

Review of phenotypic markers used in flow cytometric analysis of MGUS and MM, and applicability of flow cytometry in other plasma cell disorders

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Summary

Flow cytometric immunophenotyping is considered an indispensable tool for the diagnosis, classification and monitoring of disease in monoclonal gammopathies. The clinical sensitivity of flow cytometry is comparable with advanced molecular methods. Clinical application of flow cytometry in monoclonal gammopathies has various dimensions, such as differential diagnosis of malignant plasma cell disorder from reactive plasmacytosis, identifying the progression risk in monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic multiple myeloma (MM), and minimal residual disease detection. Flow cytometry-based clonality assessment with immunophenotyping encourages and enables the most stringent method of diagnosis and follow-up. The objective of this review is to update the malignant plasma cells phenotypic profile of MGUS and MM. The most comprehensive antigens, such as CD19, CD27, CD28, CD45, CD56 and CD117, play a significant role in the characterization of normal and malignant plasma cells. Several research groups described the putative phenotype of myeloma cell progenitors, but no remarkable suggestion could be made because of disparity. This review also focuses on the association of malignant phenotypic markers and chromosomal aberrations that identify the specific prognostic features in monoclonal gammopathies.

Keywords: MGUS, MM, myeloma precursor cells, malignant plasma cells, plasma cell proliferative disorders.

Background

In this decade, flow cytometry-based immunophenotyping has become a prominent tool for the diagnosis of monoclonal

gammopathies. Although other conventional methods, such as morphological assessment and immunohistochemistry (IHC), are satisfactory, they lack the clinical sensitivity of flow cytometry. The term monoclonal gammopathy of undetermined significance (MGUS) was coined more than 30 years ago (Kyle, 1978) to describe the most common plasma cell (PC) disorder. Its incidence rate increases with age, affecting 3% of the population older than 50 years and about 10% of population older than 70 years (Rajkumar, 2005; International Myeloma Working Group (IMWG) (2003); Kyle & Rajkumar, 2006). MGUS is characterized by the presence of monoclonal protein (M-protein) in the serum with a level not exceeding 30 g/l and <10% of PC infiltrations in bone marrow (BM) (Rajkumar, 2005; IMWG, 2003; Kyle *et al*, 2002). Asymptomatic or smouldering multiple myeloma (SMM) is a PC disorder, characterized by features, such as ≥ 30 g/l of M-protein, $\geq 10\%$ of PCs, absence of related organ or tissue damage (ROTI) and CRAB symptoms (hypercalcaemia, renal insufficiency, anaemia and bone lesions) (IMWG, 2003; Kyle & Rajkumar, 2009). Symptomatic multiple myeloma (MM) is diagnosed by malignant monoclonal proliferation of PCs, >30 g/l of M-protein, evidence of ROTI and bone lesions. Approximately one-third of MM patients are preceded by MGUS, and the interval from diagnosis of MGUS to the progression of MM is about 2.5 years (Weiss *et al*, 2008). The frequency of malignant PCs to total BMPCs is associated with the progression risk of MGUS and SMM to MM (Persona *et al*, 2007). Plasma cell leukaemia (PCL) is a different subtype of PC disorders; its characterization is based on the presence of $>20\%$ of PCs in whole leucocytes with an absolute count of 2×10^9 /l of PCs in peripheral blood (PB). PCL can be classified into primary and secondary forms. Primary PCL is identified on its presence in the leukemic phase. Secondary PCL is characterized as a leukemic transformation from MM (IMWG, 2003). In monoclonal gammopathies, two different PC populations are usually observed: one is normal, the other is malignant, and both of these populations have distinct phenotypes (Olteanu *et al*, 2008 and Ocqueteau *et al*,

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1998). The phenotypic distinction forms the base in finding prognostic features, treatment strategy and individualized follow-up in monoclonal gammopathy disorders. This review draws attention to various issues, such as multiparameter flow cytometry as a apparent tool for diagnosis and follow-up of MM patients, expression of different antigens in malignant PCs and its prognostic value, importance of normal and malignant PC enumeration for differential diagnosis between MGUS and MM, characterization of myeloma cell progenitors and association of specific PC phenotype with chromosomal aberrations. We also briefly review the applicability of flow cytometry in the diagnosis of other PC disorders (PCL and amyloidosis) and rare myeloma entity.

Flow cytometry as a diagnostic tool in multiple myeloma

Many studies have shown high clinical sensitivity of flow cytometry in the analysis of malignant PCs when compared to conventional morphology. Although significant differences in the BMPC percentages have been found between morphological and flow cytometric analysis, in many cases flow cytometry results showed less PCs than morphological analysis. Proposed reasons for this issue have been described as contamination by PB, existence of small PC clusters, fragility of PCs during sample preparation and unavailability of first pull BM aspirate for flow cytometry (Paiva *et al*, 2009a; Rawstron *et al*, 2008; Smock *et al*, 2007). A recent study reported that the frequency of BMPCs (detected by flow cytometry), together with age, were independent prognostic factors for overall survival in myeloma patients, whereas conventional morphological quantification of BMPCs failed to determine its independent prognostic value (Paiva *et al*, 2009a). However, significant positive correlation has been observed regarding PC quantification by morphological assessment and flow cytometry (Paiva *et al*, 2009a; Smock *et al*, 2007). Paiva *et al* (2009a) also determined that flow cytometric phenotyping could be used for the routine diagnosis of MM. Immunophenotyping by less configured flow cytometry and IHC staining were unable to detect the small proportion of monoclonal PCs with light chain restrictions in PC proliferative disorders. Novel multiparameter flow cytometry (six-colour analysis CD138/CD38/CD45/CD19/cytoplasmic Igk/cytoplasmic Igλ) enables increased sensitivity, has a lower requirement of samples for detection, delineation of malignant PCs and ensures routine diagnosis of PC proliferative disorders (Morice *et al*, 2007). According to European Myeloma Network (EMN) recommendation for the diagnosis of MM, MGUS and reactive conditions by flow cytometry antigens, such as CD19/CD56/CD117/CD20/CD28/CD27/CD81/CD200, must be used. In addition, antigens, such as CD45/CD56/CD117/CD28, have been identified as prognostic markers for myeloma (Rawstron *et al*, 2008).

Antigenic profile of malignant plasma cells/myeloma cells in MGUS and MM

Expression of B cell antigens in myeloma cells

CD9 and CD10 are related to pre-B cells and associated with B cell activation (Kiyokawa *et al*, 1990; Zola, 1987; Zeleznik-Le & Metzgar, 1989). In MGUS, CD10 expression is low and the expression of CD9 is high. CD22 is a B cell lineage-restricted marker expressed in/from pre-B cells to memory B cells. In normal PCs as well as in PCs of monoclonal gammopathies, CD22 expression has been reported to be undetectable (Zandecki *et al*, 1995; Hamilton *et al*, 1991; Terstappen *et al*, 1990). Negative expression of CD20 has been reported in normal PCs (Uckun, 1990). Some subgroups of MM patients PCs express CD20, and this expression is associated with small mature morphology of PCs (Robillard *et al*, 2003). CD19 is a basic and valuable marker for monoclonal gammopathies and most prominently used to identify normal and malignant PCs. An early study reported negative CD19 expression in MGUS (Leo *et al*, 1992); however contrary to this study, Harada *et al* (1993) and Zandecki *et al* (1995) defined CD19 expression in MGUS. CD19 intensity and expression on MGUS PCs was dim when compared to normal PCs, but higher than on myeloma cells. Loss of CD19 was found to be associated with tumour progression in MGUS and MM patients. Mahmoud *et al* (1999) showed that enforced expression of CD19 on myeloma cell lines lead to growth inhibition and reduced tumorigenicity. CD19 expression in MGUS PCs defines the non-malignant population; loss of CD19 by MGUS PCs in parallel with alterations of other phenotypic markers might represent stage transition or disease progression.

Expression of CD27 in myeloma cells

CD27 is a 110-kDa homodimeric transmembrane glycoprotein of the tumour necrosis factor receptor family (Camerini *et al*, 1991); it is expressed on B cell subsets and on the majority of peripheral T cells (Maurer *et al*, 1990; Van Lier *et al*, 1987). The ligand molecule for CD27 is CD70 and the interaction plays a role in the differentiation of memory B cells into mature PCs (Hintzen *et al*, 1994; Agematsu *et al*, 1998). CD27 expression on MGUS PCs are similar to normal PCs. CD27 loss in MGUS has been linked to MM progression. The lack of CD27 expression is usually coupled to the loss of CD19. Conversely, CD19 loss is not always associated with CD27 loss in monoclonal gammopathies (Guikema *et al*, 2003). Allele specific oligonucleotide-polymerase chain reaction (ASO-PCR) analysis has shown that both CD27⁺ and CD27⁻ populations can harbour clonal PCs (Guikema *et al*, 2003). CD27 negative B cells can be induced to express CD27 by ligation with CD40 (Jacquot *et al*, 1997). In contrast, the CD40 expressing myeloma U 266 cell line is not able to express CD27 even after stimulation with CD40 ligand molecules. This indicates that the loss of CD27 expression is an intrinsic

character of myeloma cells and probably not due to the absence of co-stimulatory signals. Negative CD27 expression on PCs has been found in stage II and III MM patients. In MGUS and stage I MM, half of PCs are CD27 positive, and probably all might be negative for CD19 expression (Katayama *et al*, 2003). In accordance with immunophenotyping data, gene expression profile studies also revealed that low CD27 expression in myeloma cells contrast to normal PCs with high CD27 expression (Zhan *et al*, 2003).

Expression of CD28 in myeloma cells

CD28 antigen is expressed on T lymphocytes and it is an important antigen for T cell activation. CD28 aids interaction between T and B cells via a ligand molecule CD86. The co-stimulatory interaction between CD86 and CD28 on T lymphocytes results in increased cytokine secretions, clonal expansion and boosts effector functions (Linsley & Ledbetter, 1993; Rudd *et al*, 1994; June *et al*, 1994; Guinan *et al*, 1994). CD28 is not expressed on normal PCs but consistently and brightly expressed on malignant PCs. High proliferation activity of myeloma cells has been associated with CD28 expression (Robillard *et al*, 1998). CD28 expression studies on myeloma patients have found higher reactivity in advanced disease stage (Pellat-Deceunynck *et al*, 1994). This finding is further supported by the fact that most myeloma cell lines were obtained at the terminal stage of disease or from patients with aggressive forms of myeloma cells expressing CD28 (Zhang *et al*, 1994). Forty-one percent of MM patients were found to express CD28 on BMPCs, but its expression was higher in the case of relapsed myeloma. High reactivity for CD28 expression was found in 59% of medullary relapsed patients and 93% of extramedullary relapsed patients. In contrast to relapsed and active myeloma patients, MGUS patients (19%) expressed very low levels of CD28 on PCs (Robillard *et al*, 1998). Thus, the intensity of CD28 expression on PCs correlates with stage and could be useful for diagnostic assessment of MGUS and MM patients.

Expression of myeloid antigens in myeloma cells

CD33 is a 67-kDa glycoprotein found on the myeloid cell surface. It belongs to the sialo-adhesion molecule family (Freeman *et al*, 1995). CD33 is expressed in 90% of leukaemia patients (especially in acute myeloid leukaemia, AML), but it is not detected on normal haematopoietic stem cells (Wagner *et al*, 1995). Few studies have reported CD33 expression on PCs but the reactivity of the marker has been found in 6.5–12% of myeloma patients (Mateo *et al*, 2005; Almeida *et al*, 1999). CD33 expression in myeloma patients correlates with clinical parameters, suggesting its clinicopathological significance. Patients were divided into two groups based on CD33 expression on PCs (cut-off of 20%). No significant differences were observed between the CD33⁺ and CD33⁻ group in terms of age, sex, bone lesion extension or extramedullary disease (Sahara *et al*, 2006). Prognostic mark-

ers including serum calcium level, creatinine, white blood cell counts and Bence-Jones protein (BJP) level in urine were similar in both groups. The only significant difference observed between the two groups was in the level of serum β 2-microglobulin (β 2M) and lactate dehydrogenase (LDH) (Sahara *et al*, 2006). These two parameters were higher in the CD33⁺ group compared to CD33⁻ group. Regarding the survival rate, the CD33⁺ patient group had a lower survival compared to CD33⁻ patient group.

Expression of haematopoietic stem cell antigen in myeloma cells

One of the therapeutic approaches in haematological malignancies is to treat the patients by autologous stem cell transplantation (ASCT) after high-dose chemotherapy. Therefore, it is necessary that stem cell transplants must be devoid of tumour cells and their progenitors. The well renowned marker CD34 has been widely used to isolate and enrich the stem cells (Kimlinger & Witzig, 1997). Two studies in the past decade confirmed the negative expression of CD34 antigen in monoclonal PCs (CD38/CD45^{dim/neg}) of myeloma patients (Kimlinger & Witzig, 1997; Vescio *et al*, 1994). More recently, Matsui *et al* (2004) postulated and proved that the myeloma precursors exist in CD138⁻/CD34⁻ cells. We perceived that this study disclosed indirectly that CD34 expression is not restricted to myeloma precursors. A recent investigation revealed contrasting results; that a small PC subpopulation of myeloma patients and myeloma cell lines (RPMI 8226, KARPAS 417 and U266) express CD34 (Kuranda *et al*, 2010), characterized by a CD19⁻CD274⁺ phenotype and sharing similar morphology to myeloma cells. This report also showed high expression of the proliferation marker Ki67 in CD34⁺ PCs. CD274 or B7-H1 expression in myeloma cells needs explanation because of its inhibitory function in T cells. Liu *et al* (2007) reported B7-H1 expression in MM but negative expression in MGUS and healthy individuals. The same study also revealed that γ -interferon (IFN- γ) and toll like receptor (TLR) ligand increased B7-H1 expression in MM PCs. Blocking the MEK/ERK pathway inhibited the IFN- γ - and TLR-mediated expression of B7-H1 in PCs. B7-H1 expression on malignant PCs helps in tumour evasion from immune system; so this molecule could be considered as a potential target for therapy (Liu *et al*, 2007). Because of contrasting reports, no definitive conclusions can be drawn regarding CD34 expression in myeloma cells. Their involvement in PCs potentially affects selection for ASCT. The CD34-based selection of stem cells should be accompanied by alternative markers (refer to section *Putative phenotype of myeloma precursor/progenitor cells and its drug resistance*).

Expression of adhesion molecules in myeloma cells

Adhesion molecules play an important role in the migration of cells to different sites. More than a decade ago, several groups

reported various adhesion molecule expressions on myeloma cells that were significantly elevated compared to normal PCs. In MM, changes in adhesion molecules expression have been associated with tumour growth and survival (Van Camp *et al*, 1990; Ashmann *et al*, 1992). CD44 is an adhesion molecule in myeloma cells that helps migration to the BM (Lokhorst *et al*, 1994; Barille *et al*, 1995). CD44 is encoded by a single gene and expressed as CD44 standard molecule (CD44 S) or as variant forms of the CD44 molecule (CD44 V). CD44 V isoforms are the products of alternative splicing of CD44 (Gunthert, 1993). CD44 V contributes to functions such as early haematopoiesis, endothelial cell enhancement at the site of inflammation, apoptosis and shedding (Miyake *et al*, 1990; De Grendele *et al*, 1997; Yu *et al*, 1997). Investigations showed no significant differences between normal PCs and myeloma cells regarding CD44 S expression (Pellat-Deceunynck *et al*, 1995). However, CD44 V (CD44 V9 and CD44 V10) immunophenotyping did differentiate normal PCs from myeloma cells. Normal PCs expressed CD44 V as CD44 V9⁺ V10⁺ but myeloma cells expressed CD44 V9⁺ V10⁻/CD44 V9⁻ V10⁺. Stable disease patients PCs expressed CD44 V9⁻ V10⁺ and contrasting phenotype CD44 V9⁺ V10⁻ was associated with progressive disease.

CD44 V6 is another isoform of the CD44 variant molecule. CD44 V6 expression is highly restricted in the epithelial tissue (skin, oral mucosa, oesophagus and cervix) and mostly associated with squamous cell carcinomas of the respiratory, digestive and genital tracts (Heider *et al*, 2004). CD44 V6 expression has been identified in 6% of MGUS, 17% of stage I MM, 40% of stage II/III MM and 66% of PCL patients (Liebisch *et al*, 2005) but another study reported negative CD44 V6 expression in monoclonal gammopathy patients (Driel *et al*, 1998). The difference may be due to variation in sample size or PC gating strategies. Cytogenetic studies have renewed interest in CD44 V6 expression on myeloma cells because CD44 V6 molecule was found to be highly associated with del(13q) (Liebisch *et al*, 2005). CD49 d [VLA-4 (very late antigen-4)] interaction with fibronectin has been reported to play an important role in the migration and homing of myeloma cells into the BM (Kim *et al*, 1994). CD49 d molecule was reported to be expressed in both normal PCs and myeloma cells. The rationale behind this finding is that CD49 d provides support in the process of the terminal B cell differentiation and immunoglobulin secretion by PCs (Roldan *et al*, 1992). CD44 adhesion molecule and its variant isoforms significantly correlate with tumour progression, disease burden and stages of MM.

CD138 or syndecan 1 (Syndecan is a Greek word that means 'stick together') is a molecule that prominently aids in PC identification and is important in the characterization and identification of myeloma cell phenotypes. A French group identified the applicability of CD138 in recognition of normal and malignant PCs by flow cytometry (Pellat-Deceunynck *et al*, 1994). Findings by Terstappen *et al* (1990) have shown strong association between CD38 and CD138 in PCs. CD138 mediates

the cell-to-cell adhesion by heparin binding molecules expressed by adjacent cells. In myeloma cells, CD138 expression acts as a co-receptor for various growth factors such as Baff/ April, epidermal growth factor (EGF) and interleukin (IL)-6 (De Vos *et al*, 2006). CD138 is a differentiating antigen that appears only after the plasmablastic stage (PC progenitors). PC precursors (CD138⁺) retain the ability to divide while fully matured PC (CD138⁺⁺) lack this ability (Jego *et al*, 1999). The shedding of CD138 molecule from myeloma cells has been shown as a prognostic factor. Soluble CD138 molecules in plasma reflect tumour mass and apoptotic index (Seidel *et al*, 2000; Lovell *et al*, 2005). Promising studies proved that adhesion molecule CD138 could be used as a strong therapeutic target because of its unique expression (von Strandmann *et al*, 2006; Tassone *et al*, 2004). CD38 also can be used as target but it lacks specificity (due to its broad expression); its expression in malignant PCs was lower compared to normal PCs.

CD56 (NCAM – Neural cell adhesion molecule) is mainly involved in normal embryogenesis with regard to axon growth and guidance (Walsh & Doherty, 1997). In the early 1990s several reports represented CD56 molecule expression in PCs as natural killer-associated antigen (NKH-1) and NCAM (Van Camp *et al*, 1990; Drach *et al*, 1991). CD56 mediates cell-cell and cell-matrix interactions, so loss of CD56 expression could accelerate the process of metastatic spread (Blaheta *et al*, 2002, 2004; Rutishauser *et al*, 1988). In myeloma patients, the presence of CD56 correlates with the aggressiveness of disease (the apparent impact of CD56 in the identification and differentiation of normal and malignant PCs is discussed in later section). The regulation of CD56 is controlled by a very precise mechanism. CD56 could be downregulated during migratory events and re-expressed when the cell reaches a specific site or organ (Edvardsen *et al*, 1993; Edelman & Crossin, 1991). This finding is also in accordance with study by Pellat-Deceunynck *et al* (1994) who found migrated PCs in the peritoneal cavity of MM patients. Cells localized in the peritoneal cavity expressed CD56 and have high proliferation activity. Negative expression of CD56 was found to be associated with Ig light chain λ isotype (Van Camp *et al*, 1990). Lack of CD56 expression correlated with poor prognosis in patients treated with conventional chemotherapies (Sahara *et al*, 2002). In contrast, Hundemer *et al* (2007) reported that CD56-negative expression is not a poor prognostic marker when patients are subjected to high-dose chemotherapy. Event-free survival between the CD56⁻ and CD56⁺ group treated by ASCT didn't show any statistical significant difference. However, the absence or presence of CD56 on PCs of myeloma patients enables better diagnostic value when combined with CD19 molecule.

Expression of CD45 in myeloma cells

CD45 is a leucocyte common antigen found in all haematopoietic cells with the exception of platelets and red blood cells

(Thomas, 1989). It is well known for its function as a key regulator of antigen-mediated signalling and activation in B and T lymphocytes (Trowbridge & Thomas, 1994). CD45 expression has been reported to decline *in vivo* during normal PC differentiation (Hermiston *et al*, 2002; Jensen *et al*, 1991). Two distinct PC populations can be identified based on CD45 expression in myeloma patients, characterized by dim to negative expression of CD45 and intermediate to bright expression of CD45 (Pellat-Deceunynck & Bataille, 2004; Asosingh *et al*, 2003; Bataille *et al*, 2003). CD45⁺ PCs are not associated with neovascularisation, but CD45⁻ PCs enhanced the angiogenesis and microvessel density of BM by increasing the secretion of vascular endothelial growth factor (VEGF) and anti-apoptotic factor Bcl2 (Rajkumar *et al*, 2002; Asosingh *et al*, 2004; Pellat-Deceunynck & Bataille, 2004). In accordance with this, Witzig *et al* (1999) showed reduced apoptosis of PCs in myeloma patients compared to MGUS PCs. Angiogenic switch from CD45⁺ to CD45⁻ PCs has been reported in the 5T2MM murine model (Asosingh *et al*, 2004). However, other authors reported that, in newly diagnosed myeloma and relapsed myeloma patients, CD45⁺ PCs dominate compared to CD45⁻ PCs (Kumar *et al*, 2005a). So far, no defined conclusions can be drawn regarding CD45 expression in myeloma patients. Most reports agree that CD45⁻ phenotype represents the malignant PC population in myeloma. CD56 expression strongly correlates with CD45⁻ PCs. Similarly, PC late marker CD138 is also highly expressed on CD45⁻ cells rather than on CD45⁺ cells. Conversely, CD45⁺ PCs were reported to express more often CD44 and CD11b. (Jensen *et al*, 1992). The presence of CD45⁻ PCs is associated with poorer outcome and worse overall survival rate in comparison to CD45⁺ PCs. The poor outcome of CD45⁻ phenotype might be due to other intrinsic factors influencing malignancy, such as change of ploidy or cell kinetics, especially at the time of relapse (Moreau *et al*, 2004).

Expression of CD117 in myeloma cells

CD117 (C-kit) is an essential haematopoietic growth factor receptor with tyrosine-kinase activity. Kit-mediated signal transduction was found to be critical for normal development and haematopoietic progenitor cell survival (Miettinen & Lasota, 2005). Normal PCs and tumours, including sarcomas, carcinomas and lymphomas, do not express CD117. CD117 was reported to be expressed in malignant PCs of some myeloma patients (Ocqueteau *et al*, 1996). Bataille *et al* (2008) identified CD117 expression in 36 of 122 MGUS (30%) and 169 of 617 MM (27%) cases. CD117 expression in MGUS was significantly higher compared to myeloma patients. Therefore, it is possible that CD117 could be one of the markers for transition from MGUS to MM. Myeloma patients often acquire antigen CD221 in parallel with the loss of CD117 in a process associated with disease progression and poor prognosis (Bataille *et al*, 2008). The CD117⁺ patient group had a better outcome compared to the CD117⁻ patient

group (Mateo *et al*, 2005). No significant differences were observed between the CD117⁺ and CD117⁻ groups when comparing clinical and biological parameters, such as M-protein isotype, albumin, β 2M, LDH, disease stage, response to chemotherapy and survival time (Kraj *et al*, 2004). Recently identified markers, such as CD200 and CD221, on PCs represent adverse prognosis in myeloma patients (Bataille *et al*, 2005; Moreaux *et al*, 2006). Expression of various antigens in myeloma cells and their normal distributions are summarized in Table I.

Characterization of normal and malignant plasma cells and its role in differential diagnosis of MGUS and MM

In monoclonal gammopathies, CD19 and CD56 expression on PCs can be used to differentiate malignant PCs from normal PCs. Measurement of CD56 expression and its intensity may be helpful for distinguishing MGUS, MM, extramedullary plasmacytoma and PCL (Martin *et al*, 2004; Kremer *et al*, 2005; Pellat-Deceunynck *et al*, 1998). Several groups described two clearly distinct populations of normal and malignant PCs in the BM of MGUS and MM. Normal PCs are characterized by low forward/side scatter (FSC/SSC) and high CD38 expression with CD19⁺ CD56⁻ phenotype. Malignant PCs are CD19⁻ CD56⁺ or CD19⁻ CD56⁻ with high FSC/SSC and low CD38 expression (Figs 1 and 2) (Ocqueteau *et al*, 1998; Sezer *et al*, 2001; Kovarova *et al*, 2009). The ratio between normal and malignant PCs has been identified to make differential diagnosis between MGUS and MM. In their cohort of patients, Sezer *et al* (2001) had shown that the MGUS group had $\geq 20\%$ of normal PCs to total BMPCs while no MM patients were detected to have $\geq 20\%$ of normal PCs. This study clearly showed that the normal to malignant PCs ratio could be used to differentiate the two entities, even though classical diagnosis (such as Durie & Salmon criteria: $>10\%$ of BMPC infiltration, normal IgM < 0.5 g/l, IgA < 1 g/l or IgG < 6 g/l and evidence of osteolytic lesions) fails to differentiate them (Sezer *et al*, 2001). Thus, this confirmed that immunophenotyping by flow cytometry is a sensitive and specific method to distinguish between MGUS and MM.

Prognostic features of normal and malignant PCs in MGUS, SMM and MM

Two important studies from Salamanca derived interesting independent prognostic features based on flow cytometric evaluation of BMPCs in MGUS, SMM and MM. Paiva *et al* (2009b) demonstrated that myeloma patients with $>5\%$ of normal PCs to total BMPCs had favourable prognostic features. These patients had a significant lower frequency of high-risk cytogenetic abnormalities and higher response rates than their counterparts ($\leq 5\%$ of normal PCs). Moreover, this study also found significantly increased progression-free survival and overall survival in patients with $>5\%$ of normal PCs

Table I. Expression of various antigens in plasma cells of MGUS and MM.

Cluster Designation	Normal distribution and functions	Expression in plasma cells of MGUS and MM	References
CD19	All B cells, including lymphoblasts, mature B-lymphoid cells and most of plasma cells	MGUS – normal plasma cells express CD19, rather malignant plasma cells do not MM – only negative or dim CD19 expression on plasma cells	Uckun (1990); Ocqueteau <i>et al</i> (1998)
CD20	During maturation process of pre-B cells and negative expression in plasma cells	Some subsets of myeloma patients express CD20 in plasma cells	Uckun (1990); Robillard <i>et al</i> (2003)
CD27	Aid in differentiation of mature B cells into plasma cells	MGUS – consistent high expression in plasma cells MM – expression is heterogeneous and low intensity comparing to MGUS	Agematsu <i>et al</i> (1998); Guikema <i>et al</i> (2003)
CD28	T cell activation	MGUS – only very few cases express CD28 MM – CD28 expressing plasma cells represented as aggressive phenotype. Associates always with tumour expansion	Linsley and Ledbetter (1993); Robillard <i>et al</i> (1998)
CD33	Myeloid and monocytic cells	Only few myeloma patients express CD33 on plasma cells and correlate with lower survival	Freeman <i>et al</i> (1995); Sahara <i>et al</i> (2006)
CD45	B and T cells, dim expression in precursor cells and some plasma cells	MGUS – equal distribution of CD45 ⁺ and CD45 ⁻ plasma cells MM – expression is not well characterized but survival rate is higher for CD45 ⁺ patient group comparing to CD45 ⁻ group. Also CD45 expression demonstrates proliferating compartment of normal, reactive and malignant plasma cells	Trowbridge and Thomas (1994); Kumar <i>et al</i> (2005b); Pellat-Deceunynck and Bataille (2004); Moreau <i>et al</i> (2004)
CD56	NK and NK-T cells	In plasma cell proliferative disorders CD56 consider as a valuable marker in diagnosis. MM – most of the myeloma cases express CD56. However, circulating plasma cells and extramedullary myeloma patients lack CD56 expression	Van Camp <i>et al</i> (1990); Bataille <i>et al</i> (2006); Pellat-Deceunynck <i>et al</i> (1998)
CD117	Progenitors of myeloid, erythroid and megakaryocytic lineage	MGUS – 50% of cases express CD117 MM – one-third of myeloma patients express CD117 and have better prognosis comparing to their counterpart	Bataille <i>et al</i> (2008); Kraj <i>et al</i> (2004)
CD138	Plasma cells	MGUS and MM cases express CD138 on plasma cells, but its expression is dim on malignant plasma cells	Bataille <i>et al</i> (2006)

MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; NK, natural killer cells.

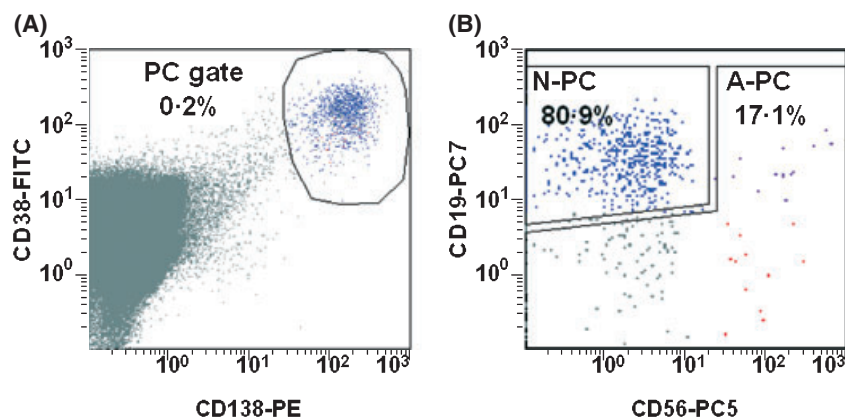


Fig 1. (A) Identification of PCs in MGUS by using the markers CD138 and CD38. (B) Showing the high frequency of normal PCs (N-PC) in MGUS comparing to the frequency of malignant /abnormal PCs (A-PC).

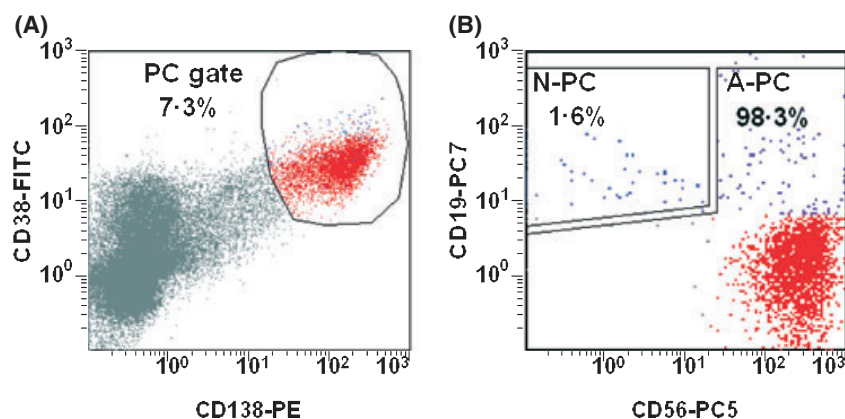


Fig 2. (A) PC infiltration in the bone marrow of MM patient. (B) Existence of high frequency of malignant/abnormal PCs (A-PC) in MM comparing to normal PCs (N-PC).

compared to $\leq 5\%$ of normal PCs (Paiva *et al*, 2009b). Their earlier study (Persona *et al*, 2007) used a different independent variable ($\geq 95\%$ malignant PCs to total BMPCs) to report the progression rate in MGUS and SMM patients for symptomatic myeloma. The study revealed the progression risk (with a cumulative probability of progression at 5 years) of MGUS and SMM as 25% and 63%, respectively, if $\geq 95\%$ BMPCs were malignant. The progression rate was significantly reduced to 5% and 8% for MGUS and SMM, respectively, if $< 95\%$ of BMPCs were malignant (Persona *et al*, 2007). This interesting study had also shown three risk groups of progression for MGUS and SMM at an interval of 5 years, by choosing independent variables, such as DNA aneuploidy and immunoparesis, along with $\geq 95\%$ of malignant PCs (progression risk based on the independent variables is summarized in Table II).

A recent interim study disclosed that, in high-risk SMM patients (defined: $\geq 95\%$ of malignant PCs, immunoparesis and either $\geq 10\%$ of BMPCs, or ≥ 30 g/l of M-protein or both), progression to MM was prolonged significantly by lenalidomide plus dexamethasone treatment when compared to

Table II. Progression risk of MGUS and SMM based on the independent variables.

Diagnostic group	Progression %	Independent variables
MGUS	2	None
	10	Either $\geq 95\%$ of malignant PCs/DNA aneuploidy
SMM	46	Both
	4	None
	46	Either $\geq 95\%$ of malignant PCs/immunoparesis
	72	Both

Source: (Persona *et al*, 2007).

MGUS, monoclonal gammopathy of undetermined significance; SMM, smouldering multiple myeloma.

delayed treatment group (Mateos *et al*, 2009). Thus, the flow cytometric evaluation of normal and malignant BMPCs in MGUS, SMM and MM aid in screening progression risk, individualization of treatment and follow-up.

Circulating clonal cells in MGUS and MM

Circulating clonal PCs and B/CD19⁺ cells can be detected in PC proliferative disorders including MGUS, SMM, MM, PCL and amyloidosis. These circulating PCs have diagnostic and predictive value for disease progression (Witzig *et al*, 1993; Boccadoro *et al*, 1988; Omede *et al*, 1990). Quantification of circulating PCs, together with M-protein and Ig isotype in MGUS predicts progression risk, prognosis and overall survival (Kumar *et al*, 2005a). One-fifth of MGUS and amyloidosis patients have circulating PCs (Witzig *et al*, 1993; Kumar *et al*, 2005a; Pardanani *et al*, 2003). Circulating PCs are found more frequently in MM – almost 80% of myeloma patients have detectable circulating PCs (Kyle *et al*, 2002). The absolute number of circulating PCs was $1 \times 10^6/l$ and $4.4 \times 10^6/l$ in MGUS and MM, respectively (Kumar *et al*, 2005a). Kyle *et al* (2002) revealed the rate of progression of MGUS to PC disorder by following 1384 MGUS patients for 35 years, from 1960–1994. Thirty-two percent of patients had >10% BMPC infiltration or >30 g/l of M-protein without symptoms of myeloma. During the follow-up, 8% of patients developed MM, primary amyloidosis, lymphoma, macroglobulinemia, plasmacytoma and chronic lymphocytic leukaemia. The probability of progression to any of these disorders is about 10% at 10 years, 21% at 20 years and 26% at 25 years. Higher progression risk was observed in patients who had IgA or IgM monoclonal protein, increased M-protein level, BJP, and elevated erythrocyte sedimentation rate (Kyle *et al*, 2002; Roodman, 2002). Some circulating B lymphocytes (CD19⁺) in MGUS and MM also carry karyotypic changes and chromosomal aberrations similar to myeloma cells. About 0.1–1.8% (MM) and 0.02% (MGUS) of CD19⁺ circulating cells carry similar chromosomal changes as BM myeloma cells (Chen & Epstein, 1996; Billadeau *et al*, 1996; Zojer *et al*, 2001).

Putative phenotype of myeloma precursor/progenitor cells and its drug resistance

In MM, considerable research efforts have been directed in characterization of the precursors, their development, and self renewal potential. In most cancers including MM, the cloning potential is less and has the ability to proliferate at lower rate. More than three decades ago, the cloning efficiency for MM was described approximately as 1 in 1000 to 100 000 cells (Hamburger & Salmon, 1977). Cancer stem cells from AML and brain tumours phenotypically resemble normal haematopoietic and neural stem cells (Lapidot *et al*, 1994; Singh *et al*, 2004). Regarding the issue of clonal MM progenitors/precursors, two different recent studies proposed different hypotheses; one reported that precursor cells of MM originate from memory B cells (CD19⁺CD27⁺CD34⁺CD138⁺) (Matsui *et al*, 2008), while other claimed that precursors derive from clonotypic B cells (CD19⁺CD34⁺CD138⁺) that are not related to a mature phenotype (Conway *et al*, 2009). However, these two studies suggested that myeloma cell

progenitors lack CD138 expression. A review article (Rasmussen, 2001) described clonal CD19⁺ cells as an ongoing differentiating population ranging from memory B cells to plasmablasts. This is comparable with the report of Matsui *et al*, 2008.

Early findings, and those reported in the late 1990s had shown that malignant PCs share identical idiotype and Ig gene sequence of B cells (Billadeau *et al*, 1993; Bergsagel *et al*, 1995; Rasmussen *et al*, 1999). The role of these B cells in the pathology of MM has remained ambiguous and needs further investigation. A recent review described the putative phenotype of myeloma precursors as CD19⁺CD20⁺CD22⁺CD27⁺CD45⁺ and CD138⁺, which shares the identity of mature B cells (Ghosh & Matsui, 2009). Matsui and colleagues have also shown that CD138⁺ cells, but not CD138⁺ cells, engrafted disease in non-obese severe combined immunodeficient mice and formed myeloma colonies *in vitro* clonogenic assays (Matsui *et al*, 2004, 2008; Ghosh & Matsui, 2009). An earlier ASO-PCR report (Rottenburger *et al*, 1999) is also in accordance with the issue of MM precursor cells. These authors screened clonotypic CD19⁺ and CD20⁺ B cells in PB of post high-dose therapy and ASCT of MM patients. Clonotypic cells in CD19⁺ fractions were higher compared to CD20⁺ fractions of patients who were in continuous remission. But clonal cells from both fractions of the former cases were significantly lower compared to patients in early relapse/progressive disease (Rottenburger *et al*, 1999). A recent study by Conway *et al* (2009) documented different myeloma precursor phenotype, which was in contrast to the previous studies. Conway *et al* (2009) found that clonotypic B cells express both lymphoid and stem cell based antigens and are characterized as CD34⁺CD19⁺CD20⁺CD38^{dim to moderate}CD138⁺ and weak light chain restriction. The phenotype of these clonotypic B cells differs from normal B cells (CD19⁺CD34⁺), malignant and normal PCs. However, a small population of cells shares similar phenotype to clonotypic B cells but those cells were defined as precursors of non-malignant cells by *IGH* arrangement and light chain restriction assays. The study also suggests that similar precursors of clonotypic B cells and normal B lymphocytes cannot be distinguished by phenotypic pattern whereas combination with light chain restriction can do. Interestingly, this study also determined no reactivity of CD117 on precursors of malignant and normal PCs and suggested CD117 could be used as selection marker for ASCT (Conway *et al*, 2009). Clonal precursors were found to be relatively resistant to the anti-myeloma agents (lenalidomide, bortezomib, glucocorticoids and alkylators) that are currently used to control the tumour mass in MM patients (Matsui *et al*, 2008). This result is similar to earlier reports (Rasmussen, 2001), suggesting that small subsets of clonal CD19⁺ cells are resistant to high-dose chemotherapies).

Several studies described that precursors of MM are the cause of malignancies, so it could be explored for therapy. Rituximab is a humanized monoclonal antibody (MoAb) and its activity has been well characterized in B cell

malignancies (Johnson & Glennie, 2003). Rituximab functions as an activator of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity effector mechanisms. Rituximab inhibitory function has been well-described in *in vitro* clonogenic myeloma cell cultures (Matsui *et al*, 2004). However, details of the clinical efficacy of rituximab in MM patients are lacking because only a few MM patients express CD20 on PCs (Treon *et al*, 2002). The disparity between clinical and *in vitro* results may be because rituximab may bind myeloma precursors but lacks the activity of elimination *in vivo* (Ghosh & Matsui, 2009). Therefore, all these studies clearly indicate that myeloma precursors exist and that they lack expression of tumour cell marker. The investigations suggest that the precursors of myeloma cells lie, in particular, in the B cell compartment but no defined conclusion can be made about the phenotypic nature because of disparity among the studies.

Flow cytometry for minimal residual disease assessment in myeloma

Minimal residual disease (MRD) screening is a standard method of assessment of the patient's status after treatment by chemotherapy or transplantation. A decade ago, responses to treatment in MM were assessed by urine or serum paraprotein (Bladé *et al*, 1998). A highly sensitive method (ASO-PCR) is currently available, which quantifies MRD with a threshold of 0.01% but apparently lacks applicability. This method is applicable only for 75% of patients due to technical reasons and is influenced by time and cost (Sarasquete *et al*, 2005; Rawstron *et al*, 2008). The possibility of detecting the malignant PCs by flow cytometry is similar to ASO-PCR, at a clinically relevant threshold of 0.01% (Rawstron *et al*, 2008). This has been also proven by comparative study between ASO-PCR and flow cytometry. Significant positive correlation ($r^2 = 0.94$, $P < 0.0001$) was observed in detection of residual malignant PCs by both methods (Lioznov *et al*, 2008; Sarasquete *et al*, 2005). Moreover, flow cytometry has greater applicability and could be used in >90% of cases, although it can fail in certain cases. This was proved by Sarasquete *et al* (2005), where flow cytometry was negative in six cases of MRD but those cases were positive by ASO-PCR. In MM, the relevance of clonality assessment in follow-up or MRD cases is less clear when compared to newly diagnosed cases. However, the EMN has suggested a method that could be applicable for both follow-up and newly diagnosed cases. The strategy combined clonality assessment with basic immunophenotyping (six-colour analysis), which enabled rapid detection of malignant PCs and excluded extended immunophenotyping. The respective screening panel was suggested by the EMN (cytoplasmic Ig λ /cytoplasmic Igk/CD19/CD56/CD38/CD45 and cytoplasmic Ig λ /CD19/cytoplasmic Igk/CD138/CD38/CD45) (Rawstron *et al*, 2008). Assessment of cytoplasmic Ig light chain expression by flow cytometry was also considered as stringent analysis for myeloma patients complete remission

status according to IMWG criteria (Durie *et al*, 2006). Taken together, MRD analysis in MM by flow cytometry is a useful technique that is comparable with ASO-PCR, provides prognostic information, and identifies patients with different progression risks.

Differential diagnosis of myeloma and plasmacytoma from lymphoplasmacytoid lymphoma and marginal zone lymphoma

The differential diagnosis of plasmacytoma from non-Hodgkin lymphoma [NHL: Marginal zone lymphoma (MZL)] and myeloma from lymphoplasmacytoid lymphoma [LPL: Waldenstrom macroglobulinemia (WM)] is required because these diseases all share the common feature of plasmacytic differentiation (Seegmiller *et al*, 2007; Hussong *et al*, 1999). One study reported that plasmacytoma, MZL and other NHL share similar morphological features and are difficult to differentially diagnose (Hussong *et al*, 1999). This information is also in accordance with flow cytometry-based diagnosis in rare plasmacytoma cases. However, flow-cytometric immunophenotyping strategy (CD19 and CD56) can differentiate plasmacytoma from MZL (Seegmiller *et al*, 2007). Immunophenotyping revealed that plasmacytoma has similar primary myeloma cell phenotype (CD19⁻CD56⁺CD45^{dim/-}), but the phenotype (CD19⁺CD56⁻CD45⁺) of MZL contrasts to that of myeloma cells. Apart from identification of PCs, the CD138 molecule also provides differential diagnosis between LPL and MM. LPL is characterized by monoclonal proliferation of lymphoplasmacytoid cells. CD138 serves as good diagnostic marker when the LPL clonal B cells (not reactive for CD138) are targeted (Chilosi *et al*, 1999). But in some cases of LPL, PCs aren't favoured for differential diagnosis because CD138 expression pattern is identical to myeloma cells (O'Connell *et al*, 2004; Morice *et al*, 2009). Another option for the discrimination of MM and LPL with regard to PCs is screening for CD19 expression (Morice *et al*, 2009). Myeloma cells have low or no reactivity for CD19 whereas LPL PCs show high reactivity to CD19. Therefore, these studies indicate that CD19 and CD56 could be used for the identification of normal and malignant PCs of monoclonal gammopathies, as well improving the differential diagnosis of different tumour entities (for detailed phenotypic nature of WM refer the section *Rare monoclonal protein associated multiple myeloma-IgM myeloma*).

CD 56/NCAM role in lytic bone lesion diagnosis

Lytic lesions are caused by an imbalance between the frequency of osteoblasts and osteoclasts. Ely and Knowles (2002) reported that the expression of CD56/NCAM (IHC method) on PCs correlated with the presence of lytic bone lesions in myeloma patients. This study documented NCAM expression in 71% of myeloma patients, 7% of MGUS and negative expression in polyclonal plasmacytoses, NHLs with

plasmacytoid differentiation and amyloidoses (Ely & Knowles, 2002). Strong restriction of myeloma cells to the BM is due to presence of adhesion molecule CD56 and homophilic binding with osteoblasts. Interactions between myeloma cells and osteoblasts lead to decreased bone matrix production and eventually to bone lytic lesions. This was documented in *in vitro* co-culturing of osteosarcoma cell lines and MM cell lines (Ranheim *et al*, 1996; Krushel *et al*, 1998; Crossin & Krushel, 2000; Cunningham *et al*, 1987; Barille *et al*, 1995). A well-noted feature of bone lytic lesions is an increase in osteoclast numbers; myeloma cells and osteoclasts interactions are limited and remote site osteoclasts are not affected (Rossi *et al*, 1990; Bataille *et al*, 1995). The increase in osteoclasts is also due to myeloma and stromal cells that secrete osteoclast activating factors, such as macrophage inflammatory protein-1 α (MIP-1 α), VEGF, tumour necrosis factor (TNF)- α , IL-1 β , parathyroid hormone -related protein, IL-6 and NF- κ B ligand (RANKL) (Podar *et al*, 2009). Conversely, certain molecules and pathways facilitate in the reduction of osteoblast activity. Major factors have been described for this reduction includes: inhibition of BM osteoblast progenitors by blocking Runt-related transcription 2/cbfa1 (Giuliani *et al*, 2006; Esteve & Roodman, 2007; Terpos *et al*, 2007), inhibition of the Wnt signalling pathway by DKK1 (Qiang *et al*, 2008) and IL-3 released by BM CD3 $^{+}$ T cells that inhibits the differentiation of pre-osteoblasts to mature osteoblasts (Giuliani *et al*, 2006). Amino bisphosphonates are used for the treatment of bone disease in MM along with targeting tumour cells. Based on osteoclast and osteoblast cells signalling cascades, several anti-bone resorption molecules have been identified, such as HDAC inhibitor PXD101, MEK inhibitor AZD6244, Resveratrol and CCR1 inhibitor MLN3897 (Podar *et al*, 2009).

Malignant PC marker and its association with genomic abnormalities in MM

Only a few studies have reported the association between genetic aberrations and phenotypic antigens in myeloma cells. One study in a large series of myeloma patients provided evidence for the systemic relation between genetic aberrations and unique myeloma phenotypes (Mateo *et al*, 2005). Del (13q) and *IGH* rearrangements were more commonly reported in CD117 $^{-}$ phenotype compared to CD117 $^{+}$ phenotype. Another abnormality, t(4;14), was also found to be associated with CD117 $^{-}$ phenotype (Mateo *et al*, 2008). Translocation t(11;14) was identified most commonly in CD20 $^{+}$ patient group. Frequency of t(11;14) was reported to be fourfold higher in CD20 $^{+}$ patients compared to CD20 $^{-}$ patients (Robillard *et al*, 2003; Mateo *et al*, 2005). CD56 $^{dim/-}$ and lack of CD117 expression have been associated in patients with t(11;14) (Mateo *et al*, 2005; Sahara *et al*, 2002; Hundemer *et al*, 2007). Non-hyperdiploid myeloma cells are more frequently associated with CD28 $^{+}$ CD20 $^{+}$ CD56 $^{-}$ CD117 $^{-}$ phenotypes compared to hyperdiploid myeloma cells (Mateo *et al*,

2005). A recent study described the additional correlation of CD28 $^{+}$ myeloma cells with t(14;16) and del(17p) (Mateo *et al*, 2008). A British group reported the specific association between phenotype (CD20 $^{-}$ CD56 $^{-}$ CD117 $^{-}$) and chromosomal abnormality t(11;14) in rare IgM myeloma cases (Feyler *et al*, 2008). Del (13) was associated with both CD33 $^{+}$ and CD33 $^{-}$ phenotypes (Sahara *et al*, 2006). Translocation t(4;14) or t(14;16) were also associated with both CD45 $^{+}$ and CD45 $^{-}$ phenotypes (Moreau *et al*, 2004). High frequency of CD221 expression on PCs was restricted to specific chromosomal aberration t(4;14) or t(14;16) (Bataille *et al*, 2005). All these studies suggest that the association of specific genomic abnormality and phenotype of PCs might encourage the identification of prognostic features for MM.

Application of flow cytometry in immunophenotyping of PCL, AL amyloidosis and rare IgM myeloma

Plasma cell leukaemia

PCL is a rare form of malignant PC disease accounting for 1–2% of MM. Earlier survey reports of PCL cases concluded that primary PCL might account for 60% and secondary PCL for 40% (Noel & Kyle, 1987). Compared to MM patients, PCL patients have adverse prognosis (lower survival rate and response to chemotherapy) (García-Sanz *et al*, 1999). Flow cytometry immunophenotypic findings have shown that the expression of antigens, such as CD38, CD138, CD2, CD3, CD10, CD13, CD16 and CD15, were similar for PCL and MM. However, the differences were observed between the two entities in expression of antigens such as, CD9, CD20, CD56, CD117 and HLA-DR (García-Sanz *et al*, 1999; Cha *et al*, 2007). CD20 expression displayed higher reactivity in PCL whereas the other four antigens (CD56, CD9, CD117 and HLA-DR) were more frequently negative in PCL compared to MM. Previous studies did not identify the markers that could differentiate primary PCL from secondary PCL, but Pellat-Deceunynck *et al* (1998) showed that the CD28 antigen could be used to differentiate primary and secondary PCL. They found that secondary PCL cases express CD28 more frequently compared to primary PCL (92% in PB, 55% in BM vs. 33% either in PB/BM). Contrastingly, CD56 expression (CD56 $^{-/-dim}$) and negativity for CD19 were homogenous in primary and secondary PCL. MM and PCL clinical features are similar. But the extensive involvement of visceral organs, fewer bone lesions and high frequency of anaemia, LDH, β 2M and thrombocytopenia identified PCL as a distinct clinical entity (Cha *et al*, 2007; García-Sanz *et al*, 1999). Different studies are concordant regarding increased BM cellularity in PCL compared to MM (García-Sanz *et al*, 1999; Dimopoulos *et al*, 1994; Cha *et al*, 2007). In conclusion, unique prognostic features and worse outcome are common in PCL in contrast to MM. Thus, it confirms that PCL patients need different clinical management and treatment.

AL amyloidosis

AL amyloidosis has been reported to be the most common form of amyloidosis in western countries, with an estimated incidence of 0.8/100 000 person-years (Kyle *et al*, 1992). Amyloidosis is a generic terminology for a final common pathway of protein sedimentation in tissues (Merlini & Bellotti, 2003; Stone, 1990). The cause of amyloidosis is protein misfolding and soluble proteins that form aggregates as extracellular amyloid fibrils and result in functional and structural organ damage (Merlini & Bellotti, 2003). Approximately 24 different proteins have been involved in the formation of amyloid deposits that might lead to localized or systemic amyloidosis. AL amyloidosis is characterized by low or undetectable level of PC proliferation, and its transformation to MM occurs infrequently (Gertz *et al*, 1989). This entity arises from Ig light chains produced by malignant PCs and is most prominently associated with λ light chain (ratio- $k/\lambda = 1:3$), which is unlike other PC disorders (Yoshida *et al*, 2008; Perfetti *et al*, 2002). AL amyloidosis clinical features are represented as progressive dysfunction in multiple visceral organs (mainly heart and kidneys) and, pathologically, as the extracellular deposition of insoluble fibrils derived from Ig light chains (Kyle & Gertz, 1995). A recent study by a Japanese group demonstrated the prominent phenotypic nature of malignant PCs in AL amyloidosis and derived the specific marker for follow-up studies (Yoshida *et al*, 2008). They reported that the phenotype of normal and malignant PCs of AL amyloidosis patients were similar to myeloma patients. Further characterization of PC subsets has been based on lineage markers, such as MPC-1, CD45 and CD49e. Lineage-specific PC subsets were described as: immature cells – MPC-1⁺CD45⁺ or MPC-1⁺CD45⁻, intermediate cells – MPC-1⁺CD45⁻CD49e⁻ and mature cells – MPC-1⁺CD45⁺CD49e⁻ or MPC-1⁺CD45⁺CD49e⁺. Immature, intermediate and abnormal PCs and serum-free light chain (FLC)/creatinine ratio were significantly higher in AL amyloidosis patients than in the control group (MGUS and other diseases). Also, a significant lower frequency of mature PCs was observed in AL amyloidosis patients compared to controls (Yoshida *et al*, 2008).

Influence of treatment and transplantation in PC phenotype of AL amyloidosis patients

AL amyloidosis patients in remission after high-dose chemotherapy [melphalan and VAD (vincristine, doxorubicin and dexamthazone)] followed by ASCT were reported to show a decrease in the frequency of malignant PCs, intermediate PCs, serum FLC/creatinine ratio and disappearance of M-protein in serum/urine (Matsuda *et al*, 2003; Yoshida *et al*, 2008). An encouraging and promising therapy was reported to target AL amyloidosis using humanized MoAb 2B6. This 2B6 MoAb targets the stable surface antigen CD32B1 isoform expressed on malignant PCs. 2B6 MoAb efficiency has been proved in

murine xenograft model of human NHL (Zhou *et al*, 2008). Highlighting intermediate PCs (MPC-1⁺CD45⁻CD49e⁻) represents a prognostic marker for diagnosis and follow-up of AL amyloidosis patients.

Rare monoclonal protein associated multiple myeloma – IgM myeloma

MM cases are more frequently associated with either IgG or IgA M-protein; the interesting entity IgM myeloma falls into an intermediate category between MM and WM. Classical MM (IgG and IgA) and WM are two distinct entities that could be easily diagnosed based on involvement of M-protein. IgM M-protein is a common feature in WM and adds incredible diagnostic value. The additional feature in the WM patient is monoclonal proliferation of lymphoplasmacytoid cells both in PB and BM (Haghighi *et al*, 1998). The clinical features of IgM myeloma have been characterized as high incidence of lytic bone lesions, decreased IgG and IgA level, renal failure, hypercalcaemia and BJ proteinuria, and these features are common findings in MM. IgM myeloma also shares typical WM clinical features, such as hyper viscosity symptoms, lymphadenopathy and hepatosplenomegaly (Zarrabi *et al*, 1981). We believe that this IgM myeloma entity might arise from IgM-specific MGUS, but a long-term follow-up study (Kyle *et al*, 2003) found that no cases of 213 IgM MGUS (diagnosed during 1960–1994) progressed to myeloma; exceptionally those patients progressed to disorders such as lymphoma, WM, primary amyloidosis and chronic lymphocytic leukaemia (Kyle *et al*, 2003).

As already discussed, IgM myeloma is an intermediate candidate between MM and WM; further immunophenotypic study revealed a hybrid phenotype of PCs in one case. The phenotype of this case was represented as CD45⁻CD38⁺⁺ cytoplasmic Ig⁺⁺CD20⁺FMC7⁺ weak surface Ig k⁺CD19⁻CD22⁻ (Haghighi *et al*, 1998). Malignant PCs of this case had an atypical phenotype of WM. Malignant phenotype of WM has been characterized as CD19⁺CD20⁺CD22⁺FMC7⁺ with strong positivity for surface and cytoplasmic Ig expression and variable expression for CD38 and CD5 (San Miguel *et al*, 1986; Kucharska-Pulczynska *et al*, 1987). A recent study of 10 IgM myeloma cases clearly showed IgM-associated MM phenotype as CD20⁻CD56⁻CD117⁻, which is in contrast to a case reported by Haghighi *et al* (1998) where CD20 expression was found on PCs (Feyler *et al*, 2008). It is difficult to comment on IgM myeloma because of lack of information about this entity but this patient group has a worse outcome when compared to IgG/IgA MM, and needs individualized care.

Conclusions

Flow cytometry in the diagnosis of plasma cell disorders has made significant impact in the tumour cell characterization in addition to determining the frequency of physiologically

normal plasma cells. As suggested in this review, the advancement of this technique optimistically influences its apparent need in clinical applications. Significant prognostic features identified in monoclonal gammopathies or in plasma cell disorders by immunophenotyping enhance malignant plasma cell targeting and specify treatment strategy. The flow cytometry-based approach of assessing MRD appears mandatory in haematological malignancies including MM. Uncertainty regarding myeloma cell progenitors are still a major concern. But the most outstanding feature regarding the myeloma cell progenitors is the establishment of Myeloma Stem Cell Network (MSCNET). This network has set a goal: to screen the progenitors by different working objectives, such as clonal analysis and tracking oncogenic events, gene expression profiling, protein expression profiling, functional characterization *in vivo* and *in vitro*. As myeloma researchers, we are hoping that progenitors of myeloma cells will be clearly identified and might have a greater impact on treatment strategy.

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