A review of Aplastic/Hypoplastic Anaemia diagnosed on bone marrow samples at the Haematology Laboratory at National Health Laboratory Service (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, Kwa-Zulu Natal.

By

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As the candidate's supervisor I have approved this thesis for submission.

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DECLARATION

I Zeenat Dawood Moorad declare that

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DEDICATION

To my wonderful mum for your unconditional love and unwavering support, and my beautiful daughters for your understanding and patience.

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OVERVIEW

Aplastic Anaemia (AA) is a bone marrow failure syndrome (BMFS) characterized by bone marrow aplasia and peripheral blood pancytopenia. The presenting symptoms in patients with AA are often those of anaemia, haemorrhage or purpura and, less frequently, infection. These symptoms usually lead to medical evaluation [1, 2]. Presentation can range from non-severe or moderate, to sometimes life-threatening cytopenias. In the Western World, the annual incidence of Acquired Aplastic Anaemia (AAA) is 2 cases per million persons [3]. A biphasic age distribution has been reported. AA primarily affects children, young adults, and those over 60 years of age. Males and females display no significant difference in incidence. The majority (70-80%) of AA cases are classified as idiopathic, as their primary aetiology is unknown [4]. Drugs or infections that precipitate bone marrow failure, can be identified in a subset of AA cases. AA is constitutional in approximately 15-20% of patients, with the commonest inherited bone marrow failure syndrome (IBMFS) being Fanconi Anaemia (FA). Five percent of idiopathic AA have undiagnosed IBMFS because the full disease phenotype has not manifest itself [5, 6].

An autoimmune pathogenesis is favored to cause stem cell depletion. Severe AA is invariably fatal without treatment. The currently available treatment options including allogeneic bone marrow transplantation (BMT), are improving patient survival in developed countries [2, 7, 8].

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare clonal haemopoietic stem cell (HSC) disorder which may arise de novo or evolve from AA. This bone marrow failure disorder manifests with haemolytic anaemia, marrow failure (peripheral blood cytopenias) and thrombophilia. AA and PNH are closely related, and small PNH clones are detectable in more than half of AA patients. PNH is also the most common clonal disorder that occurs in AA patients after treatment with immunosuppressive therapy (IST) [7, 9-18].

This project was conducted in order to delineate the demographic and clinico-haematological profile of the adult patient cohort diagnosed with AA within the Kwa-Zulu Natal (KZN) public health care sector. Inkosi Albert Luthuli Central Hospital (IALCH) in Cato Manor, Durban is the only public sector hospital in Kwa-Zulu Natal (KZN) equipped with the facilities and resources to diagnose this rare condition, as well as to treat AA. Due to the rare occurrence of this haematological disorder, there is a paucity of information regarding AA in South Africa (SA), as well as in African countries in general. To date, there have been no published studies on Adult AA from our centre or other centres within SA.

Aims and objectives: The aim of this retrospective observational study was to collect data regarding the demographics, clinical presentation, aetiology/associations, laboratory parameters and outcome of adult patients with a bone marrow diagnosis of Aplastic Anaemia at the National Health Laboratory Service (NHLS), IALCH, Durban, KZN, over a 10-year period.

The specific objectives of the study were to illustrate patient demographics, to correlate the full blood count (FBC), bone marrow aspirate and trephine (BMAT) findings and to

document disease severity. Other aims included documenting aetiological associations, in particular viruses such as Human Immunodeficiency Virus (HIV), and connective tissue disorders such as systemic lupus erythematosus (SLE). Another objective in HIV infected patients, was to establish if a relationship with the CD4 count/viral load and disease presentation, exists. The presence, or subsequent emergence, of a PNH clone and cytogenetic abnormalities, if any, were also documented. Patient outcomes, including response to treatment and overall survival, was also assessed.

Methods: BMAT biopsies with a confirmed diagnosis of Aplastic/Hypoplastic anaemia at the NHLS Haematology Laboratory at IALCH, were reviewed over a 10-year period (2005-2015). Demographic data, clinical information and laboratory parameters were recorded on a standardized data collection sheet, and then transcribed onto a Microsoft Excel spreadsheet for analysis. The demographic and clinical data was obtained from the referral forms and clinical notes. Laboratory results were extracted from the TrakCare Lab Information System (LIS).

Results: A total of 92 BMATs were reviewed. There were 42 males (M) and 50 females (F), with a M:F ratio of 0.8:1. The median age at presentation was 24 years, with an absence of a second peak of presentation in older patients. Twenty nine patients had very severe AA (VSAA), 36 had severe AA (SAA) and 27 had non severe AA (NSAA). Symptoms of thrombocytopenia (TCP) prevailed in the majority (61%) of patients at presentation, followed by symptoms of anaemia (53%). Symptoms of neutropenia, occurring in 6 patients (7.5%), were the least frequent. Ninety three percent of patients presented with pancytopenia. Thirty percent of patients had a severe anaemia (Hb <6g/dl). Sixty percent of patients had a severe thrombocytopenia (platelets < $20x10^9$/l). A severe neutropenia (ANC < 0.5 x 10^9/l) was observed in 62% of patients. Reversal of the lymphocyte: neutrophil ratio was observed in 92% of patients. All bone marrow trephines were markedly hypocellular for age. Twenty two patients had an initial failed BMAT biopsy. Cytogenetic abnormalities were noted in 2 patients at presentation. The majority of patients had idiopathic AA, with no identifiable aetiology. Two patients had FA, and a PNH clone was demonstrable in 4 patients at presentation. Thirteen patients (16.3%) were HIV positive. Four patients were pregnant at the time of presentation and their outcomes varied. Different modalities of treatment were used, including observation, androgens, immunosuppressive therapy [ciclosporin (CSA) with/without anti-thymocyte globulin (ATG)] and allogeneic bone marrow transplantation (BMT). Thirteen patients did not reach the clinical haematology unit, 35 patients are alive, 22 patients demised and 22 have been lost to follow up (LTFU). Two patients had clonal evolution to PNH and one to MDS.

Conclusion: The clinico-epidemiological profile of patients in this study is similar to that reported in the literature. A significant number of patients did not reach the clinical haematology unit at IALCH. An unusually high number of failed initial BMATs were performed at peripheral hospitals. HIV associated AA was not increased in this patient cohort.

It is my intention to provide the haematology department with this vital information and to allow comparison of similarities/differences in the findings from this study with that reported within the literature. I hope that this study will inform and educate readers on the existence of this rare condition, as well as provide much needed insight into the disease presentation and diagnosis so that patients may be referred timeously/promptly for specialist management.

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AA – aplastic anaemia

AAA- acquired aplastic anaemia

AED- antiepileptic drugs

AET- antiepileptic therapy

AIDS- acquired immunodeficiency syndrome

AL- acute leukaemia

AML- acute myeloid leukaemia

ANC- absolute neutrophil count

ANF- antinuclear factor

ART- antiretroviral therapy

ARV- antiretrovirals

ATG- antithymocyte globulin

AUG- August

AZT- zidovudine

BCSH- British Committee for standards in haematology

BM- bone marrow

BMAT-bone marrow aspirate and trephine biopsy

BMFS- bone marrow failure syndromes

BMT- bone marrow transplant

cART- combination antiretroviral therapy

CBT- chromosomal breakage test

CD4- cluster differentiation 4

CMV- Cytomegalovirus

CSA- ciclosporin

CVA- cerebrovascular accident

CY- cyclophosphamide

D4T- stavudine

DEC- December

DKC- Dyskeratosis Congenita

DNA- deoxyribose nucleic acid

DR- doctor

DVT- Deep vein thrombosis

EBMT- European group for blood and marrow transplantation

EBV- Epstein Barr Virus

EF- eosinophilic fasciitis

ESA- erythropoiesis-stimulating agents

FA- Fanconi Anaemia

FBC- full blood count

FCM- flow cytometry

FDA- Food and Drug Administration

FEB- February

FISH- fluorescent in situ hybridization

fl- femtolitres

FLAER- fluorescent labelled aerolysin

G-CSF- granulocyte colony-stimulating factor

GP 120- glycoprotein 120

GPI- glycosylphosphatidylinositol

GR- good response

GVHD- graft versus host disease

HAAA- hepatitis Associated Aplastic Anaemia

LIST OF ABBREVIATIONS

HAV- Hepatitis A virus

HBV- Hepatitis B virus

HCV- Hepatitis C virus

HB- haemoglobin

HbF%- Foetal Haemoglobin percentage

HepBSAg- Hepatitis B Surface antigen

HGF- haemopoietic growth factors

HIV- Human Immunodeficiency Virus

HLA- Human Leucocyte Antigen

HSC- haemopoietic stem cells

HSCT- haemopoietic stem cell transplant

IALCH- Inkosi Albert Luthuli Central hospital

IL 2- interleukin 2

IBMFS- inherited bone marrow failure syndromes

ICT- iron chelation therapy

ICU- Intensive Care Unit

IFI- invasive fungal infection

IFN-interferon

IFN-γ- interferon gamma

IL2- interleukin 2

IST- immunosuppressive therapy

IUD- intrauterine death

IUGR- intra-uterine growth retardation

IV- intravenous

LIST OF ABBREVIATIONS

IVH- intravascular hemolysis

JAN- January

KEH- King Edward Hospital VIII

KZN- Kwazulu-Natal

LDH- lactate dehydrogenase

LDL- lower than detectable limit

LFT- liver function test

LIS- laboratory information system

LTFU- lost to follow up

MAC- membrane attack complex

MC- myeloablative conditioning

MCV- mean cell volume

MDS- myelodysplastic syndrome

MDS-EB1- myelodysplastic syndrome with excess blasts-1

MDT- multidisciplinary team

MGMH- Mahatma Gandhi Memorial Hospital

MRI- magnetic resonance imaging

MSB- macerated still birth

MSD- matched sibling donor

NGH- Ngwelezane hospital

NFK- normal female karyotype

NHLS- National Health Laboratory Services

NMK- normal male karyotype

NO- nitric oxide

NOV- November

LIST OF ABBREVIATIONS

NSAA- non severe aplastic anaemia

NSAIDS- non steroidal anti-inflammatory drugs

NVD- normal vaginal delivery

NVP- nevirapine

OCT- October

OS- overall survival

PB- peripheral blood

PCR- polymerase chain reaction

PIGA- phosphatidylinositol glycan class A

PIH- pregnancy induced hypertension

PE- pulmonary embolism

PLT- platelet

PMMH- Prince Mshiyeni Memorial hospital

PNH- paroxysmal nocturnal haemoglobinuria

PR- poor response

PT- patient

PTB- pulmonary tuberculosis

QOL- quality of life

RA- rheumatoid arthritis

RBC- red blood cell

RCC- red cell concentrate

RIC- reduced intensity conditioning

RKK- R K Khan Hospital

RPI- reticulocyte production index

SA- South Africa

LIST OF ABBREVIATIONS

SAA- severe aplastic anaemia

SAAWP- severe aplastic anaemia working party

SBDS- Shwachman Bodian Diamond Syndrome

SDS- Shwachman Diamond Syndrome

SEP- September

SLE- systemic lupus erythematosus

SS- Sjorgens Syndrome

SX- symptoms

TA-GVHD- transfusion-associated graft-versus-host disease

TAT- turnaround time

TB- tuberculosis

TCP- thrombocytopenia

TERC- telomerase complex

TERT- telomerase reverse transcriptase

TF- transfusion

TH-1- T helper cell-type 1

TINF2- TERF-1-interacting nuclear factor 2

TNF- tumour necrosis factor

TNF α- tumour necrosis factor alpha

TOP- termination of pregnancy

TRALI- transfusion-related acute lung injury

TTV- torque teno virus

U&E- urea and electrolytes

UC- ulcerative colitis

UD- unrelated donor

LIST OF ABBREVIATIONS

USA- United States of America

VL- viral load

VSAA- very severe aplastic anaemia

WBC- white blood cell

WCC- white cell count

YR- year

3TC- lamivudine

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Aplastic anaemia (AA) is defined as a pancytopenia with a hypocellular bone marrow in the absence of abnormal infiltration or increased reticulin. The very first case of AA was described by Ehrlich in 1888. It was documented in a young woman who died of an abrupt illness with severe anaemia, haemorhage, hyperpyrexia, and a hypocellular bone marrow [19].

Acquired aplastic anaemia (AAA) is a largely immune mediated condition. It may present concurrently with clonal haemopoietic stem cell disorders, most commonly paroxysmal nocturnal haemoglobinuria (PNH). It may later evolve to myelodysplastic syndrome (MDS) in up to 15-20% of patients or acute leukaemia (AL) [2, 20]. Furthermore, there is overlap between AA and MDS in the form of the entity hypocellular MDS, which is often difficult to distinguish from AA on morphologic criteria, especially when AA is of the non-severe subtype [21].

Several inherited (genetic/constitutional) disorders may be characterized by bone marrow failure (BMF). Usually, one or more somatic abnormalities (which may often be diagnostic of the disorder) is associated with these conditions [22]. These inherited BMF syndromes (IBMFS) are heterogeneous disorders characterized by clinically significant haematological cytopaenias. The haematological features may manifest at variable periods. Some may occur in the neonatal period, however in other disorders such as Fanconi Anaemia (FA) or Dyskeratosis Congenita (DKC), cytopenias may develop later in childhood or can even present at any time in life [23].

As the exemplar of human bone marrow failure syndromes, AA has undergone paradigmatic shifts in our understanding of disease pathophysiology. This has profound implications for treating this fatal blood disease [24].

The first successful allogeneic bone-marrow transplantation for AA was performed in 1972 [25]. Prognosis of this invariably fatal illness was improved by the development of bone-marrow transplantation and potent immunosuppressive therapy in the 1970s. Although still a potentially devastating condition, prompt medical intervention has now improved survival [26].

1.2. Incidence and Epidemiology

Acquired AA has an annual incidence of approximately 2-3 million per year (all age groups) in the West (Europe). A 2-3-fold higher incidence rate was demonstrated in East Asia [27-30]. Little evidence is available for the ethnic variability in AA rates. A suggested explanation for the higher incidence in the Far East has been the easy accessibility and the increased use of medical drugs, such as antibiotics, which are available without prescription. Pesticide exposure has also been implicated [31-33, 34] .

Epidemiological studies demonstrate a bimodal **age distribution**. An early peak at 10-25 years, as well as a later peak $\geq$ 60 years has been documented [25, 31-34].

There is no significant reported gender difference in incidence between males and females [35]. The sex ratio has been close to 1:1 in most reported cases. This is unusual for immune mediated diseases, which usually demonstrate a female predominance [36].

Incidence rates in studies refer to moderate and severe AA. The severity is determined by assessing the patients initial blood counts. Although some moderate AA patients may show a worsening pancytopenia, other patients remain stable, never needing treatment. Individuals with constitutional marrow failure, either not tested for Fanconi Anaemia (FA) (based on the absence of physical anomalies) or with telomere complex mutations (for which diagnostic assays were previously not available), may be included in this group of moderate AA never requiring treatment [36].

1.3. Pathophysiology of Acquired Aplastic Anaemia

The majority of AAA cases can be pathophysiologically characterized by T-cell-mediated, organ specific destruction of bone marrow hematopoietic stem cells (HSC). In individual patients, the aberrancy of the immune response may occasionally be linked to a prior drug or chemical exposure or a preceding viral infection. Evidence for other mechanisms is rare. This may include factors such as direct stem cell toxicity or a deficiency of stromal-cell or hematopoietic growth factor function. The quantitative degree of HSC destruction, as well as the variations in immune response may sometimes explain the variability in clinical course and response to treatment [(37)].

1.3.1 Hematopoietic failure

Failure of blood cell production is responsible for the empty bone marrow of AA. The earliest observers noted the "yellow fat" and the absence of the morphologically diverse precursors of mature blood components within bone marrow spaces. This is a striking feature on bone marrow aspirate and trephine biopsy (BMAT) examination [(3)]. Vertebrae display uniform marrow replacement with fat on magnetic resonance imaging (MRI). Fluorescent-activated flow cytometry can also be used for quantification of immature haemopoietic cells. This method detects the CD34 cell antigen (an adhesion protein present on less than 1% of normal bone marrow). In AA, there is an almost complete absence of CD34 cells. There is a marked reduction of progenitor cells, which are capable of forming erythroid, myeloid, and megakaryocytic colonies. A similar consistent and severe deficit is also demonstrated in assays of very primitive, quiescent, hematopoietic cells. These are cells that are closely related, if not identical, to HSC [(38)].

Functional studies of aplastic BM have indicated, that patients with AA present with pancytopenia when stem-cell and progenitor-cell populations have been reduced to approximately 1% or less of normal. This profound deficiency has qualitative consequences, as evidenced in the shortened telomere length of granulocytes of AA patients [(39)].

1.3.2 Immune Destruction

Runt disease, in animals and transfusion-associated graft-versus-host disease (GVHD), in humans are primary examples of the efficiency of immune system at destroying blood-forming cells [(40)]. In such conditions, fatal AA is induced by a small number of alloreactive T cells. In mouse studies the stem cell destruction has been rapid and almost complete. There is abundant laboratory data which supports the hypothesis that lymphocytes are responsible for hematopoietic cell compartment destruction in AAA [(37)].

Early experiments demonstrated that lymphocytes suppressed hematopoiesis, by producing a soluble, inhibitory factor eventually identified as interferon-y (IFN-γ). There is activation of a T-helper cell type 1 (TH-1) T-cell response resulting in excessive production of interferon (IFN), tumor necrosis factor (TNF), and interleukin-2 (IL-2). Flow cytometric detection of intracellular

IFN-γ in patient samples can be performed. This may be correlated with patient response to immunosuppressive therapy (IST). It may also predict disease relapse [41].

The altered immune system results in fas-mediated destruction, specifically of CD34 cells. Intracellular pathways are activated and cell-cycle arrest ensues. The antigenic nature of the pathologic immune response is obscure. Molecularly, lymphocytes in AA show similarity to T cells in other conditions such diabetes and multiple sclerosis and other similar illnesses. The immune response is oligoclonal as demonstrated by characterization of the T-cell-receptor b chain (TCR-B) of activated lymphocytes. There are a relatively limited number of active clones infiltrating the marrow. Some immune responses may be public, meaning that it is distributed among patients with the same histocompatibility background [42].

1.3.3 Immunopathogenesis of Hepatitis Associated Aplastic Anaemia (HAAA)

Various immunological aberrations result in the development of HAAA. CD8+ kupffer cells appear as a mediator of this syndrome [43, 44]. CD8 cells residing in the BM during HAAA produce high levels of interferon gamma (IFN-γ). Tumor necrosis factor alpha (TNF-α) and IFN-γ cause onlooker damage to hepatocytes (in genetically modified mouse models). However, several studies have indicated that an increased level of soluble IL-2 receptor is a large contributor to the non-specific inflammation of HAAA [45, 46]. In children, HAAA pathogenesis has been linked to an imbalance of lymphocyte sub-populations and T lymphocyte activation [47]. There is no known genetic tendency with HAAA [48].

1.3.4 Pathogenesis of the IBMFS

Although rare, FA is the most common congenital marrow failure syndrome. It is a genetically heterogeneous multisystem disorder, in which twenty genes have been causally associated. FA is a DNA repair disorder characterized by chromosomal instability, which results from germline mutations in the DNA repair genes of the FA/BRCA pathway [23].

Germline mutations in important telomere biology genes which result in dysfunctional telomere maintenance have been implicated in DKC [23]. Mutation in TERC (gene for the RNA component of telomerase) and TERT (the gene for the telomerase reverse transcriptase catalytic enzyme), which are genes of the telomere repair complex, reduce the regenerative capacity of bone marrow. This results in gene mutation carriers being more susceptible to the development of AA [49].

1.4. Aetiology

Inherited bone marrow failure syndromes (IBMFS), of which Fanconi anaemia is the commonest, account for 15-20% of cases of AA. Approximately 5% of patients with idiopathic AA have an undiagnosed IBMFS, in which the full disease phenotype has not yet manifest [5,6].

The remainder and majority of cases of AA (70-80%) are classified as idiopathic, due to the aetiology being unknown [5,6]. An immunologic pathophysiology has been implicated in acquired AA. Similar to other autoimmune conditions, environmental triggers and individual host factors are hypothesized to determine risk. Although many drugs and chemicals have been implicated as causal agents of AA, reasonable evidence is only available for very few [25, 50-53] (See Table 1.1 below).

There has been a link to exposure to benzene and pesticides to AA [54]. Marrow failure occurring as a severe idiosyncratic complication, has been reported following the use of certain medical agents such as chloramphenicol [54]. AA can follow infections with certain viruses, as in postseronegative hepatitis [55]. AA has also been documented as a rare complication of pregnancy [56]. There are reports of clusters of AA in the literature [57-60]. However, there is a poor description of the mechanisms linking environmental triggers to bone marrow failure. Hence, most cases are labeled as idiopathic [29].

Table 1.1: Aetiology of acquired idiosyncratic AA [61]

Idiopathic	70–80% Of cases
Drugs (10)[a]	Antibiotics: chloramphenicol (no evidence for chloramphenicol eye drops), sulphonamides Anti-rheumatics: gold, penicillamine Anti-inflammatory: phenylbutazone, indomethacin, diclofenac, naproxen, piroxicam Anti-convulsant: phenytoin, carbamazepine Anti-thyroid: carbimazole (more likely to cause neutropenia), thiouracil Anti-deppressant: dothiepin, phenothiazines Anti-diabetic: chlorpropamide Anti-malarial: quinine
Chemicals	Benzene Pesticides: organochlorines e.g. lindane, organophosphates Cutting oils and lubricating agents Recreational drugs: methylenedioxy-methamphetamine, MDMA, ecstasy
Viruses	Viral hepatitis: non-A, non-B, non-C, non-G Epstein–Barr virus
PNH	Haemolytic PNH in 5%; small PNH clone can be detected by flow cytometry in at least 20–25% of AA patients at presentation
Rarely	Systemic lupus erythematosus, pregancy, thymoma, eosinophilic fasciitis

[a] Drugs currently licensed in the UK reported to have a rare association with AA.

AA- aplastic anaemia. PNH- paroxysmal nocturnal haemoglobinuria

1.4.1 Drug associated AA

Aplastic anaemia has been attributed to an idiosyncratic reaction to drug or chemical exposure. This association with AA is of great importance. Not only is it devastating to physicians and patients but it also presents serious legal implications and contributes problems in pharmaceutical drug development, government regulation and the manner in which warning labels are worded [53,54].

Of utmost importance is a careful drug history, which should document all drug exposures from 6 months before and ending one month prior to presentation [51,52].

Anticonvulsants, anti-rheumatic drugs, anti-thyroid medications, anti-tuberculosis drugs and non-steroidal anti-inflammatory drugs (NSAIDS) have commonly been implicated in the aetiology of AA. Few of the specific drugs cited include: amidopyrine, butazone, chloramphenicol, felbamate, gold salts, methimazole, penicillamine, sulfonamide, and trimethoprim/sulfamethoxazole [50, 62-64].

Many drugs reported to cause AA, can also more commonly cause mild bone marrow suppression. Idiosyncratic drug reactions are extremely rare. This may be a consequence of genetic variations in drug metabolism, alterations in major histocompatibility antigens as well as their peptide-binding abilities [37].

If the patient is taking a drug that is implicated in AA at presentation, then the drug should immediately be discontinued. Patients should not be re-challenged with such drugs after recovery of the blood counts, at a later stage [7].

Irradiation and chemotherapy-induced bone marrow aplasia are different entities. These should not be called AA [65].

Three cases of AA occurring in association with anti-tuberculosis chemotherapy have been reported [66]. The patients had been on anti-tuberculosis chemotherapy for 13, 11 and 14 months respectively, prior to the diagnosis of AA. Agents used included streptomycin, thiacetazone, isoniazid, p-aminosalicylic acid and dimethylcarbazine. Recovery from aplasia did not occur 6, 1.5 and 0.8 months, respectively, after the discontinuation of the suspect myelotoxic agents and despite the use of myelostimulatory agents. All three patients died of haemorhage secondary to thrombocytopenia. The observations reported were consistent with a protracted and probably irreversible damage of the bone marrow by anti-tuberculosis agent(s) in susceptible individuals. However, there are no other case reports/series available.

There are 5 reported cases of AA from Zambia, as a consequence of traditional herbal remedies. Three of the patients died. A feature of the haemorhagic tendency from severe thrombocytopenia was optic fundus and vitreous haemorhage [67].

A study conducted by Handoko et al, 2006, revealed that exposure to antiepileptic drugs (AED) increased the risk of AA nine fold. Felbamate was the most frequently reported drug, however Carbamazepine and Valproic acid (which are the most widely prescribed AEDs) were also commonly implicated [68].

Two cases of fatal AA associated with Clopidogrel use, have been reported in 2001 [69].

Eight cases of sulfonamide related AA, as well as six cases of nifedipine-related fatal AA have been reported [70].

A case of AA following the use of Sulphaphenazole ("Orisulf") has also been published [71].

Tabulated below are some of the currently licenced drugs which have been reported as a rare association with aplastic anaemia (Table 1.2).

Table 1.2. Currently licenced drugs which have been reported as a rare association with aplastic anaemia.

Antibiotics	Chloramphenicol*, Sulphonamides, Cotrimoxazole, Linezolid
Anti-inflammatory	Gold, Penicillamine, Phenylbutazone, Indomethacin, Diclofenac, Naproxen, Piroxicam, Sulphasalazine
Anti-convulsants	Phenytoin, Carbamazepine
Anti-thyroids	Carbimazole_, Thiouracil
Anti-depressants	Dothiepin, Phenothiazines
Anti-diabetics	Chlorpropamide, Tolbutamide
Anti-malarials	Chloroquine
Others_	Mebendazole, Thiazides, Allopurinol

*No association with chloramphenicol tablets was observed in recent study from Thailand (Issaragrisil et al, 2006). There is no evidence for an association between chloramphenicol eye drops and aplastic anaemia
(Gordon-Smith et al, 1995; Lancaster et al, 1998; Wilholm et al, 1998).
_More likely to cause neutropenia from epidemiological study in Thailand (Issaragrisil et al, 2006).

Evidence based on case reports or uncontrolled series (Young & Alter, 1994) or case control studies (Baumelou et al, 1993; Issaragrisil et al, 2006; Issaragrissil et al, 1997; Kauffmann et al, 1996) [7].

1.4.2 Toxins: Occupational and Environmental exposures

The association between the development of AA and prior exposure to drugs or industrial chemicals is a well-recognized one. Often, it is based on epidemiological evidence of the temporal relationship between an exposure and aplasia developing in the bone marrow. In about 70% of cases, there is no obvious aetiological agent. A careful occupational history of the patient may reveal exposure to chemicals or pesticides that have been associated with AA, as summarized in Table 1.3 below [(7)].

Table 1.3. Occupational and environmental exposures as potential aetiological agents in aplastic anaemia [(7)]

Benzene and other solvents (evidence based on large industrial studies (Yin et al, 1987; Smith, 1996; Yin et al, 1996; Issaragrisil et al, 2006)
Agricultural pesticides: Organochlorines e.g. Lindane, Organophosphates, Pentachlorophenol [Muir et al, 2003 (case control study), Fleming & Timmeny, 1993; Roberts, 1997 (literature reviews of case reports)], DDT and Carbamates (Issaragrisil et al, 2006)
Cutting oils and lubricating agents (Muir et al, 2003)
Non-bottled water, non-medical needle injury, farmers exposed to ducks and geese, animal fertiliser (Issaragrisil et al, 2006)
Recreational drugs: methylenedioxy-methamphetamine, MDMA, Ecstasy, [evidence based on case reports, (Marsh et al, 1994b; Clark & Butt, 1997)]

DDT- dichlorodiphenyltrichloroethane. MDMA- 3,4 methylenedioxy-methamphetamine

Solvents have been investigated as potential aetiological agents in AA [(72,73)].

Benzene is one of the oldest and most widely accepted aetiologic associations between environmental exposures and marrow failure. Exposure to benzene through occupational and other routes is the most convincingly causally related exposure to the disease. Benzene is used as a solvent in a number of industries including rubber, gum and resin production. It is also used in the dry cleaning industry and in the manufacture of dyes, leathers and shoes. Occupational exposure to benzene is anticipated to be much higher than non-occupational sources, although benzene is also a common agent found in a number of household cleaning products and exposure also occurs during the handling of petrol. High dose direct administration of benzene to animals

has shown it to be a marrow toxin producing marrow hypoplasia, hyperplasia and even malignancies [74].

There is less clear evidence for **other common solvents** having a role in the aetiology of AA. Solvents such as pure toluene or xylene are not thought to be marrow toxins [75]. The fact that benzene is concentrated in the fat of the bone marrow may partly account for such a difference in toxicity [76].

Pesticides have been investigated as aetiological agents for AA, particularly the insecticides chlordane, lindane and dichlorodiphenyltrichloroethane (DDT) [53, 77-79]. Lindane (γ-hexachlorocyclohexane) is an organochlorine. It was first was used as an insecticide in 1942. It becomes concentrated in the fatty tissue of the bone marrow [80]. "The WHO report on Lindane concluded that there was sufficient laboratory evidence that the chemical was carcinogenic in animal experiments and that it is reasonable to regard such a chemical as posing a carcinogenic risk in man" [81]. There are multiple documented reports of bone marrow injury associated with Lindane [82].

The potential dangers of **hair dye (**which are in widespread use) have been re-emphasized as reports on the carcinogenity and mutagenicity of hair colourants have emerged [83,84]. A case report from Nottingham reported a case of fatal aplastic anaemia in a 52-year-old housewife using a new hair dye [85]. The hair dye used contained paratolylenediamine (2-methyl 1.4 phenylenediamine) in a concentration of 0.03%. Phenylenediamine has been used for many years in the dyeing industry and its effects on the bone marrow are well known [85]. Two groups previously reported studies on the mutagenic and carcinogenic properties of the various paraphenylenediamine dyes [83,84]. The mutagenic properties are retained even after mixing with hydrogen peroxide, the oxidant used in the lotion developer, and appreciable quantities may be absorbed through the skin during one episode of hair dyeing. Although individual sensitivity is extremely rare, these substances used in hair dyeing must continue to be the subject of close scrutiny.

Radiation is a recognized hazard that results in bone marrow ablation [86]. The predictable pancytopenia that follows radiation exposure in therapeutic applications is not permanent. Marrow recovery ensues after completion of the exposure. Occupational radiation exposure resulting in AA is less well characterized and the medical and industrial workers are usually included [87-91].

1.4.3 Viruses and Aplastic Anaemia

An association between viral infections and BM failure implies a direct effect on the HSC.

Viruses other than hepatitis implicated as aetiological agents in AA include: Parvovirus B19, Cytomegalovirus (CMV), Epstein Barr virus (EBV), Echovirus 3, GB virus-C, Transfusion Transmitted virus, SEN virus and non-A-E hepatitis virus (unknown viruses) [37, 92-100].

Association of the Torque teno virus (TTV) was reported in a 12-year-old Japanese boy. He presented with cytopenias following acute hepatitis. Other studies have also been conducted to assess the role of TTV as an aetiological agent of HAAA [98, 101].

A documented report of AA in a 6-year-old boy, two months after the onset of the acute hepatitis demonstrated an associated with Echovirus-3 [98].

In HIV infected patients, SAA has developed after subsequent co-infection with Herpes Simplex Virus-6 (HSV- 6) [102, 103].

1.4.3.1 Hepatitis Associated Aplastic Anaemia (HAAA)

Hepatitis Associated Aplastic Anaemia is a well-recognized variant of AAA. It is rare, having been reported in 2-5% of cases in the West [96]. In the Far East, a viral aetiology has previously been documented in up to 4-10% of cases of AA. This could be in keeping with the increased prevalence of certain viral infections in the Far East such as Hepatitis and HIV [104, 105]. HAAA can occur in any age group, and has been found in children as well as adolescent and young men predominantly. There is no gender preference and also no correlation with the severity of hepatitis at the onset of AA [41, 98-99, 106].

Aetiology of the syndrome has been attributed to various agents and factors which may include pathogenic viruses, autoimmune responses, liver transplantation, bone marrow transplantation, radiation and drugs administered to control the viral replication [107].
Marrow failure and pancytopenia usually follow an acute attack of hepatitis [100, 108-109]. The pancytopenia, usually occurs two to three months "[62days: ranging from 14 to 225]" after the acute hepatitis attack [55, 63]. AA will rarely complicate a case of hepatitis that has already been diagnosed [110]. It may be acute or chronic, mild and transient, self-limiting or fulminant. The development of AA is uniformly fatal if treatment is not instituted on time [43, 100, 111]. Severe posthepatitic AA usually responds well to immunosuppressive therapy (IST). However, most of the reported cases of HAAA had fulminant hepatitis with a mortality rate of 85% [102].

Aplastic anaemia associated with viral hepatitis has a late appearance (weeks or months after the episode of hepatitis) and is markedly severe and usually irreversible [111, 112]. Of the hepatitis viruses, hepatitis A, B, C, E and G have been implicated [44, 113, 114, 115]. There has been no documented association with blood transfusion, toxins and drugs [102].

1.4.3.2 AA associated with Parvovirus B19 infection

Human parvovirus B19 has been associated with a broad spectrum of haematological diseases. It is an under-recognized hepatotrophic virus and has been documented as an offending agent in HAAA. Manifestations range from abnormal liver function to fulminant hepatic failure and AA [92]. A case of SAA was reported following asymptomatic infection with Parvovirus B19, in a previously healthy boy without any underlying diseases [116].

Further studies were conducted (1995/1996), to explore the frequency of parvovirus B19 infection in children with SAA [117]. Parvovirus B19 DNA and antibodies were investigated in thirty cases of SAA. Six cases were associated with active or recent parvovirus B19 infection and the study concluded that parvovirus B19 infection might be associated with childhood SAA and should therefore be considered as a possible aetiologic agent in some children with SAA [118].

1.4.3.3 AA and Epstein Barr Virus (EBV)

Aplastic anaemia is a rare complication of infectious mononucleosis. The pathogenic mechanisms involved in EBV infection include direct cytotoxicity or modulation of the host immune response [100, 102, 107, 119].

In a case report published in Toronto in 1987, a previously healthy 17-year-old female was diagnosed with AA on bone marrow biopsy. Her presenting symptoms were petechiae and epistaxis a week after an episode of pharyngitis. She also had cervical lymphadenopathy and fatigue. Positive results were demonstrated with the monospot test. Elevated titres of antibody to EBV capsid antigen were detected.

1.4.3.4 AA and Human Immunodeficiency Virus (HIV) infection

Various haematological alterations have been documented in acquired immunodeficiency syndrome (AIDS) patients. The prevalence of these have been reported to be increased according to progressive immunologic deficiencies and the severity of retroviral infection [120]. Cytopenias occur frequently. Amongst this, common haematological manifestation are thrombocytopenia, leucopenia, anaemia and granulocytopenia. Anaemia, of which the causes are multifactorial, is prevalent in approximately 63-95% of patients with acquired immunodeficiency syndrome (AIDS) [121, 122]. Pancytopenia is commonly evident in patients with HIV-1 infection and virtually all patients with advanced AIDS (group IV) are pancytopenic [123]. It has also been observed that antibody directed against the glycoprotein (Gp) 120 of HIV contributes to haemopoietic suppression [120].

Bone marrrow failure is multifactorial in patients with HIV/AIDS, and multiple different aetiologies may account for the varied histological findings on BM examination of these patients. Bone marrow findings may vary, and hypocellularity has been reported. One study found that 12 out of the 60 HIV positive bone marrows examined was hypocellular [124, 125].

Frank hypocellularity is exceptional and bone marrow aplasia in the form of severe aplastic anemia (SAA) extremely rare [123].

There are very few reported patients with AA in the setting of HIV, and SAA is a rare disease, that has been exceptionally reported [126]. Aplastic anaemia secondary to HIV infection has been reported in a 13-year-old female in India. This patient acquired HIV through a blood transfusion at age 5. Her AA responded very well to the ART combination composed of stavudine (D4T), lamivudine (3TC) and nevirapine (NVP) [127]. Another documented case report was that of a 34-year-old HIV positive male patient, who had received an allogeneic HSCT for AA [128].

In the setting of HIV, management of SAA remains challenging [136]. No documented evidence for the use of ATG and ciclosporin for treatment of AA in the setting of HIV, exists. AA in some patients may have an excellent haematological and clinical response to ARV therapy [129].

The observation that HIV positive patients do not receive the standard first-line drug therapy for SAA (combination of horse ATG and CSA) reflects the concern of an anticipated higher risk of severe infection with full immunosuppressive therapy in the setting of HIV infection.

It has been documented that BMT in AA associated with HIV, is not very useful [130]. However, a recent study has identified 8 patients who were diagnosed with SAA associated with HIV infection [129-130].

Four patients were offered HSCT, whereas the other 4 were managed with non-transplant therapeutic modalities. A worse outcome was observed in the non-transplanted group, were 3 of the patients demised from infections that were not HIV related. Of the 3 patients: two patients demonstrated no response (one to ciclosporin and steroids and the other to androgens) and the third patient had a partial response to anti-thymocyte globulin (ATG) associated with CSA. Only one of the non-transplanted patients demonstrated an early and durable remission on eltrombopag treatment (for a 23-month follow-up period) [131].

Conversely, long term survival with a median follow-up of 59 months (54-101 months) was documented in 3 of the 4 patients in the transplanted group. All 3 patients had received a reduced intensity conditioning (RIC) regimen. The fourth patient was the only patient who received a myeloablative conditioning (MC) regimen. He experienced veno-occlusive disease and died of multiorgan failure at 39 months' post-transplant. Hence, BMT is an approach that should possibly be considered for SAA in HIV infected patients [126].

New alternate therapies, such as eltrombopag, warrant further evaluation for the management of SAA patients who are not eligible for HSCT [126].

1.4.4 Autoimmune Disorders

Aplastic anaemia has been reported with **many** systemic **autoimmune disorders**. Amongst them are Eosinophilic fasciitis (EF), Systemic lupus erythematosis (SLE), Sjorgen syndrome (SS), coeliac disease, as well as association with Thymoma, which have all been reported. Transfusion-associated graft-versus-host disease (GVHD) is another autoimmune disorder which almost invariably results in fatal AA.

1.4.4.1 Systemic Lupus Erythematosus (SLE) and AA

Aplastic anaemia and myelofibrosis have been reported in association with fulminant cases of SLE. These are exceedingly rare events and occur much less commonly than peripheral autoantibody induced cytopenias [132, 133].

Worldwide there are at least 19 reported cases of pancytopenia, myelofibrosis and AA. A female preponderance was evident, with more than 80% of the patients being female. The age group varied vastly between five months to 74 years, with a mean age of 27.4 years. In the majority of cases, AA either preceded the diagnosis of SLE, or developed later during the course of the illness. In the minority of cases, AA is a simultaneous diagnosis with AA [134].

A case of AA presenting simultaneously with the classical features of SLE at onset, has been described in a 17-year-old Indian female [134]. There are also 2 reported cases of SLE, in which AA and pancytopenia were the sole manifestations of the disease, months prior to the appearance of other clinical features. The interval from the onset of pancytopenia to the diagnosis of SLE ranged from 3 to 12 months [135].

1.4.4.2 Primary Sjogren's syndrome (SS) and AA

There have been rare reports of SS with AA, and these were only in lymphoma patients. An exceptional case of primary SS and SAA without lymphoma was reported in a 28-year-old white male in February 1990. Cytogenetic and immunological abnormalities were documented in this case. This may also give clues to the pathogenesis of "idiopathic' AA [136, 137].

1.4.4.3 AA associated with Thymoma

AA is a rare complication of thymoma and is extremely infrequent after thymectomy. The reported incidence of AA with thymoma is 0-1.4% [136, 137]. AA is exceedingly uncommon following the surgical removal of a thymoma [138-140].

An immunologic origin is proposed in thymoma-associated haematologic dyscrasias. A case of VSAA following thymectomy, was reported in a 60-year-old female patient who was successfully treated with immunosuppressive therapy (cyclosporine A) and G-CSF [141].

1.4.4.4 Ulcerative Colitis (UC) associated with AA

Ulcerative Colitis is an inflammatory bowel disease of unknown aetiology, which predominantly affects the mucosa of the colon and rectum. Several cases of salazosulfapyridine or mesalazine-associated AA have been reported in UC patients [142]. A case of UC associated with AA was reported in a 45-year-old man who presented with thrombocytopenia before any diagnosis of UC or treatment with mesalazine [143]. The clinical course implied that the subsequent pancytopenia was not drug-induced. The AA responded well to treatment with danazol and ciclosporin (CSA), strongly suggesting an immunopathogenetic aetiology. The observation made in several case reports, indicating that the same HLA-DR2 haplotype is associated with both UC and AA also suggests a common immunologic impairment underlying the development of these diseases [140].

1.4.4.5 AA associated with Eosinophilic Fasciitis (EF)

Eosinophilic Fasciitis may sometimes be associated with haematologic disorders, particularly AA. It is not known whether AA associated with this condition is an autoimmune disease and/or if it could be the initial manifestation of an evolving clonal myeloid disorder. In a cohort of 19 patients with EF and AA, 8 patients demised from complications of AA [144-158]. Patients with EF and AA were more likely to be men (70%) and older (mean age, 56years; range 18-71 years), compared to patients with isolated EF. 4 patients with EF and associated SAA were reviewed by Masson et al in 2013 [159].

1.4.5 Pregnancy and AA

The first time that AA was described as a clinical entity was in the year 1888, in a pregnant woman. Since then, 61 documented cases of AA in pregnancy have been reported [160-164]. Although, the precise relationship between these two conditions remains a matter of speculation, pregnancy is still mentioned as a possible causative factor of AAA [161,165].

It has been reported that the pregnant state results in an increased production of factors such as oestrogen, placental lactogen and erythropoietin. Although placental lactogen and erythropoietin may stimulate increased haematopoiesis, high doses of oestrogen are suggested to inhibit bone marrow production. Hence, it is hypothesized that BM hypoplasia during pregnancy results from an imbalance of hormonal factors. This is supported by the descriptions of spontaneous haematological recovery that have been observed following termination of pregnancy, as well as the relapse of AA in subsequent pregnancies [56, 166, 167].

An epidemiological survey, to evaluate pregnancy as a possible aetiological factor in AA, was conducted in France in 1997. The 3 year prospective multicentric study documented the incidence, suspected aetiology and patient follow-up findings in 32 pregnant women. The patients' ages ranged from 15-44 years. In this cohort, 2 pregnancy-associated cases of AA (6%)

were demonstrated. This percentage (6%) is within the range of percentages found in both the patient cohort as well as in the general population [168].

Preexisting AA is sometimes worsened in pregnancy [169]. This observation may be explained by the fact that patients with AA cannot respond to the stress of pregnancy with a normal marrow response. This results in worsening of the pre-existing but undiagnosed pancytopenia. Furthermore, the bone marrow (BM) in patients with AA is still damaged and cannot respond normally to the stress of pregnancy. This results in a reduction in circulating blood cells [170].

There is no predilection for a specific gestational period in pregnancy associated AA. AA may develop in the first or subsequent pregnancies, and it may occur early or late in the pregnancy [161, 163, 164, 170].

Spontaneous haematological improvements following induced abortion or delivery, has been reported, although only in one third of cases [169]. Relapses during subsequent pregnancies has also been described, particularly in patients who have had a prior response to immunosuppressive therapy (IST). On the contrary, pregnancy does not trigger relapse in patients who have undergone successful allogeneic HSCT [170].

Increased antenatal complications have been demonstrated with AA. Maternal haemorhage and sepsis rates are escalated. Impaired oxygenation of the fetus results in growth retardation and even intrauterine death (IUD). Postpartum haemorhage (PPH), due to thrombocytopenia as well as impaired platelet function, has also been reported in patients with AA. "On review of the literature (among pregnant women with the diagnosis of AA), the rate of preterm birth was 12.1%, intrauterine death 16.7%, stillbirth 15.1%, and spontaneous miscarriage 16.7%" [167, 171-174].

There have been few reports of pregnancy following diagnosed AA. Although these are rare occurrences, successful pregnancy is possible with advances in supportive management (transfusion and treatment of infection) [162, 164, 172].

Although vaginal delivery is preferred, caesarian section (CS) should only be performed for obstetric indications [175].

1.5. Clinical presentation

Patients with AA most commonly present with symptoms of anaemia (pallor), skin or mucosal haemorrhages or visual disturbances due to retinal haemorrhages. Infection may be a presenting feature but is less common. There is no lymphadenopathy or hepatosplenomegaly (in the absence of infection), and these findings would strongly suggest another diagnosis [176]. A preceding history of jaundice, usually 2-3 months before the diagnosis is made, may indicate a post-hepatitic AA [25, 176].

A careful history and physical examination is crucial and may help to exclude inherited forms of AA, especially in children and young adults. It has recently been realized that the typical features of inherited BMFS may be absent and/or other features may be present in older patients. The findings of short stature, café-au-lait spots, abnormal thumbs and forearms, and skeletal anomalies would raise the possibility of an inherited form of AA, specifically Fanconi Anaemia (FA). However, FA may present in adults without physical anomalies. The most common presentation for patients with FA is between the ages of 3 and 14 years, although they can occasionally present later in their thirties (up to 32 years in males and 48 years in females) [19].

The findings of leucoplakia, nail dystrophy and pigmentation of the skin are characteristic of Dyskeratosis Congenita (DKC), another inherited form of AA. The median age at presentation of DKC is 7 years (range 6 months to 26 years) [177, 178].

None of these clinical features may be evident in some affected patients. The diagnosis is usually made at a later stage, when patients have failed to respond to IST [177, 179]. The family history is very important in patients with AA and an inherited TERC/TERT mutation. The pedigree may reveal avascular necrosis of bone, cirrhosis, pulmonary fibrosis, osteoporosis, low blood counts or cancer. In children or young adults, a diagnosis of Shwachman Diamond Syndrome (SDS) should be considered if there is a previous history of malabsorption or neutropenia, especially as the malabsorption often resolves in later life [25, 176].

In the context of **other primary BM disorders**, PNH usually refers to AAA. BM failure is the primary cause of anaemia in these patients. Hypocellular BMs, more severe thrombocytopenia (TCP), small PNH clones, lower reticulocyte counts, and modest or no elevation in LDH levels are more frequent in these patients. Less commonly, thrombosis may occur [180].

Subclinical PNH is asymptomatic or patients have normal/almost normal blood counts. Few (usually <10%) PNH granulocytes may be observed. These patients often have a diagnosed mild AA or have recovered hematopoiesis after treatment of AAA. Relapse of AA may be accompanied by PNH symptoms or expansion of the PNH clone [181].

1.6. Classification, Diagnostic work-up and differential diagnosis of Aplastic Anaemia

1.6.1 Diagnostic criteria:

To diagnose AA, at least two of the following criteria must be present:

(1) Haemoglobin below 10g/dl
(2) Platelet count below 100 x 10^9/l and
(3) Neutrophil count below 1.5 x 10^9/1

Bilineage or trilineage cytopenias less severe than indicated above are not classified as AA. These patients should have their blood counts closely monitored to determine whether they will develop AA later [(182)].

AA severity is graded into very severe, severe and non-severe AA, according to the blood count parameters and bone marrow findings, (see Figure 1.1 on page 18).

The classification of AA is related to the severity of depression of peripheral blood (PB) counts. The modified Camitta criteria are used to assess severity [(183)].

a) Severe Aplastic anaemia (SAA) is defined by:

-Bone marrow cellularity <25%, or if cellularity 25-50% with <30% residual hematopoietic cells

-AND any 2 out of 3 of the following:

- Peripheral blood neutrophil count < 0.5 x 10^9/l
- Peripheral blood platelet count < 20 x 10^9/l
- Peripheral blood reticulocyte count <20 x 10^9/l

b) Very severe aplastic anaemia (VSAA): As above, but absolute peripheral blood neutrophil count of <0.2 x 10^9/l

c) Non-severe aplastic anaemia (NSAA):

-Patients fulfilling the criteria for AA, but are neither severe or very severe.

Treatment decisions are based on disease severity assessment, which is also of prognostic significance.

In Severe AAA, infections are a major cause of death [(184)]. The main risk factor is the profound persistent neutropenia, which culminates in the development of invasive fungal and bacterial infections [(185)].

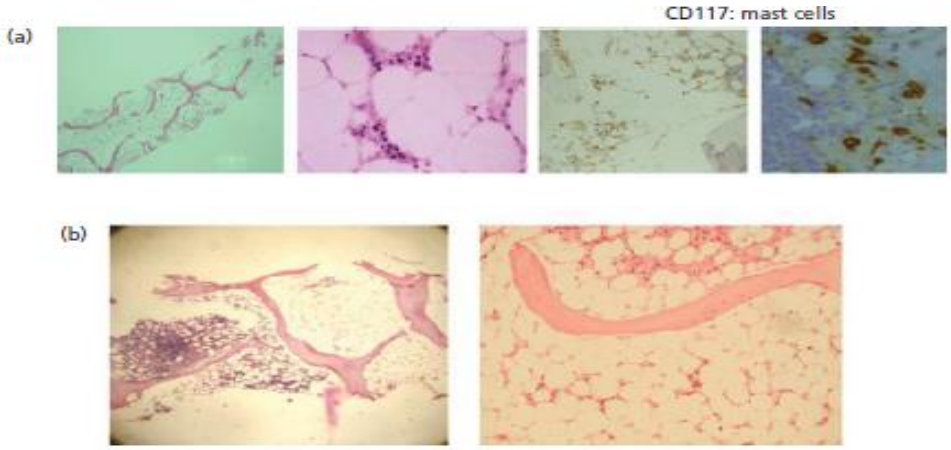


Figure 1.1 : Classification of AA a) BM trephine- severe AA b) Non-severe AA [186]

AA- aplastic anaemia. BM- bone marrow

1.6.2 Diagnosis of Hepatitis Associated Aplastic Anaemia (HAAA)

The following parameters can be used to diagnose hepatitis:

-An increase in serum Alanine Transaminase (ALT), Aspartate Transaminase (AST), by at least three times above the normal values. (which are 6 to 41U/l, 9-34U/1, 5-58U/L for ALT and AST respectively) [48, 92, 93, 104, 106, 187, 188, 189].

-An increase in serum Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and bilirubin (39-117U/1, 5-58 U/1, and 2-7 micromol/L respectively).

-Peripheral blood count can be determined by flow cytometry analysis with directly conjugated monoclonal antibodies for CD2, CD3, CD4, CD8, CD19 and HLA-DR

-Haematopoietic failure with bone marrow hypocellularity can be elucidated in terms of absolute neutrophil count (less than 500 per mm3), Platelet count (less than 20,000 per mm3), Reticulocyte count (less than 60,000 per mm^3) [189] and Prothrombin Index (%): normal value 70-100% [190].

-Various virological and serological markers are available for the detection of hepatitis A, B, C, D, E, G, TTV and parvovirus.
Among these tests, anti-HAV Ig total antibodies, HBsAg, HB core antigen, HBsIgG antibodies, various HCV recombinant antigens and hepatitis E virus IgM and IgG are being in use.
However, to determine the causative nature of all hepatitis viruses such as HCV, HDV, HEV and HGV, they can be qualitatively detected by RT-PCR reaction and DNA of parvovirus B19.

-TTV can be detected by Nested PCR [107, 189].

-IgG antibody for Cytomegalovirus, EBV and parvovirus has been found a useful tool for the diagnosis [48].

-However, serological and virological parameters for hepatitis A, B and C were found negative

in majority of the HAAA cases reported in several studies [102, 191].

Idiopathic AA is a diagnosis of exclusion. No single test reliably diagnosis idiopathic AAA. The diagnostic evaluation must include an assessment of alternative aetiologies of bone marrow failure. The "empty" marrow on histology of AA is characteristic, and is a prerequisite for diagnosis. To diagnose AA with certainty, it is important to consider and exclude other possible causes of pancytopenia with a hypocellular bone marrow such as aleukaemic leukaemia and hypocellular MDS. There is increasing recognition that IBMFS are commoner than previously thought and may present in adulthood. Hence, the correct diagnosis of IBMFS is vital for appropriate management in terms of therapeutic options, with implications for the choice of conditioning regimen and donors for HSCT. Patient education, genetic screening and counselling of family members is paramount [192].

1.6.3 Haematological investigations

1.6.3.1 Full Blood Count (FBC)

A pancytopenia is present. The haemoglobin (Hb) concentration, platelet counts and neutrophil counts are uniformly depressed. Isolated cytopenia(s) particularly thrombocytopenia, may occur in the early stages. There is preservation of the lymphocyte counts with a reversal of the neutrophil:lymphocyte ratio [7].

Blood smear examination**:** Anisopoikilocytosis is demonstrated. Macrocytosis is frequent. Neutrophils may show toxic granulation. Platelets are small in size. Abnormal platelets, blasts, dysplastic neutrophils as well as other abnormal cells, such as "hairy cells" are indicative of other causes of pancytopenia that are part of the differential diagnosis of pancytopenia with a hypocellular bone marrow [7].

1.6.3.2 Reticulocyte count

A reticulocytopenia is invariably present [7].

1.6.3.3 Bone marrow aspirate and trephine biopsy (BMAT)

Bone marrow aspirate fragments and trails are hypocellular for age. Prominent fat spaces are evident with a variable number of residual haemopoietic cells. Megakaryocytes and granulocytic (myeloid) cell series are absent or severely reduced. Dysplasia of these cell lineages is not a feature of AA. Erythropoiesis is markedly reduced or absent. Dyserythropoiesis, often marked, is a common finding that does not distinguish MDS from AA. Lymphocytes and plasma cells often appear prominent. Mast cells may also be easily seen. Early stages of AA may reveal increased macrophage activity with occasional haemophagocytosis. Background eosinophilic staining, representing interstitial oedema, may be apparent [193].

The BM trephine biopsy is uniformly hypocellular in most cases. Occasionally, hypocellularity can sometimes be patchy with residual cellular areas being present. Focal hyperplasia of erythroid or granulocytic cells at a similar stage of maturation may be evident. Small lymphoid aggregates can sometimes be seen, usually in the acute phase of AA or if the disease is associated with systemic autoimmune diseases, such as rheumatoid arthritis (RA) or SLE. Fibrosis (demonstrated by increased reticulin staining), dysplastic megakaryocytes and blasts are not seen in AA. The presence of these indicate a hypoplastic MDS or evolution to acute leukaemia [(7)].

1.6.3.4 Cytogenetics and FISH analysis

Prior assumptions stating that the presence of an abnormal cytogenetic clone was indicative of MDS and not AA, have been rectified. It has been demonstrated that abnormal cytogenetic clones are present in up to 12% of otherwise typical AA patients at diagnosis. These may be transient. They include (amongst others), del (13q) and trisomy 8. Monosomy 7, which is more often found in childhood MDS, can often be seen with adult AA. Due to insufficient metaphases, karyotyping may fail in severely hypocellular marrows. In these situations, FISH analysis for chromosomes 5, 7, 8, and 13 should be performed. Abnormal cytogenetic clones may be present at diagnosis or arise during the course of AA. The appearance of new karyotypic aberrations may provide evidence of clonal evolution [(194)].

1.6.3.5 Flow cytometry (FCM)

Flow cytometry for PNH should be performed on peripheral blood specimens of all patients with AA. This is a sensitive and quantitative test performed to detect deficient GPI anchored proteins. These proteins include CD14, CD16, CD24, as well as fluorescent aerolysin (FLAER) on white blood cells; and CD55 and CD59 on red cells. These markers are reduced/absent in PNH (see Chapter 4). Small PNH clones occurring in up to 50% of patients with AA, can be detected by FCM [(195-197)].

1.6.4 Investigations required to screen for IBMFS:

a) Peripheral blood chromosomal breakage analysis: diepoxybutane test (DEB test)

A clastogen stress test should be performed on all children with AA. A false negative test can result from somatic reversion of the FA gene mutation. This is found in at least 10% of cases [(198)]. If there is a negative stress test but the clinical suspicion remains high, the diagnosis of FA can be confirmed by testing skin fibroblasts for increased chromosomal breakage. Complementation group determination and mutational analysis can then facilitate genetic counseling. It has been recommended that this test be performed in patients younger than 50 years. However, older patients should also be screened if FA is clinically suspected. Defining an upper age limit for FA screening is difficult as cases have been reported in the fourth, and rarely

fifth decade of life [199]. All siblings of patients with FA, as well as potential transplant candidates should be screened using this test [7].

Testing for other IBMFS will depend on the degree of clinical suspicion.

b) Peripheral blood leucocyte telomere length (Flow-FISH)

The detection of very short telomeres in blood leucocytes is characteristic of DKC. This is typically less than the 1st centile for age. However, not all patients with DKC have short telomeres and short telomeres may also occur in AAA with reduced stem cell reserve. Flow cytometry with fluorescent in situ hybridization (Flow-FISH) is a laboratory technique used to screen for abnormal telomere length. It is however, unavailable for routine clinical testing [199].

Mutation screening for TERC and TERT aberrations, is an alternative method. There are probably many, as yet, unidentified mutations [200]. A negative genetic screen is hence insufficient to exclude DKC. However, these tests are useful for disease screening for telomere gene mutations in classic DKC, although less specific in adult onset AA with TERC/TERT mutations [7].

c) Molecular analysis of the Shwachman Bodian Diamond Syndrome (SBDS) gene

This test can confirm a diagnosis of Shwachman-Diamond Syndrome (SDS), however 10% of children with SDS do not have a mutation in the SBDS gene [201]. The exocrine pancreatic insufficiency that can be screened for as a child, usually improves with age and may not present in adults.

d) Emerging diagnostic tests which are not currently routine diagnostic tests, but are likely to be so within the next few years include:

Next generation sequencing, gene panels for:

- Telomere gene complex mutations
- Other IBMFS
- Acquired somatic mutations, typical of myeloid malignancies, to help distinguish AA from hypocellular MDS and for early detection of clonal evolution to MDS/AML

Single nucleotide polymorphism array karyotyping

Whole genome scanning to detect unbalanced chromosomal defects

e) Ancillary tests to support a diagnosis:

Radiological investigations are useful if an IBMFS is suspected. X-rays of the hands, forearms and feet will reveal skeletal abnormalities as in FA. If DKC or constitutional RUNX1 bone marrow failure syndrome is suspected, a high resolution CT scan of the chest is indicated. Abnormal or anatomically displaced kidneys are a feature of FA, and are evident on abdominal ultrasound scan [(7)].

HbF% (Foetal Haemoglobin)

HbF is often elevated in constitutional syndromes. As it is an important prognostic factor in children, the levels must be measured pre transfusion [(7)].

1.6.5. Investigations to exclude other aetiologies:

Vitamin B12 and Folate

A documented vitamin B12 or folate deficiency must be treated before a diagnosis of AA is made, although bone marrow aplasia as a result of vitamin deficiency is exceedingly rare [(7).]

Anti-nuclear antibody and anti-double stranded DNA

Pancytopenia in SLE may rarely be associated with a hypocellular marrow. It is usually autoimmune with a cellular bone marrow, or may occasionally be associated with myelofibrosis. In a patient with signs and symptoms of SLE, the above tests aid in the diagnosis [(7)].

Virological tests: Viral studies for hepatitis A/B/C, EBV, CMV, HIV and Parvovirus B19 are routinely performed in the work-up of patients with AA. In post-hepatitic AA the serology for the known hepatitis viruses is usually negative. If SCT is being considered, CMV should be assessed. HIV and parvovirus B19 are very rare causes of AA [(7)].

Abdominal ultrasound scan

Splenomegaly and/or lymphadenopathy warrant investigation for malignant haematological disorders that are secondary causes of pancytopenia [(7)].

Biochemistry investigations

Liver function tests (LFTs): should routinely be performed to detect antecedent/on-going hepatitis and exclude any other pathology that may be present [(7)].

Renal function tests: A deranged Urea and Electrolytes (U&E) may be present in severe sepsis [(7)].

1.7 Management of AA

Although AA is rare, it is a serious disease and the treating physician responsible for the patient must contact a haematology centre/haematologist with experience and skill in the management of AA. This must occur as soon as possible after the patient presents and has been assessed, in order to formulate a patient management plan. All patients should be offered the opportunity to be assessed at a specialist Centre. Where possible, patients with AA should be enrolled into prospective clinical trials [193].

Initial management constitutes stabilizing the patient clinically. Blood loss should be controlled and infections treated, prior to administration of disease specific therapy. Infection is an adverse factor affecting the outcome post HSCT. It may, however, sometimes be required to proceed with HSCT whilst active infections (particularly fungal), are present. In this situation, BMT may provide the best chance of early neutrophil recovery. A delay in BMT could result in progression of the fungal infection [193].

1.7.1 Supportive Care

1.7.1.1 Blood product support

a) Red blood cell (RBC) Transfusion

In patients with AA, RBC transfusion is mandatory to preserve a safe Hb level, thereby improving the symptoms of anemia and maintaining a good quality of life (QOL). It is the clinical symptoms (signs of anemia) that determine when RBC transfusion is necessary.

The patient's age as well as any cardiac, pulmonary or vascular co-morbidities must be considered. No specific pre-transfusion Hb concentration trigger has been suggested, however it is important to be symptom free and maintain QOL. Elderly patients as well as those with co-morbidities may require a higher transfusion trigger. In doing so, the optimal method of RBC transfusion involves maximizing patient outcome whilst simultaneously avoiding wastage of blood products [202].

The commonest risks associated with regular transfusion therapy include alloimmunization against red cell antigens and iron overload. The alloimmunization risk can be limited with the provision of phenotype-matched blood (for Rh and Kell blood groups) [203].

b) Platelet (plt) Transfusion

Platelet transfusion support may frequently be required for patients with AA. With the exception of one publication, literature pertaining to plt transfusion support in AA is deficient [204]. Information has been taken from studies looking at patients with reversible thrombocytopenia, and their requirement for plts [205, 206].

The following recommendations have been made by the British Committee for Standards in Haematology (BCSH):

1) Prophylactic platelet transfusions are necessary for stable AA patients on active therapy with a platelet count <10 X 10^9/1.

2) In septic patients, the platelet count should be kept >20 x 10^9/1.

3) In patients with TCP, who require invasive procedures, plt transfusions are required. The aim should be to achieve a platelet count in line with BCSH guidelines for the relevant procedures. (British Committee for Standards in Haematology, 2003) A platelet count should be checked pre-procedure.

4) Whilst on ATG treatment, worsening TCP may result as a consequence of increased platelet consumption in the presence of cross-reacting antibodies to ATG, binding to platelets. The exact threshold for platelet transfusion prior to ATG treatment is not known. However, a threshold of 20 x 10^9/1 has been suggested [207, 208].

5) HLA and non-HLA (minor histocompatibility) alloimmunization resulting from regular RBC and plt transfusion results in poor platelet increments as well as post HSCT graft rejection risk. This risk may be reduced, but not completely eliminated by leucodepletion of cellular blood components [209, 210].

6) For patients' refractory to plt transfusion due to HLA alloimmunization, the use of HLA-matched platelets is a consideration. Other causes of platelet refractoriness (such as infection) must be excluded prior to this. If HLA antibodies are absent and patients do not demonstrate an increment with the administered HLA-matched platelets, investigation and matching for human platelet antigen antibodies is warranted [203].

c) Granulocyte transfusions

In patients with severe neutropenia-related life-threatening infections, the use of irradiated granulocytes may be considered as it may be a life saving measure. There is limited data regarding the efficacy of granulocyte concentrates. There have also been reports of adverse events, such as transfusion-related acute lung injury (TRALI), alloimmunization and febrile reactions, linked to its use [211].

d) Use of irradiated cellular blood components for AA patients

Irradiation of cellular blood components prevents transfusion-associated graft-versus-host disease (TA-GVHD). Although it is a rare complication of blood transfusion, TA-GVHD has a reported fatality rate of 100%. Irradiation may also decrease the alloimmunization risk in AA [212].

Irradiation of cellular blood components is indicated for:

1) AA patients undergoing HSCT [213].
2) All granulocyte concentrates and HLA- matched platelets.
3) Patients receiving ATG. It is uncertain as to how long after ATG therapy, irradiated blood products should be continued for. It has been suggested that it should be continued while patients continue to take CSA following ATG therapy [214, 215].
4) Patients treated with alemtuzumab [213].

e) Iron chelation therapy

Tissue iron overload is a serious complication in AA patients receiving regular RBC transfusion. Although it is an unreliable parameter, the serum ferritin is still the most widely used indicator for assessment of iron overload. In HSCT patients, a raised serum ferritin reflects an adverse outcome. Cardiac and liver iron can be quantified by magnetic resonance imaging (MRI) (T2* or R2). Although this method is an extremely useful adjunct, there have been no publications regarding its utility in AA. Data regarding iron chelation therapy (ICT) in AA is lacking [216].

A study conducted by Lee et al, 2010, confirmed that ICT with deferasirox can be safely administered to aplastic anaemia patients. There was a reduction in serum ferritin levels, and drug-induced cytopenias did not occur [211]. Dose adjustments are necessary to chelate heavily transfusion dependent patients. Renal dysfunction may occur with deferasirox, which should be used cautiously in AA patients consuming CSA. Deferiprone, although extremely efficacious, cannot be used in AA patients as it is contraindicated in neutropenic patients [217]. Venesection can also be performed for iron overload. It is recommended in those patients responding to immunosuppression, and can even be used after successful HSCT [203].

1.7.1.2 Infection: prevention and treatment

The major cause of death in patients with AA is infection [218]. Neutropenia in SAA is protracted and persistent. Invasive fungal infections (IFI) and severe bacterial sepsis occur frequently. This is contrary to neutropenia experienced in cancer patients post chemotherapy [215].

a) Prevention of infections

Ideally, isolation facilities should be available in hospital for nursing severely neutropenic AA patients. Antibiotics, antifungals, mouth care products (antiseptic mouthwash such as chlorhexidine or saline), and meals with low bacterial content are common prophylactic measures. Hospitals should have local policies guiding the use of prophylactic antibiotics. Many clinicians advocate the use of two non-absorbables (e.g. colistin and neomycin) or quinolones (e.g. ciprofloxacin). An antifungal, preferably itraconazole or posaconazole, is advised depending on availability. Prophylaxis against Pneumocystis jirovecii is not routine practice. Anti-viral prophylaxis in untreated patients with AA is also not recommended [214].

Acyclovir or valaciclovir is used prophylactically during and post ATG therapy. However, sub-clinical reactivation of CMV and EBV, which is usually self-limiting, is a common occurrence during ATG therapy [203].

b) Treatment of infections

Clinicians should be guided by local hospital policy and protocols for the management of febrile neutropenia. Patients should be assessed and treated for bacterial, viral and fungal infections [219]. If an IFI is clinically suspected, empirical anti-fungal therapy should be initiated early. Granulocyte transfusions are a potentially lifesaving measure especially in conditions such as severe sepsis due to invasive fungal disease. They are also particularly important for patients due to proceed to HSCT [220].

1.7.1.3 Haemopoietic growth factors (HGF)

Erythropoiesis-stimulating agents (ESA) and granulocyte colony-stimulating factor (G-CSF) are HGF. They are often ineffective in maintaining blood counts in patients with AA [221]. However, the thrombopoietin-mimetic (eltrombopag) has demonstrated encouraging preliminary results [222].

1.7.2 Specific Therapy

1.7.2.1 Immunosuppressive therapy

a) Current standard first line IST

Standard first line IST constitutes the combination of horse ATG (ATGAM; Pfizer, New York, NY, USA) and CSA. Lymphoglobuline horse ATG, which was previously used, is no longer available [1, 7, 223]. Studies have reported significantly improved therapeutic responses, as well as survival rates with horse ATG compared to rabbit ATG [207, 224]. G-CSF is not routinely indicated for use with ATG + CSA [225]. The side effects of ATG are ameliorated with the use of prednisolone.

b) Indications

ATG + CSA is indicated as first line therapy for:

- NSAA patients who are bleeding, experiencing infections, transfusion dependent or for the preservation of lifestyle activities.
- SAA/VSAA patients with no HLA-matched sibling/donor
- SAA/VSAA patients approaching >35-50 years of age

Although there is no set upper age limit for ATG, the mortality rate is reported to be higher in patients aged >60 years treated with ATG [225, 226]. Careful co-morbidity assessment is necessary to evaluate medical fitness prior to consideration for ATG in patients aged >60 years.

Post ATG therapy, these patients complicate with bleeding and infection. This culminates in increased mortality in this age group [203].

If there is a failure to respond to therapy the first time, or if patients relapse after a first course, and even if the patient does not warrant an unrelated donor (UD) HSCT, a second course of ATG may be indicated [1, 7, 223]. Rabbit ATG may be used for a second course. As an alternative, horse ATG may also be utilized for the second course. The disadvantage is the association with more immediate and late (serum sickness) side effects [224]. Rabbit ATG, in contrast to horse ATG, is known to produce a severe prolonged lymphodepletion. In some studies, a higher infection rate has been reported with rabbit ATG making adequate prophylactic antimicrobial support fundamental with its use [203].

c) Administration of ATG:

Antithymocyte globulin is a potent immunosuppressive agent which is administered on an in-patient basis. Familiarity with the drug, its use and potential side effects is essential. Patients should be afebrile (ideally), as well as clinically and hemodynamically stable prior to starting ATG therapy.

Platelet refractoriness must be excluded with platelet count increment studies. Administration of all prophylactic antiviral, antibiotic and antifungal medications should be commenced before ATG [203].

Antithymocyte globulin has both early and delayed adverse effects. Early reactions include: fever, rash, rigors, hypo/hypertension, fluid retention, and rarely acute pulmonary oedema/adult respiratory distress syndrome and anaphylaxis. Delayed side effects include serum sickness occurring 7-14 days from ATG commencement. This commonly occurs with arthralgia, myalgia, rash and fever.

Treatment of serum sickness includes: adequate analgesia and intravenous hydrocortisone 100mg four times a day (QDS). This is usually required for a few days. Serum sickness results in platelet consumption, and additional platelet transfusions are often needed during this period. There is no reported proven benefit of using G-CSF with ATG + CSA. Neither patient response nor overall survival (OS) is improved [225].

The equine/horse ATG (ATGAM) dose is 40 mg/kg/d for 4 d, administered as an intravenous infusion over 12-18h. A prior ‘test’ dose must be administered, due to the anaphylactic risk. Each dose of ATG should be preceded with intravenous (IV) methyl prednisolone 1 mg/kg and chlorphenaramine. Platelet transfusions are necessary to maintain the platelet count >20-30 x $10^9/1$ [1, 7].

Febrile episodes, should be treated with broad-spectrum IV antibiotics, irrespective of the neutrophil count. Fluid balance is critical, especially in elderly patients, as fluid retention is a common occurrence whilst on ATG treatment. Prednisolone at a dose of 1 mg/kg/d for 2 weeks,

is started on the day after ATG is completed. Rapid tapering of prednisolone over the next 2 weeks must follow [203].

Treatment with CSA is commenced as the prednisolone dose is tapered. CSA is initiated at a dose of 5 mg/kg/d to achieve trough blood levels of 100-200 µg/l. It must be continued whilst the blood count continues to steadily improve. To reduce the risk of later relapse, slow CSA tapering (25 mg every 2-3 months) can be initiated after at least a further 12 months of therapy [227].

d) Outcomes:

Patient and disease response to ATG therapy is often delayed. It usually starts after an average of 3-4 months. Horse ATG has a 6-month response rate of approximately 70%. The five-year overall survival is age-dependent. ATG + CSA have significantly higher response rates in NSAA, compared to CSA alone [277]. Up to 35% of patients relapse after treatment with ATG. There is a risk of clonal evolution to MDS/acute myeloid leukaemia (AML) in 15% of cases. 10% of patients may develop hemolytic PNH [1, 229].

Studies report that approximately 35% of refractory AA patients and 55-60% of relapsed AA patients respond to a second course of ATG [1, 26, 31, 35, 228, 230].

e) Other immune suppressive drugs:

Other immune suppressive drugs have been used in AA, however it is recommended that expert advice be sought when considering these. Amongst those not recommended in the treatment of AA are Mycophenolate mofetil, sirolimus, corticosteroids and cyclophosphamide [203].

f) Vaccinations:

The potential risk of relapse of AA following vaccinations in responders to IST, exists. Evidence is limited, however the hypothesis that a viral insult is likely to be the trigger in the pathogenesis of AA is a crucial one [231, 232]. Vaccinations in non-transplanted patients should be avoided. This includes the influenza vaccine. Vaccinations are however, routinely recommended following HSCT for AA [203].

1.7.2.2 Haemopoietic stem cell transplant (HSCT) in AA

a) Current indications for HSCT in adults

The current indications for HSCT are guided by the European Blood and Marrow Transplantation (EBMT) SAA working party (SAAWP) [233].

HLA identical sibling donor: Up-front HSCT from a matched sibling donor (MSD) is indicated in young and adult patients, with SAA who have a MSD. Similar outcomes have been reported for patients aged 40-50 as those aged 30-40 years [230]. Co-morbidities should, however be carefully assessed, to evaluate medical fitness for up-front transplantation instead of IST for patients aged 35-50 years [203].

Unrelated donor: UD HSCT is indicated for SAA if there was a failure in response to one course of IST. No strict upper age limit has been delineated. However, this should be individualized according to co-morbidities at the transplant institute. Donors should be a 10/10- or 9/10- match, based on HLA high resolution typing for class 1 (HLA-A, -B, -C) and class II (HLA-DRBI, -DQBI) antigens [203].

Alternative donor: HSCT using either cord blood, a haploidentical family donor or a 9/10-matched UD are possible considerations, among other treatment options. They may be performed after failure to respond to IST or in the absence of a matched sibling donor (MSD) and a suitably matched UD [233, 234]. Donor screening for donor-directed HLA antibodies must be performed, as these are associated with a very high risk of graft rejection, if detected. Clear guidance on the exact indication for alternative donor HSCT is lacking, as it has been reported to be less successful than MSD or UD HSCT [203].

Syngeneic donor: In the rare circumstance that a syngeneic donor is available, HSCT should be considered in all patients irrespective of the patients age. It has been reported that the long term OS exceeds 90% [218].

b) Timing of donor search/availability

HLA tissue typing must be performed at the time of diagnosis in all newly diagnosed AA patients, who are potential transplant candidates. This facilitates a MSD HSCT to proceed as early as possible, thereby avoiding the complication of sensitization to HLA and minor histocompatibility antigens. Also, as potential availability of UDs has been realized, patients may proceed to UD HSCT should there be no response to a course of ATG and CSA. This can even be considered earlier if the patient has severe and/or recurrent infections. A response to IST is usually assessed at 3-6 months.

c) Pre-transplant work up

A multi-disciplinary team (MDT) approach is a necessity in the pre-transplant patient work up, which aims to: (i) confirm the diagnosis and exclude/document clonal evolution, (ii) evaluate co-morbidities, (iii) select the donor, conditioning regimen, stem cell dose and source, (iv) address fertility issues and (v) inform the transfusion laboratory of the potential transplant and review of transfusion requirements [203].

d) Conditioning regimens

The (i) patient's age, (ii) donor type and (iii) choice of antibody (ATG or alemtuzumab) determine the choice of conditioning regimen to be used [235-238].

The survival for adult MSD HSCT is age dependent. The OS between the ages of 30 and 50, is reported to be 70-85%. Recent analysis has indicated that outcomes after UD HSCT are no longer inferior to MSD HSCT [239-240].

1.7.2.3 Thrombopoietin mimetics: Eltrombopag

Eltrombopag, a small molecule, oral thrombopoietin receptor agonist was observed to have haematological responses, including a trilineage response, in 40% of patients in a study

performed to assess its use in AA. Most patients tolerated the therapy well. Deranged LFT's particularly, an elevated transaminase level has been observed. There have been reported concerns about clonal evolution, including monosomy 7, which requires further evaluation [226].

Eltrombopag has been Food and Drug Administration (FDA) approved in the United States of America (USA), for treatment of SAA refractory to IST. It has also been licensed for SAA refractory to IST or patients who are heavily pre-treated and unsuitable for HSCT. It should be cautiously used with meticulous long term monitoring for clonal evolution. A BMAT should be performed to exclude an abnormal cytogenetic clone typical of MDS/AA, particularly monosomy 7, prior to starting treatment [203].

1.8. Management of AA in specific populations

1.8.1 Management of AA in pregnancy

The relationship between AA may be either causal or coincidental, however it remains a serious condition which poses management challenges, and variability in clinical outcome. The diagnosis of AA may be made for the first time during pregnancy, at an early or late gestation [203].

Cytopenia(s) will often progress during the pregnancy. However, there have been reports of spontaneous disease remission after abortion (spontaneous or therapeutic) or post-delivery [170]. Relapse of AA is a common occurrence during pregnancy in patients who have previously responded to ATG. This occurs particularly in patients who demonstrate a partial response [241]. On the contrary, pregnancy does not trigger disease relapse in patients who had undergone successful HSCT [203].

A study conducted by Tichelli et al, 2002, evaluated the outcomes of 36 pregnancies in women previously treated with IST. Approximately 50% involved maternal complications, of which 3 abortions, and 2 cases each of eclampsia and maternal deaths, were reported. Fetal complications, including 5 premature deaths were also documented. Relapse of AA was evident in 19% and a further 14% needed transfusion support during delivery. The presence of normal blood counts prior to conception did not safeguard against relapse of AA during pregnancy [241].

Improvements in maternal and fetal outcome have been reported in recent years due to improved supportive care, particularly the supply of blood products [171]. Counselling regarding the potentially serious risks to both the mother and baby must be discussed with patients and their family [242]. Patients must be monitored frequently throughout the pregnancy. They can be seen monthly initially, however the frequency should be increased later according to disease severity. Close liaison with the obstetric team and haematologist is essential in the management of these

patients. The presence of a PNH clone warrants discussion with a specialist haematology centre. The mode of delivery is to be determined on obstetric grounds [203].

The mainstay of treatment of AA in pregnancy is supportive care. The platelet count should ideally be maintained above 20 x 10^9/l. Although platelet transfusions are usually necessary to achieve this, the risk of alloimmunization and platelet refractoriness need to be considered. CSA, which is safe during pregnancy, is recommended for patients who are transfusion dependent. ATG, androgens and allogeneic HSCT are not recommended for AA during pregnancy [243].

1.8.2 Management of AA in elderly patients

Compared to younger patients, the management of elderly patients with AA (aged >60 years) is considerably more complex. To compound this, patient outcomes are worse due to poor treatment tolerability [203].

Patient management should be individualized. They should be thoroughly assessed for co-morbidities and other conditions which are more common in this age group, such as hypoplastic MDS, which should be excluded. A good quality of life is an important outcome in this age cohort and patients should have their specific requests respected. Even in the very elderly, older age per se, is not a reason to withhold treatment. The treatment of choice in SAA remains immunosuppression.

In NSAA patients, the combination of ATG and CSA has demonstrated more rapid and complete responses compared to CSA alone [228]. The disadvantage is that patients must be hospitalized. The risk of acute and delayed toxicity is also higher compared to younger patients. Hence the risk-benefit ratio of treatment should be weighed up individually. The risks of infection, bleeding, cardiac failure and arrhythmias with ATG is significantly elevated in the elderly. Therefore, all patients must have a thorough assessment prior to treatment initiation. An inferior survival rate has been documented in older patients after ATG therapy compared to younger patients [226].

Allogeneic HSCT has no place as first line therapy in patients aged >60 years. HSCT, may however be considered in specific individuals with a syngeneic donor. Whilst some have reported that the treatment which is most convenient and least toxic should be administered, others considered how quickly a response is desired. Examples of these include patients with life threatening cytopenias (neutrophil count <0.2 x 10^9/1) or patients hospitalized for severe infection, who should be treated more intensely compared to those with less severe AA [203].

Alternative treatments such as CSA alone, oxymetholone (or danazol) or alemtuzumab, have been used in elderly patients [228]. CSA alone has the advantage of being convenient as it is outpatient-based treatment. Patients require close monitoring to detect nephrotoxicity and hypertension. Alemtuzumab as a single agent, is a consideration in refractory/relapsed AA.

However, very careful assessment of medical fitness in older patients prior to considering this agent is necessary [1].

In men who are intolerant or unresponsive to CSA, oxymetholone or danazol can be considered [236, 244]. Danazol, which is known to have fewer masculinizing adverse effects than oxymetholone, is a better alternative for female patients. Nephrotoxity, hepatic tumours, mood changes, cardiac failure, prostatic enlargement and raised blood lipids have necessitated careful monitoring of oxymetholone therapy. Best supportive care should be offered to patients who are intolerant of, or who decline IST [203].

1.8.3 Management of HAAA

As a result of the poor prognosis in HAAA, the standard treatment remains allogeneic BMT from HLA matched siblings [105, 235]. HLA matched donors are easier to locate because HAAA mostly occurs in children [237]. IST post BMT is effective. ATG and CSA have been safely administered without eliciting any acute side effects. Glucocorticoids have also been employed in combination with immunosuppressive medications for the treatment of HAAA patients [237].

High doses of Cyclophosphamide (CY), (a potent immunosuppressive drug which elicits its effects by readily destroying the lymphocytes and committed myeloid cells) induces a durable remission in HAAA. HSC release an enzyme called aldehyde dehydrogenase which inactivates the drug. Hence, HSC are not susceptible to CY toxicity [105].

It has been reported that the administration of antiretroviral therapy improves Parvovirus induced AA [113, 238]. Antiviral therapy for treating hepatitis B associated HAAA is not known. However, there have been studies in which the nucleoside analog, lamivudine (3TC), has been administered for AA secondary to HBV infection. Remission of AA was the result in patients with SAA accompanied by HBV infection.

Although Interferon is a known and effective treatment for both HBV and HCV infections, it has myelosuppressive effects, which limit its use as a potential therapy for HAAA [113].

1.8.4 Management of PNH

The only widely effective therapies for classical PNH patients is terminal complement inhibition using eculizumab and allogeneic bone marrow transplantation (BMT). Corticosteroids may reduce hemolysis and improve hemoglobin levels in some PNH patients. However, the long-term toxicity and limited efficacy restricts their use.

a) Eculizumab

Eculizumab is a humanized monoclonal antibody that is highly effective in treating PNH. The natural history of the disease has been altered with this drug [245-248]. By binding to C5, it blocks

terminal complement. It is the only approved treatment for PNH [249, 246, 247, 250]. It requires intravenous administration every 7 days for the first 5 weeks, and then biweekly thereafter. Eculizumab inhibits MAC formation. This compensates for the CD59 deficiency in PNH patients. There is, however, no compensation for the CD55 deficiency. Hence, this drug is highly effective in abrogating the IVH in PNH, but the mild to moderate extravascular hemolysis due to C3d deposition on the PNH red cells, continues [251].

Treatment decisions should not be based solely on the size of the PNH clone. BM failure is not alleviated by therapy with Eculizumab, hence patients with AA are unlikely to derive benefit from its use and therapy should address the BM failure. Nearly all classical PNH patients will respond to eculizumab.

b) Bone Marrow Transplant (BMT)

Given the risks of transplant-related morbidity and mortality, BMT should not be offered as initial therapy for patients with classical PNH [252, 253]. In countries where eculizumab is not available or in patents with the heterozygous c.2654G→A mutations in C5, this may be an exception [254].

Patients with the prerequisites for SAA with PNH clones, are good candidates for BMT if they are young and have a suitable donor. Myeloablative conditioning regimens are not necessary to eliminate PNH clones as cure can result from allogeneic BMT following non-myeloablative conditioning regimens [255, 256].

Non-myeloablative regimens preserve fertility and are preferred in patients with moderate organ dysfunction who may not tolerate a myeloablative regimen. Continued use and investigation of BMT in selected patients is reasonable as it is the only curative therapy available for PNH [257, 258].

CHAPTER TWO: PATIENTS AND METHODS

2.1. Study design and population

This is a retrospective study of all adult patients (according to hospital admission criteria-males and females over 12 years of age) with a confirmed bone marrow diagnosis of Aplastic/Hypoplastic Anaemia made at the NHLS Haematology Laboratory at Inkosi Albert Luthuli Central Hospital, Durban, Kwa Zulu Natal, during the period January 2005 to October 2015.

The inclusion criteria were:

All patients with a bone marrow diagnosis of aplastic/hypoplastic anaemia made at the haematology laboratory at the NHLS, IALCH were included in this study.

The exclusion criteria were:

Patients whose bone marrows were sent to laboratories other than the NHLS at IALCH, and whose diagnosis of aplastic/hypoplastic anaemia was made at other laboratories were not included in this study.

Patients not fulfilling the definition of aplastic/hypoplastic anaemia (ie. a peripheral pancytopenia together with a hypocellular/acellular bone marrow in the absence of an abnormal infiltrate or marrow fibrosis).

2.2. Methods

Data was collected retrospectively. The bone marrow aspirate and trephine (BMAT) biopsy of each patient was reviewed in great detail. Available demographic, clinical and laboratory parameters for each patient were documented on a standardized data collection sheet (Appendix 1). Data was obtained from documented bone marrow referral forms/letters, bone marrow recording journals, laboratory information computer systems and available results on TrakCare laboratory information system. Demographic and clinical data for patients who were referred to IALCH was obtained from patient hospital records on the Soarian and Meditech databases.

2.3. Data analysis

Data was recorded in an EXCEL spreadsheet and analyzed by a statistician. STATA v13.1 statistical software was used in the analysis. Missing data was excluded from the analysis. The data was summarized using frequency and percents for categorical data. Numeric data was examined using Shapiro Wilk test for normality. Means (SD) were used for data meeting the assumption of normality and medians (IQR) for others. Sub group comparisons were made using

the Chi square test for categorical data and t test or ANOVA for comparing means. A p value of < 0.05 was considered statistically significant.

CHAPTER THREE: RESULTS

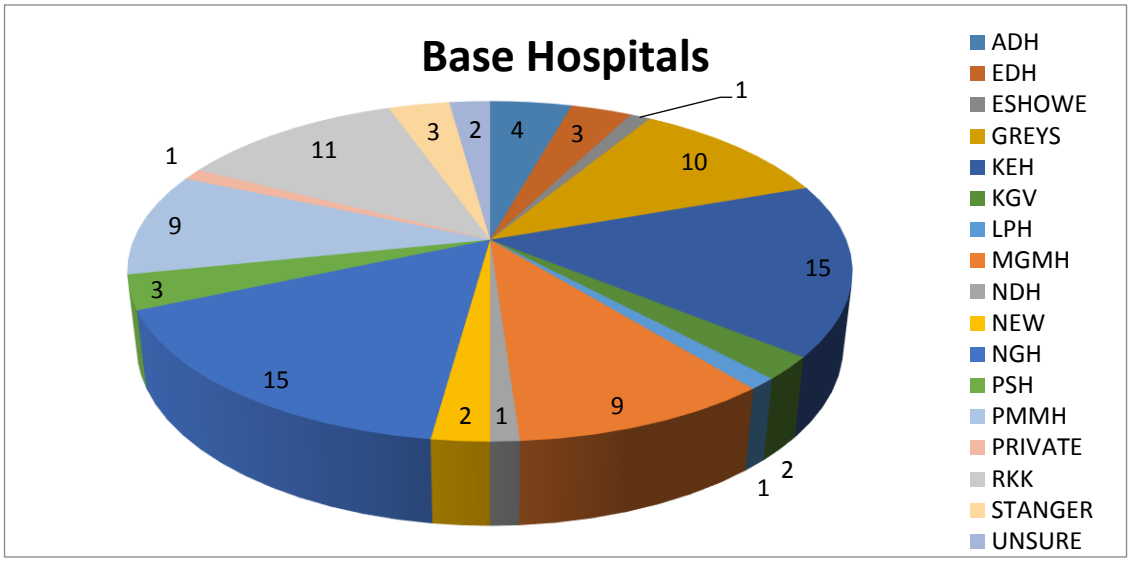
3.1 Hospital referral system

The pie chart on the following chart displays the hospitals from which the bone marrow aspirate and trephine biopsies were referred to Inkosi Albert Luthuli Central Hospital (IALCH) for assessment (Figure 3.1).

The largest number of referrals were from hospitals with a wide drainage area in terms of the number of other hospitals referring to them. These include King Edward Hospital (15 cases), Ngwelezane Hospital (NGH) (15 cases), Greys hospital (10 cases), R K Khan Hospital (RKK) (11 cases), Prince Mshiyeni Memorial Hospital (PMMH) (9 cases) and Mahatma Gandhi Memorial Hospital (PMMH) (9 cases).

Despite being diagnosed at the NHLS Haematology Laboratory at IALCH, 13 of the 92 patients did not reach the clinical team at IALCH, which is the only haematology centre in the KZN public sector that offers treatment for this condition. Of the 13 cases, 5 were from King Edward Hospital, 2 from Edendale Hospital, 2 from King George Hospital (now known as King Dinizulu hospital) and 1 each from Greys hospital, Stanger hospital, RKK and NGH. All of these patients that had not been referred to IALCH Clinical Haematology for assessment and management were categorized as Severe AA (SAA) according to their peripheral blood counts and BMATs. One patient demised at KEH. As the other 12 patients did not reach IALCH, clinical information was not available for them and their outcome is not known.

Figure 3.1: Distribution of referral hospitals



ADH- Addington Hospital. EDH- Edendale Hospital. KEH- King Edward Hospital. KGV- King George V Hospital. LPH- Ladysmith Provincial Hospital. MGMH- Mahatma Ghandi Memorial Hospital. NDH- Northdale Hospital. NEW- Newcastle Hospital. NGH- Ngwelezane Hospital. PSH- Port Shepstone Hospital. PMMH- Prince Mshiyeni Memorial Hospital. RKK- R K Khan Hospital

3.2 Patient characteristics

A total of 92 patients with Aplastic/Hypoplastic anaemia were diagnosed between January 2005 and October 2015. There were 42 males (M) and 50 females (F), with a male: female (M:F) ratio of 0.8:1.

The median age at presentation was 24 years (range 13-75 years). The inter-quartile range (IQR) was 19-35 years. The median age amongst the males was 23 years (range 15-75 years). The inter-quartile range (IQR) was 20-29 years. The median range amongst the females was 25 years (13-68 years). The inter-quartile range (IQR) was 18-43 years.

Of the 92 patients, 80% (73 of 92) were Black, 15% (14 of 92) Indian and 5% (5 of 92) Caucasian. In the cohort of Black patients, the median age at presentation was 23 years (range13-59years). The Indian patients had a median age of 33 years (range16-68years). The median age in the Caucasian population was 48 years, with a range of 17-75years.

Approximately 53% of patients (49 of 92) were between the ages of 13-25 years. Of these, 25 were female and 24 were male. 44 were of Black ethnicity, 4 were Indian and 1 was Caucasian.

Only 5.4% of patients (5 of 92) were older than 60 years. Within the cohort aged older than 60 years, 4 patients were Indian and 1 was Caucasian. 3 patients were female and 2 were male.

The number of patients between the ages of 26 and 59 years, constituted 38%. Blacks comprised 26 patients; Indians 6 and Caucasians 3 in this cohort. 19 patients were female and 16 were male. No Black patients were older than 60 years of age at presentation (see Figures 3.2a and 3.2b below).

Figure 3.2a: Histogram for Age and Ethnicity

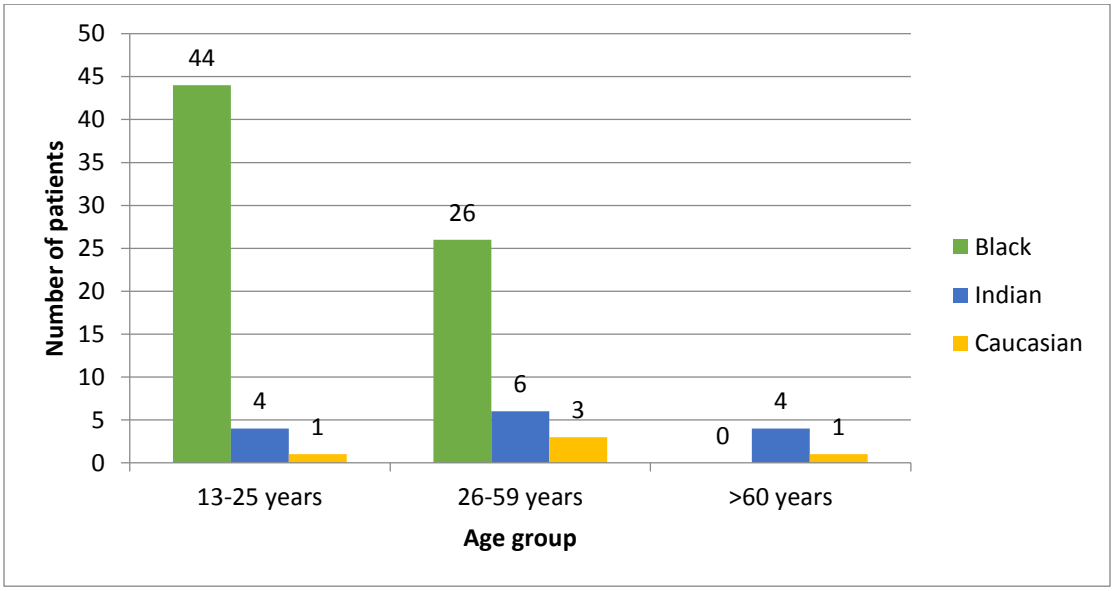
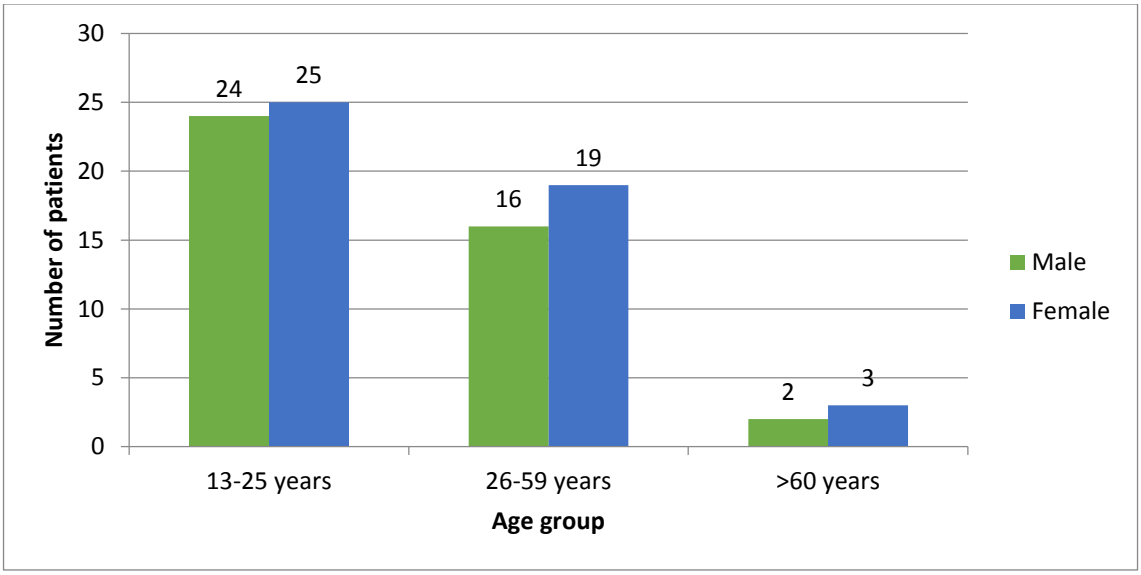


Figure 3.2b: Histogram for Age and Gender



3.3 Failed Bone Marrow Aspirate and Trephine Biopsies (BMATs)

Of the 92 BMATs received, 15 patients had a single failed BMAT at the base hospital.7 patients had 2 previous failed BMATs at the following base hospitals: Greys, KEH, PMMH (2 patients), RKK and ADH. In addition to the above mentioned hospitals, the following hospitals had 1 failed BMAT sample: NGH, and Stanger hospital (See Table 3.1 on the following page). Repeat successful BMAT biopsies were performed on all these patients at the IALCH Clinical Haematology department.

Table 3.1: Distribution of failed BMATs from referral hospitals

Hospital	Number of patients Failed x 1	Number of patients Failed x 2
NGH	5	1
RKK	4	1
GREYS	2	1
PMMH	1	2
STANGER	2	0
ADH	1	1
KEH	0	1
IALCH	0	0

NGH- Ngwelezane Hospital. RKK- R K Khan Hospital. PMMH- Prince Mshiyeni Memorial Hospital. ADH- Addington Hospital. KEH- King Edward Hospital. IALCH- Inkosi Albert Luthuli Central Hospital

3.4 Clinical features

Clinical data was available for 80 patients. 12 patients had no available clinical data.

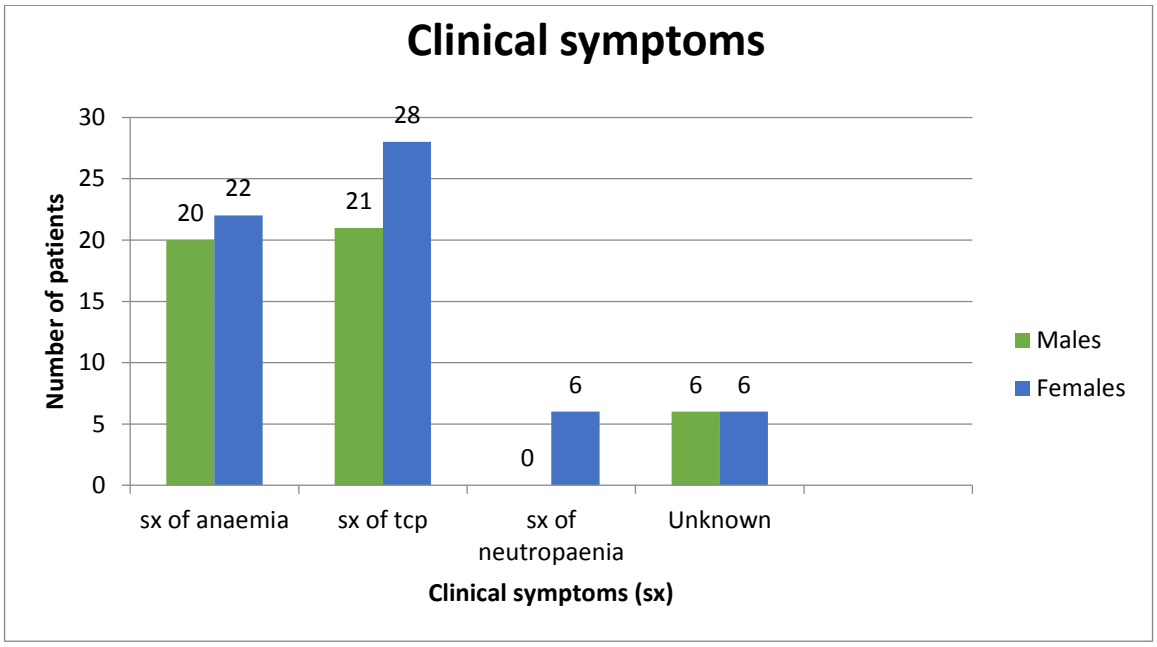
Symptoms (sx) of thrombocytopaenia (bleeding and bruising) were present in 61% of patients (49 of 80), at presentation. 34 patients presented with bleeding in the form of epistaxis, gum bleeds and menorrhagia. 1 patient had bleeding during pregnancy. Symptoms of anaemia (weakness/fatigue/dizziness) were present in 53% of patients (42 of 80) at presentation. 27 patients complained only of fatigue or weakness. 13 patients experienced bleeding as well as fatigue/weakness.

At presentation, only 7,5% (6 of 80) of the cohort had infections, presenting in the form of skin abscesses, tonsillitis and gastroenteritis. Other less common presenting features were jaundice, due to Viral Hepatitis in two patients and acute loss of vision in one patient due to CMV retinitis. One patient presented with clinical features pathognomonic of Dyskeratosis Congenita (DKC).

Figure 3.3 correlates the symptoms at presentation with the gender. No significant difference was noted between males and females.

No patients had lymphadenopathy, hepatomegaly or splenomegaly at presentation.

Figure 3.3: Histogram for clinical symptoms at presentation and gender



Sx- symptoms. Tcp- thrombocytopaenia

3.5 Patient comorbidities

Of the 92 patients, 34 had co-existing medical conditions (Table 3.2). No co-existing medical conditions were documented in the remaining patients.

Only 13 patients were HIV positive. 3 patients had a diagnosis of Hepatitis. 3 patients were known Epileptics. 6 patients had hypertension. 1 patient had pregnancy induced hypertension. 1 patient had a Cerebrovascular accident (CVA). 1 male patient had Dyskeratosis Congenita (DKC). 2 patients had Fanconi Anaemia. 1 patient had Cytomegalovirus (CMV) associated retinitis. 1 patient had Uveitis. 1 patient had Angina. 1 patient had Diabetes Mellitus. 1 patient had Pulmonary Tuberculosis and 1 patient had Psychiatric illness.

Table 3.2: Summary of patient comorbidities

Comorbidity	Number of patients
HIV	13
Drug induced Hepatitis	2
Viral Hepatitis	1
Epilepsy	3
Hypertension	6
Pregnancy induced Hypertension	1
Cerebrovascular Accident	1
Dyskeratosis Congenita	1
CMV Retinitis	1
Fanconi Anaemia	2
Uveitis	1
Psychiatric Disorder	1
Pulmonary Tuberculosis	1
Angina	1
Diabetes Mellitus	1

HIV- human immunodeficiency virus. CMV- cytomegalovirus

3.6 Patient medications

At the time of diagnosis, 56 of the 92 patients had not been consuming any regular medications in the period preceding diagnosis. 14 cases had no available information regarding use of medication. Table 3.3 illustrates the name and the number of medications consumed by the 21 patients who were on medication.

Table 3.3: Summary of patient medications

Name of medication	Number of patients
Anti-hypertensives: Ziak, Norvasc, Ridaq, Aldomet	7
Antiretrovirals: AZT/D4T/3TC, FDC	5
Anti-epileptics: Sodium Valproate, Carbamazepine, Phenytoin	3
Anti-tuberculosis treatment: Rifinah	1
Angina medication	1
Antipsychotics: Clozapine	1
Azathioprine	1
Anti-diabetic medications: Metformin	1
Digoxin	1

AZT- zidovudine. D4T- stavudine, 3TC- lamivudine. FDC- fixed dose combination

3.7 Patient occupations

No data was available for 19 patients. 24 of the 73 patients were scholars, 1 was a university student and 2 were pensioners with no preceding work history. 30 patients were unemployed and had never worked. Within the cohort of employed patients (16 of 73), no specific job description was provided for 3 patients. The following occupations prevailed: Administration workers, Receptionists, Cashiers, Chef, Security guards, Merchandisers, Painter, Miners and Factory workers (see Table 3.4 below).

Table 3.4: Summary of employment at the time of BMAT

Occupation	Number of patients
Unemployed	30
Administration	3
Merchandiser	2
Miner (coal mine)	2
Painter	1
Factory worker	1
Domestic worker	1
Cashier	1
Nurse	1
Security guard	1

3.8 Patient exposures

No identifiable exposure was noted in 69 patients. Data was not available for 16 patients. 2 patients had regular exposure to alcohol and cigarette smoke. 1 patient had exposure to herbal medications after he consulted a traditional healer for his bleeding symptoms. 1 patient had daily exposure to pesticides for 5 years. She was a nurse whose hobby was gardening and who sold rose plants.1 patient was employed as a painter. Two patients worked in coal mines with exposure to benzene.

3.9 Pregnant patients

Of the patients with AA, 5 patients were pregnant at the time of diagnosis. This included one patient who had a miscarriage at presentation of AA. This was at 6 weeks gestation. The pancytopenia was diagnosed on the presentation FBC and the diagnosis of AA was subsequently confirmed on BMAT. 4 patients had viable pregnancies at the time of presentation of AA. They were diagnosed at the following gestations: 6 weeks, 18 weeks, 20 weeks and 31 weeks respectively. Table 3.5a displays the range and the medians for the pertinent laboratory parameters in the pregnant cohort.

Table 3.5: Summary of laboratory parameters in pregnant patients

Parameter	Range	Median
Haemoglobin (Hb)	5.9-9.5 g/dl	7.8 g/dl
MCV	82-94.2 fl	93.7 fl
Platelets	6-35 x 10^9/l	15 x 10^9/l
WCC	1.26-6.06 x 10^9/l	2.14 x 10^9/l
Lymphocyte count	0.76-5.39 x 10^9/l	0.99 x 10^9/l
Neutrophils	0.14-0.91 x 10^9/l	0.28 x 10^9/l

Hb- haemoglobin. g/dl- grams per deciliter. MCV- mean cell volume. fl- femtolitres. WCC- white cell count

The pregnancy outcomes are variable and are elaborated further on in the discussion.

3.10 Laboratory Results

For normal laboratory reference ranges, see Appendix 6

The laboratory parameters reviewed in each case were as follows:

Haematology

- Full Blood Count including Mean Cell Volume, Differential Count and smear
- Reticulocyte Production Index (RPI)
- Bone marrow aspirate and trephine biopsy (BMAT) findings
- Cytogenetics (Chromosomal analysis)
- Fanconi DNA PCR testing
- Fanconi anaemia: Chromosomal breakage test (CBT)
- PNH screen
- ANF Screen

Biochemistry

- Urea and electrolytes (U&E)
- Liver function test (LFT)

Virology

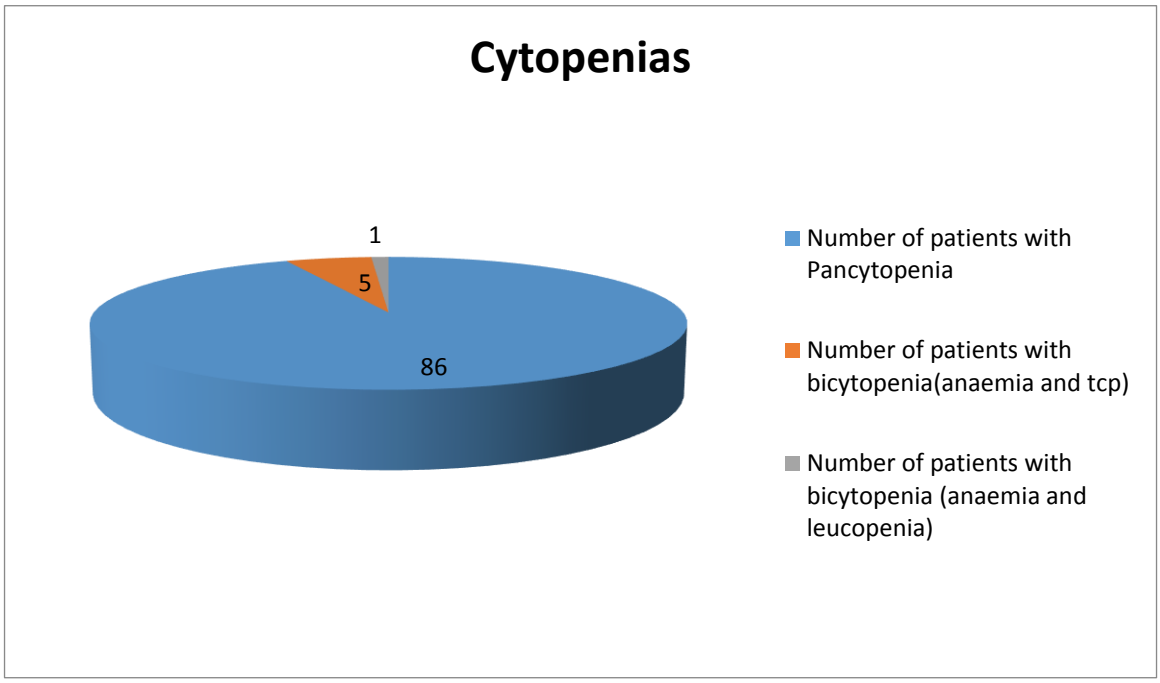
- HIV
- Hepatitis B
- Hepatitis A
- Hepatitis C
- CMV
- EBV
- Parvovirus

3.10.1 Haematology Results

a) Cytopenias

As illustrated in Figure 3.4 below, 93% of patients (86 of 92) presented with a pancytopenia. Five patients presented with a bicytopenia (anaemia and thrombocytopenia). One patient had a bicytopenia with a leucopenia (WCC of 2.51 x 10^9/l) and anaemia (Hb of 2.8 g/dl). The platelet count was 124 x 10^9/l in this patient. The BMATs were markedly hypocellular for age in the patients who did not present with a pancytopenia. All these patients had a prior transfusion record.

Figure 3.4: Distribution of cytopenias



tcp- thrombocytopenia

b) Full Blood Count (FBC) findings

Table 3.6 below depicts the relevant FBC findings. The lowest, highest and median values are tabulated as well as the interquartile range, which is expressed as a range from the 25th percentile to the 75th percentile.

Table 3.6: Summary of FBC findings

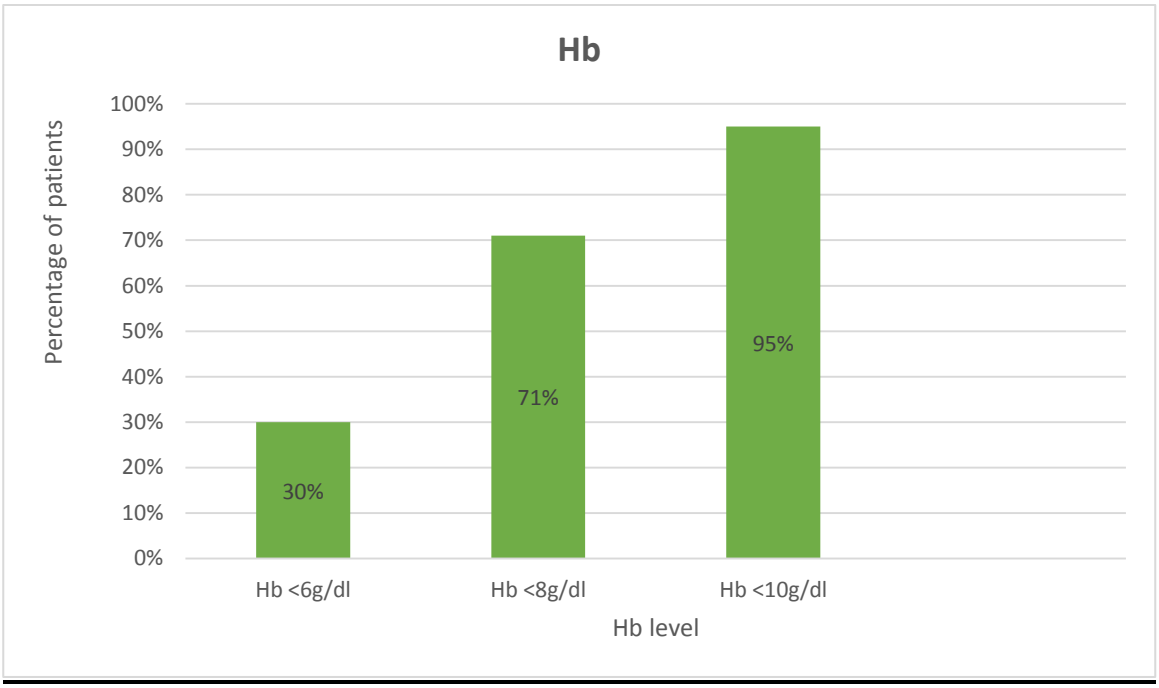
Parameters	Minimum	Maximum	p50	p25	p75	iqr
Haemoglobin	2.20 g/dl	11.90 g/dl	7.30 g/dl	5.90 g/dl	9.10 g/dl	3.20 g/dl
MCV	74.60 fl	116.20 fl	85.70 fl	80.80 fl	91.60 fl	10.80 fl
Platelets	1 x 10^9/l	136 x 10^9/l	12 x 10^9/l	5.00 x 10^9/l	28.00 x 10^9/l	23.00 x 10^9/l
WCC	0.34 x 10^9/l	6.6 x 10^9/l	2.08 x 10^9/l	1.30 x 10^9/l	2.67 x 10^9/l	1.37 x 10^9/l
Lymphocytes	0.00 x 10^9/l	5.39 x 10^9/l	1.49 x 10^9/l	1.02 x 10^9/l	1.81 x 10^9/l	0.79 x 10^9/l
Neutrophils	0.00 x 10^9/l	2.59 x 10^9/l	0.33 x 10^9/l	0.15 x 10^9/l	0.70 x 10^9/l	0.55 x 10^9/l

P50- 50th percentile. p25- 25th percentile. p75- 75th percentile. iqr-interquartile range. MCV-mean cell volume. WCC- white cell count.

The median Haemoglobin (Hb) was 7.30g/dl with a range of 2.20-11.90g/dl. The median platelet count was 12 with a range of 1-136 x 10^9/L. The WCC range was 0.34-6.6 x 10^9/l with a median WCC of 2.08 x 10^9/l.

Figure 3.5a (on the next page) demonstrates the total number of patients who presented with anaemia at the time of the BMAT biopsy. A Hb level of <6 g/dl was evident in 30% of the cohort (27 of 92 patients). 71% had a Hb level of <8 g/dl (65 of 92 patients) and 95% (87 of 92) had Hb levels < 10 g/dl. It was also noted on the BMAT request forms of 28 patients, that Red Cell Concentrate (RCC) transfusions had been administered prior to BMAT. Hence the erroneously elevated Hb levels in these patients are most likely as a result of RCC transfusion.

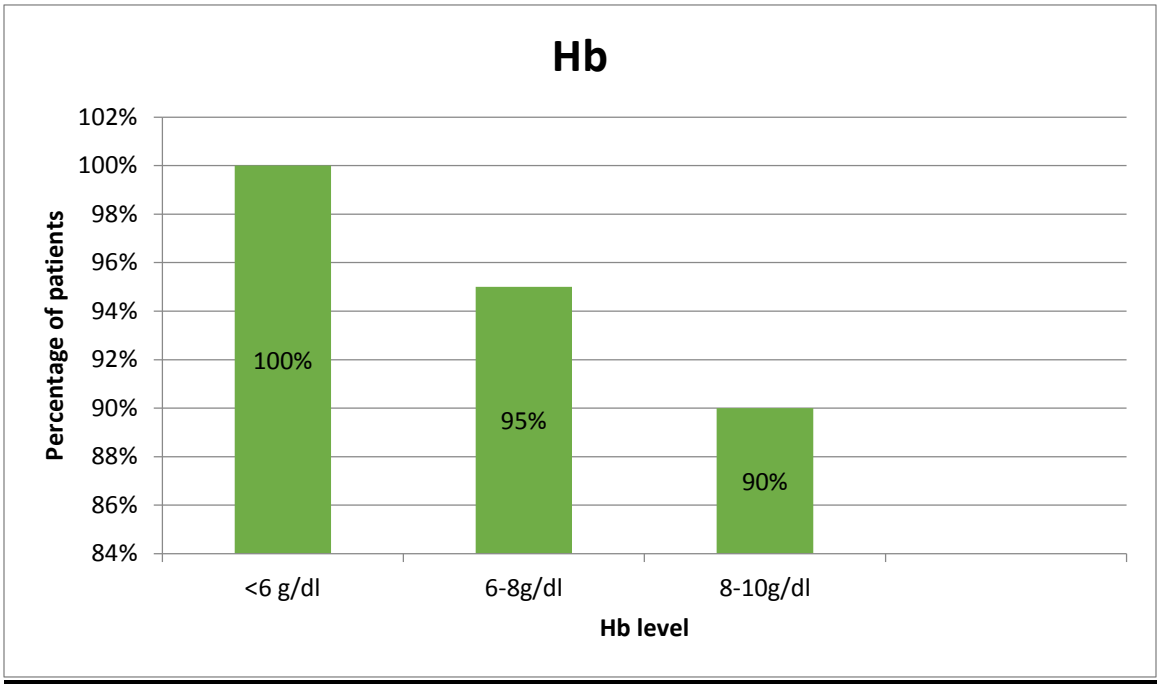
Figure 3.5a: Summary of various Hb levels in total patient cohort



Hb- haemoglobin. g/dl- gram per decilitre.

Figure 3.5b on the following page demonstrates the percentage of patients who presented with symptomatic anaemia at the time of the BMAT biopsy being performed. All (100%) of patients with an Hb level <6g/dl were symptomatic at the time of presentation for BMAT, whereas 95% of patients with an Hb between 6-8g/dl were symptomatic. This does not include the cohort of patients with an Hb of less than 6g/dl. The 90% of symptomatic patients with an Hb level of 8-10g/dl does not include the other two cohorts included in Figure 3.5b.

Figure 3.5b: Summary of symptomatic anaemia at various Hb levels



Hb- haemoglobin. g/dl- gram per decilitre.

Figure 3.5c (on the following page) illustrates the platelet counts for the total patient cohort. Forty three percent of patients (40 of 92) had a platelet count of $<10 \times 10^9$/l. A platelet count of $<20 \times 10^9$/l was noted in 60% of patients. This includes the cohort of patients with a platelet count of $<10 \times 10^9$/l. Eighteen patients had been transfused with platelets (as indicated on their BMAT request forms), yielding erroneously high platelet count values.

Figure 3.5d on the following page is a summary of the range of platelet counts in total patient cohort.

Figure 3.5c: Summary of various platelet levels in patient cohort

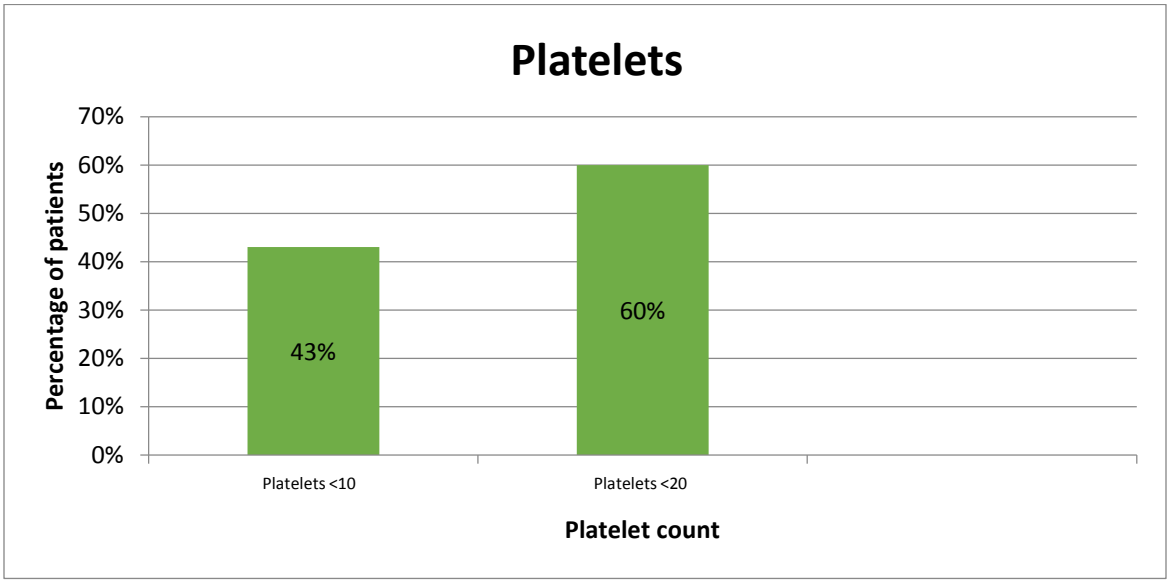
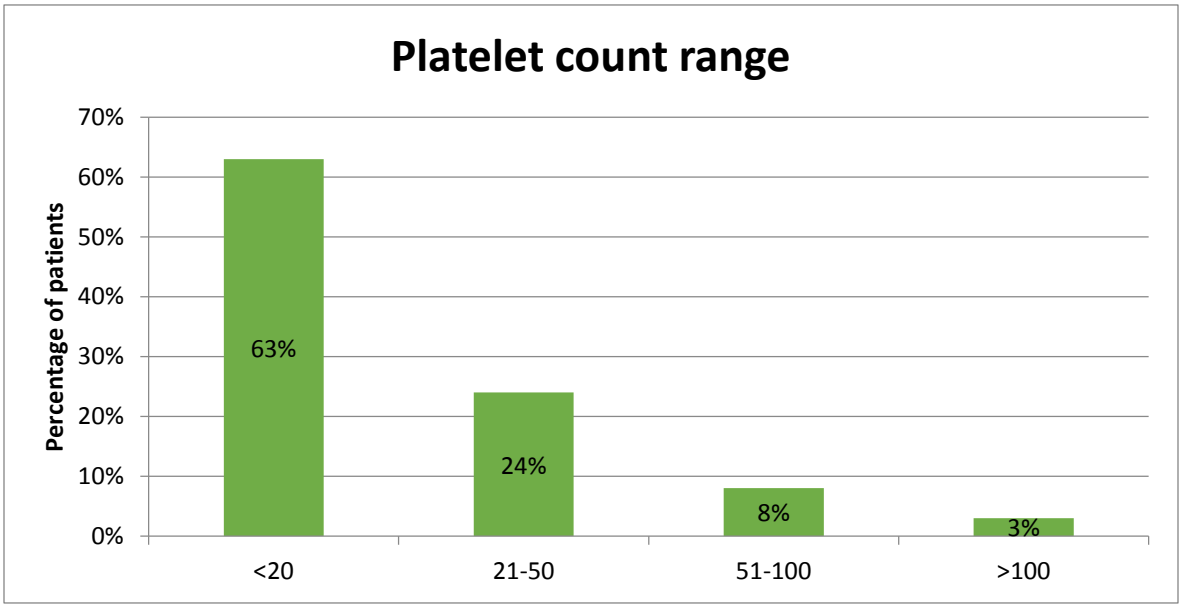


Figure 3.5d: Summary of the range of platelet counts in patient cohort



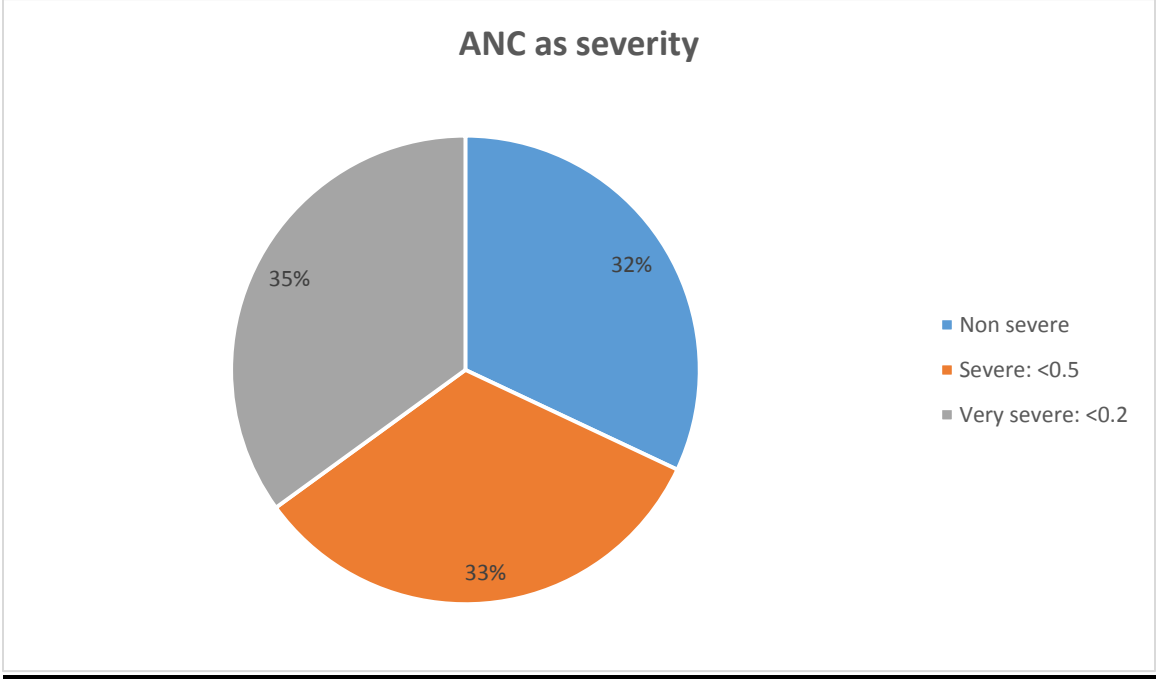
b) WBC and differential count

The WCC ranged from 0.34-6.65 x 10^9/l with a median WCC of 2.08 x 10^9/l.

With regard to the differential count, the neutrophil count ranged from 0.00-2.59 x 10^9/l with a median neutrophil count of 0.33 x 10^9/l. The lymphocyte count ranged from 0.00-5.39 x 10^9/l with a median lymphocyte count of 1.49 x 10^9/l.

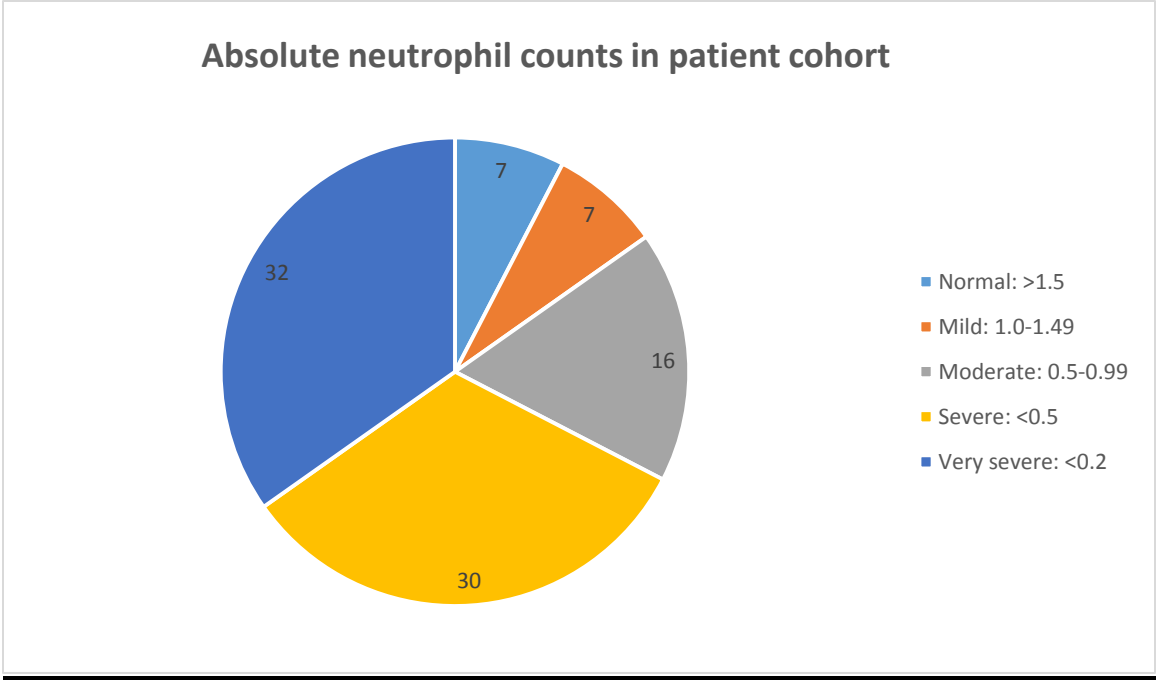
Figure 3.4e illustrates the number and percentage of the cohort categorized according to the absolute neutrophil count (ANC). Of note, is that 62 of 92 patients (67%) of patients had a severe neutropenia.

Figure 3.5e: Summary of ANC as severity



ANC- absolute neutrophil count

Figure 3.5f: Absolute neutrophil counts in patient cohort



c) Neutrophil:Lymphocyte ratio

Data was not available for 6 patients. 7 patients had a normal ratio. 92% (79 patients) had a reversal of the ratio, with lymphocyte percentages being relatively higher than neutrophil percentages.

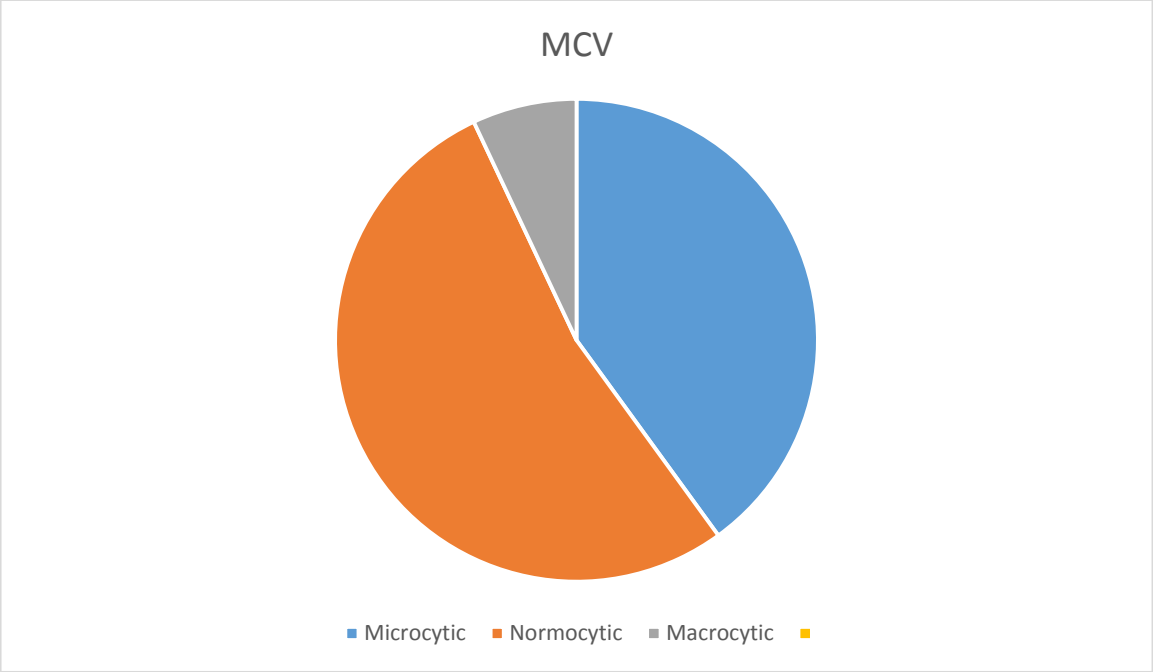
Lymphopenia was present in 40 patients at diagnosis. Of these 40 patients, seven were HIV positive. The lowest lymphocyte count and neutrophil count values, as determined by the automated FBC and differential count analyser, was 0. This is possibly due to the low WCC at diagnosis.

Nine patients had a neutrophil count of 0.0 x 10^9/l. Seventeen patients had a neutrophil count of <0.1 x 10^9/l. The lymphocyte count was 0.0 x 10^9/l in seven patients.

d) Mean cell volume and peripheral smear

The Mean Cell Volume (MCV) ranged from 74.60-116.20 fl with a median MCV of 85.70 fl. 52% of cases (48 of 92) had normocytic indices ranging from 83.6-99.7 fl. 7% of cases (6 of 92) had macrocytic indices ranging from 102.1-116.2 fl. 40% of cases (37 of 92) had microcytic indices ranging from 74.6-82.8 fl.

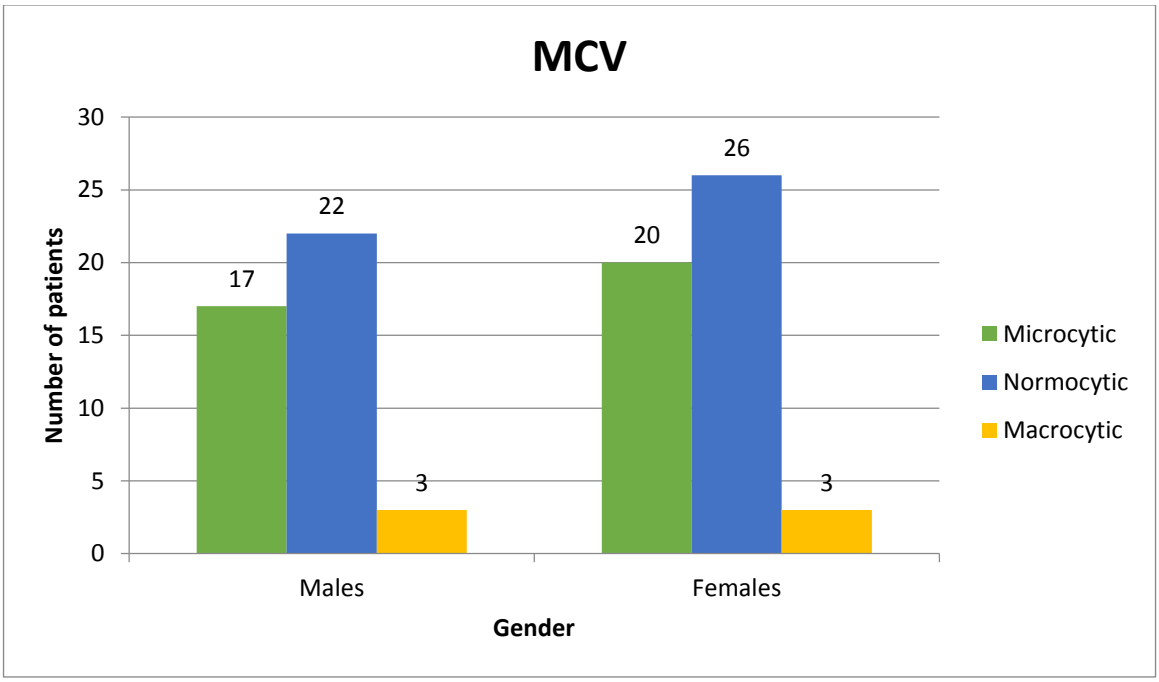
Figure 3.5g: Distribution of MCV



Smear findings were unremarkable for the patients with normocytic normochromic anaemia. Mild anisopoikylocytosis was occasionally noted. Round macrocytes were observed on the PB smear of patients with macrocytic indices. Microcytosis was not a prominent feature of the patients with microcytic indices. The microcytosis was also noted to be mild.

Figure 3.5h (on the following page) depicts the relationship between MCV and the differences in gender. Amongst the males, 17 patients were microcytic, 22 were normocytic and 3 patients were macrocytic. Females demonstrated microcytosis in 20, macrocytosis in 3 and normocytic indices in 26 patients. Iron studies, Vitamin B12 and folate levels were performed in these patients, and are discussed further in section 4.9.1.

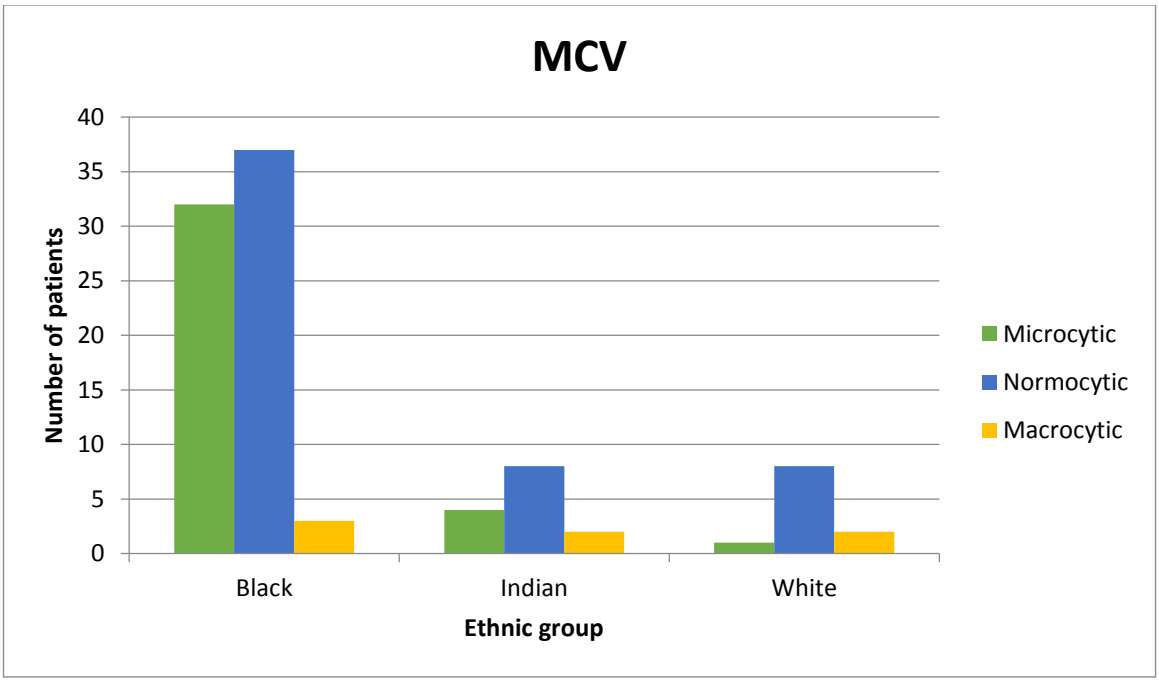
Figure 3.5h: Histogram for MCV and Gender



MCV- mean cell volume

Figure 3.5i (on the next page) illustrates the differences in MCV and Ethnicity. Amongst the Black population, 32 patients were found to demonstrate microcytic indices, 37 had normocytic indices and 3 had macrocytic indices. Within the Indian population, 4 patients' demonstrated microcytic indices, 8 patients had normocytic red cell indices and 2 patients had macrocytic indices. 1 microcytic, 8 normocytic and 2 macrocytic indices were evident in the Caucasian population. It has been reported in the literature that black females have a higher rate of microcytosis [(308)].

Figure 3.5i: Histogram for MCV and Ethnicity



MCV- mean cell volume

e) Reticulocyte Production Index (RPI)

The RPI was <1 in all cases.

f) BMAT findings

The bone marrow aspirate sample was aparticulate in 30 patients. The aspirate was mild-moderately hypocellular in 37 patients and markedly hypocellular in 25 patients.

All patients had hypocellular BM trephine biopsies. The bone marrow trephine was hypocellular (25-30% cellularity) in 23% (21 of the 92 cases) and markedly hypocellular (<25% cellularity) in 77% (71 of the 92 cases).

In patients with a PNH clone the BMATs, despite being markedly hypocellular, displayed erythroid hyperplasia with a reversal of the myeloid:erythroid (M:E) ratio, and prominent

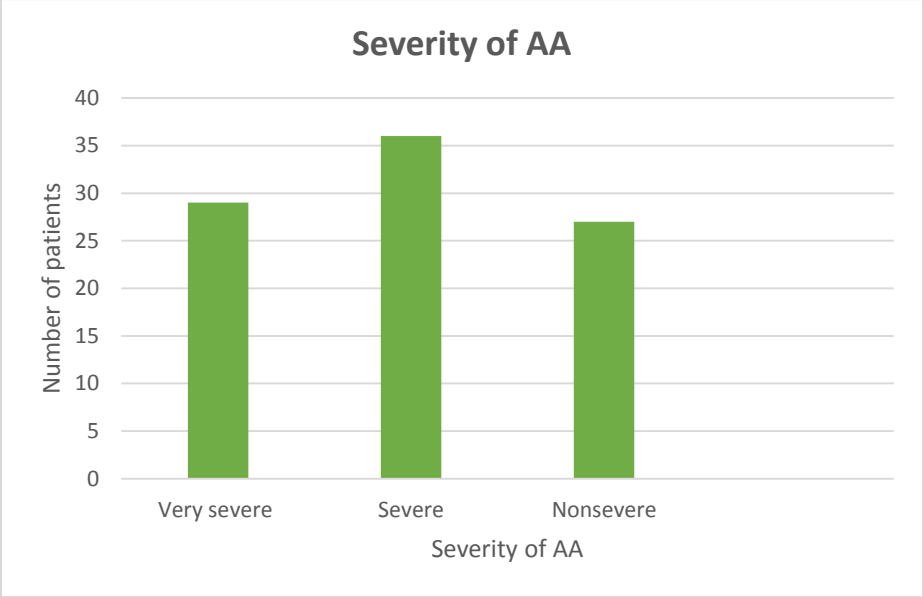
erythroid islands. There were no bone marrow granulomas detected in the patients already on TB treatment, and in any of the other patients

g) Severity of Disease

Of the 92 patients, 29 were classified as very severe Aplastic Anaemia (VSAA), 36 were classified as severe (SAA) and 27 as non-severe AA (NSAA) according to the Camitta criteria [51]. (Figure 3.6a below depicts the histogram for disease severity. There were no significant differences in gender in terms of severity.

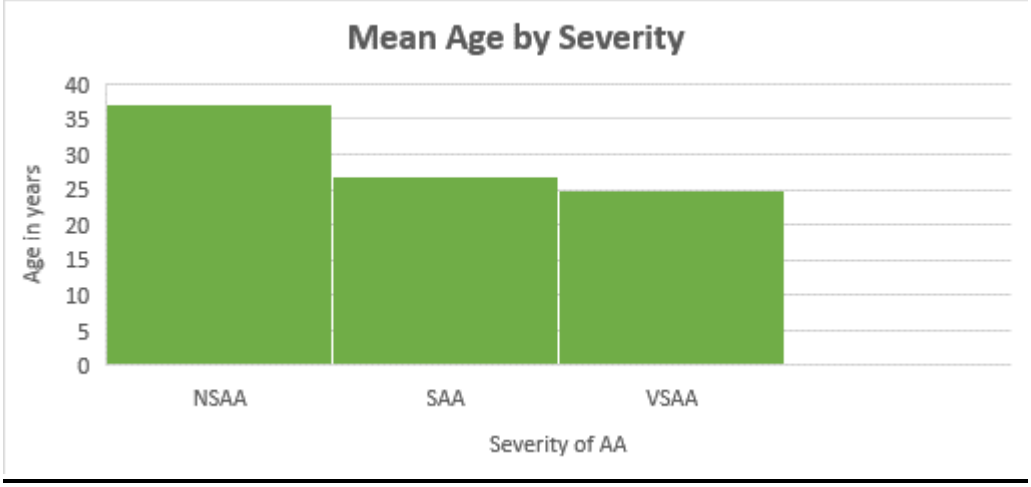
A statistically significant finding (p value=0.005) was found with disease severity and younger age patients. The patients with VSAA were significantly younger, with a mean age of 25.0 years and a SD of 11.4 compared to patients with SAA, who had a mean age of 27.0 years and patients with NSAA, who had a mean age of 37.0 years (Figure 3.6b on the next page).

Figure 3.6a: Histogram for Disease Severity



AA-aplastic anaemia

Figure 3.6b: Histogram for Mean Age by Severity



NSAA- non severe aplastic anaemia. SAA-severe aplastic anaemia. VSAA-very severe aplastic anaemia

h) Cytogenetics (Chromosomal analysis)

Cytogenetic samples were sent for 33 patients only. 16 patients had failed karyotyping due to insufficient metaphases. Results were available for 17 patients only. A normal male and female karyotype was demonstrated in 6 and 9 patients, respectively. 1 patient had a monosomy 8. 1 patient had a deletion of chromosome 7 (-7) at presentation (Fig 3.7a on the next page).

Figure 3.7a: Distribution of cytogenetic findings

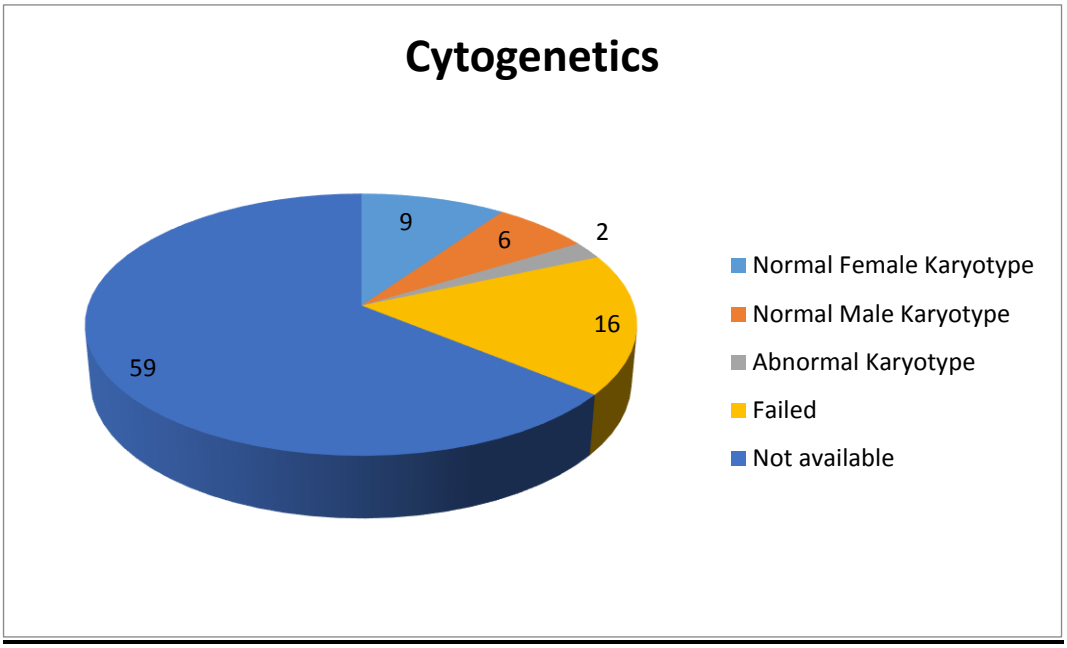
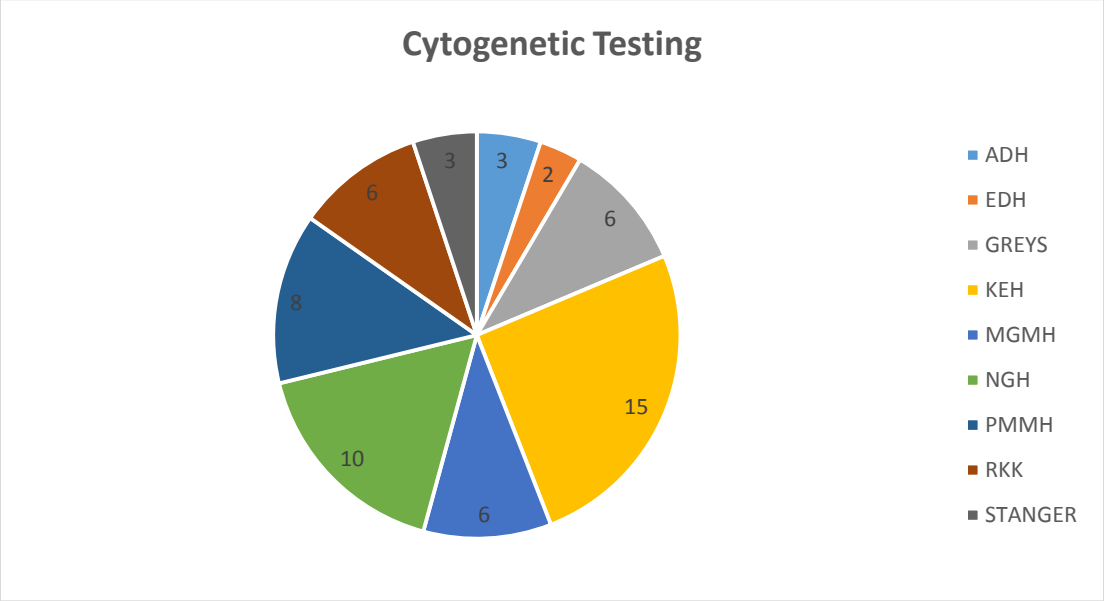


Figure 3.7b (on the next page) depicts the referral hospitals from which clinicians did not send samples for chromosomal analysis with their BMAT biopsies.

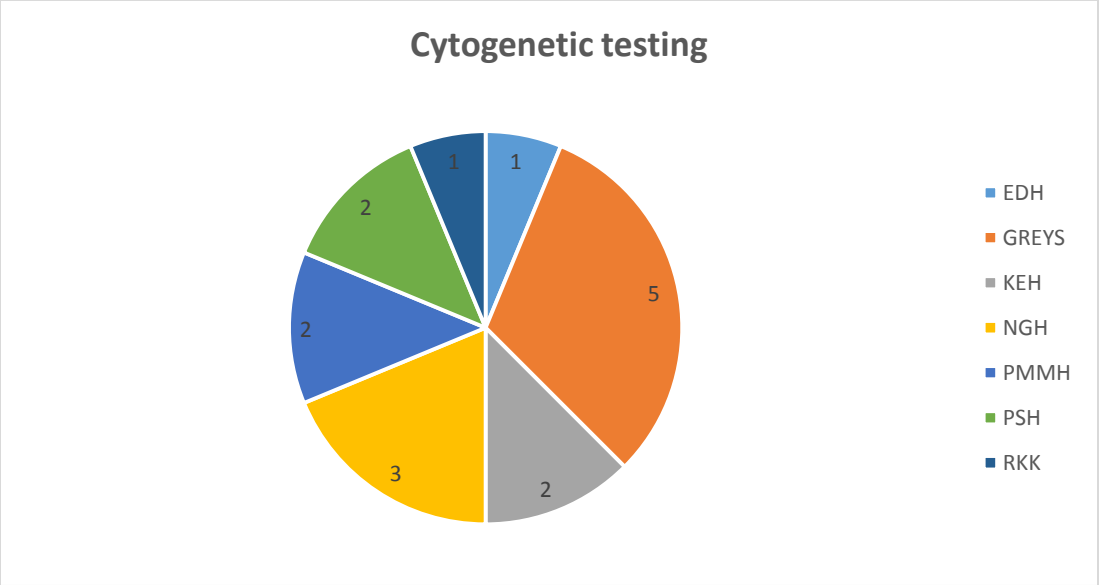
Figure 3.7b: Distribution of hospitals from which cytogenetic testing was not requested by referring doctor



ADH- Addington Hospital. EDH- Edendale Hospital. KEH- King Edward Hospital. MGMH- Mahatma Ghandi Memorial Hospital. NGH- Ngwelezane Hospital. PMMH- Prince Mshiyeni Memorial Hospital. RKK- R K Khan Hospital

Figure 3.7c (on the following page) depicts the referral hospitals from which unsuitable (failed) chromosomal analysis samples were received.

Figure 3.7c: Distribution of hospitals with unsuitable cytogenetic samples



EDH- Edendale Hospital. KEH- King Edward Hospital. NGH- Ngwelezane Hospital. PMMH- Prince Mshiyeni Memorial Hospital. PSH- Port Shepstone Hospital. RKK- R K Khan Hospital

i) Fanconi Anaemia: DNA PCR testing

Data was not available for 72 patients. The test was negative in 18 patients. 2 patients tested positive and were homozygous for the FANCG c. 637_643 deletion mutation. Somatic abnormalities in keeping with Fanconi anaemia, were evident in both these patients. One patient had 3 siblings, all of whom were diagnosed with FA. Both FA patients received steroids and androgen therapy. One patient eventually succumbed to the complications of bone marrow failure and demised from gram negative septicaemia. The other FA patient was lost to follow up (LTFU).

j) Fanconi Anaemia: Chromosomal breakage tests (CBT)

Data was not available for 78 patients. The test was unsuccessful (failed) in 7 patients, and a further 7 tested negative. The CBT was not performed in the 2 patients who tested positive for the Fanconi Anaemia DNA PCR test.

k) PNH screen

Testing for PNH was performed on peripheral blood samples. Screening for PNH was not performed in 29 patients. 55 patients tested negative for a PNH clone. 4 patients had too few

cells for analysis, and the samples were thus unsuitable for assessment by flow cytometry (FCM). A PNH clone was detected in 4 patients. Two patients had a small clone (6-8% and 7% respectively), one patient had an intermediate sized clone (20-25%) and one patient had a large PNH clone of 75%.

The patients with PNH clones were discovered between June 2014 and June 2015. All the patients presented with a pancytopenia and reduced reticulocyte count/reticulocyte production index. Iron studies were normal in these patients. Aside from a 23-year-old pregnant patient (6 weeks gestation) who was on TB treatment for Pulmonary Tuberculosis, there were no other significant comorbidities, medications consumed or exposures demonstrable in the patients with PNH. The renal and liver function tests were normal, and hemolytic or thrombotic episodes had not been reported within these patients.

Subsequent flow cytometry testing, to assess the status of the PNH clone, was not performed on these patients. Three of the patients with PNH are well and transfusion independent post ciclosporin therapy. However, the patient with a small PNH clone at baseline, has disease evolution to MDS. Follow-up peripheral blood samples for PNH flow cytometry were not requested/performed.

Of note, 2 patients with negative baseline PNH FCM results at presentation in 2011, subsequently developed large PNH clones (75-80%) in 2015. These patients were well, transfusion independent and maintaining their counts post completion of ATG and CSA therapy. The clinical presentation of both these patients, in 2015 was that of a severe symptomatic anaemia and hemolysis, requiring transfusion. PNH FCM testing subsequently confirmed the diagnosis.

l) ANF Screen

Data was not available for 23 of the 92 patients. The Anti-Nuclear Factor (ANF) screen was negative in the majority of cases (68 of 92). At diagnosis of AA, 1 patient had a positive ANF (titer of 1:50). Subsequent testing 2 months later, revealed a negative ANF test in this patient, who did not have any features of autoimmune disease.

3.10.2 Biochemistry:

a) Urea and Electrolytes (U&E)

A baseline U & E performed at presentation, demonstrated no abnormality in 88% of cases. Data was not available for six patients. Other tests such as urine protein creatinine ratio and ultrasound scans of the kidneys were not routinely performed at baseline. Renal function tests were also performed at follow up visits. The four patients that had deranged U&E results also demonstrated deranged LFTs. Two patients were clinically septic. The first patient was a 22-year-old HIV positive female with an unknown CD4 count/ viral load, not on antiretroviral

therapy (ART). Klebsiella Pneumonia was diagnosed on blood cultures and the patient had been receiving nephrotoxic antibiotic therapy (amikacin).

The second septic patient was a 14-year-old female who had been referred to the clinical haematology unit with a diagnosis of pancytopenia and sepsis. No further information was available.

The remaining 2 patients with renal dysfunction were taking chronic medications. A 40-year-old female was on psychiatric medication and had a previous nephrectomy. The other was a 26-year-old HIV positive male on cART and anti-epileptic medications.

Table 3.7 below summarizes the renal function test values as well as patient characteristics.

Table 3.7: Summary of U&E derangements

Patient	Urea	Creatinine	GFR	Patient characteristics
1.	14.1	95	Not performed	Severe klebsiella sepsis, colostomy, parvovirus IgM +
2.	16.8	638	Not performed	HIV +, Sepsis, Antibiotics, Iron overload, Deranged LFTs
3.	4.8	138	54ml/min	HIV +, cART, Antiepileptics, previous PTB, iron overload, infection (ESR >120), deranged LFTs
4.	17.2	159	31ml/min	Deranged LFT, previous nephrectomy, infection (CRP>30), multiple drugs, iron overload, previous PTB

GFR- glomerular filtration rate. IgM- immunoglobulin M. HIV- human immunodeficiency virus. LFTs- liver function test. cART- combination antiretroviral therapy. PTB- pulmonary tuberculosis. ESR- erythrocyte sedimentation rate. CRP- C reactive protein.

b) Liver Function Tests (LFT)

A baseline liver function test was performed for majority of the patient cohort at diagnosis. Data was unavailable for 11 patients. LFT's were normal in 84% of patients (68 of 81). 13 patients (16%) had a deranged LFT. Of the derangements, 23% were of a hepatitic nature (3 of 13), 46% (6 of 13) were cholestatic and 31% (4 of 13) had a mixed picture. LFT's were also performed at follow up visits in the majority of pts.

As illustrated below, many of the patients had concomitant bacterial and/or viral infections. 8 of the 13 patients (62%) were overtly septic (Table 3.8). Some were on antibiotic and/or antiviral treatment. 8 patients were iron overloaded from recurrent blood transfusion, and one patient had a history of ingestion of herbal remedies. These are some of the possible contributory factors to the LFT derangements.

Table 3.8 (on the following page) summarizes the liver function test values as well as patient characteristics.

Table 3.8 Summary of LFT derangements

Pt	Total Bilirubin	Conjugated Bilirubin	ALT	AST	GGT	ALP	Patient characteristics
1.	19.5		44	93	120	113	HIV +, Sepsis, Antibiotics, Iron overload, Renal failure
2.	35	14	52	53	127	273	Iron overload on exjade + ciclosporin
3.	16		121	69	26	43	Sepsis, Iron overload
4.	9.1		64	44	112	201	Herbal medication, sepsis
5.	456	378	381		209	229	Hepatitis, Enterobacter sepsis and Herpes simplex infection
6.	23	19	202	174	119	138	Severe klebsiella sepsis, colostomy, renal dysfunction, parvovirus IgM +
7.	16		40	77	129	164	DKC, CMV IgM+, Iron overload
8.	18		128	64	64	85	Parvovirus IgM +, Iron overload
9.	393		2016	2685	79	133	HAAA, CMV PCR +, CMV Viral Load 1315
10.	70		8	11	175		HIV +, cART, Antiepileptics, previous PTB, iron overload, infection (ESR >120)
11.	17.9		176	86	345	188	Steroids, Iron overload, Occupational exposure
12.	38		16	17	165	190	Renal dysfunction, previous nephrectomy, infection (CRP>30), multiple drugs, iron overload, previous PTB
13.	8		88		131	108	Sepsis, antibiotics

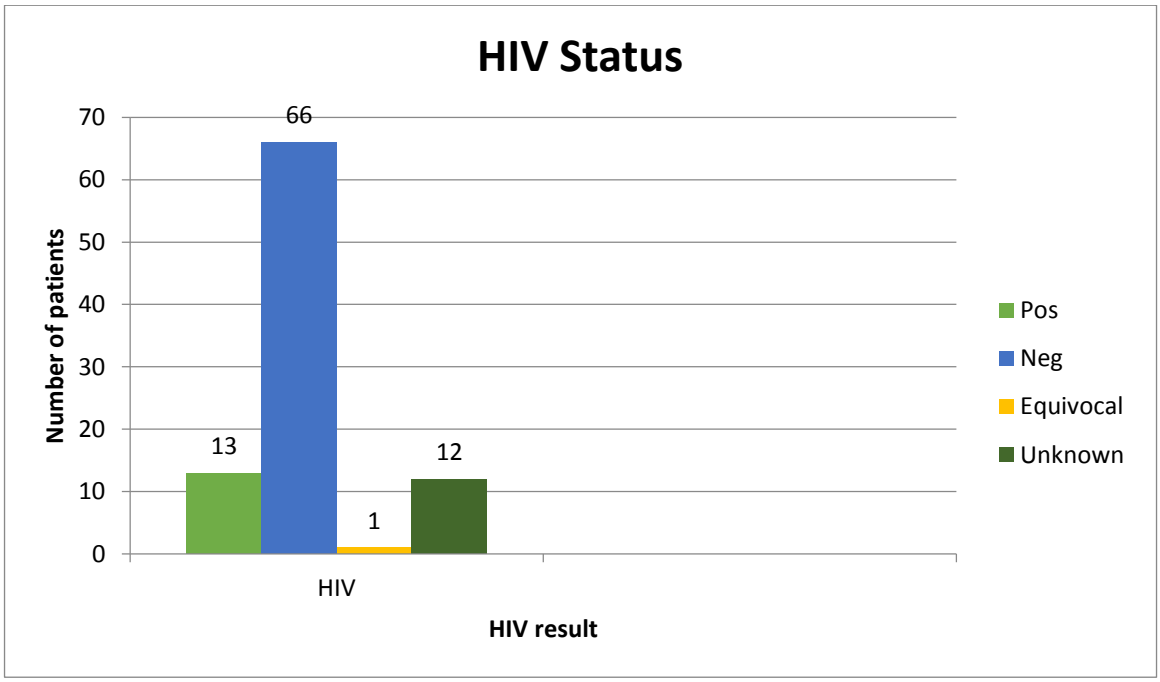
ALT- alanine aminotransferase. AST- aspartate aminotransferase. GGT- gamma glutamyl transferase. ALP- alkaline phosphatase. HIV- human immunodeficiency virus. IgM- immunoglobulin M. DKC- dyskeratosis congenita. CMV- cytomegalovirus. HAAA- Hepatitis Associated Aplastic Anaemia. PCR- polymerase chain reaction. cART- combination antiretroviral therapy. PTB- pulmonary tuberculosis. ESR- erythrocyte sedimentation rate. CRP- C reactive protein.

3.10.3 Viral Screen

a) Human Immunodeficiency Virus (HIV)

The diagnosis of HIV was made on ELISA-based assays. 66 patients were HIV negative. 12 patients had no HIV records.13 of the 80 patients (16.3%) were HIV positive at diagnosis of AA. An Equivocal HIV result was obtained in 1 patient. This could not be confirmed to be positive/negative due to the patient's subsequent demise. Findings are depicted in Figure 3.8a below.

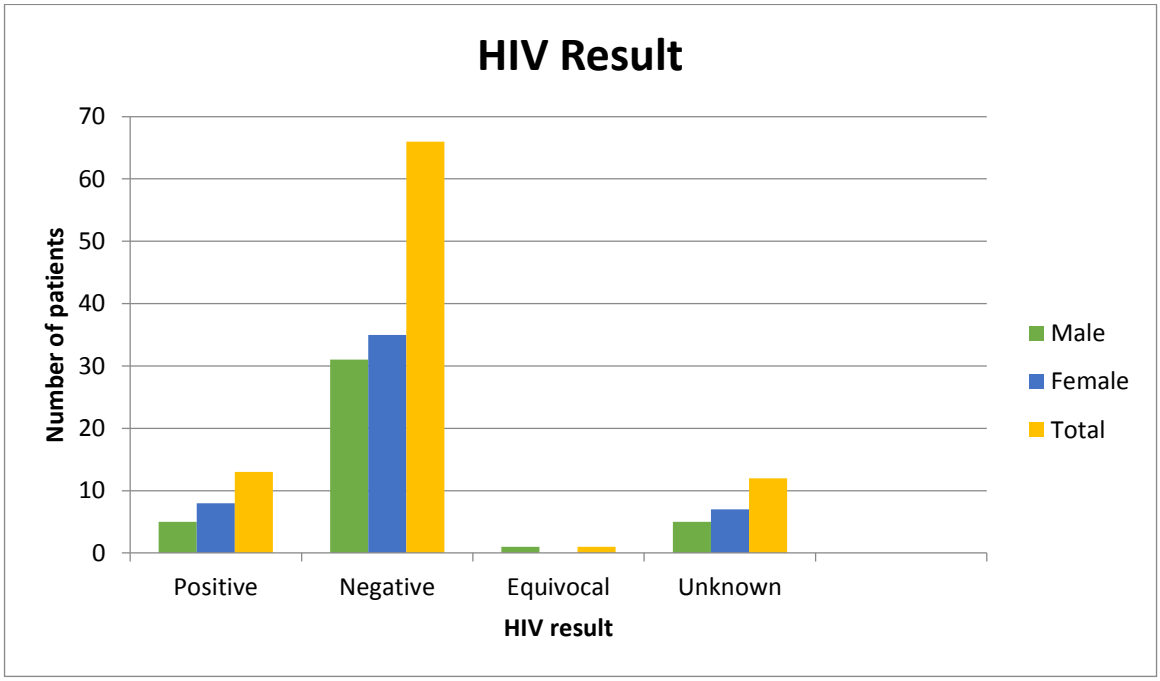
Figure 3.8a: Histogram for patient HIV status



HIV- human immunodeficiency virus. Pos- positive. Neg- negative

The HIV positive population ranged from 22-46 years of age, with a median age of 33. The M:F ratio was 0.6:1 with 8 HIV positive females and 5 HIV positive males (see Figure 3.8b below). Within the female cohort, the range was 22-46 years with a median of 33 years. The male patients ranged from 23-27 years with a median age of 26 years.

Figure 3.8b: Histogram for HIV status and Gender



The BMAT of the first HIV positive patient with AA was reviewed in 2007. The 13 HIV positive patients were consecutively distributed over the years. There was no real increase in the number of HIV positive cases over the years, or no preponderance of cases in a certain time period.

A simultaneous diagnosis of HIV and AA was made in 5 patients, when testing for HIV was performed as part of the viral screen work-up upon diagnosis of AA (see Table 3.9 below). The diagnosis of HIV was made prior to the diagnosis of AA in the remaining 8 HIV positive patients. In these patients, the duration between the diagnosis of HIV and AA ranged from just over a month to 7 years. Two patients experienced a 6 and 7-year interval between the diagnosis of HIV and the diagnosis of AA, respectively (see Table 3.10 below). 5 patients were diagnosed with AA within a year of their HIV diagnosis.

Only seven HIV positive patients had a lymphopenia. The remainder had normal lymphocyte counts.

The low viral loads in patients 1, 3 and 5 (in Table 3.9 on the next page) is interesting. It may reflect patients who have been rather recently infected or patients with a low viral multiplication rate. It may also be due to patients having already been started on cART but who had not disclosed this information. This was not investigated further.

Table 3.9: Characteristics of patients with a simultaneous diagnosis of HIV and AA

Patient	**Month and Year of Diagnosis of HIV and AA**	**CD4 count at presentation (Absolute CD4) cells/mm^3**	**Viral Load at presentation (copies/ml)**
1.	May 2008	238 cells/mm^3	<20 copies/ml
2.	Nov 2008	Not done	Not done
3.	Oct 2010	50 cells/mm^3	240 copies/ml
4.	Nov 2013	839 cells/mm^3	11730 copies/ml
5	Feb 2014	323 cells/mm^3	<40 copies/ml

HIV- human immunodeficiency virus. AA- aplastic anaemia. CD4- cluster differentiation 4. Nov- November. Oct- October. Feb- February. mm^3- millimeter cubed. ml- millilitre.

Table 3.10: Characteristics of patients with an interval between the diagnosis of HIV and AA

Pt	Month/year of diagnosis of HIV	Month/year of diagnosis of AA	Duration between diagnosis of HIV and AA	CD4 at diagnosis of HIV (Absolute CD4) cells/mm^3	Viral Load at diagnosis of HIV (copies/ml)	cART (Y/N)	CD4 at diagnosis of AA (Absolute CD4) cells/mm^3
1.	2007	2014	7 years	Unknown	Unknown	N	151
2.	2008	May 2013	6 years	Unknown	Unknown	Y	36
3.	March 2011	June 2013	27 months	228	348	Y	448
4.	April 2011	Nov 2011	8 months	500	44	Y	535
5.	Oct 2011	Feb 2012	4 months	117	Unknown	N	Unknown
6.	Sep 2012	June 2013	10 months	147	<150	N	Unknown
7.	April 2014	May 2015	11 months	388	Unknown	N	235
8.	June 2015	July 2015	1 month	381	Unknown	N	-

HIV- human immunodeficiency virus. AA- aplastic anaemia. CD4- cluster differentiation 4. cART- combined antiretroviral therapy. Nov- November. Oct- October. Feb- February. Sep- September. mm^3- millimeter cubed. ml- millilitre.

CD4 count and Viral Load

CD4 counts and viral loads (VL) were available in almost all the patients with a simultaneous diagnosis of HIV and AA. Only one of the patients with a simultaneous diagnosis of HIV and AA did not have a baseline CD4 count/viral load performed. She was diagnosed with HIV on admission, and demised shortly thereafter. The CD4 count ranged from 50-839 cells/mm^3 in the 5 patients from the simultaneous diagnosis cohort. The viral load ranged from <20-11730 copies/ml (Table 3.9 above).

Of the 8 patients with an interval between the diagnosis of HIV and AA, only 6 had available CD4 counts. The CD4 count ranged from 117-500 cells/mm^3. The 2 patients with outstanding baseline CD4 counts had been diagnosed with HIV approximately 6 and 7 years prior to the diagnosis of AA. In these patients, the CD4 count at diagnosis of AA was 36 and 151 cells/mm^3, respectively. The majority of patients did not have viral loads performed. Viral loads were only available for 3 patients, and ranged from 44-348 copies/ml. Only 3 of the 8 patients were on ARV therapy at the time of AA being diagnosed (Table 3.10 above). One of the 3 patients had defaulted therapy for 5 years, and re-initiated therapy with second line agents when AA had been diagnosed. Another had her regimen changed due to toxicity.

Follow up CD4 counts/viral loads were also performed for two of the patients at ARV clinics at their base hospitals. These patients that were lost to follow up (LTFU) at IALCH continued their base hospital visits. The follow-up CD4 counts were accessible on the TrakCare laboratory information system (LIS), and showed an improvement. The viral loads were undetectable.

b) Hepatitis B Virus (HBV)

The Hepatitis B Surface antigen was negative in the majority of patients (74 of the 92). No data was available for 14 patients. A positive HepBSAg result was documented in 3 patients. 1 patient had an equivocal result, which was not subsequently repeated. The Hepatitis B Core IgM and Hepatitis E Antigen tests were negative in these patients. The HepBSAg was persistently positive in these patients who had normal LFTs, possibly indicating a chronic carrier state. Hepatitis B viral loads were not available.

c) Hepatitis A virus (HAV)

Hepatitis A IgM:

Testing for active infection was not performed in 52 patients. 40 patients tested negative.

Hepatitis A IgG:

Testing for previous exposure to Hepatitis A virus, was not performed in 81 patients. 11 patients tested positive for Hepatitis A IgG indicating previous exposure to Hepatitis A infection.

d) Hepatitis C virus

Hepatitis C IgM

Results were available for 10 patients only. These were all negative.

Hepatitis C IgG

Results were available for 10 patients only. These were all negative.

e) Cytomegalovirus (CMV)

CMV IgM

Of the 92 patients: 26 patients had no records, 61 patients tested negative, 4 patients tested positive for acute exposure and 1 patient had an equivocal result.

CMV IgG

No records could be found for 33 patients. 4 patients tested negative, and 55 patients tested positive for previous infection/exposure.

CMV PCR

CMV PCR was not performed in 82 patients. 9 patients had a negative PCR. Only 1 patient had a positive CMV PCR. CMV serology and viral load was not available for this patient.

f) Epstein-Barr virus (EBV)

EBV IgM

Of the 92 patients, 36 patients had no records. 55 patients tested negative and 1 patient demonstrated acute exposure to EBV.

EBV IgG

No results were found for 45 patients. 6 patients tested negative and 41 patients tested positive for past exposure to EBV.

g) Parvovirus

Parvovirus IgM

Of the 92 patients, 41 had no records and 46 patients tested negative. 5 patients had a positive parvovirus IgM indicating acute exposure to parvovirus B19.

Parvovirus IgG

No records were found in 41 patients. 16 patients tested negative. 2 patients had an equivocal parvovirus IgG and 33 patients demonstrated positive results, indicating previous exposure to parvovirus B19.

Parvovirus PCR

Of the 92 patients, 78 had no parvovirus PCR results. 14 patients had negative results.

These findings are summarized in Table 3.11 below.

Table 3.11: Summary of viral investigations performed

	Hepatitis A virus	Hepatitis C virus	EBV	CMV	Parvovirus
Serology: IgM: positive			1	4	5
Serology: IgM: negative	40	10	55	61	46
Serology: IgM: not available	52	82	36	26	41
Serology: IgM equivocal				1	
Serology: IgG: positive	11		41	55	33
Serology: IgG: negative		10	6	4	16
Serology: IgG: not available	81	82	45	33	41
Serology: IgG: equivocal					2
PCR positive				1	
PCR negative				9	14
PCR not available				82	78

IgM- immunoglobulin M. IgG- immunoglobulin G. PCR- polymerase chain reaction. EBV- Epstein Barr virus. CMV- cytomegalovirus

3.11. Patient management and outcomes

The following definitions have been used as criteria for responses to therapy in aplastic anaemia[7]

Complete response:	Haemoglobin normal for age
	Neutrophil count >1.5 x 10^9/l
	Platelet count >150 x 10^9/l
Partial response:	Transfusion independent
	No longer meeting the criteria for severe AA
Failed/None:	Not meeting the above criteria
Relapse:	Loss of a complete or partial response

Thirteen patients with a bone marrow diagnosis of AA, made at the NHLS haematology laboratory at IALCH did not reach the clinical haematology unit at IALCH. All 13 patients were classified as SAA according to PB counts and BMAT findings. One of these patients demised at KEH. The outcome of the others is uncertain, though it is likely that they may have demised.

Six patients presented at the initial visit only. They were referred back to their base hospital for supportive management (blood, platelets and antibiotic therapy) after the work-up had been completed by the clinical haematology team at IALCH. Of the 6 patients, 4 had VSAA with platelets ranging from 2-5 x 10^9/l and the ANC from 0.01-0.03 x 10^9/l. The other 2 patients had SAA. It is likely that these patients who were symptomatic at presentation could have demised at home or at the base/referral institution.

A 68-year-old Indian female was diagnosed with NSAA. She was being observed, with blood product support as required.

Four patients had spontaneous recovery of the blood counts. Three had NSAA and 1 had SAA. Amongst the NSAA patients, was a 49-year-old female, whose counts recovered spontaneously after almost one year. Spontaneous recovery of blood counts also occurred in a 19-year-old female 11 months' post diagnosis of AA. The third patient who was pregnant at diagnosis, and her blood counts recovered spontaneously 3 months' post-delivery. These patients had received occasional transfusion but no CSA or ATG. The patient with SAA was HIV positive. Her blood counts improved in just over three months from the initiation of HAART.

Table 3.12 below provides a general summary of the patient outcome and management for patients in the study.

Table 3.12: Summary of patient management

Patient characteristic	**Number of patients**
Never reached IALCH	13
One visit only	6
Observation only	1
Spontaneous recovery	4
LTFU`	7
Demised	22
Disease Transformation	3
Treatment	
Ciclosporin alone:	26
Good response (Complete/Partial)	11
No response	4
LTFU (on ciclosporin)	11
Triple Therapy	10
Good response (Complete/Partial)	5
No response	5
LTFU after triple therapy	4
Androgen therapy	2

Bone Marrow Transplant	4

IALCH- Inkosi Albert Luthuli Central Hospital. LTFU- lost to follow up. ATG- anti thymocyte globulin.

Table 3.13a: Summary of patients with a response to CSA

Ciclosporin:	
a) Complete response	7
b) Partial response	4
Total	**11**

Table 3.13b: Summary of patients with a response to ATG and CSA

Triple therapy:	
a) Complete response	2
b) Partial response	3
Total	**5**

Triple therapy- ciclosporin/atg/methyprednisone

Forty patients were treated with immune suppressive therapy. CSA alone, at a dose of 5mg/kg daily with weekly monitoring of trough levels was used for 18-24 months in patients responding to this treatment modality or until the development of CSA toxicity. In patients not responding to ciclosporin, treatment was discontinued after 6 months. Triple therapy (ATG/Methylprednisolone/CSA) was used in other patients.

The term **triple therapy** denotes the use of equine ATG (ATGAM) at a dose of 40mg/kg for 4 days (administered through a central venous catheter), with the concurrent intravenous administration of 500mg methylprednisolone daily for 5 days. Patients received premedication with paracetamol and phenergan prior to ATG administration, and the platelet count was kept at $>40 \times 10^9$/l (on the days of ATG administration), by daily administration of single donor apheresis (SDA) platelets.

Ciclosporin was initiated on day 6 of the protocol at a dose of 5mg/kg daily. Weekly trough levels were monitored (and CSA doses adjusted as necessary). Renal and liver function tests were also evaluated to detect toxicity. Patients were monitored for infections and bleeding.

The 2 patients with Fanconi Anaemia (1 with SAA and 1 with VSAA) had been on androgens for several years, however response to this therapy was poor. One patient demised of sepsis and the other was LTFU. It is likely that this patient also demised.

Ciclosporin alone: Twenty-six patients received CSA alone. Of the patients on CSA alone, 11 patients achieved a favourable response and remain well. Seven patients recovered their blood counts (complete response) and four remain transfusion independent (partial response) (Table 3.13a above). One patient had no response to CSA therapy (failed response) initially, in 2005/2006. She subsequently became iron-overloaded and required iron chelation therapy (ICT). Treatment was re-initiated at higher CSA doses in 2010, to which the patient responded favorably.

No response (failed response) to CSA therapy was noted in 4 patients, 3 of whom had VSAA. 1 of these patients was subsequently LTFU. 2 patients subsequently demised. The fourth patient had a failed response to CSA therapy on two occasions. ATG had not been considered for him, the reason for which is unknown. He is currently transfusion dependent (Table 3.13 above).

Of the 26 patients, 11 patients commenced on CSA therapy were subsequently LTFU.

Of the 26 patients, 3 experienced treatment interruptions/discontinuation due to adverse effects of CSA. One patient experienced severe gum hypertrophy and was changed to tacrolimus. He is still transfusion dependent. Another experienced liver dysfunction and was LTFU whilst awaiting ATG. The third patient experienced mild renal impairment which subsequently improved. She achieved a complete response and is transfusion independent.

Triple therapy: Five patients achieved a favorable response with the combination of ATG/Methyprednisolone/CSA. Two of these five patients recovered their blood counts (complete response), and three patients had a partial response and are transfusion independent (Table 3.13b on the preceding page). One patient was subsequently lost to follow up after being in remission for one year. Two of the patients, both who had been in remission for almost 2 years, had disease evolution to PNH (Table 3.15).

Five patients had a failed response to triple therapy (Table 3.14).

One of them was a 51-year-old male with SAA who had no response to a previous course of equine ATG and CSA administered in 2016. He completed a course of rabbit ATG and CSA in July 2017. Rabbit ATG (Fresenius) was administered at a dose of 3.75mg/kg daily for 5 days. Presently, he is on CSA and prednisone. He is currently maintaining his counts without transfusion support.

Another patient, also with SAA and a poor response to ATG/CSA therapy in 2014, was subsequently treated with tacrolimus. This patient remained transfusion dependent and transfusion requirements increased in April 2017. A BMAT was performed which demonstrated disease transformation to MDS with excess blasts-1 (MDS-EB1). This patient had a PNH clone of 6-8% and normal cytogenetics at presentation in 2014. The PNH clone in April 2017 was <1% and a monosomy 7 was now evident on karyotyping.

Three patients demised after therapy with ATG/CSA. The interval ranged from <1 week to 2 months' post therapy. The cause of death in all 3 patients was septic shock.

Of the 4 patients on triple therapy who were LTFU, 1 patient had a complete response (as indicated above). The other 2 patients failed to demonstrate any response to therapy, and were still transfusion dependent at their last recorded visits. The last patient defaulted treatment and the outcome in terms of disease response to therapy, is uncertain.

Allogeneic BMT: Four patients received an Allogeneic Bone Marrow Transplant from matched sibling donors (Table 3.14). The conditioning regimen used was that of Fludarabine, cyclophosphamide and equine ATG. One patient was transplanted twice. Her initial presentation was that of a spontaneous miscarriage during which an incidental pancytopenia had been discovered. The diagnosis of AA was confirmed on BMAT. The first transplant in October 2011, was complicated by ABO blood group incompatibility. The patient was re-transplanted in February (Feb) 2013. She had a complete response to therapy and is currently well and in remission.

The other successfully transplanted patient was a 53-year-old nurse with regular exposure to pesticides. She presented in Feb 2013 and her transplant was performed in June 2013. She also had a complete response to therapy, is well and in remission at present. Both patients had their younger sisters as bone marrow donors.

The BMTs were unsuccessful in the remaining 2 patients. A 20-year-old male, diagnosed with AA in December 2012 was transplanted in March 2013. He died of treatment-related complications, namely acute-GVHD, within the first 100 days' post-transplant. A 27-year-old female diagnosed with AA in May 2011, was transplanted in July 2011. Her BMAT biopsy was still aplastic 5 months' post BMT and the patient demised in Dec 2011, from an IFI whilst on CSA therapy.

Table 3.14 (on the following page) categorizes the severity of AA and the response to different treatment modalities including CSA alone, CSA with ATG and allogeneic BMT.

Table 3.14: Disease severity with treatment response

Treatment option	VSAA	SAA	NSAA	Total
Triple therapy - Remission	3	0	2	5
Triple therapy-No Response	1	1	3	5
Triple therapy- LTFU	1			
Triple therapy-Demised			3	
CSA-Remission	2	6	3	11
CSA-No Response	3	0	1	4
CSA- LTFU	1			
CSA- Demised	2			
BMT-Remission	2	0	0	2
BMT-Demised	1	1	0	2

ATG- anti thymocyte globulin. CSA- ciclosporin. BMT- bone marrow transplant

Tabulated below are the detailed outcomes for the 79 patients that were managed by the clinical haematology unit at IALCH (Table 3.15). The 13 patients that had a BM diagnosis of AA made at the NHLS haematology laboratory, but did not reach the clinical haematology unit, are not included.

Table 3.15: Outcome of the patients referred to IALCH

Patient Outcome	Number of patients
Alive	32
Alive with Clonal evolution	3
Lost to follow up (LFTU)	22
Demised:	22
Before treatment	14
On ciclosporin, awaiting ATG	2
Post ATG therapy	3
Transplant related	2
Fanconi Anaemia	1
Total	79

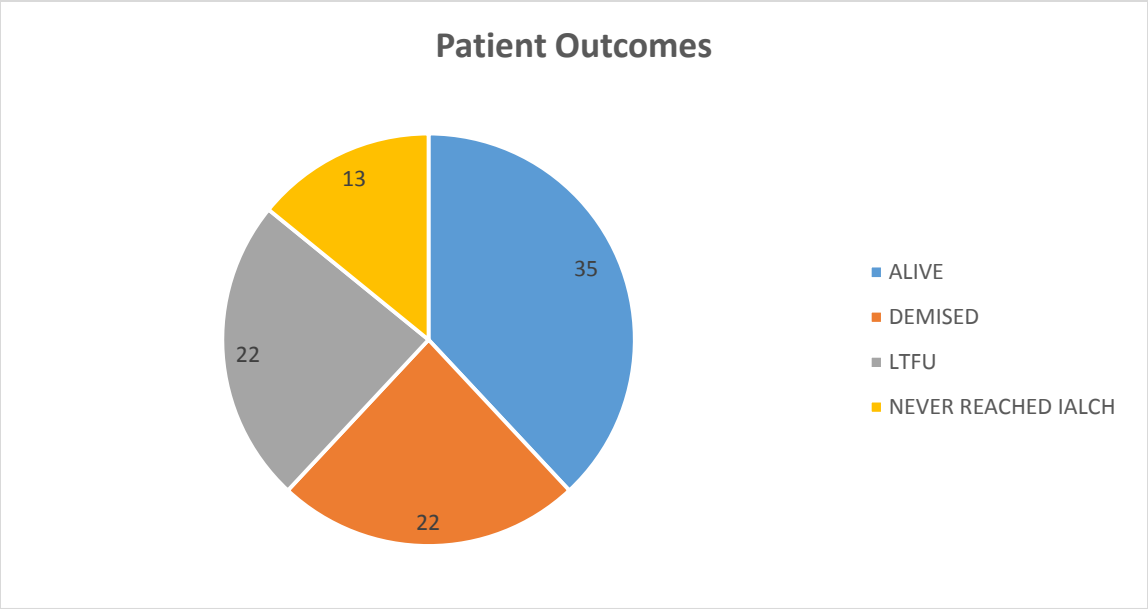
*This table does not include patients with a diagnosis of AA that did not reach IALCH

LTFU- lost to follow up. ATG- anti thymocyte globulin.

The above tabulated outcomes are depicted further in the diagrams on the following pages (Figure 3.9a-3.9i)

Figure 3.9a illustrates the outcome of the 92 patients in the study cohort.

Figure 3.9a: Patient Outcomes



LTFU- lost to follow up. IALCH- Inkosi Albert Luthuli Central Hospital.

Figure 3.9b illustrates the treatment status of the 35 patients who are currently alive.

Figure 3.9b: Characteristics of the patients who are currently alive

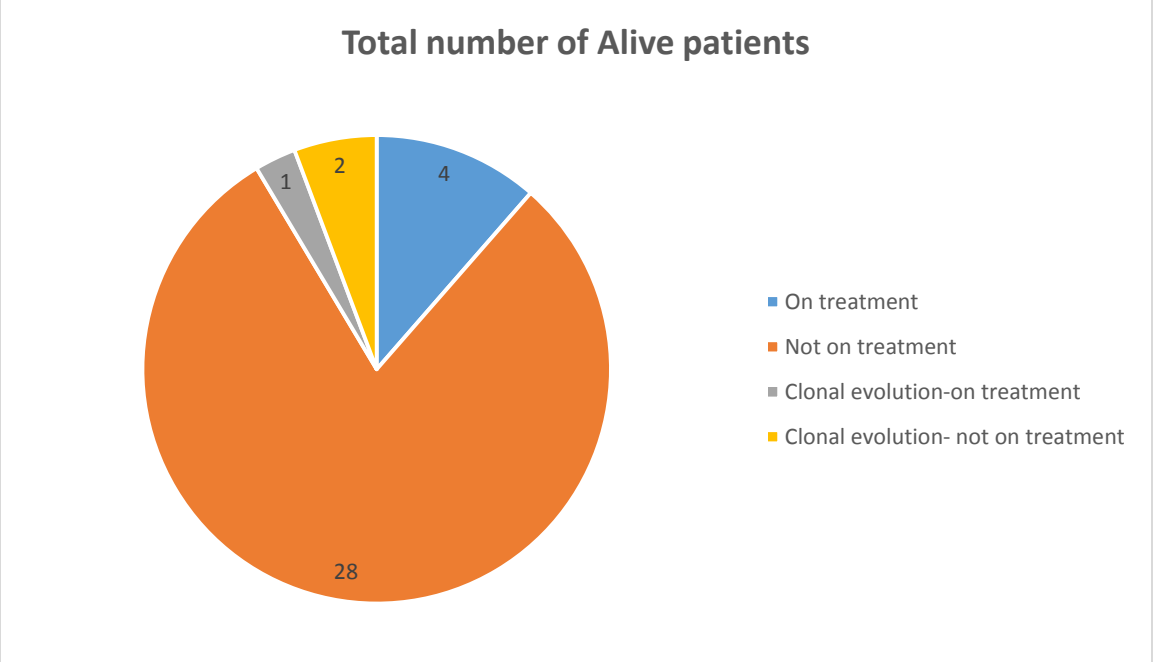


Figure 3.9c illustrates the remission status of the 5 patients who are alive and currently being managed supportively with red cell concentrate and/or plt transfusions, or with ciclosporin. Treatment of infections, with antibacterial or antifungal drugs was also part of the supportive treatment offered to patients, when required. The individual patient treatment details are tabulated in Table 3.16

Figure 3.9c: Characteristics of patients who are alive and on treatment

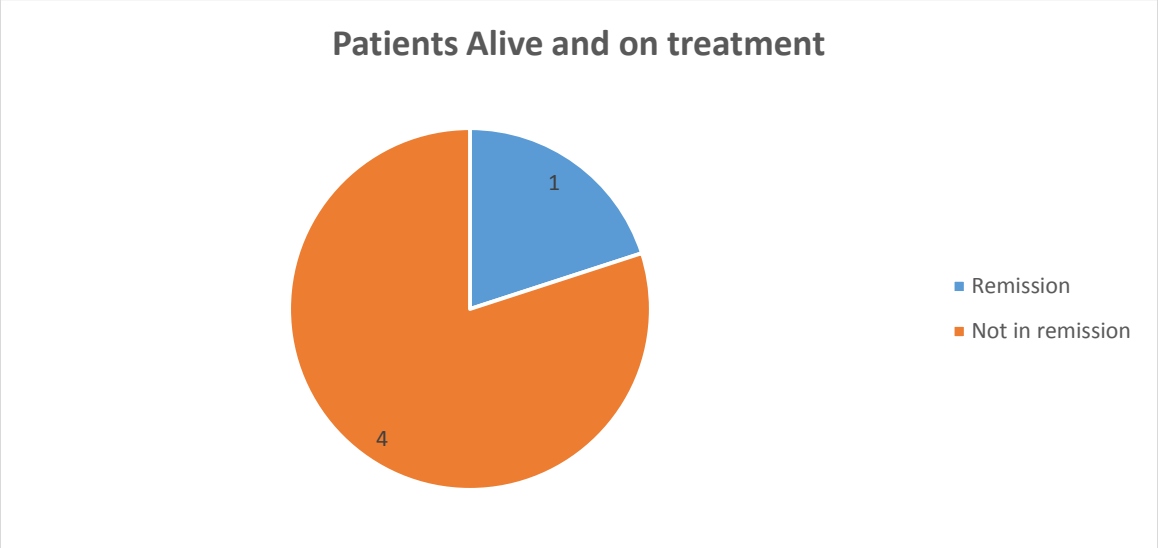
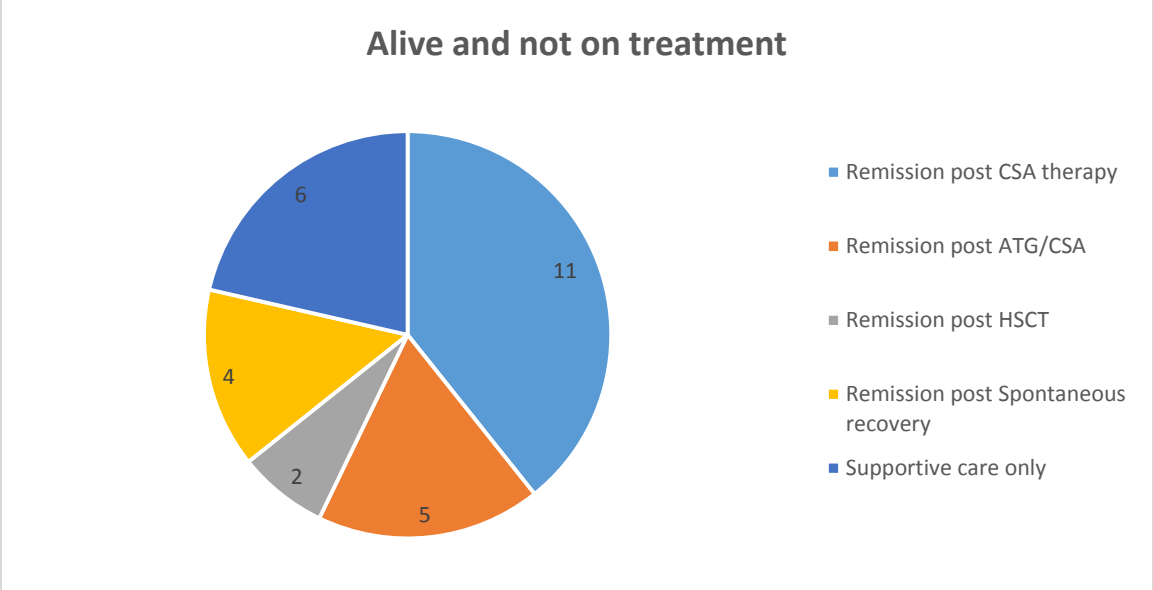


Figure 3.9d illustrates the remission status of the 28 patients who are alive and currently not on treatment. The 6 patients in the supportive care category are transfusion dependent, and receive 2-weekly red cell and/or platelet transfusions.

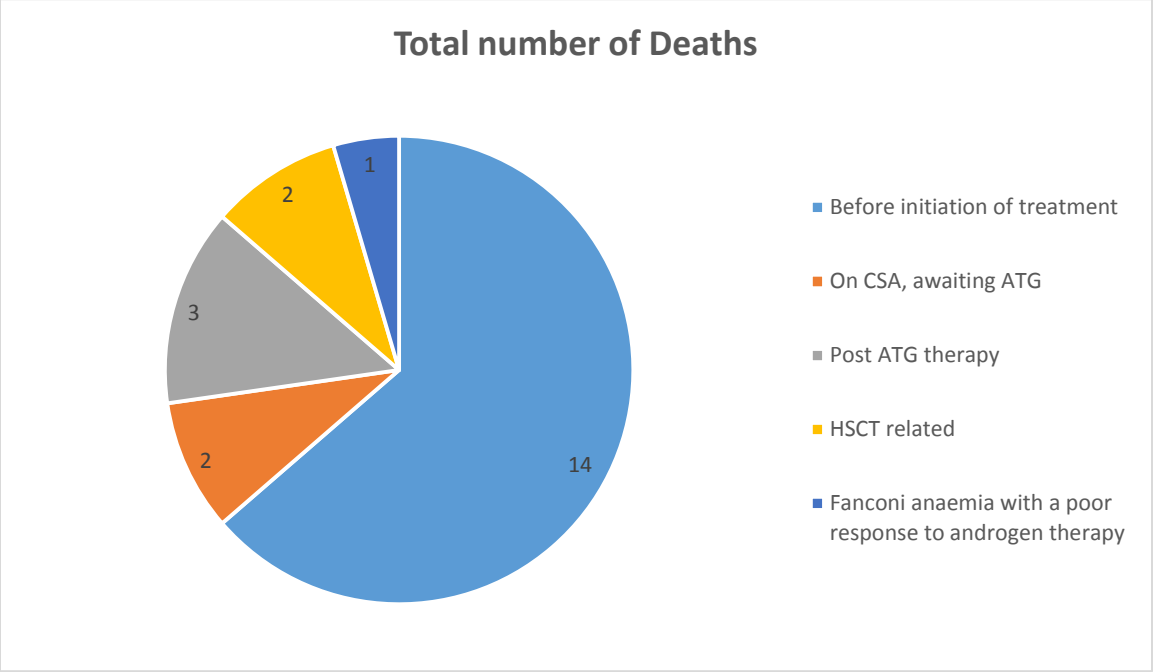
Figure 3.9d: Characteristics of patients who are alive and not on treatment



ATG- anti thymocyte globulin. CSA- ciclosporin. HSCT- haemopoietic stem cell transplant

Twenty-two of the ninety-two patients demised. Figure 3.9e demonstrates when, in relation to treatment, these patients demised.

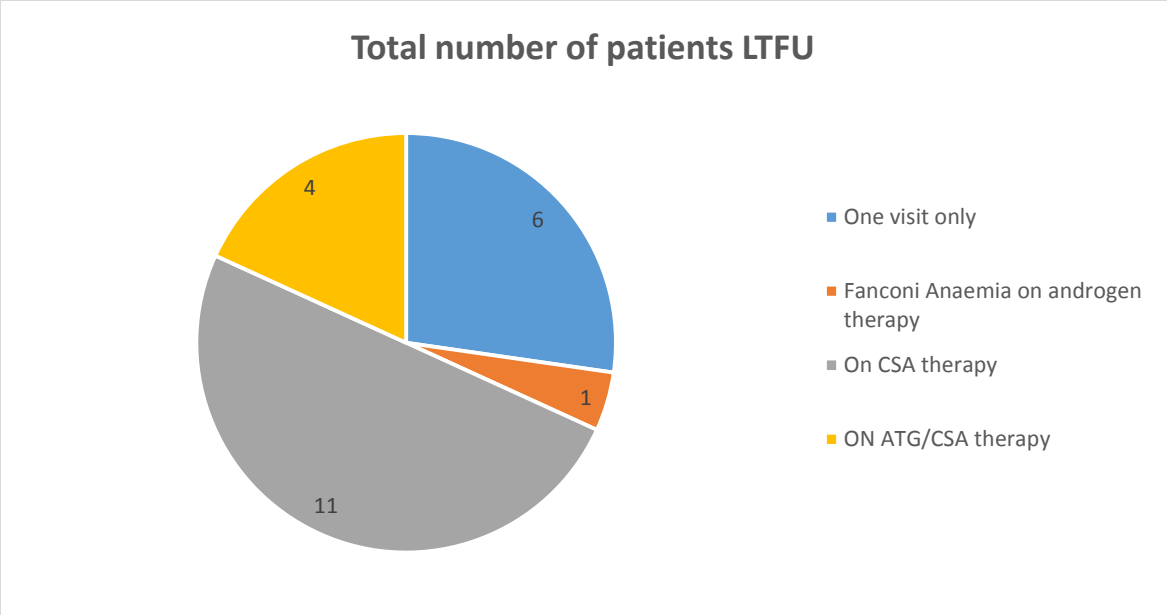
Figure 3.9e: Characteristics of patients who demised



ATG- anti thymocyte globulin. CSA- ciclosporin. HSCT- haemopoietic stem cell transplant

Figure 3.9f (on the following page) illustrates the characteristics of the 22 patients who were LTFU. Six patients had only a single visit to the haematology clinic at IALCH. They were subsequently referred to the base hospital for supportive management and/or initiation of HAART, and never returned to the IALCH clinical haematology department for their follow-up visits. One patient with FA and a poor response to androgen therapy was LTFU. Eleven patients were on CSA therapy at the time of being LTFU. 4 patients were LTFU post ATG therapy.

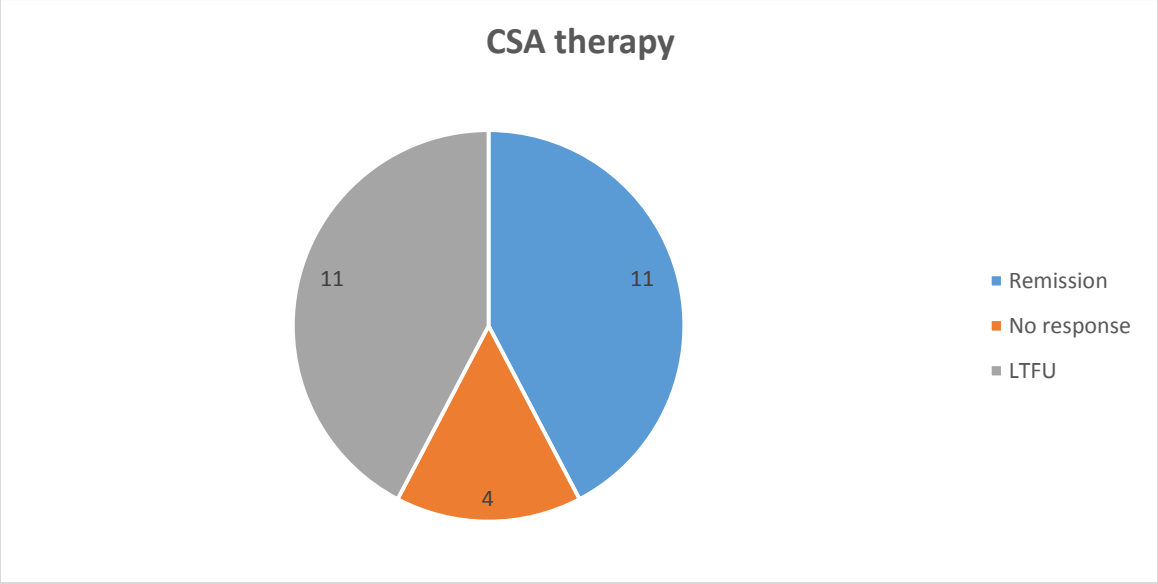
Figure 3.9f: Characteristics of patients LTFU



ATG- anti thymocyte globulin. CSA- ciclosporin.

Figure 3.9g demonstrates the outcomes of patients on CSA therapy.

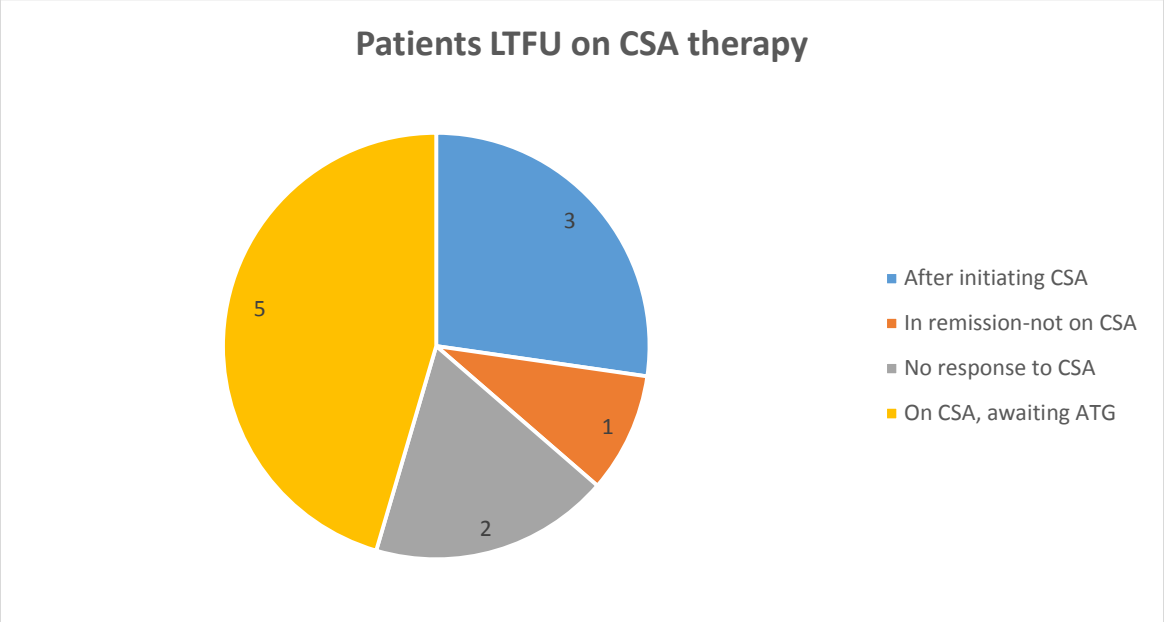
Figure 3.9g: Outcome of patients on CSA therapy



LTFU- lost to follow up.

The details of when in relation to treatment, the 11 patients that were treated with CSA were LTFU are depicted in Figure 3.9h below.

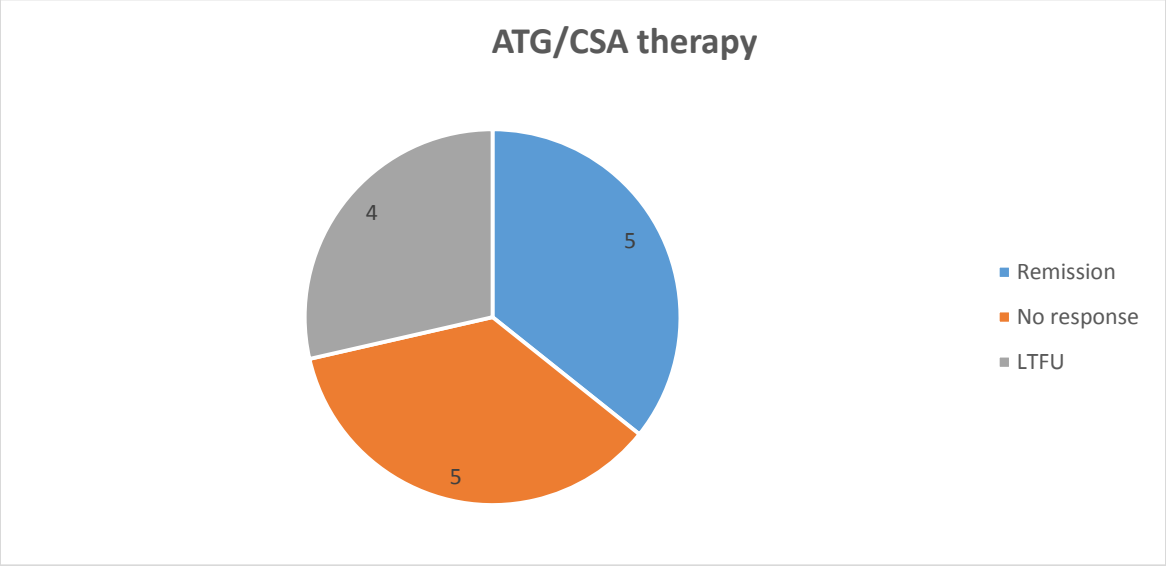
Figure 3.9h: Characteristics of LTFU patients on CSA therapy



ATG- anti thymocyte globulin. CSA- ciclosporin.

Figure 3.9i demonstrates the outcome of patients on ATG/CSA therapy.

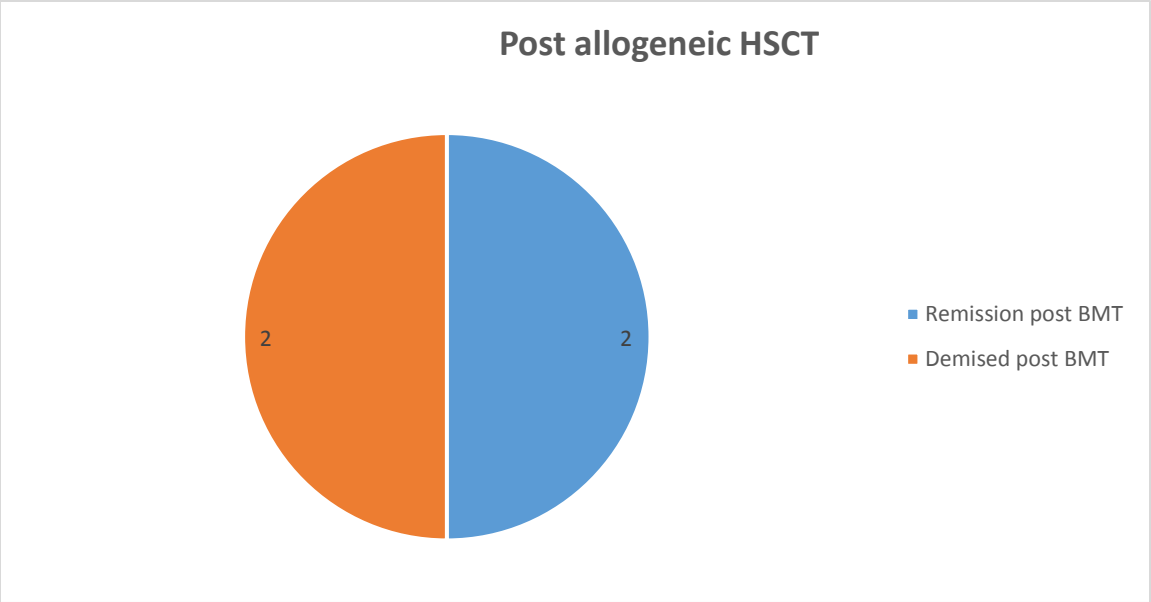
Figure 3.9i: Outcome of patients on ATG/CSA therapy



LTFU- lost to follow up.

Figure 3.9j demonstrates the outcome of patients post allogeneic HSCT.

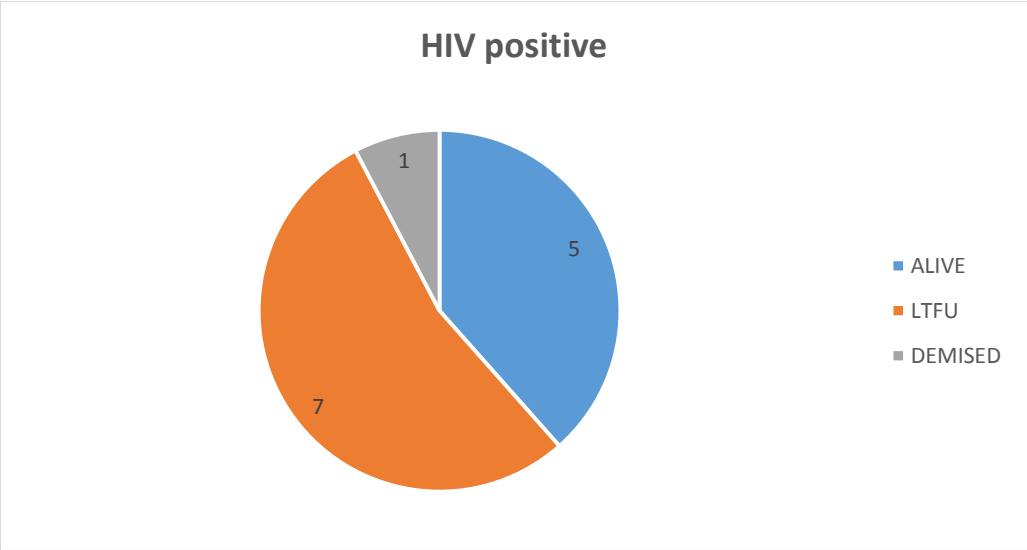
Figure 3.9j: Outcome of patients post-HSCT



HSCT- haemopoietic stem cell transplant. BMT- bone marrow transplant

Figure 3.9k (on the following page) depicts the outcome of the 13 HIV positive patients. One patient demised from overwhelming sepsis at presentation.

Figure 3.9k: Outcome of HIV positive patients



HIV- human immunodeficiency virus. LTFU- lost to follow up.

Table 3.16 (on the following page) illustrates the outcome of the HIV positive patient cohort. Included in the 7 patients who had been LTFU, was one patient who recovered her counts after initiation of ARV therapy. She was discharged from the haematology clinic and was being seen at her base hospital. Her follow-up CD4 counts showed steady improvement.

Table 3.16: Detailed outcome of HIV positive patients

a)Alive			**5**
	Well/Transfusion independent not on CSA (Partial response)	3	
	Well/Transfusion independent on CSA (Partial response)	1	
	Poor response to CSA x 2 (Failed response)	1	
b)LTFU			***7***
	After recovering counts with CSA	1	
	After initiating CSA therapy	1	
	Awaiting ATG	1	
	After initiating ARVs	1	
	After recovering counts with ARVs	1	
	After initial visit	1	
	Never reached IALCH	1	

CSA- cyclosporine. ATG- antithymocyte globulin. ARVs- antiretrovirals. IALCH- Inkosi Albert Luthuli Central Hospital.

Table 3.17: Summary of outcome of pregnant patients

Patient	Gestation at diagnosis of AA	Pregnancy Outcome	Patient outcome
1.	6 weeks	Spontaneous Miscarriage	BMT x2, currently well
2.	6 weeks	Elective Termination of pregnancy (TOP) at 8 weeks	Good response to ATG/CSA. Currently not on any meds and transfusion independent
3.	18 weeks	NVD at term, Macerated still birth (MSB) of 1.0kg	CSA initiated post-delivery, however patient LTFU
4.	20 weeks	Intrauterine death (IUD)	Patient demised of neutropenic sepsis in the Intensive Care Unit (ICU) at 22 weeks gestation
5.	31 weeks	Normal vaginal delivery (NVD) with healthy baby	Patient was still aplastic 8 months' post-delivery. Had a good response to CSA. Currently well, only on HAART

BMT- bone marrow transplant. TOP- termination of pregnancy. ATG- anti thymocyte globulin. CSA- ciclosporin. NVD- normal vaginal delivery. MSB- macerated stillbirth. LTFU- lost to follow up. IUD- intrauterine death. ICU- intensive care unit.

Table 3.18 (on the following page) below illustrates the detailed characteristics of the survivors

Table 3.18: Characteristics of survivors

Alive:	**Outcome**	**Number of patients**	**Details of patient**
A) On treatment	Remission	1	On CSA therapy
	Not in remission	1	Transfusion dependent
		1	Transfusion dependent
		1	4 months post rabbit ATG/CSA, on CSA and prednisone
	Not in remission with clonal evolution	1	Poor response to CSA, now with evolution to MDS-EB1
Total on treatment		**5**	
B) Not on treatment	Remission	11	Post CSA therapy
		5	Post ATG/CSA therapy
		2	Post HSCT therapy
		4	Spontaneous recovery
	Remission with clonal evolution	2	Remission post ATG/CSA, now with haemolytic PNH
	Supportive care	6	Transfusion dependent
Total not on treatment		**30**	

ATG- antithymocyte globulin. CSA- cyclosporin. HSCT- haemopoietic stem cell transplant. MDS-EB1- myelodysplastic syndrome with excess blasts 1. PNH-paroxysmal nocturnal haemoglobinuria

CHAPTER FOUR: DISCUSSION

4.1 Hospital referral system

The NHLS laboratory at IALCH is the only government referral centre for BMATs in KZN. Though the drainage area for the bone marrows were wide, the majority of the BMATs received were from tertiary hospitals, and there were occasional referrals from district level institutions.

The process of obtaining/performing a BMAT requires several steps:

1) The patient must be referred to an institution that can perform BMATs.
2) Referring doctors from peripheral hospitals must telephonically consult one of the haematology registrars at the IALCH laboratory, regarding the indication for BMAT.
3) If approved by the registrar, the BMAT must be "booked" with the laboratory at the base hospital.
4) The booking date is often for a later date, depending on the ability of the laboratory staff to accommodate the marrow, as well as the patient's ability to return on that specified date.
5) Once the BMAT is performed, it must be transported to the laboratory at IALCH were the sample must be received and processed. Staining of the aspirate slides is required and the trephine biopsy is sent to anatomical pathology for decalcification and slide preparation.
6) The turn-around-time (TAT) for a complete BMAT is 14 days. BM aspirates are reported within 48 hours, and trephines within 2 weeks of the sample arriving in the laboratory. Urgent bone marrow cases (AA or Leukaemia) warrant the registrar/pathologist informing the referring doctors of the diagnosis.
7) Often, the patients are out-patients and cannot be contacted to return sooner. They are usually advised to return on a certain date.

The diagnosis may have been delayed if the patient was not clinically suspected to have AA. Furthermore, a delay in any of the above numbered processes could have resulted in a potential delay in the diagnosis of AA, as well as the timely referral for management.

The alarming finding that 12% of patients did not reach the clinical haematology unit at IALCH, despite the diagnosis being made at the IALCH laboratory, may have been due to several variables. The BMATs were reported well within the expected TAT. As these patients had SAA it is likely that the patients could have been discharged and requested to return for review of results and LTFU. They may have become ill and subsequently demised at home or at the base hospital, prior to their referral to IALCH. This highlights the importance of timeous diagnosis and referral of patients with AA.

4.2 Patient characteristics

The number of patients in the study period may not be a true estimate of the prevalence of AA in KZN, because some patients present to the clinical team after being diagnosed with AA at private sector laboratories. Greys hospital, for example, were referring BMATs to private laboratories prior to the year 2012. These patients (that were diagnosed at private laboratories) were not included in the study.

An almost equal male:female (M:F) ratio (0.8-1) was evident in this study. This is similar to the demographic pattern reported in the literature. The 13-25-year age group, which is reported to be a peak age group, constituted 53% of patients in this cohort. Only 5.4% of patients were older than 60 years. There was no clear second peak in incidence in older patients.

The majority of patients (80%) were of Black ethnicity. The KZN census 2011 revealed that 86.9% of the population are of Black African ethnicity. Hence, this figure may be representative of the KZN population. However, there were too few patients from other ethnic groups to allow comparisons between the groups. The population described in this study represents public sector patients, and the patient characteristics in the private sector may differ.

Younger patients were noted to have significantly more severe disease (p value=0.005). The mean ages for patients with VSAA and SAA were 25 and 27 years, compared to the mean age of 37 years for NSAA.

4.3 Failed Bone Marrow Aspirate and Trephine Biopsies

The majority of failed BMATs were performed in non-haematology departments at the peripheral hospitals (Table 3.1). The alarmingly high number of failed BMATs is an indicator of a lack of skill in performing the procedure in the peripheral hospitals and at tertiary hospitals. This is due to the fact that there are no haematology departments/staff and consequently a lack of proficiency in performing the technique. This is also borne out of the observation that none of the BMATs performed by haematology staff at IALCH had failed, when these patients had subsequent BMATs at IALCH. Although there is a haematology department at KEH, initial BMATs for pancytopenia are performed by the department of medicine. Perhaps, this is a reflection of medical registrars not rotating through clinical haematology during their training. Hence, adequate undergraduate/postgraduate training regarding the performance of BMATs is essential.

4.4 Clinical features

Besides the patients with IBMFS, there was no clear clinical evidence pointing to a secondary cause of AA in any of the patients. Most of the patients were classified as idiopathic AA. There was a good correlation between the absence of clinical findings and idiopathic AA.

Consistent with findings in the literature, symptoms of thrombocytopenia were reported in the majority of patients, followed by symptoms of anaemia. Symptoms of neutropenia (infections) were least frequent.

Symptoms correlated with the severity of the depression in blood counts (anaemia, thrombocytopenia and neutropenia).

All patients with an Hb < 6g/dl were symptomatic. Of note, is that all the patients with an Hb level >10g/dl had symptomatic anaemia. This finding is most likely as a result of blood transfusion of these patients at the time of presentation to the base hospital. These 5 patients were possibly markedly symptomatic and required transfusion prior to BMAT.

4.5 Patient comorbidities

Comorbidities in this study that have been reported in the literature to have an association with AA include HIV infection, CMV infection, Hepatitis virus infection, FA and DKC (refer Figure 4.1 below).

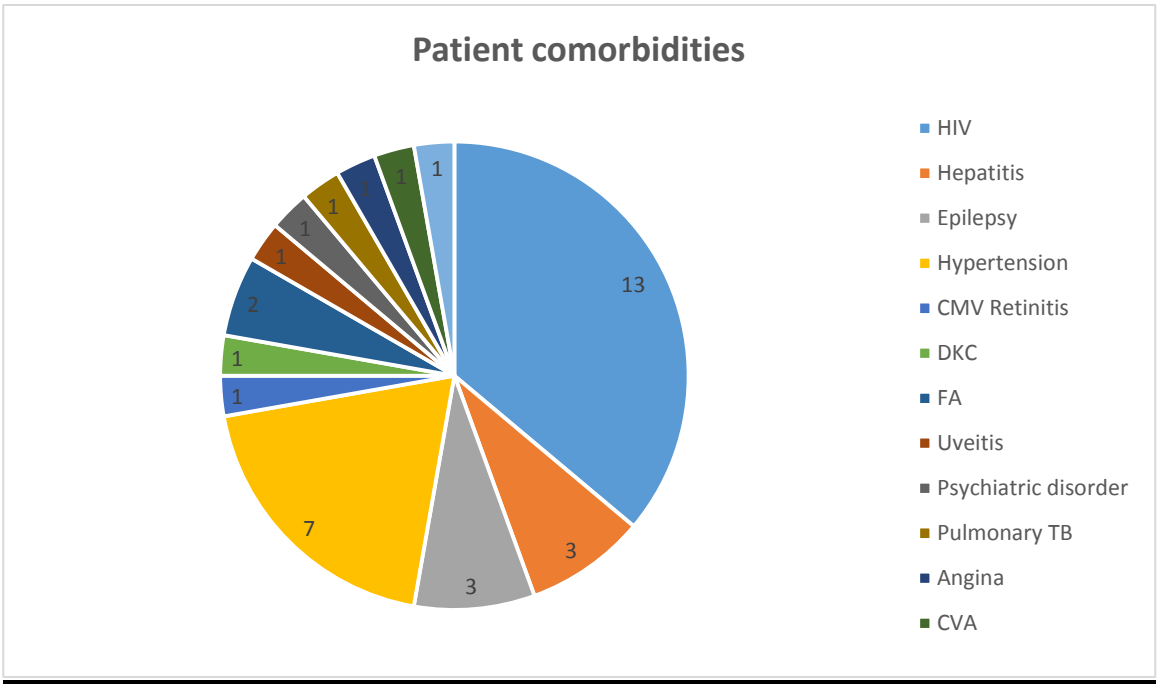
The patient with TB did not have any evidence of mycobacterial infiltration of the bone marrow. Drugs used in the treatment of TB have been implicated in AA [66]. The patient on anti TB therapy had been on therapy for 4 months prior to the diagnosis of AA.

The patient with DKC developed pancytopenia, and was diagnosed with AA at the age of 22 years. It is reported in the literature that bone marrow failure usually presents by 20 years of age in patients with DKC. Approximately 80-90 percent of patients with classic DKC experience BMF by the age of 30 [177-179]. This patient subsequently developed a CMV retinitis. He demised shortly thereafter from the opportunistic infection, and not from the AA.

The commonest comorbidity, documented in 16.3% of the patient cohort and accounting for 37% of comorbidities, was HIV infection (refer Figure 4.1 below). HIV is discussed further in section 4.9.3(a).

Three patients had a history of jaundice, liver pathology or a hepatitis picture at presentation. It is unusual that these patients should have viral hepatitis simultaneous with the diagnosis of AA. The literature states that HAAA usually presents 2-3 months after acute viral hepatitis [104, 107]. It is not clear if the LFT derangements could have been attributed to a viral hepatitis. The viral screens in these patients were negative, and there were no other clear reasons for the hepatitis, such as offending drugs and toxins.

Figure 4.1: Summary of patient comorbidities



HIV- human immunodeficiency virus. CMV- cytomegalovirus. DKC- dyskeratosis congenita. FA- fanconi anaemia. TB- tuberculosis. CVA- cerebrovascular accident

4.6 Patient medications

All of the medications tabulated in Table 3.3 (in chapter 3), aside from the angina drugs, have been reported to be causally associated with AA. It was not clear in most patients when the drugs had been initiated, however the bone marrow request forms did not suggest that the medications had been initiated at the time of presentation of AA. Table 4.2 below illustrates the drugs consumed by patients that have a causal relationship with AA.

One patient initially presented with non-granulomatous pan uveitis of the left eye which subsequently spread to the other eye. AA was diagnosed four months after starting treatment with Azathioprine (AZA). The AZA was subsequently discontinued and CSA initiated simultaneously as the patient required IST for the uveitis. Serial FBC monitoring revealed that the patient's blood counts had recovered completely within 3 months of the diagnosis of AA. The patient is currently well on CSA therapy, as the uveitis flares up with any attempt at weaning the CSA. It is unsure if the AZA treatment was clearly associated with the onset of AA (drug-

induced AA) as the simultaneous administration of CSA may have resulted in recovery of the AA.

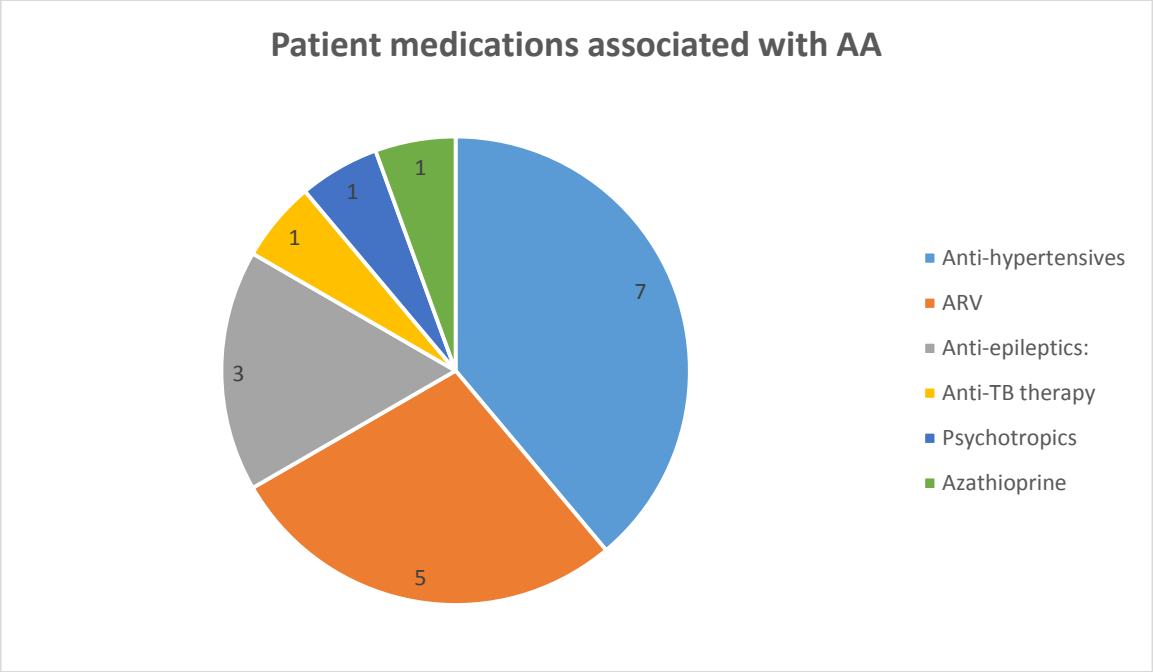
With regard to drug induced AA, a 45-year-old Caucasian female on treatment with multiple psychotropic drugs (including rivotril, effexor XR, zopiclone, as well as clozapine which was added not more than 2 months prior to the diagnosis of AA) also demised from thrombocytopenic complications of AA. The literature does indicate that although rare, psychotropic drugs may cause potentially life threatening adverse haematologic effects, and that middle-aged Caucasian women are at greatest risk for these with a mortality rate from 8%-17% [(259)].

Of the 3 epileptic patients, one was HIV positive. This patient's counts improved with a change in anti-epileptic therapy (from sodium valproate to lamotrigine), and with a change in the anti-retroviral drug (from zidovudine (AZT) to Alluvia). The other 2 patients' counts improved with changing the AED from phenytoin and carbamazapine to lamotrigine. These drugs (sodium valproate, phenytoin and carbamazepine) have been causally associated with AA, and so could the AZT in the first patient [(259)].

Seven patients had regular exposure to anti-hypertensive agents, and these medications have been implicated in the aetiology of AA but these patients had been on anti-hypertensive treatment long before the onset of AA [(260).]

The above-mentioned cases highlight the importance of correlating the onset of drug therapy with the onset of AA, and indicate the value of discontinuing possible inciting agents.

Figure 4.2: Patient medications causally associated with AA

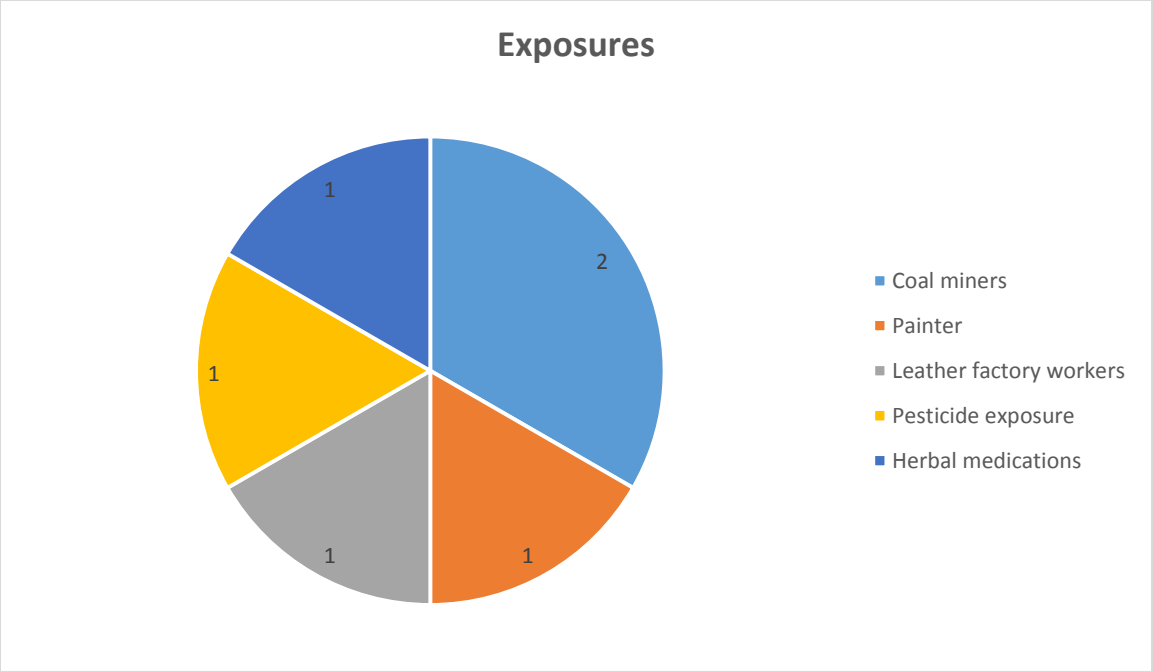


ARV- antiretrovirals. TB- tuberculosis.

4.7 Patient occupations and exposures

High risk exposures that have been reported, and which are included in this patient cohort, include painters, factory workers (shoe factory), coal miners and pesticide exposure in a nurse who was an avid gardener (Figure 4.3). Aside from the nurse who admitted to using pesticides for almost 5 years, the duration of exposure was not clear in the painter, factory worker and one of the coal miners. The other coal miner reported an occupational exposure for 2 years.

Figure 4.3: Summary of causally associated exposures/employments at the time of BMAT



4.8 Pregnant patients

Each of the five pregnant patients with AA presented at variable gestational stages. This is in keeping with the general reported findings [159, 161, 163, 164]. In some of the patients, the aetiological associations may be multifactorial. One patient had PTB and had been on anti-TB treatment at the time of diagnosis of AA. Another patient was on aldomet (methyldopa) for pregnancy induced hypertension. Although uncommon, anti-hypertensive drug therapy has been causally associated with AA [260]. One patient had coexisting HIV. A 22-year-old patient had a history of herbal ingestion prior to diagnosis of AA. The contribution of these factors, combined with pregnancy, to the development of AA, is uncertain.

Interestingly, four of these patients had AA diagnosed in their first pregnancies. One patient was diagnosed with AA during a second pregnancy. She was also HIV positive. The literature reports that AA can be diagnosed in any pregnancy and not necessarily a first one, however studies still need to be conducted to elaborate further on this [161, 163, 164, 170].

The outcomes of the pregnant patients were varied, and are summarized in the table below

Table 4.1: Summary of outcome of pregnant patients

Patient	Gestation at diagnosis of AA	Pregnancy Outcome	Patient outcome
1.	6 weeks	Spontaneous Miscarriage at 6 weeks	BMT x 2, currently well
2.	6 weeks	Elective Termination of pregnancy (TOP) at 8 weeks	Good response to ATG/CSA. Currently not on any meds and transfusion independent
3.	18 weeks	NVD at term, Macerated still birth (MSB) of 1.0kg	CSA initiated post-delivery, however patient LTFU
4.	20 weeks	Intrauterine death (IUD) at 22 weeks gestation.	Patient demised of neutropenic sepsis in the Intensive Care Unit (ICU) at 22 weeks gestation
5.	31 weeks	Normal vaginal delivery (NVD) at term, with healthy baby	Patient was still aplastic 8 months' post-delivery. Had a good response to CSA. Currently well, only on cART

BMT- bone marrow transplant. TOP- termination of pregnancy. ATG- antithymocyte globulin. CSA- cyclosporin. NVD- normal vaginal delivery. MSB- Macerated still birth. LTFU- lost to follow up. IUD- intrauterine death. ICU- intensive care unit. cART- combination antiretroviral therapy

As alluded to in the table above, pregnancy related AA is a complicated condition. In addition to complications relating to pregnancy itself, patients with AA are also more likely to experience complications of AA. These complications may affect the mother and/or the fetus.

Patient 1 (Table 4.4) experienced a complication of AA (bleeding), and the spontaneous miscarriage was her reason for seeking medical assistance, and her incidental pancytopenia being discovered. She was diagnosed with AA and prompt management with transplant instituted. Early elective TOP in patient 2 had a favorable outcome for the patient who had a good response to treatment with complete remission. Hence, early medical intervention can result in more promising and favorable patient outcomes.

Patients 3 and 4, were diagnosed in the second trimester of pregnancy. Fetal outcomes were poor in both patients, possibly resulting from fetal hypoxia and intra-uterine growth restriction. Patient 4 also experienced fatal complications of AA, in that she had neutropenic sepsis. It is evident that

the complications of pregnancy associated AA are very severe. A compounding factor is that the therapeutic options in pregnancy are limited.

Patient 5 was diagnosed in the third trimester of pregnancy. She was also HIV positive. It is possible that both pregnancy and HIV could have contributed to the diagnosis of AA in this patient. The CD4 count at the diagnosis of AA was 151. The viral load was not available. The fetal outcome was favorable, and the patient survived. Her BMAT findings were in keeping with the diagnosis of AA 8 months' post-delivery. The patient had a favorable response to CSA and HAART and is currently in remission.

Although all the pregnant patients in this study were managed supportively, CSA has been used in the treatment of AA in pregnancy [56, 171].

4.9 Laboratory Parameters

4.9.1 Haematology

a) Full Blood Count (FBC) including Mean Cell Volume

The FBC at the time of the BMAT procedure was documented. The majority of patients (93%) demonstrated a pancytopenia. Many patients had been transfused at the time of BMAT due to the severity of their initial presentation (bleeding/severe anaemia). Some patients had received multiple red cell concentrate (RCC) or platelet transfusions. This explains the occasional finding of an Hb >10 g/dl or platelet count >100 x 10^9/l. This may underestimate and not be truly representative of the severity of the AA in some patients.

The original presentation FBC from the base hospital could not be traced because of a change in the laboratory information system (LIS). This accounts for the significant number of patients with high Hb levels (5.4%), as the higher Hb levels were found in patients who had a history of RCC transfusion prior to BMAT.

Multiple attempts were made to obtain outstanding results; however, all efforts were futile. The NHLS-Information Technology Corporate Data Warehouse (CDW), was also contacted telephonically, and by email, on several occasions. Functional analysts were unable to retrieve the data.

The one patient with a normal WCC (6.6 x 10^9/l), actually had a marked lymphocytosis with a severe neutropenia. Reversal of the neutrophil:lymphocyte ratio is a common finding in AA and this was documented in 92% of patients. This emphasizes the importance of a differential count being a clue to the diagnosis of AA. Patients with a lower ANC (<0.2 x 10^9/l) demonstrated a higher degree of infections ranging from mild to severe in nature.

It is well documented that AA is a cause of normocytic-normochromic anaemia with occasional macrocytosis (MCV 95-110 fl) [186, 260]. The MCV ranged from 74.60-116.20 fl in this study cohort. Macrocytosis ranging from 102.1-116.2 was evident in only 7% of patients. Vitamin B12 and folate levels were normal in these patients. There were no cases of megaloblastic anaemia. 52% of cases had normocytic red cell indices.

Predominantly microcytic indices were demonstrated in 40% of patients, albeit a mild microcytosis. The cohort of 37 patients with microcytic indices comprised 32 Black, 1 Caucasian and 4 Indian patients. 21 were female and 16 were male. The finding that females have a higher percentage of microcytosis may possibly be due to the confounding action of blood loss during menstruation. Black females were found to have a higher rate of microcytosis. Although not statistically significant in this study (p value of 0.2), this finding has been reported in the literature [262]. This may be due to the increased incidence of co-existing iron deficiency.

Iron studies were only available for 15 patients, in which the iron was either normal or increased. Serum iron levels ranged from 16-45.2. Ferritin levels or bone marrow iron stores were available in 19 patients. Ferritin levels were normal in 10 patients and increased in 9 patients (range 332-6961). Possible reasons for the increased ferritin include liver dysfunction, iron overload, and infection etc. as ferritin is an acute phase reactant (APR).

Bone marrow iron stores were available for 10 patients only. Other marrows were inadequate for the accurate assessment of iron status. BM iron stores were normal/increased in 8 patients. 2 patients were clearly iron deficient as evidenced by the absence of bone marrow iron stores. Coexisting iron deficiency anaemia (IDA) was evident in patients with symptoms of bleeding.

In summary: the increased percentage of microcytosis was an interesting observation. Only 2 patients had iron deficiency anaemia. Despite the lack of results (iron studies/bone marrow iron stores) in the rest of the patient cohort, it is likely that the remainder of the patients had an anaemia of chronic disease. Other causes of a microcytic anaemia, such as thalassaemia, lead poisoning and sideroblastic anaemia etc. were unlikely in these patients.

b) RPI:

This was uniformly depressed in all 92 patients. None of the patients with PNH clones presented with hemolysis and an increased RPI at diagnosis of AA.

c) BMAT

An interesting observation made, was the 30 aparticulate bone marrow aspirates. This is an uncommon finding in the BMATs of patients with AA. Perhaps, the aspirates were markedly hypocellular, and as a result of the paucity of cellular elements, was misconstrued to be aparticulate. Also because AA is a rare condition and BMATs with AA are not commonly seen in the laboratory, the markedly hypocellular particles may possibly have been missed. Even with

the bone marrow aspirates that failed, the trephine biopsies were all markedly hypocellular for age. There was a good correlation between the bone marrow aspirate and trephine biopsies in all the patients whose aspirates were successful, where the aspirate cellularity was in keeping with the cellularity observed on the trephine biopsy. In patients with PNH clones, erythroid hyperplasia was prominent in the background of a hypocellular/acellular BM. Hence, erythroid hyperplasia in a patient with AA is a good indicator that a PNH clone may also be present, and should be investigated for. Myelodysplasia was not evident in any of the BMATs. There were no bone marrow granulomas detected in the patients on TB treatment and no evidence to suggest other aetiologies/infiltrates. Bone marrow findings correlated well with the PB counts. Of note, none of the patients presenting with severe pancytopenia in the context of these marrows demonstrated a reduction in only a single or two cell lineages on BM biopsy.

d) Cytogenetics (Chromosomal analysis)

Conventional cytogenetic analysis with GTG banding was performed. Karyotyping was performed according to the International System for Human Cytogenetics Nomenclature.

Cytogenetic analysis was not requested by the clinician in 64% (59 of 92) of the cohort. All of the BMATs with which cytogenetic testing was not requested, were referred from peripheral hospitals. Most likely, AA may not have been suspected at the time of the BMAT. All the BMAT samples from IALCH had chromosomal analysis requested with them. This is possibly because patients were referred to IALCH with a provisional diagnosis of AA, or because BMATs were being performed after an initial failed BMAT, at the peripheral hospital.

If AA was clinically suspected at the base hospital, it is likely that a lack of knowledge regarding the complete work-up of AA patients, by clinicians at the peripheral hospitals may explain the cytogenetics not being requested. Furthermore, the chromosomal analysis tubes require refrigeration and these tubes are not readily available in the peripheral hospital laboratories. This may have contributed to the large number of tests not requested.

Although a few patients had karyotyping performed in 2005, and this number has slowly but steadily increased through the years, it was previously thought that AA was without chromosomal aberrations [194]. These BMATs with abnormal cytogenetics were hypothesized to represent other disorders such as Myelodysplastic syndrome, hence perhaps cytogenetic testing was not routinely performed in AA in the earlier years.

Cytogenetic testing was unsuccessful in 48.4% (16 of 33) of the patients for whom it had been requested. This is an expected observation due to the insufficient number of metaphases achieved on bone marrow aspiration [196, 197]. It is likely that the scarcity of cells with mitotic potential obtained on bone marrow aspiration, result in the unsuccessful yield [263]. Sample degeneration

because of a delay in transport to the laboratory, as well as a lack of skilled laboratory staff (technologists with expertise in cytogenetics have left the department), could also have been contributory to the unsuccessful karyotyping.

The majority of the patients in whom cytogenetic testing was successfully performed (45.4%) had a normal karyotype. 2 patients (6.2%) had an abnormal karyotype (-7(deletion) and monosomy 8) that was detected on the presentation BMAT. Follow up cytogenetic testing was not performed in the 2 patients with an abnormal presentation karyotype. The repeat cytogenetics test result (performed in 2008) for the patient with a monosomy 8 stated that the test was cancelled as it was "requested in error". The BMAT findings, however, were still consistent with a diagnosis of AA.

Furthermore, many of the patients with normal karyotypes at presentation, did not have cytogenetic testing requested on subsequent BMATs. 13 patients had repeat BMATs performed to reassess disease status. Cytogenetic testing was only performed for 8 patients, of whom 5 had a normal karyotype. 1 patient had a cytogenetic aberration (monosomy7). The test was unsuccessful in 1 patient. 1 patient's result stated that cytogenetics was "requested in error", and 5 patients did not have the test requested.

The patient with -7(del) received CSA alone and the patient with a monosomy 8 received CSA with ATG. Both patients recovered their counts completely, but were subsequently LTFU. Molecular studies for FISH were not performed in any of the patients.

An interesting finding, is that a patient with an initially normal male karyotype (NMK), and a small PNH clone (6-8%) at diagnosis of AA in 2014, was found to have a monosomy 7 on follow-up cytogenetic testing performed in April 2017. This patient had received ATG in 2014. He was also given CSA, which was discontinued due to persistent gum hypertrophy. Treatment was subsequently switched to tacrolimus, however transfusion requirements persistently increased. A BMAT was performed in April 2017, which demonstrated disease evolution to MDS with Excess Blasts 1 (10%). The follow up PNH FCM reported a PNH clone of <1%. This is an important case, highlighting disease evolution with the acquisition of a new cytogenetic abnormality, as well as a change in the size of the PNH clone. It is also possible that this patient could have had a hypoplastic MDS at presentation, as this condition may be difficult to distinguish from AA with a markedly hypocellular biopsy.

The appropriate and timeous collection of cytogenetic samples (which must be requested at diagnosis as well as with follow up BMATs) to assess for possible clonal evolution, is paramount. This cannot be over-emphasized. The utility of looking for clonal evolution is important in individualizing patient management, and selecting the most appropriate therapeutic modality for a specific patient. If a patient is transplantable, and a cytogenetic aberration is detected, this allows time to work the patient up for possible allogeneic BMT, and in young patients, to look for a matched unrelated donor. Further studies on larger sample populations are

mandatory to detect and confirm cytogenetic aberrations in patients with AAA at diagnosis and follow up.

e) Fanconi anaemia: DNA testing (PCR)

Data was not available for 72 patients (78% of the study population). The FA DNA PCR test is performed in Braamfontein. It was instituted in the year 2005, after an article regarding the presence of the c.637_643delTACCGCC mutation in different tribal groups from South Africa, was published [(264)].

Testing for FA was not routinely requested or performed at IALCH, especially in older patients. It is known (and has been reported) that FA can present in adulthood and not only in children. Furthermore, as one-third of patients are reported to be phenotypically normal, the FA screening test should not be omitted in patients who are not clinically suspected to have FA. FA testing is imperative for appropriate patient management. Therapeutic modalities such as androgen therapy (danazol) are important in the management of FA patients with AA. It is also important to know if the patient has this underlying IBMFS if ATG or BM Transplantation is being considered for treatment of the AA because complications, such as secondary tumours, can arise from the use of certain chemotherapy protocols in FA patients, and FA patients are particularly sensitive to chemotherapeutic agents.

It is unclear as to why routine PCR testing for FA was not performed.

FA DNA PCR testing was positive in 2 patients, both of whom were homozygous for the FANCG c. 637_643del mutation. These patients had somatic abnormalities (skeletal abnormalities and a horse-shoe kidney), in keeping with FA. One patient had 3 siblings, all of whom were diagnosed with Fanconi Anaemia. Both patients received steroid and androgen therapy. One patient eventually succumbed to the complications of bone marrow failure and demised from gram negative septicaemia. The other FA patient was lost to follow-up (LTFU), however it is likely that this patient may have also demised, as both patients demonstrated a poor therapeutic response to androgen therapy.

f) Fanconi anaemia: Chromosomal breakage tests (CBT)

The CBT was not performed in those patients who tested positive for the FA DNA PCR test. CBT failed in 7 patients as a result of insufficient specimen yield and sample deterioration. Both the FA DNA PCR test and CBT are not performed locally but are “send away” tests that are referred to specialized centres to be performed. Hence, there are several pre-analytical variables that may have resulted in test failure. Furthermore, challenges in obtaining missing data, were encountered. Some of these challenges include changes in the LIS, results not being scanned into patient charts as well as poor record-keeping. The chromosomal breakage test was previously performed in Braamfontein. It is now being performed in Bloemfontein, for the last 6 months

approximately. Both centres were contacted for data, which has now been archived, and not readily accessible.

g) PNH

Various methods were used to detect PNH clones. Amongst them were the HAM's test, followed by the immunophenotyping of granulocytes for CD55 and CD59. The most recent method implemented for the diagnosis of PNH at the IALCH NHLS haematology laboratory, is that using fluorescent labelled aerolysin (FLAER).

All of the positive PNH cases were diagnosed in the years 2014 and 2015. This is important because the laboratory method for detecting PNH had changed in our laboratory in June 2013. The first PNH panel using FLAER was performed on the July 1, 2013.

The HAM's test is a complement based assay. It is suitable for hemolytic PNH, however it cannot detect small populations of affected red cells. The detection limit for a PNH clone, of this test ranges from 4.2%-5%. Furthermore, the HAM's test is not specific for the diagnosis of PNH and, as it was not frequently requested (as the obvious advantages of the newer tests became apparent), this test was discontinued at the haematology laboratory at IALCH in November 2015. There were 152 Ham's tests performed at our laboratory since 2003, with no documented positive result for any of these patients [265, 266].

Aside from being a rapid and easily performed test, peripheral blood FCM testing is both a reproducible and an extremely sensitive diagnostic tool to detect PNH clones in different cell populations. Although first described in 1985, it is now the gold standard for diagnosis of PNH. Type I, Type II and Type III PNH cell subtypes can be accurately identified and quantified on the GPI-AP deficient cells [266, 267].

The quantification of the estimated clone size and types of cell lineages involved is extremely important as it correlates with the varying clinical manifestations of the disease [265]. For example, it was found that in the first ten years after initial diagnosis, patients with >20% type III deficiency on red cells presented mostly with clinically significant hemolysis. Almost half of the patients with a greater than 50% GPI deficiency on granulocytes suffered from venous thrombosis [268]. Hence, it is clear that clone size of the GPI-AP-deficient population on granulocytes is an established prognostic factor for vascular sequelae, affecting prognosis as well as life expectancy.

The clone size as well as the cell lineages involved can change with time, deeming follow-up investigations mandatory to initiate appropriate therapy. Besides with the initial diagnosis of PNH, serial monitoring with FCM during the course of the disease can assist with monitoring of the clone size [9, 269, 274].

Flow cytometric analysis using a "stain-lyse-no wash" method was performed at NHLS (IALCH) until 2013. CD55 and CD59 were detected on neutrophils and monocytes. This method gave a better estimate of the clone size, however fresh blood samples (<8 hours from collection) were required rendering the test less sensitive. This issue is particularly important as many of the samples had come from peripheral hospitals and could likely have been old and degenerate at the time of processing, possibly resulting in false negative results. Sixty-one tests had been performed between 2009 to June 2013. No cases of PNH were detected whilst this method was in use.

In 2013, FLAER was added to the immunophenotyping method, using a new analyzer. Combining FLAER with multiparametric FCM further improves the sensitivity and specificity of this testing method. This highly sensitive method is more robust, and has a distinct advantage in that it can be performed on samples stored for up to 48 hours. This makes samples from peripheral hospitals suitable for assessment [270]. It can therefore be inferred that the high negative detection rate prior to the use of FLAER, was attributable to the insensitive technique where the older analyzers were only detecting CD55 and CD59.

All four patients with a PNH clone presented with features suggestive of AA. The PNH clones were incidentally discovered in the 4 patients once the FLAER method had been instituted. The PNH tests that had been performed and found to be negative prior to using FLAER, were not subsequently repeated after FLAER had been in use, except in the 2 patients discussed below. It is uncertain whether the testing done pre-FLAER could possibly be falsely negative as small PNH clones may have been missed.

Interestingly, 2 patients who had negative baseline PNH FCM results in 2011, when the older method that did not incorporate FLAER was used, where found to be positive for PNH in 2015. The newly detected clones were large in size, ranging from 75-80%. These patients presented with the hemolytic form of PNH. It is uncertain whether these patients may have had initial false negative results at baseline, or if the PNH clones were a new development as a consequence of disease evolution within the spectrum of AA-PNH.

An important observation was made in that an increased number of FCM tests requesting the PNH panel, was performed after the standardized use of the FLAER method had been instituted at our laboratory. Sixty-six PNH panels had been requested between July 2013 and April 2015. Hence, the PNH detection rate may have increased for 2 reasons. Firstly, as more patients were being tested, the detection rate increased. This may have been attributable to a change in the departmental protocol, which incorporated PNH testing in the work-up of AA. Secondly, the increased sensitivity of the new testing method resulted in the diagnosis of more cases of PNH.

There is no reported association between pregnancy or TB and PNH, and the PNH clone was discovered incidentally in these patients who had a primary diagnosis of AA.

h) ANF

The patient who tested ANF positive did not fulfil criteria for Systemic Lupus Erythematosus (SLE). As subsequent testing was negative, the low titer was therefore likely to be insignificant.

4.9.2 Biochemistry:

a) Urea and Electrolytes (U&E)

In keeping with AA, the majority of patients (96%) had normal renal function. The derangements in renal function were most likely attributable to co-existing sepsis.

b) Liver Function Tests (LFT)

Liver Function Test derangements were demonstrated in a significant number of patients (16%). The reasons for deranged LFTs are most likely multifactorial, and include underlying infections, which were identified in 62% of the cohort with deranged LFTs. Antibiotics and antivirals, amongst other drugs, also derange the LFT, and could be contributory factors. The LFTs in the patients with Hepatitis B-Sag positivity were not deranged.

It is important to perform a baseline LFT at presentation to assist with accurate assessment of the aetiology, and also prior to starting treatment with therapeutic agents such as CSA.

4.9.3 Viral screen

a) HIV

The overall prevalence of HIV differs substantially with age, sex and race. Females display a higher HIV prevalence compared to men, which was also demonstrated in this study. This is in keeping with the heterosexual mode of acquisition of HIV. While all race groups are affected by HIV, the key populations in SA are Black African females aged 20-34 years, and Black African males aged 25-49 [(270)]. However, slight differences in the median age at presentation were noted in the study population. The range in females was 22-46 years with a median of 33 years. Male patients ranged from 23-27 years with a median age of 26 years. Hence, females in this study were older, and males younger than expected from the literature. The reason for this is unexplained.

A variable range of CD4 counts was evident at presentation. Thus, an association with CD4 count and AA could not be inferred. Furthermore, CD4 counts may not be an accurate indication of HIV burden at presentation of AA, as many patients had a leucopenia, including a lymphopenia. This is further compounded by the unavailable VL data at presentation for many

patients. Although rare, cases of AA associated with HIV have been reported [127, 128, 272, 273]. More studies need to be conducted in this regard.

Of the patients with an interval between the diagnosis of HIV and AA, five of these patients, known with HIV infection, were not on combination antiretroviral therapy (cART) at the time of diagnosis of AA.

Of the 5 patients not on cART, 2 patients with CD4 counts of 381 cells/mm^3 and 388 cells/mm^3, respectively, would not have been eligible for cART at the time of presentation as the previous criteria for initiation of ART, which has subsequently been revised twice since then, was a CD4 count of <200 cells/mm^3.

One of the 5 patients not on cART, had defaulted his therapy prior to being diagnosed with AA. It is not known why the remaining 2 patients with CD4 counts <200 cells/mm^3 (CD4 count of 117 cells/$mm^3$and 147 cells/mm^3) were not on cART at the time of diagnosis of AA, despite being eligible for ARV therapy, and having had the prior diagnosis of HIV.

Unfortunately, the viral loads (at the time of diagnosis of HIV and AA) were not performed/available for the majority of patients. This information would have been of great value in assessing the actual status of HIV infection at the time of presentation with AA.

Only 3 of the patients with an interval between the diagnosis of HIV and AA were on cART at the time of diagnosis of AA.

Of the total cohort of HIV positive patients (simultaneous diagnosis and an interval between the diagnosis) 10 patients were not on cART and this may be a possible indicator of active HIV infection being associated with new onset of AA.

For some of the patients, follow up CD4 counts and viral loads were performed six months later and at various other intervals. Patients who were lost to follow up at IALCH were still continuing their visits to their respective ARV clinics. These results were accessible on the TrakCare LIS. CD4 counts showed significant improvement and viral loads were undetectable, in keeping with a good response to cART. An improvement was also noted in the FBCs of three of the patients. Two of the patients recovered their counts on cART. One patient had an FBC that normalized, however the patient was still on CSA for severe relapsing uveitis.

South Africa has the largest and highest profile HIV epidemic worldwide. There was an estimated 7 million people living with HIV in 2015 [274]. The 2008 national estimate for HIV prevalence amongst South Africans was 10.6%. In 2012, this figure increased to 12.2%. In 2015, the HIV prevalence increased to 19.2% in the general South African population. The prevalence does however vary significantly within the provinces. KZN has a prevalence of almost 40% in some areas compared to the Northern and Western Cape, which demonstrate a prevalence of 18% [275].

This study did not demonstrate an increased prevalence of AA in HIV. The prevalence of 16.3% HIV positive patients in the study population is similar to the background prevalence, or perhaps

even lower when compared to the expected prevalence of 19% and up to 40% in some areas of KZN.

Possible reasons for this figure being an underestimate include:

1) A higher threshold for performing BMATs in HIV positive patients with cytopenias. Patients with pancytopenia are often empirically started on therapy for opportunistic infections, instead of having a BMAT performed initially to accurately establish the cause of pancytopenia. AA may be under-diagnosed in this population group.
2) BMATs are only performed at tertiary level hospitals in KZN. There may be delays in the referral system, which could result in patients demising prior to obtaining a BMAT.

However, a retrospective review of a case series of 257 HIV positive adults at Chris Hani Baragwanath Academic Hospital, who underwent a BMAT examination, in order to assess the diagnostic usefulness of bone marrow examination and evaluate possible predictors of a diagnostic examination, did not implicate AA as a diagnostic BM finding in any of the patients [(276)]. Another study, conducted to assess the diagnostic utility of bone marrow biopsies performed for the investigation of fever and/or cytopenias in HIV-infected adults at Groote Schuur hospital between 2004 and 2007, found that SAA comprised only 1 out of a total of 70 cases. It was a unique diagnosis in only 1 out of 49 patients (0.6%) [(277)]. The earlier study (published in 2001), was conducted in the pre-cART era, further elucidating that perhaps, AA is not increased with active HIV infection.

An important observation was that none of the HIV negative patients being followed up until now, have contracted HIV during/after supportive therapy with blood and blood products. This is important because the risk of HIV transmission, albeit minimal, has been reported during blood transfusion. One patient had a false positive result, which was subsequently negative on confirmatory testing. Included in the management protocol for AA patients at the IALCH Clinical Haematology Unit, are routine 6-monthly viral screens. This allows monitoring for possible transfusion transmitted viral infections.

Table 4.2: Follow-up characteristics of simultaneously diagnosed HIV and AA pts

Patient	Month and Year of Diagnosis of HIV and AA	CD4 count at presentation (Absolute CD4- cells/mm^3)	Viral Load at presentation (copies/ml)	**Date of last resulted CD4 count/VL**	**Outcome**
1.	May 2008	238 cells/mm^3	<20 copies/ml	**Feb 2017: CD4 679** cells/mm^3 **VL <20 copies/ml**	**LTFU IN July 2008, after referral for cART. FBC improved with cART**
2.	Nov 2008	Not done	Not done	**N/A**	**Demised from neutropenic sepsis**
3.	Oct 2010	50 cells/mm^3	240 copies/ml	**March 2016: CD4** cells/mm^3 **VL LDL**	**Poor response to CSA x2**
4.	Nov 2013	839 cells/mm^3	11730 copies/ml	**Sep 2016: CD4** cells/mm^3 **VL undetectable**	**Well and TF independent with normal counts**
5	Feb 2014	323 cells/mm^3	<40 copies/ml	**March 2016: CD4 not available VL suppressed**	**LTFU on CSA in July 2016 with normal counts**

HIV- human immunodeficiency virus. AA- aplastic anaemia. CD4- cluster differentiation 4. ml-millilitre. VL- viral load. LTFU- lost to follow up. cART- combined antiretroviral therapy. FBC- full blood count. Nov- November. Oct- October. LDL- lower than detectable limit. CSA- ciclosporin. Sep- September. TF- transfusion. Feb- February

Table 4.3: Follow-up characteristics of patients with an interval between the diagnosis of HIV and AA

Pt	Month/year of Diagnosis of HIV	Month/year of Diagnosis of AA	CD4 at diagnosis of HIV (Absolute CD4)	Viral Load at diagnosis of HIV (copies/ml)	cART (Y/N)	CD4 at diagnosis of AA (Absolute CD4)	**Date of last resulted CD4/VL**	**Outcome**
1.	2007	2014	Unknown	Unknown	N	151 cells/mm^3	**June 2016: CD4 432** cells/mm^3 **VL <121**	**LTFU after recovering counts on CSA**
2.	2008	May 2013	Unknown	Unknown	Y	36 cells/mm^3	**No follow up CD4/VL**	**LTFU after 1 visit. Restarted on FDC.**
3.	March 2011	June 2013	228 cells/mm^3	348 copies/ml	Y	448 cells/mm^3	**Feb 2014: CD4 385** cells/mm^3	**Recovered counts on changing antiepileptics/ cART**
4.	April 2011	Nov 2011	500 cells/mm^3	44 copies/ml	Y	535 cells/mm^3	**Not available**	**LTFU on CSA**
5.	October 2011	Feb 2012	117 cells/mm^3	Unknown	N	Unknown	**May 2016: CD4 286** cells/mm^3	**LTFU post recovery on ARV**
6.	Sep 2012	June 2013	147 cells/mm^3	<150 copies/ml	N	Unknown	**Aug 2013: VL <150 copies/ml**	**LTFU before referred to IALCH**
7.	April 2014	May 2015	388 cells/mm^3	Unknown	N	235 cells/mm^3	**May 2016: CD4 457** cells/mm^3 **VL LDL**	**Well and TF independent, on CSA and ARV**
8.	June 2015	July 2015	381 cells/mm^3	Unknown	N	-	**Nov 2015: VL LDL**	**LTFU**

HIV- human immunodeficiency virus. AA- aplastic anaemia. CD4- cluster differentiation 4. ml-millilitre. VL- viral load. LTFU- lost to follow up. cART- combination antiretroviral therapy. FDC- fixed dose combination. Nov- November. Oct- October. LDL- lower than detectable limit. CSA- ciclosporin. Sep- September. TF- transfusion. Feb- February. ARV- antiretrovirals. IALCH- Inkosi Albert Luthuli Central Hospital

b) Hepatitis B

The HepBSAg was positive in three patients. One patient had chronic hepatitis with a positive HepBSAg result over 3 consecutive years. The hepatitis B core IgM and the hepatitis E antigen were consistently negative in this patient. LFTs were normal in this patient. A coincidental finding was the equivocal CMV IgM result which was obtained twice, a year apart.

In the other 2 patients, the positive HepBSAg result could indicate either acute infection or a persistent carrier state. The hepatitis B core IgM and the hepatitis E antigen were not available in these patients, limiting accurate identification of the disease state. One of the patients was HIV positive on HAART, with a CD4 count of 381 at diagnosis of AA. LFTs were normal in this patient. Deranged LFTs with a cholestatic picture, were evident in the HIV negative patient.

A 33-year-old HIV positive female with a CD4 of 151 at the time of diagnosis of AA, had an equivocal HepBSAg result. She was 31 weeks pregnant, and on antihypertensive therapy at the time of testing. Her LFTs were not deranged.

In a number of patients there were other factors that could be contributory to the liver function test derangements. There was no clear association with prior hepatitis virus infection and the subsequent onset of AA.

c) Other viruses

There was no documented co-infection of patients with EBV or Parvovirus.

Of the patients whose parvovirus IgM was positive, PCR was not performed in one patient. Parvovirus PCR was negative for the other four patients.

Majority of the patients tested negative for acute CMV infection. 1 patient had an equivocal CMV IgM result. 4 patients had evidence of recent CMV exposure, indicated by the positive CMV IgM serology. CMV IgG was positive in 3 of the patients. However, PCR was only performed in 1 of the 4 patients, and this was negative. LFTs were normal in this patient cohort.

An equivocal HIV result was documented in 1 patient with DKC and deranged LFTs. The patient with DKC was diagnosed with AA prior to the CMV infection (retinitis).

A positive CMV PCR result was obtained in a 21-year-old HIV negative female, for whom the CMV IgM and IgG were not available. In addition to symptomatic anaemia, the patient presented with jaundice, diarrhea and abdominal pain. LFTs were grossly deranged in keeping with a diagnosis of viral hepatitis. This was an unusual case of acute hepatitis with simultaneous onset of AA. As mentioned earlier, AA usually occurs 2-3 months after the hepatitis. This patient was treated with ganciclovir and is currently alive and well.

4.10 Patient management and outcomes

Important observations were made relating to:

1. The thirteen patients diagnosed with AA, but not being referred to the clinical haematology unit at IALCH timeously.
2. The six patients that had only one hospital visit and were LTFU.
3. The eleven patients that were LTFU after being referred back to their base hospitals for either transfusion, HAART or management of their opportunistic infections etc.
4. The five patients that were LTFU on CSA, or whilst awaiting ATG treatment.
5. The 2 patients that were successfully treated with BMT
6. The 3 patients who spontaneously recovered their counts
7. The one patient who recovered counts once HAART had been initiated, not requiring specific immunosuppressive treatment for the AA.
8. The one patient who recovered his counts with a change in the ARV agent as well as the AED.

Points 1-4 above highlight the issues of the scarcity of resources, which is an important limiting factor in the KZN public health sector. It also highlights the importance of treating AA timeously, as delaying treatment increases mortality.

Referral: A significant number of patients (14%) did not reach the clinical team at IALCH. Although the exact reason is unknown, it is likely that there may have been deficits in the referral of these patients or that these patients may have been very ill at diagnosis, and subsequently demised. In the peripheral areas of KZN, patients often travel long distances to see clinicians and have to wait for transportation to be booked, before they can be referred to a tertiary level hospital for work-up.

Drugs: The process of obtaining ATG therapy for patients requires motivation and approval. Patients had to wait for ATG to be approved, for beds to become available for administration of ATG, and 5 patients eventually demised while awaiting ATG therapy. There have also been instances when ATG was not available from the supplier. Addressing this pressing issue is critical for optimal patient care.

Bed constraints: The limited amount of beds in the haematology ward has always been a restrictive factor in adequate patient management. Beds are often prioritized for patients with acute leukaemia, and patients with AA wait long periods for beds to become available (especially for ATG therapy which requires in-patient management). Perhaps if this resource was more easily available, patients could have been treated at IALCH from the outset. Supportive measures such as blood transfusions could have been provided and infections treated at IALCH, and these patients would not have been LTFU. This may perhaps, have also resulted in a lower mortality rate and a greater number of patients with a favorable outcome.

Bone marrow transplantation: Although bone marrow transplant (BMT) has proved curative for 50% of the transplanted AA patients in this study, the first BMT for AA was performed at IALCH in 2011. Hence, not many patients had the option of transplantation for AA, earlier on. To date there have been 9 BMT for AA in the BMT unit at IALCH. 4 of the 9 patients have been included in this study because their diagnosis was made at the NHLS haematology laboratory during the study period. The other patients have either had the diagnosis made elsewhere (private laboratories) or they have been diagnosed with AA outside the study period (after October 2015). Although it is anticipated that the number of transplants for AA will increase in the years to come, this would to some extent depend on the availability of a fully HLA-matched sibling donor for the patient.

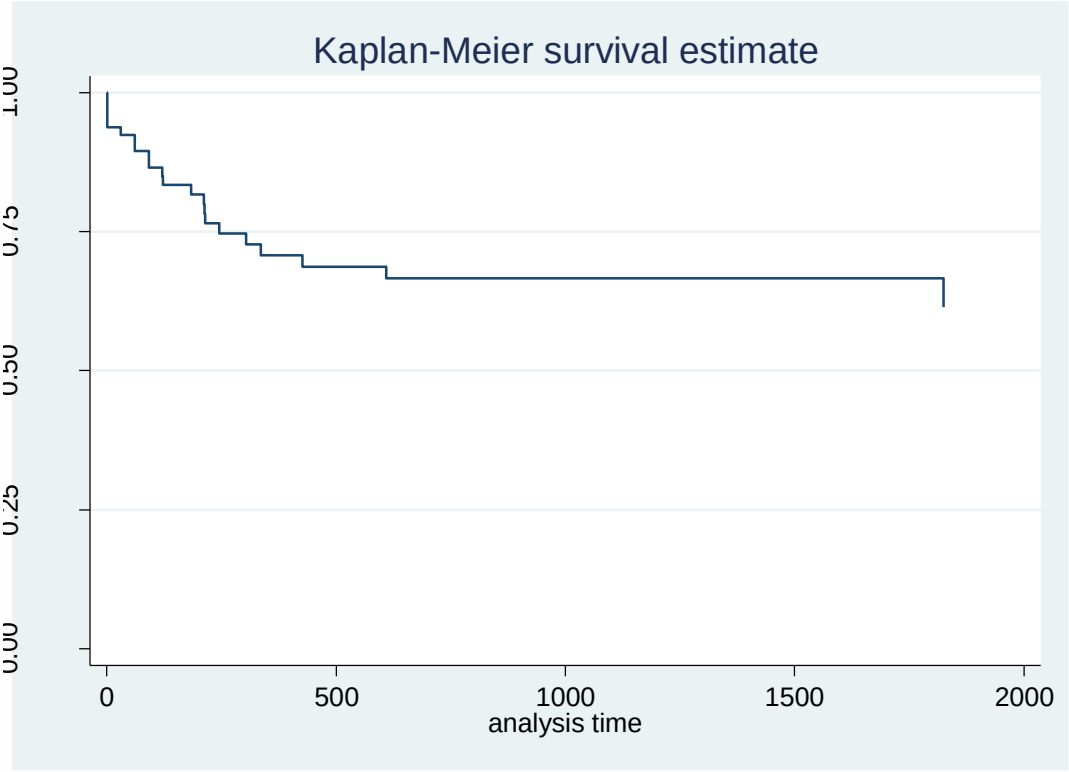
HIV infection: The institution of ARV therapy may modify the number of HIV positive patients presenting with AA. As has been reported in the literature, BM transplantation has been used as a therapeutic modality for HIV positive patients with SAA. Due to the limited resources and lack of expertise in transplanting HIV positive patients, this may not be feasible in our setting.

Mortality: It is likely that the mortality rate has been underestimated, as 24% only accounts for 22 patients in the study population. This figure does not take into account those patients who did not reach the clinical haematology unit at IALCH, as well as those patients that have been LTFU, and subsequently demised. This indicates that AA is fatal if untreated.

The steepest mortality was found to be in the first year and the sharpest decline, as illustrated on the Kaplan-Meier survival estimate graph (on the following page), is between 0- 600 days. Seventy three percent of deaths were sepsis related. Most of the deaths occurred close to presentation. Two patients demised from intracranial bleeds. One patient demised from an acute pulmonary embolus and one other patient died from acute graft versus host disease. The cause of death was not known in two patients. . None of the patients who demised had received treatment with ciclosporin or ATG.

The 2 year survival rate was 66.7% for the patient cohort, and the 5 year the survival rate was 61.5%. However, this number may not be truly representative of the entire patient cohort due to the significant number of patients (28%) lost to follow up.

Figure 4.4: Kaplan-Meier survival estimate



4.11. Study limitations

Having conducted a retrospective study, some limitations were encountered.

1. As data was retrieved from BMAT request forms and available patient records, an element of missing information was present for a number of the variables. Fortunately, the large number of patients in the cohort was sufficient to make reasonable inferences from the data.

2. The NHLS Laboratory Information System (LIS) has changed over the years, the most recent change to the TrakCare system being implemented in 2010/2011. Obtaining results prior to this time was a lengthy arduous process, with some results not being accessible. Furthermore, specialized testing is performed at specialist referral laboratories, e.g. FA DNA PCR testing is performed in Braamfontein. FA chromosomal breakage tests are performed in Bloemfontein. These results are subsequently obtained by email, often at a much later time (as the testing takes some time). Results need to be manually scanned into patient files. This process was omitted for a few patients and some patients had blank scanned documents. A better system of record keeping is required for these send away tests.

3. Prior to the year 2012/2013, PNH as well as testing for Fanconi anaemia and cytogenetics were not routinely performed. This is an important limitation in terms of assessing co-existing conditions and the accurate incidence of IBMFS.

4. The large number of patients LTFU has impacted on the accurate assessment of patient response to therapy, the toxicities and adverse effects of therapy, the complications of the disease, as well as overall patient survival/outcome.

5. This study was a review of BMATs referred to the NHLS haematology laboratory at IALCH only. Many patients with AA that were referred to clinical haematology unit at IALCH had BMATs that were referred to, and analyzed at private sector laboratories such as Heinsworth and partners, Ampath or Lancet laboratories. Hence, this study does not take into consideration patients whose diagnosis of AA was made at other laboratories, and may not be totally representative of the actual number of patients managed by the clinical haematology department.

CHAPTER 5: CONCLUSION

This study of adult patients with a bone marrow diagnosis of AA had several aims. Amongst them was a review of the epidemiological and clinic-pathological features of acquired AA in this patient cohort, as well as an assessment of laboratory investigations performed at diagnosis. The clinical outcome and management was also reviewed. The objective was to collate this data and compare these findings with that documented elsewhere in the literature.

This study has demonstrated that the presentation of AAA in adults at IALCH, NHLS, exhibits shared characteristics with that of other developed and developing countries. The patients were mostly in the younger age group. No bimodal age distribution was evident in this cohort of patients.

The majority of the cases were idiopathic AAA. Offending drugs and toxins were suspected to be causal in a few patients, some of whom in which the AA resolved upon drug withdrawal/substitution. 3 patients demonstrated IBMFS with a poor outcome. This highlights the challenges encountered in treating patients with DKC and FA.

This study did not show an increased incidence of HIV associated AA. The prevalence of HIV positive patients with AA was similar to the background prevalence of HIV in KZN. Easy access to cART and the new ARV roll-out programme could modify future findings, as some patients showed recovery from the AA with the institution of ART. This may affect the number of HIV positive patients who present with AA, and the prevalence may decrease further. However, more studies with a larger number of HIV positive patients with AA will be required to elaborate on this.

It is apparent that pregnancy outcomes are variable, and early elective TOP perhaps appears to have the safest outcome. Long term follow-up of these patients is mandatory to assess if spontaneous resolution of AA occurs.

With regard to patient outcomes, it is clear that patients with AA that are diagnosed early and managed with IST have a fairly good outcome. This elaborates on the need for an early accurate diagnosis, prompt referral and patient management, as reflected by the significant number of patients who did not reach the clinical haematology department, despite the diagnosis of AA being made.

This study has also highlighted deficiencies in the clinical and laboratory diagnostic sytems in the province of KZN:

There is an apparent lack of clinical acumen by non-haematologists in the work-up of AA patients, which is possibly a contributory factor to increased patient mortality. There is also a distinct deficit in the expertise demonstrated in performing BMATs at base hospitals, which in most cases are tertiary level institutions. This results in a delayed diagnosis of AA. Improving

undergraduate/postgraduate training programmes, with rotation of medical registrars through clinical haematology requires serious consideration for the future.

The comprehensive work-up as well as the appropriate and timely management of AA requires very close collaboration between the haematopathologist and the clinical haematologist. Specialized testing requires skilled and competent laboratory staff, and as many of the tests are send away tests, results are often not readily available culminating in delayed or even a lack of diagnosis of important conditions like FA. Our laboratory has experienced staffing difficulties in specialized field of cytogenetics testing for several years, as senior and experienced cytogenetics staff have resigned, and these posts have not yet been filled.

It is important for the clinical haematology unit to have a standardized protocol in place for the holistic work-up and diagnosis of patients suspected to have AA, as well as for the existing AA patients. This is clear from the paucity of data such as cytogenetics and FA/PNH screens in the earlier years of this study. These tests have important implications in the management and follow up of patients with AA.

The need to motivate for ATG, and more significantly, limited hospital beds is a critical issue in the management of AA patients in KZN. This contributed to the large number of patients who are LTFU, and also to the mortality rates as specific therapy is often delayed. Of the patients who were treated, many had a good response to IST. Bone marrow transplantation is also a promising therapeutic modality in the management of AA patients in our setting.

Appendix 1: The final Study Protocol

A review of Aplastic/Hypoplastic Anaemia diagnosed on bone marrow samples at the Haematology Laboratory at National Health Laboratory Service (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, Kwa-Zulu Natal.

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Student number: 200266594

Degree: MMed (Haematology)

Supervisor: Dr Fatima Bibi Fazel

Aim

The aim of this study is to collect data regarding the demographics, aetiology/associations, clinical presentation, laboratory parameters and outcomes of adult patients with a bone marrow diagnosis of Aplastic Anaemia (AA) at the National Health Laboratory Service (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal.

Specific Objectives

1) To illustrate the demographics, clinical presentation, management and outcomes in adult patients with a confirmed bone marrow diagnosis of AA.

2) To correlate the full blood count (FBC), bone marrow aspirate and trephine findings

3) To document the disease severity (using the above in 2)

4) To document aetiological associations, in particular viruses such as Human Immunodeficiency Virus (HIV), and connective tissue disorders such as systemic lupus erythematosus (SLE)

4) To establish if there is a relationship with the CD4 count/viral load and disease presentation in HIV infected patients

5) To determine the presence, or subsequent emergence, of a Paroxysmal Nocturnal Haemoglobinuria (PNH) clone in these patients

6) To determine, if present, any consistent cytogenetic abnormalities

Background

Aplastic anaemia (AA) is a rare disease that is classified as a bone marrow failure syndrome. Aplastic anaemia is a haemopoietic stem cell (HSC) disorder characterized by a markedly reduced bone marrow cellularity. Bone marrow failure may result in a peripheral pancytopenia (a decrease in all cell lines). Cytopenias involving a single myeloid lineage may dominate; lymphopoeisis is usually relatively well preserved.

Epidemiology and Pathogenesis:

Aplastic anaemia is rare in Western Europe and the United States (2 cases per million population per year), but the incidence in China, Southeast Asia and Mexico is estimated to be three to four times higher. It is primarily a disease of children and younger adults, with another peak in incidence in patients 60 years and older.

Aplastic anaemia may be inherited or acquired.

Irrespective of the aetiology, haemopoiesis is markedly reduced in all patients with aplastic anaemia, as reflected by bone marrow histology. Obligatory involvement of multiple lineages points toward HSC or very early haemopoietic progenitor cells as main targets of the pathophysiologic mechanism in aplastic anaemia. Clinical response to immunosuppressive therapy targeting T-cells (e.g. anti-thymocyte globulin) supports an immune-mediated pathogenesis for aplastic anaemia.

Aplastic anaemia is usually associated with normal cytogenetics. An abnormal karyotype in a patient with a hypocellular marrow is more consistent with a diagnosis of Myelodysplastic Syndrome, although some investigators believe that certain chromosomal abnormalities such as trisomy 8 or deletion 13q can still be consistent with a diagnosis of Aplastic anaemia.

Associations with PNH:

Paroxysmal Nocturnal Haemoglobinuria (PNH) is an acquired, chronic haemolytic anaemia in which haemolysis is largely intravascular. In addition to haemolytic anaemia, the patient may have thrombosis and pancytopenia. This triad when present is highly characteristic of PNH.

PNH is a rare disorder (estimated prevalence of less than 1 in 100 000) that can occur anywhere in the world. There is no evidence of family clustering and PNH has been observed at all ages from 1 to 72 years; however, it is most common in young adults.

The natural history of PNH is a very chronic disorder. It may afflict the patient for decades. The median survival is estimated to be about 10 years. Spontaneous recovery may occur in some

cases. Not infrequently, at some stage in the disease, the anaemia may be associated with other cytopenias. When a PNH patient becomes less hemolytic and more pancytopenic, he or she becomes very similar to a patient with AA. Conversely a patient with aplastic anaemia may subsequently develop PNH: The term “PNH-AA syndrome” has been used to designate these patients.

Aetiology:

Examples of inherited forms of aplastic anaemia include Fanconi’s Anaemia and Dyskeratosis Congenita.

Acquired aplastic anaemia (AAA) may be idiopathic or secondary to an underlying cause.

Aplastic anaemia can arise during pregnancy, with drug exposure (e.g. non-steroidal anti-inflammatories, anti-thyroid drugs, gold etc.) or in association with viral infections and autoimmune diseases. Hepatitis-associated aplastic anaemia is a variant of aplastic anaemia in which aplastic anaemia follows an acute attack of viral hepatitis. The aplastic anaemia is often fatal if untreated. Hepatitis-associated aplastic anaemia accounts for 2-5% of cases of aplastic anaemia in Europe and 4-10% of cases in East Asia. Aplastic anaemia has been reported to occur in 28-33% of patients requiring orthotopic liver transplantation for fulminant non-A, non-B, and non-C hepatitis. This seronegative hepatitis in patients with post hepatitis aplastic anaemia does not appear to be caused by any of the known hepatitis viruses and often is referred to as Hepatitis/Aplastic Anaemia syndrome. Aplastic anaemia evolves with a typical delay of several weeks to months after the episode of hepatitis, usually after improvement of liver enzymes.

Clinical presentation of Aplastic Anaemia

Idiopathic aplastic anaemia usually arises in a previously healthy patient who has no history of malignancy or collagen vascular disorder, and no exposure to cytotoxic drugs or radiation.

Symptoms and signs of aplastic anaemia are a consequence of bone marrow failure. Patients may present with pallor and fatigue due to anaemia, mucocutaneous bleeding due to thrombocytopenia, or infection due to neutropenia. More severe haemorrhage into the central nervous system or gastrointestinal tract is not typical on disease presentation.

Classification

Classification and prognosis in AA are related to the severity in the depression of peripheral blood counts.

Severe aplastic anaemia (SAA) is defined by:

-Bone marrow cellularity <25%, or 25-50% with <30% residual hematopoietic cells

-AND any 2 out of 3 of the following:

- Peripheral blood neutrophil count < 0.5 x 10^9/l
- Peripheral blood platelet count < 20 x 10^9/l
- Peripheral blood reticulocyte count <20 x 10^9/l

Very severe aplastic anaemia (VSAA):

As above, but absolute peripheral blood neutrophil count of <0.2 x10^9/l

Non-severe aplastic anaemia (NSAA):

-Patients not fulfilling the criteria for severe or very severe Aplastic Anaemia

-With a hypocellular bone marrow, with 2 out of the 3 of the following:

- Neutrophils <1.5 x 10^9/l
- Platelets <100 x 10^9/l
- Haemoglobin <10 g/l

Management of AA

Rarely, patients with aplastic anaemia can spontaneously recover normal haematopoiesis. Spontaneous remission is most often seen with drug induced aplastic anaemia and usually occurs within 1-2 months of discontinuing the offending drug.

The standard of care for moderate aplastic anaemia is not well established. Except for cases in which there is transfusion dependence, specific treatment is optional, because survival rates are not positively influenced by treatment.

Without treatment, almost all patients with severe or very severe aplastic anaemia will eventually succumb to infection or to haemorrhagic complications. Therefore, such patients require immediate supportive and eventually, specific therapy once a diagnosis is confirmed.

Besides supportive therapy with leuco-depleted red cell and single donor platelet transfusions, specific therapeutic options for patients with severe aplastic anaemia consists of allogeneic haemopoietic stem cell transplantation (HSCT) or immunosuppressive therapy with ATG and ciclosporin. At the time of diagnosis, all potential transplantation candidates should be HLA typed to identify a sibling donor or a matched, unrelated donor.

AA in developing countries such as Africa

There is a paucity of information regarding AA in Southern Africa, and an extensive literature search has shown no publications.

I have selected this topic, not only because of my own personal interest in this disease condition, but also because studies regarding aplastic anaemia are lacking in developing countries such as South Africa, and the rest of Africa. The above objectives and the data collected and analyzed will assist in providing useful epidemiological and clinical information in our local setting, as such a study has not been conducted previously.

The information obtained from this study will also provide a foundation for future research in our setting.

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6. Judith C. W. Marsh, Sarah E. Ball, Jamie Cavenagh, Phil Darbyshire, Inderjeet Dokal, Edward C. Gordon-Smith, Jane Keidan, Andrew Laurie, Anna Martin, Jane Mercieca, Sally B. Killick, Rhona Stewart, John A. L. Yin, *Guidelines for the diagnosis and management of aplastic anaemia*, 2009 Blackwell Publishing Ltd, British Journal of Haematology, 147, 43-70

7. Phillip Scheinberg and David P. Steensma, *Acquired marrow failure syndromes: aplastic anaemia, paroxysmal nocturnal hemoglobinuria, and myelodysplastic syndromes,* Chapter 17, American Society of Haematology Self-Assessment Program textbook 2013, 451-460

8. Kevin E. Brown, John Tisdale, John Barrett, Cynthia E. Dunbar and Neal S. Young, *Hepatitis-associated aplastic anaemia*, the New England Journal of Medicine 1997, Volume 336, Number 15, 1059-1064

Study Design

A retrospective review of bone marrow aspirate and trephines, laboratory investigations and clinical data of adult patients with a confirmed diagnosis of aplastic anaemia at the NHLS laboratory/Inkosi Albert Luthuli Central Hospital in Durban.

Study Population

The study population will consist of all adult patients (over the age of 12 years) with a bone marrow diagnosis of aplastic anaemia at the Haematology Laboratory (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal, South Africa, between the period 1 January 2005 - 30 October 2015.

Sampling Strategy

Bone marrow aspirate and trephine biopsies confirming the diagnosis of aplastic anaemia, that were assessed at the NHLS Haematology Laboratory from 1 January 2005 - 30 October 2015 will be included. Additional laboratory investigations and clinical data for these patients will then also be reviewed.

Sample size

Approximately 45 bone marrow aspirate and trephine biopsies and the corresponding patient investigations/clinical data will be studied.

Inclusion Criteria

All adult patients (>12yrs of age), with a bone marrow diagnosis of aplastic anaemia at the Haematology Laboratory (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, Kwa-Zulu Natal, South Africa will be included.

Exclusion Criteria

Paediatric bone marrow aspirate and trephine biopsies with the diagnosis of aplastic anaemia will be excluded from the study.

Data Collection Methods and Tools

Data collection will include a review of the following:

A) Demographic data and history submitted with the bone marrow samples by requesting doctors.

B) Relevant laboratory investigations obtained from the Trak Care Laboratory Information System:

1. Full blood count including differential and peripheral blood smear

2. Reticulocyte count

3. Bone marrow aspirate and trephine biopsies

4. Bone marrow cytogenetic studies (if available)

5. Flow cytometry for detection of a paroxysmal nocturnal haemoglobinuria clone

6. CD4 counts in HIV positive patients

7. Virological investigations including:

Human Immunodeficiency Virus Enzyme linked immunosorbent assays and viral load
Hepatitis A, B and C antibody tests
Hepatitis B Surface Antigen tests
Parvovirus Enzyme linked immunosorbent assays and Polymerase Chain Reaction tests
Cytomegalovirus Enzyme linked immunosorbent assays
Epstein Barr Virus Enzyme linked immunosorbent assays

C) Clinical data regarding the presenting symptoms, clinical course, selected management strategies and patient outcomes (such as treatment responses and overall survival).

Data will be captured onto a data capture form (Appendix 2)

Data Analysis

Data analysis techniques and statistical analysis will be done in consultation with a qualified statistician.

Study Location

Haematology Laboratory (National Health Laboratory Service) and the department of Clinical Haematology, Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal, South Africa.

Study period

The study period will include patients presenting from 1 January 2005 to 30 October 2015.

Limitations

This retrospective review will be based on data extracted from bone marrow aspirates and trephine biopsies, as well as available clinical notes of patients with AA, diagnosed at the Haematology Laboratory (NHLS). Bone marrow report and sample availability and integrity, as well as the completeness and detail of clinical notes will impact on the study.

Morphological shortfalls may also affect the study.

The sample population is exclusively from the public (state) sector, and may therefore not be an accurate representation of aplastic anaemia in the province, as it will not include patients seen in the private sector, who may have different.

Ethical considerations

No direct patient contact will be made during this study.

All data will be extracted from laboratory investigations and available clinical information.

No informed consent will be required.

All patient information is strictly confidential.

There is no financial benefit for anyone involved in the study.

This study will be conducted according to the ethical principles for medical research on human subjects as defined by the World Medical Association Declaration of Helsinki.

The protocol will be submitted to the Ethics Committee for approval, and permission will be obtained from Inkosi Albert Luthuli Central Hospital to access the relevant clinical data.

Funding

No funding is required

Reporting and Implementation

It is intended that the data be submitted for the MMed degree for Dr Zeenat Dawood Moorad

Appendix 2: Aplastic Anaemia: Data Collection Sheet

1) **Patients study number:** _____

2)

3) **Date of Diagnosis**

Episode number of bone marrow/s:

_____

4) **Age/ Date of Birth:** _____

5) **Gender:** **M** ☐ **F** ☐

6) **Ethnic Group:** **a) Black** ☐
b) Asian ☐
c) Coloured ☐
d) White ☐

7) **Occupation:** _____

Previous (Major occupations): _____

Current: _____

8) **Comorbid disease/s:**

_____

_____

9) **Relevant Medication/Exposures prior to Diagnosis**
(e.g. cytotoxic agents, radiation, pesticides, toxins):

a) Yes ☐ **No** ☐

b) If Yes, give details:

_____

_____

10) General features at Presentation:

a) Pallor:

_____

b) Bleeding:

_____

c) Petechiae/Purpura/Ecchymoses:

_____

d) Infections: _____

e) Pyrexia: _____

f) Dysmorphic features

11) Other relevant clinical findings:

_____

_____

_____

_____

12) Bone Marrow Findings:

a) Aspirate:

-Cellularity= _____

-Cell lines= _____

-Other= _____

b) Trephine:

-Cellularity= _____

-Cell lines= _____

-Other= _____

13) Cytogenetics:

Yes: ☐ **No:** ☐

If Yes, result:

14) **Flow cytometry for Paroxysmal Nocturnal Haemoglobinuria (PNH):**

a) **Peripheral blood** _____

b) **Bone marrow** _____

c) **PNH clone detected:** **Yes** **No** ☐ ☐

d) **If Yes, Size of the PNH clone:**

_____

_____

FBC: **WCC=** ____ **Hb=** ____ **MCV=** ____ **PLT=**____

15) **Differential:**

N=____ **L=** ____ **M=** ____ **E=** ____ **B=**____

16) **Reticulocyte count:** **Corrected:** _____

Absolute: _____

17) **Urea and Electrolytes:**

_____

_____

_____

18)

19) **Liver Function Tests:**

_____

_____

_____

20) **Anti-Nuclear Factor (ANF):** _____

21) Pregnant: Yes ☐ No ☐

IF YES, Gestation at diagnosis: _____

Virology

17) HIV Serology:

a) Reactive= ☐ Non-reactive= ☐

b) If reactive:

-Date of diagnosis: _____

-CD4 count at diagnosis: _____

-Viral Load at diagnosis: _____

c) Is the patient receiving ARV's? Yes No ☐ ☐

d) If yes:

-Date treatment started: _____

-Treatment regimen: _____

-Treatment complications: _____

---

e) Follow-up viral loads/CD4 counts:

DATE	VIRAL LOAD	CD4 COUNT

18) Hepatitis A Serology: **Negative** ☐

IgG + ☐

IgM + ☐

19) Hepatitis B Serology:

Hepatitis B Surface Antigen + ☐

Hepatitis B Surface Antibody + ☐

Titre: _____

20) Hepatitis C Serology: **Negative** ☐

IgG + ☐

IgM + ☐

21) Parvovirus: **Negative:** ☐

IgG + ☐

IgM+ ☐

PCR: POS ☐ **NEG** ☐

22) Cytomegalovirus: **Negative:** ☐

IgG + ☐

IgM + ☐

PCR: POS ☐ **NEG** ☐

23) Epstein Barr Virus Serology: **Negative:** ☐

IgG + ☐

IgM + ☐

24) Disease Severity: _____

25) Managemement of Patient:

Observation: ☐

Supportive care: (RBC and platelet transfusions) ☐

Ciclosporin only: ☐

ATG (Equine)/ Ciclosporin/Methylprednisolone (Triple Therapy): ☐

Bone marrow transplant from matched sibling donor ☐

26) Patient outcomes

Alive at end of study: ☐

Demised during study: ☐

Lost to follow up during study: ☐

Appendix 3: Ethical approvals

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 May 2016

Dr ZD Moorad (200266594)
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences
zmoorad@yahoo.com

Protocol: A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal.
Degree: MMed
BREC reference number: BE014/16

The Biomedical Research Ethics Committee has considered and noted your application received on **22** December 2015.

The study was provisionally approved pending appropriate responses to queries raised. Your response received 22 April 2016 to queries raised on 24 February 2016 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval.**

This approval is valid for one year from **06 May 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's **decision will be RATIFIED** by a full Committee at its meeting taking place on **14 June 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: fbfazel@gmail.com
cc postgrad: dudhrajhp@ukzn.ac.za

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

12 May 2017

Dr ZD Moorad (200266594)
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences
zmoorad@yahoo.com

Dear Dr Moorad

Protocol: A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal. **Degree:** MMed
BREC reference number: BE014/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	06 May 2017
Expiration of Ethical Approval:	05 May 2018

I wish to advise you that your application for Recertification received on 04 May 2017 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be ratified by a full Committee at its meeting taking place on 13 June 2017.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc supervisor: fblaze@gmail.com
cc postgrad: dudlira@hp@ukzn.ac.za

UNIVERSITY OF
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Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Proks/Biomedical-Research-Ethics.aspx

18 April 2018

Dr ZD Moorad (200266594)
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences
zmoorad@yahoo.com

Dear Dr Moorad

Protocol: A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal. Degree: MMed **BREC reference number: BE014/16**

I wish to advise you that your application for Amendments (addition of new site IALCH) dated 01 February 2018 for the above protocol has been **noted and provisionally approved** by a sub-committee of the Biomedical Research Ethics Committee subject to KZN DoH permission. IALCH permission noted by BREC.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

PERMISSION TO CONDUCT A RESEARCH STUDY/TRIAL

This must be completed and submitted to the Medical Superintendent/s / Hospital Manager/s for signature.

For **King Edward VIII Hospital (KEH)** and **Inkosi Albert Luthuli Central Hospital (IALCH)** studies please submit the document together with the following:

1. Research proposal and protocol.
2. Letter giving provisional ethical approval.
3. Details of other research presently being performed by yourself if in the employ of KEH, (individually or as a collaborator).
4. Details of any financial or human resource implications to KEH, including all laboratory tests, EEGs, X-rays, use of nurses, etc. (See Addendum 1)
5. Declaration of all funding applications / grants, please supply substantiating documentation.
6. Complete the attached KEH Form - *"Research Details"*

Once the document has been signed it should be returned to **Mrs Patricia Ngwenya** at the Biomedical Research Ethics Administration, Room N40, Govan Mbeki Building, Westville Campus, University of KwaZulu-Natal.

To: Chief Medical Superintendent / Hospital Manager

Permission is requested to conduct the above research study at the hospital/s indicated below:

Site 1 address:
IALCH

Investigator/s:
Principal: DR Z D Mavundla
Co-investigator: DR S. Ramsuran
Co-investigator: _____

Signature of Chief Medical Superintendent/Hospital Manager: [signature]
Date: 05/04/2006

Site 2 address:

Investigator/s
Principal: _____
Co-investigator: _____
Co-investigator: _____

Signature of Chief Medical Superintendent / Hospital Manager:

Date: _____

NB: Medical Superintendent/s / Hospital Manager/s to send a copy of this document to Natalia

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Office of The Medical Manager
IALCH

Physical Address: 800 Bellair Road, Mayville, 4058
Postal Address: Private Bag X08, Mayville, 4058
Tel: 0312401059 Fax: 0312401050 Email: ursulanun@ialch.co.za
www.kznhealth.gov.za

Reference: BE 014/16
Enquiries: Medical Management

24 January 2018

Dr Z D Moorad
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences

Dear Dr Moorad

RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hosptial, Durban, KwaZulu-Natal.**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Kindly ensure that this office is informed before you commence your research.
4. The hospital will not provide any resources for this research.
5. You will be expected to provide feedback once your research is complete to the Medical Manager.

Yours faithfully

.....
Dr N Tatmain

Dr L P Mtshali
pp **Medical Manager**

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Office of The Medical Manager
IALCH

Physical Address: 800 Bellair Road, Mayville, 4058
Postal Address: Private Bag X08, Mayville, 4058
Tel: 0312401059 Fax: 0312401050 Email: ursulanun@ialch.co.za
www.kznhealth.gov.za

23 January 2018

Dr Z D Moorad
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences

Dear Dr Moorad

Re: Approved Research: Ref No: BE 014/16: A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal.

As per the policy of the Provincial Health Research Committee (PHRC), you are hereby granted permission to conduct the above mentioned research once all relevant documentation has been submitted to PHRC inclusive of Full Ethical Approval.

Kindly note the following.

1. The research should adhere to all policies, procedures, protocols and guidelines of the KwaZulu-Natal Department of Health.
2. Research will only commence once the PHRC has granted approval to the researcher.
3. The researcher must ensure that the Medical Manager is informed before the commencement of the research by means of the approval letter by the chairperson of the PHRC.
4. The Medical Manager expects to be provided feedback on the findings of the research.
5. Kindly submit your research to:

   The Secretariat
   Health Research & Knowledge Management
   330 Langaliballe Street, Pietermaritzburg, 3200
   Private Bag X9501, Pietermaritzburg, 3201
   Tel: 033395-3123, Fax 033394-3782
   Email: hrkm@kznhealth.gov.za

Yours faithfully

.....Dr N Mtshali

PP **Dr L P Mtshali**
Medical Manager

UNIVERSITY OF
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INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
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Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

08 June 2018

Dr ZD Moorad (200266594)
Discipline of Haematology
School of Laboratory Medicine and Medical Sciences
zmoorad@yahoo.com

Dear Dr Moorad

Protocol: A review of aplastic anaemia diagnosed on bone marrow samples at the haematology laboratory at National Health Laboratory Services (NHLS), Inkosi Albert Luthuli Central Hospital, Durban, KwaZulu-Natal. Degree: MMed **BREC reference number: BE014/16**

I wish to advise you that your application for Amendments (addition of new site IALCH) dated 01 February 2018 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee subject to KZN DoH permission. IALCH permission and DOH permission (submitted on 24 May 2018) has been noted by BREC.

This approval will be noted at the next BREC meeting to be held on 10 July 2018.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix 4: Summarized diagnosis and further investigations for AA

Test	Key changes
1. Full blood count	Pancytopenia. Usually the haemoglobin concentration and neutrophil and platelet counts are uniformly depressed. In the early stages, isolated cytopenia, particularly thrombocytopenia, may occur. Lymphocyte counts are usually preserved. Presence of monocytopenia needs further investigation to exclude hairy cell leukaemia or inherited bone marrow failure due to *GATA2* mutation (Emberger/MonoMac syndrome, see section on inherited AA)
2. Reticulocyte count	Reticulocytopenia; automated reticulocyte counting will over-estimate the count compared with the levels set in the Camitta criteria (Camitta, 1984) for defining disease severity, which were defined on manual counts. This criterion has now been modified from manual percentages to absolute reticulocyte levels $<60 \times 10^9$/l as assessed by automated technologies (Rovo *et al*, 2013)
3. Blood film examination	Frequent macrocytosis and anisopoikilocytosis. Neutrophils may show toxic granulation. Platelets are mainly small in size. Exclude presence of dysplastic neutrophils, abnormal platelets, blasts and other abnormal cells, such as 'hairy' cells
4. HbF%	HbF; measure pre-transfusion in children – important prognostic factor in children. Note that the level is often elevated in constitutional syndromes
5. Peripheral blood chromosomal breakage analysis: diepoxybutane test (DEB Test)	For possible FA if patient aged <50 years, but it would also be indicated to screen older patients if FA is clinically suspected. It is difficult to set an upper age limit for FA screening, as anecdotal cases have been diagnosed in the fifth decade (unpublished observations). Screen all patients who are transplant candidates and siblings of FA patients
6. Flow cytometry for GPI-anchored proteins to detect PNH clone (6-colour methodology including FLAER)	See AA and PNH section for full description
8. Vitamin B12 and folate	Documented vitamin B12 or folate deficiency should be corrected before a final diagnosis of AA is confirmed. Bone marrow aplasia due to vitamin deficiency is exceedingly rare
9. Liver function tests	Liver function tests should be performed to detect antecedent/on-going hepatitis
10. Viral studies: hepatitis A/B/C, EBV, CMV, HIV and Parvovirus B19	AA due to hepatitis is rare, it usually occurs 2–3 months after an acute episode of hepatitis and is more common in young males (Brown *et al*, 1997). In post-hepatic AA the serology is often negative for the known hepatitis viruses. CMV should be assessed if SCT is being considered. HIV more commonly causes isolated cytopenias but is a very rare cause of AA (Wolf *et al*, 2007; Hapgood *et al*, 2013). Likewise, parvovirus B19 is more usually associated with pure red aplasia but has been reported with AA (Mishra *et al*, 2005)
11. Anti-nuclear antibody and anti-double stranded DNA	Pancytopenia in systemic lupus erythematosus may (i) be autoimmune with a cellular bone marrow (ii) associated with myelofibrosis or rarely (iii) with a hypocellular marrow
12. Chest X-ray and other radiology	Useful at presentation to exclude infection and for comparison with subsequent films. X-rays of the hands, forearms and feet may be indicated if an IBMFS is suspected. High resolution CT scan of the chest is indicated for suspected DC or constitutional *RUNX1* bone marrow failure syndrome
13. Abdominal ultrasound scan and echocardiogram	An enlarged spleen and/or lymph nodes raise the possibility of a malignant haematological disorder as the cause of the pancytopenia. In younger patients, abnormal or anatomically displaced kidneys are features of FA
14. Emerging diagnostic tests: the following are not currently routine diagnostic tests, but are likely to be so within the next few years	
Peripheral blood leucocyte telomere length:	Useful for disease screening for telomere gene mutations in classic DC; less specific in adult onset AA with *TERC*/*TERT* mutations; short telomeres may also occur in acquired AA with reduced stem cell reserve (Townsley *et al*, 2014)
Next generation sequencing, gene panels for:	• Telomere gene complex mutations • Other IBMFS • Acquired somatic mutations, typical of myeloid malignancies, to help distinguish AA from hypocellular MDS and for early detection of clonal evolution to MDS/AML (Kulasekararaj *et al*, 2014)
Single nucleotide polymorphism array karyotyping	Whole genome scanning to detect unbalanced chromosomal defects (Afable *et al*, 2011a)

HbF, fetal haemoglobin; GPI, glycerophosphatidylinositol; AA, aplastic anaemia; PNH, paroxysmal nocturnal haemoglobinuria; FLAER, fluorescent aerolysin; EBV, Epstein Barr virus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; SCT, stem cell transplantation; IBMFS, inherited bone marrow failure syndromes; MDS, myelodysplastic syndrome; AML, acute myeloid leukaemia; CT, computerized tomography; DC, dyskeratosis congenita; FA, Fanconi anaemia.

Appendix 5: Bone marrow features of Aplastic Anaemia

Bone marrow aspirate	Can be performed without platelet support, providing adequate surface pressure is applied (Kelsey, 2003), even in severe thrombocytopenia. Difficulty obtaining fragments may indicate marrow fibrosis or infiltration and should raise the suspicion of a diagnosis other than AA. In AA, fragments and trails are hypocellular with prominent fat spaces and variable numbers of residual haemopoietic cells. Erythropoiesis is reduced or absent; dyserythropoiesis is very common, often marked and does not distinguish MDS from AA. Megakaryocytes and granulocytic cells are markedly reduced or absent. Dysplastic megakaryocytes and granulocytic cells are not seen in AA. Lymphocytes, macrophages, plasma cells and mast cells often appear prominent. In the early stages of disease, there may be increased macrophages with some haemophagocytosis and background eosinophilic staining representing interstitial oedema
Cytogenetic and FISH analysis	Karyotyping may fail in very hypocellular marrows with there being insufficient metaphases. In this situation perform FISH analysis for chromosomes 5, 7, 8 and 13 It was previously assumed that the presence of an abnormal cytogenetic clone indicated a diagnosis of MDS and not AA. However it is now evident that abnormal cytogenetic clones [such as del(13q), trisomy 8 and others], which may be transient, are present in up to 12% of patients with otherwise typical AA at diagnosis (Gupta *et al*, 2006; Afable *et al*, 2011b). Although monosomy 7 may indicate the likelihood of MDS in children, in adults monosomy 7 can also be seen in AA. Abnormal cytogenetic clones may arise during the course of the disease and the appearance of a new cytogenetic abnormality may provide evidence of clonal evolution (Maciejewski *et al*, 2002)
Bone marrow trephine biopsy	A good quality trephine biopsy of at least 2 cm is essential to assess overall cellularity and morphology of residual haemopoietic cells, and to exclude an abnormal infiltrate. Care should be taken to avoid tangential biopsies because subcortical marrow is normally hypocellular In most cases the biopsy specimen is hypocellular throughout; sometimes hypocellularity is patchy with both hypocellular and residual cellular areas. Focal hyperplasia of erythroid or granulocytic cells at a similar stage of maturation may be observed. Small lymphoid aggregates may occur, particularly in the acute phase of the disease or when AA is associated with systemic autoimmune diseases, such as rheumatoid arthritis or systemic lupus erythematosus. Increased reticulin staining, dysplastic megakaryocytes (best assessed by immunohistochemistry) and blasts are not seen in AA; their presence either indicates a hypoplastic MDS or evolution to leukaemia (Bennett & Orazi, 2009)

AA, aplastic anaemia; PNH, paroxysmal nocturnal haemoglobinuria; FISH, fluorescence *in situ* hybridization; MDS, myelodysplastic syndrome.

Appendix 6: Normal Reference Ranges (for NHLS IALCH)

FBC:

Test	Sex	Age	Low	High
White Cell Count (x 10^9/L)		12.0-12.9	3.9	10.2
White Cell Count	F	13.0-999	3.9	12.6
White Cell Count	M	13.0-999	3.92	10.4
Red Cell Count (x 10^{12}/L)	F	12.0-12.9	4.05	4.98
Red Cell Count	M	12.0-12.9	4.43	5.53
Red Cell Count	F	13.0-999	3.8	4.8
Red Cell Count	M	13.0-999	4.5	5.5
Haemoglobin (g/dl)	F	12.0-12.9	11.7	14.9
Haemoglobin	M	12.0-12.9	12.5	16.5
Haemoglobin	F	13.0-999	12	15
Haemoglobin	M	13.0-999	13	17
Haematocrit (L/L)	F	12.0-12.9	.351	.436
Haematocrit	M	12.0-12.9	.368	.473
Haematocrit	F	13.0-999	.36	.46
Haematocrit	M	13.0-999	.4	.5
Mean Cell Volume (fL)		12.0-12.9	77.1	91.5
Mean Cell Volume	F	13.0-999	78.9	98.5
Mean Cell Volume	M	13.0-999	83.1	101.6

Test	Sex	Age	Low	High
Mean Cell Haemoglobin (pg)		12.0-12.9	25.8	31.7
Mean Cell Haemoglobin	F	13.0-999	26.1	33.5
Mean Cell Haemoglobin	M	13.0-999	27.8	34.8
Mean Cell Haemoglobin Concentration (g/dL)		12.0-12.9	33	35.1
Mean Cell Haemoglobin Concentration	F	13.0-999	32.7	34.9
Mean Cell Haemoglobin Concentration	M	13.0-999	33	35
Red Cell Distribution Width (%)		12.0-12.9	11.6	14.8
Red Cell Distribution Width	F	13.0-999	12.4	17.3
Red Cell Distribution Width	M	13.0-999	12.1	16.3
Platelet Count (x10^9/L)		12.0-12.9	180	440
Platelet Count	F	13.0-999	186	454
Platelet Count	M	13.0-999	171	388
Mean Platelet Volume (fL)		12.0-12.9	7	11.4
Mean Platelet Volume	F	13.0-999	7.3	11.3
Mean Platelet Volume	M	13.0-999	7.1	11
Neutrophils %		12.0-12.9	33	59
Neutrophils %	F	13.0-999	34	72
Neutrophils %	M	13.0-999	32	76

Test	Sex	Age	Low	High
Lymphocytes %		12.0-12.9	33	50
Lymphocytes %	F	13.0-999	21	56
Lymphocytes %	M	13.0-999	18	56
Monocytes %		12.0-12.9	0	6
Monocytes %	F	13.0-999	3	10
Monocytes %	M	13.0-999	4	12
Eosinophils %		12.0-12.9	0	3
Eosinophils %	F	13.0-999	0	6
Eosinophils %	M	13.0-999	0	8
Basophils %		12.0-12.9	0	1
Basophils %	F	13.0-999	0	1
Basophils %	M	13.0-999	0	2
Neutrophils (x 10^9/L)		12.0-12.9	1.5	7.4
Neutrophils	F	13.0-999	1.6	8.3
Neutrophils	M	13.0-999	1.6	6.98
Lymphocytes (x 10^9/L)		12.0-12.9	1	3.6
Lymphocytes	F	13.0-999	1.4	4.5
Lymphocytes	M	13.0-999	1.4	4.2

Test	Sex	Age	Low	High
Monocytes (x 10^9/L)		12.0-12.9	.1	.7
Monocytes	F	13.0-999	.2	.8
Monocytes	M	13.0-999	.3	.8
Eosinophils (x 10^9/L)		12.0-12.9	0	.7
Eosinophils	F	13.0-999	0	.4
Eosinophils	M	13.0-999	0	.95
Basophils (x 10^9/L)		12.0-12.9	0	.1
Basophils	F	13.0-999	0	.1
Basophils	M	13.0-999	0	.1

Reticulocyte count

Test	Age	Low	High
Reticulocyte Count Absolute (Manual) (x 10^{12}/L)	12.0-999	3.8	5.5
Reticulocyte Count (Miller) (x 10^{12}/L)	.008-999	3.8	5.5

Reticulocyte Production Index reference range:

1 - 2 Adequate response
< 1 Inadequate bone marrow response
> 2 Indicative of haemolysis, recent bleeding or nutritional support

Liver function tests

Test	Sex	Age	Low	High
Total protein (g/L)		12.0-17.9	57	80
Total protein		18.0-999	60	78
Albumin (g/L)	F	12.0-17.9	29	42
Albumin		18.0-999	35	52
Total bilirubin (umol/l)		12.0-999	5	21
Conjugated bilirubin (DBil)		12.0-12.9	0	5
Conjugated bilirubin (DBil)		13.0-999	0	3
Alanine transaminase (ALT) (U/L)	M	12.0-17.9	5	30

Test	Sex	Age	Low	High
Alanine transaminase (ALT)	M	18.0-999	10	40
Alanine transaminase (ALT)	F	12.0-17.9	5	20
Alanine transaminase (ALT)	F	18.0-999	7	35
Aspartate transaminase (AST) (U/L)	M	12.0-12.9	0	38
Aspartate transaminase (AST)	M	13.0-15.9	0	39
Aspartate transaminase (AST)	M	16.0-17.9	0	39
Aspartate transaminase (AST)	M	18.0-999	15	40
Aspartate transaminase (AST)	F	12.0-12.9	0	37
Aspartate transaminase (AST)	F	13.0-15.9	0	32
Aspartate transaminase (AST)	F	16.0-17.9	0	30
Aspartate transaminase (AST)	F	18.0-999	13	35
Alkaline phosphatase (ALP) (U/L)	M	12.0-12.9	42	362
Alkaline phosphatase (ALP)	M	13.0-15.9	74	390
Alkaline phosphatase (ALP)	M	16.0-17.9	52	171
Alkaline phosphatase (ALP)	M	18.0-999	53	128
Alkaline phosphatase (ALP)	F	12.0-12.9	51	332
Alkaline phosphatase (ALP)	F	13.0-15.9	50	162
Alkaline phosphatase (ALP)	F	16.0-17.9	47	119
Alkaline phosphatase (ALP)	F	18.0-999	42	98
Gamma-glutamyl transferase (GGT) (U/L)	M	12.0-12.9	3	22

Test	Sex	Age	Low	High
Gamma-glutamyl transferase (GGT)	M	13.0-17.9	2	42
Gamma-glutamyl transferase (GGT)	M	18.0-999	0	67
Gamma-glutamyl transferase (GGT)	F	12.0-12.9	4	22
Gamma-glutamyl transferase (GGT)	F	13.0-17.9	4	24
Gamma-glutamyl transferase (GGT)	F	18.0-999	0	39

Urea and electrolytes

Test	Sex	Age	Low	High
Creatinine (umol/L)		12.0-12.9	37	63
Creatinine		13.0-14.9	40	72
Creatinine	F	15.0-17.9	39	85
Creatinine	F	18.0-999	49	90
Creatinine	M	15.0-17.9	36	96
Creatinine	M	18.0-999	64	104
Sodium (mmol/L)			136	145
Potassium(mmol/L)		12.0-999	3.5	5.1
Chloride(mmol/L)		12.0-999	98	107
Bicarbonate(mmol/L)			23	29

Test	Sex	Age	Low	High
Anion gap(mmol/L)			9	16
Urea(mmol/L)	F	12.0-12.9	1.8	5.7
Urea	F	13.0-15.9	1.4	5.4
Urea	F	16.0-17.9	1.4	5.4
Urea	M	12.0-12.9	1.8	6.4
Urea	M	13.0-15.9	2.5	6.4
Urea	M	16.0-17.9	1.8	7.1
Urea		18.0-999	2.1	7.1

Vitamin B12, Folate, Ferritin and Iron studies

Test	Sex	Age	Low	High
Serum folate (nmol/L)			>12.2	
Vitamin B12 (pmol/L)			156	672
Ferritin (ug/L)	M	19.0-999	22	322
Ferritin	F	19.0-999	10	291
Iron (umol/L)	M	12.0-999	11.6	31.3
Iron	F	12.0-999	9	30.4
Transferrin (g/L)	M	12.0-12.9	1.73	3.8
Transferrin	M	13.0-15.9	1.71	3.74

Test	Sex	Age	Low	High
Transferrin	M	16.0-17.9	1.94	3.48
Transferrin	M	18.0-59.9	2.15	3.65
Transferrin	F	12.0-12.9	1.85	3.77
Transferrin	F	13.0-15.9	1.93	3.91
Transferrin	F	16.0-17.9	1.81	4.16
Transferrin	F	18.0-59.9	2.5	3.8
Transferrin		60.0-89.9	1.9	3.75
Transferrin		90.0-999	1.86	3.47
% Saturation (%)	M		20	50
% Saturation	F		15	50

CD4

Test	Sex	Age	Low	High
Absolute CD4 (cells/uL)			500	2010

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