Reticulated platelet fraction levels in HIV infected individuals with thrombocytopenia.

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Jenifer Leigh Vaughan

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Medicine in the branch of Haematology.

Johannesburg, 2010.

Declaration:

I, Jenifer Leigh Vaughan declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Jenifer Leigh Vaughan

…….. day of ……., 2010.

Abstract

Thrombocytopenia is common among individuals infected with HIV, with a wide range of possible causes. It often necessitates the performance of a bone marrow investigation in order to assess megakaryocyte activity and to exclude the presence of bone marrow infiltration. Unfortunately, meaningful interpretation of the bone marrow findings is often hampered by the frequent co-existence of multiple potential pathogenic processes. For example, megakaryocyte numbers are often well preserved (even in the presence of marrow infiltration), but show a degree of dysplasia, (suggesting that ineffective megakaryopoiesis is contributing to the thrombocytopenia). In addition, processes associated with peripheral platelet consumption (such as immune-mediated platelet destruction or disseminated intravascular coagulation) are also common, and the mechanism causing thrombocytopenia is therefore often obscure. Because this mechanism is of clinical interest, (in that it guides the selection of the most appropriate therapy), a functional test of megakaryocyte activity would be of potential value.

The IPF is a platelet parameter measured on the Sysmex XE-5000 haematology analyzer, which quantifies the number of reticulated platelets, and has been shown to be a good reflector of underlying bone marrow megakaryocyte activity.

The objectives of this study were therefore to measure the IPF level in HIV-positive patients with thrombocytopenia who had undergone a bone marrow investigation, and to correlate the IPF with the bone marrow morphology findings and other clinical variables of interest (including the CD4 count, the HIV viral load and the presence of opportunistic infections or malignancies). The IPF was also assessed as a tool to predict the short term platelet count response to the therapy initiated by the attending clinician.

78 patients were enrolled, of whom 38 (49%) had mycobacterial infection, 12 (15%) had ITP/TTP and eight (10.3%) had a malignancy. CD4 counts were available in 70 patients, of whom 63 (90%) had AIDS. Thirty seven patients (47%) were found to have an IPF level greater than 7.7% , with an overall population mean IPF level of 9.5%.

A strong relationship was identified between the IPF and the platelet count, with 81% of patients with grade four thrombocytopenia having an IPF level greater than 7.7% (mean IPF=14%), as compared to only 8% of grade one thrombocytopenia (mean IPF=6%). 67% of patients with hypocellular or extensively infiltrated marrow had a low IPF (≤ 7.7%)(mean=7%), as compared to only 25% of patients with ITP/TTP (mean=14.8%). A higher proportion of patients with low viral load levels had a low IPF as compared to those with higher viral loads, possibly due to the apparent sparing of patients with low viral loads from grade four thrombocytopenia. In contrast, the presence of a significant degree of megakaryocyte dysplasia, a very low CD4 count or the presence of mycobacterial infection did not affect the IPF distribution, suggesting that the underlying mechanism causing thrombocytopenia in these subgroups was heterogeneous.

An IPF level greater than 10% predicted a partial platelet count response (as defined as an improvement by greater than 50% of the baseline platelet count to a minimum level of 20x10^9/l), with a specificity of 81% and a positive predictive value of 79%, while an IPF level less than 6% had a specificity of 89% and a positive predictive value of 70% for a complete failure to show a platelet count response to the therapy instituted.

Limitations of the IPF illuminated in this study include a loss of reliability in any circumstance in which the platelet count as measured by optical fluorescence may be in question, and includes disorders associated with profound red cell fragmentation and some malignancies.

The cause of thrombocytopenia is concluded to be very heterogeneous in thrombocytopenic patients with AIDS, even among patients with a unifying diagnosis (such as mycobacterial infection). The IPF is therefore a useful tool to assist the morphologist in interpreting the bone marrow findings in this clinical setting, as well as in predicting the short term platelet count response to therapy.

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Abbreviations used in the text:

- AIDS Acquired Immunodeficiency Syndrome
- ARDS Acute respiratory distress syndrome (ARDS)
- ARV Anti-retroviral
- DIC Disseminated intravascular coagulation
- DLBCL Diffuse large B-cell lymphoma
- EBV Epstein-Barr virus
- HIV Human Immunodeficiency Virus
- HUS Haemolytic uraemic syndrome
- IL Interleukin
- IPF Immature platelet fraction
- ITP Immune thrombocytopenia
- KSHV Kaposi's sarcoma herpes virus
- MAHA Microangiopathic haemolytic anaemia
- MDS Myelodysplastic syndome
- NNRTI Non-nucleoside reverse transcriptase inhibitors
- NRTI Nucleoside reverse transcriptase inhibitor
- TB Tuberculosis
- TPO Thrombopoietin
- TTP Thrombotic thrombocytopaenic Purpura
- vWF von Willebrand factor

Chapter 1:

1.0 Introduction

1.1 An overview of HIV infection in South Africa

The human immunodeficiency virus (HIV) is a retrovirus which affects human immunity, resulting in the acquired immunodeficiency syndrome (AIDS) [1]. It infects in excess of 33 million people world-wide [2], with more than five million affected individuals residing in South Africa [3]. The CD4 molecule expressed by CD4 T-cells, monocytes and macrophages is the primary cellular receptor for HIV, and infection of these cells by the virus is associated with a slow, progressive drop in the CD4 T-cell count with an associated decline in cell mediated immunity [1]. When the CD4 count drops below a level of 200x10^6/l, the infected individual is rendered significantly immunocompromised, and is then classified as having immunological AIDS [1]. Some individuals may present with AIDS-related pathology with a relatively preserved CD4 count (i.e. a CD4 count>200x10^6/l), in which case they are classified as having clinical AIDS. HIV/AIDS may manifest as a wide range of clinical entities affecting virtually every organ system as a result of either opportunistic infection or malignancy, or a direct viral effect [1]. In South Africa, Tuberculosis (TB) is a particularly important infection in HIV-infected individuals, with 60% of patients with TB, in whom the HIV status is known, being HIV positive [4]. This is due to the extremely high incidence of TB in the population as a whole (which is in excess of 950/100 000 population [5] compared to a global incidence rate of 140/100 000 population [6]). Malignancies have an increased incidence in this setting for many reasons, including the effects of chronic antigenic stimulation and a propensity to infection with potentially oncogenic viruses (such as Kaposi's sarcoma herpes virus (KSHV) or Epstein-Barr virus (EBV)) [7]. High grade lymphomas are among the most common malignancies encountered, with the incidence of Hodgkin's lymphoma,

diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma being eight times, 60-200 times, and 1000 times more common than in the background population respectively [7].

The gold standard treatment for AIDS is a combination of anti-retroviral (ARV) drugs, including both nucleoside reverse transcriptase inhibitors (NRTIs) (such as zidovudine, lamivudine and stavudine) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (such as nevirapine and efavirenz) [8]. However, statistics from 2007 showed that less than 30% of South African's with AIDS had been initiated on anti-retroviral therapy [3].

1.2 A brief overview of platelet biology

1.2.1 What are platelets?

Platelets are small anucleate cellular structures which circulate in the peripheral blood (see Figure 1.1). They are discoid in shape, and although anucleate, their cytoplasm contains a number of organelles, granules and cytoskeletal proteins. They express surface receptors for collagen, von Willebrand factor (vWF) and fibrinogen, which mediate platelet immobilization and aggregation at the site of an endothelial breach. These interactions result in the formation of a primary platelet plug, which physically occludes the site of injury, thus preventing significant blood loss. In addition, they provide a very large phospholipid surface area for the action of phospholipid dependent coagulation proteins, and contain many procoagulant substances within their granules. They play a critical role in forming a stable thrombus in the event of a vessel wall injury, and a person with an abnormally low platelet count (thrombocytopenia) may therefore present with a propensity to bleed [9].

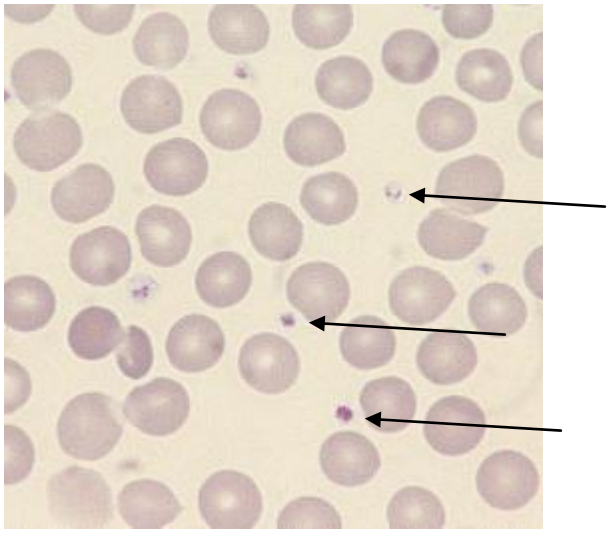


Figure 1.1: Normal blood showing red blood cells interspersed by much smaller platelets (see arrows) (May-Grünwald-Giemsa stain, 50x magnification).

1.2.2 A brief overview of platelet production.

Platelets are synthesized in the bone marrow by large polyploidy cells called megakaryocytes [9]. These extrude cytoplasmic processes (called proplatelet processes) from their cell surfaces, which dissociate from the parent megakaryocyte (as proplatelets), and then subsequently fragment further to form mature platelets [10] (see Figure 1.2). Platelets therefore are fragments of megakaryocyte cytoplasm.

Platelet production is controlled by a number of soluble haemopoietic growth factors, including thrombopoietin (TPO), interleukin (IL)-3 and IL-11. Platelets have a lifespan of between seven and ten days, and therefore need to be synthesized at a rate of ~1x10^11 per day in order to maintain a platelet count within the normal range (between ~140-400x10^9/l in healthy individuals) [11].

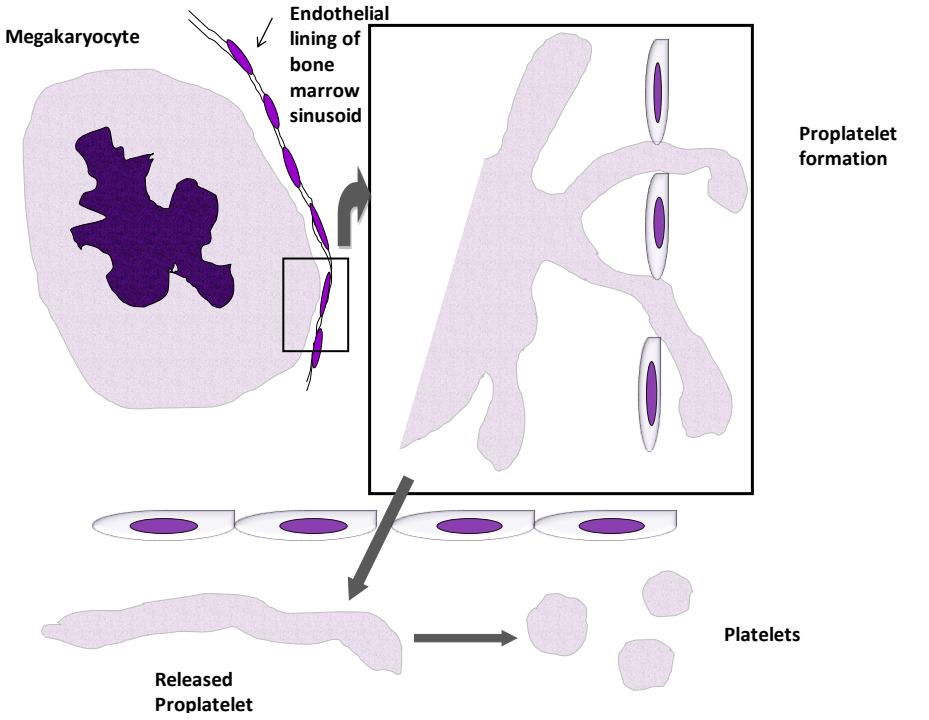


Figure 1.2. Platelets are formed from sequential fragmentation of cytoplasmic extensions (proplatelets) from the megakaryocyte surface.

Bone marrow production of platelets can be up-regulated when necessary, largely through the action of TPO, the serum levels of which are inversely proportional to the peripheral blood platelet count [12]. Two important mechanisms modulate serum TPO levels. The first is dependent on the expression of high affinity TPO receptors (Mpl) on both platelets and megakaryocytes. When platelet numbers are normal, TPO binds to its receptor on platelets and is therefore functionally unavailable for binding to Mpl on megakaryocytes. Conversely, when platelet levels are low, available TPO is increased, and is able to bind it's receptor on megakaryocytes and stimulate new platelet production [13]. The second mechanism at play depends upon increased TPO production in response to thrombocytopenia. Although TPO is synthesized by the liver, kidney and bone marrow stromal cells, an increase in TPO mRNA production in the setting of thrombocytopenia occurs only in the bone marrow stromal cells, while TPO mRNA levels in the liver and kidney are not influenced by the

platelet count [14]. One important mechanism of control of TPO production by bone marrow stromal cells is mediated by proteins present in the alpha granules of platelets, which if present, suppress TPO production [15]. TPO levels are therefore greatly influenced by the presence of mature platelets. The latter example also illustrates how the activity of megakaryocytes is dependent on the milieu in which they are located, with surrounding endothelial and stromal cells within the bone marrow microenvironment playing an essential role in maintaining normal megakaryopoiesis [11].

1.3 An overview of thrombocytopenia

Thrombocytopenia may occur as a consequence of either a failure of the bone marrow to produce sufficient platelets (i.e. a central defect), or of excessive consumption or loss of platelets at a rate which exceeds the bone marrow's rate of platelet synthesis (i.e. a peripheral disturbance). The causes of thrombocytopenia are summarized in Table 1.1.

1.4 Indication for a bone marrow investigation:

As a general rule, if a peripheral mechanism for thrombocytopenia is evident, a bone marrow investigation is not usually indicated [16]. However, if a central cause for the thrombocytopenia is suspected or there is cause for doubt about the underlying mechanism at play, a bone marrow aspirate and trephine biopsy are indicated [16]. Platelet production failure is characterized by either

abnormal megakaryocyte morphology or a reduction in their numbers, while peripheral mechanisms are usually associated with increased megakaryocyte numbers.

Table 1.1: An overview of the causes of thrombocytopenia

Platelet production failure	**Reduced platelet survival**
Hypoplastic/aplastic anaemia.	Immune thrombocytopenia (ITP)
Nutritional deficiencies -Vitamin B12 -Folic acid -Iron	Microangiopathic haemolytic anaemia (MAHA) - Disseminated intravascular coagulation (DIC) - Thrombotic Thrombocytopenic Purpura (TTP)
Bone marrow infiltration -Malignant -Infectious	Hypersplenism
Infection -Secondary hypoplastic/aplastic anaemia -Granuloma formation - Cytokine mediated haemophagocytosis -Direct viral infection of megakaryocytes	
Drug or toxin effect	
The Myelodysplastic Syndome (MDS)	

1.5 Thrombocytopenia in the setting of HIV infection:

Thrombocytopenia occurs with a prevalence of 3-30% in HIV infected individuals [17, 18]. It is more common among those with AIDS [17, 18] and may be reduced in some cases by anti-retroviral therapy [19]. This high prevalence occurs due to the frequent occurrence of pathologies associated with thrombocytopenia in this setting, including ITP [20, 21], TTP [22] and opportunistic malignancies (which may infiltrate the bone marrow or cause splenomegaly). Opportunistic infections are also highly prevalent, which may produce a myriad of effects. For instance, many viruses may cause hypoplastic/aplastic anaemia [23], and infectious agents can infiltrate the bone marrow (as may occur in miliary mycobacterial infection) or cause splenomegaly. In addition, severe sepsis may either impair platelet production, (possibly as a result of cytokine mediated haemophagocytosis within the bone marrow [24, 25]) or enhance platelet consumption by precipitating DIC [26].

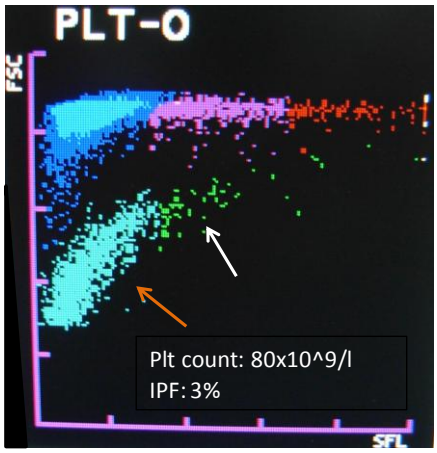
HIV positive individuals are also frequently exposed to a cocktail of medications, many of which may suppress megakaryopoiesis, cause secondary ITP or result in ineffective haemopoiesis [27-33]. Lastly, platelet production is impaired in the setting of HIV as a consequence of both stromal cell dysfunction [34] and direct viral infection of megakaryocytes by means of CD4 molecules expressed on the megakaryocyte surface [35]. As a result of one or more of these influences, the bone marrow of HIV infected persons may show hyper- or hypocellularity, with increased numbers of bare megakaryocyte nuclei (signifying increased megakaryocyte death) and morphologically dysplastic haemopoietic precursors, with or without evidence of bone marrow infiltration [29].

Using radio-isotope labeled platelets, it has been shown that immune destruction of platelets is more often seen in patients with higher CD4 counts, while those with more advanced HIV disease (although also vulnerable to immune-type thrombocytopenia) more often experience a platelet production defect [36]. Unfortunately, establishing the mechanism of thrombocytopenia in these patients on a routine basis is largely dependent on morphological assessment of the bone marrow, which is made difficult by the frequent finding of increased numbers of megakaryocytes with a degree of morphological atypia [29]. As the presence of a peripheral cause of thrombocytopenia often necessitates a specific therapeutic intervention, establishing the mechanism of thrombocytopenia is of clinical interest.

1.6 Background information about the immature platelet fraction (IPF):

Because platelets are anucleate, they are unable to synthesize new RNA after they have dissociated from the parent megakaryocyte. The presence of RNA-rich platelets (so-called “reticulated platelets”) was first demonstrated in the late 1960’s [37], and these were confirmed to be newly formed platelets in the mid 1990’s [38, 39]. A relationship between megakaryocytic activity and the percentage of reticulated platelets in the peripheral blood was first demonstrated by Kienast and Schmitz in 1990 [40], and has subsequently been confirmed on numerous occasions [41-43]. Assessment of numbers of reticulated platelets has now been automated by Sysmex, and can be measured as the “immature platelet fraction” (IPF) using a Sysmex XE-5000 haematology analyzer (Sysmex, Kobe, Japan). This parameter is measured as part of a routine full blood count, and is

reported as a proportion of the total optical platelet count. The test methodology entails the demonstration of RNA-rich platelets by means of a fluorescent RNA-binding intracellular dye, which are then quantified by means of flow cytometry [44] (see Figure 1.3).



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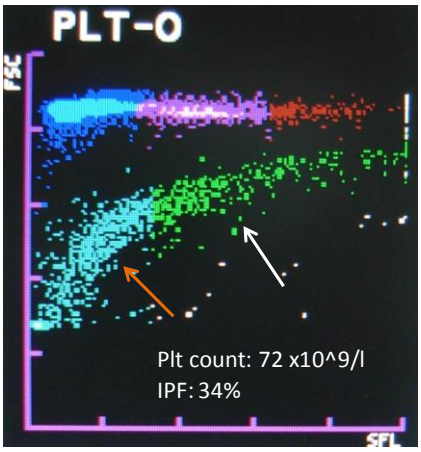


B

Figure 1.3: Optical platelet scattergrams from thrombocytopenic individuals with low (A) and high (B) IPF levels respectively. Mature platelets appear as aqua dots (see orange arrows), while reticulated platelets are depicted as green dots (see white arrows). In addition to having higher fluorescence intensities as compared to mature platelets, reticulated platelets are also slightly larger.

Like reticulated platelets quantified by other means, the IPF has been shown to correlate directly with the underlying mechanism of thrombocytopenia in a number of clinical contexts, including ITP as well as chemotherapy-related thrombocytopenia [44, 45]. Of interest, an increase in the IPF has been shown to herald a recovery in the platelet count within approximately three days in the setting of marrow hypoplasia [44]. The IPF may therefore be useful in assessing the expected time for the platelet count to recover spontaneously in this clinical setting, therefore facilitating the selection of a

group of thrombocytopenic patients who may not require a platelet transfusion in the face of imminent recovery of the platelet count.

Data from a study conducted in Japan on 137 thrombocytopenic patients from a variety of causes demonstrated that the best IPF cut-off to discriminate the patients with normal/suppressed platelet production from those with increased platelet production is 7.7%. This cutoff value was able to delineate underlying megakaryopoietic activity with a sensitivity of 86.8% and a specificity of 92.6% [44]. In this study, the normal range assessed in 129 healthy Japanese individuals (1-10.3%; mean 3.3%) was similar to the normal range established both in normal English subjects (1.1–6.1%, mean 3.4%) [45], and in a population of healthy South Africans (1.1%-5.9%, mean ~3.5%) (personal communication, M Munster).

To the author's knowledge, there has only been one publication on reticulated platelet numbers in HIV positive patients to date. This study assessed the platelets of 14 thrombocytopenic HIV infected individuals using an alternative method to the automated IPF assessment, and showed increased reticulated platelet numbers in all 14 patients [46]. However, this finding should perhaps be treated with reserve given the large body of evidence implicating impaired platelet production as an important mechanism of thrombocytopenia in HIV-positive patients, particularly in light of the small sample size.

1.7 The objectives of this MMed Research Report:

In summary, thrombocytopenia is a common finding in the setting of HIV, with many possible underlying mechanisms. This report aims to:

1) Quanititate the IPF in HIV-positive thrombocytopenic patients.

2) Assess for an association between the IPF and clinical variables (such as the CD4 count, medications, additional underlying pathology (including opportunistic infection or malignancy).

3) Assess whether there is any association between the IPF at the time of bone marrow investigation with the short-term behavior of the platelet count in response to the treatment implemented at the discretion of the treating doctor.

4) Assess the relationship between the morphological characteristics of megakaryopoiesis with the IPF.

Chapter 2:

2.0 Materials and Methods

2.1 Patients:

Participants were identified through the bone marrow register in the haematology laboratory at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). All in- hospital HIV positive patients with thrombocytopenia for whom a bone marrow investigation had been performed were considered eligible, and between August 2009 and January 2010, 78 patients were enrolled.

2.2 Ethical clearance:

Ethical clearance was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg.

REFERENCE: R14/49 Dr Jenifer Vaughan

CLEARANCE CERTFICATE: M090113

Informed consent was obtained from all participants.

2.3 Sample collection and analysis:

A venous blood sample was collected from each participant in a tube anti-coagulated with EDTA. This was used to perform a full blood count and reticulocyte count (which includes the IPF as one of the parameters measured) using a Sysmex XE-5000 haematology analyzer (Sysmex, Kobe, Japan) within 24 hours of sample collection. (The IPF has been shown to remain stable for at least 2 days after

collection if stored at room temperature [45]). If available, a sample collected from the patient on the day of the bone marrow investigation for the purpose of routine blood count analysis was used, provided that the sample was of adequate quantity and had not yet been filed for storage in the refrigerator. In the event that an adequate recent specimen was not available, a fresh sample was collected from the patient by the investigating doctor as soon as possible (and within 24 hours of the bone marrow investigation).

The platelet count measured using the Sysmex XE-5000 instrument (Sysmex, Kobe, Japan) was recorded as the baseline platelet count. When available, the platelet count measured on routine full blood count analysis in the same sample (performed in the haematology laboratory using either a Beckman-Coulter LH750 haematology analyzer (Beckman–Coulter, Brea, CA), or a Bayer Advia 2120 analyzer (Siemens Medical Solutions Diagnostics, Zurich, Switzerland) was documented, and results compared with those measured on the Sysmex XE-5000 (Sysmex, Kobe, Japan). This was done in order to verify agreement between results from the various instruments, as the follow-up platelet counts documented were those measured in the routine laboratory (see 2.1.8).

2.4 Quality control:

Quality assurance was performed using normal, abnormal high, and abnormal low quality control materials supplied by the manufacturer on a daily basis.

2.5 Precision and accuracy studies:

As there is no readily available gold standard method to assess reticulated platelets, precision and accuracy studies were performed on the Sysmex XE-5000 instrument (Sysmex, Kobe, Japan) using control materials with a designated IPF value.

2.6 Bone marrow assessment:

Analysis of the bone marrow aspirate and trephine biopsy morphological features were undertaken by a single experienced morphologist using a Nikon ECLIPSE E400 microscope (Nikon, Tokyo, Japan). The bone marrow aspirate samples were stained with the May-Grünwald-Giemsa stain, and the trephine biopsies with haematoxylin and eosin. Megakaryocyte numbers and morphology were documented in addition to any other findings judged to be pertinent (such as the presence of a malignant or infectious infiltrate). Photographs of some of the interesting pathology were taken using an Altra 20 digital camera (Soft Imaging System, Hamburg, Germany).

2.7 Clinical information:

Patient information was gathered from the hospital file and the laboratory computer system records. Information collected included details about the presenting complaint, the favored diagnosis (as judged by the treating clinician), any history of previous medications, past medical problems, treatment instituted for the primary cause of the thrombocytopenia by the treating clinician, the CD4 count, TB culture results and any other information judged to be pertinent on a case to case basis.

2.8 Platelet response to therapy:

A follow-up platelet count was recorded from the laboratory computer records as close to 1 week after the bone marrow investigation as possible, or at patient death/discharge from the hospital if the latter occurred less than 7 days after the bone marrow was performed. This was done in order to document the short-term platelet response to therapy instituted. This platelet count result was obtained from sample analysis on one of the haematology analyzers in routine use in the CMJAH haematology laboratory at this time, which included a Beckman-Coulter LH750 (Beckman–Coulter, Brea, CA) and a Bayer Advia 2120 (Siemens Medical Solutions Diagnostics, Zurich, Switzerland).

All clinical decisions and the physical management of the patient were undertaken by the attending clinician.

2.9 Study definitions:

2.9.1 Definition of Thrombocytopenia:

For the purposes of this study, thrombocytopenia was defined as a platelet count below 140x10^9/l in the absence of platelet clumping.

2.9.2 Thrombocytopenia severity grading:

Thrombocytopenia severity was graded as follows:

Platelets <25 = Grade Four Thrombocytopenia

Platelets 25-50 = Grade Three Thrombocytopenia

Platelets 51-75 = Grade Two Thrombocytopenia

Platelets 76-139 = Grade One Thrombocytopenia

This is in accordance with the National Cancer Institute common terminology criteria [47].

2.9.3 IPF cut-off:

The IPF was judged to be low/normal if less than or equal to 7.7%, and increased if more than 7.7%. This is in accordance with the recommendations made in reference 44.

2.9.4 Megakaryocyte dysplasia quantification:

Megakaryocyte dysplasia was quantified as the number of megakaryocytes judged to have abnormal or degenerate morphology out of every 30 megakaryocytes present. This was undertaken in an effort to grade the degree of megakaryocyte dysplasia noted (in accordance with the recommendations made in reference 30).

2.9.5 Response to treatment:

Response to treatment was defined according to recently published recommendations [48]. They are as follows:

- If the original platelet count was less than 30x10^9/l, the follow-up platelet count was required to have reached a minimum level of at least 30x10^9/l and to have doubled its base-line.
- If the original platelet count was between 31-50x10^9/l; the platelet count was required to have doubled from its baseline.
- If the platelet count was between 50 and 99x10^9/l, it was required to have reached a level of at least 100x10^9/l.

Because no published guidelines are available for assessment of response to treatment in patients with very mild thrombocytopenia (>100x10^9/l), a response to treatment was defined as normalization of the platelet count.

2.10 Data Analysis

Agreement in platelet count between the routinely used haematology analyzers and the Sysmex XE-5000 was assessed using the Bland-Altman method of comparison.

Parametric data (such as comparisons of the platelet count between groups) was analyzed using a student t-test, and non-parametric data (such as comparisons between the IPF distribution (high vs low) in relation to variables of interest), was analyzed using the Fisher's exact test and logistic regression. In addition, odds ratios were calculated to assess the likelihood of the IPF being high or

low in the presence of various variables of interest, both before and after correction for confounding variables by means of logistic regression. These odds ratios are expressed as crude and adjusted odds ratios respectively.

Platelet count response in relation to the IPF as well as in relation to other clinical variables of interest was analyzed by means of contingency tables and odds ratios.

P values less than or equal to 0.05 were considered statistically significant, and values between 0.05 and 0.1 were considered to be of marginal statistical significance.

Chapter 3:

3.0 Results

3.1 Precision and accuracy analysis of the IPF parameter.

This was performed using control material with a known IPF value of between 18 and 23%. Repeat analysis of this control material revealed a mean IPF of 21.7% over 11 runs, with a standard deviation of 1.16%, and a co-efficient of variation of 5.3%.

3.2 An overview of the patient cohort.

3.2.1 General information

3.2.1.1 Patient demographics (summarized in Table 3.1):

Of the 78 patients enrolled, 34 (44%) were female, and 44 (56%) were male. Their mean age was 37 years, (ranging from 21 to 67 years).

All but one of the patients were of African descent.

3.2.1.2 HIV-related information (summarized in Table 3.1):

A CD4 count was available in 70 of the patients, with a mean CD4 count of 106 (ranging from 1x10^6/l to 761x10^6/l, median = 67x10^6/l). Sixty one (87%) had a CD4 count of less than 200x10^6/l, and were therefore categorized as having immunological AIDS. In addition, two of the nine patients who had a CD4 count greater than 200x10^6/l had AIDS defining pathologies.

Twenty (26%) of the patients were on ARV therapy, five (25%) for between one week and two months duration, and 15 (75%) for a minimum of three months duration. These patients had a mean CD4 count of 84x10^6/l, with only one patient having a CD4 count over 200x10^6/l.

Viral loads were available in 21 patients, eight on ARVs, and 13 not on ARVs. Among patients on ARVs, the mean viral load was approximately 375 500 copies/ml, while the mean viral load in patients not on ARVs was over I 860 000 copies/ml.

Of the seven patients without AIDS, three had ITP, one had TTP and none had TB.

Table 3.1: A summary of patient demographic and HIV-related data.

Sex: Male: Female:	 56% 44%
Mean age:	37 years (Range: 21-67 years)
Mean CD4 count (N=70):	106x10^6/l (Range: 1-761x10^6/l, median: 67x10^6/l)
ARV therapy: Yes: < or equal to 2 months duration: > or equal to 3 months duration: No:	 26% 25% 75% 74%
Mean Viral load (N=21): On ARVs: Not on ARVs:	 375 500 copies/ml I 860 000 copies/ml

3.2.1.3 Blood counts:

The mean blood counts are displayed in Table 3.2:

Only one patient had an isolated thrombocytopenia, and 33 (42%) had a pancytopenia.

Table 3.2 Mean full blood count parameters and their ranges.

Parameter	Mean	Range
Platelet count:	56x10^9/l	4-137 x10^9/l
Haemoglobin	8g/dl	4.7-12.5 g/dl
White cell count:	4.6x10^9/l	0.6-14.5x10^9/l
Mean cell volume:	85 fl	67-101 fl

The various grades of thrombocytopenia (grade one to four) were fairly evenly represented in the cohort, with 27% having grade four thrombocytopenia, 22% grade three, 20% grade two and 31% grade one (see Figure 3.1).

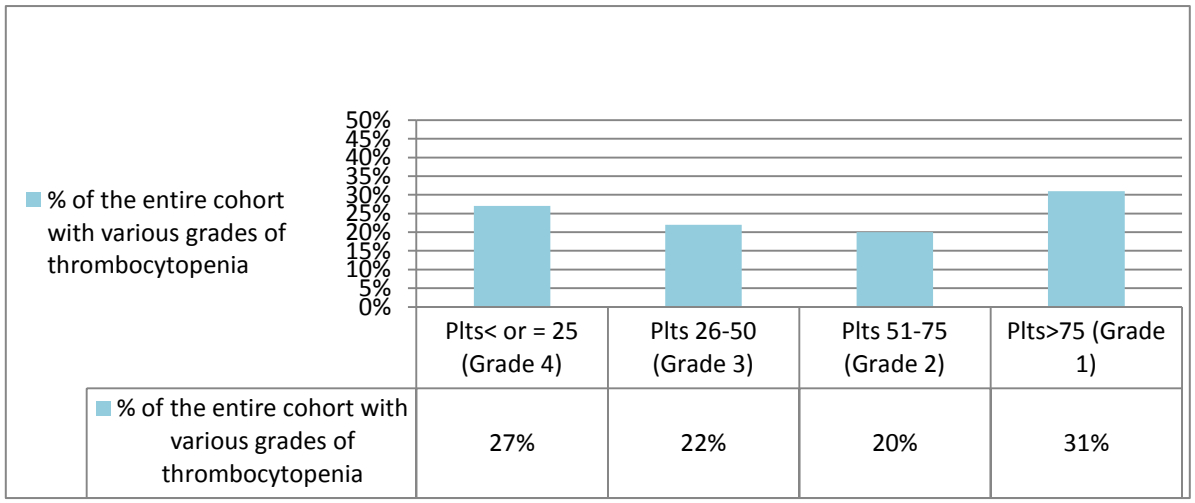


Figure 3.1: Platelet count distribution in the entire cohort of patients (N=78)

3.2.1.4 IPF data:

Of all patients, 41 (53%) had a low IPF, and 37 (47%) had a high IPF (see Figure 3.2). The mean IPF was 9,5%, ranging from 1,3-44%, with a median value of 7,6%.

In patients with grade two and three thrombocytopenia, the proportion with a high IPF was similar to the proportion with a low IPF. However, among patients with grade four thrombocytopenia, 17 (81%) had a high IPF (mean IPF 14%), while 22 patients with grade one thrombocytopenia (92%) had a low IPF (mean IPF 6%) (see Figure 3.3). The difference in IPF distribution between patients with grade one and grade four thrombocytopenia was found to be highly statistically significant (p=<0,001). In fact, the proportion of patients with a low IPF level among those with grade one thrombocytopenia was statistically different from that seen in patients whose platelet counts fell within all of the other grades of thrombocytopenia (see Table 3.3). In contrast, the different proportions of high IPF levels between patients with grade four thrombocytopenia as compared to grade three was only marginally significant (p=0.087), and the difference between grade four and grade two did not reach statistical significance at all (p=0.151) (see Table 3.4).

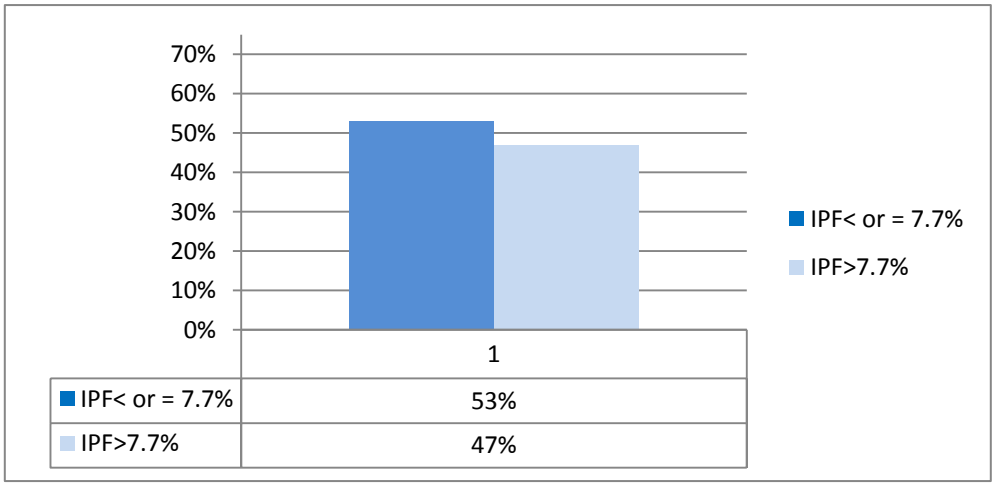
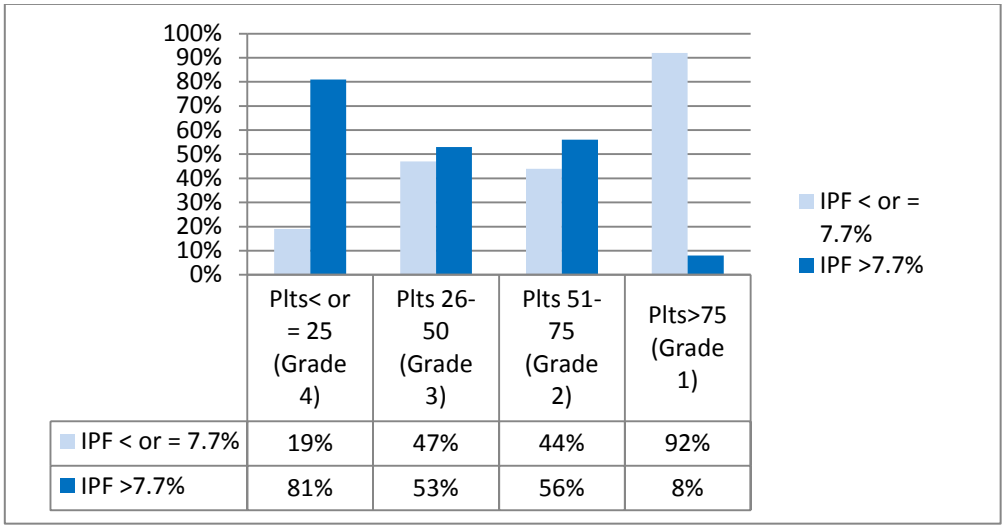


Figure 3.2: IPF distribution in the entire cohort (N=78)



	Plts< or = 25 (Grade 4)	Plts 26-50 (Grade 3)	Plts 51-75 (Grade 2)	Plts>75 (Grade 1)
IPF < or = 7.7%	19%	47%	44%	92%
IPF >7.7%	81%	53%	56%	8%

Figure 3.3: IPF distribution relative to platelet count

Table 3.3: Statistical analysis of the proportion of low IPFs in all grades of thrombocytopenia as compared to grade 1.

	Proportion of cases with a low IPF	**Odds Ratio**	**95% Confidence interval**	**p-value**
Grade 1 thrombocytopenia (N=24)	92%	14	(2.7-71.8)	0.003
Grade 2 thrombocytopenia (N=16)	44%			
Grade 1 thrombocytopenia (N=24)	92%	12	(2.4-61.5)	0.003
Grade 3 thrombocytopenia (N=17)	47%			
Grade 1 thrombocytopenia (N=24)	92%	47	(8.2-257.4)	<0.001
Grade 4 thrombocytopenia (N=21)	19%			

Table 3.4: Statistical analysis of the proportion of high IPFs in all grades of thrombocytopenia as compared to grade 4.

	Proportion of cases with a high IPF	Odds ratio	95% Confidence interval	p-value
Grade 1 thrombocytopenia (N=24) **Grade 4 thrombocytopenia (N=21)**	8% 81%	0.021	(0.004-0.22)	<0.001
Grade 2 thrombocytopenia (N=16) **Grade 4 thrombocytopenia (N=21)**	56% 81%	0.3	(0.073-1.258)	0.15
Grade 3 thrombocytopenia (N=17) **Grade 4 thrombocytopenia (N=21)**	53% 81%	0.265	(0.066-1.080)	0.087

3.2.1.5 Spectrum of Pathology:

Mycobacterial infection was diagnosed in 38 (49%) of the patients, and 31 of these had evidence of bone marrow involvement (as evidenced by either the presence of granulomatous infiltration and or acid fast bacilli visualized in the trephine biopsy, or a positive bone marrow TB culture) (see Figure 3.4). Two of these patients had *Mycobacterium avium complex* infection (see Figure 3.5), while the rest had either culture evidence or morphological characteristics more compatible with *Mycobacterium tuberculosis.*

Eight patients (10%) had bone marrow infiltration by a malignancy. Two had a large B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma (see Figure 3.6), one had plasmablastic lymphoma (see Figure 3.7), three had Hodgkin lymphoma (see Figure 3.8), one had a peripheral T-cell lymphoma (see Figure 3.9) and one had metastatic carcinoma of the breast (see Figure 3.10). Four of these patients had very extensive marrow infiltration, with marked displacement of normal bone marrow elements. The remaining four patients had focal bone marrow infiltration, with well preserved residual haemopoiesis. One of the patients with extensive marrow infiltration by large B-cell lymphoma unclassifiable with features

intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma also had pulmonary TB (as diagnosed by demonstration of acid fast bacilli in her sputum).

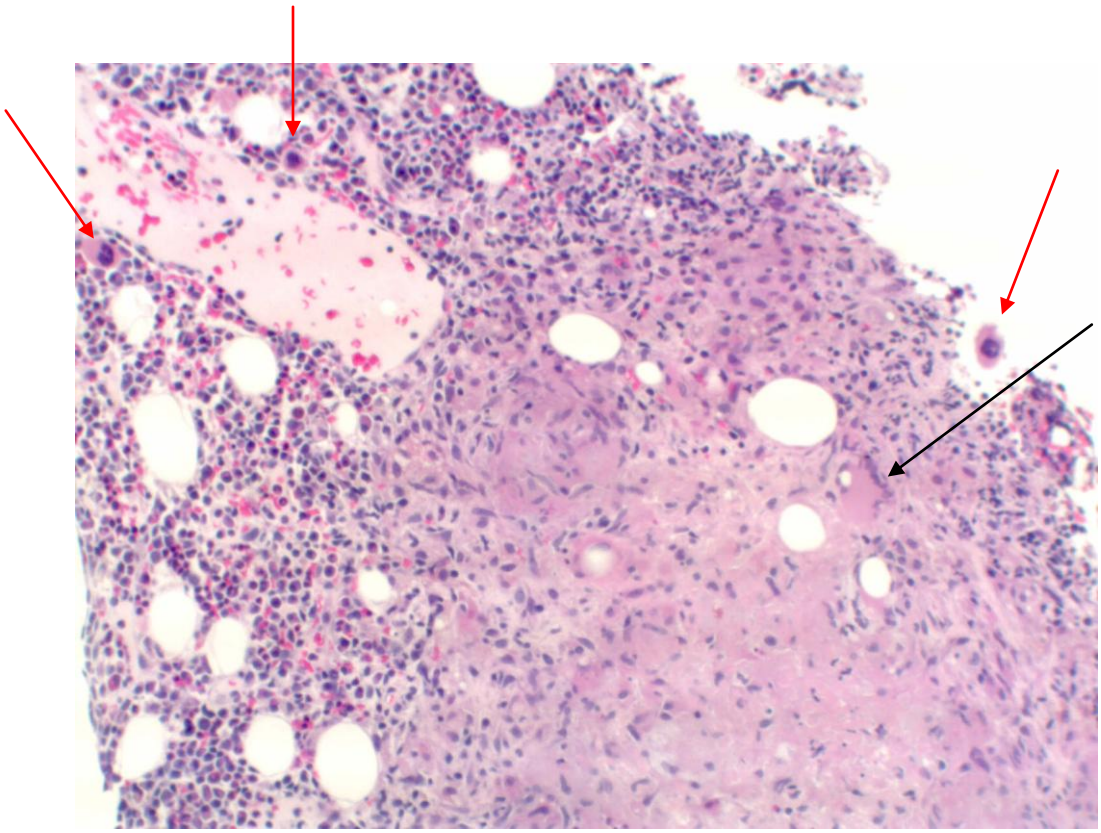


Figure 3.4: Granulomatous infiltration of the bone marrow in one of the patients with *Mycobacterium tuberculosis* infection. Normal marrow architecture is preserved on the left, while the right hand side of the image shows an infiltrate comprised of epithelioid histiocytes with caseous necrosis. The black arrow shows a Langhans giant cell, and the red arrows show megakaryocytes within the preserved bone marrow expanses (haematoxylin and eosin stain, 10x magnification).

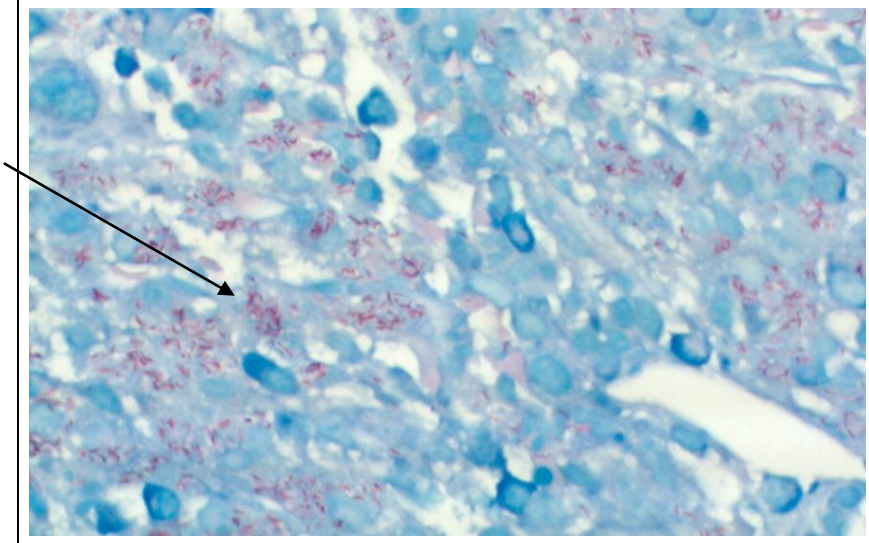
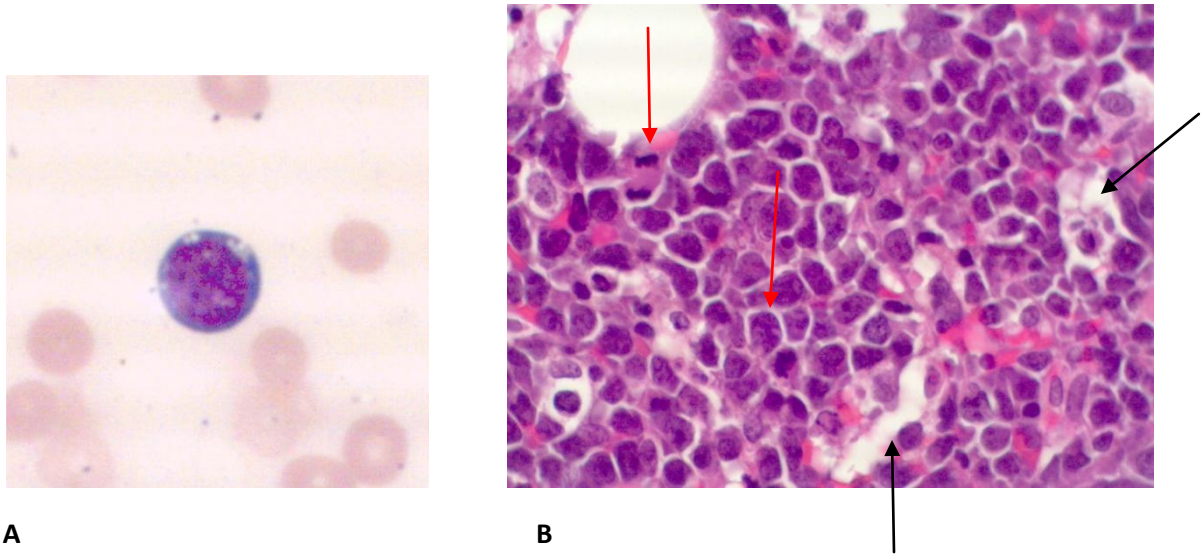
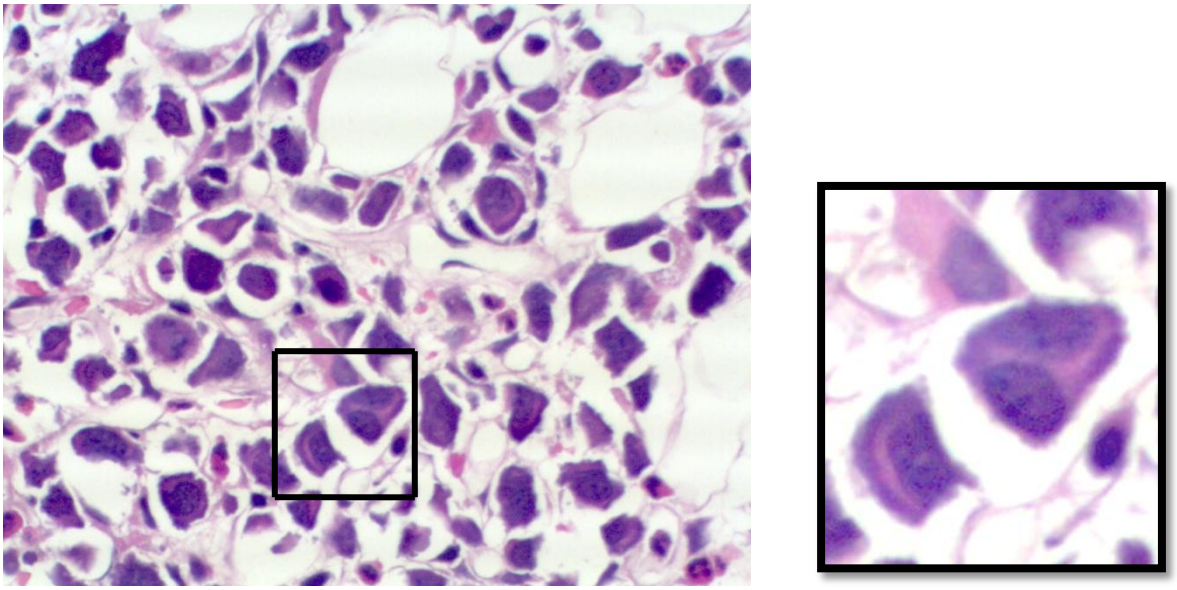


Figure 3.5: A high power view of a Ziehl-Neelsen stain showing macrophages containing numerous acid fast bacilli (see arrow) in one of the patient's with *Mycobacterium avium complex* infection (40x magnification).



A **B**

Figure 3.6: A shows the cytology of a tumour cell from one of the cases of large B- cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. The cell is intermediate in size with deeply basophillic, vacuolated cytoplasm (May-Grünwald-Giemsa stain, 50x magnification). **B** is histology of this case, showing tingible body macrophages (black arrows) and brisk mitotic activity (red arrows) (haematoxylin and eosin stain, 40x magnification).



A **B**

Figure 3.7: Histology of the case of plasmablastic lymphoma (haematoxylin and eosin stain, 40x magnification). **B** is an inset of the tumour cells, showing plasmacytoid morphology (100x magnification).

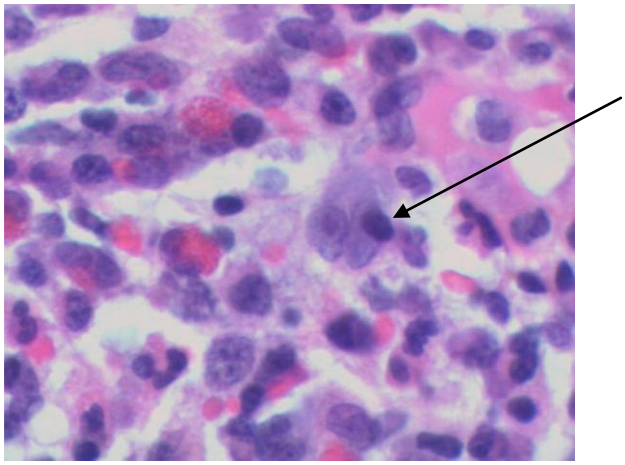
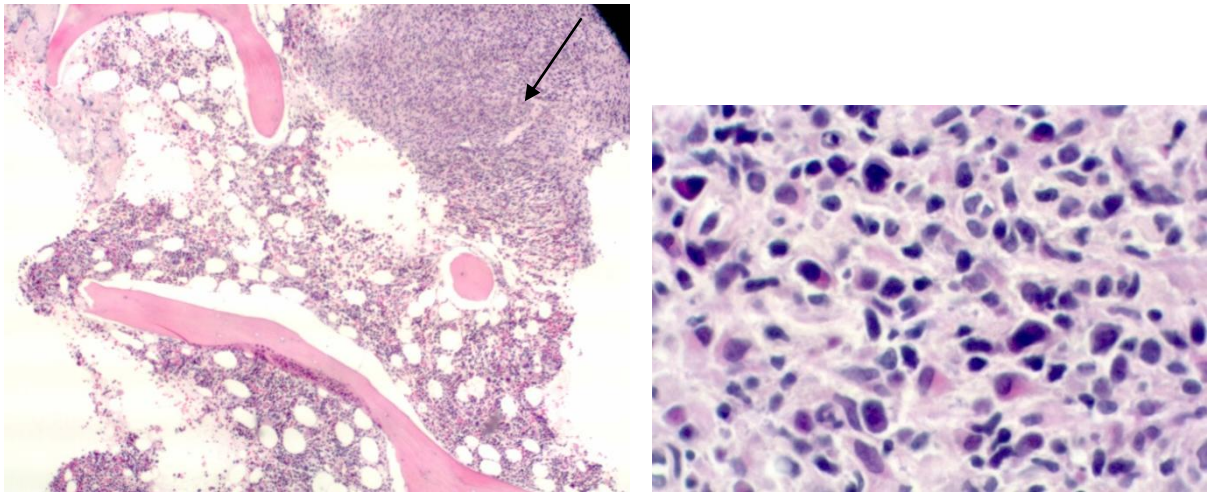
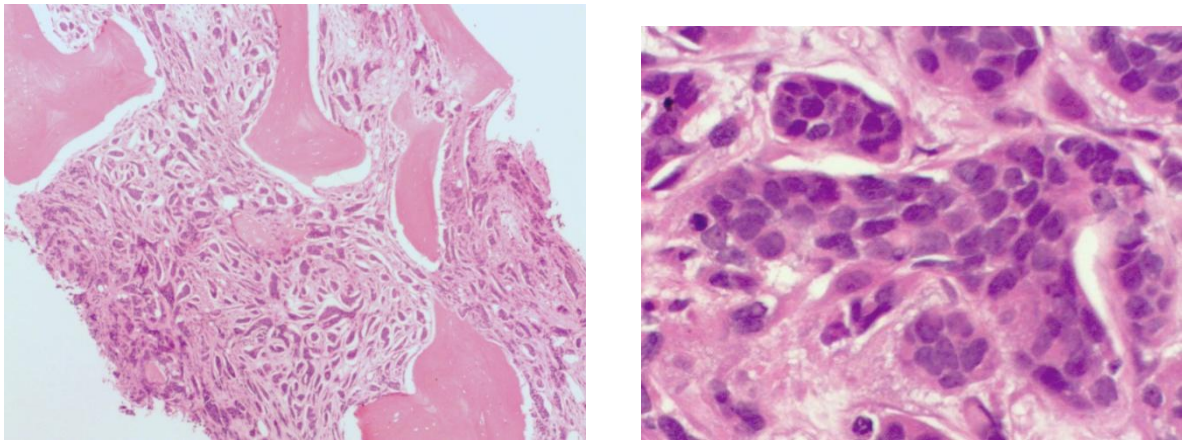


Figure 3.8: Histology of one of the cases of Hodgkin lymphoma. This picture shows a Reed-Sternberg cell (see arrow) in a rich inflammatory background (haematoxylin and eosin stain, 40x magnification).



A **B**

Figure 3.9: Histology of the case of peripheral T-cell lymphoma. In **A**, the area of infiltration is seen in the top right hand corner (see arrow), while normal haemopoiesis is preserved in the rest of the specimen (haematoxylin and eosin stain, 10x magnification). **B** shows a high power view of the infiltrate, which is comprised of hetereogeneously sized lymphocytes in a histiocyte-rich background (haematoxylin and eosin stain, 40x magnification).



A **B**

Figure 3.10 : **A** Extensive marrow infiltration by carcinoma of the breast (haematoxylin and eosin stain, 10x magnification). **B** is a high power view of non-haemopoietic cells forming pseudo-glandular structures (haematoxylin and eosin stain, 40x magnification).

Two patients were ultimately diagnosed with TTP (see Figure 3.11), and ten with ITP. One of the patients diagnosed with ITP had a co-existing peripheral T-cell lymphoma, and one of the patients with TTP also had TB complicated by a pleural effusion. Three of the patients with ITP and one of the patients with TTP had CD4 counts greater than 200x10^6/l. These four patients represent more than half of the patients without AIDS.

Fourteen patients were found to have hypocellular marrow, and in four of these, absolute megakaryocyte numbers appeared significantly reduced. Within the hypocellular group, two patients had positive bone marrow TB cultures without frank granuloma formation, and one patient had disseminated *Cryptococcus* infection (see Figure3.12).

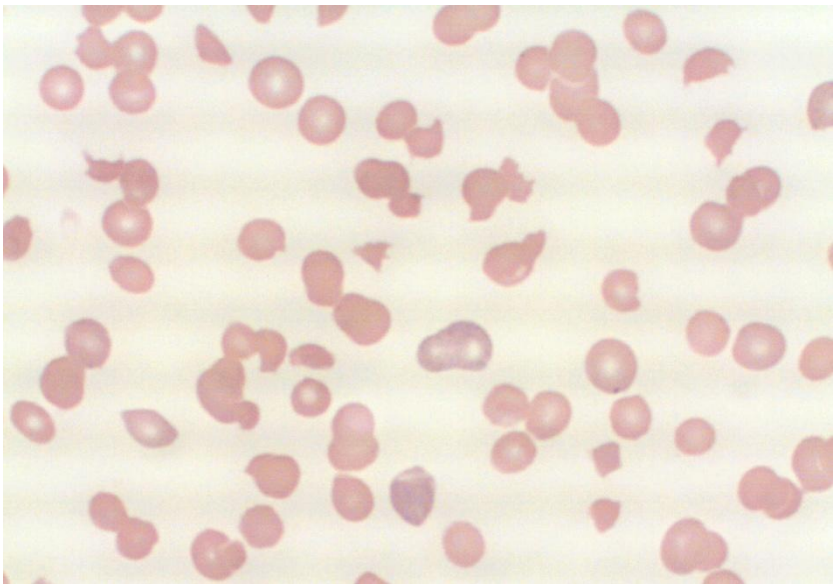
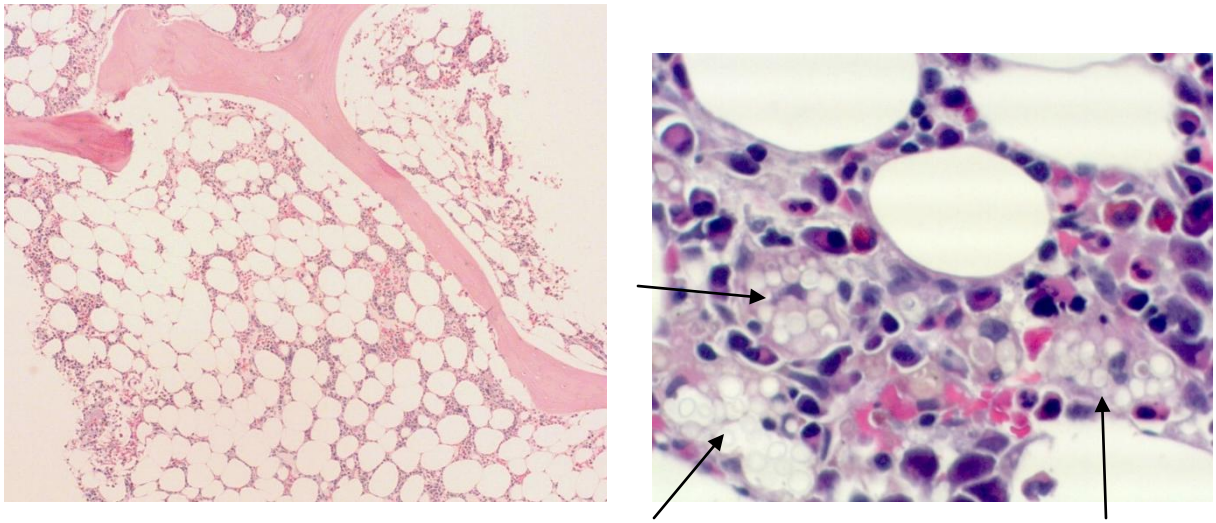


Figure3.11: Shows a case of TTP with marked red cell fragmentation and diffuse basophilia. No platelets are present in this field (May-Grünwald-Giemsa stain, 50x magnification).



A **B**

Figure 3.12: Disseminated *Cryptococcus* infection. **A** shows a low power view of the bone marrow trephine, demonstrating the hypocellular nature of the sample (haematoxylin and eosin stain, 4x magnification), and **B** shows a high power view of several macrophages laden with encapsulated fungal organisms (see arrows) (haematoxylin and eosin stain, 40x magnification).

Patients on ARVs had a similar spectrum of pathology to patients not on ARVs. Statistically, there was no difference between the prevalence of either ITP or TB between the two patient groups, while a slight trend was noted toward more malignancies in patients on ARVs, which was not found to be statistically significant (p=0.42) (see Table 3.5). Likewise, the mean CD4 and platelet counts did not differ between patients treated with ARVs as compared to those who were not (p=0.73 and 1 respectively), and their distribution of platelet grades appeared very similar (see Figure 3.13).

Table 3.5 : Distribution of pathology among patients on and not on ARVs.

	ARVs Y (N=20)	ARVs N (N=58)	Odds Ratio	p-value
BM TB (N=31)	8 (40%)	23 (40%)	1.01	1
ITP (N=10)	2 (10%)	8 (14%)	0.69	1
Malignant BM infiltration (N=8)	3 (15%)	5 (9%)	1.9	0.42

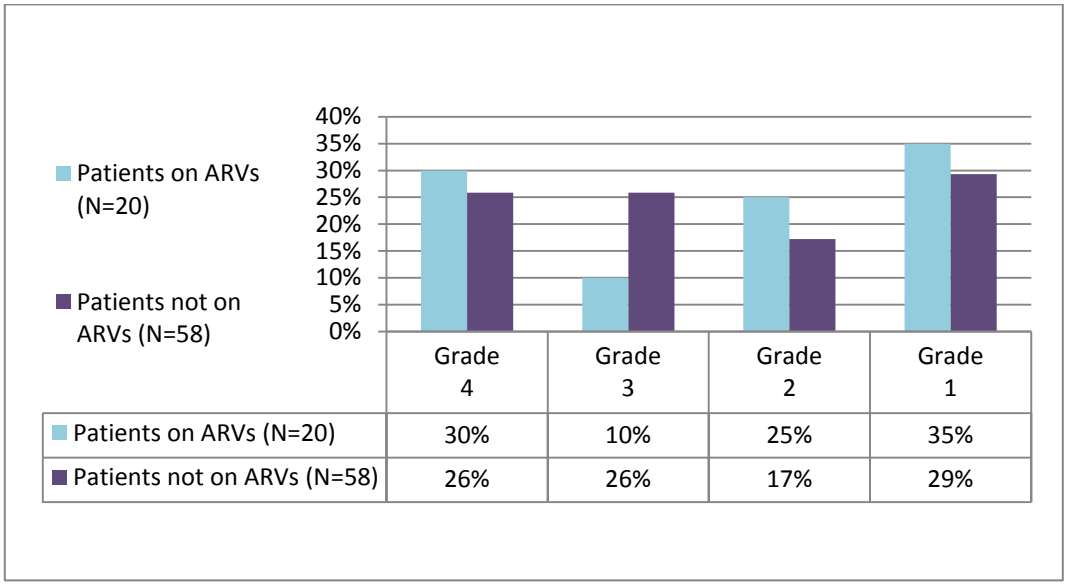


Figure 3.13: A comparison of the distribution of platelet grades among patients on ARVs as compared to those not on ARVs.

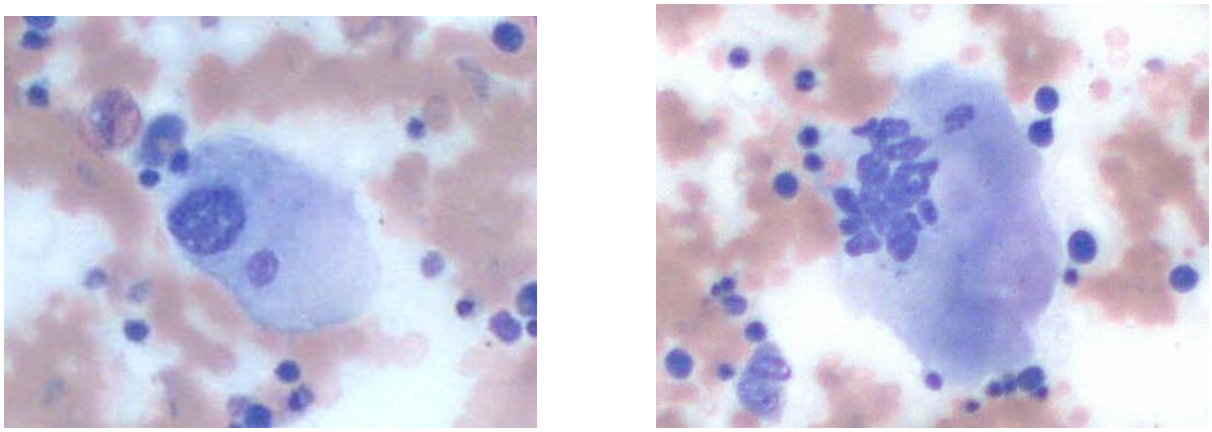
3.2.1.6: Bone marrow morphology findings:

The bone marrow aspirate and or trephine biopsy were adequate for morphological assessment in 72(92%) of the 78 patients. Among these samples, megakaryocyte numbers were considered adequate in 63 (88%). The remaining nine patients had either hypocellular marrow with an absolute reduction in all cell lines, or extensively infiltrated marrow. As a rule, megakaryocyte activity was

better judged on the trephine biopsy, with sample quality of the bone marrow aspirates rendering confident assessment of megakaryopoiesis difficult in 41 cases (57%). Megakaryocyte morphology assessment was performed if a minimum of 10 megakaryocytes were present in a specimen, and was therefore possible in 70 patients. Among these, disordered or degenerate megakaryocytes comprised greater than 10% of all megakaryocytes assessed in 53 (76%) patients, and more than 30% in 18 (26%).

Among patients with bone marrow infiltration by TB, 29 cases had sufficient megakayocytes present to assess megakarycoyte morphology. Of these, 27 (93%) were judged to have sufficient megakarycoyte numbers.

Figures 3.14, 3.15 and 3.16 show examples of abnormal megakaryocyte morphology from the cohort.



A **B**

Figure 3.14: Showing cytological examples of abnormal megakaryocytes. **A** shows hypolobation, while **B** shows hyperlobation (May-Grünwald-Giemsa stain, 50x magnification).

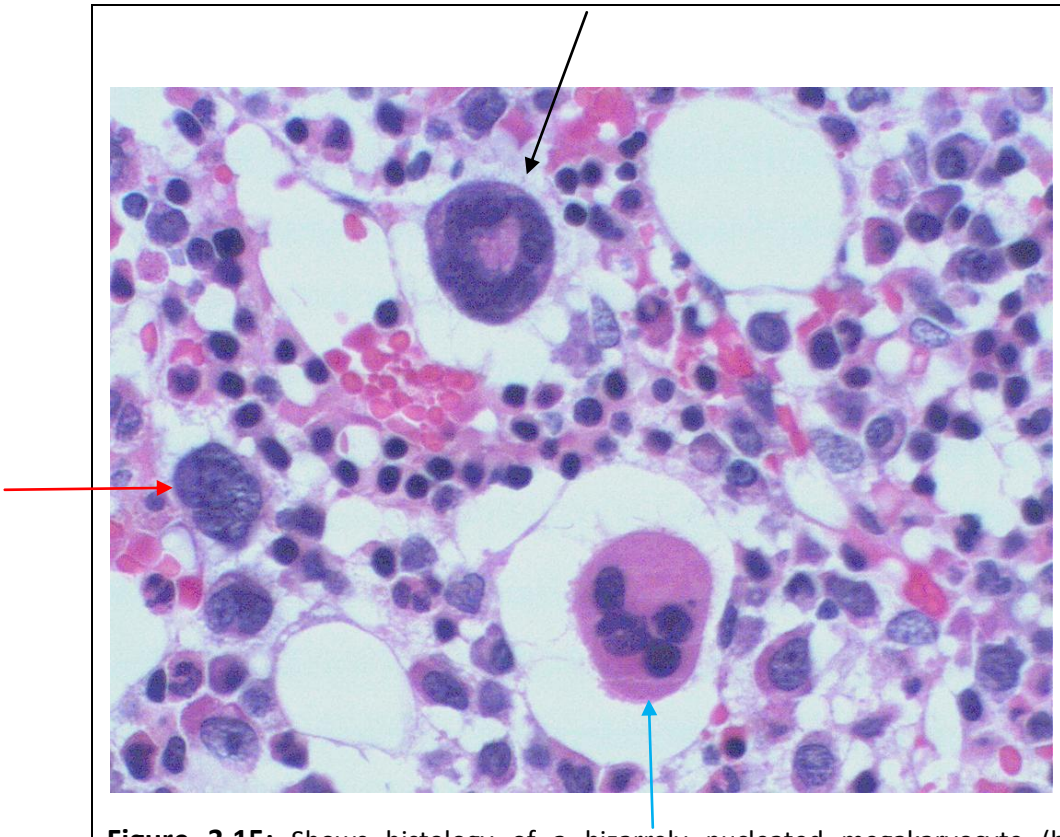


Figure 3.15: Shows histology of a bizarrely nucleated megakaryocyte (black arrow), a bare megakaryocyte nucleus (red arrow) and a morphologically unremarkable megakaryocyte (blue arrow) (haematoxylin and eosin stain, 40x magnification).

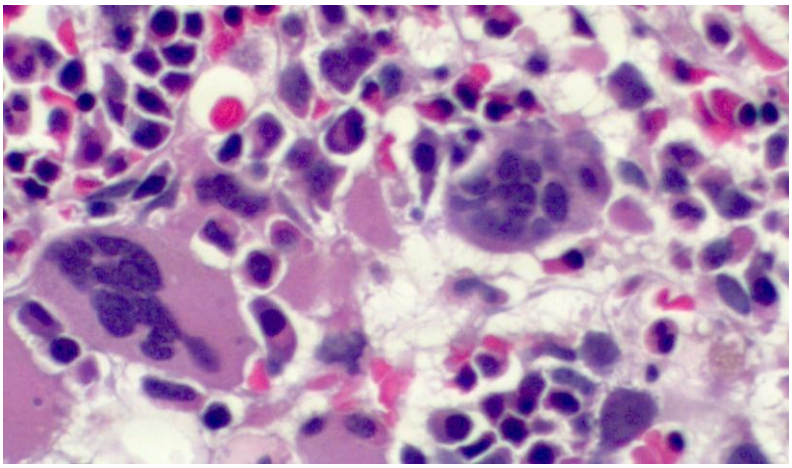


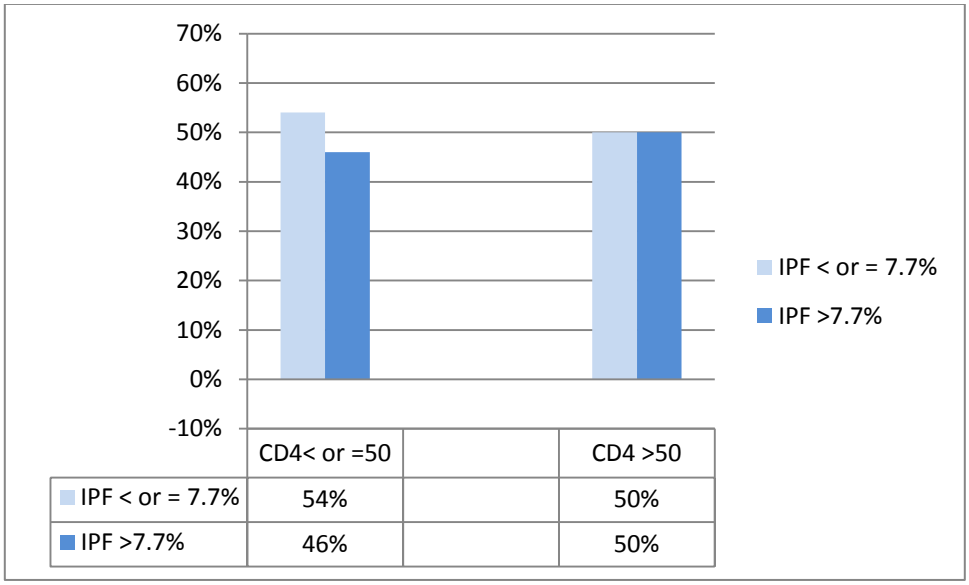
Figure 3.16: Shows histology of two hyerplobated megakaryocytes (haematoxylin and eosin stain, 40x magnification).

3.3 The IPF relationship to various variables of interest: (Summarized in Table 3.6)

Because of the very strong association between IPF and platelet count, the relationship between the IPF and all other variables of interest were adjusted for the thrombocytopenia grade by logistic regression.

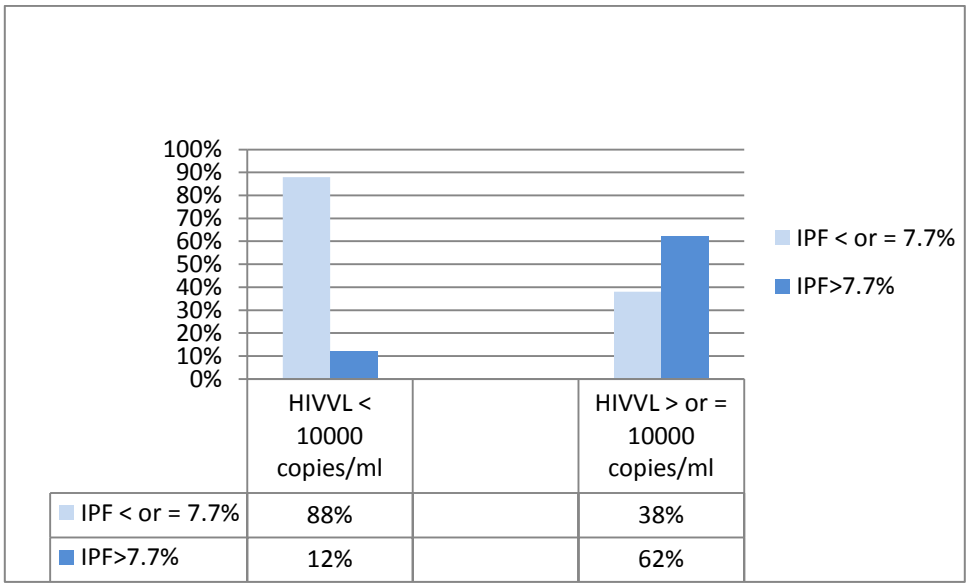
3.3.1 IPF in relation to HIV-related variables:

Because 90% of the cohort had AIDS, meaningful comparison between the IPF in patients with and without AIDS was not possible. Therefore, the IPF distribution was compared between patients with severe immunodeficiency (as defined by a CD4 count less than or equal to 50x10^6 cells/l) and patients with more moderate CD4 T-cell depletion. This revealed no difference in the proportion of patients with high versus low IPF values between these two groups (see Figure 3.17). Similarly, the IPF distribution was not different between patients on and not on ARVs. However, patients with lower viral loads showed a marginally significant tendency to have a low IPF as compared to patients with high viral loads (p=0.067) (see Figure 3.18). Unfortunately, logistic regression to eliminate the possible confounding effect of thrombocytopenia severity was not possible in this subgroup due to the very limited sample number, but the mean platelet count did not differ between the two groups (p=0.5) (see Figure 3.19).



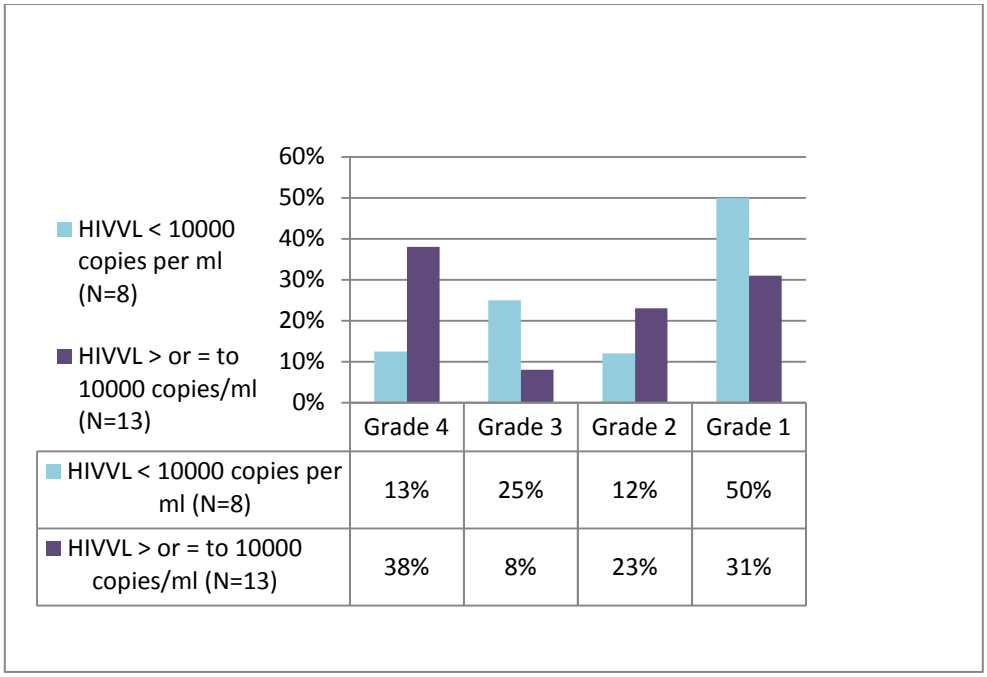
	CD4< or =50		CD4 >50
IPF < or = 7.7%	54%		50%
IPF >7.7%	46%		50%

Figure 3.17: A comparison of the IPF distribution among patients with severe immunodeficiency (CD4 < or = to 50) compared to those with more moderate immunodeficiency (CD4 >50) (N=70). This figure illustrates that the IPF distribution between these 2 groups is very similar.



	HIVVL < 10000 copies/ml		HIVVL > or = 10000 copies/ml
IPF < or = 7.7%	88%		38%
IPF>7.7%	12%		62%

Figure 3.18: A comparison of the IPF distribution among patients with low viral loads (< 10 000 copies per ml) compared to those with higher viral loads (N=21). This figure illustrates a markedly different IPF distribution between the 2 groups, with the proportion of patients having a low IPF being appreciably higher among those with low viral loads (p=0.067).



	Grade 4	Grade 3	Grade 2	Grade 1
HIVVL < 10000 copies per ml (N=8)	13%	25%	12%	50%
HIVVL > or = to 10000 copies/ml (N=13)	38%	8%	23%	31%

Figure 3.19: A comparison of the distribution of thrombocytopenia grade among patients with low viral loads (< 10 000 copies per ml) compared to those with higher viral loads. This figure illustrates a relatively marked difference between the groups, with patients with low viral loads tending to have more grade1/2 and less grade 3/4 thrombocytopenia overall.

3.3.2: IPF distribution in relation to underlying pathology:

Among patients with TB (both with and without bone marrow involvement), no difference in the IPF distribution was noted as compared to patients without TB. Patients with ITP and TTP showed a significantly higher proportion of cases with a high IPF as compared to patients without these conditions. However, when adjusted for the confounding effect of thrombocytopenia grade, this association lost statistical significance due to the high proportion of patients (8 (67%) out of 12) within this subgroup having severe thrombocytopenia. In contrast, patients with hypocellular bone marrow had a significant increase in the proportion of cases with a low IPF as compared to patients

with normal or increased cellularity, which remained statistically significant when adjusted for the effect of thrombocytopenia severity.

Unfortunately, meaningful statistical analysis was not possible among the patients with malignant infiltration of the bone marrow due to the small sample number and the heterogeneous nature of the pathologies represented. Therefore, patients in whom the broad mechanism of thrombocytopenia was likely to be similar (i.e. a product of bone marrow dysfunction as opposed to peripheral destruction or consumption) were pooled for the purposes of statistical analysis in order to counter the limitations of sample size. Among patients with either hypocellular marrow or extensive marrow infiltration, (in whom the cause of thrombocytopenia is most likely central in origin), a trend was noted towards the occurrence of a low IPF level more frequently as compared to the global cohort. This difference became statistically significant on adjustment for the effects of the thrombocytopenia grade ($p= 0.0465$).

3.3.3: IPF in relation to megakaryocyte morphology:

A trend was noted toward the more frequent occurrence of a low IPF level in patients with a significant degree of megakaryocyte disorder. However, this weak association was lost on adjustment for thrombocytopenia grade. Similarly, no relationship with the IPF could be established among patients with more gross megakaryocyte dysplasia (as defined as morphological atypia in more than 30% of megakaryocytes assessed).

Table 3.6: Summary of the relationship between the IPF and variables of interest.

	Mean IPF:	Proportion with a low IPF	Crude Odds Ratio	95% Confidence interval	p-value	Adjusted Odds Ratio	95% Confidence Interval	p-value
CD4 < or = 50 (N=28)	9.2%	54%	1.15	(0.45-3.0)	0.81	0.75	(0.23- 2.5)	0.64
CD4>50 (N=42)	9.9%	50%						
ARVs Y (N=20)	9.2%	50%	0.87	(0.30- 2.4)	0.8	0.74	(0.20- 2.7)	0.65
ARVs N (N=58)	9.6%	53%						
HIV VL > or = 10 000 cp/ml (N=13)	10.7%	39%	0.09	(0.01- 1.4)	0.067	-	-	-
HIV VL < 10 000 cp/ml (N=8)	6%	88%						
BM TB (N=31)	9%	58%	1.4	(0.52- 3.4)	0.54	0.85	(0.27- 2.7)	0.77
No evidence of BM TB (N=47)	9.9%	49%						
TB at any site (N=38)	9%	53%	1.01	(0.42-2.4)	1	0.95	(0.32- 2.8)	0.94
No evidence of current TB (N=40)	9.9%	53%						
ITP/TTP (N=12)	14.8%	25%	0.25	(0.06- 1.0)	0.058	0.57	(0.1-3.0)	0.5
Non- ITP/TTP (N=66)	8.5%	58%						
Hypocellular Marrow (N=14)	6.2%	79%	4.16	(1.12-15.0)	0.04	5.6	(1.2- 27.1)	0.032
Normo- or hypercellular marrow (N=58)	10.4%	47%						
Hypocellular OR extensively infiltrated marrow (N=18)	7%	67%	2	(0.7-6.2)	0.19	4	(1-16.2)	0.047
Normo/Hypercellular marrow without extensive marrow infiltration (N=54)	10.4%	48%						
< or = 10% Disordered megakaryocytes (N=17)	12.5%	41%	0.5	(0.16-1.5)	0.27	0.8	(0.21- 3.1)	0.75
>10% Disordered megakaryocytes (N=53)	8.6%	58%						
< or = 30% Disordered megakaryocytes (N=52)	9.9%	48%	0.69	(0.23-2.1)	0.59	1.1	(0.25- 4.6)	0.9
>30% Disordered megakaryocytes (N=18)	8.6%	61%						

Table 3.7: IPF distribution among patients with grade 4 thrombocytopenia with and without ITP/TTP.

	Proportion with a low IPF	**Odds Ratio**	**95% Confidence Interval**	**p-value**
ITP/TTP (N=8)	14%	0.48	(0.06-4.3)	1.0
Non-ITP/TTP (N=13)	23%			

3.4 Platelet count response analysis:

3.4.1 Agreement between the platelet count measured on the Sysmex XE-5000 and the platelet count measured in the routine laboratory:

A platelet count from the routine haematology laboratory was available in 68 of the 78 patients.

Bland-Altman analysis revealed no mean difference between platelet counts measured in the routine laboratory and the platelet count measured during IPF analysis, with 95% limits of agreement ranging from -19x10^9/l to 19x10^9/l (see Figure 3.20). Therefore, the instruments showed good, but imperfect agreement.

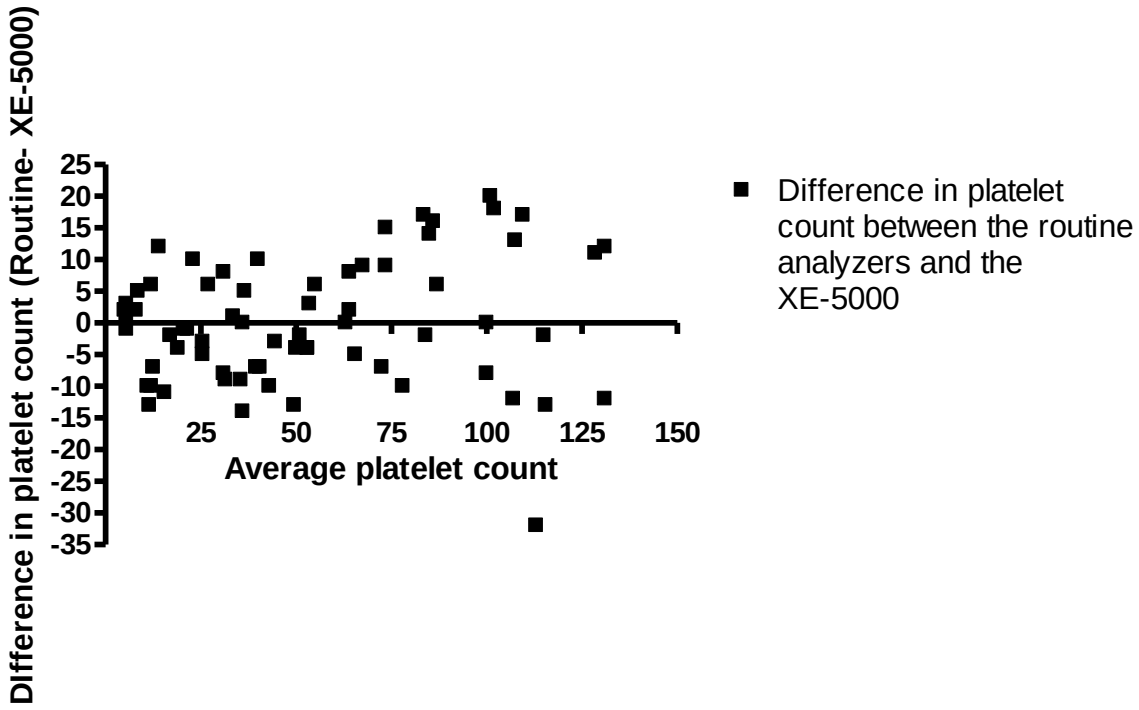


Figure 3.20: The results of Bland-Altman analysis of the difference between platelet counts measured in the routine laboratory and those measured on the Sysmex instrument relative to the average of the 2 platelet counts (N=68). The graph shows acceptable agreement between the 2 sets of instruments with very few outliers. The mean difference between platelet counts was -0,03x10^9/l, with 95% limits of agreement ranging from -19x10^9/l to 19x10^9/l.

As follow-up platelet counts were only available from the routine laboratory, response assessment was only analyzed amongst those patients for whom a baseline routine platelet count was available in the interests of maximum consistency (in view of the small differences in platelet count measurements between the two sets of instruments). The follow-up platelet count was compared with the original routine platelet count and not the platelet count measured on the XE-5000.

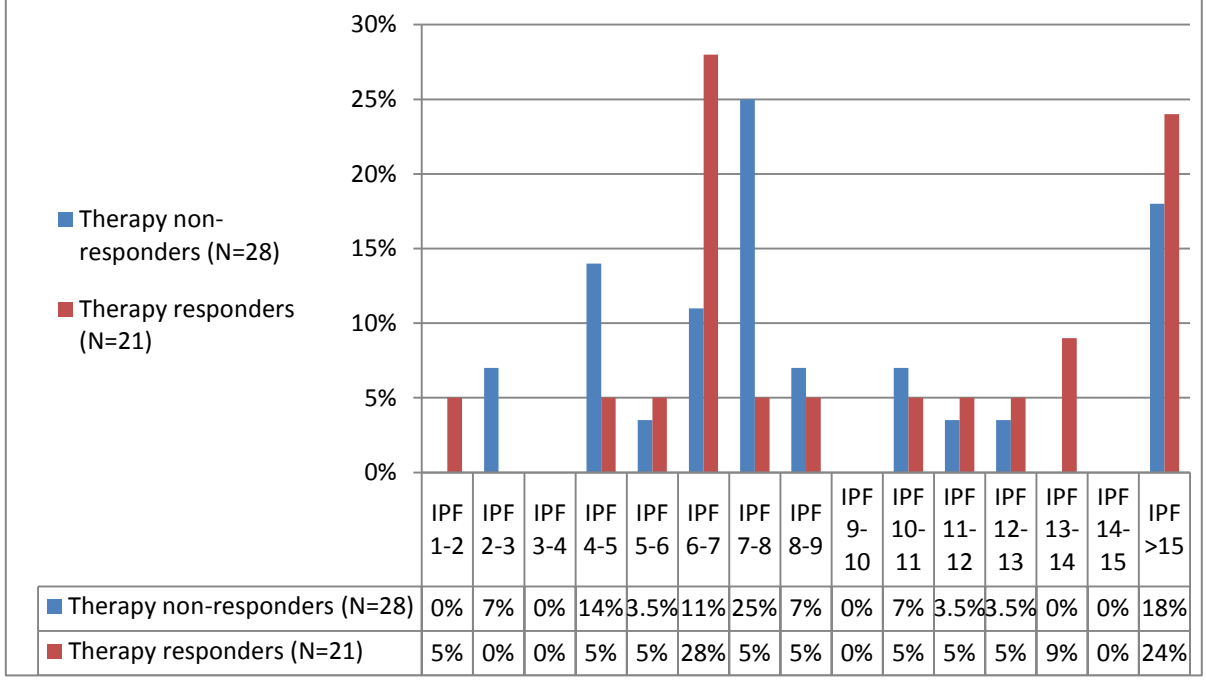
Therapy response data was therefore available in 49 patients; a minimum of 3 days after the bone marrow investigation was performed.

3.4.2 Platelet count responses:

Of the 49 patients with both baseline and follow-up routine platelet counts, 21 (43%) showed a positive response in their platelet count, and 28 (57%) did not. The IPF distribution in the 49 patients was not different from the total cohort (p=0.53).

Amongst the 21 therapy responders, the mean platelet count was 46x10^9/l and the IPF was high in 11 (52%), while the mean platelet count was 47x10^9/l with a high IPF in 13 (46%) of the 28 therapy non-responders. The proportion of patients with a high IPF was therefore not statistically different between therapy responders and non-responders.

On analysis of the spread of various IPF levels between the two groups, the distribution of very high and very low IPFs appeared similar (see Figure 3.21).



	IPF 1-2	IPF 2-3	IPF 3-4	IPF 4-5	IPF 5-6	IPF 6-7	IPF 7-8	IPF 8-9	IPF 9-10	IPF 10-11	IPF 11-12	IPF 12-13	IPF 13-14	IPF 14-15	IPF >15
Therapy non-responders (N=28)	0%	7%	0%	14%	3.5%	11%	25%	7%	0%	7%	3.5%	3.5%	0%	0%	18%
Therapy responders (N=21)	5%	0%	0%	5%	5%	28%	5%	5%	0%	5%	5%	5%	9%	0%	24%

Figure 3.21: The IPF distribution in the therapy responders (red) as compared to therapy non-responders (blue) (N=49).The distribution of both very high and very low IPFs appears similar between the 2 groups.

Further analysis of the 28 patients who failed to respond to therapy revealed that 13 (46%) actually experienced a fall in their platelet count over follow-up, while 7(25%) experienced an improvement in platelet count of less than 50%. However, eight (29%) of the patients categorized as non-responders showed a partial response to therapy, with a greater than 50% improvement in their platelet count. Four of these eight patients had severe thrombocytopenia at presentation, and had more than doubled their platelet count over the follow-up period, but had not achieved a platelet count over 30x10^9/l (see Table 3.8).

Table 3.8: Four patients categorized as therapy non-responders because their platelet count had not achieved a level above 30x10^9/l.

Original platelet count (x10^9/l)	Follow-up platelet count (x10^9/l)
6	15
7	20
10	24
6	27

Two of the remaining four patients had a baseline platelet count between 20 and 25x10^9/l, which improved in both cases by greater than 50% and achieved a level greater than or equal to 30x10^9/l, but did not double.

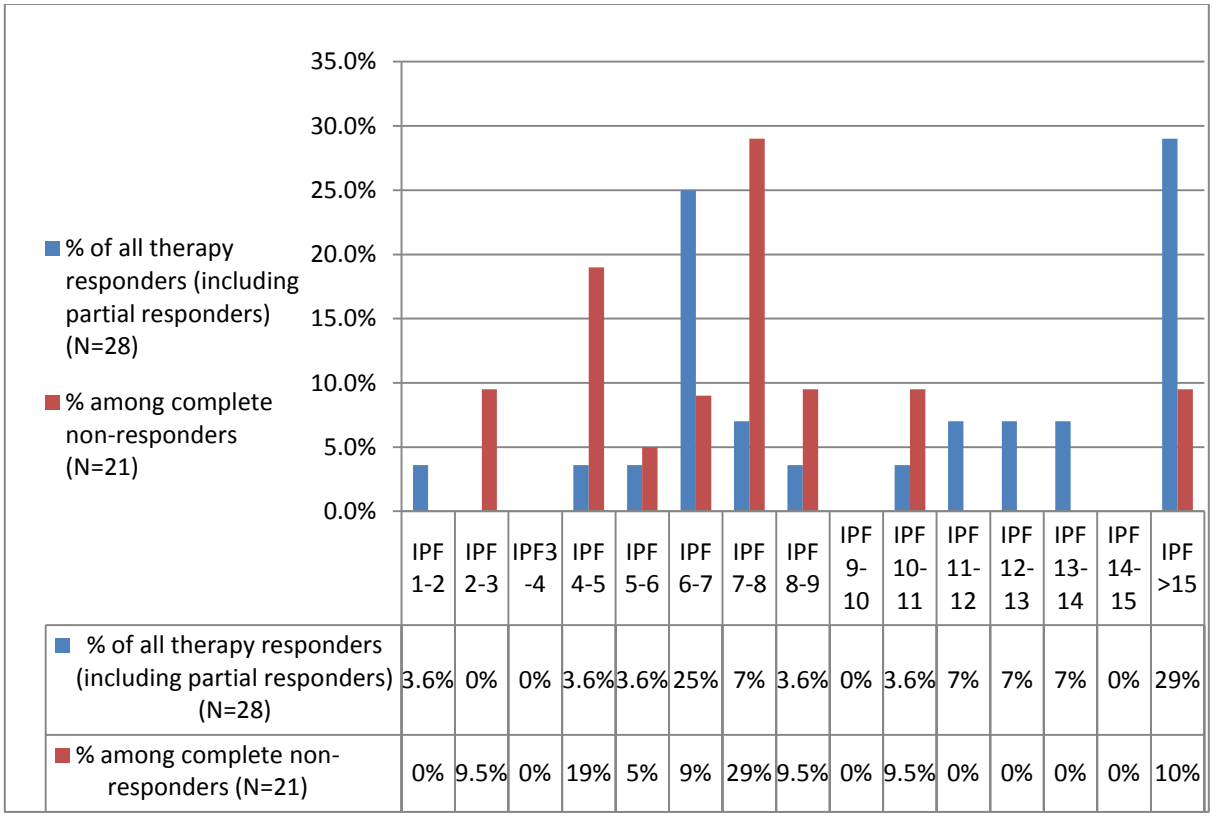
Among the eight non-responders who had experienced a minimum of a 50% improvement in their platelet count, six (75%) had a high IPF, while among the other 20 non-responders, only seven (35%) had a high IPF. In the 13 patients whose platelet counts dropped, six (46%) had a high IPF.

The mean platelet count was significantly lower in the partially responding group (mean platelet count= 19x10^9/l) as compared to the complete non-responders (mean= 53x10^9/l) (p-value: 0.012).

Because it would be clinically useful to be able to distinguish patients who were going to experience at least a partial response in their platelet count (to a minimum level greater than 20x10^9/l, which is considered to be safe from spontaneous haemorrhage [16]) from those who were not going to respond at all, further analysis was carried out to assess the power of the IPF to distinguish these two patient subsets from each other. Any patient who showed a significant change in the platelet count, but did not achieve a level greater than 20x10^9/l was classified as a complete non-responder.

Among the 28 patients who were either complete or partial therapy responders, 16 (57%) had a high IPF, while only 8 (38%) had a high IPF among the 20 patients classified as complete non-responders. Despite this apparent difference in the number of high IPF levels between the two groups, the sample numbers were insufficient to prove statistical significance (p=0.25). The mean platelet count was 41x10^9/l and 54x10^9/l respectively in these groups, and again, this difference was not found to be statistically significant (p=0.19).

Closer inspection of the data reveals that the distribution of very high IPF levels is significantly different in the two populations. Amongst the 28 patients who show at least a significant positive trend in their platelet count, 15(54%) had an IPF greater than 10%, and amongst the 21 patients with very poor responses to treatment, 15 (72%) had an IPF less than 8% (see Figure 3.22).



	IPF 1-2	IPF 2-3	IPF3 -4	IPF 4-5	IPF 5-6	IPF 6-7	IPF 7-8	IPF 8-9	IPF 9-10	IPF 10-11	IPF 11-12	IPF 12-13	IPF 13-14	IPF 14-15	IPF >15
% of all therapy responders (including partial responders) (N=28)	3.6%	0%	0%	3.6%	3.6%	25%	7%	3.6%	0%	3.6%	7%	7%	7%	0%	29%
% among complete non-responders (N=21)	0%	9.5%	0%	19%	5%	9%	29%	9.5%	0%	9.5%	0%	0%	0%	0%	10%

Figure 3.22: The IPF distribution among all therapy responders (including partial responders) as compared to complete non-responders (N=49). The IPF distribution among patients with at least a partial therapy response (blue) appears dissimilar to the IPF spread among those patients who were complete non-responders (red), with the former group showing a predominance of IPF levels greater than 10%, and the latter group generally having an IPF of <8%.

In view of the apparent predominance of patients with an IPF level greater than 10% among those who showed a positive trend in their platelet count over time, further subgroup analysis was performed using 10% as a novel IPF cut-off. Results are displayed in Table 3.9 below:

Table 3.9: Summary of the results of contingency table analysis of platelet count response in relation to IPF, with an IPF cut-off of 10%.

	IPF>10
Sensitivity	54%
Specificity	81%
Positive predictive value	79%
Negative predictive value	57%
Odds Ratio (of positive platelet response if IPF >10%)	4.9
95% Confidence Interval	1.4-17.4
P-value	0.019

To best identify patients who show complete non-response to therapy, further analysis was performed to identify the best IPF cut-off to discriminate this sub-group of patients. Results are summarized in Table 3.10 below:

Table 3.10: Results summary of contingency table analysis of complete failure to respond to therapy in relation to IPF.

	IPF <8	**IPF<6**
Sensitivity	71%	33%
Specificity	57%	89%
Positive predictive value	56%	70%
Negative predictive value	73%	64%
Odds Ratio (of no platelet response if IPF low)	3.3	4.2
95% Confidence Interval	1-10.9	0.99-17.2
P-value	0.081	0.076

3.4.3 Analysis of short –term platelet count response among various patient subsets.

Results of analysis of platelet count response (as originally defined in 2.9.5) among patient subsets of clinical interest are summarized in Table 3.11.

Briefly, analysis revealed significantly better responses in patients on anti-retroviral therapy and a trend toward worse platelet responses among patients with a CD4 count less than 50, those with high viral loads (i.e.>10 000 copies per ml), and those with bone marrow infiltration by TB. No significant differences were present between the responses in patients with platelet counts less than 50x10^9/l as opposed to those with higher platelet counts, or in those with or without a significant degree of megakaryocyte dysplasia. The latter was assessed among patients with less than or equal to 10% megakaryocyte disorder as compared to those with more than 10% disorder, as well as in patients with disorder in less than or equal to 30% of their megakaryocytes as compared to those with more than 30% morphological dysplasia.

Table 3.11: Summary of platelet count responses in various patient sub-groups.

	Proportion without a platelet count response.	**Odds Ratio**	**95% Confidence Interval**	**P-value**
Plts < or = 50 (N=30)	57%	0.95	(0.3- 3.0)	1
Plts >50 (N=19)	58%			
Megakaryocyte disorder >10% (N=31)	58%	1.62	(0.4-6.1)	0.52
Megakaryocyte disorder < or = 10% (N=13)	46%			
Megakaryocyte disorder >30% (N=10)	40%	0.47	(0.1-2.0)	0.472
Megakaryocyte disorder < or = 30% (N=34)	58%			
BM TB infiltration Y (N=19)	68%	2.2	(0.67-7)	0.25
BM TB infiltration N (N=30)	50%			
CD4< or= 50 (N=15)	67%	2	(0.6-7)	0.352
CD4>50 (N=30)	50%			
ARV treatment N (N=35)	69%	5.5	(1.2-23.9)	0.023
ARV treatment Y (N=14)	29%			
HIV Viral Load (HIVVL) >10 000 (N=7)	86%	12	(0.97-132)	0.1
HIVVL <10 000 (N=6)	33%			

Chapter 4

4.0 Discussion:

4.1 Study limitations:

- The sample number is relatively small, making statistically significant findings in patient subgroups unfeasible in some instances.
- 90% of the cohort had AIDS, which is likely to reflect the frequency of symptomatic thrombocytopenia in the setting of HIV. Asymptomatic persons with thrombocytopenia are under-represented, possibly because they are less likely to seek medical attention. The findings are therefore pertinent predominantly in thrombocytopenic patients with AIDS, and cannot necessarily be extrapolated to non-AIDS patients.

4.2 General cohort characteristics:

The findings are highly representative of the wide spectrum of pathologies that patients with AIDS are vulnerable to. In particular, the prevalence of TB in this cohort is remarkable, with nearly half of all of the patients having confirmed mycobacterial infection. Although these findings may be somewhat biased by the fact that a number of patients had a bone marrow investigation performed because TB was suspected clinically (and not to investigate the thrombocytopenia *per se*), the major burden of TB in South Africans with AIDS is nonetheless clearly highlighted. Among patients without AIDS, a very significant prevalence of immune-mediated thrombocytopenia was noted, while disseminated TB did not occur. Overall, therefore, the spectrum of disease seen in both patients with

and without AIDS concurred with the previously described pathology patterns in these respective groups [1].

The proportion of patients with AIDS in the cohort receiving ARV therapy also correlates closely with published South African epidemiological data in this regard [3]. Interestingly, although patients on ARVs showed evidence of virological control (as manifest by a reduction in their viral loads), the majority had persistently low CD4 counts, and similar prevalence's of disseminated TB, ITP and malignancies as compared to patients not on ARVs .

4.3 General IPF findings:

The distribution of high and low IPF levels in patients with various grades of thrombocytopenia was found to be significantly different, with low IPF levels being far more prevalent among patients with mild reductions in their platelet counts, and high IPF levels being more common in patients with more severe thrombocytopenia. This finding suggests that a degree of marrow suppression is the dominant cause of mild thrombocytopenia in patients with AIDS, while peripheral mechanisms predominate among patients with lower platelet counts (most notably in those with grade four thrombocytopenia). This may reflect a general tendency for peripheral mechanisms to deplete the circulating platelet mass to a greater extent than marrow suppression does in this setting. Alternatively, this finding may be a consequence of a blunting of the marrow's response to mild thrombocytopenia in AIDS, regardless of the mechanism, so that an appreciable marrow response is only evident when the platelet count falls below a certain threshold.

4.4 IPF findings in patient subgroups:

4.4.1 The IPF in patients with hypocellular or extensively infiltrated marrow and ITP/TTP:

Patients with hypocellular or extensively infiltrated marrow were significantly more likely to have a low IPF than patients with normal or increased cellularity without extensive marrow infiltration (when adjusted for the effects of thrombocytopenia grade), while patients with ITP/TTP showed a marginally significant trend toward having a low IPF level less frequently as compared to the rest of the cohort. Unfortunately, the latter finding lost statistical significance on adjustment for the platelet count as a result of the heavy prevalence of grade four thrombocytopenia in patients with ITP/TTP, (comprising 67% of cases in this sub-group). As IPF levels tended to be elevated in the vast majority of patients with grade four thrombocytopenia, (77% in those with non-ITP/TTP, see Table 3.7), the apparently higher proportion of high IPF levels in severely thrombocytopenic patients with ITP/TTP (86%) was not dramatic enough to achieve statistical significance in the context of the small sample size. Nonetheless, the findings in the above patient sub-groups illustrate very clearly that the IPF reliably reflects the mechanism of thrombocytopenia in HIV infected patients in those cases in which the cause of the thrombocytopenia is readily apparent by virtue of a combination of the clinical and morphological features (as has been demonstrated in HIV-negative patients in the literature previously [44, 45]). This finding therefore lends the IPF weight as a tool to demonstrate the mechanism causing thrombocytopenia in HIV positive patients when this mechanism is more obscure.

4.4.2 The IPF in relation to HIV-related variables:

No association was present between the IPF and either the CD4 count or ARV therapy, suggesting that the causes and severity of thrombocytopenia are heterogeneous in these two subgroups. However, a marginally significant trend toward the occurrence of a high IPF level in patients with higher viral loads was noted. On closer inspection, it is apparent that the IPF distribution in the patients with higher viral loads is similar to that of the majority of the cohort, and that the actual difference is in the significant number of low IPF levels among the patients with low viral loads. It is noted that 4(50%) of the patients with very low viral loads have grade one thrombocytopenia as compared to 4(31%) of those patients with high viral loads, while only 1(13%) patient in the group with low viral loads has grade four thrombocytopenia, as compared to 5(38%) of the patients in the group with higher viral loads (see Figure 3.17). Therefore, it would appear that the difference in IPF distribution between patients with high and low viral loads is a product of a difference in the distribution of the severity of thrombocytopenia between these two groups (despite similar mean platelet counts). As all but one of the patients with low viral loads were on ARVs (86%) as compared to only one of the 13 patients with high viral loads (8%), the possibility that ARVs mediate this protection from grade four thrombocytopenia requires consideration. However, the distribution of grade one and grade four thrombocytopenia does not appear different between patients who are and who are not on ARV therapy in the global cohort (see Figures 3.1 and 3.12). This therefore suggests that the positive effects of ARVs on the platelet count only manifest once virological control has been established. Because the CD4 count showed no relationship with the viral load ($p=0.73$), we can infer that immune reconstitution is not the principle protective factor at play. Rather, it would appear that direct viral effects of HIV itself are a very important contributor to severe thrombocytopenia.

4.4.3 The IPF in relation to underlying TB:

No significant difference was found in the IPF distribution among patients with and without TB, regardless of whether or not the bone marrow was infiltrated. Two possible explanations for this finding have been considered. The first is that unproven mycobacterial infection may be present in a significant proportion of patients included in the group without definite evidence of TB, which could be blurring any differences between the two groups. This is a very real possibility, given the imperfect sensitivity of both microscopy for acid fast bacilli and TB culture [49]. In support of this possibility is the fact that three patients included in the non-TB group had sufficient radiological and clinical evidence to merit initiation of TB treatment, and a further three patients had been on TB treatment for between three weeks and three months. Collectively, these patients comprised 15% of the patients without current, proven mycobacterial infection.

Secondly, the absence of any difference in IPF among patients with and without TB may reflect a similar degree of heterogeneity in the underlying mechanism causing thrombocytopenia between the two groups, even among patients with definite bone marrow pathology (in whom one might expect platelet production failure to be the principle mechanism at play). Indeed, patients with disseminated TB are at risk of many causes of peripherally mediated thrombocytopenia, including hypersplenism [50] and DIC [51] (the latter particularly in the context of the acute respiratory distress syndrome (ARDS) [52] and the haemophagocytic syndrome [53]). In addition, both ITP [54, 55] and TTP [56] have been reported in association with TB. In this cohort, four (11%) patients diagnosed with TB had splenomegaly, one (3%) had TTP and three (8%) had laboratory evidence of underlying DIC. The high IPF levels in many of these patients suggests that these mechanisms are dominant contributors to the thrombocytopenia in some cases, and that bone marrow stunting is not necessarily the cause for cytopenia's in patients with frank marrow infiltration. However, it is noteworthy that the IPF level was low in nearly 60% of cases with bone marrow TB, despite adequate megakaryocyte numbers in

more than 90% of those with samples adequate for assessment of megakaryopoiesis. Clearly, central mechanisms (such as ineffective megakaryopoiesis or haemophagocytosis) are important contributors to the thrombocytopenia in this setting.

4.4.4 The IPF in relation to Megakaryocyte Morphology:

The morphological impression of megakaryocyte adequacy in more than 80% of cases is disparate with the presence of a low IPF in more than half of the cohort. In addition, the presence of a significant degree of megakaryocyte dysplasia showed no relationship with the IPF (as a reflection of megakaryocyte function). These findings highlight the limitations of morphological analysis to correctly assess the underlying mechanism of thrombocytopenia, and demonstrate a possible role for the IPF in enhancing the morphologist's ability to interpret the morphological characteristics.

4.5 Short term platelet count response

4.5.1 Therapy response prediction:

The results show that an IPF greater than or less than 7,7% is a poor predictor of the short term platelet response to therapy when platelet response is defined strictly according to the recommendations made in reference 46. However, when therapy response is defined more loosely as a greater than 50% improvement in platelet count provided that the follow-up platelet count is more than 20x10^9/l, (which is generally believed to be the threshold whereat the risk of spontaneous bleeding is increased) as compared to patients who showed either very minimal changes in their platelet count or in fact a drop in their platelet count, a trend toward more high IPF levels among therapy responders becomes apparent. Although the sample number was inadequate to prove

statistical significance of this difference, the distribution of very high IPF levels appears skewed, favoring the group who showed some degree of therapy response. When the power of a higher IPF cut-off (i.e.10%) to predict platelet count response to treatment was tested, it was shown that an IPF over 10% has strong, statistically significant specificity and positive predictive value for a minimum of a partial response (both in the region of 80%). Similarly, an IPF of less than 6% had a specificity of 89% and a positive predictive value of 70% for complete non-response to therapy (p=0.068). In summary, the findings suggest that a thrombocytopenic patient with an IPF greater than 10% has a 79% chance of showing some degree of recovery in their platelet count (at least to a level greater than 20x10^9/l) in the short term. Conversely, a patient with an IPF of less than 6% has a 70% chance of showing no imminent improvement in their platelet count.

Of interest was the finding in 13 patients whose platelet counts had dropped over the course of follow-up, nearly half of whom had a high IPF. The latter finding is likely to reflect the presence of unchecked peripheral mechanisms causing the thrombocytopenia. Indeed, upon review of the clinical presentations of these patients, three of the six were found to have splenomegaly. Therefore, because splenomegaly is fairly unlikely to recede rapidly, the effects of hypersplenism are likely to persist for some time. In keeping with this surmise, is the fact that of the 7 patients in the cohort with splenomegaly and a follow-up platelet count, only 1 had a satisfactory improvement in the platelet count over the brief follow-up period.

4.5.2 Platelet count response among patient sub-groups:

Among the patient sub-groups, it is interesting to note that patients on ARVs showed significantly better platelet count responses than those not on ARVs, despite having very similar mean platelet counts and distribution of pathologies (see Table 3.5 and Figure 3.12). In fact, the relationship between ARV therapy and platelet count recovery was stronger than the association between CD4

count and the platelet count response. This finding would suggest that ARV therapy enables more rapid recovery from the underlying mechanism causing the thrombocytopenia in a manner independent of CD4 count. Because ARV therapy would be expected to reduce the viral load, the beneficial effects observed may be a consequence of reduced megakaryocyte suppression as caused by the virus *per se,* which may then produce a better degree of megakaryocyte fitness in this patient group. This hypothesis is supported by the apparently better platelet count response in patients with low viral loads (i.e. <10 000 copies per ml) as compared to those with higher viral loads. Unfortunately, the latter finding did not reach statistical significance (p=0.1), as a result of the very small number of patients with both a viral load result and a follow-up platelet count available (N=13).

Lastly, the absence of any relationship between the short term platelet response and the presence of megakaryocyte disorder is of interest, and suggests that the morphological characteristics of megakaryopoiesis are a poor reflection of megakaryocyte fitness.

4.6 Case studies illustrating some of the strengths and limitations of the IPF parameter:

In this cohort, four patients had extensive marrow infiltration by malignancies, and would therefore be intuitively expected to have centrally mediated thrombocytopenia. However, the IPF was unexpectedly found to be elevated in three of these patients. The IPF is measured as a proportion of the optical fluorescent platelet count. The latter utilizes a complex mathematical algorithm combining both the size of a cell and its fluorescent characteristics to decide its cellular nature [57]. This prevents non-cellular debris from being counted as platelets, and giant platelets from being

counted as red cells. However, it has been shown that the optical platelet count as measured on Sysmex instruments may be inaccurate in patients receiving cytotoxic chemotherapy, which is thought to be a consequence of circulating fragments of apoptotic white blood cells being erroneously counted as platelets by the analyzer [58]. Therefore, it would be logical to expect the IPF to become unreliable in any instance in which the optical platelet count cannot be trusted. In addition, as white blood cell cytoplasm is rich in RNA, a spurious elevation of the IPF would be anticipated.

Of the three patients with a high IPF despite extensive marrow infiltration, two had large B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma, with platelet counts of 5x10^9/l and 18x10^9/l, and IPFs of 7,8% and 8,8% respectively. As large B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma is a pathology associated with a high proliferative index and a brisk rate of tumour apoptosis [59], it is possible that the mildly elevated IPF levels in these cases occurred spuriously as a result of the presence of circulating fragments of apoptotic tumour cells, despite the absence of a history of chemotherapy exposure. The third example occurred in a 34 year old female patient with extensive marrow infiltration by metastatic carcinoma of the breast. The patient presented with a severe thrombocytopenia (platelets=6x10^9/l), and was initiated on both tamoxifen and corticosteroids before the bone marrow investigation was performed, at which time the IPF was found to be 15%. In view of the history of chemotherapy exposure, it seems likely that this IPF level should be treated with a degree of circumspection. However, as the IPF level was significantly elevated, the possibility remains that the pathology observed in the bone marrow sample was only focal, and that a peripheral pathology was present concurrently. This consideration is partially supported by the fact that the platelet count showed a good recovery in response to the

treatment instituted, (which included an immunosuppressive agent), rising to a level of 75x10^9/l over the next 7 days. This could reflect the presence of an associated immune-mediated process. Therefore, although the precise mechanism of thrombocytopenia remains obscure in this particular case, this example highlights one of the most compelling potential merits of the IPF in its ability to reflect global megakaryopoiesis, while a bone marrow specimen is only focally representative.

The latter example illustrates the potential value of an elevated IPF in detecting the presence of peripherally mediated thrombocytopenia. However, it must be remembered that the converse is not universally true, particularly amongst a patient population in whom multiple mechanisms may occur concurrently. In other words, the presence of a low IPF does not exclude a peripheral contributor to the thrombocytopenia. This is clearly illustrated in two cases of interest. The first, a 23 year old male patient who presented with a platelet count of 35x10^9/l and an IPF of 5.8%. His clinical and morphological features were suggestive of a diagnosis of ITP, and he responded very well to steroid therapy. However, at the time of his presentation, he was also iron deficient as a consequence of chronic blood loss (which was presumed to be a product of long standing thrombocytopenia). As iron is necessary for megakaryopoiesis [60], it is likely that his thrombocytopenia was a product of both immune mediated platelet destruction and iron depletion, while the low IPF level reflected only the dominant mechanism at play (i.e. iron deficiency in this case).

The second case of interest is one of the cases of TTP. The patient presented with a platelet count of 18x10^9/l, and had an unexpectedly low IPF of 4.5%. However, this platelet count was measured by optical fluorescence, and review of the peripheral slide revealed a severely reduced platelet count (more compatible with the impedence-based platelet count in this case, which was 7x10^9/l) (see Figure 3.10). The discrepancy between the visually assessed platelet count and the optical platelet count suggests that non-platelet structures have been designated as platelets by the instrument. In

this instance, these non-platelet structures are likely to have been small red cell fragments, (which were present in large numbers), a hypothesis which is supported by the failure of the analyzer to flag their presence. As discussed above, the IPF becomes unreliable when the optical platelet count is in doubt. Although this does not appear to be a common problem, (as no similar cases have been reported in the literature [45, 61]), a low IPF should perhaps be treated with a degree of caution in the setting of severe red cell fragmentation.

Finally, as an indirect reflector of good marrow megakaryocyte activity, the presence of a high IPF has been proposed to allow for the omission of a bone marrow investigation in certain clinical scenarios [45]. However, the wide variation in IPF levels among patients with frank marrow pathology in this cohort render this possibility untenable in the setting of HIV-associated thrombocytopenia, even when the clinical presentation is strongly suggestive of a peripherally driven pathogenic process. This is clearly demonstrated in the case of a 27 year old male patient who presented with clinical features strongly suggestive of a diagnosis of ITP and an IPF of 17%. His bone marrow aspirate showed very brisk megakaryopoietic activity, and he responded well to steroid therapy. However, examination of his trephine biopsy revealed the unexpected presence of a peripheral T-cell lymphoma. In conjunction with the presence of an elevated IPF in over 40% of the patients with evidence of mycobacterial infection in their bone marrow and in three quarters of the patients with extensive marrow infiltration by malignancy, omission of a bone marrow investigation in the setting of HIV-associated thrombocytopenia would clearly be ill advised, regardless of the IPF level.

Chapter 5

5.0 Conclusion

Thrombocytopenia in the setting of HIV has many potential aetiologies, and the exact nature of the pathophysiological mechanism at play is often not easily determined by morphological examination of the bone marrow. In this study it has been shown that the IPF level correlates well with the bone marrow findings in HIV positive patients when the cause of thrombocytopenia is readily apparent on clinical and morphological grounds (as is the case in patients with ITP/TTP or hypocellular marrow). The IPF is therefore assumed to be a fair reflector of the predominant mechanism at play when the underlying pathophysiological process is more obscure, with a high IPF suggesting the presence of peripherally-driven thrombocytopenia, and a low IPF being a consequence of inadequate megakaryocytic activity. Based on this premise, the findings of this study suggest that, in the context of AIDS, severe thrombocytopenia is significantly more frequently a consequence of peripheral mechanisms, while mild thrombocytopenia is more often a product of a depressed bone marrow response. That being said, the underlying mechanism resulting in thrombocytopenia is very heterogeneous in individuals with AIDS, even among patients with a uniform histological diagnosis (such as bone marrow infiltration by TB).

Furthermore, there is a very poor correlation between the morphological characteristics of megakaryopoiesis (including absolute megakaryocyte numbers as well as the presence or absence of varying degrees of megakaryocyte dysplasia) with the true effectiveness of thrombopoiesis (as manifest as an elevation in the IPF level). The IPF is therefore a potentially useful tool to assist the

pathologist in interpreting the morphological features in HIV-positive patients with thrombocytopenia.

IPF levels greater than 10% or less than 6% have good specificity and positive predictive values for predicting the presence and absence respectively of a clinically meaningful improvement in the platelet count over the short-term.

In addition, this study has also illuminated some of the limitations of the IPF parameter, particularly in any instance in which the optically measured platelet count is in doubt. This includes some patients with extensive marrow infiltration by malignancy as well as some patients with severe red cell fragmentation.

Lastly, some of the characteristics of HIV related thrombocytopenia have been clarified in this study, particularly with respect to the effects of ARV therapy. It was found that although the patients on ARVs included had similar spectra of pathologies and mean CD4 counts as compared to patients not on ARVs, those with evidence of good virological control were significantly less likely to develop severe thrombocytopenia, and more likely to show an improvement in their platelet count over the short term. Because these effects were seen most pronouncedly among patients who had evidence of good virological suppression without significant immunological recovery, HIV itself is implicated as a very important modifier of thrombopoiesis. Although it is well known that HIV *per se* has effects on megakaryopoiesis, (particularly as a result of direct viral infection of megakaryocytes [20]), the findings of this study indirectly illuminate the effects of that infection on megakaryocyte fitness.

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