

EVALUATION OF PROGNOSTIC MARKERS IN PLASMA CELL DYSCRASIAS

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DECLARATION

I, Sandra Havyarimana declare that this thesis is my own work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

21st day of September, 2017

DEDICATION

To the almighty God

Dedicated to my husband David, my children Theana and Ethan, my mother Capy and my sisters Pamela and Lynda.

ABSTRACT

Introduction:

Plasma cell dyscrasias (PCDs) are a heterogeneous group of diseases in which there is expansion of clonal plasma cells which may produce monoclonal immunoglobulins. CD200 is a membrane glycoprotein of the type I immunoglobulin superfamily transmembrane glycoprotein. It is postulated that CD200 is expressed by the plasma cells in a significant number of cases of multiple myeloma (MM), but normal plasma cells do not express it. It may thus assist in confirming clonality of plasma cells in PCDs.

Aims:

To evaluate CD200 expression as a potential diagnostic and prognostic marker in plasma cell dyscrasias and to compare the results with other known prognostic factors.

Methods:

CD200 expression was evaluated by immunophenotypic analysis on normal, reactive and clonal plasma cells and expressed as a mean fluorescent intensity (MFI) value. Cut-off for positive CD200 was established. A negative population was established using different methodologies to exclude reactive plasma cells. CD200 was then compared with diagnostic and prognostic markers currently in use in our laboratory.

Results:

An MFI of ≥ 30 was the cut-off for CD200 positivity. CD200 was expressed by 76.5% of PCDs cases. CD200 did not predict anaemia, hypercalcaemia or renal dysfunction which are biochemical criteria for the diagnosis of multiple myeloma. CD200 expression did not correlate with traditional prognostic markers such as beta 2 microglobulin ($\beta 2M$), lactate dehydrogenase (LDH) or genetic abnormalities (by fluorescence in situ hybridization analysis). Cases with

plasma cell CD200 expression had a higher monoclonal band level compared to those without CD200 expression.

Conclusion:

CD200 is a useful tool to evaluate plasma cell clonality; however in our settings where there is a high prevalence of infectious diseases, particularly HIV infection, CD200 might yield false positive results most likely because of small oligoclonal reactive plasma cells. CD200 as a prognostic tool in PCDs could not be confirmed in this small study.

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LIST OF ABBREVIATIONS

AHSCT: Autologous haemopoietic stem cell transplant

AKT: serine/threonine kinase

APC: Allophycocyanin

AUC: area under the curve

BCL-6: B-cell CLL/lymphoma 6

BJP: Bence-Jones protein

BMA: bone marrow aspirate

BMPCs: bone marrow plasma cells

BRAF: B-Raf proto-oncogene

CCND: cyclin D gene

CD: cluster of differentiation

C-MAF: C v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog

CMJAH: Charlotte Maxeke Johannesburg academic hospital

CRAB: Hypercalcaemia, Renal failure, Anaemia and Osteolytic Bone lesion

CT scan: computed tomography scan

FBC: full blood count

FGFR3: fibroblast growth factor receptor 3

FISH: fluorescence in situ hybridization

FITC: Fluorescein isothiocyanate

FMO: fluorescence-minus-one

HCDs: heavy-chain diseases

HIV: human immunodeficiency virus

Ig: immunoglobulin

IgH: immunoglobulin heavy chain

ISS: international staging system

JAK: janus kinase

LCDD: light-chain deposition disease

LDH: lactate dehydrogenase

LPD: lymphoproliferative disorder

MAF: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog

MAFB: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B

MFI: mean fluorescence intensity

MGUS: monoclonal gammopathy of undetermined significance

MM: multiple myeloma

MMSET: multiple myeloma SET domain

NFκB: nuclear factor κB

NHLS: National Health Laboratory Service

NK cells: natural killer cells

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PCD: plasma cell dyscrasia

PCL: plasma cell leukaemia

PD-1: programmed cell death-1

PD-L1: programmed cell death ligand-1

PE: Phycoerythrin

PerCP: Peridinin chlorophyll protein

PET scan: positron emission tomography scan

PI: proteasome inhibitor

PI3K: phosphatidylinositol 3 kinase

POEMS syndrome: Polyneuropathy, organomegaly, endocrinopathy, monoclonal paraprotein and skin changes associated with plasma cell proliferative disorder commonly MM.

RAS: rat sarcoma

ROC: receiver operator characteristic

SFLC: serum free light chain

SMM: smoldering multiple myeloma

SOP: standard operating procedure

SPEP: serum protein electrophoresis

TNF: tumour necrosis factor

VEGF: vascular endothelial growth factor

WHO: world health organisation

β2M: beta 2 microglobulin

CHAPTER 1: INTRODUCTION TO PLASMA CELL DYSCRASIAS

1.1 Plasma cells

Plasma cells are mature, fully differentiated B lymphocytes. The B cells, from which plasma cells develop, arise in the bone marrow and circulate for a short period of time during which they may encounter the cognate antigen. When naïve B cells encounter antigens that fit their surface immunoglobulin (Ig) receptors, they present this antigen to CD4⁺ T cells within the germinal centre of lymph node follicles. This interaction permits B-cell somatic hypermutation (a process of error-prone DNA replication aimed at improving B-cell receptor affinity for antigen), proliferation and maturation to both memory B-cells and long-lived plasma cells which secrete the B-cell receptors as antibodies [1].

Class switch also takes place within a germinal centre from IgM to IgG, IgE or IgA [2]. The exact mechanisms influencing the fate of the mature B cell are uncertain.

1.2 Plasma cell dyscrasia

Plasma cell dyscrasias (PCDs) are a heterogeneous group of diseases in which there is expansion of clonal plasma cells which may produce monoclonal immunoglobulins. PCDs include:

1. Monoclonal gammopathy of undetermined significance (MGUS).
2. Plasma Cell myeloma (MM). Variants of this PCD include smouldering (asymptomatic) MM (SMM), non-secretory MM or plasma cell leukaemia (PCL).
3. Plasmacytomas which are accumulations of clonal plasma cells which can involve bone or be extraosseous.
4. Immunoglobulin Deposition disorders including deposition of light chains (a type of amyloidosis) and heavy chain disease (HCDs)

1.2.1 Classification of Plasma cell dyscrasia

MGUS and SMM

MGUS is diagnosed when clonal plasma cells are present, but the abnormality does not fulfil criteria for the diagnosis of a more aggressive PCD. It may precede the diagnosis of multiple myeloma (MM), with a risk of progression to MM of 1% per year [2]. MGUS is diagnosed when:

1. A serum monoclonal paraprotein is present at levels below 3 g/dl and there are no more than 10% clonal plasma cells in the bone marrow.
2. There is no evidence of end-organ damage (i.e. CRAB - elevated calcium level, renal failure, anaemia and osteolytic lesions in bone) or Amyloidosis.

For the diagnosis of MGUS both criteria 1 and 2 must be present [2].

Smouldering myeloma may also be a precursor state for MM with a higher risk of progression than MGUS (10% /year in the first 5 years for heavy chain SMM and 5% /year for light-chain disease). SMM is diagnosed when:

1. There is either:
 - a. A serum monoclonal protein present at levels above 3g/dl or
 - b. A urinary monoclonal protein at levels above 500mg in 24 hours or
 - c. More than 10% clonal plasma cells present in the bone marrow
2. There is no evidence of end-organ damage or amyloidosis

For the diagnosis of SMM both criteria 1 and 2 must be present [2].

Multiple myeloma

MM is characterised by proliferation of malignant plasma cells with the production of clonal immunoglobulins (monoclonal paraprotein or M-protein) and end-organ dysfunction (CRAB) [3].

Plasma cell leukaemia

Plasma cell leukaemia (PCL) is diagnosed when circulating malignant plasma cells comprise more than $2 \times 10^9/l$ in total or more than 20% of cells [4]. PCL can arise de novo or be a leukaemic transformation of MM and is characterised by an aggressive clinical course [4].

1.3 Multiple myeloma

MM is a tumour of long-lived plasma cells (see figure 1 below), which secrete monoclonal immunoglobulin. The majority of cases appear to evolve from an underlying MGUS, some via a smouldering state and may transform in the later stages of the disease to a PCL (with significant peripheral blood spill over of clonal plasma cells) [5].

The cause of MM is not well established, however established risk factors include age, african ancestry, male sex, family history of MM or its precursor states [6] as well as infectious, occupational, environmental, genetic and socioeconomic factors[7].

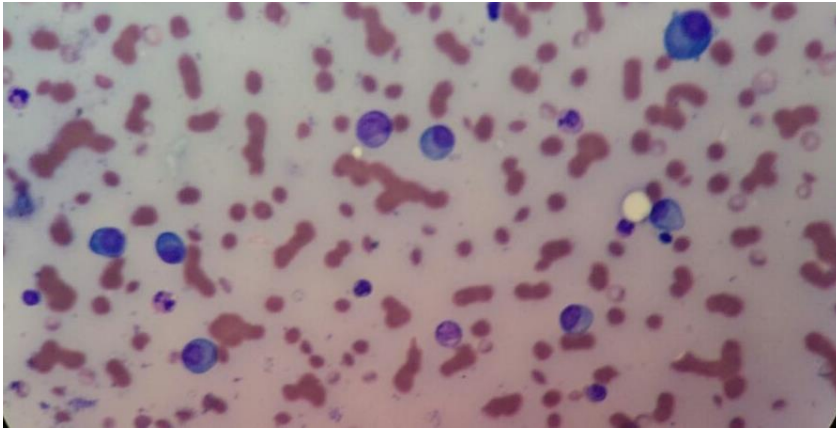


Figure 1: Bone marrow aspirate displaying plasma cells of multiple myeloma. Plasma cells are basophilic cells with an eccentric nucleus and a prominent Golgi apparatus (a perinuclear pale zone)

1.3.1 Epidemiology

Multiple myeloma (MM) accounts for 1% of all cancers and ~13% of haematological malignancies internationally [8]. Worldwide, approximately 86,000 new cases of MM occur annually [9]. The average age at diagnosis is ~70 years in higher income countries and the disease is more common in men than woman [10].

In South Africa, MM is a common haematological disease. There are limited epidemiological data available, but based on local experience the median age at diagnosis appears to be ~5-10 years younger (~60 years) than international data, with more patients diagnosed younger than 40 years of age (Verbal communication by Professor M Patel). True prevalence may be underestimated in South Africa because of limited access to health care and diagnostic services, early death and problems with reporting of data [11]. At diagnosis, patients display a more advanced stage of the disease in the South African setting (Verbal communication by Prof M Patel). In South Africa, approximately 6-6.5% of MM patients are infected with human immunodeficiency virus (HIV) (Verbal communication Prof M Patel). These patients present at a younger age than their HIV negative counterparts (~45 years) and more commonly present with

a plasmacytoma. Immunoparesis is not always evident and M-protein is not always significant (Verbal communication Prof M Patel).

1.3.2 Diagnosis

MM is diagnosed by demonstrating clonal plasma cells, in the presence of end-organ damage [12].

1. Demonstration of plasma cell clonality
 - a. The presence of a monoclonal protein (M-protein) in serum or urine (Bence Jones protein) on protein electrophoresis
 - b. The presence of serum free light chains (SFLC) especially in non-secretory or oligosecretory MM [13].
 - c. Demonstration of clonal plasma cells in the bone marrow (usually $\geq 10\%$ clonal cells by flow cytometry or in-situ hybridization / ISH)
 - d. Biopsy-proven plasmacytoma (6)
2. Myeloma-defining events (indicative of end-organ damage)
 - a. Hypercalcaemia: serum calcium >0.25 mmol/l higher than the upper limit of normal. [3]
 - b. Renal insufficiency: creatinine clearance <40 ml per min or serum creatinine >177 $\mu\text{mol/l}$ [3]
 - c. Anaemia: haemoglobin value of >2 g/dl below the lower limit of normal or haemoglobin value <10 g/dl [3]
 - d. Bone lesions: one or more osteolytic lesions diagnosed on radiography [3].

In the setting of myeloma, a bone marrow aspirate and trephine biopsy is performed for morphology, histology and for immunophenotypic analysis to assess plasma cell clonality. Flow cytometry uses monoclonal antibodies to identify a plasma cell based on the expression

of proteins on their cell membranes (such as CD19, CD38 and CD138). Flow cytometry can also diagnose plasma cell clonality which is demonstrated commonly by light chain restriction or expression of aberrant markers like CD56 [14]. (See Figure 3 in materials and methods demonstrating flow cytometry histograms used in the diagnosis of PCD).

Clonal plasma cells may not express CD56 [15] or light chain restriction and demonstration of clonality may be challenging. In these cases, use of other markers such as CD200 may be helpful in proving clonality [16].

1.3.3 Staging systems

Staging is performed in MM to assess prognosis and may also serve as a guide to therapy. 2 staging systems are commonly used: the Durie-Salmon staging system and the International Staging System (ISS) [13].

Durie-Salmon criteria

This system was developed more than 30 years ago to measure the burden of multiple myeloma disease. The tumor burden is correlated with the patient's clinical, laboratory and radiographic features. Patients are classified as stage I to III according to their haemoglobin, calcium and M protein levels as well as the presence or absence of bone lesions. This system has limitations including the fact that lytic lesions on X-rays, an important feature in Durie-Salmon staging, are observer dependent [13].

International staging system (ISS)

The ISS was developed more than 25 years ago as a more objective staging system. It is easily reproducible and readily available in the laboratory and uses beta 2 microglobulin ($\beta 2M$) as a measure of cellular turnover and albumin levels. Although it has many advantages like the possibility of comparing different clinical trials, it has several limitations such as in MGUS or

SMM where it has no role. The ISS cannot differentiate the latter diseases from MM and it cannot provide a good estimate of tumour burden [13].

Alternative staging systems, in the future, are likely to focus on molecular prognostication markers. These include genetic aberrations as well as biochemical markers which estimate disease burden.

1.3.4 Genetics of MM

MM is a tumour of terminally-differentiated B lymphocytes. During class switch or somatic hypermutation in the germinal centre, the mutations promoting oncogenesis in PCDs may arise. Many of these mutations are also seen in premalignant states and may be necessary but not sufficient for MM to develop [17].

All MM cases are believed to have a baseline of cyclin dysregulation, which may occur through a variety of mechanisms (e.g. translocation t(11;14) involving cyclin D1 or MAF (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog) mutation. MM is divided into 2 distinctive subgroups with different prognoses. In the first subgroup, chromosomal translocations at the level of immunoglobulin (Ig) switch region on chromosome 14q32 (heavy chain locus/ *IgH@*) are thought to be early or driver mutations. These cases are typically non-hyperdiploid and have a poorer prognosis. Several oncogene partners have been described in this group, including cyclin D1 and D3, MM SET domain (MMSET), fibroblast growth factor receptor 3 (FGFR3), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (C-MAF) and MAFB [8].

The other subgroup is characterized by hyperdiploidy and accounts for 50-60% of all cases. Associated with this group, is the trisomy of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21 (the odd number chromosomes), a better prognosis and typically the absence of *IgH@* rearrangements (other than those involving cyclins). See table 1.

Table 1: Comparison of different molecular classifications of multiple myeloma

Group	Translocation	Gene	Effect	% cases	Prognostic value and associated clinical features
Cyclin D Translocation	11q13 6q21 12p13	CCND1 CCND3 CCND2	CCND1 upregulated	15% 2% <1%	Favourable prognosis; bone lesions.
MMSET Translocation	4p16	MMSET/FGFR3	MMSET upregulated, FGFR3 upregulated in 75%,	15%	Unfavourable prognosis with conventional therapy; bone lesions less frequent
MAF Translocation	16q23 20q12 8q24	c-maf mafB mafA		5% 2% <1%	Poor prognosis
Hyperdiploid	D1 D1+D2	CCND1 CCND1+CCND2		33% 7%	More favourable prognosis, Associated with IgG kappa, seen in older patients
Other	None D2	No CCND CCND2		2% 18%	Many subtypes and some with overlap

CCND: Cyclin D gene, MMSET: Multiple myeloma SET domain, MAF: Transcription factor which controls cyclin expression and other genes.

Adapted from Chesi and Bergsagel [18]

Other recurrent genetic changes are well-described and include secondary translocations, acquired mutations and epigenetic changes. These may be secondary events and/or either

involved in the progression of the disease from MGUS to MM or PCL. Important acquired mutations include activating mutations of oncogenes like rat sarcoma (RAS) and the B-Raf proto-oncogene (BRAF) and loss or mutations of tumour suppressor genes such as TP53 (on chromosome 17p) and retinoblastoma 1 (RB 1) on chromosome 13q14 [19]. Other important genetic changes include gain of chromosome 1q (genes are not fully explored but this region contains many candidate oncogenes); loss of chromosome 1p (may contain candidate tumour suppressor genes); activated NF-kappa B pathway (the transcription repressor B-cell CLL/lymphoma 6 (BCL-6) is a proto-oncogene in MM whose expression is significantly upregulated in the context of the bone marrow microenvironment through the IL-6/STAT3, Janus kinase (JAK), and tumor necrosis factor (TNF)- α /canonical nuclear factor (NF)-kB pathways) and deletion of chromosomes 12p, 6q and 8p [19].

1.3.5 Prognosis

Genetic abnormalities in myeloma plasma cells predict tumour aggressiveness. Patients with MM are classified as having high, intermediate, or standard risk disease on the basis of genetic findings [20].

High risk disease

This category includes patients with translocations t (14; 16) and t (14; 20) and deletion of chromosome 17p (17p-). 17p- is aggressive with a shorter overall survival and requires more intensive therapy [20].

Intermediate risk disease

This includes patients with translocation t (4; 14), which was previously thought to be high risk, but the prognosis approaches that of standard risk MM, with appropriate therapy [21].

Standard risk disease

This includes MM patients with no high or intermediate risk genetic abnormalities. The median survival for patients with standard risk MM is currently 8 to 10 years [21].

1.3.6 New biomarkers

Sophisticated tools have been developed to study the bone marrow micro-environment. Several molecules on plasma cells and in the microenvironment have been found to be novel potential targets in the treatment of multiple myeloma. Those molecules expressed on myeloma plasma cells may also be used as markers in the diagnosis of the disease. These include [22]:

CD200:

CD200 is a membrane glycoprotein, which is expressed on endothelial cells, dendritic cells, neurons, B and T lymphocytes. A high proportion of clonal plasma cells express CD200, whilst normal plasma cells are thought not express this marker. [23].

PD-1(programmed cell death 1) and PD-L1 (programmed ligand death 1):

PD-1 is expressed on Natural Killer (NK) cells and interacts with PD-L1 on myeloma cells. This inhibits NK cell function. . PD-L1 is present on the majority of myeloma cells but is rarely expressed in MGUS or on normal plasma cells [24].

CD74 (invariant chain):

CD74 is associated with the major histocompatibility class Ia and β chains. It functions in the transport of these complexes to endosomes and lysosomes and also serves as a receptor for macrophage migration-inhibitor factor. The majority of myeloma cells express CD74 (90% of patients) [25].

CD40:

CD40, a member of the TNF-receptor family, is normally expressed on dendritic, epithelial, endothelial and B cells. It is strongly expressed by myeloma cells and upon interaction of CD40-CD40L, myeloma proliferation and migration take place through the PI3K/AKT/NF- κ B signalling pathway. Myeloma cells are stimulated to adhere to bone marrow stromal cells via stimulation of CD40. This leads to increased production of IL-6 and VEGF [26].

1.3.7 Approach to treatment

In the early 1960s before the introduction of chemotherapy, median survival was ~1 year after diagnosis. Almost 10 years ago in 30% of patients diagnosed before the age of 60 years, a 10-year survival was observed, because of availability of proteasome inhibitors and immunomodulatory agents and introduction of autologous stem cell transplant [27].

Management includes supportive and specific treatment. Supportive measures consist of treating end-organ damage including cytopenias (such as anaemia), hypercalcaemia, neurological signs, bone pain, infections and assuring adequate hydration to improve renal function [8] (Verbal communication by Prof M Patel). Specific treatment includes chemotherapy and transplantation (autologous and allogeneic stem cell transplantation). Management is determined by the age of the patient, the presence of co-morbidities and performance status (see table 2 below).

Table 2: Management of multiple myeloma [8]

Patients	Ideal treatment	Combination chemotherapy	Stem cell transplant
<65 years	Proteasome inhibitor (PI) and immunomodulatory drugs	Cyclophosphamide, vincristine, Adriamycin and dexamethasone	Autologous haemopoietic stem cell transplant
>65 years	Combination containing Melphalan and prednisone	Melphalan, prednisone with bortezomib or thalidomide	Usually not an option
Patients without end-organ damage	Considered to have asymptomatic myeloma, therefore no treatment offered.		

Patients in whom stem cell transplantation is not possible may benefit from assessment of CD200 marker expression, as if this is found to be positive, anti-CD200 may be given as a potential therapy. This is still in trials, but appears promising [22]. In these patients, an accurate and early diagnosis of a plasma cell dyscrasia is paramount.

1.4 Introduction to CD200

1.4.1 Definition

CD200 (or the OX-2 antigen) is a type I immunoglobulin superfamily transmembrane glycoprotein. It is normally expressed by thymocytes, B cells, T cells, dendritic cells, endothelial cells and neurons [28]. The gene locus for CD200 is located at chromosome 3q12. CD200 interacts with the CD200 receptor (CD200R), which is an inhibitory receptor present on myeloid/monocyte lineage cells and certain populations of T cells. It has an immunosuppressive effect [29, 30].

1.4.2 Utility of CD200

CD200, evaluated by immunophenotyping or immunohistochemistry, is expressed by a number of haematological tumours. CD200 is helpful in differentiating between several malignancies such as in the differential diagnosis of the CD19/CD5 co-expressing tumours (i.e. chronic lymphocytic leukaemia where it is expressed as compared to Mantle cell lymphoma where it is reported to be negative)[31]. Approximately 86% of clonal plasma cells express CD200 when analysed by flow cytometry [32], however reactive plasma cells are reportedly CD200 negative. CD200 can thus assist in confirming clonality [32-34].

The expression by clonal plasma cells is variable from weak to strongly positive. CD200 expression is stable in myeloma cells before and after chemotherapy or before and after stem cell transplant [34, 35]. Therefore CD200 can be used for minimal residual disease monitoring [34].

The prognostic function of CD200 is uncertain in myeloma with some studies suggesting absence of expression predicts progression and others that CD200 expression by myeloma cells is associated with inferior survival [32-34]. Myeloma cells which do not express CD200 may

be more susceptible to certain drugs like bortezomib, lenalidomide and thalidomide [36] and CD200 may be a useful indicator for the choice of myeloma treatment [36]. CD200-expressing plasma cells, on the other hand may be susceptible to treatment with anti-CD200 monoclonal antibodies [22]. CD200 expression has not been assessed in most other clonal plasma cell disorders probably because of the scarcity of these entities. CD200 expression in MM has not been assessed in the South African context.

1.5 Aim of the study

This study aims to evaluate CD200 expression as a potential diagnostic and prognostic marker in plasma cell dyscrasias and to compare the results with other known prognostic indicators including ploidy analysis (by flow cytometry or cytogenetic techniques), FISH (fluorescence in situ hybridization) results, cytogenetics, beta 2 microglobulin, serum protein electrophoresis, lactate dehydrogenase, and Bence-Jones protein.

Specific objectives

- To optimize the CD200 assay by immunophenotyping, using controls without evidence of a clonal B cell process.
- To assess CD200 expression in bone marrow aspirate (BMA) samples received for routine flow cytometry analysis where there is no evidence of B-Cell clonality.
- To assess the expression of CD200 in clonal plasma cells in BMA or peripheral blood samples received for flow cytometry.
- To evaluate the expression of CD200 as a diagnostic and prognostic marker compared with other available prognostic tools in plasma cell dyscrasias in the South African setting.
- To describe the epidemiological and pathological profile of PCD in the South African setting.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design

A prospective study to evaluate CD200 expression as a diagnostic and prognostic marker in plasma cell dyscrasias by flow cytometry.

2.1.1 Ethics

The Human research ethics committee (medical) approved the study (clearance certificate number M130278 (please see appendix 2)). Patient confidentiality was maintained throughout the study by allocating a study number to samples.

2.1.2 Funding

Funding was received from the faculty research committee (FRC) as individual research grants in 2013 and 2014 (0012548468101512110500000000000000004990).

2.2 Sample type and target population

Residual bone marrow aspirate (BMA) or peripheral blood material submitted for diagnostic testing was used in this study.

2.2.1 Study inclusion criteria

2.2.1.1 Patients:

All adult patients (>18 years) diagnosed using WHO criteria (2) with a Plasma cell dyscrasia (PCD), were included in the study, provided there was sufficient good-quality material remaining, after routine diagnostic work-up, for additional testing. The diagnosis of PCD was made by flow cytometry, clinical, morphological, radiological and biochemical findings.

The diagnosis of Plasma Cell clonality by flow cytometry included the following criteria [37]:

- a) Aberrant antigen expression (e.g. bright aberrant CD56 expression on plasma cells).

- b) Plasma cells displaying light chain restriction
- c) Aneuploidy of the plasma cell population.

During the period June 2013 to August 2016, 102 appropriate patients were identified.

2.2.1.2 Controls:

All adults, HIV negative or positive, who had routine immunophenotypic screening to exclude a B-Cell Lymphoproliferative disorder (LPD) and who showed no evidence of clonal disease were eligible for inclusion. 27 control samples were included and used to evaluate the antibody and to establish CD200 expression in reactive samples as a baseline for comparison.

2.3 Methods

2.3.1 CD200 optimisation

This study evaluated CD200 expression by flow cytometry. A monoclonal antibody against CD200 conjugated to allophycocyanin (APC) was sourced commercially (BioLegend, San Diego, USA). This reagent was optimised for use in the laboratory.

In order to determine the optimal quantity of reagent, the antibody was titrated according to standard operating protocol (HAE0066). Briefly, control cells were incubated at 5 different volumes of antibody (2,5ul, 5ul, 10ul, 20ul and 40ul). The optimal volume was considered the lowest volume at which the mean fluorescence intensity (MFI) remained stable. In this case, a volume of 10ul was considered optimal (figure 2).

For inclusion in a 3-colour antibody panel, spectral overlap between the 3 conjugates (FITC, PE and APC) was minimised by performing fluorescence-minus-one (FMO) analysis. A control sample was incubated with PE and APC, FITC and PE, FITC and APC monoclonal

antibodies. The removal of a single antibody enabled accurate separation of populations for each of the monoclonal antibody stains. It also enabled accurate separation of positive and negative populations.

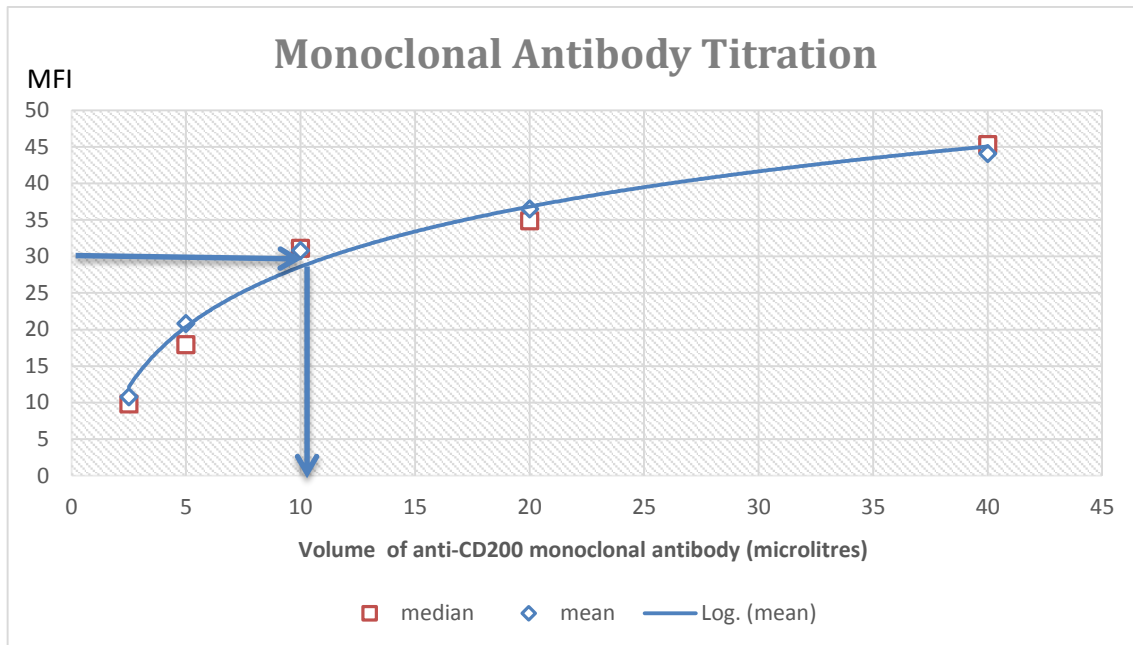


Figure 2: Anti-CD200 titration – this shows an optimal antibody volume for the APC anti-CD200 antibody of 10ul

2.3.2 Sample processing

Samples (patients and controls) were processed as per standard laboratory procedures (SOP number HAE0066). Peripheral blood or bone marrow cells underwent high-density Ficoll/Histopaque (St Louis, Missouri, USA) centrifugation. Sample was layered onto a high-density reagent and then centrifuged at 3500rpm for 15 minutes to enable isolation of a peripheral blood or bone marrow mononuclear cell (PBMC/BMC) population. This concentrates the cell population of interest and prevents unnecessary reagent wastage. If necessary, any remaining red cells were removed by lysing using Ammonium chloride. A pellet of cells was obtained after centrifugation at 3000rpm for 3 minutes. Phosphate -buffered saline (PBS) was used to resuspend the cells at an approximately concentration of 1 million cells per ml (determined by visual inspection of sample turbidity). Pre-titrated monoclonal antibodies were then added to the cell pellet; the sample was mixed by vortexing and then incubated in

the dark for 15 minutes. Unbound monoclonal antibodies were removed by washing the sample in PBS.

A 4 colour FACS Calibur flow cytometer (Becton-Dickinson / BD, San Jose, USA) was used to acquire flow cytometry data, utilizing CellQuest software (BD Bioscience). The Data was analyzed using Paint-a-gate (BD) or Modfit software (Topsham, Maine, USA).

The following antibody panels were used in the study at the discretion of the pathologist performing the routine analysis (please see Table 3 for monoclonal antibody supplier and provenance):

- a) Anti-kappa light chain FITC, Anti-lambda light chain PE, Anti-CD19 PerCP and Anti-CD5 APC
- b) Anti-CD19 FITC, Anti-CD138 PE, Anti-CD45 PerCP and Anti-CD200 APC
- c) Anti-CD38 FITC, Anti-CD56 PE, Anti-CD45 PerCP
- d) Isotype monoclonal antibody controls
- e) Propidium iodide for ploidy analysis

A set screening panel of antibodies was performed to exclude a B lymphoproliferative disorder (B-LPD) in controls (anti-kappa FITC, anti-lambda PE, anti-CD19 PERCP and anti-CD5 APC abbreviated to K/L/19/5) to exclude clonal B-Cells in the sample. A Plasma Cell panel was added to define plasma cells (PC) for assessment of CD200 (i.e. Anti-CD19 FITC, Anti-CD138 PE, Anti-CD45 PerCP and Anti-CD200 APC). Isotypic controls were used to exclude non-specific binding of the monoclonal antibody.

Table 3: Monoclonal Antibodies used in this study

Antibodies	Conjugate	Manufacturer (location)
Anti-Kappa	FITC (Fluorescein isothiocyanate)	Dako (California, USA)

Anti-Lambda	PE (Phycoerythrin)	Dako
Anti-CD19	FITC or PERCP(Peridinin chlorophyll protein)	Becton-Dickinson (San Jose, USA)
Anti-CD5	APC (Allophycocyanin)	Becton-Dickinson
Anti-CD10	APC	Becton-Dickinson
Anti-CD38	FITC	Dako
Anti-CD56	PE	Beckman-Coulter (California, USA)
Anti-CD138	PE	Beckman-Coulter
Anti-CD45	PERCP	Becton-Dickinson
Anti-CD200	APC	BioLegend (San Diego, USA)
Isotopic controls IgG1 κ isotype	APC	BioLegend

2.3.3 Immunophenotypic analysis

CD200 expression was assessed by a single operator on a target population by measurement of Mean Fluorescence Intensity (MFI).

2.3.3.1 Establishing positive cut-offs for CD200 MFI

The use of CD200 to differentiate reactive and clonal plasma cells has been documented in the literature [34]. To determine the diagnostic utility in a South African population, positive and negative cut-off MFI values for CD200 needed to be determined. Initially it was decided to determine the background MFI of CD200 APC on a control population known to be CD200 negative. The control population in this study was reactive plasma cells (defined as no

immunophenotypic evidence of clonal plasma cells). Normal B lymphocytes and plasma cells do not express CD200 [38].

Unfortunately, MFI levels on reactive plasma cells were unreliable. In most individuals, reactive plasma cells are present in low quantities making assessment of the MFI on these cells inaccurate. Where higher levels of plasma cells were present, the patients were often clinically unwell and the MFI range generated in these cells was broad.

To obtain a more reliable negative population, it was decided to utilise 3 methodologies which had been utilised in previous studies to establish a negative MFI value:

1. The MFI of an APC isotypic control on clonal plasma cells [32]
2. The MFI of CD5 on clonal plasma cells (CD5 is a T-cell specific marker included routinely in panels to assess B cell clonality and is measured on APC)
3. The autofluorescence of APC in plasma cells [39]

Using these 3 methods, samples with an MFI of >30 were considered positive, those with an MFI of <20 were negative and samples with a MFI between 20 and 30 were considered indeterminate as previously described [39].

2.3.3.2 Immunophenotypic identification of plasma cells

Plasma cells were identified by Paint-a-gate software. Plasma cells were identified initially by their light scatter characteristics as large cells with high internal complexity (figure 3A). These cells express high levels of CD138 and may express the pan-B cell marker CD19 (figure 3B). Clonality is identified as expression of aberrant CD56 (figure 3C) or light chain restriction (only kappa or lambda light chain expression – figure 3D)

The anti-CD38, anti-CD56 and anti-CD45 plot is used to assess clonality of the plasma cells by virtue of aberrant CD56 expression (figure 3C).

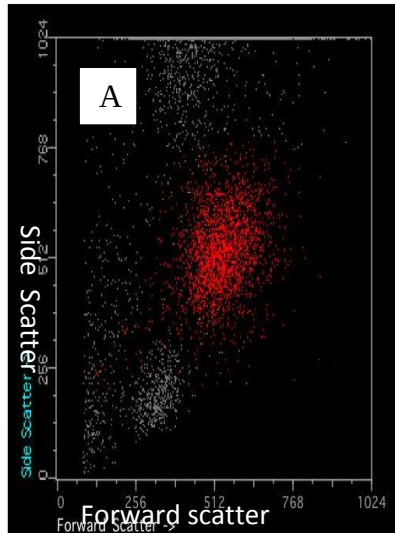


Fig 3A: Forward versus side scatter plot. Plasma cells are large with high internal complexity (red population)

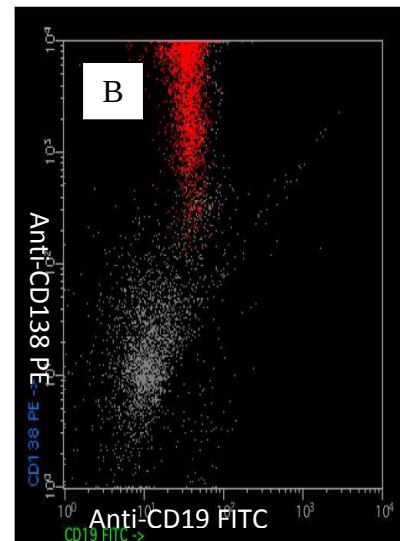


Fig 3B: Anti-CD19 (FITC) versus anti-CD138 (PE). Plasma cells show bright CD138 expression with variable CD19 expression (red population)

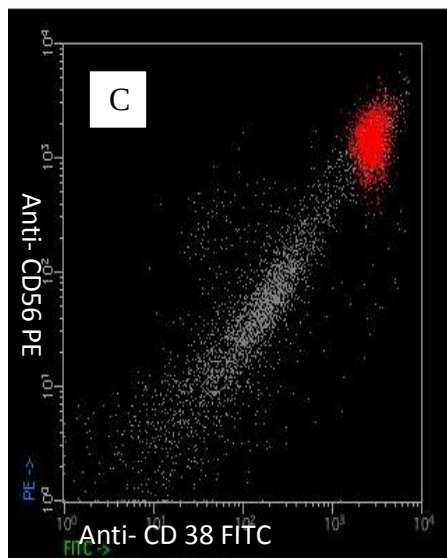


Fig 3C: Anti-CD38 (FITC) versus anti-CD56 (PE). Plasma cells express bright CD38. CD56 is an aberrant marker (red population)

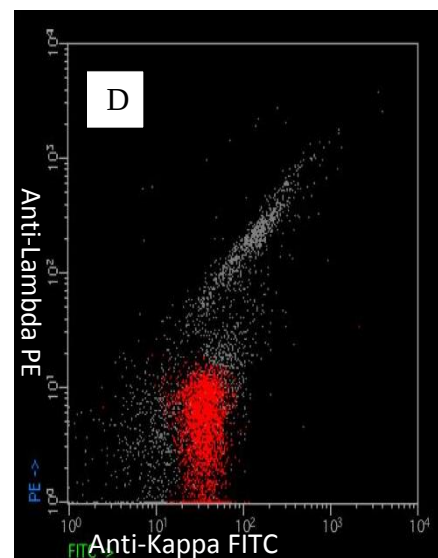


Fig 3D: Anti-kappa (FITC) versus anti-lambda (PE) light chains. Malignant Plasma cells show light chain restriction (red population)

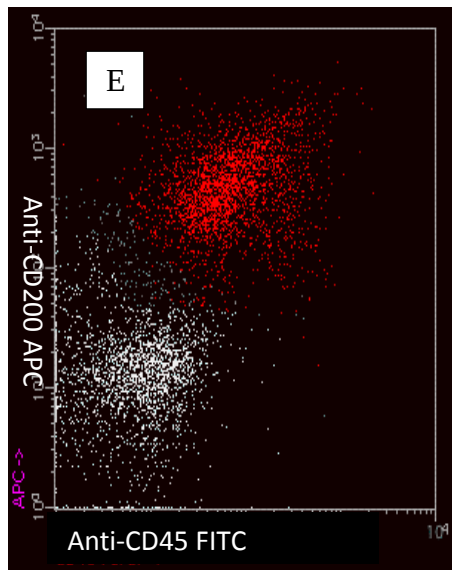


Fig 3E: Anti-CD45 (PERCP) versus anti-CD200 (APC) light chains. Malignant Plasma cells show CD200 expression with an MFI of >60 (red population)

Figure 3: Diagnosis of Multiple myeloma, flow cytometry histograms.

Plasma cells are large cells with high internal complexity (A) and express bright CD138 (B) and CD38 (C). Clonal plasma cells may show aberrant CD56 expression (C) and /or monoclonal light chains expression (in this case kappa light chains – D)

2.4 Data collection

Patient data was collected from the Laboratory information system (TRAKCARE and DISA) as per the data collection sheet (Appendix 1) and a database was created. Patient epidemiological information collected included gender, race and age.

Clonal plasma cells with production of monoclonal proteins (M-band) and end-organ damage must be present in order to make the diagnosis of multiple myeloma [3]. Plasma cell clonality and numbers were assessed on the bone marrow aspirate and trephine biopsy and by immunophenotyping. M-band and monoclonal gammopathy were assessed by serum protein electrophoresis and confirmed by the presence of clonal serum free light chains or urinary Bence-Jones proteins which constitute clonal immunoglobulin light chains.

In addition to diagnostic information, the following clinical and prognostic data were collected (where available)

- a. A full blood count (FBC),
- b. Cytogenetics (CG/ karyotyping): This is used to detect numerical and/or structural chromosome abnormalities in metaphase cells,
- c. Fluorescence in situ hybridization (FISH): Fluorescently labeled oligonucleotide probes are used to detect numerical and/or structural genetic defects. A number of cytogenetic abnormalities have been linked to prognosis and treatment response in myeloma [21]. Risk stratification on FISH analysis are as follows:
 1. High and intermediate risk genetic defects: t (14;16), t (14;20), del 17p13, t (4;14), 13q- or monosomy 13, gain of 1q and hypodiploidy.
 2. Standard risk genetic defects: t(11;14) and all patients who lack high or intermediate risk genetic abnormalities. Hyperdiploidy is classified as a standard risk [20, 40].
- d. Beta2 microglobulin (β 2M): This is a protein which is a component of the major histocompatibility class I and acts as an indicator of tumour volume in multiple myeloma in which significantly elevated levels can be found [41]
- e. HIV status.
- f. Lactate dehydrogenase (LDH): This is a marker of cellular damage and serves as an indicator of prognosis [42].
- g. Calcium: Multiple myeloma (MM) causes bone loss and the breakdown of bone in MM can lead to hypercalcaemia (from calcium stored in bones).

See appendix 1 for data collected.

2.5 Statistics

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Baseline demographic and laboratory characteristics, for continuous variables, were described using mean, median and range. For categorical variables, frequencies and percentages were used.

Continuous data were assessed by a Mann-Whitney *U* test. Receiver operator curves (ROC) – *XL STAT*; Add-in for Microsoft Excel (Addinsoft SARL, NY, USA.) were used to assess the value of CD200 in differentiating reactive and clonal plasma cells. Linear regression analysis was utilized to correlate CD200 expression with other prognostic markers and ANOVA was used with more than 2 parameters. Statistical significance was defined as a two-sided P value of <0.05 [43].

CHAPTER 3: RESULTS

3.1 Epidemiology

Plasma cell dyscrasia (PCD) patients were sourced from cases received by the flow cytometry department at Charlotte Maxeke Johannesburg academic hospital (CMJAH). During the time period June 2013 to August 2016, 102 appropriate patients were identified. Of these, Multiple Myeloma was diagnosed in 101 patients and Plasma Cell Leukaemia was diagnosed in one patient.

Flow cytometry demonstrated the clonality of plasma cells: aberrant CD56 expression was present in 94 patients (85%) and absent in 8 patients (15%). Light chain restriction was documented with SPEP in 75 patients: 48 (64%) had kappa and 27 (36%) lambda light chain restriction.

The male to female ratio was 1:1.5 in this cohort. Overall, the median age for patients was ~57.5 years (range: 31-86 years). The age at presentation was not statistically different between males and females (57 years and 59 years respectively, $p=0.40654$). See table 4.

Table 4: Epidemiological findings in myeloma patients

	Median age (range) years [n/N (%)]	Documented HIV status n/N (%)	HIV positive n/N (%)
All patients	57.5 (31-86)	80/102 (78%)	20/80 (25%)
Males	57 (31-81) [41/102 (40%)]	32/41 (78%)	8/32 (25%)
Females	59 (35-86) [62/102 (61%)]	48/62 (77%)	12/48 (25%)
HIV negative patients	60 (30-77) ¹ [59/79 (75%)]		
HIV positive patients	52 (35-68) ¹ [20/79 (25%)]		

¹ p-value: 0.00596

Only 80/102 (78%) of patients were tested for HIV infection. Of these, 20 (25%) were HIV positive and 1 patient had an equivocal test result. HIV positive patients presented at a statistically significant younger age than their HIV negative counterparts (p-value: 0.00596). Please see table 4.

During the same time period 27 controls were included in the study. The median age for this group was 40 years (range 21-64 years). 25/27 controls (93%) had an HIV test. Among these 23/25 (92%) were HIV positive.

3.2 CD200

The primary aim of the current study was to assess the diagnostic value of CD200 i.e. its ability to distinguish between reactive and clonal plasma cells in the diagnosis of plasma cell dyscrasias in the South African setting. A secondary aim was to assess if CD200 correlates with other prognostic markers in PCD.

3.2.1 CD200 in the diagnosis of PCD

The median CD200 MFI expression in plasma cells in patient samples with plasma cell dyscrasias was 129.5 (range: 1.54-3837; mean: 272.4). This is significantly higher than the MFI values for CD200 documented in the plasma cells in control samples: median MFI 24.37 (range: 1.2- 76.1; mean: 31.25) (p-value <0.0001). See Figure 4.

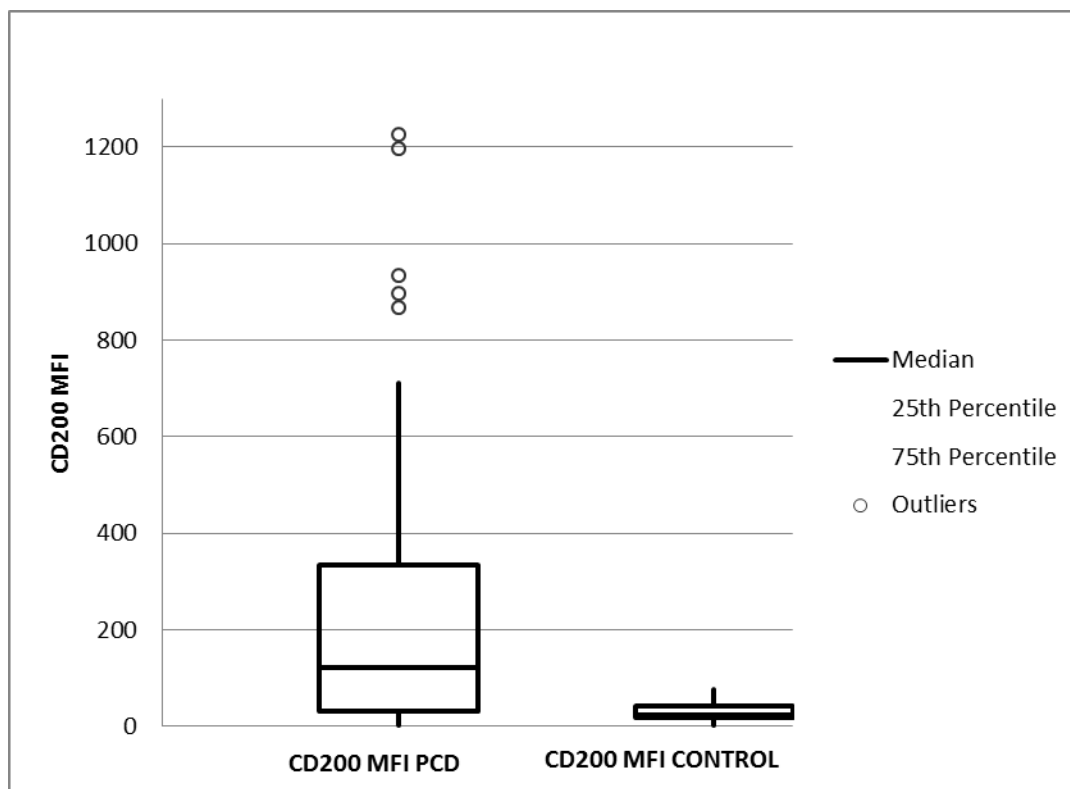


Figure 4: Box plot comparing CD200 MFI in PCD and control samples

(p-value < 0.0001).

MFI: mean fluorescence intensity; PCD: plasma cell dyscrasia

Extreme outliers have been excluded.

A receiver operating characteristic (ROC) curve performed showed an area under the ROC curve (AUC) of 0.7908 (95% confidence interval / CI 0.7163 to 0.8654). See Figure 5.

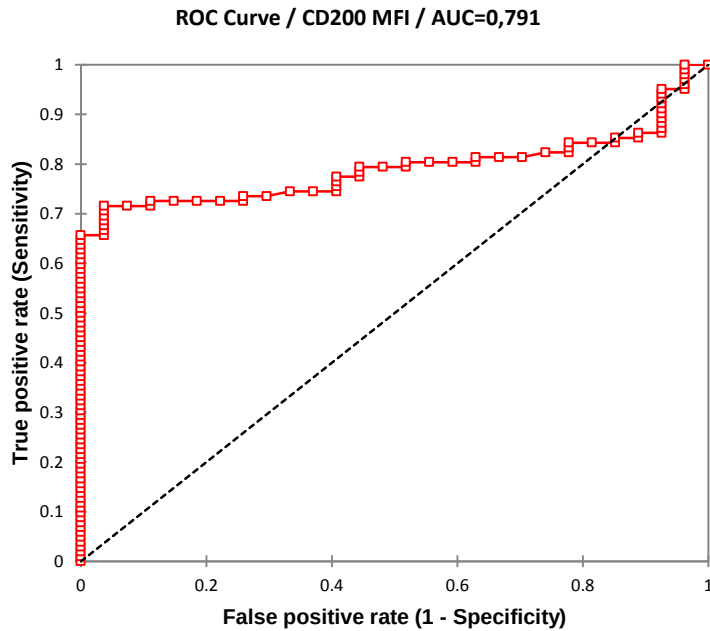


Figure 5: Receiver operating characteristic (ROC) curve

AUC 0.7908; p-value < 0.0001

In addition, based on previous studies [39] and using 3 different methodologies (i.e. MFI of an APC isotypic control on clonal plasma cells; MFI of an APC CD5 on clonal plasma cells; and autofluorescence of APC in plasma cells) a cut-off for confirming CD200 expression was set as an MFI of 30. Thus samples with an MFI of >30 were considered positive, those with an MFI of <20 were negative and samples with an MFI between 20 and 30 were considered indeterminate. Please refer to Figure 6 for a representative flow cytometry scatter plot showing CD200 expression on plasma cells.

Based on this analysis, the clonal plasma cells expressed CD200 in 76.5% of patients (78/102) with a median MFI value of 197 (range: 31-3837). In 17.65% of cases (18/102), the clonal plasma cells were negative for CD200 expression and 5.88% (6/102) of cases fell in the indeterminate range (see table 5). Positivity for CD200 expression did not correlate with gender or age.

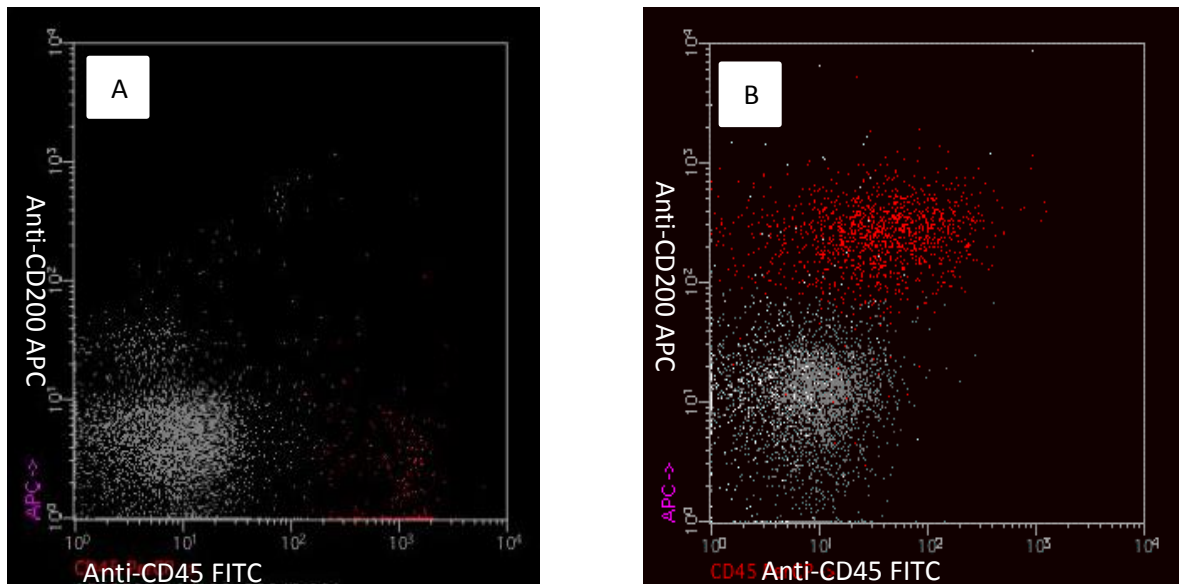


Figure 6: A representative flow cytometry scatter plot showing CD200 expression on plasma cells

Fig 6A: CD45 PERCP and CD200 APC: CD200 negative; MFI 3.47

Fig 6B: CD45 PERCP and CD200 APC: CD200 positive; MFI 301.01

40.74% (11/27) of plasma cells in the control sample showed CD200 expression, 25.92% (7/27) were negative and 33.3% (9/27) were indeterminate. (See table 5). Despite the presence of CD200 positivity in some control samples, the median MFI in positive control samples compared to that noted in patient samples was significantly lower ($P < 0.0001$).

Table 5: CD200 expression in patient and control groups

	CD200 positive Median MFI (range) [n/N (%)]	CD200 negative Median MFI (range) [n/N (%)]	CD200 indeterminate Median MFI (range) [n/N (%)]
Myeloma patients	196.9 (31-3837.1) [78/102 (76.5%)]	8.4 (1.5-16.85) [18/102 (17.7%)]	26.2 (21.98-29.86) [6/102 (5.9%)]
Control samples	47.88 (36.64-76.09) [11/27 (40.7%)]	13.31 (1.19-15.86) [7/27 (25.9%)]	22.26 (20-29.2) [9/27 (33.3%)]

Values have been rounded to one decimal point. MFI – mean fluorescence intensity

The presence of CD200 expression differentiated PCD patients from normal controls using our defined cut-offs with a sensitivity of 81.25% (95% CI 72%-88.49%) and with a specificity of 38.89% (95% CI 17.30% to 64.25%) (see contingency Table 6). The positive likelihood ratio was 1.33 (95% CI 0.91-1.95) with a negative likelihood ratio of 0.48 (95% CI 0.24-0.98).

Table 6: Contingency table: CD200 expression in patients and controls

	PCD (patients) n = 96	Non-PCD (controls) n = 18
CD200 positive	TP 78	FP 11
CD200 negative	FN 18	TN 7

Excludes samples with an indeterminate CD200 expression.

PCD: Plasma cell dyscrasia

TP: True positives; TN: True negatives; FP: False positives; FN: False negatives

3.2.2 Correlation of CD200 with other diagnostic criteria in PCD

CD200 expression was correlated with other diagnostic criteria for multiple myeloma (MM) which indicate organ involvement. These factors include anaemia (measured by haemoglobin concentration), renal failure (urea and creatinine levels) and hypercalcaemia. CD200 did not predict anaemia, renal failure or hypercalcaemia in this population.

3.2.2.1 Haemoglobin

In multiple myeloma anaemia is a diagnostic criterion [3]. The median haemoglobin level for all patients was 8.4 g/dl (range 2.1-17.1), and did not differ statistically between those patients with plasma cell CD200 expression and those without (p-value: 0.68916) (See table 7).

3.2.2.2 Calcium

In multiple myeloma calcium is a diagnostic criterion, measuring the extent of bone involvement (3). The median calcium level in the study population was 2.57 mmol/L (range: 1.17-4.72); this is mildly elevated (normal calcium level 2.2 to 2.5 mmol/L). This did not differ statistically between those patients with plasma cell CD200 expression and those without (p-value 0.8181) (See table 7).

3.2.2.3 Urea and creatinine

Urea and creatinine are diagnostic criteria measuring the extent of renal failure in multiple myeloma [3]. The median urea level was 9.4 mmol/L (normal range: 2.5 to 7.1) and that of creatinine was 135 umol/L (normal range: 74.3 to 107). There was no statistical difference between those with CD200 expression and those without for urea and creatinine (P-value: 0.61006 and 0.38978 respectively). (See table 7).

Table 7: Correlation of CD200 and haemoglobin, calcium, urea and creatinine

	CD200 MFI >30	CD200 MFI<20
Haemoglobin (range) g/dl	8.4 (3.9-17.1)	8.4 (5.1-13.7)
Calcium (range) mmol/L	2.57 (2-4.72)	2.59 (2.18-3.98)
Urea (range) mmol/L	9.1 (3.1-57.1)	10.6 (3.6-21.8)
Creatinine (range) umol/L	133 (41-1574)	147 (68-1441)

3.2.2.4 Bence-Jones proteins (BJP)

These are monoclonal immunoglobulin light chain proteins secreted in urine. The test is used mainly to diagnose and monitor multiple myeloma

(<https://www.urmc.rochester.edu/encyclopedia>). Only 66 (64.7%) patients had BJP testing performed.

Of the 66 patients, 20 produced IgG kappa, 7 produced IgG lambda, 4 produced IgA kappa and 2 produced IgA lambda. Free kappa light chains were found in 12 patients, free lambda in 10 and a negative BJP was found in 4 patients. In one patient, quantification was done with no light chain specification.

Where it was possible to quantify the monoclonal protein (57 patients), the median concentration level of the BJP was 2.2 g/L (range of 1 to 6.6 g/L) in 23 patients and the monoclonal protein measured <1 g/L in 34 patients. The median CD200 MFI in patients with positive BJP was 127.29 (range: 1.54-3837.11) and the median CD200 MFI in the 4 patients with negative BJP was 180.43 (range: 29.86-444.31).

3.2.3.1 Beta 2 microglobulin

Beta 2 microglobulin, a component of the major histocompatibility class I protein, acts as an indicator of tumour volume in multiple myeloma in which significantly elevated levels can be found [41]. Only 52.9% (54/102) patients had beta 2 microglobulin performed. The median level was 8.85 mg/L (range 3-52.2). The normal reference range for B2M is 1.1-2.5 mg/L. The median CD200 MFI in those samples where beta 2 microglobulin was measured was 155.23 (range 3.45-3837.11).

In patients expressing CD200 on their plasma cells, the median beta 2 microglobulin level was 8.7 mg/L and 11.55 mg/L in those without CD200 expression. There was no significant association between beta 2 microglobulin level and CD200 MFI (p-value: 0.3149).

3.2.3.2 Lactate Dehydrogenase (LDH)

LDH measures tissue damage caused by injury or disease. In multiple myeloma patients, it is a prognostic indicator [42]. Only 64% (65/102) of patients had an LDH performed. The median level was 284 U/l (range 105-2913) with a normal reference range of 110-210 U/l. The median LDH level for patients with CD200 expression was 264 (range: 113-800) and in those without CD200 expression, the median LDH level was 350 (range: 197-2913); no statistically significant difference was seen between LDH levels of CD200 expressing cases and those without (p-value: 0.20408). The median CD200 MFI in those samples where LDH was measured was 20 (mean: 72.3 and range 7 -190). No significant association was noted between LDH and CD200 MFI (p-value: 0.1909).

3.2.3.3 Age

In PCD there has been an association between age and a poorer prognosis [44]. There was no significant association between age and CD200 MFI (p-value: 0.3120).

3.2.3.4 Serum protein electrophoresis

95/102 (93.1%) patients had a documented serum protein electrophoresis (SPEP) and 7 did not. Of the 95 patients with a SPEP, 50 produced an IgG monoclonal protein (52.6%) and 25 an IgA monoclonal protein (26.3%). For the remaining 20 patients; SPEP was negative in 1 case, 3 had a monoclonal protein which was not subtyped (into IgG or IgA), 10 were reported as free kappa light chains and 6 as free lambda light chains. Comparison between patients with IgG and IgA expressing PCD found no statistically significant difference in CD200 expression (p-value: 0.8259). The median CD200 MFI appeared lower on samples which produced only free light chains; however, the sample size precluded accurate statistical analysis. Of the 75 patients

where the monoclonal protein was typed, 48 showed kappa light chain restriction (64%) and 27 showed lambda light chain restriction. In patients expressing CD200 on their plasma cells, no statistically significant difference was found between those expressing Kappa or Lambda light chains (p-value: 0.1802). See table 8.

Table 8: CD200 and SPEP findings

	IgG monoclonal protein (n= 50)	IgA monoclonal protein (n=25)	Kappa or Lambda free light chains (n=16)	Kappa light chain expression (n=48)	Lambda light chain expression (n=27)
CD200 Median (range)	168.65 (1.54-3837.11)	157.56 (7.96-1798.18)	50.14 (6.4-582.83)	182.45 (1.54-3837.11)	113.94 (3.4-1225.64)

3.2.3.5 Quantification of the monoclonal band

A serum protein electrophoresis (SPEP) was performed in 95/102 patients (93.1%). Among the 95 patients, monoclonal band quantification was possible in 83 patients (87.4%); in 8 patients, there were no results as it was difficult to quantify and in 4 patients the test yielded negative results.

The median level of the monoclonal band was 29.4 g/L (range 1 to 91) and its associated median plasma cell CD200 MFI for these patients was 145.76 (range 1.54-3837.11).

A comparison between monoclonal band in those samples expressing CD200 and those without was performed. The median of the monoclonal band with CD200 positive was 30.66 g/L (range 1-84.8) and the median level of those with CD200 negative was 10.44 g/L (range 1-37.5), see table 9. A comparison between the monoclonal band levels of those with and without CD200 expression revealed a statistically significant difference (p-value 0.0232).

Table 9: CD200 expression and monoclonal band

	MB of CD200 positive	MB of CD200 negative
Median	30.66	10.44
Range	1-84.8	1-37.5

MB: monoclonal band

A comparison between the monoclonal protein size of IgG and IgA, showed an elevated M protein in the IgG subtype as found in the Zhang *et al.* study [45], however no statistically significant difference was found (p-value of 0.18352). Although in previous studies [45], it was found that IgA subtypes have a more severe anaemia, in our study there was no difference between the haemoglobins of IgG and IgA subtypes (p-value of 1). See table 10.

Table 10: Comparison of IgG and IgA

	IgG	IgA
Monoclonal protein median (range) g/L	36.25 (4.72-91)	28.24 (5-70.5)
Haemoglobin median (range) g/dl	8.8 (2.1-17.1)	8.7 (4.5-13.5)

3.2.3.6 Fluorescence in situ hybridization

In 102 patients, only 64 (62.7%) had fluorescence in situ hybridization (FISH) done. Hyperdiploidy was diagnosed by flow cytometry in 3 cases but FISH was not done. 15/64

(23%) showed intermediate or high risk FISH findings, 40/64 (63%) showed standard risk FISH findings and 12 could not be accurately classified because a complete FISH panel was not performed (refer to table 11).

Table 11: FISH findings

Classification	Number of cases	CD200 MFI median (range)
High/Intermediate risk		138.8 (7.5-894.87)
13q-	10	
17p-	2	
t(4;14)	3	
Total	15	
Standard risk		157.85 (7.96-3837.11)
Negative FISH	31	
Trisomy 17	3	
Trisomy 18	3	
Hyperdiploidy	3	
Total	40	
Not classified		Not applicable
Negative 17p-	7	
Negative t(4;14)	1	
IgH rearrangement but negative for t(4;14)	3	
Extra IgH	1	
Total	12	
Cases without FISH	35	
Grand total	102	

IgH: Immunoglobulin heavy chain rearrangement

In standard risk group, the median CD200 was 157.85 and in non-standard risk group (high/intermediate risk) represented by t(4;14), 17p- and 13q-, the median CD200 was 138.8. When comparing CD200 MFI between standard and high /intermediate risk FISH groups, no significant difference was found (p-value=0.83).

CD200 expression seems to differ in various molecular subtypes, although the numbers in each subtype were very small. See table 12.

Table 12: CD200 and different molecular subtypes

	13q- (n/N)	17p- (n/N)	t(4;14) (n/N)	IgH neg t(4;14) (n/N)
CD200 positive	9/10	2/2	3/3	1/3
CD200 negative	1/10	0/2	0/3	2/3

IgH neg t(4;14): IgH rearrangement with negative t(4;14).

3.3 Correlation between other diagnostic and prognostic criteria in MM

Linear regression analysis demonstrated no association between calcium and haemoglobin (p-value: 0.4742), urea (p-value= 0.0727), creatinine (p-value= 0.1264) or age (p-value= 0.9229) in this cohort. Haemoglobin level (anaemia), however, showed a statistically significant inverse relationship with urea (p-value= 0.0060) and creatinine (p-value= 0.0007) level (renal dysfunction). Urea and creatinine also showed a statistically significant association (p-value: < 0.0001) and an increased urea was associated with age (p-value: 0.0467). See Figure 7.

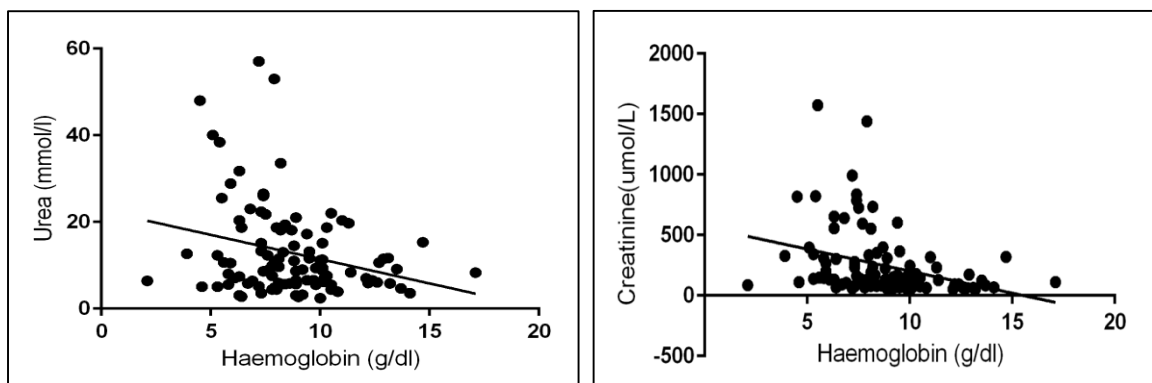


Figure 7: Linear regression analysis demonstrating a statistically significant association between anaemia (haemoglobin) and renal dysfunction [urea (p-value: 0.0060) and creatinine (p-value: 0.0007)]

In addition, there was a statistically significant association between beta 2 microglobulin level and other prognostic markers including haemoglobin (inverse relationship p-value= 0.0239);

urea (p-value < 0.0001); and creatinine (p-value < 0.0001). No significant association was found with age (p-value: 0.3543) or calcium (p-value= 0.1249).

No statistically significant association was found between LDH and other prognostic markers including haemoglobin (p-value= 0.5874); urea (p-value= 0.8564); creatinine (p-value= 0.8407) and Beta 2 microglobulin (p-value= 0.3969). There was, however, a significant association between LDH and calcium (p-value= 0.0057).

No significant difference was noted in other prognostic markers (Hb, Urea, Creatinine, LDH, B2 microglobulin or age) between IgA and IgG expressing PCD and between Kappa and Lambda expressing PCD. IgA PCDs were, however, associated with significantly higher calcium levels (median of 2.96mmol/L in IgA vs 2.38 mmol/L in IgG; p-value: 0.00038).

3.4 HIV and PCD

Comparison between HIV positive and HIV negative patients showed a significantly younger age at presentation in HIV positive patients (median ages of 52 years and 60 years respectively, p-value of 0.00596). (See table 4). HIV positive patients had a significantly lower calcium level (median of 2.33 mmol/L and 2.64 mmol/L respectively; p-value= 0.01732).

The median CD200 MFI level in HIV positive patients appeared to be lower compared to HIV negative patients; however no statistically significant difference was noted (median of 46.04 and 136.13 respectively, p-value of 0.08726). Other parameters such as haemoglobin, urea, creatinine, beta 2 microglobulin and LDH did not differ significantly between HIV positive and HIV negative patients. See table 13.

Table 13: Comparison of demographic and laboratory findings in HIV positive vs HIV negative patients with PCD

	HIV positive	HIV negative	P value
Age, median (range) Years	52 (35-68)	60 (31-72)	0.00596
Calcium, median (range) mmol/L	2.33 (1.17-3.98)	2.64 (2.07-4.21)	0.01732
CD200, median (range) MFI	46.04 (3.4-1798.18)	136.13 (3.45-3837.11)	0.08726

CHAPTER 4: DISCUSSION

Multiple myeloma is a common haematological malignancy in South African patients [46]. Infectious diseases can stimulate reactive plasma cell proliferation [47]. Because of a high prevalence of infectious diseases including HIV infection in our settings, a reliable indicator of plasma cell clonality would be useful to distinguish malignant and reactive plasma cells. This study aimed to evaluate the transmembrane glycoprotein CD200 as a marker of plasma cell clonality. A secondary aim was to assess the prognostic significance of CD200 expression when compared to traditional, validated prognostic markers which are performed in patients with PCD at the CMJAH haematology facility.

Our cut-off for CD200 expression was set as an MFI of 30. Hence samples with an MFI of >30 were considered positive, those with an MFI of <20 were negative and samples with an MFI between 20 and 30 were considered indeterminate. In our cohort of 102 patients, the median CD200 MFI in patient samples was 129.5 (range: 1.54-3837; mean: 272.4) which is higher and significantly different compared with the median MFI in control samples; 24.37 (range: 1.2-76.1; mean: 31.25) (p -value <0.0001). In the control patients, according to our cut-off for CD200 positivity, an estimated 40.74% patients were positive for CD200 expression, 26% were negative and 33.3% were indeterminate. The MFI of CD200 in positive control samples (samples from control patients with no evidence of clonal disease, but which were positive for CD200 expression) was significantly lower than that noted in patient samples (47.88 vs 196.9 with $P < 0.0001$). This is thought to be the result of the possible presence of oligoclonal plasma cells that do not qualify for a diagnosis of PCD, especially in those patients with HIV infection. Due to this finding, CD200 may not be predictive of plasma cell clonality in our setting.

In patients with multiple myeloma, 76.47% of malignant plasma cells expressed CD200, which is consistent with previous reports which demonstrated CD200 expression on 61.8-78% [23, 32, 33] of plasma cells in multiple myeloma patients. Based on our cut-off, 5.88% patients had an indeterminate result. This yielded a sensitivity of 81% and a specificity of 39% for the identification of clonal plasma cells. Specificity is poor suggesting that there may be other causes of plasma cell CD200 expression. This could include infectious diseases in our setting such as HIV (present in 25% of the myeloma patients and 92% of the selected control samples). The specificity for CD200 expression was significantly lower than other studies (92%) although this may reflect different methodologies and CD200 cut-off values [48].

CD200 expression did not correlate in this study with other markers of end-organ dysfunction including anaemia, creatinine and urea levels (markers of renal failure) or hypercalcaemia (bone damage). Other studies suggested that CD200 expression can independently predict haemoglobin, serum creatinine and calcium levels [23, 48, 49]. This discrepancy may reflect the small sample size of the current study cohort.

The second aim of this study was to evaluate the association of CD200 expression with any other traditional prognostic markers. In this study, CD200 was compared with expression of B2M, LDH and genetic markers assessed by FISH. Unfortunately, not all patients in this study had a full profile of prognostic markers which limits analysis.

There was no relationship between elevated B2M or LDH and CD200 MFI levels. Olteanu *et al.* [23] reported no statistically significant difference between CD200 positive and CD200 negative patients in their study concerning B2M; this is however in contrast with the findings of Osman *et al* where patients with plasma cell CD200 expression had significantly elevated

B2M levels [48]. In our study, due to small numbers, an accurate comparison could not be done for B2M levels in patients with and without CD200 plasma cell expression.

Only 64% of patients had LDH performed. The relationship between LDH and CD200 MFI is controversial with some studies reporting a positive correlation and others a negative correlation [23, 48].

For the purposes of this study, genetic abnormalities obtained by FISH analysis were divided into standard risk and non-standard risk comprising intermediate and high risks. CD200 MFI levels showed no significant difference amongst these two risk groups.

The epidemiological characteristics of the patients with myeloma recruited into this study, differed in some respects from international data. At diagnosis the median age for patients in this study was 57.5 years, with males and females showing no statistically significant difference (57 years and 59 years respectively, $p=0.40654$). This is approximately 10 years younger than published international data, which may reflect the younger age structure of African populations in general (<https://www.brandsouthafrica.com/investments-immigration/africanews/africa-s-youth-population-can-lift-the-continent>), a higher burden of infectious diseases in the local context and / or onset at an earlier age in black populations [7, 46]. These results are, however, concordant with previously published South African data [50] (verbal communication by Prof M Patel).

In our cohort, more women than men were diagnosed with Multiple myeloma as shown by a male to female ratio of 1:1.5. This differs from other multiple myeloma studies locally and internationally where men predominate [7, 34]. This could be due to a bias in the data and/or differences in treatment seeking behaviour where females are more likely to present for investigation and treatment [51, 52].

In our cohort 80 PCD patients had an HIV test performed. PCD are not thought to be HIV associated [50]. There are only a few published studies documenting multiple myeloma in HIV- infected patients. Of the patients tested, 25% (20) were HIV positive. This is a much higher prevalence of HIV infection in our cohort compared to other local studies which range from 5% [50], (Verbal communication by Prof M Patel) to 14% [11] in myeloma patients. This may be a coincidental finding given the very high HIV prevalence in South African populations. It could also be explained by a testing bias where a patient who has stigmata of HIV is much more likely to be tested, giving rise to an inflated prevalence figure. The site of the study (tertiary academic referral hospital) with more complicated cases may have also contributed to the elevated HIV prevalence. Compared to HIV negative patients, HIV positive patients in our cohort presented at a younger age (median age= 52 years in HIV positive and 60 years in HIV negative, p-value= 0.00596). This may reflect decreased tumour immunosurveillance or increased antigenic burden with a dysregulated B-cell population [53] in HIV positive patients. HIV positive patients appeared to have a lower median CD200 MFI than HIV negative patients, however statistical significance was not demonstrated (p-value= 0.08726).

Although outside the scope of this study, we also compared markers of end-organ dysfunction with prognostic markers including B2M. Markers of end-organ damage correlated with each other as expected.

Haemoglobin level (anaemia) showed a statistically significant inverse relationship with urea (p=0.0060) and creatinine (p=0.0007) level (renal dysfunction). There was also a statistically significant association between beta 2 microglobulin (B2M) level and other markers including haemoglobin (inverse relationship p-value= 0.0239); urea (p-value= 0.0001); and creatinine

(p-value= 0.0001). Renal function deterioration and tumour burden growth increase B2M level, therefore B2M is a sensitive indicator of glomerular filtration [54].

There was also a significant association between LDH and calcium ($p= 0.0057$). In some studies of plasma cell dyscrasias, increased mortality has been associated with higher LDH and calcium levels [55].

Patients with IgA monoclonal proteins appears to show higher calcium levels than those with IgG monoclonal proteins (median 2.96 in IgA vs 2.38 in IgG; $p= 0.00038$) Higher calcium levels have been reported in IgA subtype myeloma by other studies [45]. Hypercalcaemia reflects lytic bone lesions. This may suggest increased lytic lesions in our patients with IgA myeloma although this could not be confirmed.

CHAPTER 5: CONCLUSION

Plasma cell dyscrasias are a group of clonal plasma cell disorders, with multiple myeloma being the most common entity. CD200 is thought to be expressed only on clonal plasma cells and may thus be useful in differentiating clonal and reactive plasma cells. There are some limitations in our setting, with a high prevalence of infectious diseases, particularly HIV infection, CD200 might yield false positive results most likely because of small oligoclonal reactive plasma cells.

Although approximately 76% of the myeloma patients in our cohort showed CD200 expression on plasma cells, a significant proportion of the control patients also showed plasma cell CD200 expression. This may limit the use of CD200 in confirming clonality in our high HIV prevalence setting.

CD200 expression did not correlate with markers of end-organ dysfunction including anaemia, creatinine and urea levels (markers of renal failure) or hypercalcaemia (bone damage) or with prognostic markers like LDH, beta 2 microglobulin (both markers of cellular turnover) and genetic abnormalities obtained by FISH. This may reflect the small cohort sizes as results for all of these investigations were not available for all patients.

CD200 may be useful as an additional marker in the diagnosis of plasma cell dyscrasia but demonstrated no clear prognostic value in the current study cohort. In future, it could be used to monitor minimal residual disease and for monitoring of novel anti-myeloma drugs including the anti-CD200 antibody therapy (currently in trials). Ideally, a larger study with comprehensive clinical information should be conducted for more conclusive data on the utility of CD200 for prognostication in patients with multiple myeloma and other plasma cell dyscrasias.

APPENDIX

Appendix 1: Data collected

	Patient 1	Patient 2	Etc.
Age			
Sex			
Race			
FBC			
Urea and creatinine			
HIV			
FC			
FISH			
CG			
β2M			
SPEP			
MB			
LDH			
Calcium			
BJP			

FBC: full blood count, FC: flow cytometry, FISH: fluorescence in situ hybridization

CG: cytogenetics, β2M: beta 2 microglobulin, SPEP: serum protein electrophoresis

MB: monoclonal band, LDH: lactate dehydrogenase, BJP: Bence-Jones protein

Appendix 2: Human Research Ethics Clearance Certificate



M130278

R14/49 Dr Sandra Havyarimana

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130278

NAME:
(Principal Investigator)

Dr Sandra Havyarimana

DEPARTMENT:

Molecular Medicine & Haematology
National Health Laboratory Services

PROJECT TITLE:

Evaluation of Prognostic Markers in Plasma
Cell Dyscrasia (under blanket approval
M090688)

DATE CONSIDERED:

Ad hoc

DECISION:

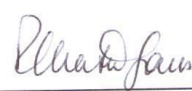
Approved unconditionally

CONDITIONS:

SUPERVISOR:

Professor Lesley Scott

APPROVED BY:



Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 10/06/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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ONLINE REFERENCES

<https://www.brandsouthafrica.com/investments-immigration/africanews/africa-s-youth-population-can-lift-the-continent>

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