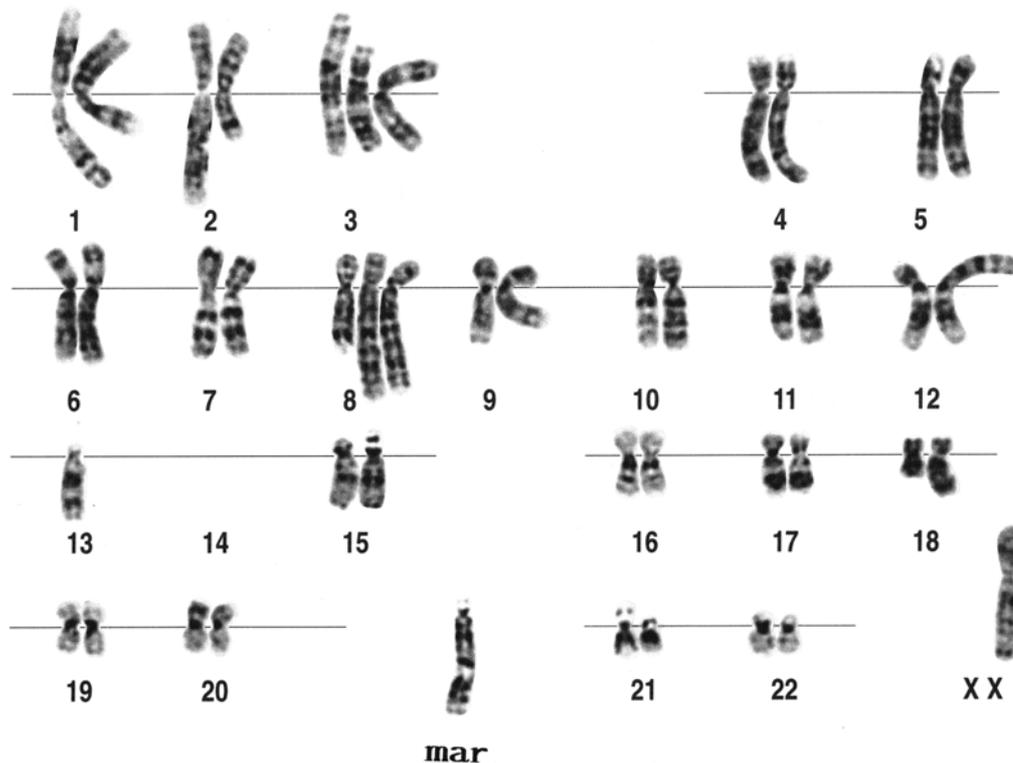


Figure 6. Complex karyotype in MM: 45,X,-X,der(1)t(1;3)(q21;p23), add(2)(q21),+del(3)(p11p21),+8,+add(8)(q24)x2,+add(9)(q13),der(12)t(1;12)(q21;p13),-13,?add(13)(q22),-14,-14,del(18)(q21),+mar.

Several characteristic numerical as well as structural chromosomal changes have been identified in MM (II; Mitelman et al., 2004a). The most common numerical aberrations (Figure 7) are +9 (27% of karyotypically abnormal cases), -13 (27%), +15 (24%), +19 (22%), +11 (20%), and -Y (20%) (II). The frequencies of some of the numerical abnormalities differ significantly in relation to age or gender. For example, trisomy 11 and monosomy 16 are more common among men, trisomy 5 is more frequent in elderly patients, and monosomy 14 is more common in younger patients (II). It may be noteworthy that similar numerical chromosomal abnormalities, i.e. +3, +7, +9, +11, also are found, using interphase FISH, in MGUS, providing indirect evidence for a close

relationship between MGUS and MM (Drach et al., 1995a,b; Zandecki et al., 1995, 1997; IV).

Structural chromosomal abnormalities are very frequent in MM. A few are cytogenetically balanced, such as  $t(4;14)(p16;q32)$ ,  $t(11;14)(q13;q32)$ , and  $t(14;16)(q32;q22-23)$ , but most are genomically unbalanced, often resulting in losses of 1p, 6q, and 13q, and gain of 1q (Avet-Loiseau et al., 1997; Kuehl & Bergsagel, 2002; II). Age- and gender-related differences have also been noted for some MM-associated structural rearrangements. For example,  $t(8;22)(q24;q11)$  seems to be more common in men (Yamamoto et al., 1998; Mitelman et al., 2004a) and  $t(11;14)$  may possibly be more frequent in younger MM patients (Fonseca et al., 2002c). Although all chromosomes have been shown to be involved in structural aberrations in MM, some chromosome bands are more frequently involved than others: 14q32 (29% of cytogenetically abnormal MM), 11q13 (16%), 1q10 (14%), 8q24 (10%), 1p11 (8%), 19q13 (6%), 1p22 (5%), 6q21 (5%), and 17p11 (5%) (II). These chromosomal regions are hence likely to harbor genes of importance in the development and/or progression of MM.

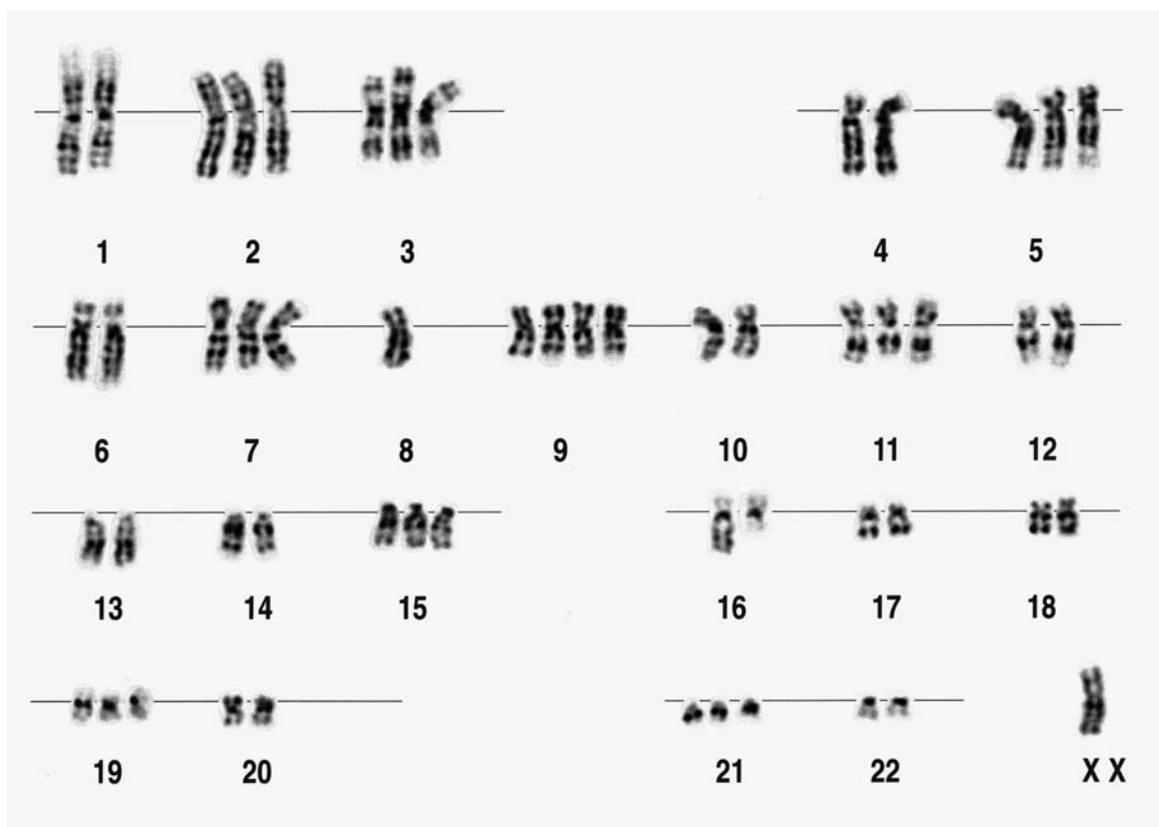


Figure 7. MM case with mainly numerical abnormalities, with the karyotype  $54,-X,+2,+3,+5,+7,-8,+9,+9,+11,+15,add(16)(q22),+19,+21$ .

### **Molecular genetic abnormalities**

The molecular genetic era of MM started in the mid 1990s, when Bergsagel et al. (1996b) reported that the vast majority of MM, mainly cell lines, harbor illegitimate *IGH* rearrangements, such as translocations involving the switch region. To date, numerous studies have corroborated this finding, showing such *IGH* aberrations in most primary MM cases as well (Nishida et al., 1997; Bergsagel & Kuehl 2001; I; IV; V). It should be stressed that cytogenetic analyses identify abnormalities of 14q32 (*IGH* locus) in only 20-40% of the cases but that interphase FISH and molecular genetic investigations reveal *IGH* rearrangements in 60-80% (Bergsagel et al., 1996b; Nishida et al., 1997; Hallek et al., 1998; Rao et al., 1998; Avet-Loiseau et al., 1999a; Bergsagel & Kuehl, 2001; Pratt, 2002; IV; V). Illegitimate recombination of the *IGH* gene also occurs in MGUS, but in a slightly lower frequency (about 50%) (Avet-Loiseau et al., 1999b; Fonseca et al., 2002a; IV; V). Recently, similar rearrangements of the *IGK* and *IGL* genes have been found in a small subset of MM and MGUS (Fonseca et al., 2002a; Kuehl & Bergsagel, 2002). The rearrangements involving the *IGH* gene are, in most cases, simple reciprocal translocations, but recently more complex recombination events, such as insertions and duplications, have been described (Bergsagel & Kuehl, 2001; Küppers & Dalla-Favera 2001; Fenton et al., 2004).

The *IGH* translocations result in deregulation of the partner genes. At the cytogenetic level, more than 40 different partner loci have been reported in MM (Mitelman et al., 2004a), and to date, 10 target genes have been identified (Table 1).

Table 1. Genes deregulated by *IGH* in MM/MGUS

Genes	Locus	Reference
<i>FGFR3, WHSC1</i>	4p16	Chesi et al., 1997, 1998b
<i>ARHH</i>	4p13	Preudhomme et al., 2000
<i>IRF4</i>	6p23-25	Iida et al., 1997
<i>CCND3</i>	6p21	Shaughnessy et al., 2001
<i>MYC</i>	8q24	Avet-Loiseau et al., 2002
<i>CCND1</i>	11q13	De Boer et al., 1993
<i>MAF</i>	16q22-23	Chesi et al., 1998a
<i>BCL2</i>	18q21	Bergsagel et al., 1996b
<i>MAFB</i>	20q11-13	Hanamura et al., 2001

The most common *IGH* translocations in MM are t(4;14), t(11;14), and t(14;16), occurring in approximately 15%, 15-20%, and 5-10% of the cases, respectively (Avet-Loiseau et al., 1999a; Finelli et al., 1999; Ho et al., 2001; Pratt, 2002; Fonseca et al., 2003). These three translocations have also been identified in MGUS, in which the frequencies are slightly lower (2-9%, 25%, and 5%, respectively) (Fonseca et al., 2002a; Keats et al., 2003).

The t(4;14) activates both the *WHSC1* and *FGFR3* genes, the latter often harboring point mutations as well (Chesi et al., 1997, 1998b, 1998; Richelda et al., 1997; Stec et al., 1998). The t(4;14) is rather unique in the sense that it simultaneously deregulates the *FGFR3* on the derivative chromosome 14 and the *WHSC1* on the derivative chromosome 4 (Chesi et al., 1997; Kuehl & Bergsagel et al., 2002; Soverini et al., 2002). The t(11;14), which also occurs in chronic lymphocytic leukemia and mantle cell lymphoma (Mitelman et al., 2004a) and which deregulates the *CCND1*, may be molecularly more complex than the cytogenetic features suggest (Fenton et al., 2004). The t(14;16) leads to dysregulation of the *MAF* gene, which encodes a transcription factor (Bergsagel & Kuehl, 2003). Interestingly, the *MAFB* gene, targeted by the t(14;20)(q32;q11) found in a few MM cases, displays functional similarities with the *MAF* gene (Kuehl & Bergsagel, 2002; Hanamura et al., 2001).

The pathogenetically important molecular genetic consequences of the numerical changes are, as in other neoplastic disorders (Heim, 1992), unknown. It remains to be elucidated whether the genomic gains and losses in MM result in altered expression of only a few genes or whether they lead to deregulation of a vast number of genes. Although some cDNA microarray studies of MM have been reported (see below) none have specifically addressed this issue. However, it has been shown that AML cases with +8 as the sole anomaly overexpress numerous genes located on this chromosome (Virtaneva et al., 2001). Furthermore, an expression analysis of high-hyperdiploid childhood ALL cases revealed that many of the aberrantly expressed genes map to either chromosome X or 21, the chromosomes most often gained in this ALL subtype (Yeoh et al., 2002). It is reasonable to assume that there is a similar gene-dosage effect of trisomies in MM.

Because losses of chromosome 13 material – monosomy or deletions involving 13q – are common in MM and MGUS (Avet-Loiseau et al., 2000; Königsberg et al., 2000a; Shaughnessy et al.,

2000; Zojer et al., 2000; Fonseca et al., 2002; Nomdedéu et al., 2002; II; Elnenaei et al., 2003a; IV; V) and have been associated with poor prognosis (see below) several studies have focused on the pathogenetically essential molecular genetic outcome of these changes (Avet-Loiseau et al., 1999b; Königsberg et al., 2000a; Shaughnessy et al., 2000; Facon et al., 2001; Fonseca et al., 2002). Although the extent of the 13q deletions may vary substantially, a commonly deleted region – 13q14 – has been delineated. This chromosome band harbors the *RB1* gene, but there is no evidence for bi-allelic inactivation of this tumor suppressor gene in MM/MGUS (Avet-Loiseau et al., 2000; Königsberg et al., 2000a; Shaughnessy et al., 2000; Fonseca et al., 2001b; Elnenaei et al., 2003a). In fact, to date no candidate gene has been identified in this region.

Point mutations, deletions, and hypermethylation of different neoplasia-associated genes, e.g. *CDKN2A*, *HRAS*, *KRAS2*, *NRAS*, *PTEN*, and *TP53*, have also been detected in MM and MGUS. However, the available data indicate that these abnormalities are mainly involved in disease progression rather than in tumorigenesis (Drach et al., 1998; Avet-Loiseau et al., 1999c; Bezieau et al., 2001; Kalakonda et al., 2001; Pratt, 2002; Elnenaei et al., 2003b).

Microarray analyses of MM and MGUS have revealed clear differences between normal PCs and neoplastic PCs, and the expression pattern in PCs in MGUS has been shown to be more similar to the one observed in MM than the one seen in normal BM, again indicating the close relationship between these two disorders (Claudio et al., 2002; de Vos et al., 2002; Tarte et al., 2002; Davies et al., 2003). Furthermore, there are preliminary data suggesting that different cytogenetic subgroups of MM have distinct expression profiles (Zhan et al., 2002; Magrangeas et al., 2003; Santra et al., 2003; Shaughnessy et al 2003b).

## PROGNOSTIC FACTORS

Two fundamental questions in MGUS and MM are: which MGUS cases will transform to MM and is it possible to identify patients with MM with particularly good or poor prognosis? Numerous attempts have been made in order to identify both non-genetic and genetic prognostic factors, of which some are summarized below.

### **Non-genetic factors in multiple myeloma and MGUS**

Whereas little is known about clinical and laboratory parameters of importance in MGUS to MM transformation, several studies have identified factors that may provide important prognostic information in MM, e.g. age, beta-2-Microglobulin (B2M), creatinine, IL-6, PC morphology, PCLI, thymidine kinase, and stage (Turesson et al., 1999; Fonseca et al., 2001; Kyle & Rajkumar, 2003).

The B2M concentration primarily reflects the tumor mass. Creatinine is one of the most important prognostic factors in MM, with increased levels indicating BJP-associated nephrotoxicity. A high level of IL-6 and a plasmablastic PC morphology are also poor prognostic signs. Finally, a high proliferation of the neoplastic PCs, seen as increased PCLI as well as with higher serum levels of thymidine kinase, is associated with a dismal prognosis (Turesson et al., 1999; Fonseca et al., 2001).

### **Genetic factors in multiple myeloma and MGUS**

During the last few years, the MM/MGUS-related genetic aberrations have increasingly proved to be prognostically important.

Several investigations of MM have suggested that the finding of an abnormal karyotype as such is associated with a poor prognosis (Liang et al., 1978; Weh et al., 1993; Cigudosa et al., 1994; Zandecki et al., 1996). Because many groups have reported that chromosomal abnormalities are more common in MM cases with high PCLI and/or high PC percentages (Cigudosa et al., 1994; Lai et al., 1995; Cuneo et al., 1996; Calasanz et al., 1997a; Rajkumar et al., 1999), the prognostic impact of an abnormal karyotype may simply reflect a high proliferation and/or a great tumor burden. However, it should be stressed that some studies have not found any significant correlations between karyotypic features and the degree of BM PC infiltration (Hernández et al., 1998; IV; V).

Some specific karyotypic patterns and chromosomal changes in, particularly, MM have also been associated with outcome. For example, it has repeatedly been shown that hypodiploidy is significantly correlated with shorter survival (Calasanz et al., 1997b; Smadja et al., 2001; Fassas et al., 2002), whereas hyperdiploidy and trisomies of chromosomes 9, 15, 19, and 21 have been associated with a better prognosis (Seong et al., 1998; Smadja et al., 2001). Among the structural chromosomal

abnormalities, most studies have focused on the prognostic impact of 13q losses and the clinical significance of the translocations t(4;14), t(11;14), and t(14;16).

With a few exceptions, monosomy 13/del(13q) has been reported to confer a poor prognosis in MM, seemingly irrespective of treatment (Tricot et al., 1995; Seong et al., 1998; Carlebach et al., 2000; Desikan et al., 2000; Fassas et al., 2002; Fonseca et al., 2002b; Shaughnessy et al., 2000; Kaufmann et al., 2003; Nakagawa et al., 2003), and 13q losses have also been associated with other risk factors, such as increased BM angiogenesis (Schreiber et al., 2000) and high concentrations of B2M (Facon et al., 2001; Tricot et al., 2002). However, it is debated whether the prognostic impact of cytogenetically identifiable 13q losses, seen in approximately 30% of the MM cases (II), and deletions found by interphase FISH (40-90%) differs, with the prognosis perhaps being worse in the former group (Tricot et al., 1995; 2002; Zojer et al., 2000; Nomdedéu et al., 2002; II; Shaughnessy et al., 2003b,c; IV; V). Furthermore, -13/13q- is more common in some cytogenetic subtypes also shown to be associated with poor prognosis, i.e. hypodiploidy, t(4;14), and t(14;16) (Fonseca et al., 2001a; Moreau et al., 2002), making it difficult ascertain the specific impact of chromosome 13 aberrations. Interestingly, losses of 13q have been suggested to play a major role in the transition of MGUS and SMM to MM (Avet-Loiseau et al., 1999b,d; Carrió et al., 2003), although this has been questioned (Fonseca et al., 2001a).

The t(4;14) and t(14;16) are both associated with poor prognosis as well as with -13/13q- (Moreau et al., 2002; Fonseca et al., 2001a; Keats et al., 2003; Chang et al., 2004). It is noteworthy that the 13q losses may occur prior to these translocations (Sawyer et al., 1998; Moreau et al., 2002), suggesting that they are earlier events than previously thought (Sawyer et al., 1998; Fonseca et al., 2001a). MM cases with the t(4;14) often have immature PCs, a great tumor burden, and an IgA M component, and are frequently hypodiploid (Fonseca et al., 2001; Garand et al., 2003; Smadja et al., 2003; Chang et al., 2004).

The prognostic significance of t(11;14) is uncertain. A few years ago, t(11;14) was strongly associated with poor outcome (Fonseca et al., 1998). However, a recent reevaluation suggested that it, in fact, is associated with a favorable prognosis (Fonseca et al., 2002c; Moreau et al., 2002). Needless to say, prospective

studies are needed to clarify this issue. Morphologically, t(11;14)-positive MM cases often have a lymphoplasmacytoid appearance or small PCs with cleaved nuclei (Weh et al., 1995; Hoyer et al., 2000; Garand et al., 2003), and the t(11;14) is said to be the hallmark of IgM, IgE, and nonsecretory MM (Avet-Loiseau et al., 2003a).

Cytogenetic analyses of MM patients who have received chemotherapy may also provide important information as regards the possible development of treatment-related MDS/AML. Such secondary myeloid malignancies are characterized by quite specific chromosomal abnormalities, such as hypodiploidy with whole or partial losses of chromosomes 5 and 7 (Mauritzson et al., 2002; III). In a few instances, however, MDS/AML-associated chromosomal changes, e.g., der(1;7)(q10;p10), t(1;3)(p36;q21), del(5q), -7, +8, and del(20q) (Figure 8), are detected in PCDs without any morphologic features of MDS or AML (Dewald et al., 1985; Clark et al., 1989; Johansson et al., 1995; Weh et al., 1996; Amiel et al., 1999a; Fonseca et al., 2000; Blann et al., 2002; Ferro et al., 2002; I; III). The pathogenetic as well as prognostic importance of such abnormalities remains to be elucidated.

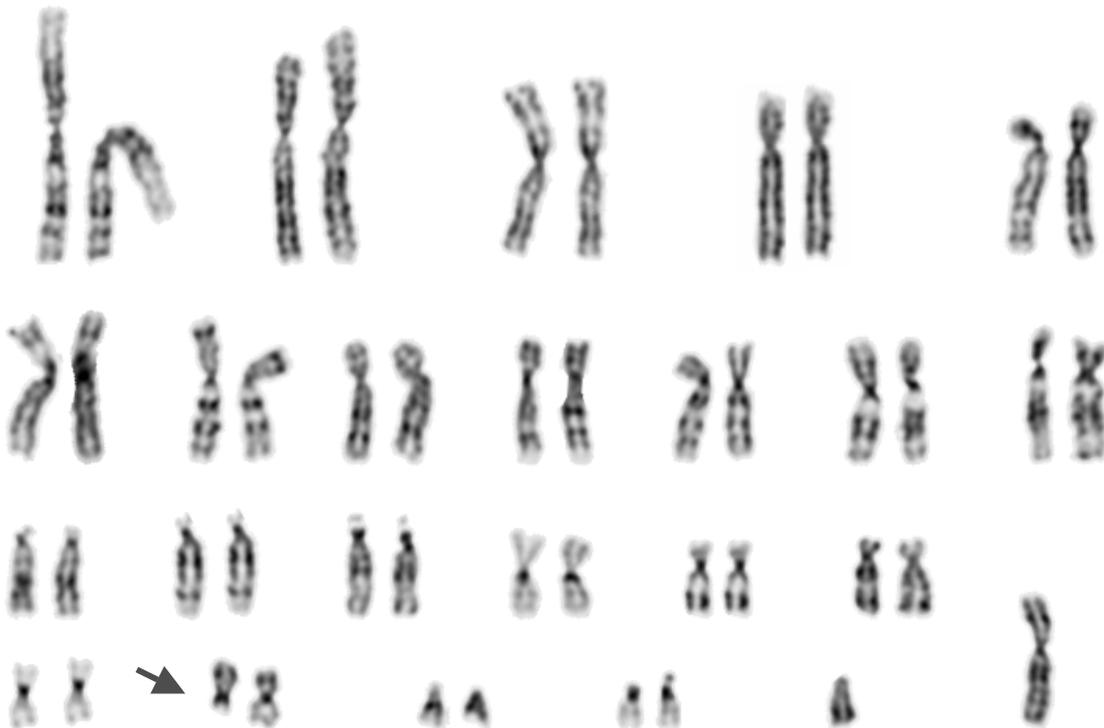


Figure 8. MGUS case with 46,XY,del(20)(q11).

## THE PRESENT STUDY

### **Objectives**

The major aims of the present thesis were to:

- Evaluate different culture procedures of multiple myeloma and related plasma cell dyscrasias.
- Identify and characterize chromosomal abnormalities and karyotypic patterns in these disorders.
- Increase our knowledge of multiple myeloma-associated genetic aberrations and to correlate these with clinical parameters.

### **Patients, materials, and methods**

Between 1978 and 2002, a total of 171 patients with PCD (150 MM, 18 MGUS, 3 Plasmacytomas) have been cytogenetically analyzed at the Department of Clinical Genetics, Lund, Sweden, and 279 cultures were investigated as part of the present thesis. With a few exceptions, the following data could be collected in all cases: stage according to the DS classification, gender, age, B2M, treatment, and M component. The Durie and Salmon system (Durie & Salmon, 1975) subgroups MM into three stages. In stage I, all of the following should be present: hemoglobin >100 g/l, S-calcium normal or <2.60 mmol/l, normal bone structure or a solitary bone plasmacytoma only at X-ray, IgG <50 g/l or IgA <30 g/l, and BJP <4 g/24h. Stage II is defined as neither I nor III. In stage III, one or more of the following should be present: hemoglobin <85 g/l, S-calcium above or equal to 3.00 mmol/l, advanced lytic bone lesions, IgG >70 g/l or IgA >50 g/l), and BJP >12 g/24h.

Various culture methods were tested. In article I, the BM cells were adjusted to a concentration of 1.0-1.5 million cells/ml medium and cultured for 24 and/or 48 hours in conventional McCoy 5A medium with 20% fetal calf serum and antibiotics, and/or for 72, 96, and 120 hours in Chang D medium supplemented with 20% serum, antibiotics, and recombinant human IL-6 (0.02 microgram/ml medium, Genzyme corporation, Cambridge, MA) and GM-CSF (0.06 microgram/ml medium, Leucomax®, Schering-Plough, Novartis, Basel, Switzerland). In article IV, direct chromosome preparations (DCP) were performed on BM cells after exposure to different Colcemide concentrations, i.e. 30, 100, or 200 ng/ml RPMI 1640 medium. The time of the BM aspiration as well as the time of arrival of the samples were

registered, thus enabling exposure times to be calculated. In article V, the BM cells were adjusted to a concentration of 1.0-1.5 million cells/ml medium and cultured in plastic tissue culture flasks or glass flasks for 3-12 days in Chang D medium supplemented with serum, antibiotics, and IL-6 (0.02 microgram/ml medium) and GM-CSF (0.06 microgram/ml medium). Accumulation of metaphase cells was achieved by exposure to Colcemide (100 ng/ml medium) for 30 minutes before harvesting (with the exception of article IV). The cells were exposed to 0.075 M KCl for 15 minutes in room temperature, followed by a gradual exchange of the hypotonic solution for methanol: acetic acid fixative (3:1). Chromosome preparations were made in a standard manner, and G-banded with Wright's stain. Whenever possible, 25-100 metaphases per sample were analyzed. The clonality criteria and karyotypic descriptions followed the recommendations of ISCN (1995).

Metaphase and/or interphase FISH investigations were performed in the studies reported in articles I, III, IV, and V. The samples used were cells remaining from the cytogenetic analyses. The samples had been stored in methanol: acetic acid fixative at -18°C for 0-24 years. The protocols used have been described in article I and in Barbouti et al. (2003). As many metaphases as possible were analyzed on the metaphase level, and 200 nuclei, only mononuclear cells, were scored whenever possible. The cut-off values for the various probes were determined based on control studies. In general, the probe for centromere 4 was used as a control for ploidy when using the 13q14 probe for detection of complete or partial losses of chromosome 13, because chromosome 4 is rarely involved in numerical abnormalities in MM/MGUS/SMM (II, Mitelman et al., 2004a). For details on probes used, please see articles I, III-V.

The genetic findings in articles I, IV, and IV were correlated with the culture conditions, Colcemide concentrations, gender, age, disease phase, and PC percentages.

Article II was a review of the cytogenetic patterns of MM, including cases from our own Department as well as all published cases (Mitelman et al., 2004a). The following features were collected: ploidy levels, karyotypic complexity, common numerical as well as structural chromosomal abnormalities, genomic imbalances, and breakpoints (bp). Cases with clones harboring different number of aberrations were grouped in relation to the simplest clone present, and cases that were incompletely

karyotyped were included in the cytogenetically complex group. In the cytogenetic classification and in the breakpoint and imbalance maps, constitutional chromosomal abnormalities were disregarded. For the breakpoint map, if more than one copy of the same chromosome aberration was found in the same, or related, clone, the bp involved was plotted only once, but if the same bp was involved in different aberrations, it was plotted once per aberration. Furthermore, rearrangements with an uncertain bp localization, e.g. add(14)(q3?2) or del(6)(q13-16), were not included. The imbalances were ascertained according to the following criteria: the net results of the aberrations were always registered with regard to the nearest ploidy level. When additional chromosomal changes were acquired during clonal evolution, only novel abnormalities were included, i.e. if the same imbalance was found in more than one related clone, it was recorded only once. When the same chromosome was involved in several aberrations, only the total net imbalances were plotted. In cases in which a particular chromosome segment was involved in different types of imbalances, only the largest imbalance was registered. Clones with loss of chromosome Y as the sole anomaly were not included in the imbalance map. The cytogenetic features were correlated with age (61 years or younger or 62 years or older) and gender. In an attempt to focus on pathogenetically important changes, only genomic bps and imbalances found in more than 5% and 10%, respectively, were included in the statistical analyses.

## RESULTS

### **Article I**

A total of 95 samples (186 cultures) from 68 MM, 3 plasmacytomas, and 3 MGUS cases were cytogenetically analyzed. Metaphase FISH for *IGH* rearrangements could be performed in 57 of the 74 cases, revealing 14q32 aberrations in 10 cases not seen by conventional G-banding. Taken together, abnormal karyotypes were detected in 77 (41%) of the 186 cultures, 46 (48%) of the 95 samples, and in 41 (55%) of the 74 patients, and a total of 20 chromosomal aberrations previously not reported in PCDs were identified. There was no evidence that gender, age, disease phase, culture time, or cytokine stimulation significantly influenced the karyotypic features.

## **Article II**

The cytogenetic features were ascertained in 783 cytogenetically abnormal MM cases. Hyperdiploidy was most common (39%), followed by hypodiploidy (27%), pseudodiploidy (24%), and tri-/tetraploidy (10%). Most cases were complex, with a median of eight changes per patient. The distribution of modal numbers differed between younger and older patients, with hyperdiploidy being more common in elderly patients. No gender- or age-related differences regarding the number of anomalies were found. The most frequent genomic breakpoints were 14q32, 11q13, 1q10, 8q24, 1p11, 1q21, 22q11, 1p13, 1q11, 19q13, 1p22, 6q21 and 17p11. Breaks in 1p13, 6q21 and 11q13 were more common in the younger age group. The most frequent imbalances were +9, -13, +15, +19, +11 and -Y. Trisomy 11 and monosomy 16 were more common among men, while -X was more frequent in women. Loss of Y as the sole change and +5 were more common in elderly patients, and -14 was more frequent in the younger age group.

## **Article III**

Comparing cytogenetic features in all published MM (n = 993) and t-MDS/t-AML post-MM (n = 117) revealed significant differences regarding complexity, ploidy levels, and most genomic changes. Thus, these features can often be used to distinguish between MM and t-MDS/t-AML. However, we identified 6 MM and 3 MGUS/SMM that displayed myeloid abnormalities, i.e. +8 (1 case) and 20q- (8 cases) as sole anomalies, without any evidence of MDS/AML. One patient developed AML, whereas no MDS/AML occurred in the remaining 8 patients. In one MGUS with del(20q), FISH analyses revealed its presence in CD34+CD38- (hematopoietic stem cells), CD34+CD38+ (progenitors), CD19+ (B cells), and CD15+ (myeloid cells).

## **Article IV**

DCP was used to investigate 47 bone marrow samples from 39 patients with MM/MGUS/SMM. Abnormal karyotypes were detected in 15 (63%) of the 24 MM and in 4 (50%) of the 8 MGUS/SMM cases that could be successfully cytogenetically analyzed. Age, sex, or degree of bone marrow PC infiltration did not influence the karyotypic patterns. However, the frequencies of aberrant karyotypes varied in relation to the Colcemide concentrations used – 7% (30 ng/ml) versus 69% and 67% (100

and 200 ng/ml) ( $P=0.01$ ). Combining the G-banding and FISH results, abnormalities were detected in 29/31 (94%) MM and in 6/8 (75%) MGUS/SMM patients. Thus, cytogenetic and FISH analyses after DCP using 100-200 ng Colcemide/ml identify aberrations in the most MM/MGUS/SMM, irrespective of PC percentages.

#### Article V

A total of 53 samples from 49 patients with MM (n=41), MGUS (n=7), or SMM (n=1) were investigated after culturing as solid tumors. Abnormal karyotypes were detected in 18/39 (46%) MM and in 4/8 (50%) MGUS/SMM patients that could be successfully cytogenetically analyzed. The karyotypic features (abnormal vs. normal/failure and proportions of abnormal karyotypes) did not vary significantly in relation to culture time or degree of BM PC infiltration. FISH revealed *IGH* rearrangements in 22/36 (61%) MM and in 3/8 (38%) MGUS/SMM, whereas 13q losses were identified in 34/40 (85%) MM and in 5/8 (63%) MGUS/SMM. Combining the G-banding and FISH results, abnormalities were detected in 40/41 (98%) MM and in 7/8 (88%) MGUS/SMM patients. Thus, neoplastic MM/MGUS cells can attach to plastic substrate (Figure 9), and cytogenetic analyses of such cultures reveal clonal chromosomal abnormalities in approximately 50% of the cases.

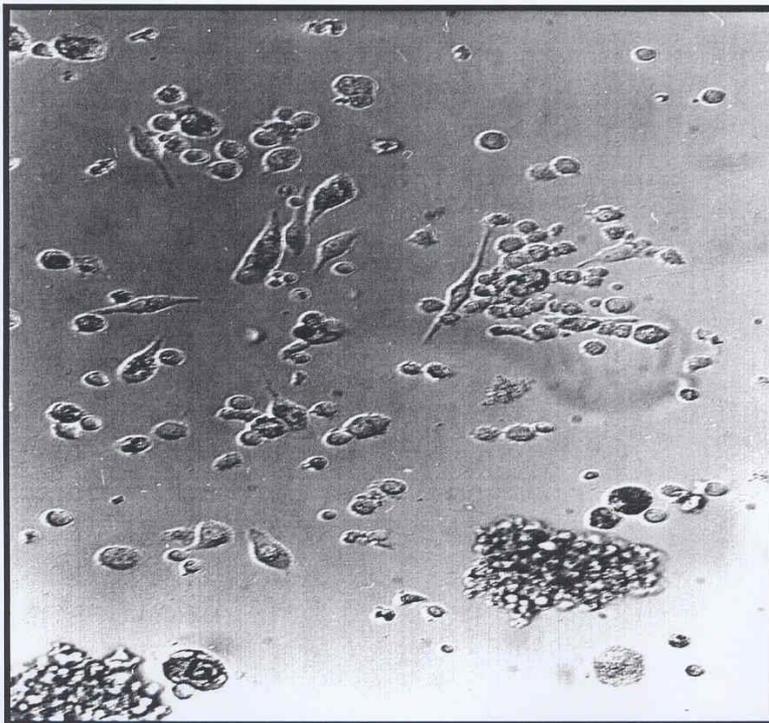


Figure 9.  
Cultured cells  
from a MM case  
plating to the  
surface of a  
tissue culture  
plastic flask.

## DISCUSSION

The neoplastic PCs in MM and related PCDs have proved notoriously difficult to culture, hampering cytogenetic analyses in a large proportion of the cases. In fact, chromosomal abnormalities are found in only 30%-50% of MM (Facon et al., 1993; Cuneo et al., 1996; Calasanz et al., 1997a), despite the fact that interphase FISH analyses reveal abnormalities in close to 100% of the cases (Drach et al., 1995a,b; Flactif et al., 1995; Tabernero et al., 1996; Nishida et al., 1997; IV; V). This strongly suggests that the nonneoplastic cells have a growth advantage *in vitro* and that the “wrong” cells are investigated (Weh et al., 1990). One consequence of this is that the interest in performing conventional G-banding analyses of MM seems to have declined in recent years. Instead, several groups use FISH or molecular genetic methods only when studying MM (Tabernero et al., 1996; Pérez-Simon, et al., 1998; Königsberg et al., 2000b; Rasillo et al., 2003). Although such studies may yield important clinical and biological information, it should be emphasized that these techniques only identify abnormalities that are already known and for which appropriate probes are available. Considering that only approximately 1 000 cytogenetically abnormal MM cases have been published (Mitelman et al., 2004a), it is highly likely many MM-associated chromosomal aberrations remain to be identified. For this reason, it is definitely premature to stop performing karyotypic investigations. However, due to the low cytogenetic success rate, it is imperative to develop and evaluate new culture methods, and it has been suggested that the culture of myeloma PC can be considered as the holy grail of myeloma research (Barker et al., 1993).

A number of different culturing conditions have been used in order to increase the number of abnormal metaphases in MM. Culturing the cells in different media (e.g. McCoy 5A, Chang D, and RPMI 1640), with or without cytokines (for example IL-3, IL-4, IL-6, and GM-CSF) and for different amounts of time, as well as to harvest the cells with no prior culture are some of the methods that have been used. However, twenty years of hard work by several groups working with MM can be summarized as follows: 20-55% are abnormal when cultured for 24-48 hours without cytokines, 55-65% are abnormal after culture for 72 hours without cytokines, 30-55% are abnormal when cultured for 72-144 hours in media with GM-CSF, IL-3, and/or IL-6, 50-65%

are abnormal after culture for 120 hours with IL-4, and 50-60% are abnormal when direct chromosome preparations are used (Liang et al., 1979; Philip et al., 1980; Ferti et al., 1984; Dewald et al., 1985; Chen et al., 1986; Gould et al., 1988; Lisse et al., 1988; Clark et al., 1989; Gutensohn et al., 1992; Facon et al., 1993; Lai et al., 1995; Sawyer et al., 1995; Smadja et al., 1995; Weh et al., 1995; Cigudosa et al., 1994; Tricot et al., 1995; Cuneo et al., 1996; Solé et al., 1996; Brigaudeau et al., 1997; Calasanz et al., 1997a; Hernández et al., 1998; Seong et al., 1998; Rajkumar et al., 1999; Gutiérrez et al., 2000; Facon et al., 2001; I; IV; V). Thus, it seems as if approximately 50% of MM cases are cytogenetically abnormal, irrespective of culture method used. Novel approaches to culture the neoplastic PCs in MM and related PCDs are hence badly needed. An attempt in that direction was to culture the samples as solid tumors (V).

There are several reasons why one should continue to collect cytogenetic information in PCDs. First, unless cytogenetic analyses are performed no “surprising” abnormalities will ever be found, such as the seemingly common occurrence of del(20q) as the sole anomaly in MM and MGUS (III); an aberration characteristic for myeloid malignancies (Greenberg et al., 1997). Second, the identification of recurrent structural abnormalities will undoubtedly result in the detection and characterization of genes of importance in the genesis or progression of PCDs (Mitelman et al., 2004b). Third, further studies will undoubtedly identify important clinico-genetic associations, akin to what has been achieved in the acute leukemias (Grimwade, 2001; Harrison, 2001a).

## SVENSK SAMMANFATTNING (SWEDISH SUMMARY)

Elakartade (maligna) blodsjukdomar uppstår i den blodbildande benmärgen, vilken är innesluten i hålrummen i de skelettdelar som kan sägas täckas av en badmössa och en gammaldags baddräkt. En av de celltyper som bildas i benmärgen är plasmacellen, en vit blodkropp som utgör mindre än 2% av benmärgscellerna och som tillverkar specifika antikroppar vid infektioner. Vid monoklonala gammopatier, exempelvis monoklonal gammopati av oklar signifikans (MGOS) och multipelt myelom (MM), ansamlas abnorma, tumöromvandlade, plasmaceller i benmärgen.

MM och MGOS, som kan sägas vara ett förstadium till MM, förekommer hos c:a 5-6 respektive 200-500 personer per 100 000/år. Patienter med MM uppvisar varierande grad av symtom och fynd, vilka beror på ett ökat antal sjuka plasmaceller och de antikroppar de producerar. I MM kan den sjuka plasmacellen jämföras med en kopieringsmaskin som dels ideligen gör beställningar på nya, identiska kopieringsapparater, dels ser till att dessa kopieringsapparater löper amok och spottar ut likadana papperskopior (antikroppar). Denna överproduktion av celler och antikroppar ger upphov till en mängd olika sjukdomsyttningar. De sjuka plasmacellerna kan "ta över" benmärgsutrymmet och tränga ut de friska cellerna, med resultatet att patienten blir känslig för infektioner (minskat antal normala vita blodkroppar), blödningsbenägen (färre trombocyter) och trött (ont om röda blodkroppar vilka transporterar syre). Allvarliga skador såsom spontana frakturer sker då benceller påverkas av sjuka plasmaceller och njurarna skadas då enorma mängder identiska antikroppar ansamlas. I dagsläget finns det ingen säker bot för MM. Dock kan flertalet symtom lindras avsevärt.

Multipelt myelom kan se ut som beskrivits ovan men kan ha helt andra ansikten med färre symptom; vissa patienter har nästan inga symptom alls. Det finns inte några säkra möjligheter att förutspå hur hårt drabbad en enskild patient kommer att bli. Då det är välkänt att förvärvade kromosomavvikelser i de maligna cellerna kan ge värdefull klinisk information vid leukemier och besläktade sjukdomar, torde kromosomundersökningar av MM och MGOS också kunna vara av betydelse för att, exempelvis, identifiera personer med MGOS som har förhöjd risk att insjukna i MM och förutspå hur allvarlig sjukdomen kommer att bli. Vår kunskap om kromosomala förändringar i MGOS och MM är dock

yttre bristfällig. En orsak till detta är att det är mycket svårt att odla MGOS/MM-celler inför kromosomanalys.

Målsättningen med detta avhandlingsarbete, vilket resulterat i fem vetenskapliga artiklar (I-V), var dels att utveckla och testa olika odlingsmetoder, dels att identifiera och karakterisera typiska kromosomavvikelser i MGOS och MM.

I artiklarna I, IV och V utvärderades om tillsats av olika tillväxtfaktorer och varierande odlingstider kan öka andelen fall med identifierbara kromosomavvikelser. Förutom sedvanlig kromosombandningsanalys utfördes även s.k. FISH, en metod som gör det möjligt att studera avvikelser i icke-delande celler. De genetiska fynden jämfördes med olika kliniska fynd, såsom kön, ålder, frekvens plasmaceller och sjukdomsstadier. Dessa studier visade att avvikelser kan ses med klassisk kromosomanalys i c:a 50% av fallen. Dock påvisades kromosomförändringar i nästan alla fall med FISH. Sålunda behövs båda dessa metoder för att i detalj karakterisera monoklonala gammopatier.

Artikel II är en sammanfattning av ett stort antal MM – både egna fall och samtliga fall som publicerats. Deskriptiva och statistiska analyser av materialet påvisade dels frekventa avvikelser, dels intressanta köns- och ålderskillnader. Genom att identifiera återkommande kromosomavvikelser och vilka kromosomregioner som är involverade får man värdefulla ledtrådar till kromosomsegment som torde innehålla gener av stor betydelse för uppkomst alternativt utveckling av MM. De genetiska skillnader som noterades mellan män och kvinnor och mellan olika åldersgrupper talar för att yttre faktorer kan ha betydelse för uppkomst av vissa MM-associerade kromosomförändringar.

Ett intressant fynd, som rapporteras i artikel III, var att en tämligen stor andel av MGOS och MM uppvisar en kromosomavvikelse (förlust av ett segment från kromosom 20; 20q-), som tidigare endast beskrivits i andra typer av blodsjukdomar, s.k. myeloiska maligniteter. Dock förelåg inte – med ett undantag – några tecken på myeloisk malignitet hos dessa patienter. Detta talar för att 20q-, i motsats till vad man tidigare förmodat, kan förekomma utan att den leder till myeloiska leukemier. Detta trots att utökade analyser visade att 20q- uppstod på stamcellsnivå.

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