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FANCG 637-643 DELETION MUTATION: FREQUENCY IN BLACK PATIENTS WITH ACUTE MYELOID LEUKAEMIA OR APLASTIC ANAEMIA AND THE CLINICAL PHENOTYPE OF HOMOZYGOTES

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Tabitha Haw

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science in Medicine in Genetic Counselling.

Johannesburg, South Africa, 2005

DECLARATION

I, Tabitha Haw declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine (Genetic Counselling) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Tabitha Haw

4th day of May 2005

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POSTER PRESENTATIONS ARISING FROM THIS STUDY

Haw T, Krause A (2003) Fanconi anaemia gene mutations in patients with acute myeloid leukaemia, aplastic anaemia and myelodysplasia. Tenth biennial congress of the Southern African Society of Human Genetics held in Durban, South Africa, 11-14 May 2003.

Haw T, Krause A (2004) *FANCG 637-643* mutation in black patients with acute myeloid leukaemia, aplastic anaemia or myelodysplasia and defining the clinical phenotype in homozygotes. Faculty of Health Sciences Research Day held in Johannesburg, South Africa, 4 August 2004.

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ABSTRACT

Fanconi anaemia (FA) is an autosomal recessive disorder characterised by aplastic anaemia (AA) and a high risk of developing acute myeloid leukaemia (AML). It is unknown whether heterozygote carriers are also predisposed to developing these disorders.

The black South African population group is ideal for FA mutation screening because the presence of a founder mutation, *FANCG 637-643,* makes screening relatively straight forward. Three individuals with AML (115 screened) and one with AA (78 screened) were found to be heterozygous for the black South African founder mutation. From our data it seems unlikely that this mutation places heterozygous carriers of the mutation at high risk of developing AML or AA. Three children with AA out of 26 screened, were homozygous for the mutation. This finding reiterates the importance of screening all children with AA for FA.

The frequency of certain congenital abnormalities in black South African FA patients was compared to patients described by other research groups. The frequencies of the abnormalities were similar to other FANCG cohorts described but significant differences to a group of FA patients from unspecified complementation groups were found. This difference could be because different complementation groups are associated more or less strongly with specific abnormalities.

It was found previously that particular congenital abnormalities in FA patients are associated with a poor haematological outcome. We concluded that black South African FANCG patients have a high risk of early development of AA even though they do not have a high frequency of congenital abnormalities.

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NOMENCLATURE

χ^2	Chi square
°C	degrees Celsius
µg	microgram(s)
µl	microlitre(s)
A	Adult (older than 18 years)
AA	aplastic anaemia
AML	acute myeloid leukaemia
A-T	Ataxia Telangiectasia
bp	base pair(s)
CHB	Chris Hani Baragwanath Hospital
CT	computed tomography
df	degrees of freedom
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetra-acetic acid
EUFAR	European Fanconi Anaemia Research Group
FA	Fanconi anaemia
FANCA (B-L)	Fanconi anaemia complementation group A (likewise for groups B to L)
F exact	Fischer's exact test
g	gram
HIV	Human immunodeficiency virus
JHB	Johannesburg Hospital
l	litre
MDS	myelodysplasia
mg	milligram(s)
ml	millilitre(s)
N	number
Non-FANCG	Belonging to any complementation group but FANCG
P	paediatric (18 years or younger)
p	probability
PCR	polymerase chain reaction
rpm	rotations per minute
SDS	sodium dodecyl sulphate
SA	South African
TBE	Tris borate EDTA
TE	Tris-EDTA solution
U	units
USA	United States of America

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1 INTRODUCTION

Fanconi Anaemia (FA) is a rare genetically heterogeneous disorder that has a wide variety of possible clinical manifestations. FA is characterized by bone marrow failure and a predisposition to malignancy, especially acute myeloid leukaemia (AML) (reviewed in Tischkowitz and Hodgson 2003). Eleven genetic subtypes of FA have been described but only nine of the genes have been cloned (Levitus et al. 2004). It is thought that the protein-products of these eleven genes function together in a DNA damage response pathway (reviewed in Nisbet-Brown 2004).

The aim of this project was to determine whether there was an increased frequency of the *FANCG 637-643* deletion mutation in patients with aplastic anaemia (AA), AML or myelodysplasia (MDS). This study also aimed to determine whether there are a significant number of individuals with FA who present atypically with AA, MDS or AML as the primary manifestation of the disease. The second part of the study aimed to delineate the clinical features of black South African patients with mutations in FANCG. This included determining the most frequently occurring congenital abnormalities in these patients and calculating the percentage of patients likely to develop AA before ten years of age.

In this introduction the clinical presentation of FA is discussed with special reference to the haematological disorders listed above. The genetics of FA and the functions of the FA proteins are then reviewed. The incidence and presence of founder mutations in certain population groups is relevant to this study and is therefore reported in some detail. The breakage disorders are discussed in terms of the predisposition to cancer they elicit in their heterozygous carriers.

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1.1 CLINICAL FEATURES OF FANCONI ANAEMIA

Fanconi anaemia (FA) is an autosomal recessive or X-linked recessive disorder with diverse clinical features that can include developmental anomalies affecting the skeleton, kidneys, heart and other major organ systems (Glanz and Fraser 1982, Meetei et al 2004b). Based on groups of genetically heterogeneous patients, the most frequently occurring skeletal anomalies are radial ray defects such as hypoplasia of the thumbs and radial hypoplasia. Congenital hip dislocation, scoliosis and vertebral anomalies are also often seen. Renal abnormalities are present in approximately one third of patients and include renal aplasia, renal hypoplasia, horseshoe kidneys and double ureters. Males with FA have a high incidence of genital abnormalities including small genitalia, undescended testes, hypospadias and infertility. Females with FA can have underdeveloped genitalia and uterine anomalies. They also have sparse and irregular menses but are usually able to become pregnant (reviewed in Tischkowitz and Hodgson 2003).

Individuals with FA frequently have café-au-lait marks as well as areas of skin hypopigmentation. A low birth weight and subsequent short stature are common features of FA due to prenatal and postnatal growth retardation. Micropthalmia, microcephaly and developmental delay are also often evident. Conductive deafness is not uncommon (Glanz and Fraser 1982). Less common features include gastrointestinal defects like atresia, imperforate anus and tracheo-oesophageal fistula. Hydrocephalus and neural tube defects as well as cardiac defects are also described (Tischkowitz and Hodgson 2003).

The clinical hallmark of FA is bone marrow failure that begins in childhood and affects all blood lineages. This failure results in progressive pancytopaenia (Butturini et al. 1994). The onset of AA is usually at about eight years of age (Alter 2003). It has been estimated that the percentage of individuals with FA in the USA, who die from aplastic anaemia

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reaches 11% at age 48 (Rosenberg et al. 2003). The percentage of patients who die in South Africa is unknown but is likely to be higher than in the USA because accessibility to medical care in South Africa is often limited. There is also a high risk of neoplasia, particularly leukaemia, developing in patients with FA. By the age of 48 years, 10 % of individuals with FA have developed AML (Rosenberg et al. 2003). The risk of an individual with FA developing MDS has been estimated to be 5% (Alter et al. 2000).

In an American study aimed at determining whether particular congenital abnormalities are associated with the early development of AA, the frequency of ten congenital abnormalities in a cohort of 144 FA patients was established (Rosenberg et al. 2004). The abnormalities chosen were easy to recognise and manifested early in life (Rosenberg et al. 2004). Six of the abnormalities, including abnormalities of the radius, developmental delay, cardio-pulmonary abnormalities, renal abnormalities, hearing impairment, and abnormalities of the head were identified as being predictive of the early development of AA (Rosenberg et al. 2004).

Patients with FA are susceptible to solid tumours especially vulvar cancer, oesophageal cancer and head and neck cancers (Rosenberg et al. 2003). It has been calculated that if an individual with FA survives until 48 years of age, the chance that they will develop a solid tumour is as high as 1 in 3 (Rosenberg et al. 2003). Due to bone marrow failure and susceptibility to malignancy the life expectancy of an individual with FA is significantly reduced to an average age of 20 years (Joenje and Patel 2001).

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1.2 HAEMATOLOGICAL MANIFESTATIONS IN PATIENTS WITH FANCONI ANAEMIA

The haematological abnormalities seen in FA are considered to be the most significant clinical feature of the disease because 98 % of affected individuals will develop haematological complications (Butturini et al. 1994). The full blood count of an individual with FA is usually normal at birth. However, thrombocytopaenia and neutropaenia usually develop early, with pancytopaenia becoming evident between five and ten years of age (Butturini et al. 1994). AA is the most common haematological finding in individuals with FA. AML often develops after the AA and is a major complication of FA (Alter 2003).

It is still to be established whether a percentage of individuals who present primarily with AML or MDS are homozygous for mutations in one of the FA genes. It is possible that one of these disorders may be the only manifestation of FA and therefore the individual is unlikely to be tested for and diagnosed with FA.

In this section general information about AA, AML and MDS will be given before their presentation in patients with FA is discussed.

1.2.1 Aplastic Anaemia

Bone marrow failure syndromes in general consist of a diverse number of diseases presenting with single or multi-lineage cytopaenias (D'Andrea et al. 2002). AA is a bone marrow failure syndrome characterized by decreased formation of erythrocytes, granulocytes and platelets due to hypoplastic bone marrow. The blood cell count determines the presentation and prognosis of the condition. A low erythrocyte count

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results in fatigue, dyspnea or cardiac symptoms, thrombocytopaenia results in bruising and mucosal bleeding while neutropaenia is evidenced by a susceptibility to infection (Young 2002). It has been suggested that bone marrow failure is a phase in the development of leukaemia although there is some controversy regarding this relationship (Butturini et al. 1994).

The onset of AA in the general population has been associated with different illnesses, biologic states such as pregnancy, radiation exposure, chemical exposure especially to benzene or chloroamphenicol, as well as viral infections (Guinan 1997). It is now thought however that the majority of acquired cases of AA have an immunological component that may arise because of exposure to a viral or chemical agent (Young 2002).

1.2.1.1 APLASTIC ANAEMIA IN PATIENTS WITH FANCONI ANAEMIA

FA is considered to be the commonest inherited bone marrow failure syndrome because the majority of individuals with FA develop AA (Rosenberg et al. 2003). Frequently FA patients without other clinical signs of the disease, present with AA. It is therefore important to screen all children with AA for FA because of the familial and treatment implications (Guinan 1997). Liu and Auerbach (1991) described the case of three siblings who had no dysmorphic features, however the eldest sibling presented with AA which led to all the children being diagnosed with FA. This case raises the question of whether adult patients with AA should also be screened for FA.

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1.2.2 Acute Myeloid Leukaemia

Leukaemia is a progressive proliferation of abnormal leukocytes (white blood cells) found in haemopoietic tissue. The result is an uncontrolled increase in the production of leukocytes and their precursors and "crowding out" of other blood cell types in the bone marrow. Acute leukaemia differs from the chronic form of the disease in that it has a sudden onset and aggressive course because maturation of the poorly differentiated and immature blast cells is interrupted (Waugh and Grant 2001).

Acute myeloid leukaemia is the result of a block in the differentiation of primitive myeloid precursor cells which normally give rise to basophils, eosinophils, neutrophils and monocytes (Waugh and Grant 2001). This clonal disorder is driven by the accumulation of DNA mutations. These mutations are inherited in only about 5% of cases, the remainder are acquired (Langmuir et al. 2001). In the USA, approximately 500 children per year develop AML, with the incidence peaking at 2 years of age when 12 cases per million are diagnosed. Patients with AML usually present with recurrent infections due to neutropaenia or bleeding due to thrombocytopaenia (Langmuir et al. 2001).

AML is diagnosed on bone marrow aspirate or histology sections. Individuals with AML are divided into risk groups, M_0 to M_6 according to karyotype studies and the patient's response to induction therapy (Langmuir et al. 2001). In the First World approximately 50% of newly diagnosed patients with AML will die from the disease (Langmuir et al. 2001).

Factors implicated in causing acquired acute leukaemia include ionising radiation, chemicals such as organic solvents, *in utero* exposure to ethanol, dietary topoisomerase

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inhibitors, viral infection and parental exposure to pesticides (Greaves 1997, Langmuir et al. 2001). Large, acute doses of ionising radiation have been shown to cause acute leukaemia whereas evidence for an association with smaller doses, such as for radiographic procedures is less convincing. Radiotherapy and chemotherapy agents used to treat disease can be genotoxic and can therefore also cause AML (Greaves 1997).

Certain conditions are known to predispose affected individuals to developing AML. Individuals with Down syndrome are 10 to 18 times more likely to develop leukaemia than the general population. This is evidenced by the fact that 10% of all paediatric AML cases are in individuals with Down syndrome (Langmuir et al. 2001). Neurofibromatosis Type I is associated with a 20 fold increased risk of myeloid disorders (Langmuir et al. 2001).

1.2.2.1 Acute Myeloid Leukaemia in Patients with Fanconi Anaemia

AML frequently occurs in individuals with FA and it has been suggested that all FA patients may be considered to be pre-leukaemic (Auerbach and Allen 1991). The median age for a child with FA to present with leukaemia is 14 years old. It is interesting to note that 94% of cases of leukaemia in individuals with FA are myeloid and 6% are lymphoid whereas in non-FA patients, 84% of leukaemia cases are lymphoid and the remainder myeloid (Alter 2003). The prognosis for an individual with FA who develops AML is very poor (Butturini et al. 1994). An especially adapted low dose treatment needs to be employed because these patients are unable to tolerate conventional chemotherapy (Langmuir et al. 2001). Bone marrow transplantation with a compatible donor should be done as soon as possible because of the poor prognosis (Butturini et al. 1994).

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It is thought to be very rare that an individual with FA presents primarily with AML and in most cases bone marrow failure has already been established in these patients (Cavenagh et al. 1996). The majority of cases in which FA has presented primarily with AML, have been described in patients younger than 21 years of age (Butturini et al. 1994). Cavenagh et al. (1996) reported a patient with FA who first presented at 28 years of age with AML. The patient also had a vestigial right thumb which lead to the diagnosis of FA being made. It is unlikely that individuals presenting with AML and no other features of FA would be screened for the disease. It is therefore unknown whether FA may present primarily with AML in children or adults, albeit rarely.

1.2.3 Myelodysplasia

MDS is a disorder of the bone marrow that results in the proliferation of abnormal stem cells. It is characterised by low blood counts due to ineffective haematopoiesis (Hirai 2003). MDS can present with hypocellular bone marrow and it can be difficult to distinguish from AA. The detection of clonal cytogenetic abnormalities however, suggests a diagnosis of MDS (Guinan 1997). MDS is typically a disease of the elderly and does not have a paediatric peak. The incidence of MDS rises slowly until after 50 years of age (Alter 2003).

Treatment for MDS in the elderly is supportive involving transfusions and antibiotic therapy but in paediatric cases it is treated with bone marrow transplantation. There is little known about the pathogenesis of MDS but as for AML, exposure to environmental or occupational toxins are thought to cause a large number of MDS cases (Hirai 2003). It is known that MDS can arise after cytotoxic treatment used for other malignancies (Hirai 2003).

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The clonal proliferation of cells seen in MDS is as a result of acquired somatic mutations which confer a proliferative advantage to the cell. These haematopoietic stem cells are susceptible to genetic lesions and MDS can therefore be considered to be a pre-leukaemic state (Alter et al. 2000). MDS can remain stable for a long period but frequently progresses to AML (Hirai 2003).

1.2.3.1 MYELODYSPLASIA IN PATIENTS WITH FANCONI ANAEMIA

The risk of an individual with FA developing MDS has been estimated to be 5% (Alter et al. 2000). The relationship between MDS and leukaemia in patients with FA has not yet been clearly established. Initially, it was suggested that MDS is frequently not a prelude to AML in these patients (Alter et al. 2000). However, Rosenberg et al. (2003) determined that the risk of an individual with FA developing AML after MDS has been diagnosed is approximately 9.4% per year. The researches suggest that due to the cytogenetic and clinical heterogeneity of MDS in FA, the natural history of the disorder may well vary between patients with not all clones necessarily progressing to AML (Rosenberg et al. 2003).

1.3 THE HETEROGENEITY OF FANCONI ANAEMIA

1.3.1 Genetic Heterogeneity of Fanconi Anaemia

FA has been shown by cell fusion experiments to have at least eleven different complementation groups (Levitus et al. 2004). Complementation groups are thought to reflect genetic heterogeneity meaning that each group relates to a distinct gene. They are

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assigned by determining whether the function of a mutant cell can be restored by another defective cell. If function is restored then it is deduced that the cells carry mutations in different genes, whose products play a part in the same biochemical pathway. The pathway can function because the cells replace (complement) each other's missing proteins (Buchwald 1995). This technique fails however, if the endpoint used to assess correction is only distantly related to the primary genetic defect, as was found to be the case in studies on ataxia telangiectasia. The endpoint used to assess complementation in FA is the correction of hypersensitivity to the cytotoxic effect of DNA crosslinking agents (Buchwald 1995). The FA genes cloned so far have been shown to correspond to the complementation groups previously assigned by cell fusion experiments although FANCD was subdivided after the initial work-up into FANCD1 and FANCD2.

The eleven complementation groups identified in FA to date have been named FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ and FANCL (reviewed in Nisbet-Brown 2004). Six of these genes, FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG, had been cloned by 2001 and have been shown to be widely dispersed throughout the genome (Strathdee et al. 1992, Lo Ten Foe et al. 1996, de Winter et al. 1998, de Winter et al. 2000a, de Winter et al. 2000b, Timmers et al. 2001).

Recently the FANCD1 locus has been shown to be allelic with the BRCA2 locus, one of the genes known to predispose carriers to breast cancer (Howlett et al. 2002). Complementation group FANCL is thought to be PHF9, a protein with E3 ubiquitin ligase activity (Meetei et al. 2003). FANCI and FANCJ complementation groups have been identified, but as yet the genes have not been cloned (Levitus et al. 2004). Most recently it

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has been shown that FANCB is in fact a previously identified protein, FAAP95 which is localized at Xp22.31 (Meetei et al. 2004b). Table 1-1 shows the chromosomal locations of each FA gene where known, as well as the reference describing the discovery of each complementation group or gene.

Table 1-1: The chromosome locations of the eleven different FA genes

Complementation Group	Chromosome Location	Reference describing discovery
FANCA	16q24.3	Pronk et al. 1995
FANCB	Xp22.31	Meetei et al. 2004b
FANCC	9q22.3	Strathdee et al. 1992
FANCD1 / BRCA2	13q12-13	Howlett et al. 2002
FANCD2	3p25.3	Timmers et al. 2001
FANCE	6p21-22	de Winter et al. 2000a
FANCF	11p15	de Winter et al. 2000b
FANCG / XRCC9	9p13	de Winter et al. 1998
FANCI	?	Levitus et al. 2004
FANCJ	?	Levitus et al. 2004
FANCL / PHF9	2p16	Meetei et al. 2003

The prevalence of mutations in the different complementation groups varies with ethnic origin (Faivre et al. 2000). FA complementation group A (FANCA) is the most common form of FA worldwide and accounts for 60-65% of FA patients (Morgan et al. 1999). All types of mutations have been identified in the FA genes making it very difficult to screen

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for mutations. There seem to be no significant hot spots in the FA genes and over 100 mutations have been identified in FANCA alone (Wijker et al. 1999). This heterogeneity means that unless a founder mutation has been identified in a particular population group, molecular testing is laborious and expensive because DNA sequencing is necessary.

In South Africa, three of the population groups namely the white Afrikaners, the Ashkenazi Jews and the black Africans have commonly occurring founder mutations (Verlander et al. 1995, Krause et al. 2001, Tipping et al. 2001). This is discussed in more detail in section 1.5 and a summary of the mutations in the groups is given in Table 1-2. The presence of common mutations makes it relatively easy to screen individuals from these population groups for FA mutations unlike in other populations where no common mutations have been identified.

1.3.2 Clinical Heterogeneity of Fanconi Anaemia

The range and variability of manifestations associated with FA have yet to be fully elucidated (Tischkowitz and Hodgson 2003). Giampietro et al. (1997) reported that as many as a third of patients with FA do not have congenital malformations but often do have alterations in growth parameters, skin pigmentation abnormalities or microphthalmia. However, there are case reports of patients being diagnosed with FA only after developing secondary malignancies.

A recent article describes a case of a male infant presenting with medulloblastoma and no dysmorphic features. FA was suspected because a sibling had died of AML and the diagnosis was confirmed by chromosome breakage studies (Tischkowitz et al. 2004). Kwee et al. (1997) described a fifty-six-year-old woman with microcephaly, short stature

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and congenital deafness. FA was suspected because of clinical findings in her brother and was confirmed by hypersensitivity to mitomycin C. These rare cases highlight the variability of symptoms seen in individuals with FA.

The variable manifestations seen in FA individuals may to some extent be influenced by which gene is involved and the type of mutation present (Faivre et al. 2000). For example, it has been shown that FA caused by mutations in FANCG is associated with an increased incidence and earlier onset of AML or MDS and more severe cytopaenia than mutations in other FA genes (Faivre et al. 2000). It therefore seems that there is also variability in the age of onset of haematological abnormalities and survival between the different FA complementation groups (Kutler et al. 2003). Further, homozygosity for null mutations in FANCA patients has been associated with an earlier onset of anaemia and a higher incidence of leukaemia than when the mutations present produce an altered protein (Faivre et al. 2000). It is therefore likely that the type or position of the mutations causing FA may also affect the clinical variability of the disease (Kutler et al. 2003).

To add further complexity to the situation, the Ashkenazi Jewish population carries a founder mutation in FANCC, which is known to result in a severe phenotype with multiple congenital anomalies and an early onset of bone marrow failure (Gillio et al. 1997). However, this same mutation is found in the Japanese population but the phenotype is less severe (Futaki et al. 2000). This seems to indicate that population specific modifying genes or possibly environmental factors may also influence the variability of FA.

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1.4 THE FANCONI ANAEMIA PROTEINS

The similarities that are present in the phenotypes between patients that belong to different FA complementation groups are evidence that the gene products of the groups interact in a common pathway (Nisbet-Brown 2004). This common pathway contributes to genomic stability by DNA repair (Joenje and Patel 2001). It is hypothesised that the FA pathway is involved in repairing double stranded breaks by non-homologous end joining. It is also possible that the FA proteins play a role in DNA damage signalling pathways by mediating cell cycle checkpoints (reviewed in Tischkowitz and Hodgson 2003)

FANCA, FANCC, FANCE, FANCF and FANCG were the first FA proteins shown to form a nuclear multiprotein complex that is disrupted in FA patients belonging to one of these five complementation groups. The nuclear complex is essential for activation of FANCD2 by mono-ubiquitination, which results in specific localisation of FANCD2 in the nucleus with BRCA1 (Reviewed in Grompe and D'Andrea 2001). It has very recently been shown that FANCB is also a component of the nuclear multiprotein core complex (Meetei et al. 2004b).

It has been found that individuals with FA who belong to complementation group D1, have bi-allelic mutations in BRCA2. It has therefore been concluded that FANCD1 is in fact BRCA2 (Howlett et al. 2002). BRCA1 and BRCA2 are known to be involved in homologous recombination and DNA double-strand break repair. Both of these proteins are important components of the FA pathway (Zdzienicka and Arwert 2002). BRCA2 mutations have also been identified in individuals with FA from complementation group B (Howlett et al. 2002). Because of this, it was originally thought that FANCD1 and FANCB complementation groups may be one and the same, although for several reasons

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this was unlikely to be the case. The recent finding that the FANCB protein is localized to Xp22.31 resolves this ambiguity (Meetei et al. 2004b).

The six FA proteins in the multiprotein complex do not have recognizable ubiquitin ligase motifs and the catalytic subunit had until last year not been identified. The discovery of FANCL/PHF9 and the fact that it has ubiquitin ligase activity, solved this dilemma (Meetei et al. 2003). It has been shown that FANCL is required for mono-ubiquitination of FANCD2 and therefore FANCL is also likely to be part of the nuclear complex (Meetei et al. 2004a).

The two most recently identified complementation groups are FANCI and FANCJ (Levitus et al. 2004). Cells with homozygous FANCI mutations are not able to undergo mono-ubiquitination of FANCD2 indicating that FANCI operates upstream of this step in the FA pathway. This was not the case in cells with FANCJ mutations however, indicating that FANCJ operates downstream in the pathway (Levitus et al. 2004). An illustration of the proposed FA pathway can be seen in Figure 1-1.

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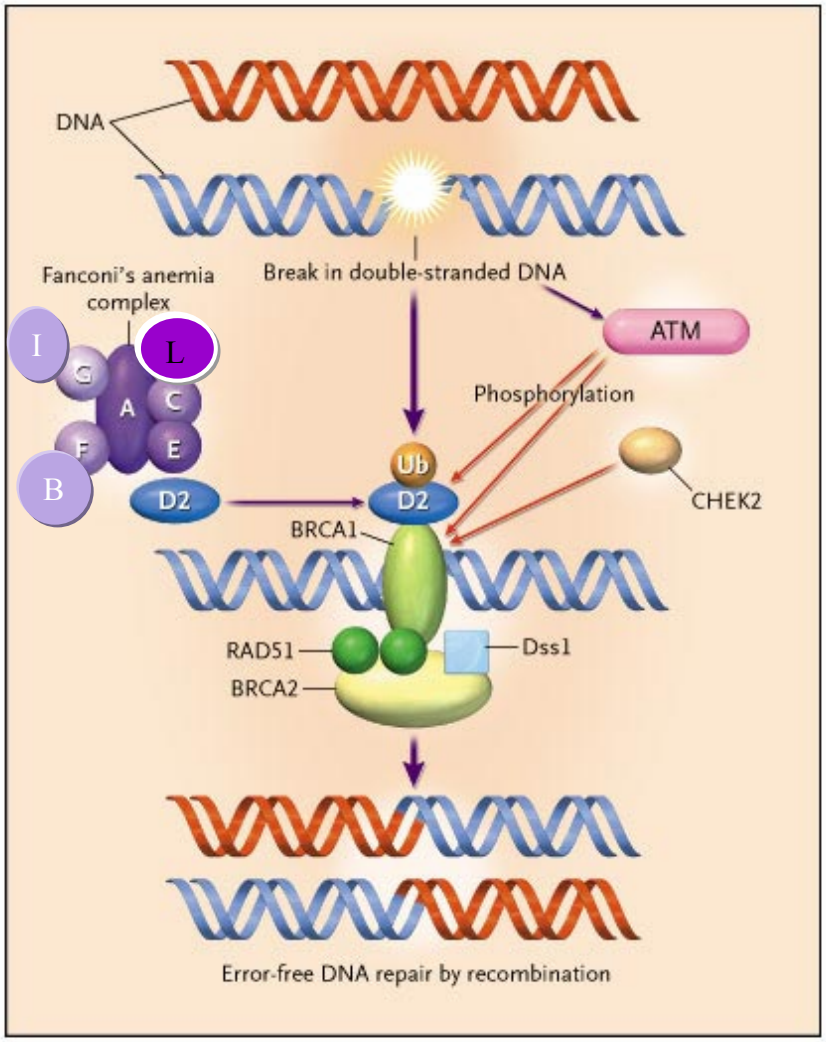

Figure 1-1: A diagramatic representation of the hypothesised FA pathway*.

* FA proteins A, B, C, E, F, G, L and I form a nuclear complex which activates FANCD2 in response to DNA damage. Activated FANCD2 then colocalizes with BRCA1 which has been phosphorylated by ATM and CHEK2. FANCD1/BRCA2 brings the DNA-recombination enzyme RAD51 to the site of repair. It is thought that FANCJ works downstream of the mono-ubiquitination of FANCD2. It has not been included in the diagram. Image reproduced and modified (FANCB, FANCL and FANCI included) from Venkitaraman 2003

Cultured lymphocytes from FA patients show spontaneous chromosome instability and are hyper-responsive to chromosome breaking and anti-proliferative effects of crosslinking agents such as mitomycin C. The presence of broken and rearranged chromosomes in cells from patients with FA shows the importance of the FA pathway in DNA damage response (reviewed in Joenje and Patel 2001). The impaired capacity of FA cells to maintain

genomic integrity is likely to lead to an accumulation of mutations, which could account for FA patients' predisposition to malignancy (Joenje and Patel 2001).

1.5 THE PREVALENCE AND CARRIER FREQUENCY OF FANCONI ANAEMIA

FA is found in all races and ethnic groups but with a variable prevalence. There is a general worldwide prevalence of 1-5 per million and the heterozygote carrier frequency in Europe and the United States has been estimated at 1 in 300 (Swift 1971, Schroeder et al. 1976). The heterozygote carrier frequency has been found to be higher than this in some of the other ethnic groups studied (Rosendorff et al 1987, Morgan et al. 2005). The population groups with a higher incidence of FA due to founder mutations and genetic drift include the Ashkenazi Jewish people (Verlander et al. 1995), the German population (Demuth et al. 2000), the white Afrikaners of South Africa (Tipping et al. 2001), and the black population of South Africa (Morgan et al. 2005). A summary of the common mutations seen in three of the South African populations can be seen in Table 1-2.

A single splice site mutation in *FANCC (IVS4 + 4 A → T)* has been found to account for a high percentage of FA cases in the Ashkenazi Jewish population. The carrier frequency of this gene mutation in the population is approximately 1 in 89 (Verlander et al. 1995). The German population has been shown to have a founder mutation in the FANCG gene (*E105X*). This mutation accounts for 44% of mutant alleles in individuals with FANCG in the German population (Demuth et al. 2000).

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The incidence of FA in the white Afrikaans-speaking population of South Africa is 1 in 22000 with an estimated carrier frequency of 1 in 77 (Rosendorff et al. 1987). Three founder mutations causing FA, have been found in the FANCA gene in the Afrikaans-speaking population. The most prevalent, *Del E12-31*, is a deletion of exons 12 to 31 and accounts for 60% of FANCA mutations in this population group (Tipping et al. 2001). The other two mutations account for an additional 20% of FA mutations in this population. The first, *Del E11-17*, is a deletion of exons 11 to 17 which accounts for 13% of FANCA mutations and the second, *3398delA*, is a deletion of an adenine residue at nucleotide position 3398 which accounts for 7% (Tipping et al. 2001).

A seven base pair deletion of nucleotides 637-643 in exon five of the FANCG gene has been identified in the South African black population (*FANCG 637-643*). 300 healthy black South African individuals were screened for the mutation and three were found to be carriers. The carrier rate of this mutation in the population has therefore been estimated to be 1 in 100 (Morgan et al. 2005). The deletion mutation was found in 33/41 (64/82 mutant alleles) black South African patients confirmed to have FA by cytogenetic analysis but of unknown complementation group. Morgan et al. (2005) concluded from this that the deletion mutation accounts for about 78% of all FA mutations in this population group. From these figures the expected prevalence of FA for all complementation groups was calculated to be 1 in 24 000 (Morgan et al. 2005).

Macdougall et al. (1990) estimated the minimum prevalence of FA homozygotes in the black South African population to be 1 in 476 000. This was based on the number of patients presenting for treatment and the South African population census of 1980 which was used to estimate the number of black children under 15 years of age. It is interesting

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to note that this prevalence is significantly lower than would be expected from the reported carrier frequency in this population (Morgan et al. 2005). This lends support to the hypothesis that a proportion of FA homozygotes are not diagnosed as such, possibly because of variable manifestations.

Table 1-2: Founder Mutations in three South African population groups and their respective carrier frequencies.

Population Group	Mutation	Carrier frequency	Reference
Afrikaans-speaking	***FANCA (Del E12-31)* - accounts for 60 %**	**1/77 (Carrier freq of FA)**	**Tipping et al. (2001)**
	***FANCA (Del E11-17)* - accounts for 13 %**		
	***FANCA (3398delA)* - accounts for 7 %**		
Black South African	***FANCG 637-643***	**1/100**	**Morgan et al. (2005)**
Ashkenazi Jewish	***FANCC (IVS4 + 4 A → T)***	**1/89**	**Verlander et al. (1995)**

1.6 CANCER RISKS TO HETEROZYGOUS CARRIERS OF CHROMOSOME BREAKAGE DISORDERS

FA, Ataxia-Telangiectasia (A-T) and Bloom's syndrome all exhibit chromosome fragility that results from a defect in the cellular DNA repair mechanism and can thus all be called chromosome breakage disorders (Roux 1996). These three disorders all show characteristic chromosome abnormalities in lymphocytes and cells from affected individuals are unusually sensitivity to particular environmental agents. Probably most

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striking however is that individuals with these disorders have a predisposition to developing malignancies (Taylor 2001).

A-T is an autosomal recessive disorder resulting in progressive cerebellar ataxia with skin and conjunctival telangiectasia (Welshimer and Swift 1982). Affected individuals have a high risk of developing leukaemia, lymphoma and solid tumours (Athma et al. 1996). The features of Bloom syndrome include erythema and telangiectasia in a butterfly distribution on the face, photosensitivity and prenatal onset of growth retardation. Individuals with Bloom syndrome have a high incidence of malignancies including leukaemia, carcinoma and embryonic tumours (Taylor 2001). As discussed in section 1.1 individuals with FA are predisposed to developing AML as well as solid tumours (Rosenberg et al. 2003).

An interesting aspect to the breakage disorders is whether heterozygote carriers of these disorders are at an increased risk of cancer development due to less effective DNA repair mechanisms. It is important to identify if these carriers are predisposed to serious common diseases due to the relatively high frequency of carriers in a population (Swift et al. 1980). It would be particularly relevant in South Africa because of the high carrier frequency of FA in three of our population groups, namely the Afrikaners, black group and Ashkenazi Jews.

Heterozygous carriers of A-T have been shown to have a predisposition to developing malignancies, especially breast cancers. Although the carrier rate of A-T is 1%, it is estimated that 6.6% of all breast cancer cases in the USA occur in these carriers (Athma et al. 1996). A-T heterozygotes have also been shown to be predisposed to developing acute lymphoblastic leukaemia (Liberzon et al. 2004).

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It has been reported that there is an excess of patients with colorectal cancer among Ashkenazi Jewish heterozygote carriers of Bloom syndrome (Gruber et al. 2002). However, recent research has questioned this association and further investigation is needed to clarify the cancer risks to heterozygote carriers of Bloom syndrome (Cleary et al. 2003).

Petridou and Barrett (1990) found that heterozygote carriers of FA have skeletal proportions that are significantly different from normal, as well as mild haematological abnormalities. It was concluded from these findings that there is partial expression of the FA gene in heterozygote carriers. As previously discussed, exposure of cells from individuals with FA to cross-linking agents induces chromosome breakage *in vitro.* This property is used to give a definitive cytogenetic diagnosis of FA. Cells from heterozygote carriers, however, cannot be differentiated from normal cells by their reaction to cross-linking agents (Rosendorff and Bernstein 1988).

Initially it was reported that heterozygote carriers of FA were three times more likely to die from a malignant neoplasm than members of the general population (Swift 1971). This finding was based on only 8 families however, and the results were not replicated when a larger sample was used and no association between carriers and increased cancer risk was found (Swift et al. 1980). At the time of both of these studies the FA genes had not been identified which meant that heterozygote carriers of FA could not be specifically identified. The incidence of cancers in heterozygotes was estimated by comparing the number of individuals with cancer in an affected individual's family with the general population incidence of cancer. A more recent study by Tischkowitz (2003) also looking

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at cancer incidences in families with individuals affected by FA has also not found a significant increase. Meyer et al. (2004) have recently reported that they did not observe an increased frequency of pathogenic FANCG mutations in sporadic childhood AML cases.

It is known that heterozygous carriers of BRCA2 have a high risk of developing breast and other cancers (Breast Cancer Consortium 1999). The recent discovery that BRCA2 is in fact one of the FA proteins and is a component of the FA pathway, means that heterozygous germline mutations in any of the FA genes may confer a cancer risk similar to that for families with BRCA1 or BRCA2 mutations (Zdzienicka and Arwert 2002). It is possible however, that different BRCA2 mutations may have variable cancer risks (Howlett et al. 2002). Seal et al. (2003) have shown that heterozygous mutations in FA genes other than BRCA2, are unlikely to result in a highly penetrant breast cancer predisposition. Whether heterozygous carriers of FA mutations have an increased cancer risk at all is still to be elucidated (Howlett et al. 2002).

1.7 OBJECTIVES OF THIS STUDY

Individuals with FA are highly likely to develop AML, AA or MDS as well as solid tumours if they survive the haematological complications. Individuals affected by other breakage disorders, such as Bloom syndrome and A-T, have similarly high cancer risks and it has been shown that heterozygote carriers of these disorders are also predisposed to developing malignancy (Athma et al. 1996, Taylor 2001, Gruber et al. 2002). The aim of this project was to determine whether there is an increased incidence of heterozygote

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carriers of the *FANCG 637-643* deletion among black South African patients with AML, AA and MDS.

This project also aimed to elucidate further the variability in manifestations of FA. The fact that the incidence of homozygotes is lower than that expected from the carrier frequencies, indicates that individuals homozygous for a FA mutation may not be recognised as having FA perhaps due to the absence of other symptoms or an atypical presentation. By screening individuals with AML, AA or MDS, there was the possibility of detecting homozygotes who had not been diagnosed with FA, but who presented primarily with these conditions.

The black South African population group is ideal for FA mutation screening because of the founder mutation in the population. Individuals with AML, AA or MDS were tested for the known founder FA mutation to determine whether it is more prevalent in individuals with these disorders. Both homozygous and heterozygous individuals were of interest in this research project. An increased incidence of homozygous individuals indicates the variability in primary manifestations of FA, while an increased incidence of heterozygous individuals may indicate a predisposition of carriers to certain haematological disorders.

In the second part of this study we aimed to delineate the clinical manifestations of black South African FANCG patients. FA is known to be clinically heterogeneous and different mutations are thought to be associated with variable risks of abnormalities (Kutler et al. 2003). We aimed to determine which congenital abnormalities occurred most frequently in a group of black South African FANCG patients. We also aimed to determine the risk of

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AA developing in these patients before ten years of age and compare it to that found in other studies.

The aims of the study were to:

1. Screen samples from black patients with AML, AA or MDS for the common deletion mutation in the *FANCG* gene.
2. Compare the frequency of mutations found among the AML, AA and MDS patients with the mutation frequency in a healthy control group matched for ethnicity.
3. Determine the frequency of ten clinical features of FA in black South African *FANCG* patients and compare them to previous studies.
4. Compare the percentage of black South African FA patients presenting with early onset AA to that found in a previous study.

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2 SUBJECTS AND METHODS

2.1 SUBJECTS IN THE FANCG MUTATION SCREEN

Ethics approval for this study was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Appendix 3). Written, informed consent was obtained from the patients from whom samples were collected prospectively.

Samples from 199 South African black individuals with AML, AA or MDS were obtained. Of the group studied, 115 individuals had AML, 78 had AA and 6 had MDS. These particular disorders were chosen because individuals with FA are predisposed to them and because there are case reports of FA mutations being identified in individuals, who have presented with these disorders as the primary manifestation of FA (refer to section 1.3.2). It was thought that if heterozygous carriers of FA are predisposed to developing a disease, it is likely to be the same condition that homozygous individuals are predisposed to.

Initially we hoped to also screen 150 white patients with AML, AA or MDS for the common founder mutation in FANCA in the Afrikaans population. However, due to technical problems, discussed in section 2.3, and the fact that only a limited number of white patients attend state hospitals, it was decided to focus our study on the black population.

Different tissue types were used in this research depending on what was available from particular patients. Different tissue types could be used because genomic DNA was extracted and analysed. Fresh samples were preferable for analysis however, because the

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DNA is easier to extract and of better quality. Collecting samples prospectively also allowed for the individual to be contacted and referred to the Genetic Counselling Clinic for full counselling by the primary researcher if FA mutations were detected. Detecting a homozygous individual during the mutation screening could have significant implications for the individual's medical care as well as their family in terms of risks for FA and malignancies in other siblings.

It was often not possible to collect samples prospectively because many patients had died and some had had bone marrow transplants. In these cases bone marrow trephines and bone marrow aspirate samples obtained at the time of diagnosis of the haematological illness were the most readily available source of DNA. Another factor making it difficult to collect all samples prospectively was that AML, AA and MDS are rare diseases and therefore a very limited number of patients were available to us at the time of the study. Collecting samples both prospectively and retrospectively allowed for the largest possible sample size. A pie chart showing the number of samples derived from each source is shown in Figure 2.1. The percentage of the total number of samples is indicated in parenthesis.

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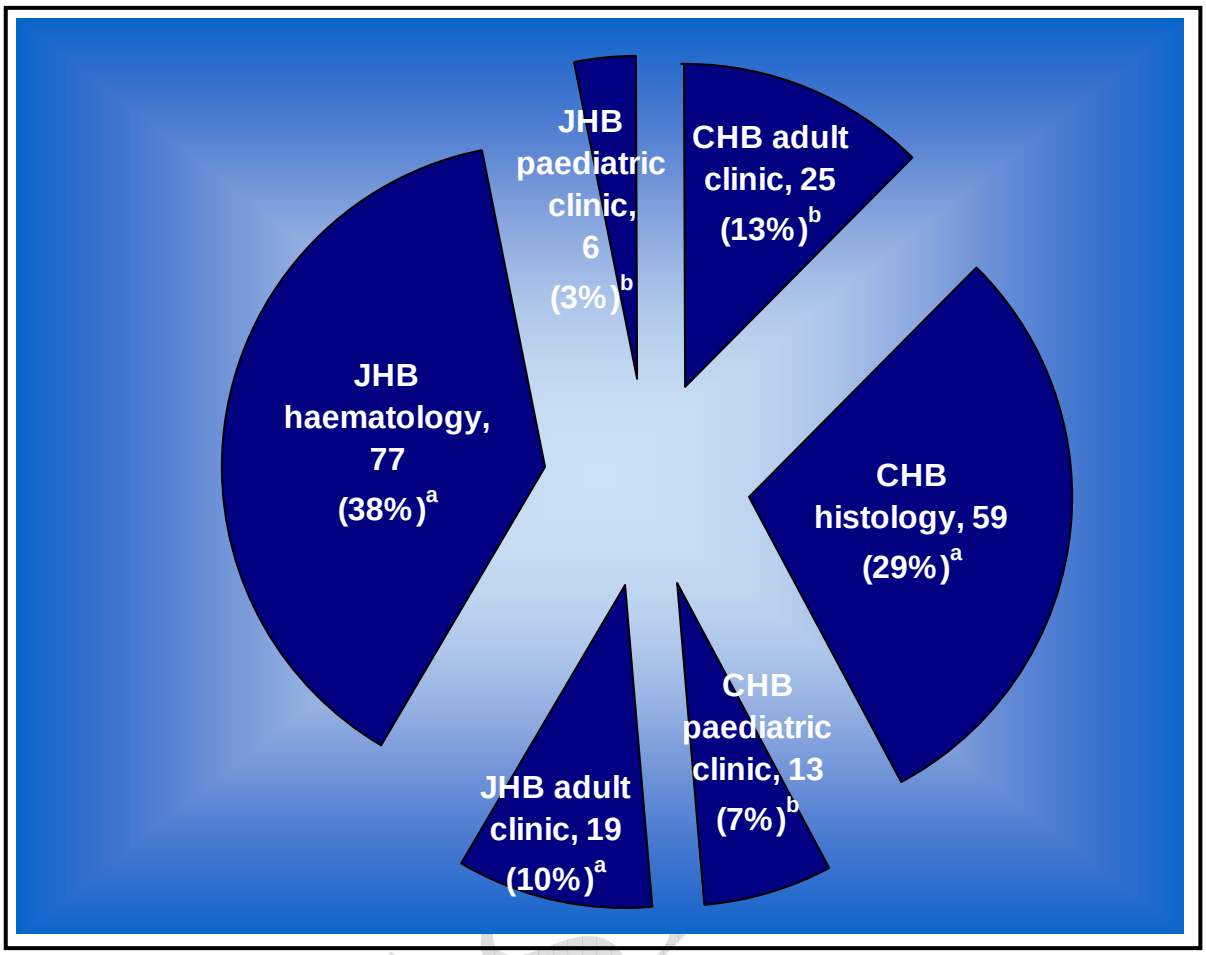

Figure 2-1: Pie chart showing the number of patient samples obtained from each of the six sources.

JHB is Johannesburg Hospital
CHB is Chris Hani Baragwanath Hospital.
[a] retrospectively collected specimens
[b] prospectively collected specimens

Each sample was accompanied with the patient's name, hospital number, diagnosis, ethnic group, gender and age. We also obtained contact information from the patients in the prospective arm of the study so that we could contact them in the event of a mutation being identified.

2.1.1 Retrospective Sample Collection

A total of 155 samples (77.9 %) were collected retrospectively. These included bone marrow trephines from the Histology Department at Chris Hani Baragwanath Hospital, bone marrow aspirate specimens from the Department of Haematology at Johannesburg Hospital and full blood count samples stored for one week, also in the Haematology Department at Johannesburg Hospital. The samples were anonymised for the analysis but patient names were collected to ensure that there was no duplication of samples.

2.1.1.1 *Bone Marrow Aspirate Slides from Johannesburg Hospital*

Names of patients with a diagnosis of AML, AA or MDS were collected from the flow cytometry records and the bone marrow aspirate records in the Department of Haematology at Johannesburg Hospital dating from January 1999 to March 2004. The hospital database was then used to find the bone marrow aspirate laboratory numbers and the unstained spare slides were obtained from the archive room in the laboratory. Slides from many patients could not be located presumably either because there were no spare slides at the time of processing or because they had been filed incorrectly. The number of bone marrow aspirate samples eventually obtained and used in this study was 77.

2.1.1.2 *Full Blood Count Samples from Johannesburg Hospital*

Patients were also ascertained from a weekly list of patients who attended follow-up at the Haematology/Oncology Clinic at Johannesburg Hospital. Blood is taken routinely at this clinic for a full blood count but only a very small amount of the sample is used. The remainder is stored in a fridge in the Department of Haematology for one week. The

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stored blood from 19 selected patients was available for us to use and was collected between August 2003 and March 2004.

2.1.1.3 Paraffin-embedded Bone Marrow Trephines from Chris Hani Baragwanath Hospital

At Chris Hani Baragwanath Hospital, 59 patients with AML or AA were ascertained from records kept in the Histopathology Department at the hospital from the years 2000 to 2003. The histology report on the bone marrow trephine from each sample identified was obtained so that the diagnosis could be confirmed and a list of selected patients was made. The technical staff in the laboratory then cut sections from the stored bone marrow trephine histology blocks selected and placed the sections in 1.5 ml Eppendorf tubes for use in this study.

2.1.2 Prospective Sample Collection

The prospective samples were blood specimens collected at adult and paediatric follow-up haematology and oncology clinics at Johannesburg and Chris Hani Baragwanath Hospitals. All patients were spoken to individually in the clinics about their disorders and information was given to them about this study. If consent to take part in the study was obtained, an additional sample of 5-10 ml of peripheral blood was collected from them usually at the time of other investigations. We arranged with patients that we would contact them with a result if a mutation were detected.

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2.1.2.1 PAEDIATRIC ONCOLOGY CLINIC AT CHRIS HANI BARAGWANATH HOSPITAL

13 patients were ascertained from Chris Hani Baragwanath Hospital Paediatric Oncology Clinic. These patients were identified either by the consultants or from records of patient's diagnoses kept in the department. The files of the identified patients were then located to determine when their follow-up appointments were so that we could attend the clinic on the appropriate day to speak with the patient about taking part in the study and collect a specimen.

2.1.2.2 ADULT ONCOLOGY CLINIC AT CHRIS HANI BARAGWANATH HOSPITAL

25 patients from Chris Hani Baragwanath Hospital adult oncology outpatients' clinic took part in the study. As patients arrived at the clinic, their files were checked to determine their diagnoses. Those patients with a diagnosis of AML, AA or MDS were spoken to about the project and consent to take part in the project obtained. A list of the patient's names and hospital numbers was made and given to the staff in the Haematology Department at Chris Hani Baragwanath Hospital. They then put the EDTA tubes that had been taken for routine full blood counts aside for us to collect later in the week, once they were finished with them.

2.1.2.3 PAEDIATRIC ONCOLOGY CLINIC AT JOHANNESBURG HOSPITAL

6 patients from Johannesburg Hospital paediatric oncology clinic in ward 296 were identified by the consultants at the clinic. The consultants spoke to these patients about the project and took blood if the patient consented. If a mutation was found during screening, the results were communicated to the consultant involved.

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2.2 METHODS USED IN THE *FANCG 637-643* SCREEN

The recipes for all solutions that are used in these methods appear in APPENDIX A on page 86.

2.2.1 DNA Extraction

The DNA extraction procedure varied according to the type of tissue available. Three different extraction protocols were used in this study, one for whole blood, one for bone marrow aspirate specimens and the last for paraffin embedded bone marrow trephines.

2.2.1.1 DNA EXTRACTION FROM WHOLE BLOOD

Genomic DNA was extracted from leukocytes in peripheral blood according to the salting-out procedure described by Miller *et al.* 1988. The blood was collected in EDTA tubes to prevent it from clotting and then transferred into 50 ml polypropanol tubes. The blood was frozen at –20 °C until the DNA extraction was performed.

The blood samples were allowed to thaw before the extraction was begun. Each tube was filled with chilled Sucrose-Triton-X lysing buffer and inverted. The tubes were then centrifuged in a *Beckman GS-6R Centrifuge* for 10 minutes at 2300 rpm and the supernatant discarded. The pellets were washed a second time in Sucrose-Triton-X lysing buffer and placed on ice for five minutes. The samples were centrifuged again for five minutes at 2300 rpm and the supernatant discarded. The remaining pellet was then incubated overnight at 42 °C with 1.5 ml T20E5, 0.1 ml 10% SDS and 0.25 ml Proteinase-K mix.

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The following day, 0.5 ml saturated NaCl was added to the samples and they were shaken vigorously. The samples were put on ice for 10 minutes before being centrifuged for 30 minutes at 2500 rpm. The supernatant containing the DNA was then transferred to a new tube and two volumes of absolute ethanol added. The DNA was spooled out with a sterile, disposable pipette tip. This DNA was washed in ice-cold 70 % ethanol and air-dried before being re-suspended in 1 x TE buffer. The volume of buffer varied from 75 µl to 250 µl depending on the amount of DNA spooled out. If the DNA did not become visible after the addition of ethanol, a precipitation procedure was followed.

Precipitation of the DNA in the samples was begun by incubating the tube at -20°C overnight. After centrifuging the samples for 20 minutes at 2400 rpm in the *Beckman GS-6R Centrifuge,* the supernatant was discarded and approximately 10 ml of 70 % ethanol was added to wash the pellet. The samples were centrifuged again at 2400 rpm for 10 minutes before the supernatant was again discarded and the pellet allowed to air-dry. The pellet was finally re-suspended in 75 µl of 1 x TE buffer.

2.2.1.2 DNA EXTRACTION FROM BONE MARROW ASPIRATE SLIDES

The tissue from the bone marrow aspirates was scraped off the microscope slides using 200 to 400 µl lysis buffer (containing 4 M Urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) from the *Boehringer Mannheim High Pure PCR Template Preparation* kit. The lysis buffer was used to soften the tissue and a sterile, disposable pipette tip could then be used to scrape the tissue off the slide into an Eppendorf tube. The suspensions were then incubated with 40 µl of 40 mg/ml proteinase-K at 55°C overnight.

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The following day, 200 µl of Binding Buffer (containing 6 M guanidine-HCL, 10 mM urea, 10mM Tris-HCl, 20 % Triton® X-100, pH 4.4) from the *Boehringer Mannheim High Pure PCR Template Preparation* kit was added to each tube and incubated for 10 minutes at 72 °C. 100 µl isopropanol was then added to the samples and the entire volume pipetted into a filter tube provided in the kit. The filter tubes containing the samples were centrifuged in an *Eppendorf 5415C Centrifuge* for 1 minute at 8000 rpm and the flow-through discarded. 500 µl Wash Buffer (containing ethanol, 20 mM NaCl, 2mM Tris-HCl, pH 7.5) from the same kit was then spun through each column at 8000 rpm twice to clean the DNA. The DNA was eluted with 100 µl of 1 x TE buffer pre-warmed to 72 °C.

2.2.1.3 DNA EXTRACTION FROM PARAFFIN-EMBEDDED BONE MARROW TREPHINES

The technical staff in the Histology Department at Chris Hani Baragwanath Hospital sectioned the paraffin-embedded bone marrow trephines using a microtome. The blade and blade-holder of the microtome were swabbed down with alcohol before each block was sectioned to avoid contamination between the different samples. We requested that 10 to 20 sections of 10 µm were cut from each block. This was not possible when only a small amount of tissue was present and in these cases the technologist cut as many sections as possible without compromising the integrity of the tissue in the block. The sections cut from each block were placed in a 1.5 ml Eppendorf tube after sectioning.

On receiving the paraffin-embedded sections, 1 ml xylene was added to each tube and incubated at room temperature for approximately 7 minutes to dissolve the wax in the sample. After centrifugation in the *Eppendorf 5415C Centrifuge* at 14 000 rpm for 3 minutes, the supernatant was pipetted off with a thin disposable glass pipette and

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discarded. If wax was still present in the sample, another 1 ml of xylene was added and the procedure described was repeated up to 3 times. Absolute alcohol was then added to the sample to remove the xylene remaining in the tube. After centrifugation for 2 minutes at 14000 rpm, the supernatant was again pipetted off and discarded. The pellet was then air-dried before the lysis buffer from the *Boehringer Mannheim High Pure PCR Template Preparation* kit was added. From this point, the extraction procedure was identical to the one outlined above for bone marrow aspirate slides in section 2.2.1.2.

2.2.2 Assessment of DNA Quality

All of the extracted DNA samples were run on a 0.8% agarose gel to approximate the concentration and quality of the DNA. The principle of agarose gel electophoresis is described in section 2.2.4. 1µl to 4µl of each DNA sample was loaded with 5µl Ficoll dye into wells in the agarose gel. If the DNA was not easily visualised on the gel, a larger volume of the DNA sample was used in the polymerase chain reaction (PCR).

2.2.3 The Polymerase Chain Reaction for FANCG 637-643

All samples from black individuals were tested for the common seven base pair deletion in FANCG by PCR and agarose gel electrophoresis. The PCR method was originally described by Saiki et al. in 1985 and subsequently simplified by the discovery of a thermostable Taq polymerase in 1988 (Saiki et al. 1985, Saiki et al. 1988). The conditions for the PCR and the primers for the detection of the *FANCG 637-643* deletion were designed by Professor Chris Mathew at Guys Hospital in London, United Kingdom.

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The sequences of the PCR primers for detecting the deletion mutation in FANCG were:

Forward primer 5'-CCC AGG GAT TGA AGG ATG TC-3'

Reverse primer 5'-GCA TGA GAC TGG AGG ACC AC-3'

The PCR reaction occurred in a 25 µl volume. The reaction mix was made up as follows: 1µl DNA (100 – 1000 ng), 2.5µl of the 10 x concentrated commercial Taq polymerase buffer, 2.5 µl dNTP mix (25µM), 0.5µl (5pmole) of the forward and 0.5µl (5pmole) of the reverse primer, 1 unit of Boehringer Mannheim Taq polymerase enzyme and 17.8µl of distilled water. If the DNA sample had been shown by agarose gel electrophoresis (section 2.2.2) to have a low concentration, a larger volume of DNA sample was used and the volume of water used in the reaction decreased accordingly.

The PCR was carried out in the Applied Biosystems Gene Amp Thermocycler under the following conditions:

Denaturing step: 94 °C for 1 minute

Annealing step: 60 °C for 1 minute

Extension phase: 72 °C for 1 minute

This thermo-cycling was repeated 30 times. The program included an initial denaturing step at 94 °C for 5 minutes at the beginning of the reaction and a final extension phase of 5 minutes at 72 °C at the end of the thermo-cycling.

The PCR products from this reaction were 105 bp if the deletion mutation was not present and 98 bp when the deletion was present. The controls included in each PCR run were DNA from a homozygous normal individual (called N/N), DNA from a known heterozygote carrier individual (called N/M) and DNA from an affected individual

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homozygous for the mutation (called M/M). A blank control with no DNA added to the PCR mix was also included to ensure that there was no contaminating DNA present. The PCR products were separated using agarose gel electrophoresis.

2.2.4 Agarose Gel Electrophoresis

Electrophoresis can be used to separate DNA fragments of different sizes. The procedure involves inducing a potential difference across a gel medium through which DNA can move. DNA is negatively charged and so migrates towards the positive electrode. The rate at which the DNA fragments move is dependent on the extent to which they are retarded by the gel medium in which they are moving. Smaller fragments will move through the medium faster because they encounter less resistance. Each DNA molecule will therefore move to a unique position on the gel depending on its size and the fragments will in this way be separated (Sambrook et al. 1989).

Metaphor® gel was used to separate the PCR fragments detecting the *FANCG 637-643* deletion because there was only a 7 bp difference in the size of the two fragments. Metaphor® is a refined agarose gel and results in better separation of two similar sized fragments than low grade White Sci® agarose gel does. A 4% Metaphor® gel was made by slowly dissolving 16 g Metaphor® powder in 400 ml chilled 1 x TBE buffer. The mixture was then boiled and allowed to cool slightly before ethidium bromide (EtBr) was added to a final concentration of 0.03 mg EtBr per 100 ml gel. EtBr allows the DNA in the gel to be visualised by intercalating between the bases of the DNA and fluorescing under ultraviolet light (Sambrook et al. 1989).

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The gel was then poured into a mould with a comb placed at one end and allowed to set for approximately one hour before the comb was removed. The gel was cooled and placed in a trough of 1x TBE buffer and the PCR or DNA sample loaded into the wells with 5 µl of Ficoll dye. The cathode was attached to the trough at the same end as the wells so that the DNA would move towards the anode when the current was applied. The Gibco® 1 kb plus molecular weight marker (see APPENDIX A) was loaded into the first well of the gel to aid sizing of the DNA fragments. A constant voltage of 120V was applied across the gel. Electrophoresis was ended after about three hours when the fragments were adequately separated. The gel was then photographed under UV light. Figure 2.2 illustrates a schematic diagram of the agarose gel after separation of the PCR products.

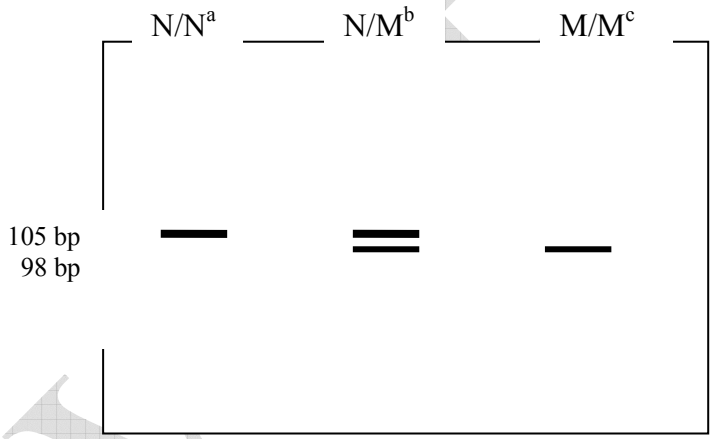

Figure 2-2: A diagrammatic representation of the PCR products obtained in the assay to detect *FANCG 637-643*, the common mutation in black South Africans.

[a] an individual that does not carry the mutation
[b] an individual who is heterozygous for the mutation
[c] a homozygous affected individual

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2.3 METHODS IN THE FANCA MUTATION SCREEN

Many attempts to optimise the PCR to detect the common Afrikaner mutation, a deletion of exons 12 to 31 in the FANCA gene, were made. In order to detect this large deletion, a 1.3kb fragment of the gene needs to be amplified. The large size of the PCR product means that the PCR is very sensitive to the quality of the DNA being used. The majority of the samples available to us were collected retrospectively and were therefore a few years old and paraffin-embedded. This meant that the DNA was frequently degraded and long sequences were near impossible to amplify. The DNA concentrations in our samples were also often low because either only a small amount of tissue was available for extraction or in the case of blood samples, patients had low white blood counts due to AA.

Due to time constraints, difficulty obtaining samples from white patients and technical problems extracting and amplifying DNA from the samples we did have, it was decided to focus on the collection of samples from black South African patients and analysis of the deletion mutation in FANCG.

2.4 SUBJECTS WITH FANCONI ANAEMIA USED TO ASSESS THE FREQUENCY OF CONGENITAL ABNORMALITIES

An initiative to collect clinical data on individuals with FA had been ongoing in the Department of Human Genetics, University of the Witwatersrand, Johannesburg, since 2000 in collaboration with the European Fanconi Anaemia Research Group (EUFAR). A clinical data sheet, designed by EUFAR (Faivre et al. 2000) was sent to clinicians requesting FA testing on a patient if the result showed homozygosity or heterozygosity for the *FANCG 637-643* mutation or if the patient had clinical features strongly suggestive of a

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diagnosis of FA. The data sheet contained questions about physical manifestations in the patient and about haematological abnormalities. The data sheet is attached (Appendix B on page 89).

A total of 34 data sheets had been completed by June 2004. Of these patients, 25 were shown to be homozygous or heterozygous for the *FANCG 637-643* deletion mutation while the other 9 had features strongly suggestive of FA. We divided the patients into two groups, the first group of patients were known to belong to FA complementation group G (FANCG patients) and the second group of patients were presumed to belong to one of the other complementation groups (non-FANCG patients).

Patients in the first group had been found to be either homozygous for the *FANCG 637-643* mutation (N = 21) or heterozygous (N = 4) with clinical features of FA. We assumed that patients with only one *FANCG 637-643* mutation were in fact compound heterozygotes with a second unidentified mutation and did in fact belong to complementation group G. This assumption was made because it is highly unlikely that an individual with FA would carry a mutation in FANCG as well as being homozygous for mutations in another complementation group, due to the rarity of FA mutations.

The second group included patients who did not carry the common FANCG mutation but had clinical features of FA. It was assumed that if patients did not carry at least one *FANCG 637-643* mutation, they were highly unlikely to belong to the FANCG goup because of the very low frequency of heterozygotes in the affected group. Therefore these patients were assigned to the non-FANCG group, which was a group of patients from undefined complementation groups.

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2.5 METHODS IN THE DELINEATION OF THE FANCG PHENOTYPE

2.5.1 Frequency of Congenital Abnormalities in Black South African Patients

The frequency of manifestations chosen for analysis was determined for the black South African FANCG and non-FANCG groups. The included manifestations were radial ray abnormalities (also separated individually into thumb abnormalities and abnormalities of the radius), developmental delay, heart abnormalities, renal abnormalities, hearing disability, growth retardation, lower limb abnormalities, pigmentation anomalies, head abnormalities and other skeletal abnormalities. Head abnormalities included dysmorphic facial features as well as microcephaly. Most of the manifestations were chosen because they are relatively easy to recognise and they manifest early in life. Also, Rosenberg et al. (2004) used some of the same manifestations in their study which allowed comparison between the patient groups to be made. We also compared the frequencies of the manifestations in our FANCG sample to a cohort of FANCG patients described by Faivre et al. (2000).

Comparing the frequency of manifestations in the three cohorts was sometimes made difficult because of poorly defined medical terminology. The definition of radial ray abnormalities, for example, is not clear and the term is often used to describe different manifestations. Rosenberg et al. (2004) separate thumb abnormalities from what they term radial ray abnormalities, while Faivre et al. (2000) group thumb and abnormalities of the radius together under this heading. In our study, we separated thumb and abnormalities of the radius when comparisons were made to the Rosenberg et al. (2004) study and grouped

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them together when comparing the frequency of radial ray abnormalities to the Faivre et al. (2000) study.

The numbers of patients with each manifestation in the American study was calculated using the percentage data given in the paper and the known total number of patients in the sample (Rosenberg et al. 2004).

2.5.2 Association of Congenital Abnormalities with Haematological Outcome

Rosenberg et al. (2004) determined that besides abnormalities of the radius, five of ten congenital abnormalities they studied were specifically associated with the early development of AA. These abnormalities were developmental delay, heart, kidney, hearing and head abnormalities. Rosenberg et al. (2004) determined that the risk of AA developing in a particular patient at any given age could be predicted by the presence or absence of abnormalities of the radius and the so-called CAB5 score, which is the number of the above five abnormalities present in a particular patient. They constructed graphs plotting the cumulative incidence of developing AA against age for CAB5 scores of 0, 1, 2, 3, 4, and 5 (the complete set of graphs were requested from the author). Because none of our patients had abnormalities of the radius, the graphs used in our study were those for a RAD score of 0 (i.e. the patients from Rosenberg et al. (2004) study that did not have abnormalities of the radius).

The cumulative incidence of AA at age ten was read from the five graphs and a straight line graph was approximated (Figure 2-3). The equation of the graph was found to be $y = 4.98x + 17.22$. The average CAB5 scores in our cohorts of FANCG and non-FANCG

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patients were determined. The percentage of patients expected to develop AA by ten years of age from the American study was then calculated from the equation for the straight line graph. The incidence predicted with our average CAB5 score was compared to the observed incidence reported in our patients using a one sample test on proportions (refer to section 2.6.5).

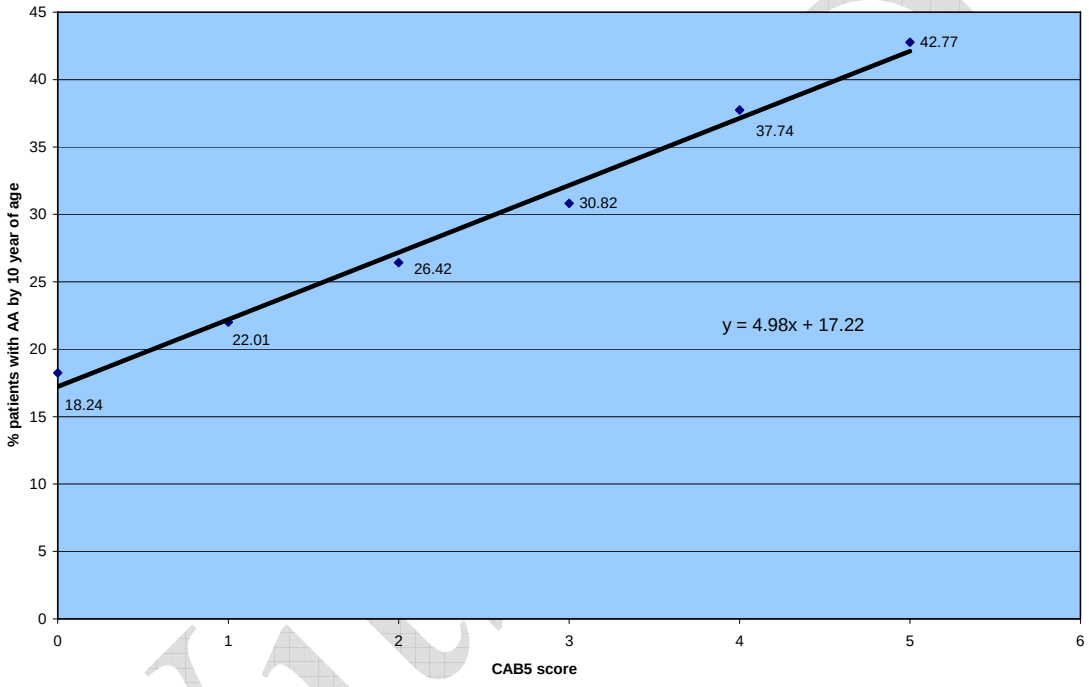

Figure 2-3: The percentage of American patients with Fanconi anaemia to have developed aplastic anaemia by ten years of age for each CAB5 score[a].

[a] Data were requested from the authors of Rosenberg et al. (2004) in order to construct the graph.

2.6 DATA ANALYSIS

2.6.1 The Chi-Square Test

The chi-square test is used to determine the probability that chance alone could produce the deviation seen between two groups. The null hypothesis states that the two groups are from the same population and the chi-square test gives the probability that the different

frequencies seen in the two sample groups is due to random sampling of one large population.

Yates' correction, which is used to improve the approximation of the *p* values when the sample sizes are small, was used for all chi-square tests done in this study. When more than 20 % of the expected numbers in any class are less than five, Yates' correction is recommended to make the chi-square test approximation more accurate (Motulsky 1999). The chi-square values with Yates' correction were calculated using a program available on the World Wide Web (GraphPad Prism). The chi-square test was not used in cases where one or more of the expected numbers in any class was less than one due to the inaccuracy of the test under these circumstances.

The chi-square test was used to determine whether significant differences in frequency of the FANCG mutation allele existed between the control group, which represents the population frequency, and the patients with AML or AA. The chi-square test was also used to compare the frequency of ten clinical features in a cohort of South African patients with FA to data described by Rosenberg et al. (2004) and data described by Faivre et al. (2000).

2.6.2 Fisher's Exact Test

Fisher's exact test (F exact test), like the chi-square test, measures whether there is a significant difference between two data sets. In the F exact test however, the two data sets are combined and random samples drawn from them. The probability of the original two data sets being drawn is then calculated and this indicates the chance of these two sets

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being samples from one population. The F exact test was done using a program available on the World Wide Web on the same data sets as the chi-square test (GraphPad Prism).

The F exact test is thought to be a better test than the chi-square test because it gives a more accurate probability value than the chi-square test, which only gives an approximation. The F exact test is especially useful when sample sizes are small and the chi-square value cannot be accurately calculated. The chi-square value was still calculated however, because this test has traditionally been used and lends further support to the results obtained.

2.6.3 Power Calculations

The power of a test is the probability that the null hypothesis is rejected when it is truly false. The power of a test is directly related to the size of the experimental sample group. We used a calculator available on the World Wide Web to determine the percentage increase in frequency of FANCG mutation carriers in our patient groups that we would have been able to detect with our sample size (Proportion Difference Power / Sample Size Calculation Page).

2.6.4 The Wilcoxon Two Sample Test

The Wilcoxon two sample test was used to compare the number of abnormalities in the South African FANCG group and the non-FANCG group. A program available on the World Wide Web was used to do the calculations (Wilcoxon Two Sample Test).

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2.6.5 The One-Sample Test on Proportions

The one-sample test on proportions (Clarke and Cooke 1998) was used to establish whether the number of patients expected to develop AA by ten years of age using the American model (Rosenberg et al. 2004) was significantly different to that which was observed. The probability of obtaining the observed data is calculated after assuming the established data in the American study (Rosenberg et al. 2004).

The formula for the one-sample test on proportions is:

$$Z = \frac{[\, p_1 - p_0 \,]}{\sqrt{[\, p_0(1 - p_0) / n \,]}}$$

where p_0 = the number presenting in the control population, p_1 = the number observed in the research sample and n = the number of samples in the research group. $\sqrt{}$ is the square root of the divisor. The *p* value is then read off a Z table (Clarke and Cooke 1998).

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3 RESULTS

3.1 THE *FANCG 637-643* DELETION MUTATION SCREEN

The common mutation in the black population in South Africa is a deletion of seven base pairs in FANCG from nucleotide position 637 to 643 (Morgan et al. 2005). This mutation was detected using PCR and agarose gel electrophoresis (refer to section 2.2.3). An image of an agarose gel containing the PCR products from homozygous normal individuals, an individual heterozygous for the deletion mutation and an individual homozygous for the deletion mutation can be seen in Figure 3.1.

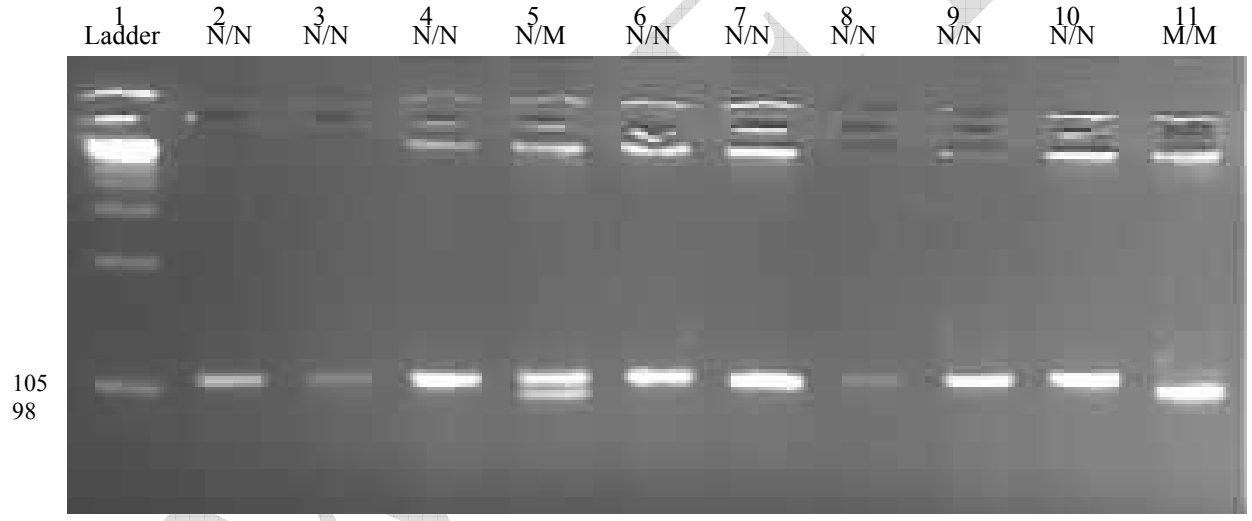

Figure 3-1: A 4 % Metaphor® agarose gel showing the PCR products of the region including the *FANCG 637-643* mutation.

Lane 1 contains a 1 kb plus ladder, lane 5 contains PCR products from a heterozygous carrier of the deletion mutation, lanes 2 to 4 and 6 to 10 contain PCR products from individuals homozygous for the absence of the deletion mutation, while lane 11 contains PCR products from an individual homozygous for the deletion mutation. The PCR product sizes (measured in bp) are shown on the left-hand side of the gel.

The results of the screen for the deletion mutation in black South African patients are shown in Table 3.1. The number of individuals with each genotype in each of the three disease groups screened is shown and further divided into paediatric and adult patients. Paediatric patients have been defined as those eighteen years and younger (≤ 18), while adult patients are those older than eighteen years (> 18). In a previous study it was found that the carrier frequency of the *FANCG 637-643* mutation in the black South African population was 1% (3 heterozygotes were found in a sample of 300 random individuals) (Morgan et al. 2005). This was used as our control group and these figures have therefore been included in Table 3-1.

Eighteen years of age has been used to divide the adult and paediatric patients in the sample because adolescents eighteen years and younger are still treated by the paediatric oncologists at Chris Hani Baragwanath Hospital and not the adult oncologists (Dr L. Wainwright 2004, Department of Paediatrics, Chris Hani Baragwanath Hospital - personal communication). Although the course of the diseases and their treatment are similar in both adult and paediatric cases, this division (for our own comparison) is important to determine whether the *FANCG 637-643* mutation has different implications for diagnosis in adults and children.

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Table 3-1: The results of the *FANCG 637-643* deletion assay in black South African patients with aplastic anaemia, acute myeloid leukaemia or myelodysplasia.

			Genotypes			Alleles	
DISEASE	Age	**Sample size**	**M/M**	**N/M**	**N/N**	**M**	**N**
Aplastic anaemia	**≤ 18**	26	3 (0.12)	1 (0.04)	22 (0.85)	7 (0.13)	45 (0.87)
	> 18	52	0 (0.00)	0 (0.00)	52 (1.00)	0 (0.00)	104 (1.00)
	Total	78	3 (0.04)	1 (0.01)	74 (0.95)	7 (0.04)	149 (0.96)
Acute myeloid leukaemia	**≤ 18**	37	0 (0.00)	1 (0.03)	36 (0.97)	1 (0.01)	73 (0.99)
	> 18	78	0 (0.00)	2 (0.03)	76 (0.97)	2 (0.01)	154 (0.99)
	Total	115	0 (0.00)	3 (0.03)	112 (0.97)	3 (0.01)	227 (0.99)
Myelodysplasia	**>18**	6	0 (0.00)	0 (0.00)	6 (1.00)	0 (0.00)	12 (1.00)
	Total	6	0 (0.00)	0 (0.00)	6 (1.00)	0 (0.00)	12 (1.00)
Control group[a]		300	0 (0.00)	3 (0.01)	297 (0.99)	3 (0.01)	597 (0.99)

[a] The control group data is from Morgan et al. (2005)
Frequencies are shown in brackets

The combined adult and paediatric results for AML, AA and the control group are shown graphically in Figure 3-2 below. The percentages of patients in each group with an N/M or M/M genotype at the *FANCG 637-643* locus are compared in the figure. MDS was not included in the figure because no mutations were identified in the sample.

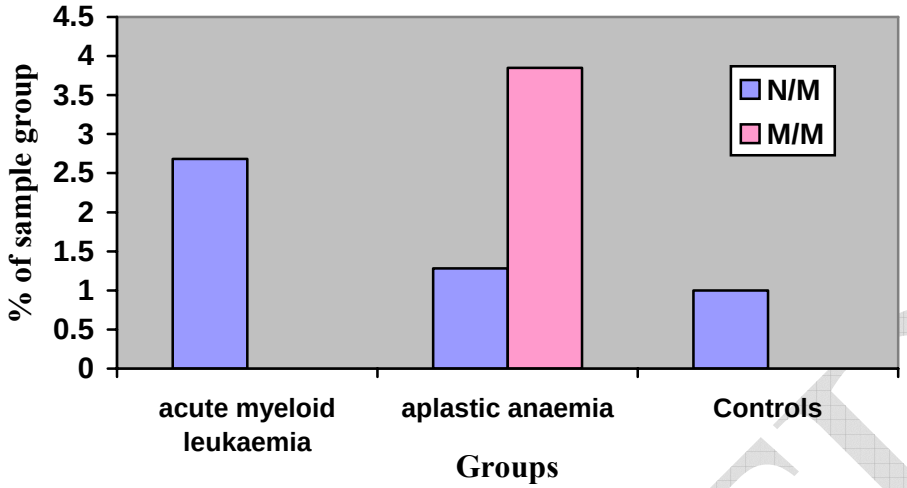

Figure 3-2: The percentage of individuals from the control group and the groups with acute myeloid leukaemia or aplastic anaemia, heterozygous (blue) or homozygous (pink) for the *FANCG 637-643* mutation.

We compared our data to the control group data to establish whether individuals with AML or AA are more likely to be heterozygous for the above-mentioned mutation. MDS was not included in the study because the small number of samples collected made it impossible for meaningful comparison to the control group.

Significant differences were found using the Chi-square test with Yates' correction and the F exact test. A comparison was made firstly between the number of observed genotypes and then between the observed number of alleles. The results of the comparisons are shown in Table 3-2. The Chi-square test was not done in cases where one or more of the expected numbers in any class had a value less than one. This occurred when the genotype numbers of the group of paediatric patients with AA and the combined AA group were compared to the control group. This was due to the presence of M/M individuals in the

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sample group and the absence of M/M individuals in the control group. Therefore when comparing the numbers of genotypes for these two groups only the F exact test was used.

Table 3-2: **Comparison of the frequency of the *FANCG 637-643* mutation in black South African patients with aplastic anaemia and acute myeloid leukaemia patients to a control group[a].**

		Genotypes				**Alleles**			
		Chi-square test			F exact test	Chi-square test			F exact test
DISEASE	Age	χ^2	df	*p*	*p*	χ^2	df	*p*	*P*
Aplastic Anaemia	**≤ 18**	-	-	-	**$<10^{-3}$**	44.996	1	**$<10^{-3}$**	**$<10^{-3}$**
	>18	0.009	1	0.926	1	0.009	1	0.927	1
	Total	-	-	-	0.054	12.180	1	**0.001**	**0.001**
Acute Myeloid Leukaemia	**≤ 18**	0.01	1	0.922	0.373	0.01	1	0.922	0.373
	>18	0.271	1	0.602	0.275	0.270	1	0.604	0.276
	Total	0.592	1	0.442	0.354	0.588	1	0.443	0.356

[a] Control goup data is from Krause et al. (2001)

Because FA is characterized by the development of AA in most cases, it was expected that there would be paediatric AA cases detected which were homozygous for the FANCG mutation. To determine whether there was an increased number of heterozygous carriers of this mutation in paediatric patients with AA, it was necessary to remove the three homozygous individuals found in this study and then compare the data again. The

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comparison between the paediatric group without M/M individuals and controls can be seen in Table 3-3**.**

Table 3-3: Result of the Chi-square and F exact test comparing the frequency of heterozygotes for the *FANCG 637-643* mutation in black South Africans (<18 years old) with aplastic anaemia to a control group[a].

		Chi-square test: Genotypes			**Chi-square test:** Alleles			**F Exact test**
Disease	Age	χ^2	df	*p*	χ^2	Df	*p*	*P*
Aplastic Anaemia	≤ 18	0.177	1	0.673	0.176	1	0.675	0.256

[a] Control goup data is from Krause et al. (2001)

3.2 COMPARING THE PHENOTYPES OF PATIENTS WITH FANCONI ANAEMIA

Data was collected on the clinical features reported in black South African patients with FA and compared to features reported in two previous studies. Firstly, the frequencies of the clinical manifestations reported in the groups were compared. Secondly, we attempted to determine whether the risk of developing AA could be predicted in black South African patients based on the number of congenital abnormalities present as Rosenberg et al. (2004) had done in their study. Rosenberg et al. (2004) refer to AA as bone marrow failure in their study. We collected patients with a diagnosis of AA in our study and therefore referred to bone marrow failure as AA throughout the study.

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3.2.1 Frequency of Congenital Anomalies in Patients with Fanconi Anaemia

The number of patients reported with the chosen clinical manifestations can be seen in Table 3-4. In the table the number of patients with each manifestation is shown for South African FANCG and non-FANCG patients. Also included in the table is the number of patients identified with each manifestation in the American Cohort (Rosenberg et al. 2004) and in the cohort reported by Faivre et al. (2000). Rosenberg et al. (2004) did not determine which complementation groups the patients in their cohort belonged to. Faivre et al. (2000) identified a group of patients, the majority not South African, known to belong to complementation group G which is the group that was used in this study for comparison.

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Table 3-4: Congenital abnormalities in four groups of patients with Fanconi anaemia.

Manifestation	South African FA Cohort[a]		American Cohort[b] $N^d = 125$	FANCG Cohort[c] $N^d = 24$
	Non-FANCG patients $N^d = 9$	FANCG patients $N^d = 25$		
Abnormalities of the radius	0/9 (0)	0/25 (0)	18/144 (0.125)	Not counted separately
Thumb abnormality	6/9 (0.667)	15/25 (0.600)	81/144 (0.563)	Not counted separately
Radial ray anomaly[e]	6/9 (0.667)	15/25 (0.600)	99/144 (0.688)	13/24 (0.540)
Developmental Delay	1/9 (0.111)	1/25 (0.040)	39/144 (0.271)	2/24 (0.083)
Heart abnormalities	0/9 (0)	4/25 (0.160)	27/144 (0.188)	1/24 (0.042)
Kidney abnormalities	3/9 (0.333)	5/25 (0.200)	58/144 (0.403)	4/24 (0.166)
Hearing impairment	0/9 (0)	1/25 (0.040)	60/144 (0.417)	1/24 (0.042)
Head abnormalities	9/9 (1)	22/25 (0.880)	57/144 (0.396)	17/24 (0.708)
Growth retardation	8/9 (0.889)	22/25 (0.880)	105/144 (0.729)	18/24 (0.750)
Lower limb abnormalities	1/9 (0.111)	0/25 (0)	10/144 (0.69)	Not counted separately
Skeletal abnormalities	1/9 (0.111)	1/25 (0.040)	23/144 (0.16)	3/24 (0.125)

[a] The South African cohort is divided into patients known to belong to complementation group G (FANCG patients) and patients belonging to other complementation groups (Non- FANCG patients).
[b] The American cohort was described by Rosenberg et al. (2004)
[c] The FANCG patient group was described by Faivre et al. (2000)
[d] N is the number of patients screened in each group
[e] Radial ray abnormalities include individuals with either thumb abnormalities, abnormalities of the radius or both abnormalities together.

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We compared the frequency of the clinical manifestations in the two South African FA groups using the chi-square test with Yates' correction and Fischer's exact test. The results of these statistical tests are shown in Table 3-5. The chi-square test was not done in cases where one or more expected numbers in any class had a value less than one.

Table 3-5: A comparison of the frequency of clinical manifestations in black South African FANCG patients and black South African non-FANCG[a] patients.

	Chi-square test:			**F-Exact test**
	χ^2	**df**	***p***	***p***
Abnormalities of the radius	-	-	-	1
Thumb abnormalities	0.125	1	0.724	1
Radial Ray abnormalities[b]	0.125	1	0.724	1
Develop Delay	0.604	1	0.437	0.465
Heart abnormalities	0.455	1	0.500	0.554
Kidney abnormalities	0.123	1	0.726	0.649
Hearing	0.371	1	0.543	1
Head abnormalities	0.162	1	0.687	0.549
Growth retardation	0.005	1	0.943	1
Lower limb abnormalities	0.293	1	0.588	0.265
Skeletal abnormalities	0.604	1	0.437	0.465

[a] The non-FANCG patients belong to complementation groups other than FANCG.
[b] Radial ray abnormalities include individuals with either thumb abnormalities, abnormalities of the radius or both abnormalities together.

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No statistically significant differences in the frequency of clinical features were identified between the South African FANCG patients and those patients belonging to other complementation groups. However, the small number of non-FANCG patients restricts the power of the statistical comparisons and limits the conclusions that can be drawn from them. The frequency of the clinical manifestations in the FANCG South African patients was compared to the frequency of manifestations described in the American cohort by Rosenberg et al. (2004) and those described for FANCG patients by Faivre et al. (2000). Again the chi-square test with Yates' correction and Fischer's exact test were used. The results of these statistical tests are shown in Table 3-6. A correction for false discovery due to multiple testing was not done because the *p* values that are significant are not borderline significant and therefore are unlikely to shift to non-significance.

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Table 3-6: Statistical analysis comparing the frequencies of manifestations seen in the South African FANCG cohort compared to an American cohort and a group of non-South African FANCG patients.

Manifestation	Group	Chi-square test			F-Exact test
		χ^2	df	*p*	*P*
Abnormalities of the radius	American cohort[a]	2.307	1	0.129	0.078
	Non-SA FANCG[b]	-	-	-	-
Thumb abnormality	American cohort[a]	0.017	1	0.896	0.828
	Non-SA FANCG[b]	-	-	-	-
Radial Ray abnormalities[c]	American cohort[a]	0.398	1	0.528	0.488
	Non-SA FANCG[b]	0.015	1	0.902	0.776
Developmental Delay	American cohort[a]	5.070	1	**0.024**	**0.010**
	Non-SA FANCG[b]	0.001	1	0.971	0.609
Heart abnormality	American cohort[a]	0.002	1	0.962	1
	Non-SA FANCG[b]	0.803	1	0.370	0.349
Kidney abnormality	American cohort[a]	2.929	1	0.087	0.072
	Non-SA FANCG[b]	0.091	1	0.763	1
Hearing impairment	American cohort[a]	11.520	1	**0.007**	$\boldsymbol{p < 10^{-3}}$
	Non-SA FANCG[b]	0.001	1	0.977	1
Head abnormality	American cohort[a]	18.161	1	$\boldsymbol{p < 10^{-3}}$	$\boldsymbol{p < 10^{-3}}$
	Non-SA FANCG[b]	0.345	1	0.557	0.446
Growth retardation	American cohort[a]	1.850	1	0.174	0.135
	Non-SA FANCG[b]	0.649	1	0.420	0.289
Lower limb abnormality	American cohort[a]	0.809	1	0.369	0.361
	Non-SA FANCG[b]	-	-	-	-
Skeletal abnormality	American cohort[a]	1.620	1	0.203	0.209
	Non-SA FANCG[b]	0.319	1	0.572	0.349

[a] The American cohort was described by Rosenberg et al. (2004)
[b] The non-South African group of FANCG patients was described by Faivre et al. (2000)
[c] Radial ray abnormalities include individuals with either thumb abnormalities, abnormalities of the radius or both abnormalities together.

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The frequencies of developmental delay, head abnormalities and hearing disabilities were significantly different in the American and black South African cohorts. Developmental delay and hearing impairment occurred more frequently in the American cohort while head abnormalities occurred more frequently in the South African cohort (Rosenberg et al. 2004). There were no significant differences between the frequency of manifestations in the South African FANCG cohort and the non-South African FANCG group described by Faivre et al. (2000).

3.2.2 Association of Congenital Abnormalities with Haematological Outcome

A congenital abnormality score (CAB10 score) was calculated for each patient in our cohort by counting the number of clinical manifestations, out of the ten chosen, present in each patient. The average CAB10 score occurring in the 25 black South African FANCG group was 2.84, ranging from a minimum of 1 to a maximum of 5. The average number of abnormalities in the 9 patients belonging to other complementation groups in the South African cohort was 3.22, ranging from 1 to 5. No significant difference between these two averages was found using the *Wilcoxon two sample test* ($p = 0.520$). The average number of abnormalities in the 144 patients from the American study was found to be 3.3, ranging from none to 9 (Rosenberg et al. 2004). It was not possible to compare our averages to the American average because we did not have access to their raw data.

Rosenberg et al. (2004) determined that besides abnormalities of the radius, five of the ten congenital abnormalities were specifically associated with the development of AA. These abnormalities were developmental delay, heart, kidney, hearing and head abnormalities.

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Therefore patients were given CAB5 scores which was the number of the above abnormalities they had.

In the black South African FANCG group, 23 of the 25 patients presented with AA and 19 of them did so before the age of ten years. One patient was 2 years old at the time of the study and was healthy. He was diagnosed with FA after a sibling was confirmed to be homozygous for the deletion mutation. Two patients in the FANCG group had MDS diagnosed, both before ten years of age. One of them was diagnosed with AA prior to MDS developing. In our group of FANCG patients, 19 of 24 patients (79 %) had developed AA before ten years of age. The total number of patients is not 25 because one patient was only 2 years old at the time of study and had not yet developed AA.

The average CAB5 score in our sample was 1.32 (ranging from 0 to 3) with no abnormalities of the radius reported. Using the cumulative incidence curves in Rosenberg et al. (2004) study, we calculated that for a CAB5 score of 1.32 with no abnormalities of the radius, the model predicts that 23.8 % (range from 18.2 %, if CAB5 = 0, to 30.8 %, if CAB5 = 3) of our patients should develop AA before ten years of age. We have established that 79 % of our patients develop AA before this age. This difference in frequency of patients presenting before ten years of age is statistically significant ($p << 10^{-3}$). Table 3-7 shows a summary of this analysis.

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Table 3-7: The number of South African FANCG patients presenting with aplastic anaemia before ten years of age, the average CAB5 score of the cohort and the percentage of patients predicted to present with aplastic anaemia before ten years.

Cohort	Patients with aplastic anaemia <10 years	Average CAB5 score[b]	% predicted to present <10 years[c]	One sample proportion test[d]
FANCG N[a] = 24	19 (79 %)	1.32 (0-3)	23.8 %	$p < 10^{-3}$

[a] The number of patients in the FANCG group
[b] The CAB5 score was described by Rosenberg et al. (2004). The range of scores observed is given in parenthesis.
[c] The percentage predicted to develop aplastic anaemia before ten years of age is calculated from Rosenberg et al. (2004) study.
[d] The one sample proportion test compares the observed number of patients presenting with aplastic anaemia before ten years and the predicted number

In the non-FANCG group, 8 of the 9 patients presented with AA and five of them did so before ten years of age. One patient in the non-FANCG group was diagnosed with acute leukaemia at ten years and ten months old. Therefore, 56 % (5 of 9) of the non-FANCG patients developed AA before ten years of age. The average CAB5 score in this group was 1.5 (ranging from 1 to 3). From figure 1 in the American model, 25.5 % (from 22 % for CAB5 score of 1 to 30.8 % for CAB5 score of 3) of the group would be expected to develop AA before ten years of age (Rosenberg et al. 2004). The small number of patients in this group does not allow for statistical testing.

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4 DISCUSSION

4.1 THE COLLECTION OF SAMPLES FOR THE *FANCG 637-643* SCREEN

An important part of this study was the collection of samples from individuals with AML, AA or MDS. Sample collection will be discussed here in some detail because the way in which samples are collected has direct bearing on the conclusions that can be drawn from the data.

The diagnosis of each patient was obtained when the sample was collected as discussed in section 2.1. It is possible that erroneous diagnoses were obtained in some cases, as full clinical data were not available for every patient. Also, occasionally diagnoses are not initially clear and are established or changed after subsequent testing. We assume that this would not have been the case with a significant number of our specimens as the diagnoses of AML and AA especially, are fairly unequivocal.

4.1.1 The Black South African Patient Group

It was a time-consuming part of this research study collecting 199 samples from black South Africans with AML, AA or MDS. These disorders are rare and therefore few fresh samples were available to us. However, collecting retrospective samples was also challenging because many specimens were missing or miss-filed. Also diagnoses were often not recorded and over-worked laboratory staff were relied on to section the histology specimens, which limited the number of specimens that could be reasonably requested. Another stumbling block was that records of all samples collected prior to 2000 at

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Johannesburg Hospital are stored on a database that was inaccessible for two months during the study because the computer system had mal-functioned.

Although the sample size is significant when considering the small numbers of patients affected with the disorders of interest, its size does limit the conclusions that can be drawn from the data. An analysis of the data collected is discussed below.

4.1.2 The Black South African Control Group

The control group used in this study was 300 randomly chosen black South African individuals (Morgan et al. 2005). It was assumed that none of them had AML, AA or MDS because these are rare disorders that are generally clinically obvious. There were no data on the ages of the individuals in the control group but we assumed that the carrier frequency of the *FANCG 637-643* mutation would be the same in healthy individuals in both adult and child populations. The same control group data was therefore compared to both the adult and paediatric patient samples.

4.1.3 The White South African Patient Group

Our intention initially was to also screen 150 white patients with AML, AA or MDS for the common FANCA mutation in the Afrikaans population. This part of the study was abandoned, as already discussed in section 2.3, because of difficulty with the PCR amplification of large fragments from poor quality DNA and because white patients with the disorders of interest are extremely rare in the state sector. We did not have access to patients or specimens in the private sector, which meant that the number of samples available to us was severely limited.

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4.2 *FANCG 637-643* SCREEN IN PATIENTS WITH APLASTIC ANAEMIA

4.2.1 Patients homozygous for FANCG 637-643

Although individuals with FA were not selected for in this study, the fact that AA is an almost inevitable complication of FA (Butturini et al. 1994) meant that it was likely that some individuals homozygous for the *FANCG 637-643* mutation would be detected among our paediatric sample group. Therefore, it was not surprising that a significant difference in the frequency of the FANCG mutation was found in patients with AA when compared to the control group (refer to Table 3-2). On investigation, we found that all the patients in our study shown to be homozygous for the mutation had previously been diagnosed with FA.

None of the adult patients with AA screened for the mutation were found to be homozygous, which suggests that individuals with FA present early in life with their AA. This supports the findings of previous research studies that have observed that the majority of FA patients present with AA in childhood, the median age of onset being 7 years (Kutler et al. 2003, Rosenberg et al. 2003).

4.2.2 Heterozygote Carriers of FANCG 637-643

In order to establish whether an increased number of FANCG heterozygote carriers were present in the group with AA, the homozygous individuals were excluded from the statistical analysis (Table 3-3). After exclusion of the homozygotes, one heterozygous carrier of the mutation was present in the group.

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On investigation it was found that this mutation carrier was an eight-year-old female individual who had died from an intra-cranial haemorrhage, presumably secondary to AA. The patient had hypo- and hyper-pigmented skin patches, café-au-lait spots, almond-shaped eyes, thumb abnormalities and growth retardation. These clinical features are strongly suggestive of a diagnosis of FA. Chromosome breakage studies were performed and were also indicative of a diagnosis of FA. This patient was probably a compound heterozygote with an unidentified mutation in the other FANCG allele and was actually affected with FA.

It can be seen in Table 3-3 that there is no significant increase in the number of heterozygous carriers of the FANCG mutation in the patients with AA compared to the control group. If the only heterozygote carrier identified actually had FA, as we suspect, then none of the patients with AA were heterozygous for the FANCG mutation.

The size of the sample (N=78) tested was fairly small but the data analysis indicates no increase in the number of heterozygote carriers among patients with AA. Because of our sample size, the minimum carrier frequency that would have been detected 80 % of the time is 8.4 %. Therefore an increase in the frequency of heterozygous individuals below 8.4 % among patients with AA would not have been detected as significantly different from the control group in this study. We therefore cannot rule out a small effect of the mutation on the development of AA in heterozygous carriers although there is no evidence to suggest this.

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4.2.3 Other Causes of Aplastic Anaemia

FA is not considered to be a common cause of AA, especially in adults because FA is a rare disease. However, the percentage of carriers of FA in the black South African population is significant at 1 % (Morgan et al. 2005). Although an increase in the frequency of *FANCG 637-643* heterozygotes was not detected in patients with AA, it is possible that other causes of AA are overwhelming any effect that the FANCG mutation may have in the population. If another factor in the population is common or strongly associated with AA, this factor might over-shadow any effect that the relatively rare FANCG mutation may have had on predisposition to developing AA.

It is known that tuberculosis infection of the bone marrow can cause AA and pancytopaenia, usually not involving the bone marrow, can be secondary to HIV infection (Rosenfeld and Young 1991). As these diseases are common in our population it is possible that a proportion of our sample group with AA may have had the haematological condition secondary to tuberculosis or HIV infection. There are also other known causes of AA, such as radiation and chemical exposure, discussed in section 1.2.1. There is no way of knowing whether our sample group had been exposed to these agents but it seems unlikely that a significant number would have been. Most commonly the cause of AA is never identified in an affected individual.

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4.3 *FANCG 637-643* SCREEN IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA

Due to the hypothesis that the number of individuals diagnosed with FA in South Africa is less than expected from the reported carrier frequency, it was proposed that some individuals with FA are not identified because of unusual presentation, for example with AML (Morgan et al. 2005). No patients with AML were found to be homozygous for the FANCG mutation in our study and it can therefore be deduced that there are not a significant number of individuals with FA who present primarily with AML in the South African black population. It has been estimated that approximately 1 % of FANCG patients (mutation not specified) present with AML in childhood (Meyer et al. 2004). Based on this estimation and our findings, it is probably unnecessary to screen individuals with AML, especially adults, for the FANCG mutation.

4.3.1 Heterozygote Carriers of FANCG 637-643

Three patients (N=115) with AML were found to be heterozygous carriers of the *FANCG 637-643* mutation. This was not a significantly different carrier frequency to the one found in the control group although it is 2.61 times higher. It was calculated that a 7.17% carrier frequency would have been detected 80 % of the time with our sample size. Therefore an increase in the carrier frequency below 7.17 % among patients with AML would not be detected as significantly different to the control group. The study had insufficient power to detect a small effect of the FANCG mutation on the development of AML in mutation carriers. To prove the 2.61 times increase that we found was statistically significant, a sample of 761 patients and 1986 controls would have been needed. Meyer et al. (2004)

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however, also recently concluded from their research that pathogenic FANCG mutations do not play a major role in sporadic childhood AML.

Of the three patients with AML found to be heterozygous for the FANCG mutation, one patient was initially suspected to have FA while the other two did not have any clinical features of FA.

Patient one

The first adult patient identified as a heterozygous carrier of *FANCG 637-643* had an unconfirmed diagnosis of myelofibrosis. She was initially included in the study because of the possibility that she did in fact have AML. She was the only patient to our knowledge in the study with an unconfirmed diagnosis of AML. Primary myelofibrosis is a separate entity to AML but almost invariably transforms to an AML. It is also possible for myelofibrosis to arise as a secondary complication of AML.

The patient was a 42-year-old woman who presented with anaemia and mild hepatomegaly. The report of the examination of the bone marrow histology states that the likely diagnosis is myelofibrosis but that transformation to AML cannot be excluded. The bone marrow aspirate report states that a haemopoietic malignancy is more likely than a reactive state. These are the only reports available on this patient. It seems likely that the patient had primary myelofibrosis which was transforming into AML at the time the specimens were taken.

It is difficult to know whether this patient should in fact have been included in the sample group due to the uncertainty of her diagnosis. However, myelofibrosis is a disorder within

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the spectrum of AML and for this reason it has been included. There is unfortunately no other clinical data on this patient to allow us to establish whether her clinical presentation was unusual.

Patient two

The second patient with AML found to have the FANCG mutation was a 49 year old male. His clinical presentation was not reported to be unusual. He developed disseminated intravascular coagulation, not unusual in patients with AML, thought to be secondary to an infection. A CT brain scan was done because the patient exhibited fluctuating levels of consciousness. It showed age inappropriate involutional changes probably caused by chronic alcohol abuse reported in the history. The patient was lost to follow-up after being discharged from the hospital in remission. Therefore, as far as we know the patients clinical course was not unusual in any way. Establishing whether heterozygous carriers of FA who have AML have a different clinical course, will require further investigation.

Patient three

There was one paediatric AML patient, aged nine years old, found to carry the FANCG mutation. Interestingly she was suspected to have FA by the haematologists because of some subtle clinical features. She reportedly has small eyes and a thumb abnormality. She is however of normal stature and is responding well to her chemotherapy treatment, which is usually not the case in individuals with FA (Alter 1996). It would be interesting to perform chromosome breakage studies on this patient to determine whether she is a likely compound heterozygote for a FANCG mutation. Unfortunately chromosome breakage studies are unreliable in patients who have AML due to the associated increased chromosome instability in lymphocytes. FA is clinically heterogeneous and this patient

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may have a milder phenotype, perhaps because of a second milder mutation. Follow-up of this patient will hopefully allow clarification of her disease.

4.4 *FANCG 637-643* SCREEN IN PATIENTS WITH MYELODYSPLASIA

The number of cases of MDS found for this study was small with no paediatric cases and only six adults screened for the FANCG mutation. The reason that so few cases of MDS were found is possibly because the diagnosis of MDS is rare and subject to inter-observer variability (Butturini et al. 1994). MDS is extremely rare in children, which is most likely why no cases of the disorder were seen in our paediatric sample group. MDS is a known complication of FA but because we did not detect any children with MDS it is not possible for us to comment on whether FANCG mutations are associated with MDS development (Butturini et al. 1994). Unfortunately we were unable to perform any statistical analysis because our sample of MDS patients was so small.

As discussed, MDS often progresses to AML in the general population but this has not been found to be the case in FA patients (Alter 2003). It has been suggested that MDS in FA patients may be different to primary MDS (Alter et al. 2000). It was therefore decided not to include the patients with MDS in our sample of patients with AML.

4.5 CLINICAL MANIFESTATIONS OF FANCONI ANAEMIA

FA is known to be an extremely clinically heterogeneous disorder (reviewed in section 1.1). It is interesting that even when the mutation is the same, variable phenotypes can occur in different population groups. This is best illustrated by the mutation in FANCC,

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which is known to result in a severe phenotype in the Ashkenazi Jewish population (Gillio et al. 1997) but a relatively mild phenotype in the Japanese population (Futaki et al. 2000).

Many research groups have studied the frequency of the various clinical manifestations in FA individuals. We compared the frequency of clinical features of FA in a group of black South African patients to that described by Rosenberg et al. (2004) and to a group of FANCG patients described by Faivre et al. (2000). We also applied the method described by Rosenberg et al. (2004) to predict the risk of the first adverse event to determine whether the risk of AA in the black South African FA group was as predicted for American patients.

Rosenberg et al. (2004) described the clinical manifestations reported in a group of 144 American FA patients and their association with the development of AA, AML and solid tumours. Their patient cohort was not divided into complementation groups. Faivre et al. (2000) determined the frequency of clinical features in each of the commoner FA complementation group.

4.5.1 Phenotype of Fanconi Anaemia in Black South African Patients

Our sample group consisted of 34 black South African FA patients, 25 of these patients belonged to FA complementation group G and 9 to other undefined complementation groups. No statistically significant differences in the frequency of clinical features were identified between the two South African groups. However, the small size of the non-FANCG group restricted the power of the comparison.

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The patients in our FANCG group were mainly homozygous (N = 21) with a few patients heterozygous (N = 4) for the *FANCG 637-643* deletion mutation. We therefore had a sample of patients from one complementation group with a specific mutation, which allowed more accurate genotype phenotype correlations to be made because the group was homogeneous. The diagnosis of FA in the American cohort was made by chromosome breakage studies and therefore patients were not divided by complementation group or mutation type. Faivre et al. (2000) identified a group of FANCG patients by cell fusion experiments, by detecting at least one pathogenic mutation or by the existence of a sibling already classified.

On comparing the frequencies of manifestations in the American and black South African FANCG cohorts, it was noted that the occurrence of developmental delay, hearing disabilities and head abnormalities were significantly different. Developmental delay and hearing impairment occurred more frequently in the American cohort while head abnormalities occurred more frequently in the South African group (Rosenberg et al. 2004). There were no significant differences between the frequency of manifestations in the South African FANCG cohort and that described by Faivre et al. (2000). Significant differences in the frequency of hearing impairment and head abnormalities were also found between the cohorts described by Faivre et al. (2000) and Rosenberg et al. (2004). This suggests that the clinical features in FANCG patients may be fairly specific irrespective of the specific mutation involved.

Developmental delay is not specifically tested for in FA patients in South Africa and it can be a fairly subjective diagnosis. Personal communication with a paediatrician treating many patients with FA confirmed that although it is acknowledged that many patients with

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FA are not severely developmental delayed; they are often on the slower side of normal (Dr J. Poole, Department of Paediatrics, Johannesburg Hospital 2004). It is therefore possible that there is underreporting in our data set especially as developmental delay was not asked about specifically in our data collection. Hearing disability is again not tested for in FA patients and so may be underreported in our cohort although Faivre et al. (2000) also showed a significantly lower incidence of hearing impairment in their FANCG group.

Head abnormalities, including microcephaly and dysmorphic features, were reported more frequently in the South African cohort. This supports the finding from a previous study that microcephaly and eye anomalies occur more frequently in black South African FA patients than in other population groups that have been studied (Macdougall et al. 1990). The Faivre et al. (2000) study also showed an increase in the frequency of head abnormalities in patients with FANCG. Interestingly microcephaly is usually associated with developmental delay and the fact that microcephaly was detected in our sample but not developmental delay indicates that developmental delay may have been underreported as suggested in the previous paragraph.

The definition of radial ray abnormalities, as previously discussed, is not clear and the term is often used to describe different manifestations. Rosenberg et al. (2004) do not include thumb abnormalities in their definition of radial ray abnormalities, while Faivre et al. (2000) group thumb and abnormalities of the radius together under this heading. In our study, we separated thumb and abnormalities of the radius when comparisons were made to the Rosenberg et al. (2004) study and grouped them together when comparing the frequency of radial ray abnormalities to the Faivre et al. (2000) study.

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Although the difference between the frequency of abnormalities of the radius in the American and South African patients was not quite significant ($p = 0.078$), it is interesting that abnormalities of the radius rarely manifest in the South African cohort. It was confirmed by personal communication with a paediatrician (Dr J. Poole, Department of Paediatrics, Johannesburg Hospital 2004) treating many patients with FA, that black South African FA patients rarely have abnormalities of the radius. As the *FANCG 637-643* mutation is so common in the population, it can be assumed that this mutation is not associated with high rates of abnormalities of the radius. On the contrary, thumb abnormalities seem to manifest commonly in the black South African FA group (60 %) as they do in the cohort described by Rosenberg et al. (2004) (56 %).

Pigmentary changes including café-au-lait marks, hypo and hyper-pigmentation were observed in 80 % (20/25) of the black South African FANCG patients. Pigmentary changes are very obvious especially in black patients and are therefore noted. Rosenberg et al. (2004) excluded pigmentary findings from their study because they are age and sun-exposure-dependent. Faivre et al. (2000) however found that 74 % (124/167) of their FANCA group had pigmentary manifestations and 66 % (16/24) of their FANCG group had pigmentary manifestations. No significant differences were found between the groups in terms of the frequency of this manifestation.

It is very difficult to know whether the differences between the frequencies of clinical manifestations in the different population groups are real or simply due to a reporting bias. In the American study the information was collected from the patients themselves or from parents, while in the South African study the clinical features were reported by the medical professional treating the FA patients. One would suspect that professionals might report

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the clinical features more accurately. However, often the patient would not be present, meaning features may have been forgotten because they are subtle, not asked about specifically or don't impact on the haematological diagnosis. Because parents generally know their children's features well, they are probably less likely to miss specific features when asked about them. However they may under-report developmental delay and microcephaly unless they have been made aware of them. Because we did not see the questionnaire used in the American study, it is difficult to be aware of the possible shortcomings.

Faivre et al. (2000) collected information about their cohort of FANCG patients by administering the same clinical data sheet (Appendix B on page 89) that we used to collect our data. Our results can probably be more meaningfully compared to those of Faivre et al. (2000) because there is less likely to be ascertainment bias. This may account for why no statistically significant differences in frequencies of congenital abnormalities were found between our cohort and theirs. However, the group of patients described by Faivre et al. (2000) belonged to complementation group G as ours did. It is probably more likely that no statistically significant differences in congenital abnormalities were found because patients from complementation group G have similar clinical features which are specific to this group.

4.5.2 Association of Congenital Abnormalities and Haematological Outcome

Of the ten clinical manifestations of FA chosen by Rosenberg et al. (2004), five were shown in their study to be associated with the development of AA. These five included developmental delay, heart, kidney, hearing and head abnormalities. Interestingly, the

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frequency of developmental delay, hearing impairment and head abnormalities were the three clinical features found to differ significantly in frequency between the South African FANCG group and the American cohort.

Each patient was given a CAB5 score, which indicated how many of the five chosen clinical features they had. The risk of developing AA by the age of ten was shown to vary from 18 % to 83 % in the American population depending on the CAB5 score and the presence or absence of an abnormality of the radius (Rosenberg et al. 2004).

The average CAB5 score in our sample of FANCG patients was 1.32 with no abnormalities of the radius reported. The number of these patients presenting with AA before ten years of age (79 %) was found to be significantly greater than predicted (24.5 %) by the American model (Rosenberg et al. 2004). It would appear therefore that black South African patients with FANCG mutations do not fit well into the model described by Rosenberg et al. (2004). This finding supports that of Faivre et al. (2000) who found that patients with mutations in FANCG are at high-risk of a poor haematologic outcome.

Macdougall et al. (1994) also found that the black South African FA patients (assumed to be mostly FANCG) in their sample were more likely than the white patients (assumed to be mostly FANCA) to present with severe anaemia. Although Macdougall et al. (1994) suggested that the difference may be due to cultural, educational and socio-economic differences rather than differences in the disease process, their results support the more recent findings that FANCG is associated with a poor haematological outcome.

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The black South African non-FANCG patients seem to fit the model described by Rosenberg et al. (2004) more closely. The average CAB5 score in this group was 1.5 (ranging from 1 to 3). The percentage of patients presenting with AA before ten years of age was 56% (5 of 9 patients). The predicted percentage calculated using Figure 2-3 is 24.69 %. Because of the small sample size the difference between the observed and expected number of patients presenting with AA before ten years of age in this group could not be tested statistically.

The American study did not divide their sample into complementation groups. It is known that different complementation groups carry different risks for the development of haematological abnormalities (Faivre et al. 2000). The American study does not account for this except to say that the presence of the different congenital defects may be associated with different genes or mutations that also influence the risks of adverse events (Rosenberg et al. 2004). Mutations in FANCA account for the vast majority of FA patients, about 70%, while mutations in FANCG account for only about 9.8 % (Faivre et al. 2000). These figures do vary within different populations however, but it can be assumed that the majority of FA patients in Rosenberg et al. (2004) study belonged to FANCA. Therefore it is possible that some of the complementation groups are associated with specific outcomes but go undetected because they make up such a small part of the sample of FA patients.

The reason that the non-FANCG patients appear to fit the American model better than the FANCG group is probably because the patients in the non-FANCG sample belong to different complementation groups and have different mutations. The South African non-FANCG sample is therefore potentially heterogeneous like the American sample and likely to be dominated by the globally common FA complementation groups. The FANCG

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sample is homogeneous and the risk of early AA development and the clinical features identified are likely specific to FA complementation group G or more specifically to the *FANCG 637-643* mutation.

The American study found that abnormalities of the radius are the biggest risk factor for the development of AA (Rosenberg et al. 2004). In our study, no patient had this abnormality yet the risk of early development of AA was very high. Therefore abnormalities of the radius may not be associated with *FANCG 637-643* even though this mutation appears to confer a high risk of AA. Because FANCG is rare in America, its clinical features and prognosis are likely to be overshadowed in a sample of FA patients that probably mostly belong to FANCA (Morgan et al. 1999).

The American study only considered an individual to have AA if severe enough to warrant bone marrow transplantation or result in death (Rosenberg et al. 2004). We did not think that this was appropriate in our setting because bone marrow transplants are done extremely rarely in the state sector mainly due to financial restrictions. Therefore even when bone marrow transplant is indicated, it is extremely rarely done. We considered an individual to have AA if they had pancytopaenia, bone marrow involvement and were being treated with androgen therapy, corticosteroids and blood transfusions. This may be the reason that the number of patients with AA is higher in our sample than in the American group.

From our observation that 79 % of black South African FANCG patients develop AA before ten years of age, there is little doubt that patients with FANCG are at high risk for developing AA at a young age. The question then remains, why are only 1 in 476 000

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black South African patients diagnosed with FA (Macdougall et al. 1990) when the number expected from the carrier frequency should be 1 in 24 000 (Morgan et al. 2005)?

It is possible that biased ascertained of our sample resulted in the finding that FANCG patients have a poor outcome. This is because our FANCG patients were collected from clinics where they were being treated for FA and therefore were severely affected. The possibility that less severely affected patients do not present at these clinics cannot be excluded. Because FANCG is seemingly a severe form of FA (Faivre et al. 2000), it is probably unlikely that patients are presenting with atypical or milder features although the possibility of a bias in the acsertainment of patients cannot be entirely ruled out.

A possible reason that patients are not diagnosed with FA in South Africa is that a large part of the population does not have readily available access to health care and so many FA patients die without seeking medical attention, particularly if they have a severe form of the disease. It is not possible to know whether these patients are likely to have more or less severe congenital abnormalities. Another reason patients may not be diagnosed with FA is that doctors in primary health care clinics are most likely unfamiliar with FA and when these patients are seen, a diagnosis of FA is not made. A delay in making the diagnosis may result in the child dying and the diagnosis being missed.

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4.6 CONCLUDING COMMENTS

4.6.1 FANCG 637-643 Screen in Black South African Patients with AML or AA

No statistically significant increases in the frequency of heterozygous carriers of the *FANCG 637-643* deletion mutation were detected in black South African patients with AML or AA, although there may be a slightly increased frequency in patients with AML. From our data it seems unlikely that this mutation predisposes heterozygous carriers to developing AML or AA but further studies are required to confirm this deduction. A small increase in the carrier frequency would not have been detected in this study due to the relatively small sample size.

4.6.1.1 PATIENTS WITH ACUTE MYELOID LEUKAEMIA

The prevalence of FA homozygotes in the South African population is significantly lower than would be expected from the reported carrier frequencies (Morgan et al. 2005). We hypothesised that a proportion of FA homozygotes may be presenting with AML and the diagnosis of FA missed due to the unusual presentation. However, no AML patients homozygous for the FANCG mutation were identified indicating that this is probably unlikely. From our data, it seems unnecessary to screen black South African patients with AML for the *FANCG 637-643* mutation.

4.6.1.2 PATIENTS WITH APLASTIC ANAEMIA

Three individuals with AA out of the 78 screened (3.8 %) were found to be homozygous for the FANCG deletion mutation and one individual with features of FA was found to be a

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heterozygous carrier. This relatively high incidence of FA among our cohort of patients with AA supports the findings of previous studies (Guinan 1997) describing the importance of screening all children with AA for FA.

4.6.2 Clinical Manifestations of Fanconi Anaemia in Black South Africans

4.6.2.1 CONGENITAL ABNORMALITIES IN FANCONI ANAEMIA

The frequency of certain clinical features of FANCG in black South African patients was compared to a group of FA patients described by Rosenberg et al. (2004) belonging to undefined complementation groups, and secondly to a group of FANCG patients described by Faivre et al. (2000). The frequencies of the features were similar to the FANCG cohort described by Faivre et al. (2000). The frequency with which developmental delay and hearing impairment occurred was found to be significantly higher in the study by Rosenberg et al. (2004) than in the South African group. The frequency of head abnormalities including dysmorphic features and microcephaly however, occurred more frequently in the South African cohort.

The difference in frequencies of the clinical manifestations could be due to the fact that the different complementation groups are associated more or less strongly with specific abnormalities. Our findings could therefore be explained by the fact that the group described by Faivre et al. (2000) had mutations in FANCG like our South African group, while the cohort described by Rosenberg et al. (2004) were probably mainly patients with mutations in FANCA, unlike our group. However, we were unable to rule out the possibility of reporting biases and biases in patient ascertainment. Further studies on the

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correlation between phenotype and genotype will elucidate whether the different FA genes are responsible for different manifestations.

4.6.2.2 Association of Congenital Abnormalities with Haematological Outcome

Rosenberg et al. (2004) found that in their cohort of patients abnormalities of the radius were associated with a poor haematological outcome. They also found that the number of certain congenital abnormalities in a patient was associated with the risk of early AA development. From our study we concluded that black South African FA patients with the *FANCG 637-643* mutation have a high risk of early development of AA even though they do not have a high frequency of abnormalities of the radius or a large number of the congenital abnormalities described by Rosenberg et al. (2004). Our findings support those of Faivre et al. (2000) and Macdougall et al. (1994) which described early onset of FA and the poor haematological outcome of patients with FANCG mutations.

The poor prognosis may mean that patients die before being diagnosed and this may provide an explanation for the discrepancy between the frequency of clinical diagnoses and the frequency predicted from the gene frequency. It is probably unlikely that black South African FANCG patients are missed because they present differently or have a milder phenotype.

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The URLs for the programs used for the statistical analysis are as follows:

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APPENDIX A: SOLUTIONS AND RECIPES

All solutions were made up with distilled deionised water and stored at room temperature unless otherwise stated.

STOCK SOLUTIONS

dNTPs (10 x)
125 µl dATP (10 mM Li-Salt)
125 µl dTTP (10 mM Li-Salt)
125 µl dGTP (10 mM Li-Salt)
125 µl dCTP (10 mM Li-Salt)
Add 500 µl water.
Aliquot into 1.5 ml Eppendorf tubes and store at -20°C

Ethidium bromide (10mg/ml)
Dissolve 100 mg ethidium bromide in 10 ml water by shaking overnight. Filter and store in aluminium foil.

0.5 M Ethylenediamine-acetic acid (EDTA)
Dissolve 9.31 g EDTA in 40 ml water. Adjust pH to 8.0 with concentrated NaOH. Make up to 50 ml with water and autoclave.

1 M $MgCl_2$
Dissolve 20.33 g $MgCl_2$ $6H_2O$ in 90 ml water. Make up to 100 ml and autoclave.

Proteinase K (10 mg/ml)
Dissolve 100 mg Proteinase K in 10 ml water. Divide into 1 ml aliquots and store at -20°C.

1 M Tris-HCl (pH 8.0)
Dissolve 121 g Tris in 900 ml water. Adjust with HCl to pH 8.0 and make up to 1l.

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SOLUTIONS

DNA molecular marker
Mix together: 250 µl GIBCO® 1 kb plus DNA ladder
125 µl Ficoll dye
2.1 ml of 1 x TE
Store at 4°C. Use 5 µl in agarose gels.

70 % Ethanol
Add 700 ml of 100% ethanol to 300 ml water.

Ficoll dye
50 g of 50 % sucrose crystals
100 µl of 0.5 M EDTA
0.1 g of 0.1 % bromophenol blue
10 g of 10 % ficoll powder
Make up to 100 ml with water. Filter through Whatman no. 1 filter paper. Aliquot into 1.5 ml Eppendorf tubes and store at 4°C.

Metaphor® gel (4 %) for agarose gel electrophoresis
Slowly add 16 g Metaphor gel powder to 400 ml chilled 1 x TBE buffer and dissolve. Heat until dissolved, swirling at intervals. Allow gel to cool slightly before adding 12 µl of EtBr and then pour into a gel mould. Wrap gel in cling wrap and tin foil and store at 4°C.

Saturated NaCl
40 g NaCl was slowly added to 100 ml water until the solution was totally saturated, ie. some salt precipitated out of solution.

Proteinase K working solution (2 mg/ml)
Make up just before use
Mix together: 1.4 ml water
0.4 ml Proteinase K stock
0.2 ml 10% SDS
8 µl 0.5 M EDTA

10% Sodium dodecyl sulfate (SDS)
Dissolve 2.5 g SDS in 20 ml water by stirring at low heat. Make up to 25 ml and dispense into 5 ml aliquots.

Sucrose-Triton X Lysing Buffer
Mix together 10 ml 1 M Tris-HCl (pH 8.0), 5 ml 1 M $MgCl_2$ and 10 ml Triton X-100. Make up to 1 l and autoclave. Add 109.5 g sucrose just before use.

1 x TE (pH 8.0)
Mix together 1 ml of 1 M Tris-HCl (pH 8.0) and 200 µl of 0.5 M EDTA. Make up to 100 ml with water and autoclave.

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10 x TBE
108 g Tris-base, 55 g Boric Acid, 7.44 g EDTA in 1 l water to pH 8.0.

T20E5
Mix together 2 ml 1 M Tris-HCl (pH 8.0) and 1 ml 0.5 M EDTA (pH 8.0) and make up to 100 ml with water.

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APPENDIX B: EUROPEAN FANCONI ANAEMIA REGISTRY – INITIAL FORM – V1.0 – 25/08/99

Physician name: _____
Hospital:
Unit:
Address:
Country: _____
Fax: _____
Email:

_____
Patient identification number:

_____
Initials: |_|_| |_|_|
First name Family name

Date of Birth: |_|_| |_|_| |_|_|
Day Month Year

Sex: 1) M 2)F |_|

Spontaneous chromosome breakage: |_|
0) No 1) Yes 9) Not done

Increased chromosome breakage with Cross-linking agents |_|
0) No 1) Yes 9) Not done

Abnormal cell cycle study |_|
0) No 1) Yes 9) Not done

1) Familial history

Consanguinity in parents |_|
0) No 1) Yes 9) Not done

Number of siblings: |_|_|

Positive familial history: |_|
0) No
1) Abortion
2) Still birth
4) Other family members
9) Unknown

If yes, number of affected kinship: |_|_|

Familial history of cancer: |_|
0) No 1) Yes 9) Unknown

If yes, precise: _____

Date of Diagnosis: |_|_| |_|_| |_|_|
Day Month Year

2) Clinical manifestations

Cutaneous manifestations: |_|_|
0) No 1) Café au lait spot
2) Leukoplakia 4) Hyperpigmentation
8) Hypopigmentation 16) Nail dystrophy
32) Other: _____

Head abnormalities: |_|
0) No 1) face 2) eye 4) ear 8) neck
Precise: _____
16) Other: _____

Skeleton abnormalities: |_|
0) No 1) thumb 2) radius
4) Other: _____

Organ abnormalities: |_|_|
0) No _____
1) Kidney _____
2) Uro-genital _____
4) Gastro-intestinal tractus _____
8) Cardiac _____
16) Neruological _____
32) Other _____

Other abnormalities: |_|_|
0) No _____
1) Endocrinopathy _____
2) Low birthweight _____
4) Growth retardation _____
8) Other _____

3) Hematological complications

Date of first abnormal hemogram:

|_|_| |_|_| |_|_|
Day Month Year

First abnormal hemogram:
Hemoglobin (g/dl) |_|_|.|_|
Reticulocytes (G/l) |_|_|_|
V.G.M |_|_|_|
Leukocytes (G/l) |_|_|_|.|_|
Neutrophils (G/l) |_|_|_|.|_|
Platelets (G/L) |_|_|_|_|.|_|
Marrow blasts (%) |_|_|

Date(s) of first hemogram(s) with:

Hemoglobin < 10 g/dl |_|_| |_|_| |_|_|
Hemoglobin < 8 g/dl |_|_| |_|_| |_|_|
Platelets < 100 G/l |_|_| |_|_| |_|_|
Platelets < 20 G/l |_|_| |_|_| |_|_|
WBC < 4 G/l |_|_| |_|_| |_|_|
ANC < 1 G/l |_|_| |_|_| |_|_|
ANC < 0.5 G/l |_|_| |_|_| |_|_|
Day Month Year

Marrow clonal cytogenetic abnormality |_|
0) No 1) Yes 9) Unknown
If yes, date: |_|_| |_|_| |_|_|
Day Month Year
If yes, type: _____

Did you observe modifications over time |_|
0) No 1) Yes 9) Unknown
If yes, precise: _____
Date of modification: |_|_| |_|_| |_|_|
Day Month Year

Myelodysplastic syndrome (MDS) |__|
0) No 1) Yes
If yes, type: _____

Date of diagnosis |__|__| |__|__| |__|__|
Day Month Year

Acute Leukaemia (AL) |__|
0) No 1) Yes
If yes, type: _____

Date of diagnosis: |__|__| |__|__| |__|__|
Day Month Year

Treatments (for MDS or AL) |__|
0) none
1) high-dose chemotherapy
2) low-dose chemotherapy
4) growth factors: _____
8) other: _____

Result: |__|
0) no response
1) complete remission
2) partial remission

Treatments for pancytopenia
0) None 1) Corticosteroids 2) Androgens |__|
Date of beginning:
- Androgenotherapy |__|__| |__|__| |__|__|
Day Month Year
- Corticotherapy |__|__| |__|__| |__|__|
Day Month Year
Growth factors:
0) None 1) G-CSF 2) GM-CSF 4) Epo |__|
Date of beginning: |__|__| |__|__| |__|__|
Day Month Year
Immunosuppressors:
0) None 1) Cyclosporine 2) ATG/ALG |__|
Date of beginning: |__|__| |__|__| |__|__|
Day Month Year
Other(s): _____

First RBC transfusion: |__|__| |__|__| |__|__|
Day Month Year
Number of RBC transfusions:
0) No 1) <20 2) >20 9) unknown |__|

First platelet transfusion: |__|__| |__|__| |__|__|
Day Month Year
Number of platelet transfusions:
0) No 1) <20 2) >20 9) unknown |__|

3) Allogeneic Stem Cell Transplantation |__|
0) No 1) Yes 9) Programmed
If yes, date of SCT: |__|__| |__|__| |__|__|
Day Month Year
If yes, with an: |__|
1) HLA matched sibling donor
2) Other HLA matched related donor
3) HLA mismatched donor
4) Unrelated HLA matched donor
5) Unrelated HLA mismatched donor

Please if the patient has received an allogeneic stem cell transplantation fill the specific form.

4) Other complications
Liver adenomas |__|
0) No 1) Yes before androgens 2) after androgens

Hepatocellular carcinoma |__|
0) No 1) Yes, before androgenotherapy
2) Yes, during androgenotherapy
4) Yes, with before adenoma
Date of diagnosis |__|__| |__|__| |__|__|
Day Month Year

Other malignant diseases
0) No 1) Yes
Date of diagnosis |__|__| |__|__| |__|__|
Day Month Year
Localization: _____

Histological subtype: |__|
1) squamous cell carcinoma
2) adenocarcinoma
3) other: _____
4) unknown

5) Last follow-up

Date: |__|__| |__|__| |__|__|
Day Month Year
Status 0) Alive 1) Dead |__|

Hematological status |__|
0) Normal counts
1) Aplastic phase 2) MDS 3) Acute Leukemia
Hemoglobin (g/dl) |__|__|.|__|
Transfused: 1) yes 0) no |__|
Leukocytes (G/l) |__|__|__|.|__|
Neutrophils (G/l) |__|__|__|.|__|
Platelets (G/L) |__|__|__|__|.|__|
Transfused: 1) yes 0) no |__|
Marrow blasts (%) |__|__|

Therapeutic status
0) None
1) RBC transfusions
2) Platelet transfusions
3) Androgens
4) Corticosteroids
5) Growth factors
Precise: _____
32) Chemotherapy
Precise: _____

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APPENDIX C: ETHICS APPROVAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Haw

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M03-02-16

PROJECT *Fanconi Anaemia* Geme Mutations in Patients with Acute Myelogenous Lukemia, Aplastic Anaemia and Myelodysplasia

INVESTIGATORS Ms T Haw

DEPARTMENT School of Pathology, NHLS

DATE CONSIDERED 03-02-28

DECISION OF THE COMMITTEE Approved unconditionally

Unles otherwise specified the ethical clearance is valid for 5 years but may be renewed upon application This ethical clearance will expire on 1 January 2008.

DATE 03-03-26 CHAIRMAN.....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Prof A Krause
Dept of School of Pathology, NHLS
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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress form. I/we agree to inform the Committee once the study is completed.

DATE 14/04/03 SIGNATURE T S Haw

PLEASE QUOTE THE PROTOCOL NO IN ALL QUERIES .: M 03-02-16

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES