Comparison of protocols for multiple myeloma and its precursor diseases

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HÁSKÓLI ÍSLANDS

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Samanburður greiningarprófa fyrir mergæxli (multiple myeloma) og forstig þess

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Abstract

Plasma cells (PCs) are antigen producing B cells that develop from hematopoietic stem cells. The main site for B cell and PC maturation is the bone marrow, spleen, and lymph nodes. PCs are the functional active cells of the B cell maturation lineage, and secrete antibodies. PC maturation needs to be highly controlled otherwise a serious pathological condition can occur like multiple myeloma.

Multiple myeloma is defined as elevated clonal bone marrow PCs or extramedullary plasmacytoma together with end-organ damage (CRAB criteria), ≥60% clonal bone marrow PCs or abnormal free light chain ratio. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma are precursor forms of multiple myeloma. Non-IgM-MGUS, IgM-MGUS, and light chain monoclonal gammopathy, are disorders categorized as MGUS. Smoldering myeloma is defined by an elevated monoclonal protein concentration of IgG or IgA type, or elevated urinary monoclonal protein concentration, 10-60% clonal bone marrow PCs, with the absence of the end-organ damage.

The aim of this project is to compare protocols for multiple myeloma and its precursor diseases MGUS and smoldering myeloma in bone marrow samples, on aspirate smear, in biopsy sections, and by flow cytometry. To examine the proportion of normal and abnormal PCs within the bone marrow (abnormal PC/BMPC) compartment by flow cytometry, and compare it to the aspirates and biopsy and finally to compare samples analyzed in different flow cytometers, FACS Cal and MACS Quant.

The results showed a highly significant correlation, between different flow cytometers. The comparison between flow cytometry and morphology showed fewer PCs obtained by flow cytometry than morphology. The correlation between flow cytometry and bone marrow aspirate was not significant and there was no relationship between these two methodologies. In the case of flow cytometry and biopsy, as well as aspirate/biopsy there was a significant but weak correlation. The discrimination between the ratio of normal and abnormal PCs obtained with flow cytometry was not successful.

Immunophenotypic analysis and the use of flow cytometry for characterization, diagnosis and MRD analysis of malignant hematological diseases have become an important analyzing method in modern laboratories. For an efficiency of the PC burden in patients with multiple myeloma, smoldering myeloma and MGUS which are important for prognosis and outcome for patients, further analysis is needed.

Ágrip

Plasma frumur framleiða mótefni og tilheyra B eitilfrumum (B frumur). Þær þroskast út frá stofnfrumum í beinmerg. Þroskun B eitilfruma og plasma fruma á sér aðallega stað í beinmerg, milta og eitlum. Þroskun þeirra þarf að lúta ströngu eftirliti svo ekki fari illa, annars geta hlotist af alvarlegir sjúkdómar eins og til dæmis mergæxli.

Mergæxli er skilgreint sem hækkun illkynja plasma fruma í beinmerg, utan beinmergs plasmafrumuæxli (extramedullary plasmacytoma), ásamt einu eða fleiri eftirfarandi viðmiða; kalsíum hækkun í blóði, blóðleysi, nýrnabilun eða sáramyndun í beinum (CRAB einkenni), eða ≥60% illkynja plasma frumur í beinmerg, óeðlilegt hlutfall af léttum keðjum. Mergæxli þróast út frá einkennalausri góðkynja einstofna mótefna-hækkun í blóði (monoclonal gammopathy of undetermined significance (MGUS)) og mallandi mergæxli (smoldering myeloma). MGUS er einkennalaust og skiptist í: non-IgM MGUS, IgM MGUS og MGUS með léttum keðjum. Mallandi mergæxli er einnig einkennalaust og skilgreint út frá hækkandi styrk einstofna mótefnis í blóði af gerðinni IgG eða IgA, ásamt hækkun einstofna mótefna í þvagi og 10-60% illkynja plasma fruma í beinmerg.

Tilgangur þessa verkefni var að bera saman mismunandi greiningapróf fyrir mergæxli og forstig þess MGUS og mallandi mergæxli í beinmergs sýnum, í stroki úr beinmerg, með vefjalitun og með mótefnalitun fyrir frumuflæðigreiningu. Sérstök undirmarkmið verkefnisins voru að skoða hlutfall illkynja plasmafruma af heildarfjölda plasmafruma í beinmerg sem fæst með frumuflæðigreiningu og bera saman við hlutfall plasma fruma sem fæst í beinmergsstroki og með vefjalitun. Einnig að bera saman sýni keyrð í tveimur mismunandi frumuflæðisjám, í FACS Cal og MACS Quant.

Helstu niðurstöður voru þær að sterk jákvæð fylgni var á milli tveggja frumuflæðisjáa. Mun færri plasma frumur fengust með frumuflæðisjá heldur en beinmergsstroki og vefjalitun. Engin fylgni var á milli frumuflæðigreiningar og beinmergsstroks. Á milli frumuflæðigreiningar og vefjalitunar var veik fylgni, sem og á milli beinmergsstroks og vefjalitunar. Aðgreining á hlutfalli illkynja plasma fruma af heildarfjölda plasma fruma í beinmerg, með frumuflæðigreiningu gekk ekki sem skyldi.

Mótefnalitun fyrir greiningu og eftirfylgni illkynja blóðsjúkdóma með notkun frumuflæði eru orðnar mikilvægar í nútíma rannóknastofum. Mikilvægt er að geta fylgst með fjölgun illkynja plasma fruma hjá sjúklingum með MGUS, mallandi mergæxli og mergæxli upp á framgang og horfur sjúkdómsins. Til þess að svo geti orðið þarf frekari rannsókna við.

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List of abbreviations

Abnormal	
PC/BMPC	Proportion of abnormal bone marrow plasma cell among the bone marrow plasma cell compartment
APC	Allophycocyanin
BD	Bioscience
BMPC	Bone marrow plasma cells
Ca	Calcium
CD	Cluster of Differentiation
CIMA	Centro de Investigación Médica Aplicada
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraaceticacid
FACS	Fluorescence activating cell sorting
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
CRAB	Ca, renal, anemia, bone lesion
FO	Follicular
H&E	Hematoxylin-eosin staining
IL-	Interleukin
k	Kappa
λ	Lambda
LIS	Laboratory information system
MGUS	Monoclonal gammopathy of undetermined significance
MRD	Minimal residual disease
MZ	Marginal zone
PC(s)	Plasma cell(s)
PE	Phycoerythrin
PerCp	Peridinin chlorophyll protein
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B Ligand
RNA	Ribonucleic acid
SSC	Side scatter

1 Introduction

1.1 From B cells to plasma cells

Plasma cells (PCs) are derived from the B cell lineage that develops from hematopoietic stem cells in the bone marrow. The main site for B cell and PC maturation is the bone marrow, spleen and lymph nodes (1) .

In the first stages of maturation which takes place in the bone marrow, the B cells go through several stages of maturation, from progenitor B cells to precursor B cells, and finally to immature B cells ready to leave the bone marrow. During this maturation, rearrangement of the immunoglobulin genes takes place to form the immunoglobulin heavy-chains, the light-chains, the B cell receptor, and immunoglobulin IgM with help from several transcription factors (1, 2). The B cells leave the bone marrow as immature B cells and finalize their early maturation stage in the spleen. In the spleen, the B cells continue their maturation and diverge into Transitional T1 and T2 B cells. The transitional cells then further mature into marginal zone (MZ) B cells and follicular (FO) B cells depending on their role (see **Figure 1**). Some of the MZ B cells from the T1 stage do not circulate but they have the ability to respond quickly to blood borne pathogens entering the circulation (1, 2). These cells are thought to be long-lived B cells and can mature further into memory B cells, that can have a similar life span as the host they are living in (3). FO B cells on the other hand get their name from the follicles in secondary lymphoid organs where they reside. The FO B cells circulate between the bone marrow, the lymph nodes and the spleen in order to meet an antigen for further maturation. The FO B cells do not live as long as the MZ B cells and die if they do not encounter an antigen or get survival signals from T cells (1, 3, 4).

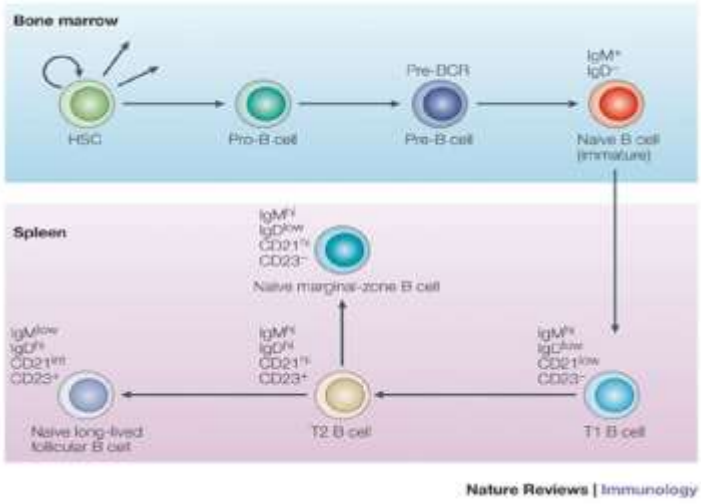

Figure 1 B cell development

This figure shows in a schematic way how B cell maturation takes place in a few stages within the bone marrow, from a hematopoietic stem cell to an immature B cell ready to leave the bone marrow. The cells enter the spleen after leaving the bone marrow where they are divided into T1 and T2 transitional stages. Some of the T2 stage B cells mature further into MZ B cells, and the others mature into FO B cells (1).

Within the germinal center in the follicles of the lymphoid and spleen, further maturation of the B cell occurs that leads to formation of plasma blasts. When these plasma blasts leave the germinal center they mature into Immunoglobulin secreting PCs and memory B cells. The bone marrow is thought to contain mostly long lived PCs secreting antibodies. The long lived memory B cells do not secrete antibody, but circulate between the spleen and lymph nodes (1, 5).

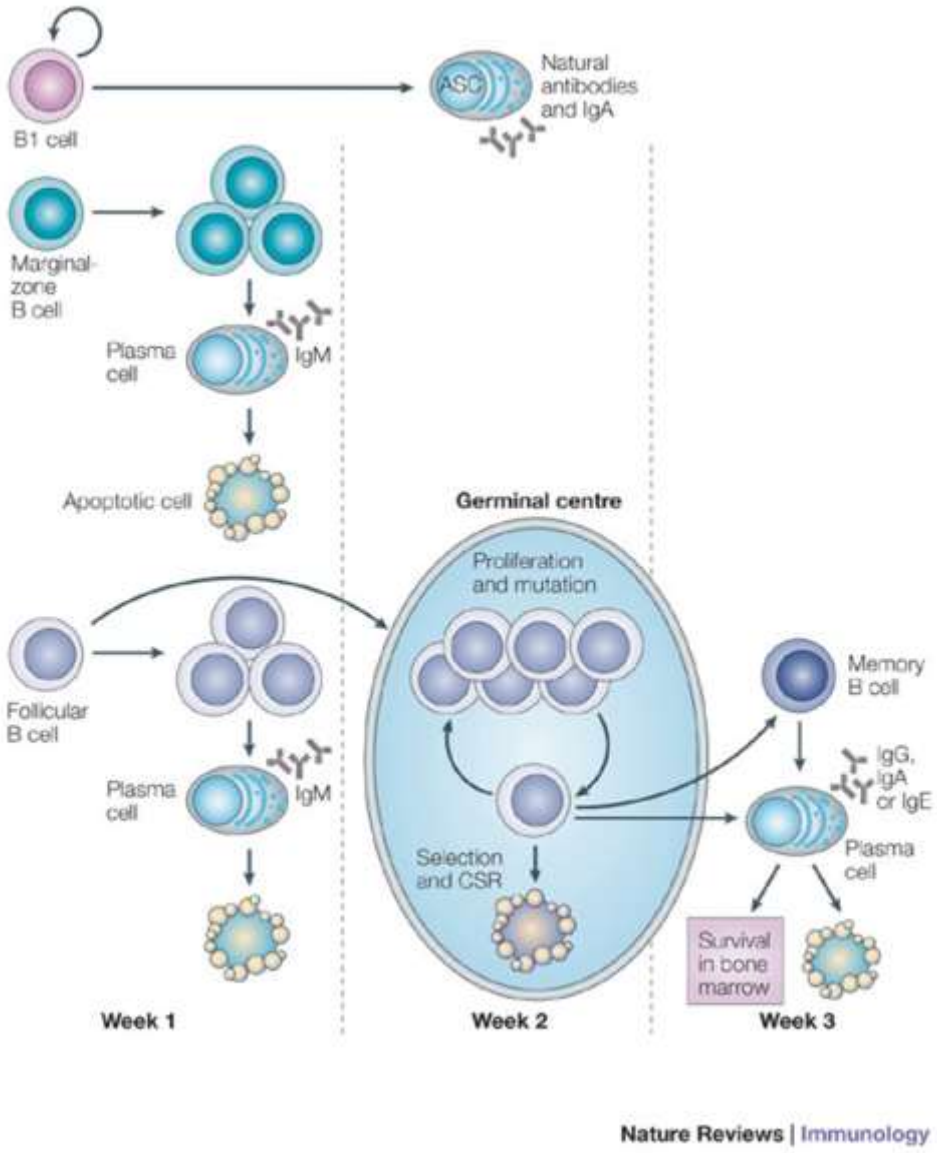

Figure 2 Plasma cell formation

This schematic figure shows the development of the B cells that spans about three weeks. In the first week, B1 cells can develop into an antibody secreting cells immediately without help from a foreign antigen, and in the intestine they can act to pathogens by secreting IgA. Maturation into plasma cells (PCs) can be derived from both marginal zone (MZ) B cells and follicular (FO) B cells, but these cells have a short life time and will not mature further, but go into apoptosis. The second week, some of the FO B cells go to the germinal center for further proliferation and a class switch recombination of the Immunoglobulins. In the germinal center they either go into apoptosis or develop further into memory B cells and PCs. The memory B cells can proliferate into PCs. PCs derived from the germinal center are thought to be long lived PCs producing Immunoglobulins and relocated to the bone marrow (1).

PCs are the functional active cells of the B cell maturation lineage, and the only antibody secreting cells in the body (6). Fully mature PCs have lost almost all of the B cell receptor and membrane Immunoglobulin, but obtained a steady-state of the Immunoglobulin heavy and light chains (5). The PCs are mainly found in tissues like the spleen and lymph nodes (6). PCs located in the bone marrow are thought to be long lived PCs (5). The morphological appearance of the PCs is quite different from other cells and can easily be recognized by their small eccentric nucleus and large cytoplasmic area.

They can also be recognized by their immunophenotypic expression of syndecan-1, a membrane bound protein (cluster of differentiation, CD138), and their bright expression of CD38 (6, 7).

The maturation process has to be regulated and directed towards the right pathways for an efficient control. If not, several diseases can develop, such as multiple myeloma (1).

The Immunoglobulin classes are five and are composed of two heavy and two light chain types (**Figure 3**). The heavy chains are IgG, IgA, IgM, IgD and IgE (also designated as gamma, alpha, mu, delta and epsilon), and the light chain types are: kappa (k) and lambda (λ). Each antibody molecule is composed of two Immunoglobulin heavy chains and two light chains bound to the heavy chains, which would be either κ or λ (8, 9).

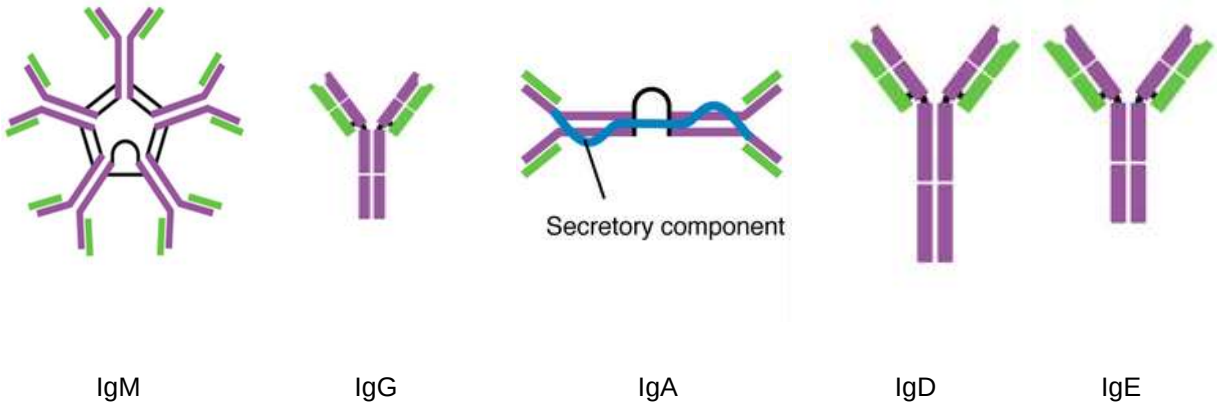

Figure 3 Immunoglobulin structure

This figure shows the immunoglobulin structure of five main Immunoglobulin heavy chains, i.e., IgM, IgG, IgA, IgD and IgE (purple color) and the Immunoglobulin light chains (shown in green, is either κ or λ) bound to the heavy chains (10).

1.2 Monoclonal gammopathies

Monoclonal gammopathies are a synonym for different diseases characterized by the production of monoclonal protein in PCs (also called immunoglobulinopathies, dysproteinemias and paraproteinemias) (11). Primary amyloidosis, chronic lymphocytic leukemia, B cell lymphoma, Waldenström macroglobulinaemia, and multiple myeloma are diseases derived from monoclonal gammopathies of undetermined significance. Rearrangement of the immunoglobulin genes is the main characteristic of monoclonal gammopathies that lead to an excessive secretion of the monoclonal protein (12).

Monoclonal gammopathy can be either benign or malignant. Benign monoclonal gammopathy can be seen in inflammatory rare situations, in reactive rare processes and as secondary to other diseases, such as certain infections. Benign monoclonal gammopathy can progress to a malignant disease. Monoclonal gammopathy can progress to multiple myeloma, Waldenström macroglobulinaemia, chronic lymphocytic leukemia, primary amyloidosis, and B cell lymphoma (11, 12).

1.3 Epidemiology

Multiple myeloma affects all races, and is just below 1% of all cancers in the world (13), and about 10-15% of hematological diseases (14). People of Asian origin are those who are least likely to get multiple myeloma, but Europeans, Australians and Americans have a higher incidence rate with African Americans having the highest (15). Other factors that are thought to increase the risk of developing monoclonal gammopathy of undetermined significance and multiple myeloma are chemicals, pesticides used in agricultural work, and ionizing radiation (15, 16), autoimmune disease and familiality (17, 18).

Multiple myeloma accounts for 1% of all malignant tumors in the Icelandic population, with an average diagnosis of 18 individuals per year during the years 2010 to 2014. The mean age at diagnosis is 70 years (see **Table 1**) (19).

Cases where more than one individual has developed multiple myeloma have been reported in some families (20). First degree relatives of Swedish multiple myeloma patients show a higher risk of developing monoclonal gammopathy of undetermined significance, multiple myeloma, hematological diseases and solid tumors (21). In a population,- and cancer-registry-based study, Helga Ögmundsdóttir et al. have described an increased risk of developing multiple myeloma or other hematological diseases in Icelandic female relatives of multiple myeloma patients (22).

The most common immunoglobulin type among Icelandic patients diagnosed with monoclonal gammopathy of undetermined significance is IgG in 55% cases, IgM in 32% cases, and IgA in 13% cases (23).

Table 1 Incidence of multiple myeloma in the Icelandic population

Multiple myeloma incidence in the Icelandic population (19).

Overview (2010-2014)

	Males	**Females**
Mean incidents per year	9	9
Ratio of all cancers	1.2%	1.3%
Mean age at diagnosis	67 years	71 years
Mean deaths per year (during the years 2010-2014)	6	4
Number of patients alive, at the end of 2014	62	49

1.4 Monoclonal gammopathy of undetermined significance

Monoclonal gammopathy of undetermined significance (MGUS) is a condition that causes elevation of the monoclonal protein (see **Figure 4**). Patients with MGUS are without any symptoms related to the monoclonal gammopathy and the condition is usually detected due to work up for other reasons (12). The definition for MGUS patients is that they are without physical symptoms like calcium elevation, renal insufficiency, anemia, and bone lesion, symptoms referred to as the CRAB criteria (see **Table 4**). MGUS can be categorized as following: Non-immunoglobulin M MGUS (non-IgM MGUS),

immunoglobulin M MGUS (IgM-MGUS) and light chain immunoglobulin MGUS. Development into multiple myeloma is about 80% from non-IgM MGUS and 20% from light chain MGUS. IgM MGUS usually develops into Waldenström macroglobulinaemia (see **Table 2**), but can evolve into multiple myeloma in rare cases. Solitary plasmacytoma, both with and without PC involvement, can develop into multiple myeloma, as well as systemic amyloidosis (24).

The prevalence of MGUS varies between age, gender and race (25-27). MGUS mainly affects the elderly, and the older they get, the higher is the risk. Approximately 3.2% individuals have MGUS at the age of 50, 5.3% at the age of 70 and 7.5% of those 85 years and older among Caucasians. Also, a 1.3% higher prevalence rate is seen in males compared to females (27). The prevalence of MGUS among African Americans is higher or over twofold compared to Americans of European ancestry. The risk of progression to multiple myeloma or a related disorder is approximately 1% per year (25, 26).

The most common immunoglobulin clonal types are usually IgG, followed by IgM, IgA, and the most uncommon ones are bi-clonal types. Among the light chain types k is more common than λ (27). The same trend between males and females is also seen among African ancestry, i.e., males are at a higher risk of having MGUS, and the immunoglobulin type shows the same pattern with IgG the most common one, followed by IgM, and then IgA. However among the light chains, λ is more common than k (25).

Table 2 Characterization of monoclonal gammopathy of undetermined significance

Different features of MGUS, (24, 28).

	Description	Progression rate per year	Progression event
MGUS (IgG and IgA), non-IgM type	S-monoclonal protein <30 g/L. <10% clonal PCs in bone marrow, and absence of the CRAB symptoms All 3 criteria above must be met	1%	Multiple myeloma, solitary plasmacytoma, immunoglobulin amyloidosis (AL, AH, AHL)
MGUS, IgM type	S- IgM monoclonal protein <30 g/L. lymphoplasmacytic infiltration <10% in bone marrow CRAB symptoms absent All 3 criteria above must be met	1.5%	Waldenström macroglobulinaemia, Immunoglobulin amyloidosis (AL, AH, AHL). Multiple myeloma in rare cases
MGUS, light chain type	<10% clonal bone marrow PCs/<500 mg/24 hours urinary monoclonal protein No expression of the immunoglobulin heavy chain Free light chain ratio is abnormal (normal ratio is 0.26-1.65) κ increases with a high free light chain ratio (>1.65), and λ increases with a low free light chain ratio (<0.26) CRAB/ amyloidosis absent All criteria above must be met	0.3%	Multiple myeloma light chain type Immunoglobulin light chain amyloidosis

S-monoclonal protein = serum monoclonal protein. IgM = Immunoglobulin M. PCs = Plasma cells. AL = immunoglobulin light chain amyloidosis. AH = Immunoglobulin heavy chain amyloidosis. AHL = Immunoglobulin heavy- and light chain amyloidosis. CRAB = Calcium elevation, renal insufficiency, anemia, bone lesion.

1.5 Smoldering multiple myeloma

Smoldering multiple myeloma is also an asymptomatic condition like MGUS, a stage between MGUS and multiple myeloma. Smoldering myeloma patients are without symptoms related to the CRAB criteria (see **Table 4**), patients have IgG or IgA type of monoclonal protein concentration ≥30 g/L or urinary monoclonal protein ≥500 mg per 24 hours and/or 10-60% clonal bone marrow PCs, both of

these criteria must be met. Patients with ≥60% clonal PCs in the bone marrow progress faster to multiple myeloma, or within two years. Smoldering myeloma has a progression rate of 10% per year, the first five years, and a 5% progression rate among light chain smoldering myeloma (11, 24, 29) (see **Table 3**). Approximately 14% of patients have smoldering myeloma according to a population based study from Sweden (30). Patients diagnosed with smoldering myeloma are usually not treated until progression occurs. In a recent publication it is recommended that high risk patients with the minimum of 60% PCs within the bone marrow PC compartment (BMPC) should be diagnosed as having multiple myeloma. Thus, these patients should receive therapy to delay progression to multiple myeloma (11, 24).

Table 3 Characterization of multiple myeloma

Different features of smoldering- and multiple myeloma (24).

	Smoldering myeloma	**Multiple myeloma**
S-monoclonal protein	IgG or IgA ≥30 g/L or urinary monoclonal protein ≥500 mg per 24 hours and/or bone marrow PCs 10-60%	Not necessarily
Clonal PCs in the bone marrow	10%-60%, >60% for high risk smoldering myeloma	≥10% or extramedullary plasmacytoma
Biomarkers of malignancy		>60% clonal bone marrow PCs
CRAB symptoms or Amyloidosis events	Absent	Yes, one or more of the symptoms
Free light chain ratio		≥ 100 mg/L
Progression rate to multiple myeloma per year	10%	

S-monoclonal protein = serum monoclonal protein. PCs = Plasma cells. CRAB = Calcium elevation, renal insufficiency, anemia, bone lesion

1.6 Multiple myeloma

Symptomatic multiple myeloma is a malignant PC disease that has progressed from smoldering myeloma and MGUS (31). Until recently the criteria for multiple myeloma has been the existence of monoclonal protein in serum or urine, ≥10% clonal PCs in the bone marrow and one of the symptoms related to the CRAB criteria (11). In 2014 the criteria for multiple myeloma and its precursor diseases was revised (24).

Multiple myeloma does not need the requirement of monoclonal protein in its criteria, it can be either secretory-multiple myeloma or non-secretory multiple myeloma. Multiple myeloma is characterized as having at least one of the CRAB symptoms (**Table 4**), extramedullary plasmacytoma, bone marrow clonal PCs ≥10%, and/or ≥60% of bone marrow clonal PCs, serum free light chain ratio of ≥100 mg/L, and the minimum of one focal lesion on magnetic resonance imaging of ≥5 mm in size (24). Non-secretory myeloma is a condition that is seen in about 3% of multiple myeloma patients. These patients do not have any monoclonal protein in the serum or urine. Free monoclonal light

chains in serum can be in approximately 67% of the patients (32, 33). Non secretory multiple myeloma patients are treated the same way as other multiple myeloma patients (11).

The annual incidence of multiple myeloma is 5.6 cases per 100.000 individuals in the western part of the world (31). Median age at diagnosis is approximately 70 years old, and males have a higher incidence rate compared to females, 7.1 versus 4.8 (34).

Table 4 CRAB symptoms

This table shows the end organ damage that is caused by multiple myeloma (11, 24).

Calcium level elevated	s-calcium ≥3 mmol/L (11 mg/dL)
Renal insufficiency	s-creatinine >177 µmol/L or creatinine clearance <40 mL per min
Anemia	Hemoglobin <100 g/L (or >20 g/L below the lower normal limit)
Bone lesion	One or more osteolytic bone lesion, >one focal lesion (≥5 mm)

CRAB = calcium, renal insufficiency, anemia, bone lesions. S = serum

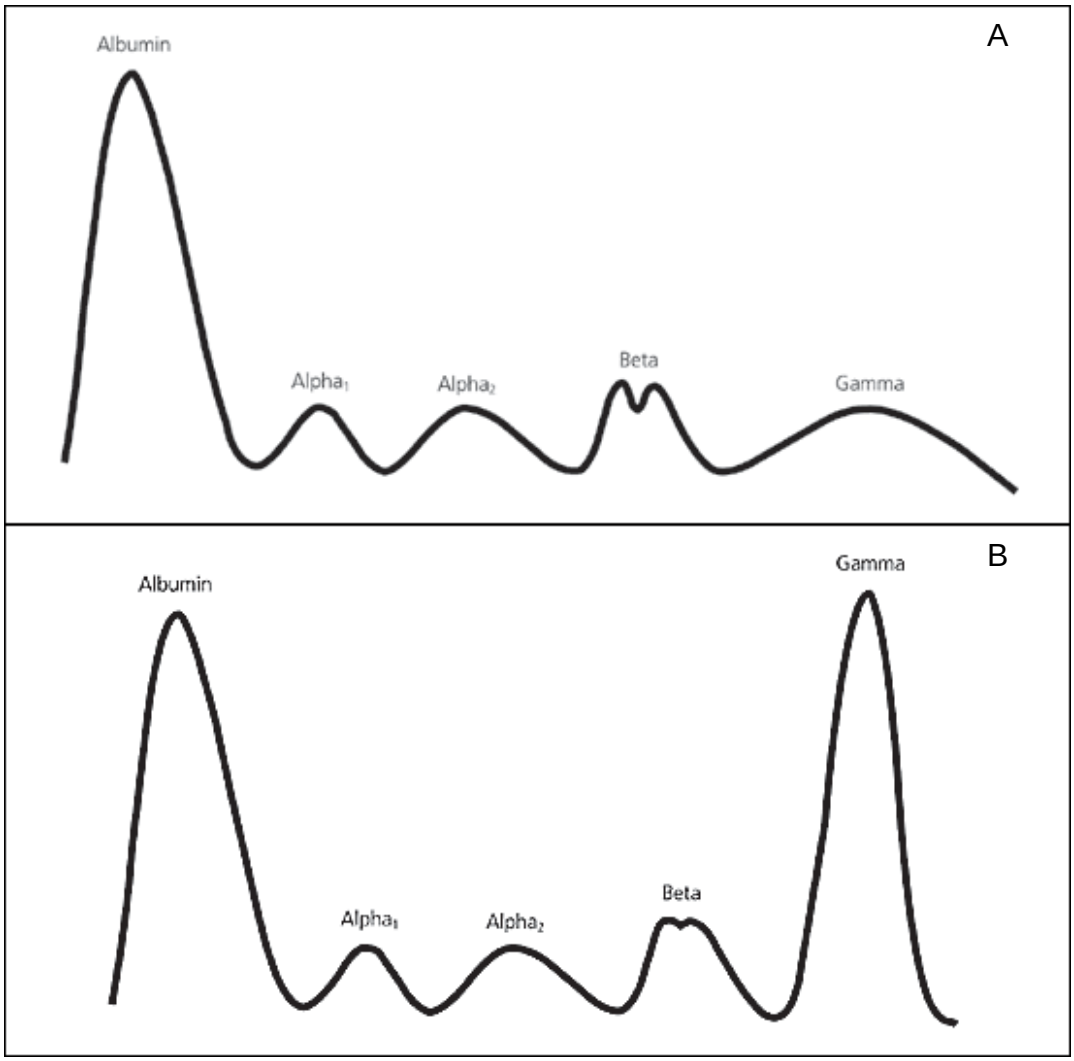

Figure 4 Pattern of serum protein electrophoresis

This figure shows a serum electrophoresis pattern. A) A normal distribution of the serum protein. B) Abnormal serum protein. Multiple myeloma patients show a large spike in the gamma region (35).

1.7 The bone marrow microenvironment in multiple myeloma

Over 80% of multiple myeloma patients are affected by bone lesions, and 40-60% of the patients suffer from fractures during the time of their disease (36). In multiple myeloma patients the osteoclast activity is increased at the bone resorbing surface next to abnormal PCs and osteoblastic formation is inhibited and the bone becomes lytic (37).

The normal bone marrow is mainly controlled by bone forming cells called osteoblasts and the osteoclasts that resorb bone tissue. The mechanism of bone formation and resorption has to be highly controlled. Receptor activator of nuclear factor κB (RANK) is expressed on immature osteoclasts, and its ligand receptor activator of nuclear factor κB ligand (RANKL) is expressed on osteoblasts. Osteoclasts formation occurs when RANKL and RANK bind together.

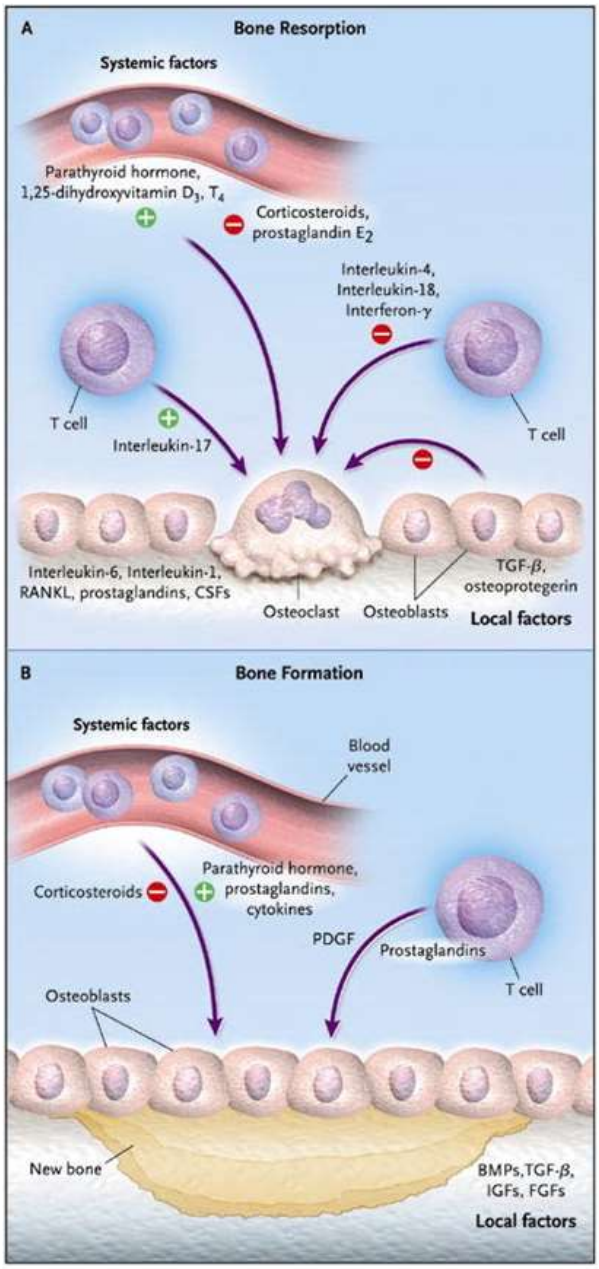

Figure 5 Normal bone formation and resorption

This figure shows the formation and resorption of normal bone. Factors that induce osteoclast activity and formation, are shown with a (+) sign, and factor that inhibit osteoclasts formation are shown with a (–) sign.

A) Osteoclasts are formed and activated by an activator nuclear factor κB ligand (RANKL) on marrow stromal cells and osteoblasts, and by Interleukins (IL-1, and IL-6), colony stimulating factor and prostaglandin produced by osteoblasts. Hormones from the circulation, i.e., 1.25-dihydroxyvitamin D_3, thyroxin (T_4) and parathyroid hormone (PTH) stimulate osteoclasts activity through RANKL. Factors that inhibit the formation of osteoclasts are Interferon γ, IL-18 and IL-4 produced by T cells.

B) Osteoblasts are expressed through stimulation from prostaglandins, cytokines, platelet derived growth factor and PTH. Corticosteroids can prevent bone formation (37).

Thyroxin, 1.25-dihydroxyvitamin D_3, parathyroid hormone (PTH), and hormones from the blood circulation stimulate osteoclast formation and activity through RANKL. Interleukin (IL-6, and IL-1), colony stimulating factors and prostaglandins, produced by osteoblasts also help with formation of osteoclasts. Interferon-γ, IL-18 and IL-4 are cytokines produced by T cells that can prevent osteoclast formation see **Figure** 5 (37).

Osteoprotegerin, a decoy receptor expressed by stromal,- and osteoblastic cells, along with RANK and RANKL are crucial factors in osteoclasts function (38). Osteoprotegerin/RANK/RANKL, maintain normal bone homeostasis (36). RANK is expressed by myeloid precursor cells. When RANKL binds to RANK on myeloid precursor cells, the cells differentiate into osteoclasts. The binding of osteoprotegerin to RANKL inhibits the activation of RANK, which down regulates osteoclast genesis. The soluble form of RANKL can be released from the cells at the tumor site and dispersed to new bone sites where they activate osteoclasts and spread out bone lesions see **Figure 6** (36).

The Wnt signaling pathway is necessary for the growth and function of osteoblasts (39). Dickkopf-1 and frizzle-related protein, are proteins produced by abnormal PCs, and among the factors inhibiting the Wnt-signaling pathway (36). Dickkopf-1 protein binds to Wnt receptors on osteoblasts and disturbs

osteoblastic formation. The expression of RANKL is increased by Dickkopf-1 and the expression of osteoprotegerin is decreased in osteoblasts. The frizzle-related protein also inhibits osteoblastic differentiation by inhibiting the Wnt signaling pathway (36, 39).

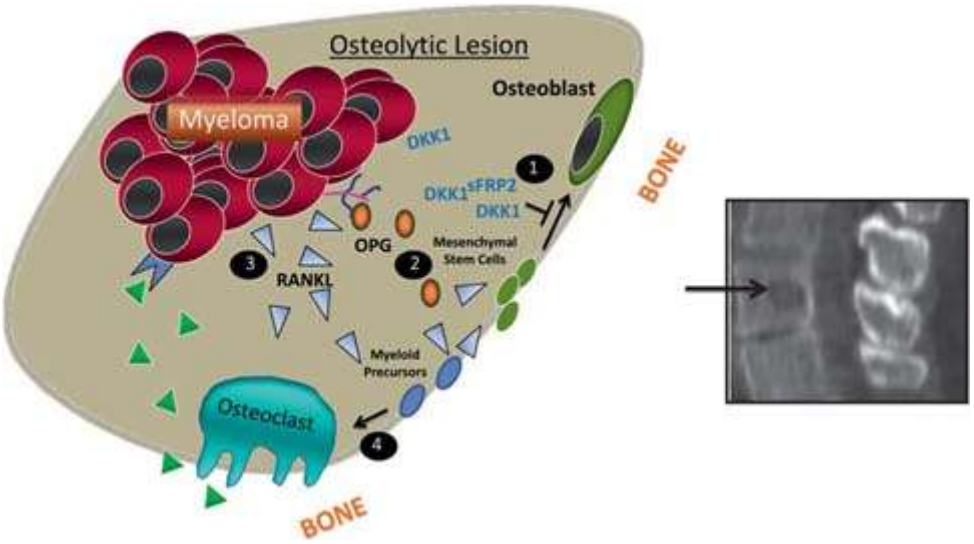

Figure 6 Multiple myeloma bone destruction

This figure shows in a schematic way how bone destruction occurs in multiple myeloma. A) Osteoblast genesis is blocked, because Wnt-signaling pathway has been inhibited by Dickkopf-1 and Frizzle-related proteins. B) The expression of RANKL on bone marrow stromal cells and osteoblasts is increased and osteoprotegerin expression is decreased. CD138 and osteoprotegerin bind together on abnormal PCs and increase osteoclast genesis. C) RANKL is expressed in high levels of abnormal PCs, both soluble and cell surface. D) The differentiation of myeloid precursors into osteoclasts is stimulated by a high level of RANKL in the lesion area. When the osteoclasts become mature they degrade bone and stimulate abnormal PC growth by releasing factors. The result of this is osteolytic lesions at the abnormal PC site which can be detected with an X-ray (36).

RANKL = blue triangles. Osteoprotegerin = orange circles. Factors released by osteoclasts = green triangles. Wnt signaling pathway is a process needed for the bone marrow mesenchymal stem cell to differentiate into osteoblasts. Dickkopf-1 protein prevents Wnt-binding to its receptor with competition. Frizzle-related protein disturbs osteoblast differentiation and inhibits the Wnt pathway.

1.8 Morphology

Bone marrow PCs fraction is critical for classification and optimal clinical management among patients with plasma cell disorder (40). One of the standard and major criteria for quantifying PC infiltration is the morphological evaluation with a microscopy examination of May-Grünwald Giemsa stained bone marrow aspirate and the use of bone marrow biopsy for evaluation of the proportion of bone marrow PC infiltration, used for differentiating between MGUS, multiple myeloma and solitary plasmacytoma, with over 10% of PC infiltration in multiple myeloma. Bone marrow biopsies are thought to give more accurate evaluations of the PC infiltration. These two methods only take into account the proportion of PC infiltration without paying attention to the morphological characterization of the PCs (41).

1.9 Fluorescence activating cell sorting

Fluorescence activated cell sorting (FACS) or flow cytometry technology has broad application possibilities and offers the opportunity of fast and accurate results for many analysis, like microorganisms, DNA/RNA contents, as well as hematological aberrancies (42, 43).

The flow cytometer is mainly composed of fluidics, optics and electronics. In the fluidics, the cells in the sample pass through the flow cell one at a time, and into the cytometer due to pressure difference and eventually the cells end up in the waste tank (44). If a sorting mechanism is included, the cells can be harvested and cultured further (45). Within the optic system the cells pass through a laser beam obtaining the forward scattered light (FSC) and side scattered light (SSC) parameters that reflect their physical properties, i.e., size and characterization of internal complexity (see **Figure 7**).

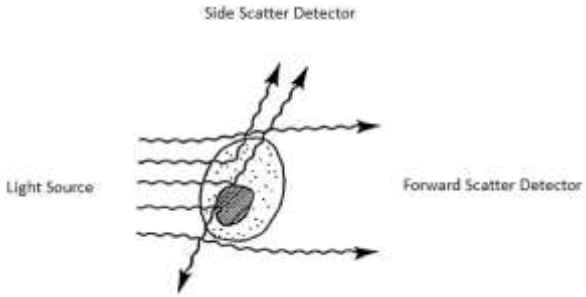

Figure 7 Light scatter properties

This figure shows in a schematic way, a forward scattered (FSC) light, which is proportional to the cell surface, thus giving us the size of the cell. The side scattered light (SSC) is proportional to the internal complexity and gives us information about the granular content of the cell (46).

The FACS Cal flow cytometer from BD Bioscience has two lasers:

- 488 nm blue argon laser
- 635 nm red diode laser which can excite dyes at this wavelength.

- The detectors are FL1 – FL4, also named after their Fluorchrome:
  - FL1 Fluorescein isothyocyanate (FITC) detects green at 515-545 nm
  - FL2 Phycoerythrin (PE) detects orange at 564-606 nm
  - FL3 Peridinin chlorophyll protein (PerCp,) detects red at 653-669 nm
  - FL4 Allophycocyanin (APC) detects red >670 nm

The electronic system collects light from the cells by photodiode detectors and changes their signals from analog signals to electrical pulses or digital signals. The height of each pulse is measured and then it is changed into digital numbers ranging from 0 to 1023 that can be read out on a computer screen (**Figure 8**) (43, 44).

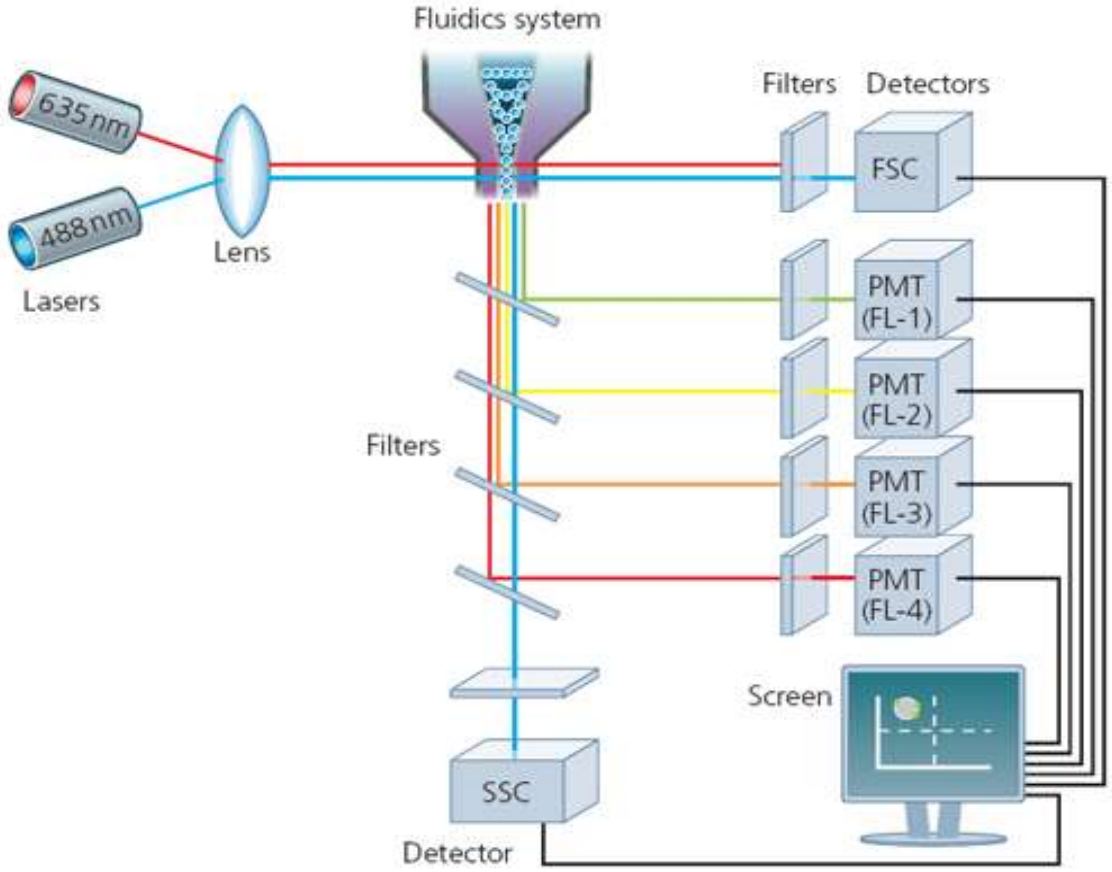

Figure 8 Flow cytometer setup

This figure shows in a schematic way how the 635- and 488 nm laser beams are directed through the lens. The laser beam hits the flowing cells that pass through one by one. The optic system is composed of filters and mirrors that collect the light and direct it to the detectors that change the signals into digital data (47).

1.10 Cluster of differentiation (CD molecules)

CD molecules are antigens on the surface of the leukocytes, also called CD markers. Fluorescence labeled antibodies recognize these cell surface antigens as well as intracellular proteins, and can bind to them. Different fluorescent dyes can be bound to the antibody, yielding a different population of cells either in peripheral blood or bone marrow (42, 48).

CD138 or syndecan-1 is a receptor belonging to the transmembrane heparan sulfate proteoglycan family. Its function is to provide adhesion between cells, and between matrix and cells. Its expression is on epithelial cells as well as normal PCs and abnormal PCs. PCs are the only cells within the hematopoietic system that expresses CD138, thereby making it a specific marker for PC identification. The molecule mediates adhesion of PCs to the bone marrow stromal matrix (41, 49, 50). CD38 is a transmembrane glycoprotein with an enzymatic activity expressed both on hematopoietic and non-hematopoietic cells. What makes it unique is the strong expression on normal PCs which fades out on abnormal PCs, thus making it a “special PC marker” (49). CD19, also called the pan-B-cell marker (49), is expressed on B cells from their early maturation stages (51). The CD19 molecule regulates

intracellular signals (52), and its expression is lost in about 98% of myeloma cases (49). The neural cell adhesion molecule CD56, is expressed on natural killer cells and some T lymphocytes (53). Its expression can be found in several cancers as well as in multiple myeloma (54). It is one of the most important CD markers for identification of abnormal PCs, but weak expression can also be found on normal PCs. CD56 and CD19 alone give great information on the aberrancy of PCs, whereas $CD19^{+}/CD56^{-}$ are normal but $CD19^{-}/CD56^{+}$ would be aberrant (51). CD45 is a common leukocyte antigen found on all nucleated cells in the hematopoietic system. This protein is a transmembrane protein-tyrosine phosphatase that lymphocytes use for their development and activation (49, 55). The expression of CD45 on normal B cells in the bone marrow increases during maturation, and then remains stable (56). Most abnormal PCs lose their CD45 expression (49).

1.11 Immunophenotyping

Immunophenotyping analysis by flow cytometry is one major method for the diagnosis of hematological malignancies as well as minimal residual disease (MRD) analysis (57, 58). The flow cytometry technique has many qualities beyond the microscopy and gives the possibility of using many CD markers at the same time, semi quantitative analysis of fluorescence intensity, high sensitivity and a greater amount of cells analyzed in each sample (41). Information about cell properties like size and internal complexity in bone marrow and peripheral blood can be viewed in flow cytometry by measuring the scatter properties of cells, i.e., FSC and SSC (see **Figure 7**), thus different populations can be seen (59).

One of the most useful tools for differential diagnosis, outcome and prognostic factors of MGUS, smoldering myeloma and multiple myeloma is the immunohistochemical staining and evaluation of the proportion of abnormal and normal PCs (abnormal PC/BMPC) of bone marrow samples. MGUS patients have a higher proportion of normal PCs (8) or >3%, but smoldering myeloma and multiple myeloma patients usually show abnormal PC/BMPC of ≥ 60% (7, 8, 24, 60).

In the bone marrow of MGUS and smoldering myeloma patients, two plasma cell populations assessed by flow cytometry can be identified by gating $SSC/CD38^{+++}$ (strong positive) cells. A normal or polyclonal population and the aberrant monoclonal population can be identified as demonstrated in **Figure 9**. The normal population shows a phenotypic expression of $CD38^{+++}$ cells, $CD56^{-}$, and $CD19^{+}$ and an abnormal phenotype showing $CD38^{+}$, $CD56^{+}$ and $CD19^{-}$ population. The proportion of normal plasma cells is higher in MGUS patients than smoldering myeloma or multiple myeloma patients (57). This can be achieved by using only four to five antibodies against CD markers such as CD38/CD56/CD45/D19 and CD138. The proportion of abnormal cells and the bone marrow plasma cells (abnormal PCs/BMPCs) gives important information about the disease progression (7). Additional CD markers that are considered helpful are for example: CD117/CD81/CD28/CD27 and CD20 (see **Table 5**), as well as both the light chains κ and λ (51, 57). Both normal and abnormal PCs usually express syndecan-1 (CD138). The leukocyte common antigen CD45 is usually missing on abnormal PCs (51), and the pan-B-cell marker CD19 which is expressed on all B cell maturation stages is usually lacking. Yet another usually positive marker and one of the most valuable ones is the natural killer cell marker CD56 (41, 51, 61).

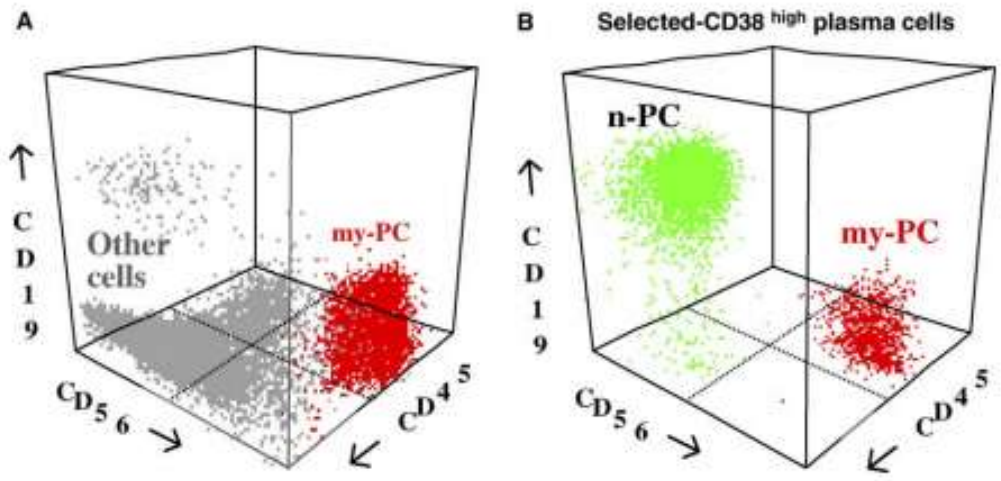

Figure 9 Two populations of PCs, normal PC and abnormal PC.

This figure shows two, three dimensional dot-plots of a bone marrow sample from the same patient diagnosed with multiple myeloma, at diagnosis and after stem cell transplantation. A) The phenotype of the abnormal PCs (red), show $CD38^{+}CD56^{+}CD19^{-}CD45^{-}$ at diagnosis. B) The phenotype of both abnormal PCs (red) and normal PCs (green) after treatment using the same CD markers ($CD38^{+}CD56^{-}CD19^{-/+}CD45^{+)}$. These results are similar to those seen in MGUS patients (41).

1.12 Immunophenotyping in minimal residual disease

Immunophenotyping by flow cytometry with the use of CD56/CD45/CD38 and CD19 has become a very useful and cheap technique in minimal residual disease (MRD) analysis. Patient follow-up during and after treatment gives a rapid way to evaluate whether there are residual malignant cells left in the patient, and is thought to be very efficient. The flow cytometry can detect as few as 0.01% of PCs (7, 41).

Table 5 Useful antigens for PC monitoring

List of necessary surface antigens, as well as some of those recommended as useful antigens for normal PC and abnormal PC monitoring (60, 62).

CD antigen	Normal PC expression	Abnormal PC expression	Required
CD38	yes	yes dim	necessary
CD138	yes	yes	necessary
CD45	yes	no	necessary
CD19	yes	no	necessary
CD56	no	yes, strong pos	necessary
CD117	no	yes	recommended
CD28	weak pos	yes, strong pos	recommended
CD27	yes, strong pos	no, weak/neg	recommended
CD20	no	yes	recommended

dim = dim expression, pos = positive expression, neg = negative expression

2 Aim of this project

The aim of this project is to compare diagnostic protocols for multiple myeloma and its precursor diseases MGUS and smoldering myeloma in bone marrow samples, on bone marrow aspirate smears, in immunohistochemically stained biopsies and by flow cytometry.

The specific tasks of the project are as follows:

1. To examine the proportion between abnormal PCs and normal PCs within the bone marrow PC (BMPCs) compartment (abnormal PCs/BMPCs), obtained by flow cytometry and compare it to a bone marrow aspirate and a bone marrow biopsy.
2. To compare samples analyzed in FACS Calibur (FACS Cal) from Becton & Dickinson, and MACS Quant, from Miltenyi.

3 Material and methods

3.1 Participants

Seventeen patients who were all suspected to have MGUS, smoldering myeloma or multiple myeloma were included in this study. Total of 17 patients, 8 males and 9 females were included in the study. Mean age was 68 years old (median: 70 years), ranging from 40 years to 88 years old (see **Table 9**).

The concentration (**Table 8**) and the type of the serum monoclonal protein from these patients was obtained from the Laboratory information system (LIS) at the core-laboratory at Landspítali University Hospital, Reykjavík (see **Table 9**). The patient samples were given a specific ID number in the LIS at arrival to the laboratory, for personal protection.

Ethical statement: The research in this thesis falls under Research Permit no 14-047 granted to Professor Sigurður Y. Kristinsson in 2014 by the National Bioethics Committee of Iceland.

3.2 Sample collection

A total of 20 bone marrow samples from 17 individuals were collected between March 2013 and July 2014. The samples were taken with a puncture at the posterior iliac crest by physicians (see **Figure 10**) and placed in 4 ml K_3 EDTA (Ethylenediaminetetraaceticacid) anticoagulation tubes from Greiner Bio-one GmbH (4550 Kremsmünster, Austria), and sent to the laboratory. Samples from three of these patients came twice during this period of time.

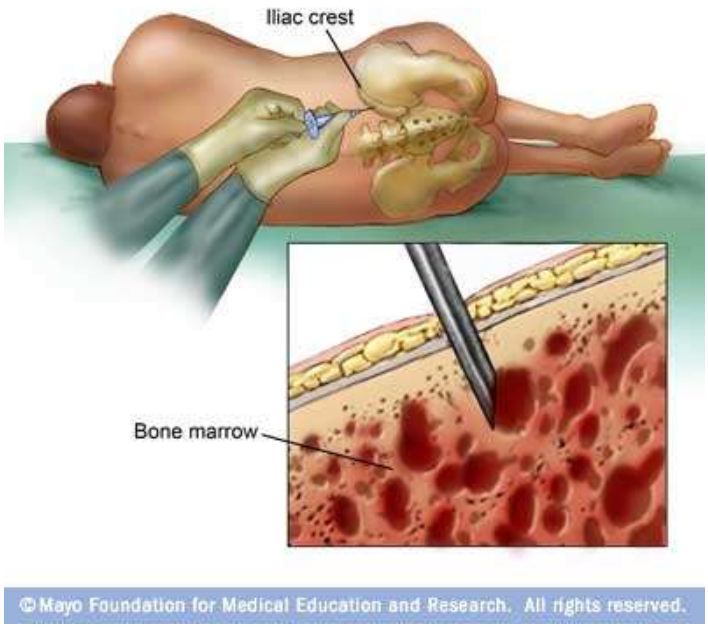

Figure 10 Bone marrow aspiration and biopsy

This figure shows how the bone marrow aspirate and biopsy samples are collected by a puncture at the posterior site of the iliac crest (63).

3.3 Staining procedure

3.3.1 Immunophenotype staining

50 µl of bone marrow samples were placed in four tubes with 5 µl of each monoclonal antibody (see **Table 6**), vortexed, and incubated for 10 minutes in the dark. Lysing of the erythrocytes was done by adding 1 ml of red blood cell lysing solution (BD Bioscience) and incubating for another 10 minutes in the dark. They were then centrifuged at 1300 rpm for 6 min. The supernatant was discarded and the sediment washed by adding 1 ml of washing solution (cell wash, BD Bioscience), vortexed and centrifuged again at 1300 rpm for 6 min. The supernatant was discarded and the sediment re-suspend in 0.4 ml of Cell fix (BD Bioscience) and ready for acquisition.

In cases of surface κ and λ Immunoglobulin light chains, the procedure was the same except the samples were washed twice with cell wash before adding the antibodies to the tube.

3.3.2 May-Grünwald Giemsa staining of aspirates

Bone marrow aspirate samples were processed and stained with May-Grünwald-Giemsa, at the routine core-laboratory at Landspítali University Hospital, Reykjavík. The slides were fixed for morphological evaluation in methanol (SIGMA Aldrich®) for 20 min., then stained in May-Grünwald (SIGMA Aldrich®) for 5 min., in Giemsa (SIGMA Aldrich®) for 15 min., and finally rinsed with pure water. The morphological PC count was obtained by a differential count of total 700 cells in a light microscope (Nikon Eclipse 50i).

3.3.3 Immunohistochemical staining of biopsy sections

The bone marrow biopsy sections were processed and immunohistochemical- and hematoxylin-eosin (H&E) staining performed at the Histopathology Department at Landspítali University Hospital, Reykjavík. The samples were placed in formalin for fixation overnight, then subjected to a zinc chloride fixation for 3-4 hours, decalcified in Decal liquid for 40 min., and finally stained with H&E and CD138 (Dako, clone MI15).

Table 6 Fluorchrome antibodies

Antibody	Fluorchrome	Isotype	Clone	Producer	Specificity
mIgG1	FITC	Mouse $IgG_{1,}k$	x40	BD	no specific binding
mIgG1	PE	Mouse $IgG_{1,}k$	x40	BD	no specific binding
mIgG1	APC	Mouse $IgG_{1,}k$	x40	BD	no specific binding
mIgG1	PerCp	Mouse $IgG_{1,}k$	x40	BD	no specific binding
CD38	APC	Mouse $IgG_{1,}k$	HB7	BD	Exp. of CD38 antigen
CD56	FITC	Mouse $IgG1_{2b,}k$	NCAM-16.2	BD	Exp. of CD56 antigen
CD56	PE	Mouse $IgG1_{2b,}k$	NCAM-16.2	BD	Exp. of CD56 antigen
CD19	PerCp	Mouse $IgG_{1,}k$	4G7	BD	Exp. of CD19 antigen
CD45	PerCp	Mouse $IgG_{1,}k$	2D1	BD	Exp. of CD56 antigen
CD138	FITC	Mouse $IgG_{1,}k$	MI15	BD	Exr. of syndecan-1
*Anti-kappa	FITC	IgG1-k	TB28-2	BD	Exp. of κ-light chain
*Anti-lambda	PE	IgG-λ	1-155-2	BD	Exp. of λ-light chain

*Simultest. Exp = Expression. BD = Becton and Dickinson Bioscience.

3.4 Protocols

In optimizing a protocol for immunophenotyping, and the evaluation of abnormal PC/BMPC for differential diagnosis between MGUS, smoldering myeloma and multiple myeloma (by gating on SSC/CD38 bright expression of CD38, where PCs are included), three different protocols where used (see **Table 7**).

Table 7. Protocols for PC detection

Three different protocols were used. In protocol 1 PerCp-CD19 is in tube 3 with k and λ. In protocol 2 PerCp-CD19 was added to tube 2 and protocol 3 had a different combination of the CD markers. Isotype controls were in separated tubes (not shown).

Protocol-1

Fluorchrome	1	2	3
FITC	CD56	CD138	kappa*
PE		CD56	lambda*
APC	CD38	CD38	CD38
PerCp	CD45		CD19

Protocol-2

Fluorchrome	1	2	3
FITC	CD56	CD138	kappa*
PE		CD56	lambda*
APC	CD38	CD38	CD38
PerCp	CD45	CD19	CD19

Protocol-3

Fluorchrome	1	2	3
FITC	CD138	CD138	kappa*
PE	CD56	CD56	lambda*
APC	CD38	CD38	CD38
PerCp	CD45	CD19	CD45

In protocol 3, CD19 has been exchanged by CD45

3.5 Equipment and software

All the samples were acquired on FACS Cal (Becton & Dickinson, San Jose, California, USA), and MACS Quant® analyzer from Miltenyi Biotec (Bergisch Gladbach, Germany). Total of 1 x 10^4 cells were acquired. Data analysis of the results from FACS Cal was done using Cell Quest™ Pro, the premier acquisition and analysis software, version 6.0 and data analysis of the results from MACS Quant® was done using FlowJo software, version 8.6.6 (FlowJo, LLC, Data analysis software, 385 Willliamson Way, Ashland, OR 97520).

For acquiring images of the aspirate and biopsies, the slides were viewed in a Leica DM LB light microscope connected to a Leixa DFC 310 FX camera and the images were processed with the Leica application suite V3 3.0 software.

3.6 Evaluation of results and gating

For evaluation and phenotypic detection of PCs, a gate was drawn on SSC/CD38 bright expressions of CD38, where PCs are included in a dot plot. To distinguish between positive and negative expression, quadrants were drawn around the negative isotype control to place them in the left corner of the dot plot and the positive expression would then be in the upper left-, upper right- and lower right corner of the dot plot (see **Figure 11**: A, B and C).

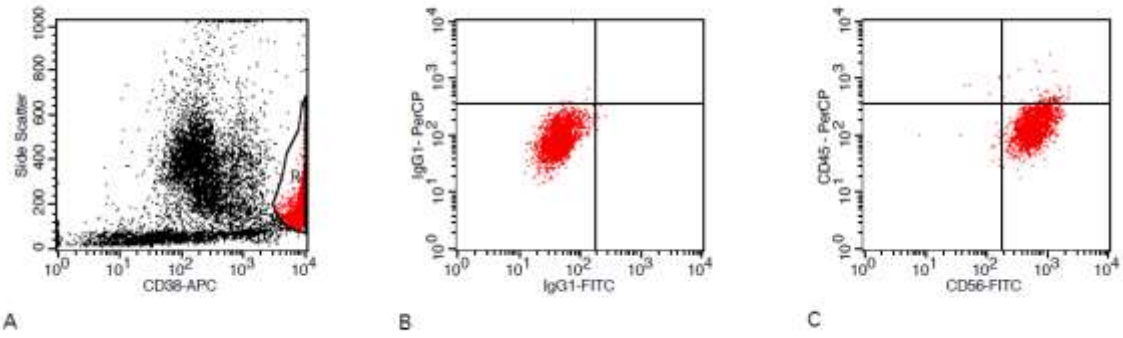

Figure 11 Interpretation of PC gating

This figure shows the interpretation of how the PC gating was done. A) A gate was drawn on the brightest SSC/CD38 expressing cells. (B) Quadrants were drawn to evaluate negative expression of isotype controls and (C) a positive expression of a single antibody.

3.7 Data and statistical analysis

All the results were documented in Microsoft Excel® 2010 (Microsoft Corporation, WA, USA), as well as chart building. Correlation and p value calculation was done using the data analysis tool pack in Microsoft Excel. The calculated p value of less than 0.05 was thought to be significant. Other calculations were mean, median and percentage.

4 Results

4.1 Patient classification

A total of 20 samples from 17 patients were analyzed by immunophenotypic staining procedure, by the count of May-Grünwald Giemsa stained bone marrow aspirate and by evaluation of immunohistochemically stained bone marrow biopsy. The monoclonal concentration is shown in **Table 8**. The mean monoclonal protein concentration was 14 g/L (median: 6 g/L) ranging from 0 to 48 g/L.

Table 8 Participants

Participants are arranged in a numerical order from 1 to 17, their age, sex, monoclonal concentration, sample type and disease definition.

Patient	Sex	Age	Monoclonal protein g/L	Immunoglobulin type	Sample type	Disease definition
1	female	79	1	IgG-κ	Bone marrow	MGUS
2	female	88	27	IgG-κ	Bone marrow	Myeloma
3	male	70	48/5,5	IgG-κ	Bone marrow	Myeloma
4	male	65	10	IgG-λ	Bone marrow	Myeloma
5	female	71	1	IgG-κ	Bone marrow	MGUS
6	female	64	5	IgG-κ	Bone marrow	Myeloma
7	male	40	1	IgG-λ	Bone marrow	MGUS
8	female	83	43,5	IgG-λ	Bone marrow	Myeloma
9	male	80	28	IgM-λ	Bone marrow	Myeloma
10	male	83	0,2	IgM-κ	Bone marrow	MGUS
11	male	55	45/6	IgG-λ	Bone marrow	Myeloma
12	female	73	1/0	Free light chain-λ	Bone marrow	Myeloma
13	female	60	0	None	Bone marrow	Myeloma
14	male	58	0	None	Bone marrow	Myeloma
15	female	57	10	IgG-κ/IgA-κ	Bone marrow	MGUS
16	male	82	17	IgM-κ	Bone marrow	Waldenström
17	female	49	27	IgG-λ	Bone marrow	Myeloma

Two samples were obtained from three patients, marked as 3, 11 and 12.

The characteristics of the immunoglobulin heavy and light chain types from these patients were obtained from the LIS at Landspítali University Hospital in Reykjavík and are shown in **Table 8** and **Table 9**. The most common immunoglobulin type was IgG in 59% cases, followed by IgM in 18% cases and the light chain type was κ in 41%, and λ in 35% cases. One patient had a bi-clonal type of IgG-kappa/IgA-kappa (5%). Two of the patients are without a monoclonal protein and one of them has a monoclonal protein concentration of 1 g/L in the first sample that arrived, and 0 g/L in the second sample, this patient has free λ chains (see **Table 9**).

Table 9 Patients gender, age and immunoglobulin classification

This table shows the proportion of males and females among participants, their mean and median age. The proportion and type of the monoclonal protein, as well as the immunoglobulin light chain type.

Characterization	n = 17
Males (%)/female (%)	8 (47%)/9(53%)
Age, years, mean	68
Age, years, median (range)	70 (40-88)
Myeloma Immunoglobulin type (%)	
IgG	10 (59%)
IgM	3 (18%)
IgL chain type (%)	
Kappa	7 (41%)
Lambda	6 (35%)
Bi-clonal, IgG-κ/IgA-κ	1 (6%)
Free λ chains	1 (6%)
Without monoclonal protein	2 (12%)

4.2 Correlation and calculation

4.2.1 Comparison of correlation

Immunophenotypic staining was performed on EDTA K_3 bone marrow samples and acquired in FACS Cal and MACS Quant flow cytometers by setting the primary gate on SSC/CD38$^+$ cells where PCs are included. The samples were acquired soon after the staining procedure, first in FACS Cal and then in MACS Quant. The results are shown in **Figure 12**. A comparison of the correlation between SSC/CD38$^+$ cells in FACS Cal and the PC count in May-Grünwald Giemsa stained bone marrow aspirates can be viewed in **Figure 13** A and B. In **Figure 14** A and B, the correlation between SSC/CD38$^+$ gated cells in FACS Cal and bone marrow biopsies are shown, and the correlation of bone marrow aspirate and bone marrow biopsy samples can be seen in **Figure 15**.

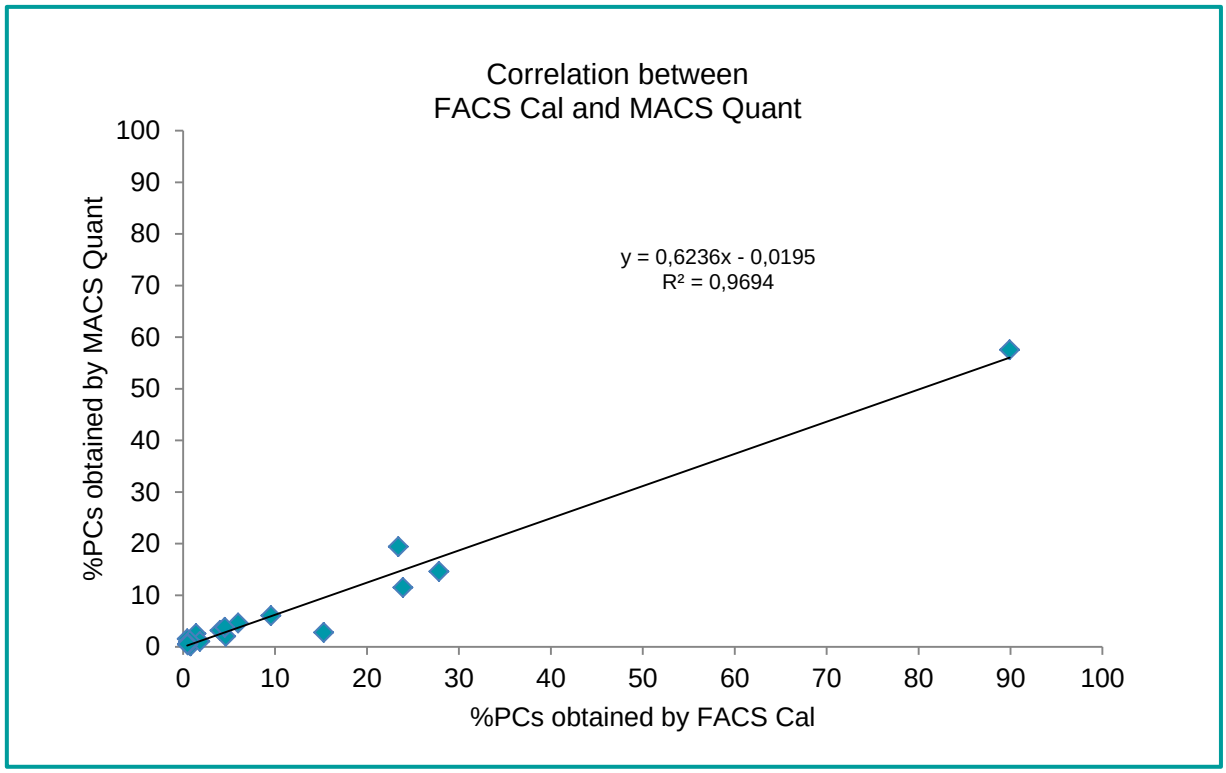

Figure 12 Correlations between FACS Cal and MACS Quant

The results in this figure are based on SSC/CD38$^+$ gated cells obtained by flow cytometry and show the comparison of the correlation between FACS Cal and MACS Quant flow cytometers, after immunophenotypic procedure of bone marrow cells.

The results in **Figure 12**, show that the correlation between FACS Cal and MACS Quant is significantly positive and that there is a very strong linear relationship (R^2 = 0.9694) with a calculated p-value of $4.41 * 10^{-15}$ whereas $p < 0.05$ was thought to be significant.

4.2.2 Correlation between FACS Cal and bone marrow aspirate

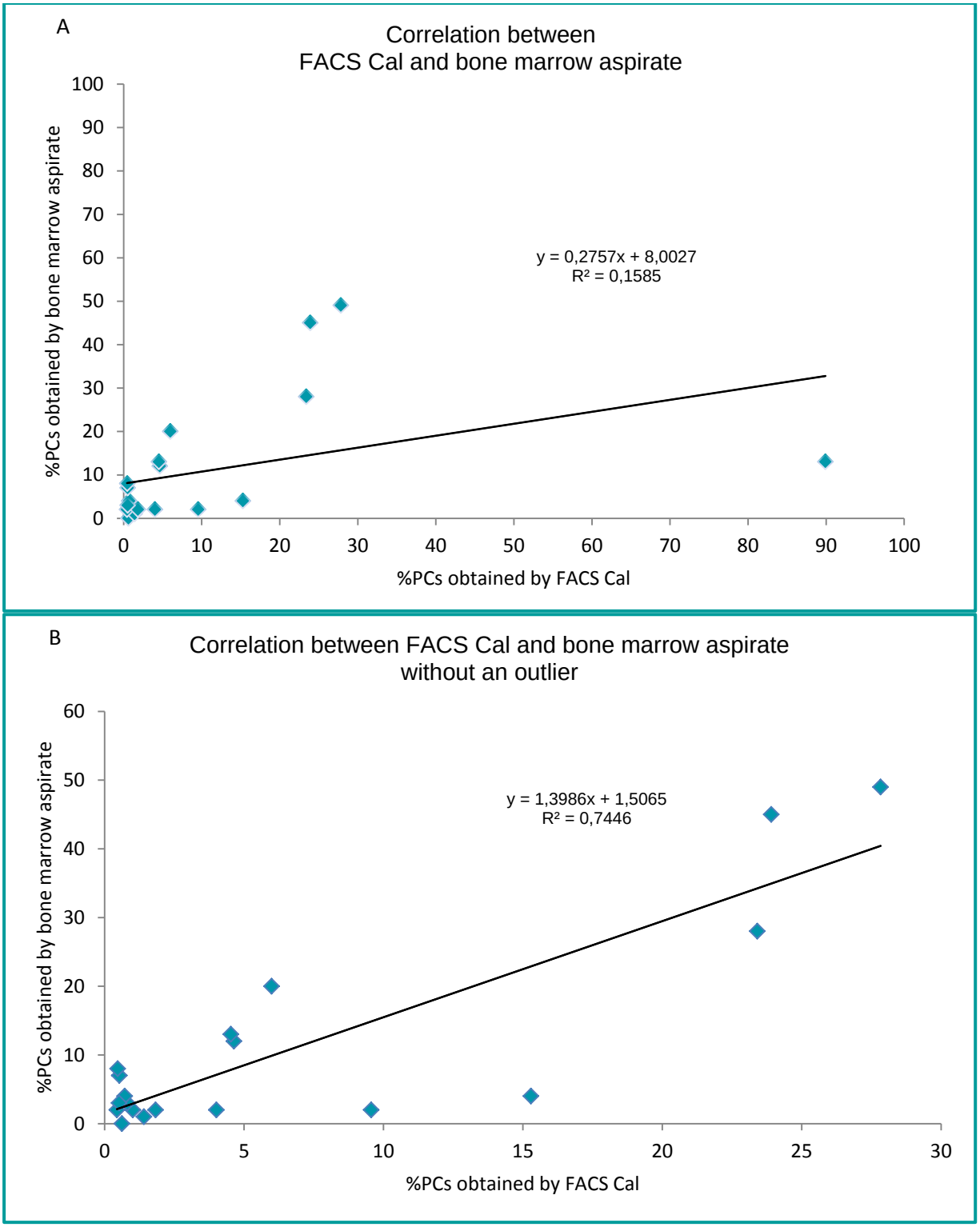

Figure 13 Correlation between FACS Cal and bone marrow aspirate

The results in this figure are based on SSC/CD38^{+} gated cells obtained by FACS Cal flow cytometry, and PC count of May-Grünwald Giemsa stained bone marrow aspirate. A) Shows the correlation between FACS Cal and bone marrow aspirate and figure B) the same results are shown after an outlier has been removed.

The results in **Figure 13** A show the correlation of SSC/CD38^{+} cells between FACS Cal and bone marrow aspirate. These results obtained are insignificant since calculated p-value was 0.082 and $p < 0.05$ was thought to be significant. The calculated R^2 is 0.1585 showing a very weak relationship. These results also show an outlier. After removing the outlier and recalculating the results, the changes of the results can be seen in **Figure 13** B, and the calculated p-value becomes 1.98×10^{-6}, which then becomes significant, and the calculated R^2 strengthens and becomes 0.7446.

4.2.3 Correlation of PCs obtained by FACS Cal and bone marrow biopsy

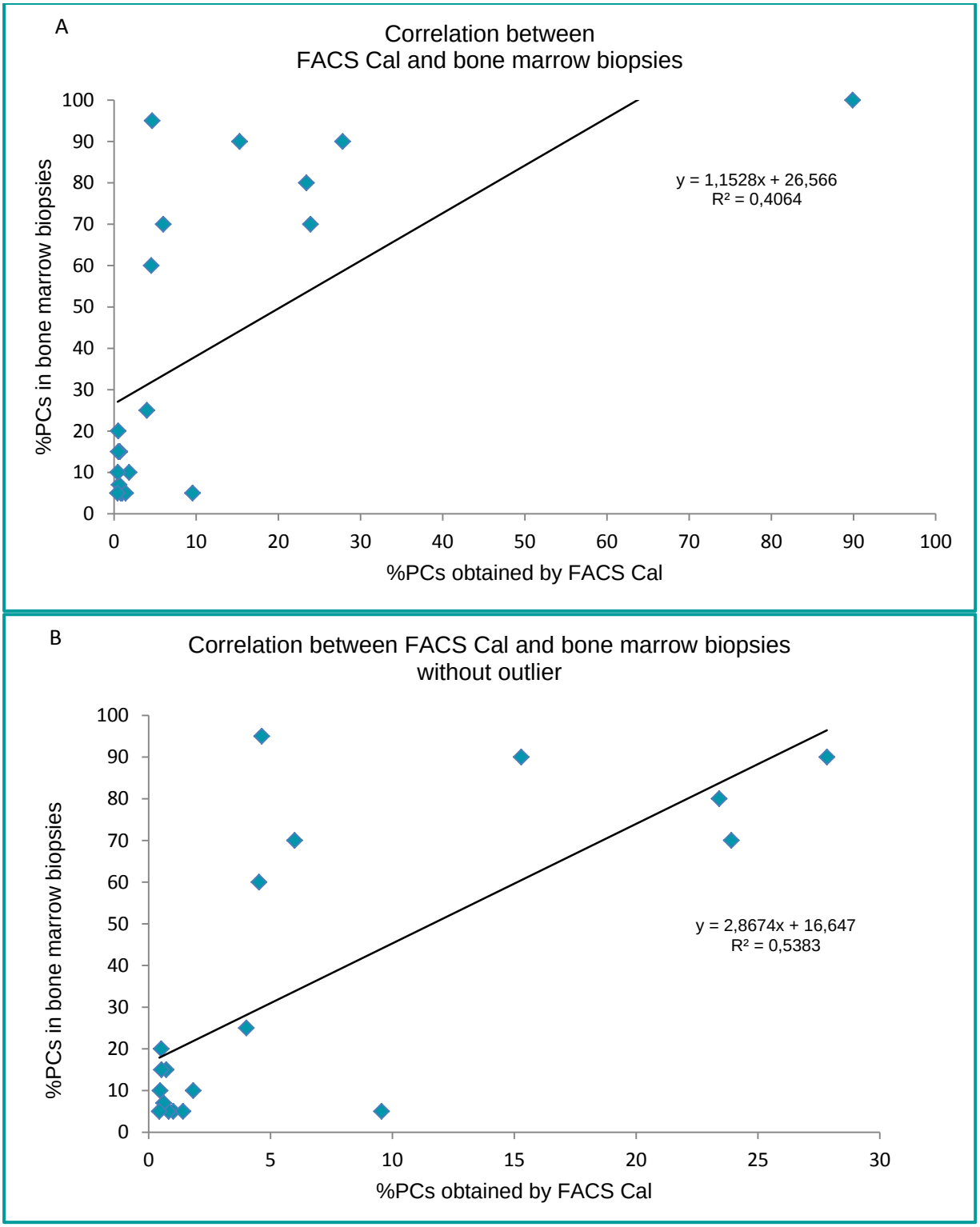

Figure 14 Correlation between FACS Cal and bone marrow biopsies

This figure is based on results of SSC/CD38^{+} gated cells obtained by FACS Cal and evaluation of immunohistochemically stained CD138^{+} cells in bone marrow biopsies. A) Shows the correlation between FACS Cal and bone marrow biopsies and B) shows the same correlation after an outlier has been removed.

The calculated p-value in **Figure 14** A, obtained by FACS Cal and biopsies was 0.003, whereas p <0.05 was thought to be significant. These results are significant, but there is a weak relationship between these two methods (R^2 = 0.4064). PCs obtained by the biopsies are of a much higher percentage than those obtained by FACS Cal. **Figure 14** B, shows the same results after an outlier has been removed. The recalculated p-value is 0.0003. This strengthens the relationship between these two methods and the R^2 rises from 0.4064 to 0.5383.

4.2.4 Correlation between PC count in bone marrow aspirate and biopsy evaluation

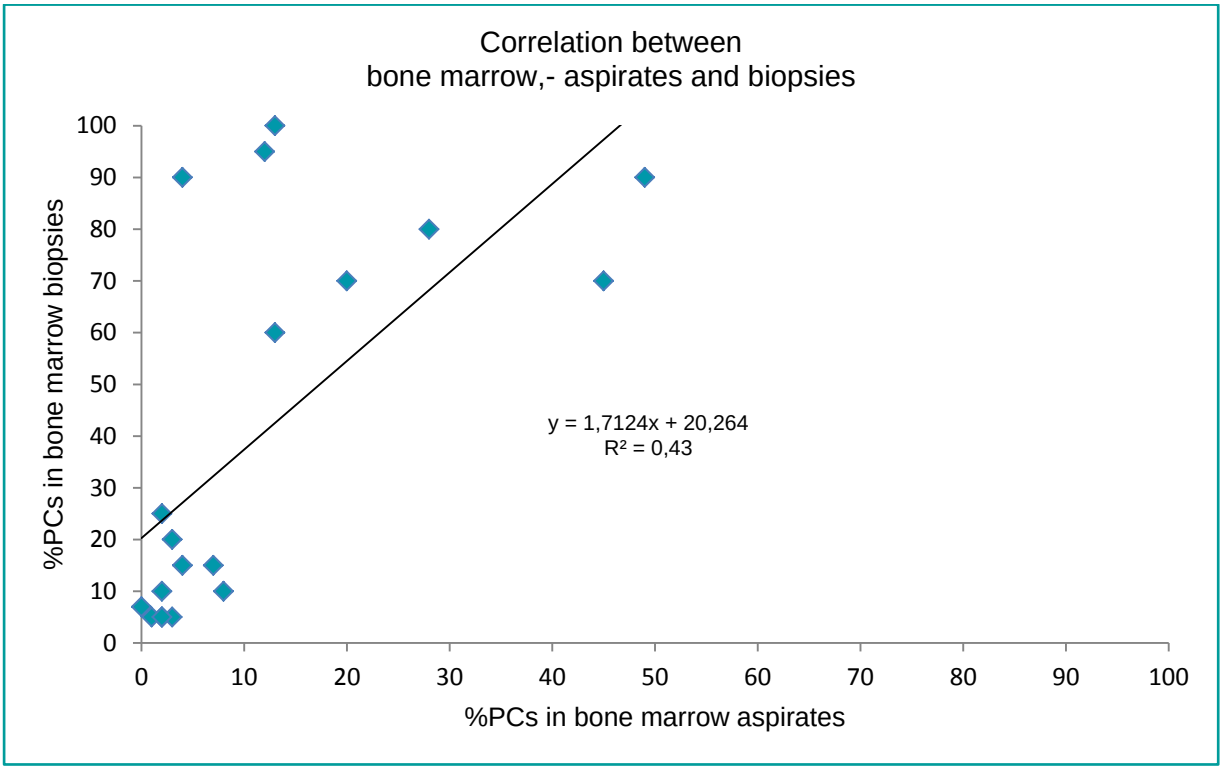

Figure 15 Correlation between bone marrow aspirates and bone marrow biopsies

This figure is based on the percentage of PC count obtained by May-Grünwald Giemsa stained bone marrow aspirates and the percentage evaluated in $CD138^+$ immunohistochemically stained biopsies.

In **Figure 15** the calculated p-value was 0.002 whereas p <0.05 was considered significant. These results show a weak positive relationship (R^2 = 0.43). The percentage of PCs obtained by bone marrow biopsies is much higher than that obtained by bone marrow aspirates.

4.3 Phenotypic interpretation

Table 10 Phenotypic aberration

This table shows the evaluated phenotypic aberration among 20 samples obtained in FACS Cal, by gating SSC/CD38 postive cells. The expression for the CD marker CD56, CD45, and CD19, was evaluated within each quadrant as negative $^{(-)}$, dim, positive $^{(+)}$ or strong $^{(++)}$ expression, the monoclonal concentration and disease definition.

Sample	CD56	CD45	CD19	Monoclonal concentration g/L	Definition
1	++	-/dim	-/dim	1	MGUS
2	++	-/dim	-/dim	27	Myeloma
3	+/++	-/dim	-	48	Myeloma
4	+/++	+/++	-/dim	10	Myeloma
5	-/+	dim/+	-/dim	5,5	Myeloma-MRD
6	+/++	+	-/dim	1	MGUS
7	++	-/dim	-/dim	5	Myeloma
8	-/dim	dim/+	-/dim	1	MGUS
9	+/++	dim	-/dim	43,5	Myeloma
10	++	-/dim	-/dim	28	Myeloma
11	+	-/dim	-/dim	0,2	MGUS
12	+/++	dim/+	-/dim	45	Myeloma
13	+/++	dim/+	-/dim	1	Myeloma
14	-/++	-/dim	dim	0	Myeloma
15	+/++	-/dim	-	0	Myeloma
16	+/++	-/dim	-/dim	10	MGUS
17	+/++	dim/+	dim/+	17	Waldenström
18	+/++	-/dim	-/dim	6	Myeloma MRD
19	+/++	-/dim	-/dim	27	Myeloma
20	+/++	dim/+	dim	0	Myeloma MRD

Colors are drawn around samples indicating samples from the same patient. Number 3 and 5 (red) number 12 and 18 (green) and number 13 and 20 (blue).

Table 10 shows evaluation of SSC/CD38^{+} gated cells based on the expression of three CD markers, i.e., CD56, CD45 and CD19 within each quadrant as negative, dim, positive or strong positive. For almost all the samples, expression of CD56 is $^{+}$ to $^{++}$, except for one sample (sample number 8, see **Table 10**) which shows a negative to dim expression. For the expression of CD45 there are 18 out of 20 samples showing -/dim expression (two samples indicated as no. 4 and 6) show a $^{+}$ to $^{++}$ expression. The expression for CD19 is negative to dim in all cases. The monoclonal concentration of each sample is in g/L, and disease definition according to the monoclonal concentration is in the far right column.

Table 11 Evaluation of PC contents of different methods

This table shows calculated p-values, expressing the pairwise differences between different methods used, i.e., between FACS Cal and bone marrow aspirate, FACS Cal and bone marrow biopsy and finally bone marrow aspirate and bone marrow biopsy. Calculated p-values was done by using Microsoft Excel. The p value p <0.05 was considered to be of significance.

	Correlation p-value
FACS Cal and MACS Quant	$4{,}40 \times 10^{-15}$
*FACS Cal and bone marrow aspirates	0,082
**FACS Cal and bone marrow biopsies	0,002
Bone marrow aspirates and bone marrow biopsies	0,002
*FACS Cal and bone marrow aspirates without an outlier	$1{,}98 \times 10^{-06}$
**FACS Cal and bone marrow biopsies without an outlier	0,00035

* FACS Cal and bone marrow aspirate = indicate measurement before and after the outlier was removed. ** FACS Cal and bone marrow biopsies = indicates measurement before and after the outlier was removed

In **Table 11** the calculated p-values are summarized. The calculated p-value for correlation was significant in all cases except for one. Recalculated p-value after an outlier has been removed, gives a significant calculation.

4.4 Plasma cell count

Plasma cells were obtained with three different methodologies; the immunophenotyping approach, flow cytometry acquisition, by May-Grünwald Giemsa staining of bone marrow aspirates and by immunohistochemical staining of bone marrow biopsy sections where the percentage of PCs was estimated among the bone marrow cell compartment.

The additional column, showing the proportion of abnormal PC/BMPC for four samples, was done by sending the results obtained from FACS Cal to Pamplona, Spain. The samples were analyzed in the laboratory of Centro de Investigación Médica Aplicada (CIMA), by using the Infinicyt™ software and the results can be seen in **Table 12**.

Table 12 Plasma cell count by different methods

The percentage of PCs obtained from 20 samples. SSC/CD38^{+} gated PCs obtained in flow cytometry, counted PCs from bone marrow aspirates and the evaluation of PCs in bone marrow biopsies. The proportion of abnormal PC/BMPC for four samples is also shown, as well as the disease definition.

Sample number	FACS Cal	Bone marrow aspirate	Bone marrow biopsy	Monoclonal concentration g/L	Proportion of abnormal PC/BMPC	Disease definition
1	1.01	2	5	1		MGUS
2	23.91	45	70	27		Myeloma
3	23.41	28	80	48		Myeloma
4	4.01	2	25	10		Myeloma
5	9.56	2	5	5.5		Myeloma-MRD
6	0.82	3	5	1		MGUS
7	0.62	0	7	5		Myeloma
8	1.41	1	5	1		MGUS
9	27.83	49	90	43.5		Myeloma
10	4.64	12	95	28		Myeloma
11	0.44	2	5	0.2		MGUS
12	15.29	4	90	45		Myeloma
13	89.91	13	100	1		Myeloma
14	1.83	2	10	0		Myeloma
15	5.99	20	70	0		Myeloma
16	0.72	4	15	10		MGUS
17	0.53	7	15	17	0,42%BMPC/all normal	Waldenströms macroglobulinaemia
18	0.52	3	20	6	0,46%BMPC/69%abnormal	Myeloma-MRD
19	4.53	13	60	27	6%BMPC/100%abnormal	Myeloma
20	0.47	8	10	0	0,21BMPC/83abnormal	Myeloma-MRD

Colors are drawn around samples indicating samples from the same patient: number 3 and 5 (red), 12 and 18 (green) and number 13 and 20 (blue). BMPC = bone marrow PCs, MRD = minimal residual disease

Table 12 shows PC count by different methods obtained from FACS Cal SSC/CD38^{+} gated cells, bone marrow aspirate and bone marrow biopsy. Also shown is the proportion of abnormal PC/BMPC for four samples, indicated no. 17, 18, 19 and 20, that were analyzed at CIMA, Pamplona, Spain. The last column shows a disease definition for each individual defined by the monoclonal concentration.

4.4.1 Number of PCs between methods

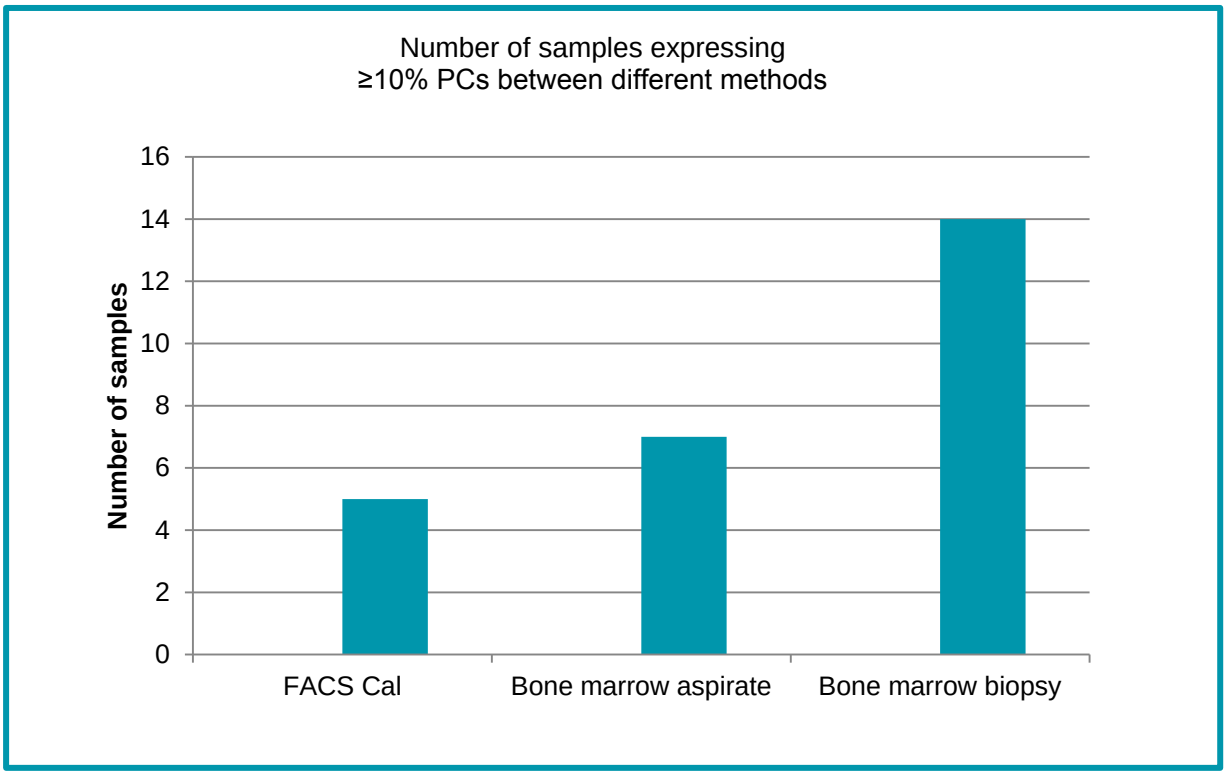

Figure 16 samples expressing ≥10% PCs

This figure shows a histogram of the number of samples that express ≥10% PCs between different methodologies, i.e., FACS Cal, bone marrow aspirate and bone marrow biopsy samples.

Figure 16 shows a histogram of the number of samples that express ≥10% PCs between these different methodologies based on the expression from **Table 12**. The number of samples was found by using a cut of value of ≥10% PCs expressed in each methodology.

4.5 Gated cells

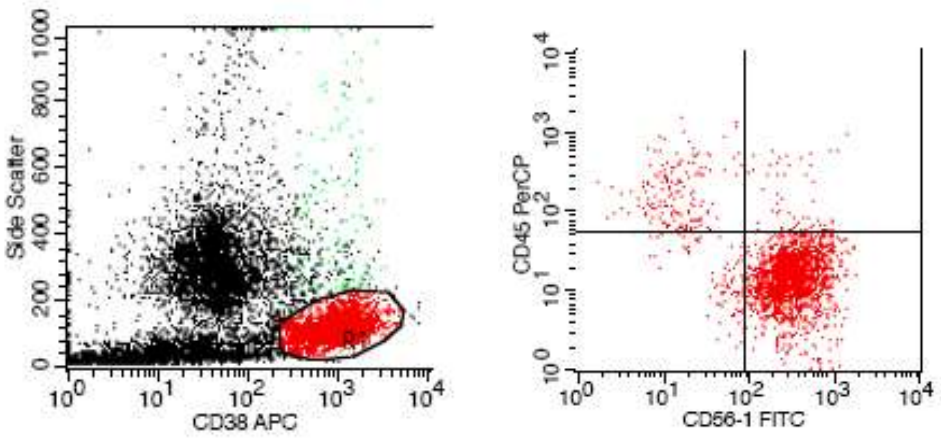

Figure 17 SSC/CD38 gated cells

This figure shows how PCs were obtained by gating SSC/CD38$^+$ cells (left figure), and how the quadrants were drawn to evaluate positive/negative CD markers.

Figure 17 shows how a gate was drawn around the brightest SSC/CD38$^+$ cells (red) where PCs are included. This figure is from patient number 3 and shows that the percentage of gated primary cells was 23.41% (2341 events). The CD138 expression was 18.99% and the expression of CD56 was 21.1%.

4.6 Bone marrow from MGUS patient

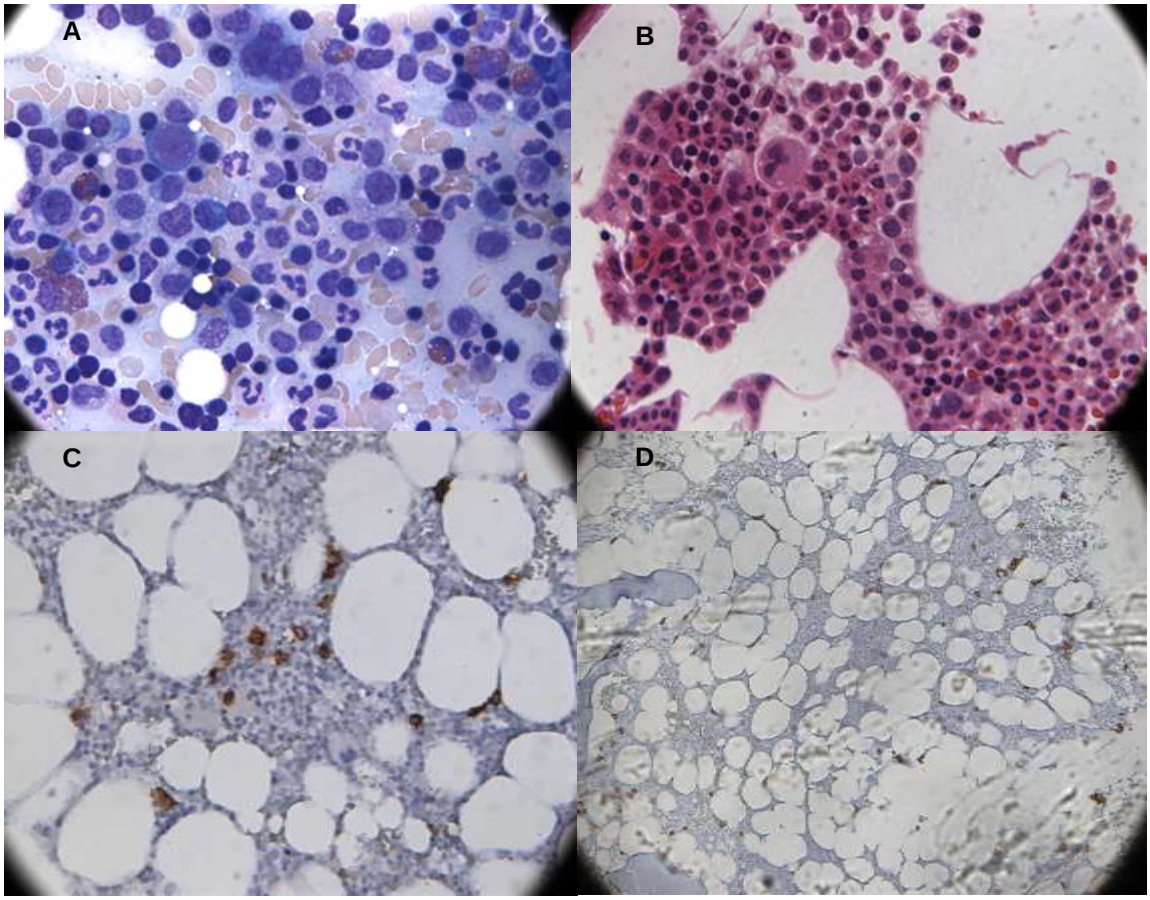

Figure 18 Bone marrow from a MGUS patient

These figures are based on bone marrow from a MGUS patient. Figure A) May-Grünwald Giemsa stained bone marrow aspirate (x 400). B) H&E stained bone marrow biopsy (x 400). C) CD138 stained bone marrow biopsy section (x400), and D) is x100.

Figure 18 shows, four different pictures of bone marrow from a MGUS patient with little PC infiltration, taken in a light microscopy, from the same individual. A) May-Grünwald Giemsa stained bone marrow aspirate (x400), counted percentage of PCs was 1%. B) H&E stained bone marrow biopsy section (x400). C) CD138 stained bone marrow biopsy section (x400) and D) CD138 stained bone marrow biopsy section (x100), estimated bone marrow biopsy section was 5%. SSC/CD38^{+} gated cells obtained by FACS Cal from this same patient was 1.41%. Since there is little PC infiltration, the appearance of the bone marrow is more like normal bone marrow.

4.7 Abnormal bone marrow

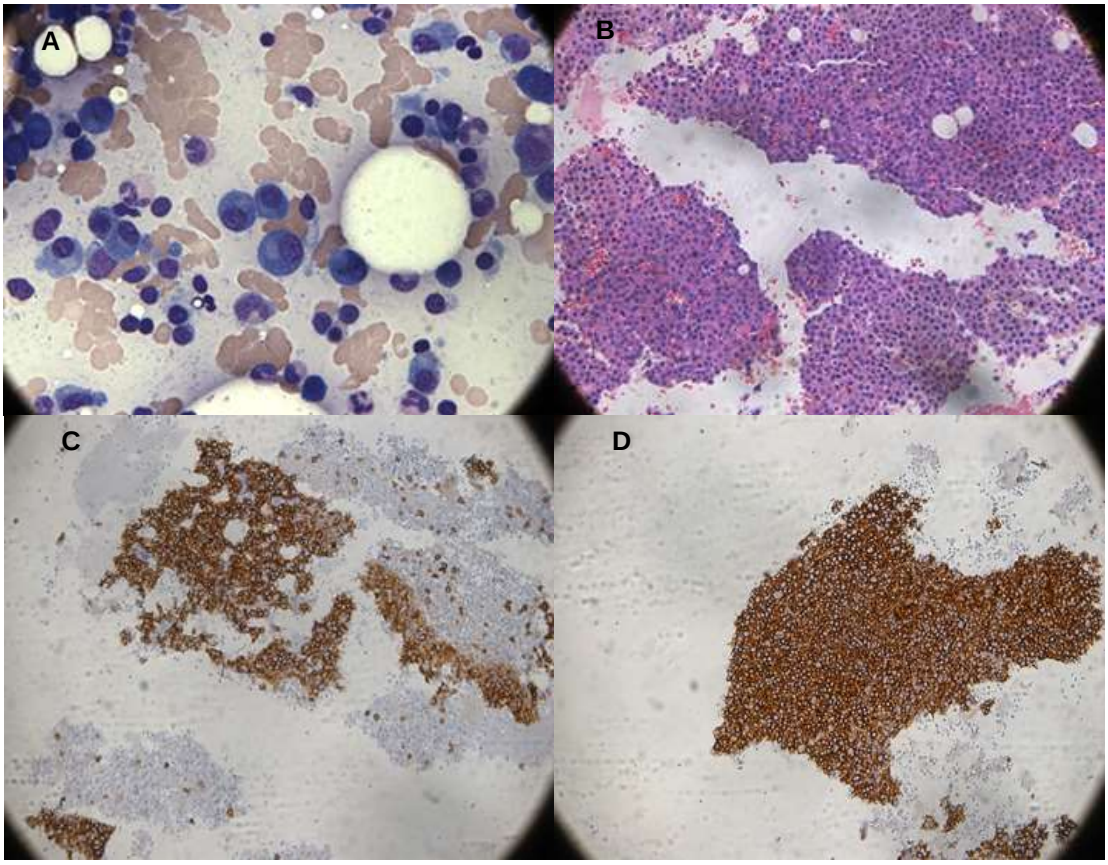

Figure 19 Abnormal bone marrow

This figure shows how PCs accumulate in the bone marrow of a patient with multiple myeloma and how the bone marrow has been taken over by PCs. A) May-Grünwald Giemsa stained bone marrow aspirate (x400) that shows an increased count of PCs. B) H&E stained bone marrow biopsy. C and D) CD138 stained bone marrow biopsy sections.

The cells in **Figure 19** are abnormal bone marrow PCs from a patient that has been diagnosed with multiple myeloma. These figures are taken in a light microscopy. Almost all the cells have been replaced by PCs. A) May-Grünwald Giemsa stained bone marrow aspirate (x400) counted percentage of PCs was 49%. B) H&E bone marrow biopsy (x400). C and D) CD138 bone marrow biopsy section x100, evaluated PCs amount was 90%. SSC/CD38^{+} obtained from FACS Cal was 27.83%.

5 Discussion

The purpose of this project was to compare different methods to analyze the malignant PC diseases, multiple myeloma and its precursor forms, MGUS and smoldering myeloma. Also to compare immunophenotypic protocols in two different flow cytometry equipment. Plasma cells are the functional active B cells and as such they are in the final differentiation stage of the B cell lineage. MGUS is an asymptomatic disorder of proliferative PCs, characterized by a consistent monoclonal protein in serum, accumulation of PCs in the bone marrow as abnormal free light chain ratio or as solitary plasmacytoma and systemic amyloidosis see **Table 2**. Smoldering myeloma is also asymptomatic with the existent of monoclonal protein in serum or urine in patients with IgG or IgA monoclonal gammopathy and a higher proportion of abnormal PCs in the bone marrow. Patients with smoldering myeloma are without symptoms related to the CRAB criteria. Multiple myeloma is characterized by an increased level of bone marrow abnormal PCs, extramedullary plasmacytoma and one or more of the symptoms related to the CRAB criteria mentioned in **Table 4**, or as bone marrow clonal PCs over 60%, abnormal serum free light chain ratio, and more than one focal lesion (**Table 3**).

Immunophenotyping of PC disorder in order to give a better diagnosis and classification has become essential in the last decade with further understanding of its phenotypic aberrancy (8).

5.1 Correlation of flow cytometry equipment

This project started with an immunophenotypic procedure of 20 bone marrow samples from 17 individuals who were all suspected to have MGUS, smoldering myeloma or multiple myeloma. Immunophenotypic staining was done on the samples and then they were acquired in FACS Cal and MACS Quant as described in chapter 3.

The results for the comparison between FACS Cal and MACS Quant showed a highly significant correlation between the equipment as shown in **Figure 12**, calculated correlation p-value is shown in **Table 11**. R^2 shows a strong relationship between the two flow cytometer equipment. These results are up to expectation, and strengthen the opinion of using MACS Quant flow cytometry as backup equipment in routine leukemia analysis.

5.2 Flow cytometry and morphology

5.2.1 FACS Cal, bone marrow aspirate and bone marrow biopsy

The results obtained for FACS Cal and bone marrow aspirate were not significantly correlated (see **Table 11**) for p value calculation and **Figure 13** A for calculated correlation. There is no relationship (R^2) between these two methodologies. It should not be a surprise that the correlation for these samples is insignificant. These results are based on a very few samples (n=20). **Figure 13** A contains an outlier and if it is removed and the data recalculated it becomes significant, and the relationship strengthens as shown in **Figure 13** B. In contrast, the results obtained for FACS Cal and the bone marrow biopsies were significantly correlated see, **Table 11** for calculated p value, and **Figure 14** A for calculated correlation. The relationship (R^2) between these methodologies was stronger, even though it was not very strong. The same trend, as for FACS Cal and bone marrow aspirate was seen

in FACS Cal and bone marrow biopsy, i.e., an outlier. After removing the outlier, the results strengthen as before and the p value is much lower and the relationship between FACS Cal and bone marrow biopsy becomes stronger, see **Figure 14** B.

It is well known that PCs obtained by flow cytometry show markedly lower percentage of PC count than PCs based on slide morphology (8, 64-67). Despite of lower PC count obtained by flow cytometry, the results are thought to yield more reliable information than morphology predicting patient outcome (60, 65). A few things have been mentioned as possible causes for the discrepancy between lesser PCs obtained by flow cytometry than by morphology. Bone marrow aspirate is a “first pull” bone marrow sample used for morphological analysis, but bone marrow samples for immunophenotypic analysis are usually secondary aspirate and contaminated with peripheral blood (60, 67). It has also been speculated that this difference might originate in the characterization and biological properties of the abnormal PCs, and that the abnormal PCs accumulate near the bone surface (64) The biological mechanism of bone destruction is not completely understood but it has been suggested that the cells communicate through a complex molecular pathway that includes cytokines, adhesion molecules, growth factors, chemokines, and receptors particularly RANK, RANKL and osteoprotegerin which are thought to be key factors in regulating osteoclasts activity (38, 68).

Smock et al. performed a study, analyzing 30 cases of bone marrow samples that had ≥10% PCs determined by morphology on bone marrow aspirate and compared to PCs obtained by flow cytometry. The results showed markedly lower PC enumeration obtained by flow cytometry than the percentage of PCs obtained with the bone marrow aspirate (66). Similar results and a significant correlation between flow cytometry and morphology was seen in a study of 765 newly diagnosed patients on a first pull aspirate, stained with May-Grünwald Giemsa where the median percentage of bone marrow PCs obtained by microscopy was 40% (range: 5%-100%) compared to 11% (range: 0.5%-95%) obtained by the flow cytometry (67). Yet another study showed that PCs obtained from flow cytometry was 40% lower than PCs obtained by morphology (64).

5.2.2 Aspirate and biopsy

The calculated correlation results between bone marrow aspirate and bone marrow biopsy were thought to be significant see **Figure 15**, for correlation and **Table 11** for calculated p values. The relationship (R^2) between these two methodologies does not seem to be very strong.

It should be considered that the bone marrow aspirates were counted by a biomedical scientist that is used to blood cell differentiating but has little experience in evaluating bone marrow aspirate slides, and that there is a great difference of PC enumeration between areas under the microscope, whereas some areas were covered with PCs and other areas that had only a few or perhaps no PCs, however the bone marrow biopsy slides were estimated by a pathologist who is experienced in his work. Despite that there was a significant positive correlation, as mentioned earlier. Stifter et al. compared conventional differential count in May-Grünwald Giemsa stained bone marrow aspirate and CD138 stained bone marrow biopsy microscopically and by computer-assisted digital image analysis in a study from 59 patients at Rijeka University Hospital Center (during the years 2001-2008), who had been diagnosed with multiple myeloma. Their results showed greater PC infiltration in CD138 stained

bone marrow biopsy, 50% (ranging from 5%-100%) compared with 29% (ranging from 3%-68%) in bone marrow aspirate assessed by a pathologist, compared to 39% (range 1%-99%) in bone marrow biopsy when using the computer-assisted digital image analysis (40).

5.3 Phenotypic interpretation

Table 10 shows an interpretation of the phenotypic aberration for the samples. All the patients diagnosed with multiple myeloma, have a strong expression of CD56, a dim to negative expression for CD45 and CD19. MGUS patients also show a similar pattern, i.e., a rather strong expression of CD56 and a negative to dim expression of CD45 and CD19.

Similar results were obtained in a study of 500 patients diagnosed with MGUS and smoldering myeloma at the University Hospital of Salamanca, where 50% of the patients showed a strong expression of CD56 and a negative expression of CD45 and CD19. They were also able to identify two groups of patients with phenotypically abnormal PC/BMPC at diagnosis, which enables the prediction of the risk of progression to multiple myeloma among MGUS and smoldering myeloma patients (7).

5.4 Plasma cell count

In **Table 12**, a list of the PCs obtained by different methodologies is presented, the monoclonal concentration in g/L, the proportion of abnormal PC/BMPC for four samples (indicated samples number 17, 18, 19 and 20), and the diseases definition. The discrimination between the two groups of PCs obtained by FACS Cal was very difficult to do, i.e., the proportion of abnormal PC/BMPC.

Samples number 17-20 obtained by FACS Cal were analyzed by the use of the Infinicyt™ software, at CIMA, Pamplona, Spain and show the proportion of total PCs among bone marrow cells and the proportion of abnormal PCs within BMPC. It is interesting to see that sample number 17 has a total of 0.42% BMPC obtained and all of them show a normal phenotype. This patient is known to have lymphoid infiltration and is diagnosed with Waldenströms macroglobulinaemia. This patient is the only patient that showed any sign of CD19 expression as revealed in **Table 10**. The phenotypic expression in Waldenström macroglublinaemia is thought to be more like the phenotype of chronic lymphocytic leukemia, so these patients do not lose the expression of CD19 and CD45 as they do in multiple myeloma. The PC markers CD138 and 38 are not expressed in Waldenströms macroglbulinaemia (69). Sample number 18 is defined as MRD (multiple myeloma patient also number 12), his results show 0.46% BMPC obtained and 69% are considered abnormal PCs. The results in sample number 19 yield 6% BMPCs and all of them show an abnormal phenotype. Sample number 20 is also a MRD patient with 0.21% BMPC obtained, whereas 83% of them show an abnormal phenotype.

5.5 Light chain expression

The expression of surface immunoglobulin light chains κ and λ was calculated for 15 samples. The results showed very little expression (except for one sample, data not shown), the median expression was 0.29% (range: 0.03% - 12.09%). It is interesting to see that patient no. 13 is expressing the most

of surface immunoglobulin k. This is of special interest since the patient has been diagnosed with serum free immunoglobulin λ chains. Cytoplasmic staining would have been more appropriate in this project, since the expression of surface light chains are usually weak in PCs (70). In cases of very few abnormal PCs with a low level of cytoplasmic k and λ light chains it can be difficult to confirm the existence of the light chains as well as with bi-clonal patients expressing both k and λ (8).

5.6 Plasma cell enumeration

In this project the number of cells acquired was 1 x 10^4 cells (except for one sample, which was 5 x 10^5 cells acquired) see chapter 3. This leads to a very low number of cells obtained in some cases as shown in **Table 12**, where there are eight samples that have 101 cells or less obtained. The European myeloma network et al., recommends acquiring the minimum of 100 malignant PCs for a suitable amount of cells to be analyzed, acquired in two steps, and by creating an electronic live gate on the cells desired. This means that 1 x 10^6 cells or more need to be acquired for each sample, which is especially important for MRD analysis that requires at least 20 malignant PC events. This means that the acquisition of 1 x 10^6 cells is recommended (8, 60, 71).

5.7 Expression of $CD38^+$ and $CD138^-$ cells

The dim expression of SSC/$CD38^+$ cells is especially seen in samples from patients who have multiple myeloma. These samples are designated as number 2, 3, 9, 12 and 13 (**Table 10**). But there is a great difference between the amount of SSC/$CD38^+$ cells and $CD138^+$ expression. This difference may be explained by the fact that aberrant PCs lose their bright expression of CD38 and become more like other cells expressing CD38, e.g., precursor B cells and activated T cells. About 80% of multiple myeloma patients show a downregulated CD38 expression (8, 49).

Another reason to consider is the activation of the heparanase enzyme which decreases CD138 expression (8). The heparanase enzyme can be found on a subpopulation of abnormal PCs and in bone marrow PC aspirates among multiple myeloma patients. Heparanase splits up heparan sulfate chains and causes shedding of CD138 expression, this may lead to angiogenesis and metastasis (72-74)

Yet another interesting point that Lin et al. have pointed out in a study of 306 cases of multiple myeloma is that a difference in $CD138^+$ cells can be seen between two lysis methods, where the loss of $CD138^+$ cells were seen when using FACS Lyse compared to NH_4CL (75).

5.8 Different population groups

One of the main goals in this project was to use the flow cytometry to discriminate between normal and abnormal PC populations in order to determine the ratio of abnormal PCs/BMPCs, within the bone marrow. The discrimination between these two PC populations is far from being obvious, and therefore deciding exactly whether a patient is a MGUS,- smoldering myeloma or a multiple myeloma patient is not very clear.

One of the most useful prognostic factors is the proportion of abnormal PCs/BMPC in the bone marrow of MGUS and smoldering myeloma patients (7, 60). MGUS has a different progression rate

depending on the definition of MGUS (see **Table 2**). Smoldering myeloma patients who are diagnosed with >60% abnormal PC/BMPC are considered to have multiple myeloma and should therefore receive treatment for their disease, to delay progression to symptomatic multiple myeloma (24). The ratio can therefore be used as an indicator of the disease's progression (7, 60). The Multiple Myeloma Working Group has suggested a new goal for multiple myeloma patients, i.e., by using MRD analysis, complete response instead of partial response after transplantation, without evidence of the aberrant monoclonal protein in the bone marrow and normalization of the light chain ratio (76).

A group of experts in the fields of flow cytometric and molecular diagnostics called the EuroFlow Consortium has created a fully standardized "all-in-one" pipeline, that consists of reagent panels, sample preparation protocols, instrument settings and a software for disease classification and data analysis (77). For a better discrimination between plasma cells with low SSC values, and granular lymphocytes, a transformed SSC is preferred to SSC (71), which could not be achieved in this project.

5.9 Recommended PC protocols and future perspective

Accurate, fast and sensitive flow cytometry diagnosis of hematological aberrancies have been developed by the EuroFlow Consortium formed in 2005. The EuroFlow Consortium has developed 8 color antibody protocols to be used in flow cytometry with 3 lasers (78). For PC identification, it is recommended to use 12 markers in two tubes, four backbone markers for PC identification and eight markers that show a unique phenotypic pattern. The backbone markers are useful for recognizing both normal and abnormal PCs, these markers are: CD138, CD38, CD45 and CD19. Eight additional markers are CD56, cytoplasmic immunoglobulin light chains, i.e., κ and λ, β_2-micro globulin, and the CD markers: CD117, CD81, CD28 and CD27 (**Table 13**) (79).

Table 13 Useful markers for PC identification

CD markers designed by the Euro Flow consortium for PC identification. The backbone markers identify both normal and abnormal PCs, and should be used in both tubes. Additional CD markers are common abnormal markers and markers to distinguish between clonal cells (79).

	Backbone markers	Tube one	Tube two
	CD38	cytoplasmic Ig-κ	CD117
	CD138	cytoplasmic Ig-λ	CD81
	CD45	CD56	CD28
	CD19	B_2-micro	CD27

Backbone markers are for PC identification and need to be in both the tubes. Common abnormal CD markers: CD138, CD45, CD19, CD56, secondary level markers: CD117, CD81, CD28, CD27, B_2-micro and cytoplasmic κ/λ ratio for clonal classification.

Immunophenotyping by flow cytometry has become a major tool for classification and diagnosis of hematological aberrancies as well as PC disorder, for MRD monitoring and prognostic evaluation in bone marrow samples, peripheral blood and body fluids (80).

For identification of PCs and other hematological malignancies in large panels that show a complex pattern of both abnormal and normal phenotyping the EuroFlow consortium has developed a novel multidimensional software the “Infinicyt” that is able to overcome complexity of immunophenotyping. This software is a multidimensional analyzing tool that recommends the use of at least an 8 color flow cytometry equipment with three lasers (78, 81).

This project has focused on flow cytometry evaluation of bone marrow PCs and the comparison between flow cytometry, bone marrow aspirate and bone marrow biopsy. The discrimination of the proportion between abnormal PCs/BMPCs with the use of flow cytometry, by gating SSC/CD38^{++} cell has not been possible in this project. One of the biggest problems is thought to be due to software disability. Perez-Anders et al., recommend the use of “Transformed SSC” instead of SSC, since the transformed SSC is better capable of discriminating between granular lymphocytes and PCs with low SSC values (71).

6 Conclusion

Immunophenotypic analysis and the use of flow cytometry for characterization, diagnosis and MRD analysis of malignant hematological disease in bone marrow, peripheral blood and other body fluids, has become a very important analyzing method in modern laboratories. Multiple myeloma and its precursor diseases, MGUS and smoldering myeloma are aberrant hematological conditions related to the final product of the B cell proliferation, the PCs. Flow cytometry analysis is a valuable tool for the diagnosis and quantification of PCs in bone marrow samples and patients prediction and outcome.

The aim of this project was to compare protocols for multiple myeloma and its precursor diseases MGUS and smoldering myeloma in bone marrow samples, on bone marrow aspirate, in bone marrow biopsy and flow cytometry. Special and very important tasks were to examine the proportion between abnormal PCs and normal PCs within the BMPC obtained by flow cytometry and to compare it to bone marrow aspirate and bone marrow biopsy, to compare different flow cytometry equipment and to set up a protocol for MGUS, smoldering myeloma and multiple myeloma.

Results showed that much less PCs were obtained by flow cytometry than by bone marrow aspirate and bone marrow biopsy. A good positive correlation and a strong relationship were seen between the two flow cytometry equipment, i.e., FACS Cal and MACS Quant placed at the hematology laboratory at Landspítali University Hospital in Reykjavík. An insignificant weak correlation was seen between FACS Cal and bone marrow aspirate, a weak positive correlation between FACS Cal and bone marrow biopsy as well as bone marrow aspirate and bone marrow biopsy. The attempt to discriminate between abnormal and normal PCs in bone marrow samples and decide the proportion of abnormal PCs/BMPC could not be done, and is thought to be due to software problems.

The use of 8 color flow cytometry is thought to be the minimum equipment required by laboratories for development and quantification of PC burden, as well as for other hematology malignancies. It would be a very important thing to do, to keep up with other laboratories, to set up a protocol for MGUS, smoldering myeloma and multiple myeloma in at least an 8 color flow cytometry application. Such work will have to wait a better time.

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